Interactions Between Inbreeding and Environmental Stressors: Implications for Ecotoxicology

Submitted to the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: A Ross Brown

ABSTRACT

In this thesis the effects of individual and multiple environmental stressors (physical and chemical) are examined in inbred and outbred zebrafish (*Danio rerio*, Hamilton), a model species used in ecotoxicology and environmental risk assessment (ERA). The central question addressed is, are inbred laboratory animals representative and protective of wild populations? That is, are inbred fish equally or more sensitive to chemicals and other stressors compared with more outbred (wild) fish? A combination of tools and approaches incorporating traditional (eco)toxicology and population genetics have been employed, together with more contemporary molecular genetics and population modelling, to compare and contrast a range of responses in inbred and outbred zebrafish exposed to the endocrine disrupting chemical clotrimazole and/or temperature elevation in the laboratory. The choice of test species was based on our broad understanding of its basic biology, extending from the molecular level to the population level, and its wide use as a model organism in (eco)toxicology. Selection of the test chemical clotrimazole and temperature was based on a shared mode of action, aromatase inhibition, and therefore their ability to block oestrogen production, impair reproduction, promote male development and skew population sex ratios in zebrafish.

A cascade of responses were compared in inbred and outbred zebrafish, including changes in the levels of expression of genes for gonadal aromatase and other steroidogenic enzymes, circulating sex steroid hormones, gonadal sex differentiation and development (via gonadal histopathology) and reproductive fitness (female fecundity, paternity and viability of embryos). Amongst the most striking results were directional skews in sex ratio towards males in response to clotrimazole (Chapter 5) and elevated temperature exposure (Chapter 7). Inbred fish were generally more responsive compared to outbreds, which showed evidence of physiological and developmental compensation, resulting in lower male-sex skews and superior fitness in terms of male reproductive success (paternity and viability of embryos). The greater effects observed in inbred fish were attributed to inbreeding×environment interactions and the amplification of inbreeding depression. Although no empirical genetic evidence of this mechanism is presented (loss of heterozygosity at quantitative trait loci and concomitant loss of heterosis and/or the expression of recessive, deleterious alleles in homozygotes), supporting evidence was provided by increased phenotypic variance in some apical endpoints in inbred fish, including specific growth rate and fecundity. This increased variance also has the potential to counteract the higher levels of response observed in inbreds, because the power to detect statistically significant changes in responses is reduced. This trade-off was demonstrated for specific growth rate.

Crucially, significant male-sex ratio skews (>80%) were induced at substantially lower clotrimazole exposure concentrations (1.7 μg l^{-1}) in combination with elevated temperature (33°C), compared with exposure concentrations (43.7 μg l^{-1}) generating similar sex ratio skews at the standard test temperature of 28°C. These temperatures represent current and predicted 2100 (elevated) mean temperatures in the zebrafish's native India and Bangladesh. Although the lowest observed effect concentration was an order of magnitude above the predicted environmental concentration for clotrimazole, it is conceivable that combined environmental exposures to similarly acting chemicals (e.g. other azole compounds used in crop protection, veterinary and human medicine) could produce similar effects to those we observed. The consequent effects of sex ratios skews and reduced fitness (fecundity and embryo viability) on per capita population growth rate (r) and extinction probability were predicted in inbred versus outbred zebrafish populations using stochastic population viability analysis. The results showed that the observed male-skews >80% threaten small zebrafish populations with fewer than 100 breeding adults (<20 adult females). However, small reductions of 2-3% in embryo-juvenile (age 0+) survivorship (including simulated inbreeding depression) were more influential on r and extinction probability than large sex ratio skews and/or reduced female fecundity.

The results presented in this thesis support the contention that chemical effects may be exacerbated by other environmental stressors, but also illustrate the importance of considering biological (genetic), as well as physical and chemical interactions in cumulative ERA. Greater sensitivity of inbred versus outbred organisms to the effects of environmental stressors on sexual differentiation and reproductive fitness offers a margin of safety to ERA and the protection of wildlife populations (excluding those that are severely inbred and critically endangered). This is because, as originally stated, laboratory organisms used in ERA are generally more inbred than their wild counterparts. Nevertheless, more attention should be paid to the origin, breeding history and genetics of laboratory strains. This will help to ensure consistency between studies and testing laboratories and provide more confidence in extrapolating the results to wild populations.

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1 CHAPTER 1: INTRODUCTION

1.1 Inspiration and relevance of research

Organisms need to respond and adapt to their constantly changing physical, chemical and biological environment to ensure survival and avoid extinction (Hoffman and Parsons, 1991). At the molecular level each response involves the modulation of gene expression, and this includes interactions within and between genes (at quantitative trait loci) and the environment. Crucially the maintenance of genetic variation that enables adaptation to environmental change, takes place at the population level (Reed et al., 2003a).

Considerable scientific progress has been made since the discovery of Deoxyribose Nucleic Acid (DNA) by Watson and Crick (1958), including how this self-replicating and evolving (mutating) genetic code is translated and expressed in organisms depending on the environmental conditions to which they are exposed. At the interface between genetics and the environment some environmental chemicals (genotoxins) have been shown to cause DNA damage leading to permanent and/or heritable sequence alteration (Preston and Hoffman 2001). Other chemicals (mutagens) have been shown to increase genetic variation, including the frequency of deleterious mutations, which are selected against, causing bottlenecks, impairing gene flow (reviewed in Van Straalen and Timmermans, 2002) and eroding reproductive fitness (reviewed in Bickham et al., 2000; Bickham, 2011). Chemicals (e.g. endocrine disrupting chemicals) may also affect evolutionary processes indirectly, without selection on adaptive traits, by reducing effective population size (N_e) or by reinforcing the reproductive isolation of exposed populations (Nacci and Hoffman 2008). Recent research has shown that translation of DNA may be modified by chemicals (without sequence alteration) via epigenetic changes to the chromatin structure and this may be the proximal mechanism behind many gene×environment interactions leading to adverse effects including disease development (Liu et al, 2008).

As a result of higher-throughput DNA sequencing the entire genomes of numerous model organisms have been assembled and the identification of key functional genes (encoding proteins), modulating genes (transcription factors) and beneficial versus deleterious alleles (gene variants) is progressing rapidly (reviewed in Allendorf et al., 2010; Queitsch et al., 2012); and is being extended to non-model organisms (Nawy, 2012; Hoffman et al., 2012). This empirical work in molecular genetics offers great insights for numerous other fields of biological science including medicine, agriculture, aquaculture and environmental protection. However the extent to which information from (usually highly inbred) model organisms reared in an often constant, artificial laboratory environment reflects the situation in the wild is uncertain (Flint and Mackay, 2009; Nawy, 2012). The combination of contemporary molecular genetics[1] with more traditional population genetics[2] offers exciting prospects for investigating the influence of genetic variation on responses to environmental stress and *vice versa*, but in working with laboratory animal populations we must be careful to distinguish the "the rule from the exception".

In this thesis the effects of individual and multiple environmental stressors (physical and chemical) are examined in inbred (laboratory) and outbred (wild) zebrafish (*Danio rerio*, Hamilton), a model species used in ecotoxicology and environmental risk assessment (ERA). The central question addressed is "Are inbred laboratory animals representative and protective of wild populations?" A combination of tools and approaches incorporating traditional ecotoxicology and population genetics are applied together with more contemporary molecular genetics and population modelling to compare and contrast a range of responses in inbred and outbred zebrafish to endocrine disrupting chemical (clotrimazole) exposure and/or temperature elevation. Close attention is also given to individual response variation in inbreds versus outbreds and the consequent effects on statistical power and reliability (repeatability) of regulatory ecotoxicology studies, as well stochastic uncertainty in population dynamics and consequent impacts on population viability.

[1]Molecular genetics: study of the structure, function and expression of genes.

[2]Population genetics: study of the maintenance and distribution of genetic variation influencing quantitative traits (heredity variation) across time and space in species and populations.

1.2 Defining responses to environmental stress

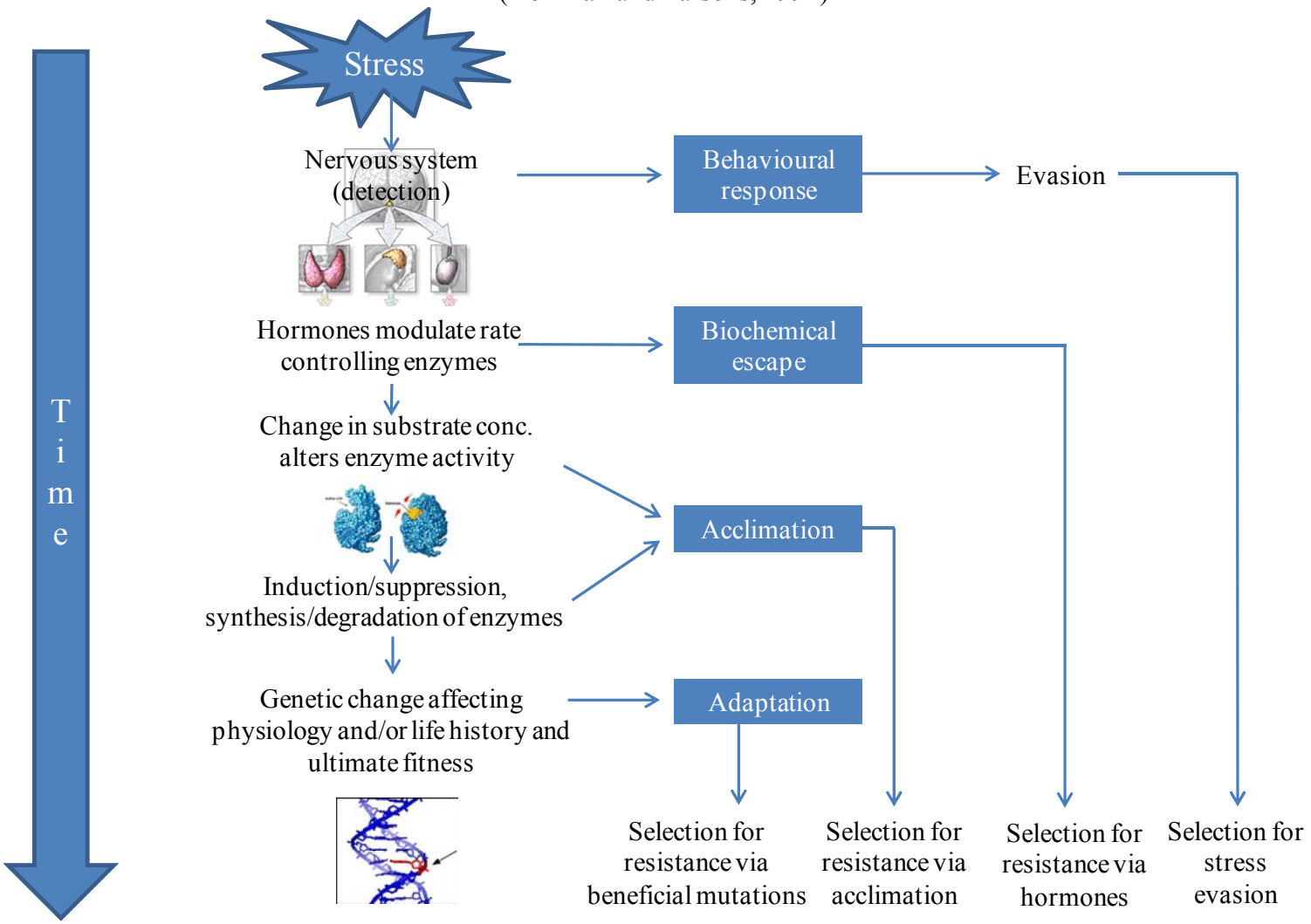
In biological systems '*stress'* may be defined as "a measurable alteration of a steady state (cytological, biochemical, physiological or behavioural), which is induced by environmental change and which renders individuals, populations or communities more vulnerable to further environmental change" (Bayne, 1975). This definition illustrates the concepts of *'cumulative risk'* resulting from the combined action of multiple stressors, and *'adverse effect'* capable of tipping the individual, population or community beyond the point of no return. The internationally agreed definition of an 'adverse effect' in environmental and human health terms is virtually identical to the definition above: "a change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences (IPCS/OECD, 2004).

Biological responses to environmental stress are many and varied, depending on the nature, level and duration of stress and the existing status of the exposed organism(s) (Hoffman and Parsons, 1991). Nevertheless responses (Figure 1) generally take place in the following sequence: i) behavioural responses for stress evasion mediated by the nervous system; ii) biochemical escape i.e. changes in metabolic rate mediated by the endocrine system; iii) acclimation/ compensation i.e. changes in metabolic scope involving substrate changes, enzyme synthesis or degradation; iv) changes in vital rates of survivorship, growth and reproduction affecting individual fitness i.e. the probability of individuals passing their genes on to the next generation. Although all response categories constitute processes of selection, only the last category can be considered irrefutably to constitute an adverse effect (see above).

Differences in the ability of individuals to respond to stress may be induced by environmental variation, reflect underlying genetic variation, or both (Hoffman and Parsons, 1991). Genetic variation is key to understanding and predicting organism, population and community responses to environmental change.

Figure 1: Sequence of stress response in organisms

(Hoffman and Parsons, 1991)



As stated above, the effects of environmental stressors are determined by their interaction with genes or *'genotypes'* possessed by different individuals, which are translated into a corresponding range of *'phenotypes'* (including physiological, morphological or behavioural life-history traits). The extent to which environmental variation can modify the expression of a genotype leading to phenotypic change or variation is known as *'phenotypic plasticity'* (Bradshaw, 1965) and plasticity in stress response may also be governed by genetic variation (Hoffman and Parsons, 1991).

Unravelling *'genotype×environment'* interactions presents a major scientific challenge (Ellegren and Sheldon, 2008). Although in some circumstances these interactions may centre on genes of "large effect" (e.g. the cytochrome P450 super-family of enzymes involved in xenobiotic metabolism, homeostasis and development), phenotypic traits, particularly those affecting fitness are often governed by multiple genes at *'quantitative trait loci'* (QTLs). For well studied quantitative traits (showing continuous variation) in humans and mammalian models, including height, immune-competence or susceptibility to disease (e.g. Alzheimer's and type 1 diabetes), the current model of large numbers of loci, each of "small effect", is generally applicable, making it difficult to link phenotypes with definitive genotypes (Flint and Mackay, 2009). This rule may also apply to some extent to non-quantitative (discontinuous) phenotypes such as phenotypic sex in hermaphroditic fish, including zebrafish (Section 1.7.2).

In the broadest sense standing genetic variation in wildlife populations (often measured at non-coding, non-selected, neutral loci) has been correlated directly with population fitness (Reed and Frankham, 2003) and adaptability to environmental change (Hoffman and Parsons, 1991; Lande and Shannon 1996; Reed et al., 2003a), including novel chemical exposure (reviewed in Chapter 2). This is likely to be because rare alleles which are markers for, or directly responsible for, novel stress resistance are more likely to occur within larger populations containing more genetically diverse individuals (Hoffman and Parsons, 1991). However, not all genetic variation is beneficial and there may be trade-offs whereby genotypes underlying a certain phenotypic trait may be advantageous in coping with a particular environmental stressor but not with another. So it follows that trade-offs limit

adaptation (Futuyma and Moreno, 1988), and at the same time, help to maintain genetic variation by allowing species to occupy different ecological niches (Clark et al., 2007).

The use of modern molecular genetics alongside traditional population genetics in laboratory and field investigations offers three main approaches for studying the influence of genetic variation on responses to environmental stress (Figure 2). i) The "classical top-down" quantitative population genetics approach begins with examining the phenotypic variance in a stress response. This variance is then partitioned into genetic and environmental components by identifying extreme phenotypes and then performing controlled breeding and exposure studies to help isolate the corresponding genotypes. This is achieved using *genetic linkage mapping*, which relies on the use of model organisms with detailed pedigrees and suitable genetic marker(s) (chromosomal or DNA) linked to the locus in question. ii) A more "targeted bottom-up" approach starts with a genetic polymorphism identified in a model or wild-type organism (e.g. allozyme[3] or single nucleotide polymorphism (SNP[4])) potentially related to a given stress response and then attempts to correlate the polymorphism to variation in the stress response. iii) A third "global" approach used widely in the study of human disease is *association mapping* (a.k.a. linkage disequilibrium mapping or genome wide association studies). Studies are performed by scanning the entire genome for statistically significant associations between a panel of markers (e.g. SNPs) and a particular phenotype. For any one of these approaches loci may subsequently be verified using genetic manipulation or gene silencing (e.g. morpholino knockdown) in order to show that they either; a) contribute to the trait of interest directly, or b) are linked to (in linkage disequilibrium) with a quantitative trait locus (QTL) that contributes to the phenotype of interest. However, genetic variation (i.e. different combinations of alleles) at different loci may contribute to similar phenotypes and these phenotypes may vary between geographically distinct populations due to gene×environment interactions (Ellegren and Sheldon, 2008).

[3] Allozymes are variations of an enzyme that differ in amino acid sequence (e.g. indicated by electrophoretic mobility) from other forms of the same enzyme. They are encoded by specific alleles at a single locus.

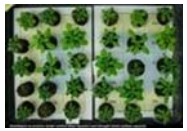
[4] Single Nucleotide Polymorphisms (SNPs) are the most abundant source of genetic variation in genomes. They have a low enough mutation rate to effectively preclude recurrent mutations, making them largely bi-allelic (Krawczak, 1999) and analytically highly tractable, whilst being amenable to high-throughput genotyping (Brumfield et al. 2003).

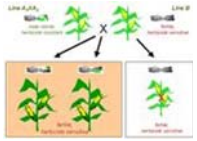
Figure 2: Approaches for studying genetic variation in stress response

(After Hoffman and Parsons, 1991; Ellegren and Sheldon, 2008)

1) Deduce genotype from phenotype (classical "top down" approach)

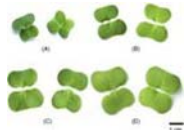
Study phenotypic variation for stress response → Quantify genetic and environmental variance via controlled breeding & exposure → Identify extreme phenotypes & associated genotypes via "genetic linkage mapping"

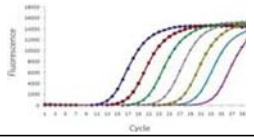


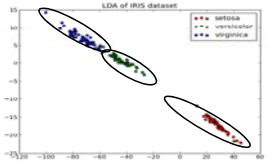


2) Confirm phenotype is linked to a known genetic polymorphism (targeted "bottom up" approach)

Identify phenotype with a known genetic basis potentially related to stress response → Quantify genetic polymorphism at target locus across study population (e.g. using qPCR) → Correlate polymorphism with variance in stress response

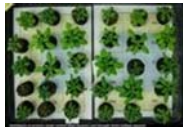


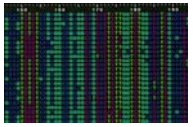




3) Link phenotype to genetic polymorphism(s) via genome wide analysis (global approach)

Sample individuals across population(s) showing variable phenotype → Conduct a genome-wide sequencing on tissue samples → Identify "linkage association" between genetic markers e.g. SNPs and phenotype





1.3 Genetic variation in wildlife –threats and trends

Genetic diversity is a fundamental component of biological diversity or biodiversity (CBD, 1992). Rates of biodiversity loss across the globe have reached unprecedented levels in the last century, coinciding with the industrial revolution and associated loss of natural habitat, environmental pollution, accelerated climate change and the spread of invasive species (UNEP, 2005). According to the World Conservation Union (IUCN) 750-1000 species have gone extinct in the last 400-500 years (approaching 2 species per year) and current extinction rates are 1000 times higher than indicated in the fossil record. A prioritised assessment of 65,000 species (less than 5% of the world's described species) shows that 40,429 are currently threatened with extinction (including critically endangered (CR), endangered (EN), or vulnerable (VU) species). All exist below minimum genetic effective population sizes (Ne), making them vulnerable to further environmental change (IUCN, 2012a). Limited successes in maintaining genetic diversity in these and commercially important species populations via habitat protection and rehabilitative breeding programmes have highlighted our lack of knowledge concerning the interaction of genes and the environment. The precarious state of many flagship species populations including the cheetah (Acinonyx jubatus) plagued by congenital heart disease and immune deficiency syndromes (O'Brien, 1994), and the continued decline of commercial fish stocks, despite restocking from fish farms, highlight the importance of 'genetic quality' as opposed to genetic diversity (Neff et al., 2011). Impacts on these declining populations may exacerbated by subsequent outbreeding between genetically distinct individuals, leading to the dilution of adaptive genetic variation and/or the breakdown of beneficial (pleiotropic or epistatic) gene×gene interactions in their offspring. This can reduce individual fitness and is known as outbreeding depression (Templeton, 1986; Lynch, 1991; Monson and Sadler, 2010).

Inbreeding between related individuals can also lead to fitness depression due to the increased frequency of homozygotes and the expression of deleterious, recessive alleles (or lethal

equivalents[5]) and, to a lesser extent, the loss of heterozygote superiority (Charlesworth and Willis, 2009). Inbreeding depression is considered to be the fastest acting and most significant biological mechanism contributing to the loss of genetic variation within wildlife populations (Brook et al., 2002), reducing N_e and further increasing the likelihood of inbreeding and genetic drift[6] (Lande 1976, Gilpin and Soulé, 1986). Whilst sexual reproductive behaviours generally favour outbreeding (Greenwood, 1980; Pusey and Wolf, 1996; Hosken and Blanckenhorn, 1999) between individuals with optimal levels of genetic divergence, as shown in fish (Neff, 2004), birds and mammals (Amos et al., 2001), inbreeding may be unavoidable in small wildlife populations which have experienced geographical isolation or bottlenecks caused, for example, by disease outbreaks (O'Brien, 1994) or pollution incidents (Bickham et al., 2000). Estimates for the minimum N_e[7] required for maintaining equilibrium between loss of genetic variation due to genetic drift (and inbreeding) and its replenishment by mutation (and outbreeding/immigration) and ultimately for maintaining self sustaining, genetically viable populations vary between 500-5000 (500-1000, Franklin and Frankham, 1998; 1000-5000, Lande, 1995; Lynch, 1996; Lynch and Lande, 1998). Many wildlife populations do not meet these thresholds. However, it is recognised that most genetic problems in endangered populations have accumulated over tens or hundreds of generations and a low N_e for several generations will not necessarily lead to irreversible

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Definition from Morton et al. (1956):

[5] The number of **lethal equivalents per diploid genome** estimates the average number of lethal alleles per individual in the population if all deleterious effects of inbreeding were due entirely to recessive lethal alleles. LE of 1 may equate to two recessive alleles per individual, each of which confer a 50% decrease in survival, or it may be some other combination of recessive deleterious alleles which equate in effect with one lethal allele per individual.

Definitions from Lande (1976):

[6] **Genetic drift** is the random change in allele frequencies and loss of heterozygosity (in the absence of selection, mutation and immigration) due to the random segregation of genes in gametes and unequal reproduction among individuals.

[7] **Effective population size (N_e)** is the size of the 'ideal' population that would undergo the same amount of genetic drift as the actual population.

[7.1] The '**ideal' population** is a population of constant size consisting of non-overlapping generations (no inbreeding) of diploid hermaphroditic individuals that reproduce by random sampling of gametes from an infinite pool to which each individual contributes equally.

genetic damage (Amos and Balmford, 2001). Consequently World Conservation Union criteria (IUCN, 2012b) include demographic population structure, geographical distribution, temporal trends and generation time, as well as breeding population numbers for defining endangered (N_e = 250-2500) and critically endangered species (N_e = 50-250). Levels of inbreeding (*F*) are inversely related to effective population size N_e and increase with generations (t) (Equation 1).

Equation 1: Inbreeding coefficient (Falconer and Mackay, 1996)

$F = 1 - (1 - 1/[2N_e])t$

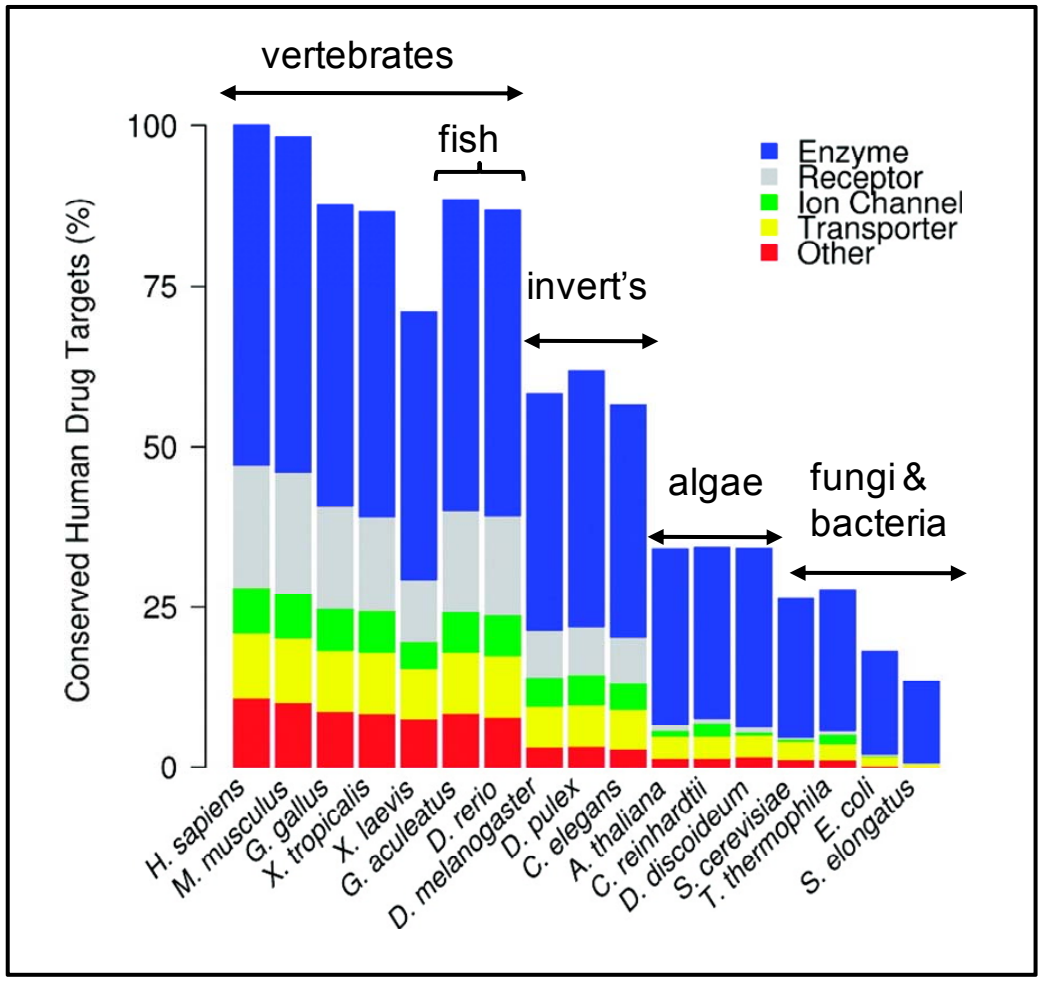
1.4 Laboratory plant and animal models – relevance in environmental research and regulatory testing

Considerable scientific research is being directed towards understanding the *'genetic architecture of fitness'* in plant and animal models by using their genome sequences to map QTLs and their polymorphisms (allelic sequence variations) that influence evolutionarily, commercially (and medically) important quantitative traits (Mackay, 2001, Flint and Mackay, 2009). At the same time, research effort is being devoted to identifying orthologs[8] (conserved gene sequences) at QTLs and confirming their function in different model species and strains (Figure 3). This is aiding extrapolation (read across) between species and phyla, helping to streamline research and ensuring the reliability and relevance of experimental results or highlighting the need for species-specific testing. The use of model species in genetic research is advancing our understanding of gene×environment interactions relating to the aetiology of environmentally-related diseases (Liu et al., 2008; Patel and Butte, 2010), the evolution of pesticide (Raymond et al., 2005) and antibiotic resistance (Remold and Lenski, 2001) and the effects of both genetic strain and culturing/breeding regimes on the sensitivity of model organisms to environmental toxicants (Baird et al., 1991; Coutellec and Lagadic, 2006).

[8] Orthologs are genes in different species that have evolved from a common ancestral gene and often (but not always) retain the same function.

Figure 3: Conservation of human drug targets in different laboratory models

(Gunnarsson et al. 2008)



Animal models used in mammalian toxicology are often deliberately inbred in order to minimise genetic variance so that observed phenotypic variance can be attributed mainly to "environmental" exposure to the toxicant under study (Equation 2), improving detection of dose response with minimal experimental replication/ animal use (Chia, 2005; NAS, 2007).

Equation 2:

Phenotypic variance (σ_P^2) = genetic variance (σ_G^2) + environmental variance (σ_E^2)

Conversely animal models used in QTL mapping are selectively bred to maintain heterozygosity. Crossing these heterozygous strains with homozygous strains facilitates the identification (karyotyping) of breakpoints, formed by recombination, along chromosomes that may then be used to identify loci associated with quantitative traits (Chia et al., 2005). Similarly in ecotoxicology outbreeding is required to ensure that selected individuals/models are representative of "generally outbred" wild populations and potentially a diverse range of similar species (Chapter 2). The limitations of using outbred strains in terms of increased genetic variance may be balanced against the risk of failing to indentify, in inbred strains, important variations in phenotypes and underlying genotypes that aid QTL mapping and the detection of adverse effects in divergent subpopulations. This applies to phenotypic traits with additive genetic variance (Equation 3) as opposed to traits that are governed by dominant alleles, which mask other alleles (dominance variance).

Equation 3: (Falconer and Mackay, 1996)

Genetic variance (σ_G^2) = $2pqa^2$

Where p and q = allele frequencies, a = homozygous effect. Therefore in progeny derived from a cross of two inbred lines (e.g. with genotypes AA and BB), the frequency of all segregating alleles (A and B) is always p = q = 0.5, whereas in an outbred population (e.g. with genotypes AA, Aa, aa, BB, Bb, bb) allele frequencies can vary throughout the entire range.

Despite best efforts, outbred laboratory animal strains are still considerably more inbred and less genetically diverse than their wild counterparts, as indicated by neutral and quantitative

markers of heterozygosity in trematode worms (Stohler et al., 2004), dipteran insects (Woods et al., 1989; Mukhopadhyay et al., 1997; Norris et al., 2001; Nowak, 2007), amphipod crustaceans (Duan et al., 1997), rodents (Razzoli et al., 2003) and zebrafish (Coe et al., 2009; Whitely et al., 2011). This is due to practical constraints limiting N_e in laboratory populations, resulting in genetic drift and the loss of genetic diversity (and quality) over time, despite regular outbreeding between laboratory strains or substrains. The generation of new wild-type laboratory strains is also hampered at the outset by founder events or genetic bottlenecks in which the founders carry only a limited proportion of the initial allelic variation in the source populations (Nowak et al., 2007b). It is sobering to know that the most outbred (heterozygous) zebrafish strain used routinely in ecotoxicology today, the Wild Indian Karyotype (WIK) strain, was derived originally from a single pair mating of second generation wild-caught Indian zebrafish (WIK11) in the mid 1990's (Rauch et al., 1997) (see Section 1.7.1 for more information on zebrafish).

As with inbred wildlife populations (Section 1.3), inbred laboratory populations are susceptible to inbreeding depression and this may be amplified under simulated environmental stress. Consequently there is a growing body of evidence for a range of model species suggesting that inbred animals are more susceptible to adverse chemical effects than outbreds (see following Chapters and supporting references). However, the effect is not universally observed. In a meta-analysis of 34 studies quantifying the severity of inbreeding depression in a range of species in laboratory and semi-field environments, severity increased by 69% (on average) following exposure to environmental stressors, including chemicals (Armbruster and Reed, 2005). However, the frequency of occurrence of inbreeding depression was less than 50% among the reviewed cases and this was attributed to inconsistencies in the distinction of benign control conditions relative to stress treatments, as well as differences in genetic variation between test species, populations and lineages (Armbruster and Reed, 2005). Thus, inbreeding effects are conditional on genetic ancestry and environmental conditions and variations in the responses of different study populations may be due to different frequencies of deleterious alleles. Purging of these alleles can take place in the laboratory following increased stocking densities and competition, leading to increased reproductive fitness in fruit flies (*Drosophila melanogaster*) (Swindell & Bouzat, 2006). In contrast, the purging of

deleterious alleles is less likely in wild populations, including the fruit fly (Bijlsma et al. 1999), under low rates of inbreeding and variable selection pressures (Miller and Hedrick 2001). Consequently a universal, quantitative relationship between the loss of genetic diversity, reduced adaptation and fitness is lacking (Nacci and Hoffman 2008).

1.5 Population models – use in environmental research and regulation

Wildlife populations are widely regarded as the minimum units for species conservation (Reed et al., 2003a; UNEP, 2005) and environmental protection (Barnthouse et al., 2008; EFSA, 2010). However, as mentioned earlier (Section 1.3) defining protection goals based on a minimum effective population size (N_e) requires the integration of a number of influential factors, including demographic population structure, geographical distribution and gene flow, temporal trends and generation time. These factors all contribute to the resilience or susceptibility to environmental change of discrete populations or inter-linked meta/sub-populations in the wild. Adding the dimension of time substantially increases complexity further and presents a major technical challenge, since the full impact of genetic and environmental degradation on population viability may not become apparent for tens of generations (Reed et al., 2003b). Consequently computational population models capable of rapidly simulating population dynamics (overall population numbers and age-related demographic structure) over space and time are being exploited increasingly in the assessment of habitat fragmentation and environmental degradation (Summers, 1989; Morita and Yokota, 2002) and environmental risk assessment (ERA) of chemicals (Spromberg and Birge, 2005; Topping et al., 2005).

Population models describe the essential life history of a species in the wild i.e. “vital rates” of birth, maturation/reproduction and death. There are four broad types, which increase in complexity from type 1 - 4 (Table 1). These models account for vital rates directly or via energy budget calculations, and incorporate various levels of demographic and/or geographic structure and temporal resolution.

Table 1: Population model types

Model Type	Sub-type/ example	Input control data		Input effects data
		Intrinsic (Life History)	**Extrinsic (Environmental)**	
1. Single stage logistic models	Simple logistic[a]	Adult survivorship (l_a) and fecundity (m_a)	Carrying capacity (K)	Adjust vital rates: survivorship and fecundity accordingly
2. Age/stage-based demographic models	Two-stage[b]	Juvenile (l_j), adult survivorship (l_a) and fecundity (m_a)	Density dependence functions can be applied to l_x and m_x	
	Matrix[c]	Age/stage specific survivorship (l_x), fecundity (m_x)		
	Delay differential equation[d]	Daily survivorship and fecundity		
	Meta-population[e]	l_x, m_x, sex ratio, age/length at maturation, movement/ dispersal	Spatial variation in l_x, m_x due to habitat and food availability, immigration/ emigration	Adjust vital rates, extent /level of spatial factors
3. Physiology-based energy budget models	Simple dynamic[f]	Net energy intake versus requirement for homeostasis (basal metabolism) and reproduction	Resource availability (food and space), temperature (Arrhenius equation)	Additional energy requirement for xeno-biotic metabolism or homestatic control
	DEBTox zebrafish model[g]	Net energy intake versus specific energy budget requirements		
4. Individual-Based (IB) models	Zebrafish IB mechanistic model[h]	Daily mortality: (embryonic, back-ground, senescence), growth, length at maturation, competition for breeding habitat, fecundity	Habitat, and food availability, duration of breeding season, density dependent growth and mortality, density independent predation	Survival, growth, development, sex ratio, fecundity, behavioural effects
	Population Viability	Age & sex-specific mortality, fecundity,	Carrying capacity (habitat and food	Adjust vital rates, sex ratio

	Analysis (PVA) model Vortex©[i]	age at maturation, sex ratio, fraction of each sex breeding, inbreeding depression of age 0+ survival, purging of deleterious/ lethal alleles	availability), dispersal and gene flow between meta - populations, natural catastrophes	and standard deviations. Major (acutely toxic) chemical releases can be modelled as catastrophes

References: a Barnthouse et al., 1987; b Sibly and Calow, 1986; c Caswell, 2001; d Brown et al., 2005; e Akçakaya et al., 2008; f Kooijman, 1993; g Augustine, 2012; h Hazlerigg et al., 2013; i Lacy et al., 2005.

Model types 1 and 2 focus on females only, whereas types 3 and 4 can differentiate between males and females and enable modelling of stressor effects on growth and sex ratio, as well as survival and fecundity. In stage-based models (types 1 and 2) individuals of equivalent stage or age (e.g. cohort) are modelled collectively, whereas in individual-based (IB) models (type 4) each individual is modelled separately with trait values being input randomly from the available data distributions. In the former case population age and size structure are stipulated from the outset, whereas in IB models population dynamics emerge from interactions between individuals and environmental variables. Thus IB models enable the incorporation of mechanistic data linking behaviour and physiological/pathological biomarkers to vital rates and these models are being used increasingly in ERA (Grimm et al., 2009). Physiology-based dynamic energy budget (DEB) models (type 3) also offer the possibility of individual based modelling, but the approach is based on universally applicable metabolic processes and responses to toxicants, rather than depending on prescribed mechanisms of toxicity (Martin et al., 2012). However, DEB models require very detailed and specific input data that are rarely generated in standard ectoxicity tests and where they are available they may require complex lab to field extrapolation e.g. due to temperature-related variations in energy partitioning between physiological processes. Temperature is a key environmental variable for exotherms including fish, not only affecting metabolic function, growth and development (Pitcher and MacDonald, 1973), but also acting as a behavioural cue for breeding (Spence et al., 2008) and

influencing larval dispersal (O'Connor et al., 2007) and the geographical range of species (Perry et al., 2005).

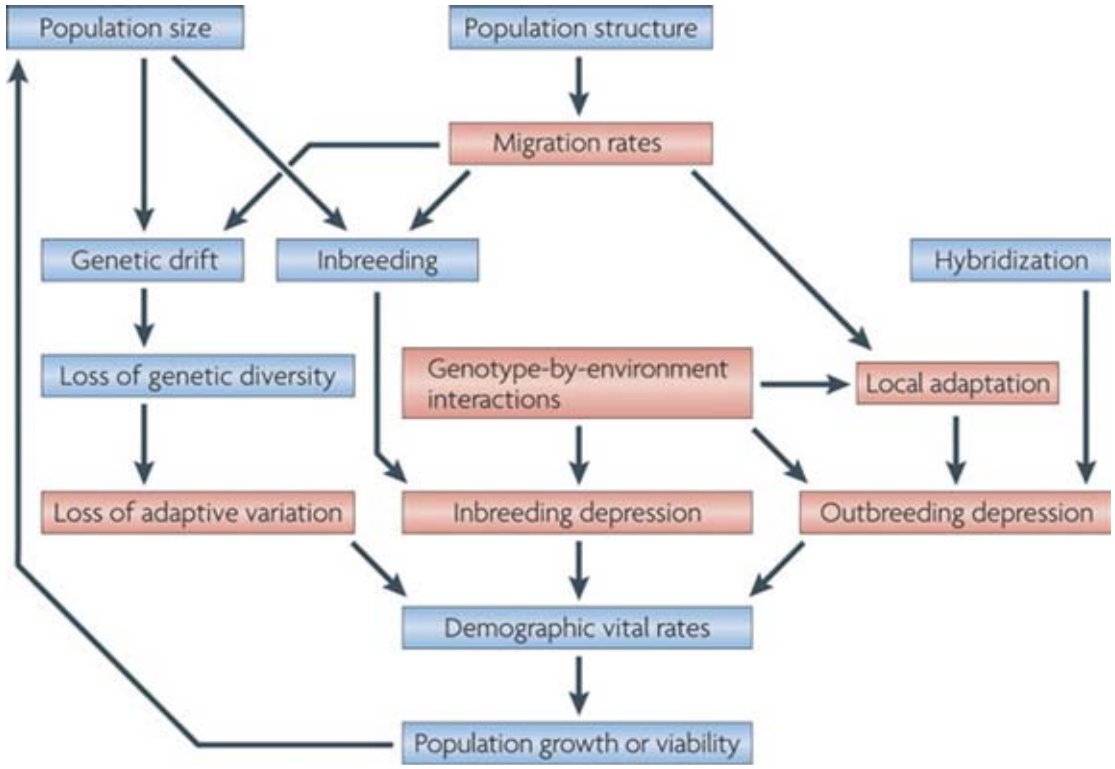
Each of the model types (Table 1) offers the ability to simulate asymptotic population growth, limited by the availability of food and space. This may be achieved by incorporating a ceiling carrying capacity in logistic models (Barnthouse et al., 1987; Miller and Lacy, 2005) or a finite energy budget (Kooijman, 1993) (both providing negative feedback only) or via density dependence functions (providing positive and negative feedback) for each vital rate in age/stage-based models (Beverton and Holt, 1957; Ricker, 1975) or IB models (Hazlerigg et al., 2013). Once parameterised baseline predictions can be made with a "control model" and verified using population census data from wild populations to confirm population size and/or demographic structure. Control predictions can then be compared with "effects model" predictions, after appropriate adjustment of the input parameters. Thus mean treatment effects are superimposed on any background effects already incorporated in the control model, leading to an overall deterministic prediction of population number and growth rate (Equations 4-7). Alternatively, models incorporating variability due to intrinsic (genetic[9] and/or demographic[10]) and extrinsic (environmental[11]) stochasticity (Shaffer, 1981; Goodman, 1987; Lacy et al., 2005) can provide probabilistic predictions of population decline (e.g. interval decline or extinction risk) covering a range of possible scenarios. In conjunction with these latter models, sensitivity analysis can also be used to identify life history traits (and, in mechanistic IB models, associated biomarkers of effect) that are most influential on the trajectory of the population over successive generations, helping to identify cause and effect or direct further testing.

[9] Genetic stochasticity refers to changes in the genetic composition of a population due to genetic drift (in the absence of selection, inbreeding, or migration). It inevitably leads to the loss of heterozygosity, which reduces genetic variation and adaptive capacity and at the same time increases the chance of deleterious recessive alleles being expressed in homozygotes. Therefore although random by its very nature, genetic stochasticity is correlated with systematic inbreeding in small populations and both can lead to depression in fitness (Miller and Lacy, 2005).

[10] Demographic stochasticity is the random fluctuation in survival, sex ratio and fecundity, which in small populations can result in large deviations from mean values, increasing the risk of extinction (Goodman, 1987).

[11] Environmental stochasticity typically results from climatic variation, affecting habitat size and quality, abundance of food and preditors etc. and ultimately adding to demographic stochasticity.

Figure 4: Schematic diagram of interacting factors in conservation of natural populations



Traditional conservation genetics, using neutral markers, provides direct estimates of some interacting factors (blue). Conservation genomics can address a wider range of factors (red). For example, traditional conservation genetics can estimate overall migration rates or inbreeding coefficients, whereas genomic tools can assess gene flow rates that are specific to adaptive loci or founder-specific inbreeding coefficients.

Population viability analysis (PVA) models offer additional confidence by incorporating population genetics (heterozygosity, inbreeding depression and gene flow[12]) along with population demographics (survival, fecundity and generation time) in the prediction of population growth and viability (Figure 4).

Minimum Viable Population size (MVP) equates to a population size with 99% probability of persisting for 40 generations and can be expressed in total population number (MVP_i), adult population number (MVP_A) or effective population number (MVP_{Ne}). Population viability analysis on over 100 vertebrate species has shown that MVPs do not vary significantly among major taxa or with trophic level or latitude (Reed et al., 2003b). However, MVP is correlated with extinction risk (+ve), temporal variation in population size relative to generation time (+ve) and population growth rate per generation (-ve) (Vucetich et al., 2000; Reed et al., 2003b). Regression of the >100 species dataset indicates that MVP_A is 13,500 when population growth rate is minimal (R_0 = 1, 1:1 replacement) in non-pristine habitats, and MVP_A is 2220 when population growth rate is maximal (R_0 = 2.72, more than exponential i.e. >doubling) in pristine habitats (Figure 5).

Equation 4: Exponential population growth in continuously breeding populations (overlapping generations)

$N_t = N_0 e^{rt}$

Where r = per capita population growth rate (r>0 indicates population growth), N is population number at time t and time 0.

Equation 5: Geometric population growth in seasonally breeding populations (non-overlapping generations e.g. discrete annual cohorts)

$N_t = N_0 \lambda^t$

Where λ = finite population growth rate (λ>1 indicates population growth). λ is equivalent to net reproductive rate (R_0) and, if generation time is 1 yr, λ approximates to e^r (in Equation 4).

[12] Gene flow due to immigration/emigration can be simulated by adjusting age-specific birth and death rates. However, predictions of minimum viable population size usually assume populations are closed (zero gene flow).

Since N_t and N_0 cannot be measured reliably in the field, "r" is normally calculated by solving Equation 5 iteratively as follows:

Equation 6: Estimation of geometric population growth (Lotka, 1925)

$\sum (e^{-rx} l_x m_x) = 1$

Where l_x= mean survivorship (probability of survival to age/stage x), m_x = mean fecundity (no. of viable eggs per age/stage x). $l_x m_x = R_0 = \lambda$. NB For exponential population growth the products of each generation are integrated rather than summed.

Equation 7: Logistic population growth in populations with a ceiling carrying capacity (K)

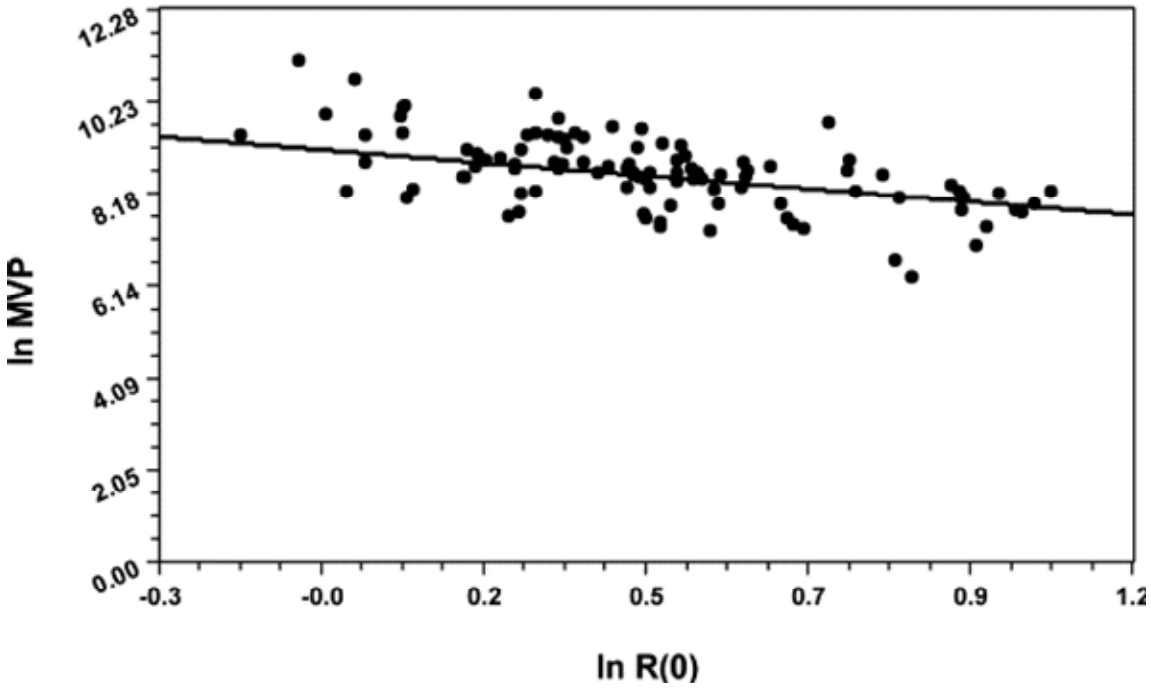
$\frac{dN}{dt} = r N \left[1 - \frac{N}{K}\right]$ i.e. population size limit at which dN/dt = 0

Table 2: Applicability of the PVA model Vortex compared to alternative models

***Vortex* is less appropriate**	***Vortex* is more appropriate**
High fecundity	Low fecundity
Short lifespan	Long lifespan
Polyploid	Diploid
Genetic effects of little interest	Changes in genetic variation of interest
Local population (*N*) > 500	Local population (*N*) < 500
> 20 populations modelled	< 20 populations modelled
Demographic rates not estimable	Age-specific fecundity & survival rates estimable
Stage- or size-dependent demography	Age-dependent fecundity and survival rates
Demographic rate fluctuations not estimable	Fluctuations in rates can be estimated
No catastrophic events of interest	Catastrophic events modelled
Only polygamous breeding	Polygamous or monogamous breeding
Random breeding	Random or non-random distribution of fecundity
Population starts at stable age distribution	Starting population not at stable age distribution
Constant sex ratio	Unequal sex ratio
No trends in habitat expected	Trends projected in habitat quality or area
No manipulation of animal numbers	Managed removal, supplementation, translocation
Fish, amphibian, invertebrate, or plant	Bird, mammal, or reptile

Attributes applicable to modelling the combined impacts of genetic inbreeding and reproductive fitness effects of environmental stressors on wild fish populations. Large, highly fecund, stage-structured populations (fish, amphibians, invertebrates, plants) can be modelled in Vortex© (Lacy et al., 2005).

Figure 5: Minimum viable population size (ln MVP) for vertebrates (breeding adults) required for 99% probability of persistence for 40 generations at a given population growth rate per generation (ln R_0)



Regression coefficient $r^2 = 0.351$, $P<0.0001$.

Notes: (Reed et al., 2003b)

lnR_0 is population growth rate per generation (NB: lnR_0/t equates to per capita population growth rate, where t is generation time).

MVP_A was predicted using Vortex© (Table 1) assuming that: i) populations were closed (no immigration or emigration); ii) carrying capacity was constant (no habitat gain or loss); iii) density dependent population growth was limiting at high population densities (approaching carrying capacity), with no compensation and no "Allee"/depensatory effects at low population densities); iv) inbreeding depression amounted to 5 lethal equivalents per diploid genome affecting juvenile survival in the first year.

1.6 Environmental chemicals of concern – endocrine disrupting chemicals

Reproduction is critical to the maintenance of populations, both in terms of numbers (demographics) and genetic variation. Reproduction and reproductive development are mediated by steroid hormones. Numerous endocrine disrupting chemicals (EDCs), discharged into the environment have been shown to mimic steroid hormones and reduce N_e in wildlife populations (reviewed in Goodhead and Tyler 2008; Vos et al. 2000). One of the first examples documented was the impact of the antifouling agent tri-butyl tin on the dog whelk (*Nucella lapillus*) leading to "imposex" (the growth of male genitalia in females) impeding or completely blocking their reproductive tract and causing local population extinctions in busy harbour areas (Bryan et al. 1988). Similar population impacts have been observed in other species' populations following their exposure to other EDCs: DDT in Western gulls (Fry and Toone, 1981), PCBs in common seals (Reijnders, 1986), dicofol and DDT in alligators (Guillette et al., 1994), mercury, DDE, PCBs in Florida panthers (Facemire et al., 1995), E1, E2, EE2 in fish (Jobling et al., 1998). In each case, adverse ecological effects were first observed in the field, rather than in the laboratory. Therefore in each case, the EDCs evaded traditional ERA.

Predicting the sublethal impacts of EDCs on vertebrate populations, including fish, has become a possibility, because in the past 10-15 years, there have been major advances in knowledge regarding reproductive ecotoxicology and endocrine disruption (Goodhead and Tyler 2008; Vos et al. 2000). ERA testing guidelines recognise the peculiarities of EDCs (Table 3) and unlike other classes of chemicals, they require assessment of their potential fate and effects, irrespective of quantities released into the environment (EMA, 2006; EC, 2009; ECHA, 2011). Endocrine disruption is an effect rather than a mode of action. The basic definition of an EDC is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function (EC, 1996).

Table 3: ERA challenges presented by endocrine disrupting chemicals

Challenge	Description
Potency	EDCs are highly potent and can elicit adverse developmental, reproductive, or behavioural effects at very low environmental concentrations.
Unusual dose-response	Effects may follow a non monotonic dose response, making them difficult to detect in typical laboratory range-finding studies — low exposure concentrations may elicit an entirely different response to that observed at high concentrations.
Multiple effects	The consequences of endocrine disruption may be multi-faceted (e.g., alterations in sexual maturation, sexual differentiation (intersex), sex reversal, sex ratio, male and female fertility, mating behaviour.
Cumulative effects	Pre-exposure of embryos may infer greater resistance or conversely greater sensitivity to continued exposure of juvenile fish. The degree of intersex tends to increase with the age of an individual, which may be related to susceptibility, as well as exposure duration.
Trans-generational effects	Endocrine effects may be passed on to future generations via the accumulation of some EDCs in the egg yolk, effects on the germ-line and/or epigenetic changes.

Adapted from Brown et al. (2009)

EDCs can alter normal hormone functioning via several mechanisms in a range of phyla, including: (i) binding with hormone receptors and acting as receptor agonists or antagonists (Andersen et al., 2006; Kelce et al., 1995); (ii) altering hormone receptor expression, amplifying or reducing the effects of endogenous hormones (Chen et al., 2008); (iii) interfering with the biosynthesis (Afonso et al., 1999; Ankley et al., 2007), metabolism and transport (Guengerich, 1999; Hegelund et al., 2004) of endogenous hormones. In fish, sex determination and differentiation are processes that have been shown to be particularly susceptible to endocrine disruption (reviewed in Delvin and Nagahama, 2002; Orban et al., 2009). Exposures to various synthetic and natural oestrogens from sewage effluent discharges have been associated with significant impacts on male reproductive development (i.e. feminisation) and function in wild fish populations (Jobling et al., 2002; Kidd et al., 2007; Länge et al., 2009; Harris et al., 2011). Anti-androgens have also been implicated in the

feminisation of males in freshwater fish populations in the UK (Jobling et al., 2009). As yet there is no evidence linking these observations to significant fish population declines or localised extinctions, but sewage effluent exposure and feminisation have been correlated with genetic impoverishment in roach (*Rutilus rutilus*) populations, potentially threatening their long-term viability (Hamilton et al., 2013).

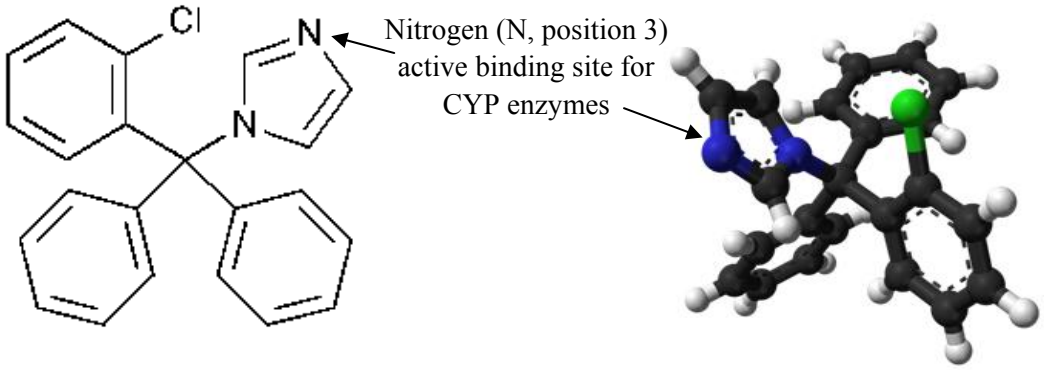
Other EDCs including synthetic androgens, anti-oestrogens (Ankley et al., 1998) and aromatase inhibitors (Ankley et al., 2002; Uchida et al., 2004) capable of impairing reproduction, lowering fecundity and crucially inducing male-biased sex ratios in wildlife populations could potentially have a more rapid and dramatic impact on population numbers. This is because reproduction generally involves far fewer eggs than sperm (Helfman et al. 1997; Warner 1997), so the loss of females is more limiting than the loss of males. Indeed only one male is required for all females to have a finite chance to breed, but the reverse is not true (Miller and Lacy, 2005). Furthermore male-biased sex ratios are often induced by environmental stress. In fish populations this may result from stunted growth and growth-related sex allocation favouring males (Parker, 1992), which may be mediated by the endocrine system and feedback between the synthesis of growth hormones and sex steroids (Holloway and Leatherland, 1998) and the stress hormone cortisol (Knapp et al., 2011). Environmental changes accompanying projected climate change, including increased temperature, dissolved oxygen stress, reduced nutrition and overcrowding, capable of promoting male-biased sex ratios in fish (Section 1.7.2), could act in combination with masculinising EDCs. Laboratory studies on sex determination in zebrafish in response to dietary exposure to the aromatase inhibitor fadrozole (≥10 μg fadrozole g^{-1} food) and temperature elevation (≥35°C) point towards some common mechanisms underlying male development (Uchida et al., 2004). These include apoptosis of primordial germ cells (Baroiller et al., 2009), apoptosis of immature (presumptive) oocytes and localised inhibition of estrogen production via gonadal CYP 450 aromatase (Uchida et al., 2004).

1.6.1 Clotrimazole as a model EDC (aromatase inhibitor)

Clotrimazole (Figure 6), CAS no. 23593-75-1, belongs to the diverse family of imidazole fungicides (Table 4). It is used globally in veterinary and human medicine for the treatment of fungal infections and oestrogen responsive cancers. Approximately 10 tonnes of the compound is produced in the EU annually (OSPAR, 2005). Clotrimazole is persistent with an environmental half-life of >60 days, bio-accumulative (bio-concentration factor of 610), and toxic to aquatic life (NOEC = 10 μg l^{-1} for 21 day *Daphnia magna* reproduction test) (OSPAR, 2005). This leads to a borderline 'PBT' classification for clotrimazole according to the EU Technical Guidance Document for environmental risk assessment and its inclusion in the Oslo-Paris Commission Priority Hazardous Substance list (OSPAR, 2005; OSPAR, 2009). Exposure data are limited for sewage treatment plants and receiving surface waters (OSPAR, 2009). However, recent research results along the Swedish West coast suggest that the low measured environmental concentrations of clotrimazole were sufficient to highlight risks of disturbance of growth and reproduction of microalgae, the basis of the ocean's food chain (Porsbring et al., 2009). Despite low solubility (490 μg l^{-1} in freshwater at 25°C), clotrimazole has a predicted environmental concentration (PEC_{local}) of 0.2 μg l^{-1} versus a predicted no effect concentration (PNEC) 1 μg l^{-1} (OSPAR, 2005).

Figure 6: Clotrimazole molecular structure and active site

2D structure 3D structure



The antifungal action of imidazole compounds involves the competitive binding of the N3 nitrogen of the imidazole ring (Figure 6) to the haeme moiety of cytochrome P-450 (CYP51) enzyme lanosterol 14-α-demethlyase, effectively blocking the enzyme and preventing the conversion of its substrate lanosterol to ergosterol, an essential component of fungal cell walls (Rodrigues et al., 1987). This enzyme is highly conserved in a wide range of phyla and plays a key role in cholesterol biosynthesis (Debeljak et al., 2003) and in the production of meiosis-activating sterols (Zarn et al., 2003).

1.6.2 Effects of clotrimazole on sexual differentiation and development in vertebrates

Imidazole compounds, including clotrimazole, are potent ligands of the haeme iron atom in numerous P-450 enzymes other than CYP51 (Figure 7) (Zhang et al., 2002). In particular clotrimazole is a potent inhibitor of aromatase (CYP19) (Trosken et al., 2004; Hinfray et al., 2006; Table 4), which catalyses, among other reactions, the demethylation of the methyl carbon 19 of androstenedione and testosterone to produce estrone and estradiol respectively in mammals (Conley and Hinshelwood, 2001) and other vertebrates including fish (Noaksson et al. 2003; Hinfray et al. 2006). Aromatase inhibition is the basis for tumour chemotherapy involved in breast cancer treatment (Trosken et al., 2004). Additionally clotrimazole is effective at nanomolar concentrations at treating various tumor types by: i) blocking cell signaling via calmodulin dependent Ca^{2+}-activated potassium (IK) channels; ii) preventing cell proliferation by depleting intracellular Ca^{2+} stores, reducing glycolytic flux and adenosine tri-phosphate (ATP) synthesis; iii) inducing apoptosis (Ito, 2002; Meira et al, 2005; Zancan et al., 2007). Whilst IK channel blockers including clotrimazole appear to be protective against several forms of apoptosis in a range of "normal" cell types (reviewed in Yu, 2003), including mammalian oocytes and granulosa cells (Perez et al., 2000), there may be exceptions. For example, porcine granulosa cells have been shown to undergo apoptosis following exposure to the broad spectrum IK channel antagonist clofilium, decreasing cell number and potentially affecting oocyte maturation (Manikkam et al., 2002). Similarly clotrimazole induces wild-type p53-mediated apoptosis in gliomas (tumors in glial cells in the brain) both *in vitro* and *in vivo* rat studies (Khalid et al., 2005).

Figure 7: – Inhibitory action of clotrimazole ⊘ on steroidogenic pathways in gonadal cells (Leydig cells in males and granulosa/theca cells in females)

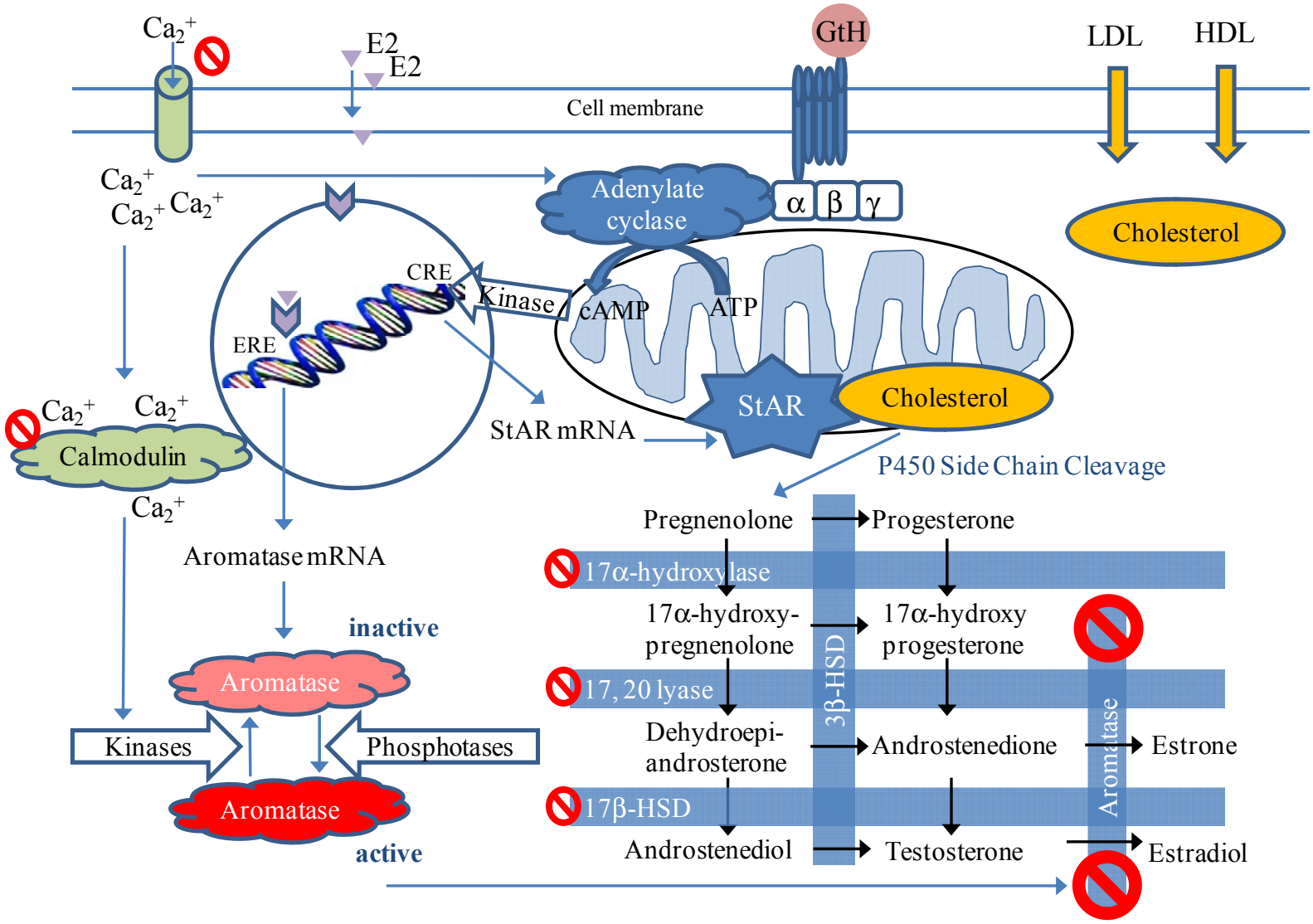


Table 4: Potency of imidazole versus triazole fungicides for inhibiting aromatase activity

Rank	Compound	Inhibitory Concentration IC50 (µM)		
		Human CYP19 enzyme	Trout brain microsomes	Trout ovary microsomes
1	Fadrozole	0.0045*		
2	Bifonazole	0.24		
3	Prochloraz	0.44	1.3	1
4	**Clotrimazole**	**1.2**	**0.011**	**0.016**
5	Imazalil	3.6	0.43	0.32
6	Flusilazole	7.7		
7	Miconazole	8.2		
8	Penconazole	47		
9	Myclobutanil	47		
10	Hexaconazole	96		
11	Propiconazole	199	0.9	0.9
12	Ketoconazole	281		
13	Triadimefon	483		
14	Tebuconazole	609		
15	Triadimenol	972	11	26
16	Bitertanol	>300		
17	Fluconazole	>300		
18	Cyproconazole	≈100		
19	Itraconazole	≈100		
20	Difenoconazole		70	29
21	Fenarimol (pyrimidine)		6	18
22	Fenbuconazole		1.3	0.21
	References	Trösken et al., 2004	Hinfray et al., 2006	

Key: Imidazoles in blue, Triazoles in red, Pyrimidine in black

Compounds ranked for human CYP19.

N.B. Inhibition in fish appears to be greater for clotrimazole than indicated by human CYP19 data

* Inhibitory concentration in rat ovary (Browne et al., 1991)

1.6.3 Effects of clotrimazole on xenobiotic metabolism

Hepatic cytochrome P-450 enzymes (P-450s1) constitute a superfamily of haemoproteins that play a major role in the metabolism of endogenous compounds and in the detoxification of xenobiotic molecules. CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 are the most important forms in humans, mediating the metabolism of about 70% of therapeutic drugs and endogenous compounds (Shimada et al., 1994). Imidazole compounds including clotrimazole can bind and inhibit each of these CYP enzymes. In particular clotrimazole is a potent inhibitor of CYP3A4 in human hepatic microsomes (Zhang et al., 2002), an enzyme accounting for nearly half of the total abundance of microsomal CYP in the human liver (Evans et al., 1999) and thought to be solely responsible for metabolism of 50% of prescription drugs (Xiao et al., 2011). Clotrimazole is also a potent binder of the pregnane X-receptor (PXR) (Xiao et al., 2011), a promiscuous nuclear receptor which serves as a generalised sensor of hydrophobic toxins and which is primarily responsible for the induction of genes from the cytochrome P-450 3A family (Moore and Kliewer, 2000). The binding of clotrimazole to CYP3A4 and PXR in the liver has major consequences for human drug metabolism. In turn this may potentially lead to adverse drug–drug interactions (Zhang et al., 2002; Xiao et al., 2011) and increase susceptibility to other environmental influences/stressors. Clotrimazole has been shown to activate the ligand-binding domain of PXR, involved in CYP3A signalling, in zebrafish *in vitro* hepatocyte cultures (Kliewer et al., 2002).

1.7 Gaps in ERA and ecotoxicity testing guidelines and opportunities for research

Recent scientific progress and technological advancements in understanding the effects of environmental stressors on biological systems have highlighted a number of areas in which ecotoxicology and ERA guidelines could be updated and improved (EC, 2012). For example there is considerable scope for *in silico* (computational) and *in vitro* (cell culture) models. However it is recognised that targeted *in vivo* testing is still required for example for EDCs

(Section 1.6). Currently data used to estimate the likelihood of adverse ecological effects typically include responses of survival, growth, or reproduction in usually highly inbred individuals (Flint and Mackay, 2009) measured under constant and typically favourable laboratory conditions (Forbes et al., 2008). These organism-level endpoints are far removed from the ecological features that ERA aims to protect, i.e. the long-term persistence of populations of species in space and time under naturally varying field conditions and in the presence of other stressors (Forbes et al., 2008). More consideration is being given to identifying key environmental exposure pathways for chemicals (including mixtures) in order to focus effects testing on realistic exposure scenarios and relevant species and trophic levels (EC, 2012). Similar attention should be paid to intra-species variation, in particular the genetic relevance and consistency of individuals and populations being used in effects testing. Existing test guidelines may require the stipulation of the genetic strain being used and the supplier, or in the case of a wild strain its provenance. However, detailed pedigrees or genetic screening to verify the consistent maintenance/outbreeding of strains and to confirm their genetic diversity (c.f. wild populations) are not currently required.

A critical review of the available scientific literature (Chapter 2) identified several studies showing that inbreeding can lead to significant differences between laboratory strains of invertebrate ecotoxicology model species (with outbreeding mating systems) in terms of their sensitivity to chemical and physical stressors: cadmium effects on midges *Chironomous riparius* (Nowak et al., 2007a; Nowak et al., 2007b); temperature effects on midges (Vogt et al., 2007); insecticide effects on pond snails *Lymnaea stagnalis* (Coutellec and Lagadic, 2006) (also see review by Armbruster and Reed, 2005). However, apart from some studies on the implications of inbreeding in fish husbandry, e.g. trout *Oncorhynchus mykiss* (Kincaid, 1983), studies concerning genetic variability versus inbreeding in vertebrate ecotoxicology models are limited. The lack of studies on inbreeding×chemical interactions in fish may be due in part to practical limitations presented by their longer generation times compared to many invertebrates. Until now no such studies have been published on zebrafish, despite the many potential benefits of this animal model, including an in depth understanding of its genetics and life history (Section 1.7.1).

1.7.1 The zebrafish as a laboratory animal model

The zebrafish is one of the most important vertebrate model organisms used in genetics and developmental biology research (reviewed by Grunwald and Eisen, 2002), with wide ranging commercial applications in human disease modelling (Rubinstein, 2003; Amsterdam and Hopkins, 2006), human toxicology (Spitzbergen and Kent, 2003; Rubinstein, 2006) and ecotoxicology (Scholtz et al., 2008). The complete diploid genome "Zv9" for the Tübingen strain (Sanger Institute, 2010) is contained within 25 chromosomes and comprises 1,412.47 mega bases and 28,700 genes, of which 27,404 encode proteins. Despite an additional ancestral genome duplication event (R3) in fish (Meyer and Van de Peer, 2005) this is approximately half the size of the human diploid genome GRCh37.p11 (Genome Reference Consortium, 2012), which contains 3,095.69 mega bases, 37,150 genes, 33,868 protein encoding genes distributed amongst 24 chromosomes. Nevertheless, zebrafish share 86% orthology with humans for over 1300 genes constituting medicinal drug targets (Figure 3), which is less than mammalian models such as the mouse (*Mus musculus* 97%), but comparable with avian models like the chicken (*Gallus gallus* 85%). A virtual comparative map identifying highly conserved syntenic[13] regions across the zebrafish and mammalian model genomes is now available (http://www.apps.niehs.nih.gov/centres/public/res-core/ctr740-2933.htm). In addition the zebrafish has several practical advantages over these other vertebrate models (Table 5).

Laboratory zebrafish also appear to be substantially more genetically diverse than mammalian models with an estimated 425,000 protein encoding SNPs represented in seven common inbred laboratory strains (Table 6). This is four times greater than in outbred rat strains and higher than in any vertebrate genome sequenced to date (other than the human genome) (Guryev et al. 2006). Although this may be a disadvantage in terms of increasing experimental variation in treatment response, requiring greater levels of replication and animal use in human toxicology (NAS, 2007), the relevance of genetic variation to ecotoxicology is inescapable (see Chapter 2) and the same can be argued for human health assessment.

[13] Synteny is the preserved co-localization of genes on chromosomes of different species

Table 5: Advantages of the zebrafish compared to some other vertebrate models

Attributes	Advantages
Small size (adults are generally <40 mm standard length, excluding tail fin).	Large numbers can be maintained in the laboratory for replication in studies and maintaining genetic diversity in brood stock.
Generation time is relatively short (3-4 months).	Life-cycle or multi-generation studies are feasible in the laboratory and semi-field mesocosms.
Year-round breeding (females spawn every 2-3 days and a single clutch can contain several hundred eggs).	Possible to generate organisms from the same parental gene pool for consistent high through-put testing.
Fertilisation is external.	Live embryos are accessible to manipulation including genetic manipulation via microinjection.
Eggs are relatively large (≈0.7 mm diameter) and optically transparent.	
Embryo development is rapid with precursors to all major organs developing within 36 hours of fertlisation.	Embryogenesis & organogenesis can be observed using visible light microscopy.
Larvae are optically transparent up to 10 mm in total length, 30 days post fertilization (dpf).	Developmental and behavioural toxicological endpoints can be studied using light microscopy and imaging techniques.
Larvae rapidly display food seeking and active avoidance behaviours within 5 dpf.	Some tests e.g. behavioural tests and fluorescent reporters (in transgenic strains) are amenable to automation and miniaturization in micro-well plates.
Work on embryos and larvae up to 5 dpf does not require licensing under the Animals in Scientific Procedures Act (ASPA, 1986, UK).	*In vivo* research work can be performed in unlicensed laboratories and universities etc.
Numerous wild-type and transgenic strains are available and can be sourced from wholesalers (pet shops) or recognised laboratory suppliers.	Wild-type strains are more representative of wild populations, whereas inbred and transgenic strains offer sensitivity and specificity for the detection of adverse chemical effects and biomarker responses.
Genome is fully sequenced and annotation and confirmation of gene function is progressing rapidly.	Molecular basis of development, disease and quantitative traits can be studied…
Genome is highly similar to other vertebrates with orthologs for many key functional genes eg. human drug targets.	…and extrapolated to other species.
Ecological life history is well documented.	Population models can be constructed for the zebrafish enabling population-level risk assessment (Section 1.7.3)

Attribute information obtained from review by Spence et al. (2008) and www.zfin.org.

Table 6: Common laboratory zebrafish strains used in human health and environmental research

Name	Description
*AB *AB *AB	Derived originally from two lines purchased by George Streisinger from a pet shop in Albany, Oregon in the late 1970s.
AB/Tübingen	An 'official' line maintained as a cross but the term is also applied to crosses where the two parental lines are maintained separately.
C32	Derived from laboratory strains at the Zebrafish International Resource Centre in Oregon.
Cologne	Isolated at the Reugels/Campos-Ortega Laboratory, University of Cologne.
Darjeeling	Collected in Darjeeling in 1987. Highly polymorphic and fast swimming.
Ekkwill (EKW)	From Ekkwill breeders in Florida and maintained in Grunwald lab, University of Utah.
Hong Kong	Stock obtained from a Hong Kong fish dealer.
HK/AB	Hybrid of Hong Kong and AB wild-type lines
HK/Sing	Hybrid of Hong Kong and Singapore wild-type lines.
India	Stock obtained from expedition to Darjeeling (wild isolate).
Indonesia	Stock obtained from Indonesian fish dealer.
Nadia	Wild-caught about 40 miles east of Calcutta. Stock derived from initial breeding of approximately ten individuals.
Singapore	Stock obtained from Singapore fish dealer.
SJA	An inbred line of *AB isolated at the Stephen L. Johnson Lab, Washington University Medical School (at least 85% monomorphic).
SJD	Isolated at the Stephen L. Johnson Laboratory.
TL	Tübingen long fin. Homozygous for *leot1*-recessive mutation causing spotting and *lofdt2*-dominant mutation causing long fins.
TM1	Derived from zebrafish acquired from a pet store in 1986 and is now >30 generations removed from that point.
TU	Tübingen Wild-type short fins. Strain cleaned up to remove embryonic lethal mutations and used by Sanger for the zebrafish sequencing project.
WIK	Derived from wild catch in India and commonly used for genome mapping.

Strains sequenced by: Guryev et al., 2006 (n=2 individuals per strain); Whiteley et al., 2011 (n<20 individuals from distinct populations, N=16); Anderson et al., 2012 (n≥100 siblings from distinct families, N=1 or 2).

A comprehensive list of wild-type, inbred and genetically manipulated (mutant) strains can be found at www.zfin.org.

For example, initial assessments of variation within the human genome has revealed over 1.4 million protein encoding SNPs in a sample size of just 24 individuals representing different ethnicities across the world. This is equivalent to one SNP for every 2000 base pairs, of which each of us has approximately three billion (Sachidanandam et al., 2001). However, this is likely to be a gross underestimate of genetic variation within our species due to considerable immigration and emigration and consequently high levels of outbreeding between human populations (Levy et al., 2007). Assessment of genomic variation within animal models is somewhat lagging behind. Nevertheless the mapping of SNPs and/or restriction site associated (RAD-tag) markers in zebrafish is progressing via relatively fine scale sequencing ($n \geq 100$ siblings from N=1 or 2 distinct families) to examine inter-individual variations within laboratory strains (Anderson et al., 2012) and wild fish (Bhartiya et al., 2010), and broader scale sequencing ($n < 20$ individuals from N=16 distinct populations) to examine variations between lab strains and wild populations across the Indian sub-continent (Whiteley et al., 2011).

1.7.2 Reproduction and reproductive development in zebrafish

Laboratory studies have demonstrated that reproduction in zebrafish is sensitive to environmental stressors, including EDCs (Nash et al., 2004; Goodhead and Tyler, 2008). Timing and duration of exposure have been shown to greatly influence sensitivity of zebrafish to 3,4-dichloroaniline and lindane in the following order: life-cycle F_0-F_1 generation exposure > juvenile exposure > adult exposure (Ensenbach and Nagel, 1997). This suggests that reproduction incorporating the direct exposure of male and female gametes to these stressors during external fertilisation represents a particularly sensitive exposure window. This may be because the exposed gametes are susceptible to epigenetic changes induced directly by the environmental media surrounding them (Piferrer et al., 2012).

The process of sexual development in zebrafish is also highly plastic and sensitive to environmental factors (see reviews by Delvin and Nagahama, 2002; Ospina-Alvarez and Piferrer, 2008; Baroiller *et al.*, 2009). Initial sex determination is thought to be genetic, but as

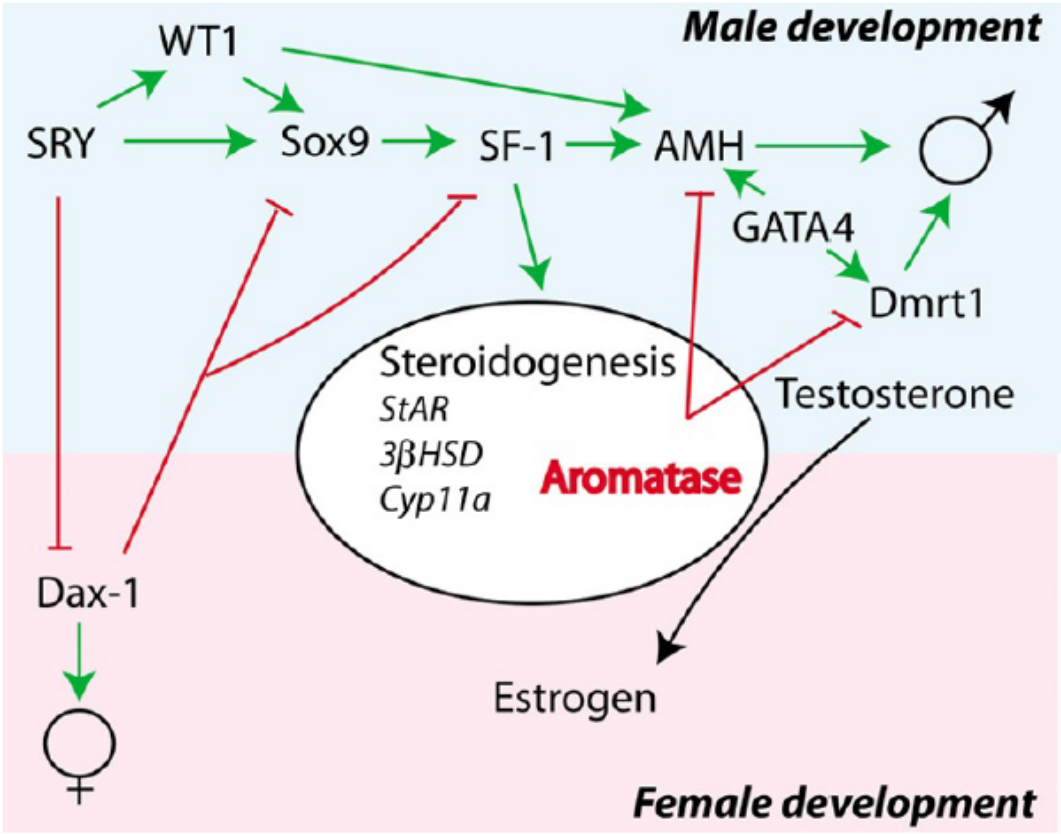
yet no definitive sex determining gene has been found. The probability of being male or female appears to be heritable with consistently different sex ratios being generated by different zebrafish strains/lines under consistent laboratory conditions (Drew et al., 2012; Liew et al., 2012). Furthermore biases may be reinforced within family lines by repeated selection for breeding pairs with predominantly male or female offspring (Liew et a., 2012). The lack of consistent 1:1 sex ratios in these laboratory studies and absence of a universal molecular sex marker indicate that there is no specific sex chromosome and no single sex determining gene in zebrafish (Liew et al., 2012). Instead sex determination appears to be poly-genic (von Hofsten and Olsson, 2005; Liew et al., 2012) and may be associated with different genes in different zebrafish strains (encompassing inbred laboratory and outbred wild strains) (Whitely et al., 2011; Anderson et al., 2012). Furthermore different genes may become more important under different environmental conditions (Anderson et al., 2012). Indeed genetic sex determination appears to be highly labile in the zebrafish and may be influenced by environmental factors such as temperature, as well as genetic factors (Ospina-Álvarez and Piferrer, 2008). Male-biased sex ratios are induced in zebrafish by: increased temperature (≥35°C between 5-48 hpf (Abzoaid, 2011) and between 17-27 dpf (Uchida et al., 2004)); reduced dissolved oxygen (0.8 mg O_2 l^{-1} (Shang et al., 2007)); reduced nutrition (50% of normal food ration (Lawrence et al., 2008)); overcrowding (starting density of 100 larvae per 1.5 litres (Liew et al., 2012)); inbreeding via 3 generations of selective full-sibling mating generated all male populations but no all female populations (Liew et al., 2012). Such environmental changes are likely to take place in response to climate change, increasing pollution and scarcity of freshwater (Vorosmarty et al., 2000; Confalonieri et al., 2007). Further study of these gene×environment interactions is required to aid our understanding of the evolution of sex determining mechanisms in fish (Pifferer et al., 2012). Although evidence is lacking in the zebrafish, the evolution of adaptive variation in sexual differentiation in another teleost fish, the Atlantic silverside (*Menidia menidia*), has been demonstrated in response to geographical variations in temperature in the Atlantic ocean (Conover and Heins, 1987). However, oceanic temperature gradients have in recent evolutionary time been relatively constant, whereas predicted variations in ground-level air (and shallow surface water) temperatures are considerable (Confalonieri et al., 2007),

providing little scope for directional selection. The amplitude of variations, as well as the rate of environmental temperature change will determine the success of evolutionary adaptation in fish, including the zebrafish (Ospina-Alvarez and Piferrer, 2008).

In contrast with the considerable uncertainty surrounding sex determination in fish, the genes ultimately involved in sexual differentiation are well known and appear to be highly conserved between species and between fish and other vertebrates (Pifferer et al., 2012). These genes may be broadly categorised as steroidogenic enzymes, sex steroid receptors, transcription factors and growth factors (van Hofsten and Olsson, 2005; Piferrer and Guiguen, 2008) (Table 7, Figure 8). Among these genes, *cyp19a1* plays a pivotal role (Guiguen et al., 2010) as the only steroidogenic enzyme responsible for the conversion of male hormones (androgens) to female hormones (estrogens) (Figure 7). This is key since zebrafish are juvenile hermaphrodytes. Their gonads are initially ovary-like and subsequent differentiation of testes versus ovaries is under hormonal control, making the species particularly sensitive to EDCs (Maack and Segner, 2004), including aromatase inhibitors. Two aromatase enzymes a and b are coded by *cyp19a1a* and *cyp19a1b* respectively[14], the former gene being highly expressed in the zebrafish ovary, while the latter is equally highly expressed in the adult zebrafish male and female brain (radial glial cells). Thus continual interactions between the brain and the gonad via the hypothalamus-pituitary-gonadal axis may permit sex change, potentially throughout the life of the zebrafish (reviewed by Le Page et al., 2010). The *cyp19a1a* gene locus (NC_007129.5) is located on chromosome 18 between base pairs 38057823 to 38073559, while the *cyp19a1b* locus (NC_007136.5) is located on chromosome 25 between base pairs 4903097 to 4916748 (Zv9, Sanger Institute, 2010). With respect to steroidogenic pathway regulation, the 5'-flanking region (regulatory/promoter region) for *cyp19a1a* has a steroidogenic factor-1 (SF-1) binding site, while the 5'-flanking region of the *cyp19a1b* gene has two estrogen response elements (EREs), an ERE half-site (ERE1/2) and a GATA-2 gene neural specific enhancer. Flanking regions for both genes also possess multiple potential SRY/SOX binding sites (8 and 16 in *cyp19a1a* and *cyp19a1b*, respectively) (Callard et al., 2001; Tong and Chung, 2003) and NR5A (Suzawa and Ingraham, 2008).

[14] These genes have recently been renamed and were previously a) *cyp19a1* and b) *cyp19a2*

Figure 8: Interaction of genes involved in sexual differentiation in mammals and other vertebrates including fish



In sex chromosome (XY/XX) systems SRY is the key regulator of sex determination, its absence leads to activation of Dax1 and female development. Conversely, the presence of SRY results in a hierarchy of activation of genes leading to the development of testis. In this hierarchy SF-1 (FTZ-F1) is a key regulator of steroidogenesis and AMH, demonstrating its central role in sex determination and differentiation.

⊥: Inhibition, ↓: stimulation.

In autosomal, polygenic systems SRY is missing and sexual differentiation relys onnumerous other genes, illustrated above and listed in Table 7, interacting with other and the environment.

The auto-(feedback) regulation of *cyp19a1b* expression has been demonstrated in the zebrafish brain by 17β-estradiol mediated by EREs (Menuet et al., 2005; Tong et al., 2009). Additionally three putative cyclic-Andenosine Mono-Phoshate (cAMP) responsive elements (CREs) in the 5'-flanking region of *cyp19a1a* in zebrafish (Kazeto et al., 2001) offer the potential that transcription in ovary may be regulated by gonadotropins via cAMP, as in mammals (Figure 7). Similarly, the transcription factor *foxl2* has been shown to bind to the promoter region of gonadal *cyp19a1* in Tilapia (*Oreochromis niloticus*) activating expression, while inhibition of gonadal *cyp19a1* is promoted by *dmrt1* (Wang et al., 2010).

Table 7: Genes influencing sexual differentiation in fish including zebrafish

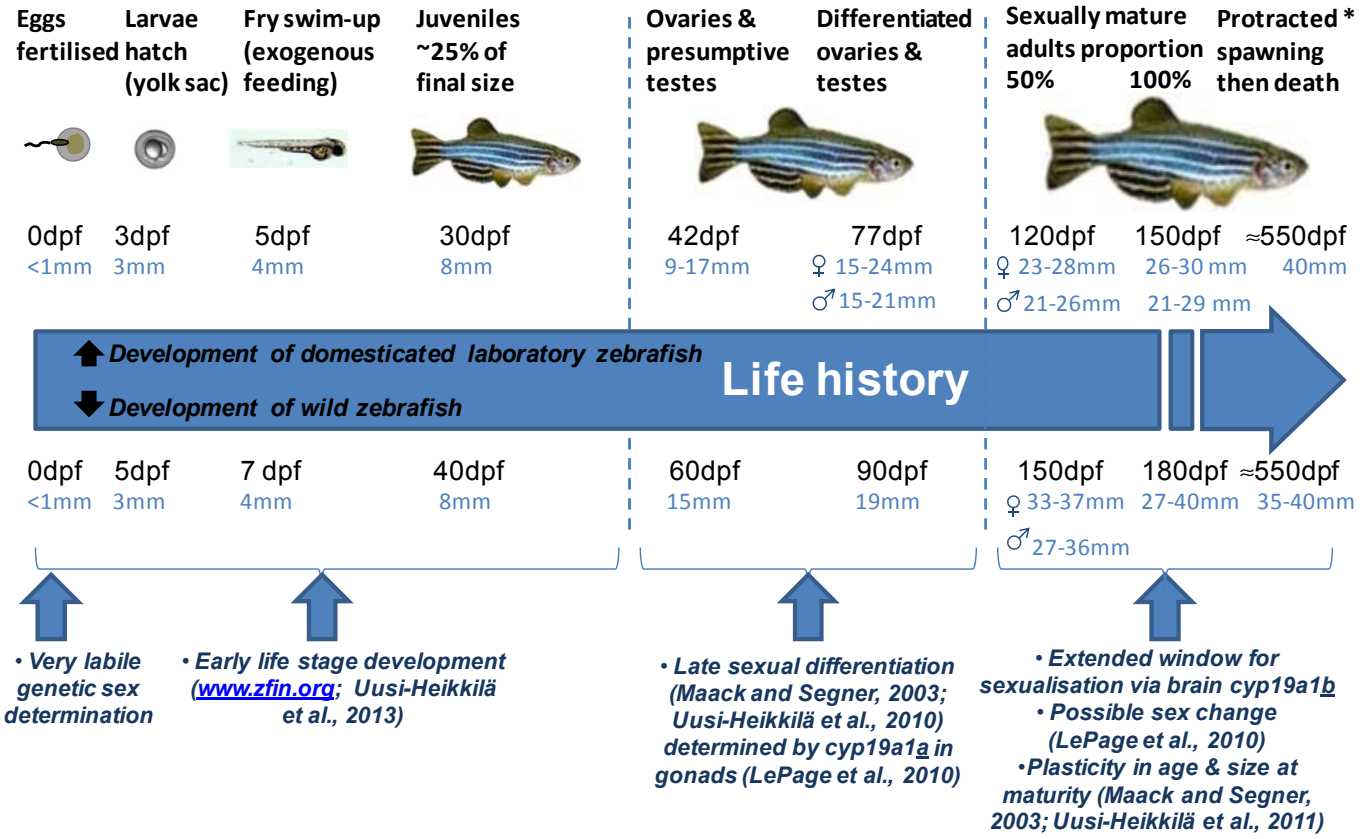
Gene	Description
Cytochrome P450 aromatase (*cyp19a1*)	Enzyme solely responsible for conversion of androgens to estrogens. Two isoforms present in zebrafish: *cyp19a1a* in the gonad and *cyp19a1b* in the brain (radial glial cells)[a]
Sry-related homeobox gene 9 (*sox9*)	Transcription factor activating *amh*. Sox9a expressed in zebrafish testis, sox9b expressed in ovary[b]
Anti-Müllerian hormone (*amh*)	Hormone (growth factor) involved in male gonad development – inhibits expression of *cyp19a1* in zebrafish[c]
Double-sex mab-related transcription factor (*dmrt1*)	Transcription factor inhibiting *cyp19a1* in Nile tilapia[d]
Steroidogenic factor 1 (*sf-1*) represented by four ortholgs: ff1a, b, c and d	Also known as Fushi Tarazu factor-1 (*ftz-f1*). Regulates inter-renal development and steroidogenesis in medaka[e] Binding sites in *cyp19a1* regulatory region in zebrafish[f] - potentially activated by cAMP signalling-response elements in this region[g]
GATA-4	Transcription factor regulating *sry* and *amh* in mammalian sexual development. Binding sites in *cyp19a1* regulatory region in zebrafish[f]
Forkhead transcription factor 2 (*foxl2*)	Up-regulates *cyp19a1* expression by binding to the *cyp19a1* promoter region, as well as interacting with Ad4 binding protein/*sf-1* in Nile tilapia[h]
Dosage-sensitive sex reversal gene (*dax-1*)	Nuclear receptor protein that suppresses *cyp19a1* expression in medaka ovarian follicles[i]
Wilms Tumour suppressor gene 1 (WT1)	Transcription factor required for steroidogenic inter-renal development[j] in combination with sf-1 (ff1b)[k] in zebrafish

Table 7 references: a) Menuet et al., 2005; b) Chiang et al., 2001; c) Rodriguez-Mari et al., 2005; d) Wang et al., 2010; e) Watanabe et al., 1999; f) Tong and Chung, 2003; g) Suzawa and Ingraham, 2008; h) Wang et al., 2007; i) Nakamoto et al., 2007; j) Drummond et al., 1998; k) Hsu et al., 2003.

Although azole aromatase inhibiting chemicals are potent ligands which bind to and inactivate aromatase enzymes (Section 1.6.1), it is important to note that they may also inhibit *cyp19a1* gene expression as shown by the effects of fadrozole on *cyp19a1a* in zebrafish gonads (Fenske and Segner, 2004) and *cyp19a1b* in fathead minnow (*Pimephales promelas*) brain (Ankley et al., 2002). The masculinizing effects of high temperature are also invariably mediated by inhibition of *cyp19a1* expression as well as enzymatic activity (Guiguen et al., 2010). Recent epigenetic studies in the European sea bass (*Dicentrarchus labrax*) have linked methylation of the *cyp19a1* promoter region with inhibition of aromatase in response to elevated temperatures, demonstrating the importance of this locus, and its methylation as a proximal causal link between environmental temperature and sex determination in this species (Navarro-Martín et al., 2011).

Under optimal laboratory conditions ontology of sexual differentiation of the zebrafish gonad (Figure 9) begins with the formation of presumptive ovaries at 28 dpf, with more defined ovaries forming between 35-42 dpf and initial formation of the male testes from 45 dpf (Maack and Segner, 2003). However, according to laboratory studies on a heterozygous WIK strain and a more homozygous inbred zebrafish strain, a small proportion of individuals (ranging in total length from 12-23 mm and 13-22 mm in the respective strains) may not sexually differentiate (transforming from ovary to testis) until 74-77 dpf (Maack et al., 2003). Therefore under constant environmental conditions (simulated in the laboratory) commitment to sexual differentiation appears to take place between 45-77 dpf. Indeed exposure of zebrafish to 500 μg fadrozole g^{-1} of food during this critical period of sexual differentiation (35-71 dpf) led to inhibition of *cyp19a1a* mRNA expression in the gonad, and testicular differentiation (Fenske and Segner, 2004). Furthermore gonadal masculinisation was found to be persistent for a further 90 days after the fadrozole exposure ceased. However, gonadal *cyp19a1a* mRNA expression in fadrozole-treated phenotypic male fish developed a dimorphic distribution indicating that physiological masculinisation was not persistent. Unfortunately, due to the absence of sex-linked markers in zebrafish it was not possible to demonstrate if those fish that returned to a female-like gonadal expression of *cyp19a1a* after the removal of fadrozole, were genetic females (Fenske and Segner, 2004).

Figure 9: Life history highlighting ontology of sexual development in domesticated and wild zebrafish



Notes:

Time measured in days post fertilisation. Length (mm) is total length measured from mouth to end of tail.

* Spawning continual in the laboratory (spawning interval lengthens with old age) but is seasonal in the wild (limited by water levels and food availability) (Spence et al., 2008).

↕Domesticated zebrafish (Maack and Segner, 2003) and wild F_1 zebrafish (Uusi-Heikkilä, 2012a) and wild F_3 zebrafish (Uusi-Heikkilä, 2012b) were raised under laboratory conditions (25°C, 12:12 hrs light:dark and 25°C, 14:10 hrs light:dark respectively).

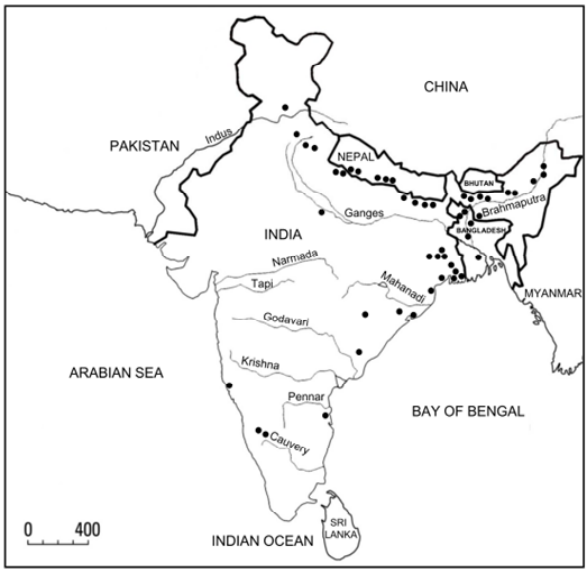
1.7.3 The ecology of the zebrafish and its use as a population model and environmental sentinel

The zebrafish (*Danio rerio*) belongs to the Cyprinidae, the largest family of freshwater fishes and the most species-rich of all vertebrate families (Nelson, 1994), containing 237 genera and 44 species within the *Danio* genus alone (Fang, 2001). Its ecological life history (see below) has been compiled from various field data (Spence et al., 2008) and supplemented by laboratory data, which provide greater detail on reproduction, early life stage survival, growth, metabolism, development and sexual differentiation (Section 1.7.2). Collectively these data have enabled detailed population models to be developed for the zebrafish (Table 1: Augustine, 2012; Hazlerigg et al., 2013).

Zebrafish are valuable environmental sentinels. They are relatively small (<40 mm) species with no commercial value (other than as an ornamental fish) and therefore subject to minimal (if any) fishing pressure. They are widely distributed across the fertile floodplains of the Indian subcontinent, from the foot of the Himalayas to the cape (Figure 10), preferring shallow, standing or slow flowing water bodies including ponds, lakes, channels and rice paddies with seasonal connections to main arterial rivers (McClure et al., 2006; Spence et al., 2006; Engeszer et al., 2007a). Consequently water temperatures vary considerably within the natural range of the zebrafish, from as low as 6°C in winter in the north to over 38°C in summer in the south. The main spawning season during the summer monsoon (June to September) coincides with extended day length and substantial rainfall (leading to a sudden fall in water temperature and a rise in water levels). However, gravid females have been found outside the monsoon season, indicating that reproduction may be cued more by food availability (Spence et al., 2006). Zebrafish are generally considered to be group spawners and egg scatters, displaying no parental care. Sexual dimorphism is minimal with males being marginally smaller than females (Pyron, 2003a) (Figure 9) and neither sex possessing obvious secondary sexual characteristics, other than yellowish colouration in sexually mature males and a small genital papilla in front of a more rounded anal fin, which is visible in adult females. Nevertheless, during spawning there is some evidence of intra-sexual competition i.e. female mate preference for unrelated and/or larger males (Uusi-Heikkilä, 2010; Pyron

2003b) and competition for optimal spawning substrate (reviewed in Spence et al., 2008). Daily spawning activity is limited to a short period at dawn (Spence et al., 2007a) and preferred sites consist of an aerated gravel spawning substrate and submerged vegetation that offers protection from predators (Engeszer et al., 2007b). There are no field data accurately quantifying reproduction in zebrafish. However, spawning intervals are likely to be longer and egg clutches smaller than mean measured laboratory values for 6, 12 and 15 month old fish: spawning interval = 1.5, 1.9 and 2.7 ± 2 days; clutch size = 158, 185, 195 ± 150 (Eaton and Farley, 1974b). Eggs are non-adhesive and demersal, lying on the substratum for 48-72 hours post fertilisation, whereupon the larvae hatch at a length of 3 mm. Growth rate of wild zebrafish is rapid during the first three months, reaching up to 19 mm (Uusi-Heikkilä et al., 2010), then gradually slows, total length reaching a mean of 25 mm after 12 months and a maximum of 35 mm after 18 months, after which growth is virtually zero (Spence et al., 2008; Uusi-Heikkilä et al., 2011) (Figure 9). Length frequency analysis reveals two distinct age classes during the summer spawning season, representing age 0+ juveniles and age 1+ adults, with few individuals surviving to age 2+ and a second spawning season (Spence et al., 2007b). Adult sex ratios in wild zebrafish populations appear to be 1:1 (Spence et al., 2007b). The species co-occurs with a number of competitor species including other *Danios* and predatory species. Adult zebrafish also predate zebrafish eggs (Engeszer et al., 2007b).

Figure 10: Distribution of wild zebrafish populations



Major river systems indicated. Black dots indicate recorded occurrences. (Spence et al., 2008)

1.8 Thesis objectives and experimental approaches

Chapter 2: Literature review

Effective breeding population size (N_e) is limited during the founding and maintenance of laboratory strains and consequently inbreeding and random genetic drift are likely to occur over time (Section 1.4). Without suitable intervention (including appropriate outbreeding – see Templeton, 1986; Lynch, 1991; Monson and Sadler, 2010), this may lead to significant differences between strains in terms of their fitness and sensitivity to toxicants (Chapter 2). In Chapter 2 a critical review of the available literature was undertaken on the considerations for ecotoxicology of genetic variation, inbreeding and chemical exposure and the findings identified the following research questions, which were subsequently addressed in the following experimental chapters:

1. "Are pass/fail criteria in current testing guidelines sufficient to identify genetic problems or inconsistencies in laboratory animals?"
2. "Are there differences in sensitivity between distinct strains?"
3. "Are inbred laboratory animals more sensitive than related outbred animals to environmental stressors, including novel chemical exposure and natural stressors?"
4. "Are inbred laboratory animals representative and/or protective of wild populations?"

Throughout the following experimental work, the zebrafish was used as the experimental model. The effects of genetic variation (between strains and with various levels of inbreeding within strains) were examined on sexual development, sex ratio and reproduction (population relevant endpoints). Inbreeding effects were also assessed in combination with chemical exposure (the aromatase inhibitor clotrimazole – Section 1.6.1) and a key environmental variable affecting sexual development and reproduction in fish (temperature – Section 1.7.2).

Chapter 3: Methods

Detailed methods are provided for molecular analysis, hormone analysis, histopathology and population viability analysis. Exposure methods are summarised in each experimental chapter.

Chapter 4: Differences in sexual development in inbred and outbred zebrafish (*Danio rerio*) with implications for chemical testing

This chapter examined sexual differentiation and development in different zebrafish strains and related family lines representing different levels of inbreeding under (control) laboratory conditions.

Hypotheses

I) Growth (body size) will be depressed and variability increased with increasing levels of inbreeding due to inbreeding depression and/or loss of heterosis (Charlesworth and Willis, 2009) and the unveiling of recessive characters i.e. additive genetic variance (Falconer and MacKay, 1996) respectively. **II)** In accordance with growth-related sex allocation (Parker, 1992), increasing levels of inbreeding will lead to impaired growth and increasingly male-biased sex ratios.

Experimental approach

Early life stage (0-63 dpf) survival, growth and sexual development (gonadal differentiation and development) were assessed in the absence of chemical exposure in an established, domesticated WIK strain and a WIK/wild hybrid strain with various levels of inbreeding (0, 1 and 2 consecutive generations of full-sibling mating). Experimental design, conditions and endpoints were based on a new OECD Fish Sexual Development Test Guideline 234 (OECD, 2011) (Figure 11). Results were related to test validity criteria for the stated endpoints, including sex ratios, in each of the strains. Variability in each of the endpoints was also examined and the implications for statistical sensitivity and experimental replication were considered.

Figure 11: Fish sexual development test design with breeding as the treatment

Time (dpf)	Endpoint	W/W$_{outbred}$	W/W$_{inbred}$	W/W$_{inbredx2}$	WIK
0	Sample embryos destructively for microsatellite analysis	N=11 microsatellites in an additional n=8 viable embryos			
0	Day 0, start with viable embryos (0-4 hpf)	N=4 families in separate aquaria, each with n=30 viable embryos			
0-5	Embryo hatch (%)				
5-28	Larval survival (%)				
29-63	Fry survival (%)				
63	Standard length (mm)				
63	Wet weight (mg)				
63	Fix tissue for histological assess –ment of gonadal sex & stage				

Test design was based on OECD fish sexual development test (OECD, 2011)
Breeding treatments:

- WIK is the domesticated "Wild Indian Karotype" strain
- W/W is WIK/Wild hybrid strain with different levels of inbreeding (0, 1, 2 consecutive generations of full-sibling mating) - all W/W fish were F_4 generation (following hybridisation at F_0)

Chapter 5: Are toxicological responses in laboratory (inbred) zebrafish representative of those in outbred (wild) populations? – case study with an endocrine disrupting chemical

This chapter examined the effects of exposure to the aromatase inhibitor clotrimazole on sexual differentiation and development in related inbred and outbred lines of a semi-wild (WIK/Wild) zebrafish strain.

Hypotheses

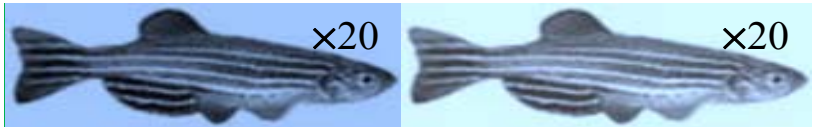
III) Inbred zebrafish will be more susceptible than outbred zebrafish to the effects of clotrimazole on steroidogenesis, sexual differentiation and development due to inbreeding depression and/or loss of heterosis. **IV)** Development will be skewed more towards males in exposed inbred treatments due to greater inhibition of steroidogenic enzymes and/or reduced capacity to compensate for this inhibition via up-regulation of key functional genes. **V)** Phenoypic variance will be greater in exposed inbred treatments due to the unveiling of recessive, conditionally expressed characters in homozygotes.

Experimental approach

The effects of clotrimazole exposure (nominally 0, 5, 50 μg l^{-1})[15] on growth and sexual development were assessed from adolescence to adulthood (40-136 dpf) in N=20 inbred and outbred family lines of a WIK/Wild hybrid strain resulting from 1 and 0 generations of full-sibling mating respectively. Gonadal (germ cell and interstitial cell) development and sex ratios were related to circulating levels of the male steroidal hormone 11-ketotestosterone and gonadal transcripts for key functional genes implicated in steroidogenesis (*cyp17a1, cyp19a1a, cyp51, hsd17b3*) and gonadal growth and development (*igf1*) (Figure 12). Variability in each of the endpoints was also examined and the implications for statistical sensitivity and experimental replication were considered.

[15] Low-level clotrimazole exposure (nominal concentration 5 μg l^{-1}; mean measured concentration 2.9 μg l^{-1}) compares with a predicted environmental concentration (PEC_{local}) of 0.2 μg l^{-1} (OSPAR, 2005).

Figure 12: Partial life-cycle test designed to assess the combined effects of inbreeding and exposure to clotrimazole



Time (dpf)	Endpoint	$W/W_{outbred}$			W/W_{inbred}		
40	Exposure start (µg clotrimazole l^{-1})	**0 µgl^{-1}**	**5 µgl^{-1}**	**50 µgl^{-1}**	**0 µgl^{-1}**	**5 µgl^{-1}**	**50 µgl^{-1}**
		N=20 families in separate aquaria, with n=8 fish					
40-136	Specific growth rate & condition based on std length, wet weight (40-47-52-66-88-136 dpf)	N=20 families, with n=3 fish sampled randomly					
88, 136	11-ketotestosterone in males (via radio-immunoassay of 1-2 µl plasma)	N≥10 families, with n=1-4 fish sampled depending on sex ratio					
136	Gonad weight (right gonad)	N≥10 families, with n=1-2 fish sampled depending on sex ratio					
88, 136	Gonad sex and sex ratio (left gonad sampled for histology)	N=20 families, with ALL n=8 fish sampled					
136	Gonad (germ cell) development stage	N≥10 families, with n=1-2 fish sampled depending on sex ratio					
136	Gonad transcripts for target genes (right testis only via qPCR)	N≥10 families, with n=1-2 fish sampled depending on sex ratio					

W/W signifies WIK/Wild hybrid strain, represented by inbred and outbred family lines produced by 1 and 0 generations of full-sibling mating - all W/W fish were F_3 generation (following hybridisation at F_0)

Chapter 6: Interactive effects of inbreeding and endocrine disruption on reproduction in a model laboratory fish

This chapter examined the effects of prior exposure to the aromatase inhibitor clotrimazole on reproductive fitness in related inbred and outbred lines of a WIK/Wild zebrafish strain.

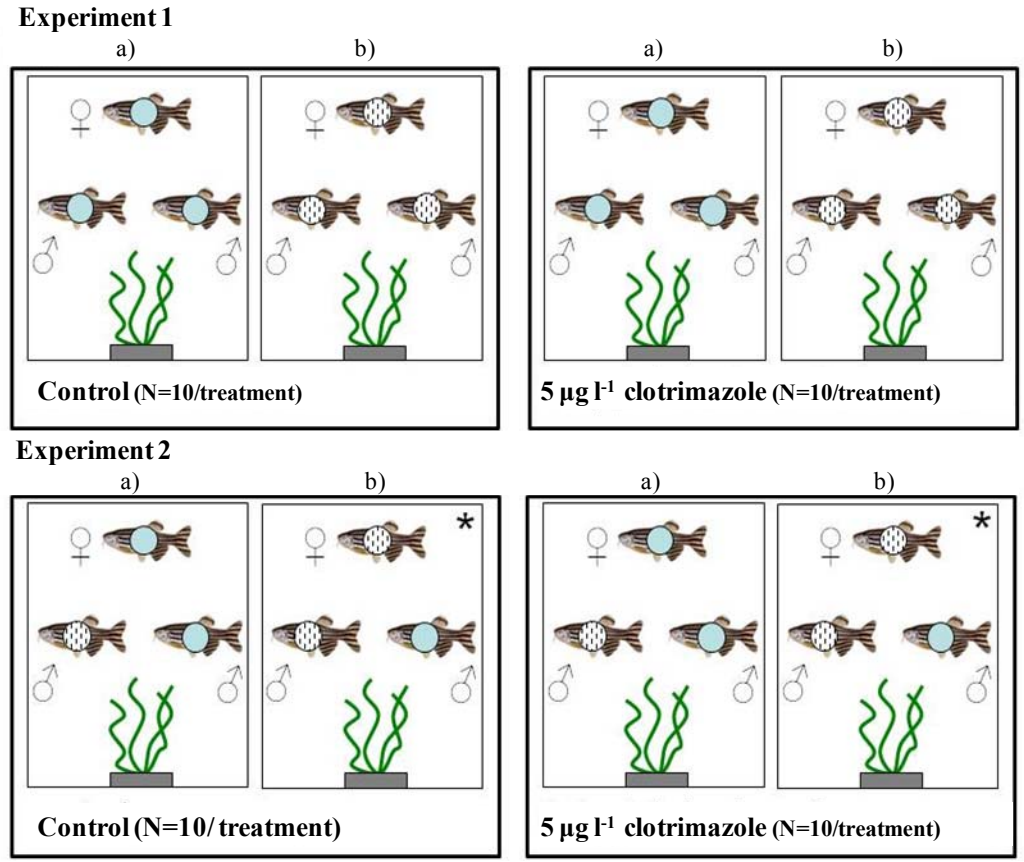
Hypotheses

VI) Prior exposure to clotrimazole will affect (reduce) reproductive output to a greater extent in inbred females compared to outbred females, due to inbreeding depression and/or loss of heterosis. **VII)** Inbred males will be less reproductively successful compared to outbred males. **VIII)** Male fitness components (paternity and plasma 11-ketotestosterone concentration, a marker for social dominance) will suffer greater inbreeding depression than female fitness (i.e. fecundity), as evidenced in other species and phyla (Armbruster & Reed, 2005; Bijlsma et al., 1999; Meagher et al., 2000; Keller and Waller, 2002).

Experimental approach

Adult fish remaining from the 0 (control) and 5 μg l^{-1} clotrimazole exposure treatments in the previous study (Chapter 5) were "grown out" in clean water. Effects of prior exposure (40-136 dpf) on adult male and female fitness were assessed in inbred and outbred fish from both the control and exposed treatments after being assigned to N=10 spawning triads, each containing two males and one female. Experiment 1 (163 – 172 dpf) was designed to assess comparative fitness 1a) between N=10 inbred families and 1b) between N=10 outbred families. Experiment 2 (173 – 182 dpf) was designed to assess comparative fitness between inbred and outbred males competing for either a) an unfamiliar inbred female (N=10) or b) an unfamiliar outbred female (N=10) (Figure 13). Measures of male fitness included paternity ratio and circulating levels of 11-ketotestosterone (reflecting reproductive success and social dominance), while female fitness was measured in terms of fecundity (eggs/triad/day) and egg viability. Fertilisation success was taken to be a product of male and female fitness.

Figure 13: Spawning triads used to assess reproductive fitness



Fish used in spawning triads were WIK/Wild hybrids "grown out" following the study on reproductive development (Chapter 6). **Experiment 1**: all fish in each spawning triad were either inbred ( ) or outbred ( ), and had either been previously maintained under control conditions or exposed to clotrimazole (5 µg l^{-1}); **Experiment 2**: spawning triads contained one female (either inbred or outbred) and two males (one inbred and one outbred), and all fish in each spawning group had either been previously maintained under control conditions or exposed to clotrimazole (5 µg l^{-1}). *denotes treatment used for subsequent parentage analysis.

Chapter 7: Skews in sex ratio induced by elevated temperature combined with an endocrine disrupting chemical are compounded by inbreeding in zebrafish

This chapter examined the effects of exposure to clotrimazole and/or elevated temperature on sexual differentiation and development in related inbred and outbred lines of a wild zebrafish strain.

Hypotheses

IX) Sex ratios will be increasingly skewed towards males with increasing concentrations of clotrimazole and with elevated temperature, and the effects of these environmental factors will be additive due to a common, principal mode of action (assumed to be aromatase inhibition). **X)** Inbred zebrafish will be more susceptible to masculinisation due to reduced growth rate, consequently skewing growth-related sex partitioning towards males and/or due to inbreeding depression or reduced heterosis (hybrid vigour and homeostatic capability), following reduced heterozygosity and allelic loss at the aromatase locus, a gene of "large effect" on phenotypic sexual differentiation (downstream of a suite of possible genes implicated in genetic sex determination).

Experimental approach

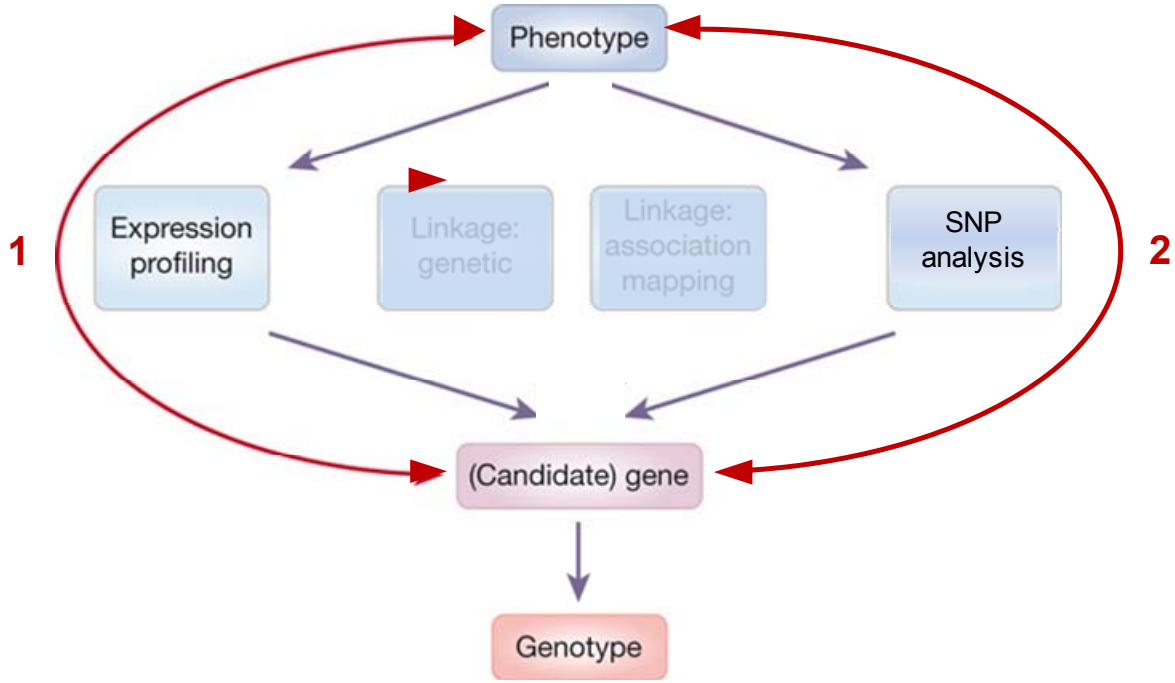
The combined effects of clotrimazole (nominally 0, 2, 10 μg l^{-1}) and temperature (28, 33°C) exposures on growth and sexual development were assessed from adolescence to adulthood (40-100 dpf) in N=20 inbred and outbred family lines of a wild Bangladesh strain resulting from 1 and 0 generations of full-sibling mating respectively (Figure 14). Sex ratios and gonadal (germ cell) development were related to a) expression levels of gonadal transcripts for aromatase (*cyp19a1a*) normalised to the house-keeping gene ribosomal protein l8 (*rpl8*) in phenotypic males and females and b) SNPs identified in cDNA products derived from the aromatase transcripts (Figure 15).

Figure 14: Partial life-cycle test designed to assess the combined effects of inbreeding, elevated temperature and exposure to the aromatase inhibitor clotrimazole

Time (dpf)	Endpoint	$Wild_{outbred}$ 28°C			$Wild_{inbred}$ 28°C			$Wild_{outbred}$ 33°C			$Wild_{inbred}$ 33°C		
40	Exposure start (µg clotrimazole l^{-1})	**0** $\mu g l^{-1}$		**8** $\mu g l^{-1}$	**0** $\mu g l^{-1}$		**8** $\mu g l^{-1}$	**0** $\mu g l^{-1}$	**1.7** $\mu g l^{-1}$	**8** $\mu g l^{-1}$	**0** $\mu g l^{-1}$	**1.7** $\mu g l^{-1}$	**8** $\mu g l^{-1}$
40-100	Specific growth rate based on std length, wet weight (equivalent time points in degree days underlined)	←	(40, 55, 70, 80, 100 dpf) →					←	(40, 54, 70, 74, 91, 100 dpf) →				
			N=20 families, each represented by n=4 fish (i.e. all fish in study)										
100	Gonad weight (right gonad)		↓			↓			↓			↓	
100	Gonad sex and sex ratio (left gonad sampled for histology)		↓			↓			↓			↓	
100	Gonad (germ cell) development stage		↓			↓			↓			↓	
100	Gonad transcripts for target genes (right gonad only via qPCR)		N≥6 families, each with n=1-2 female fish										
100	SNP analysis on aromatase gene (cDNA from liver tissue)		N≥6 families, each with n=1-2 male and female fish										

The wild zebrafish strain was represented by inbred and outbred family lines produced by 1 and 0 generations of full sibling mating - all fish were F_3 generation (following generation from wild great grandparents at F_0). Equivalent degree day periods underlined.

Figure 15: Steps taken to confirm candidate gene explaining variation in sex ratio (resistance of female phenotype) between treatments



Chapter 8: Population-level consequences of masculinisation in zebrafish induced by the combination of inbreeding chemical exposure and elevated temperature

In this chapter long-term predictions of the effects of clotrimazole and/or elevated temperature exposure on the viability of inbred and oubred zebrafish populations were compared.

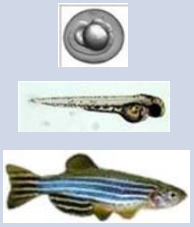
Hypotheses

XI) Inbred populations are more susceptible to the deterministic effects of clotrimazole exposure and elevated temperature, which act independently and additively to skew sex ratio increasingly towards males (Chapter 7), reducing effective population size and increasing extinction risk. **XII)** Genetic stochasticity (due to emergent expression of lethal/ deleterious alleles) will combine with demographic and environmental stochasticity (inter-individual and inter-annual variation in survival, sex ratio and fecundity) to further increase extinction risk.

Experimental approach

The individual-based population viability analysis model Vortex© (Lacy et al., 2005) was employed to predict population growth rate and extinction risk for inbred versus outbred wild zebrafish populations. Simulations were based on "control" life history data for wild zebrafish populations (Section 1.7.3; Hazlerigg et al., 2013) and laboratory study data (Chapter 7) quantifying the combined "effects" of clotrimazole exposure and temperature elevation on survival, sex ratio and fecundity in inbred and outbred zebrafish (Figure 16). Vortex© is a stochastic model incorporating demographic and environmental stochasticity in annual survival, sex ratio, fecundity and carrying capacity (Section 1.5). Genetic stochasticity i.e. inbreeding depression on juvenile survival (age 0+) was based on an estimated 5 lethal equivalent alleles per diploid genome in zebrafish (McCune et al., 2002; McCune et al., 2004). Populations were assumed to be small (100-200 adults) and "closed", and breeding was limited to the four month monsoon season in India. Consequently model simulations were conservative and were repeated 100 times, enabling probabilistic risk calculations and sensitivity analysis (to identify the most influential input parameters).

Figure 16: Parameterisation of the Vortex PVA model for inbred and outbred zebrafish populations

Year	Age group	Month	Life history stage	Individual fecundity ±SD	Sex ratio (%) ♀	Population number	Inbred annual survivor ship (%)	Outbred annual survivor ship (%)
x=1	0+	Jun Jul Aug Sept			Ceiling =	5000 ±1000 3840	4 ± 3	9 ± 3
x=2	1+	Jun Jul Aug Sept	x15 spawns x15 spawns x15 spawns x15 spawns	50 ± 20 50 ± 20 50 ± 20 50 ± 20	x 18.1 ± 1.8	x 90 = eggs	90 ± 10	90 ± 10
x=3	2+	Jun Jul Aug Sept	x15 spawns x15 spawns x15 spawns x15 spawns	50 ± 20 50 ± 20 50 ± 20 50 ± 20	x 18.1 ± 1.8	x 81 = eggs	0	0

1.9 References (Chapter 1)

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2 CHAPTER 2: LITERATURE REVIEW

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Review

Genetic variation, inbreeding and chemical exposure—combined effects in wildlife and critical considerations for ecotoxicology

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Exposure to environmental chemicals can have negative consequences for wildlife and even cause localized population extinctions. Resistance to chemical stress, however, can evolve and the mechanisms include desensitized target sites, reduced chemical uptake and increased metabolic detoxification and sequestration. Chemical resistance in wildlife populations can also arise independently of exposure and may be spread by gene flow between populations. Inbreeding—matings between closely related individuals—can have negative fitness consequences for natural populations, and there is evidence of inbreeding depression in many wildlife populations. In some cases, reduced fitness in inbred populations has been shown to be exacerbated under chemical stress. In chemical testing, both inbred and outbred laboratory animals are used and for human safety assessments, isogenic strains (virtual clones) of mice and rats are often employed that reduce response variation, the number of animals used and associated costs. In contrast, for environmental risk assessment, strains of animals are often used that have been selectively bred to maintain heterozygosity, with the assumption that they are better able to predict adverse effects in wild, genetically variable, animals. This may not necessarily be the case however, as one outbred strain may not be representative of another or of a wild population. In this paper, we critically discuss relationships between genetic variation, inbreeding and chemical effects with the intention of seeking to support more effective chemical testing for the protection of wildlife.

Keywords: chemicals; inbreeding; resistance; populations; wildlife

1. INTRODUCTION

Consistent with the fundamental population genetics theory (Falconer 1989), an increasing number of studies find that wildlife populations with low genetic variation appear less able to adapt to changes in environmental conditions, such as physical climate change, biological threats, including disease outbreaks (O'Brien & Evermann 1988; Lande & Shannon 1996; Frankham 2003), transient or variable exposure involving combinations of different physico-chemical stressors (Reed *et al.* 2002) or novel chemical exposure (Bijlsma *et al.* 1999; Kovatch *et al.* 2000; Van Straalen & Timmermans 2002; DeSalle & Amato 2004; Kristensen *et al.* 2003). Furthermore, species threatened with extinction tend to have lower levels of genetic variation than related non-threatened species (Spielman *et al.* 2004). Environmental change is a source of strong selection on wildlife, and the exposure of organisms to novel, man-made chemicals is a relatively new pressure. Wildlife may be exposed to multiple chemicals with the potential to cause acute as well as long-term chronic toxicity via various physiological routes (reviewed in Escher & Hermens 2002), some of which may induce characteristic phenotypic (biomarker) responses. At the molecular level, each response involves the modulation of gene expression, and this includes interactions within and between gene loci and the environment. It has been shown that sustained chemical exposure can select on wildlife populations and promote the evolution of resistant genotypes. Studies of wildlife populations exposed to contaminants such as heavy metals (reviewed in Klerks & Weis 1987), persistent organic pollutants Meyer & Di Giulio (2002, 2003) or both (reviewed in Guttman 1994) provide evidence of this. More rapid adaptation has also been observed in wildlife with the evolution of pesticide and antibiotic resistance (reviewed in Roush & McKenzie 1987; Futuyma 1998; Palumbi 2001).

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In considering how chemicals potentially affect evolutionary processes, some chemicals (genotoxins) can affect DNA integrity directly, leading to heritable changes. These genotoxic chemicals increase the frequency of DNA damage and thus increase the risk of replication and transcription of altered DNA sequences. The probability of DNA damage being converted into a permanent and/or heritable sequence alteration depends on the type of damage, the repair pathway recruited, the rate of repair and the fidelity and completeness of repair (Preston & Hoffman 2001). In some instances, mutagenic chemicals have been shown to increase genetic variation (albeit at neutral markers) (Chen *et al.* 2003; Berckmoes *et al.* 2005; Eeva *et al.* 2006), but in other cases, to reduce it. This reduction can have negative impacts on wildlife by increasing the frequency of deleterious mutations that are selected against, causing bottlenecks, impairing gene flow (reviewed in Van Straalen & Timmermans 2002) and, less dramatically, lead to cumulative erosion of reproductive fitness (reviewed in Bickham *et al.* 2000). Less obvious effects of chemicals on the genome are also possible but may be latent and may accumulate over generations. Some so-called endocrine-disrupting chemicals (EDCs), for example, can impose heritable reproductive effects via epigenetic processes affecting the germ line, without altering underlying gene sequences (Anway & Skinner 2006; Nilsson *et al.* 2008). Chemicals may also affect evolutionary processes indirectly by reducing effective (breeding) population size (Ne), with or without selection on adaptive traits and/or by reinforcing the reproductive isolation of exposed populations (Nacci & Hoffman 2008). Some EDCs have been shown to reduce effective breeding population size in wildlife populations (reviewed in Vos *et al.* 2000; Goodhead & Tyler 2008) and documented examples include population declines and local extinctions both in the dog whelk (*Nucella lapillus*) following tri-butyl tin exposure (Bryan *et al.* 1988) and in raptors owing to eggshell thinning resulting from the bioaccumulation of DDT and its metabolites (Ratcliffe 1967).

Populations are widely regarded as the minimum units for species conservation (UN Millennium Ecosystem Assessment and World Resources Institute 2005) and environmental protection (Barnthouse *et al.* 2008). It is generally accepted that a minimum Ne is required to maintain a genetically viable population (Frankham 1995*a*; Lynch & Lande 1998). Populations with low Ne contain low levels of genetic variation, lowering the chances for recombination and maintaining beneficial mutations (other than those under very strong selection) (Pasteur & Raymond 1996; Futuyma 1998). Other problems that can arise because of declining population size include increasing genetic drift where mildly deleterious alleles can reach appreciable frequencies (Lande 1994), and perhaps more significantly, the enhanced likelihood of inbreeding and inbreeding depression (Frankham 1995*b*; Amos & Balmford 2001; Brook *et al.* 2002). Ne is a key factor in considerations for conservation and environmental protection.

In this review, we evaluate the importance of genetic variation in wildlife populations for coping with environmental, including chemical, stressors and consider the implications for ecotoxicology. We focus on the importance of inbreeding as a factor affecting individual fitness and ultimately the viability of populations, and we assess potential interactions between inbreeding and chemical toxicity. Assessing the strength of these chemical–ecogenetic interactions is critical to the future refinement of the environmental risk assessment (ERA) process and for environmental realism in predicting the effects of chemicals in nature.

2. THE GENETIC BASIS FOR CHEMICAL RESISTANCE

There are examples in the wild of selective effects of chemicals on populations. Numerous field studies have shown historical metal pollution has imposed selection on exposed wildlife populations, with directional evolution in neutral marker loci along spatial and temporal gradients of exposure (Klerks & Weis 1987; Groenendijk *et al.* 2002; Klerks 2002; Peles *et al.* 2003). Increasing levels of metal contamination are often associated with reduced genetic variation at the population level. At the same time, there is also evidence that individuals that are more genetically diverse (greater heterozygosity and lower 'internal relatedness') within populations derived from exposure areas are more able to tolerate higher levels of exposure than those from less contaminated areas (Bourret *et al.* 2008). Tolerance to mercury exposure in mosquitofish (*Gambusia affinis*) has been related specifically to genetic polymorphism at the glucose isomerase locus, and the distribution of genotypes in natural and artificial contaminated environments were shown to be consistent with this (Heagler *et al.* 1993; Tatara *et al.* 1999).

(a) *Heritable variation and evolution of chemical resistance*

Adaptation to organic pollutants also implies heritable variation in resistance, and this has been demonstrated in fish populations exposed long term to some organic pollutants (e.g. polyaromatic hydrocarbons (PAHs) and polychlorinated bi-phenyls (PCBs); Atlantic killifish, *Fundulus heteroclitus*; Meyer & Di Giulio 2002; Nacci *et al.* 1999). However, PAH resistance has been associated with reduced fitness when individuals were challenged with other environmental stressors (Meyer & Di Giulio 2003). Other studies of natural populations resistant to specific toxicants have also shown associations between resistance to one chemical and heightened sensitivities to other stressors, or sometimes a general reduction in performance in the absence of contaminants (Carrière *et al.* 1994; Shaw 1999; Nacci & Hoffman 2008). A further experimental study on the sheepshead minnow (*Cyprinodon variegatus*) found heritability of chemical resistance was reduced with exposure to increasing numbers of chemical pollutants, suggesting that resistance to any one chemical may develop more slowly during exposures to complex chemical mixtures, as experienced by most wildlife in the natural environment (Klerks & Moreau 2001).

Exposure to more contemporary and emerging contaminants, including pesticides and antibiotics, has also been shown to lead to the evolution of chemical resistance. The resistance mechanisms in these cases are often highly conserved across phyla (bacteria, fungi, higher plants, insects and vertebrates), despite the fact that these chemicals have been developed to selectively target specific groups of animals, and include target site insensitivity, reduced chemical uptake and increased metabolic detoxification and sequestration (Eckert *et al.* 1986; Feyereisen 1995). In the case of synthetic chemical pesticides, resistance at the molecular level is conferred by point mutations in the ion-channel component of a GABA receptor subunit (for cyclodiene insecticides) or within the sodium-channel gene (for DDT and pyrethroid insecticides), via mutations in the region coding for the active site of acetylcholinesterase (organophosphorus and carbamate insecticide resistance), by mutations leading to the upregulation of detoxification enzymes such as cytochrome P450 and glutathione-*S*-transferases (for many classes of insecticides), or via amplification of esterase genes (for organophosphorus and carbamate insecticides) (Feyereisen 1995; Pasteur & Raymond 1996) (table 1).

Resistance to some chemicals evolves via mutations in a single gene (monogenic resistance). For example, cyclodiene resistance in insects is conferred by a single base pair substitution (ffrench-Constant *et al.* 2000). The finding that cyclodiene resistance in pest insects accounts for over 60 per cent of reported cases of insecticide resistance (Georghiou 1986) indicates just how important monogenic resistance can be. Single-point substitutions also confer some herbicide and fungicide resistance, and again the resistance-associated mutations are often highly conserved across species (Gressel 1986). Although the evolution of resistance to synthetic pesticides generally begins with single-point mutations, some of which may confer cross resistance to multiple classes of compounds (Cochrane *et al.* 1998), high-level resistance generally requires combinations of such mutations (Mutero *et al.* 1994). Indeed, pesticide resistance mechanisms conferred by elevated carboxylesterases (in aphids (Devonshire & Field 1991) and mosquitoes (Mouches *et al.* 1986)), and target site-mediated resistance, including insensitive acetylcholinesterases (Russell *et al.* 2004), are associated with a series of distinct mutations, each one increasing resistance. Resistance to fungicidal ergosterol biosysnthesis inhibitors and polyene antibiotics are also both polygenic (Eckert *et al.* 1986). In addition to physiological adaptation, altered morphology may also contribute to increased pesticide resistance. For example, DDT resistance in mosquitoes is facilitated by the evolution of a protective footpad cuticle, reducing DDT uptake (Guillaumot 2006). Recent work in evolutionary developmental biology suggests that adaptive mutations affecting morphology may occur in protein-coding regions as well as in *cis*-regulatory regions of genes (Hoekstra & Coyne 2007), which helps explain the apparent multi-trait DDT adaptation seen in mosquitoes. These examples highlight the importance of genetic variation at multiple loci in the evolution of chemical resistance.

(b) *Adaptation to chemicals in wild populations*

The work described above has been derived from laboratory or controlled field studies. Fewer investigations document direct evidence of adaptation to chemical pesticides in wild populations, and in those that do, phenotypic evolution is generally all that is assessed, with the specific genes and molecular mechanisms underlying these adaptive changes largely unknown (Merila & Crnokrak 2001). The evolution of resistance to *Bacillus thuringiensis* (Bt) toxins in insects is an exception to this. Bt toxins are used widely in insect pest management and kill insects by creating pores in mid-gut membranes. In the diamondback moth (*Plutella xylostella*), resistance to Bt toxins Cry1Aa, Cry1Ab, Cry1Ac and Cry1F has been shown to occur through a single autosomal recessive gene (Tabashnik *et al.* 1997).

It appears to be the case, however, that emergence of pesticide resistance from de novo mutations in susceptible, wild-type populations is rare (Pasteur & Raymond 1996; Tabashnik *et al.* 2003). Resistance is more likely to develop owing to emigration (gene flow) from pre-existing resistant populations (Pasteur & Raymond 1996; Futuyma 1998). Between-population gene flow and the resulting recombination, which increases genetic variation, are considered to be primary mechanisms for the development and spread of chemical resistance in wild populations (Leslie & Watt 1986). Recombination is also more frequent, thus, more likely, than de novo point mutation: King & Jukes (1969) estimated the substitution (mutation) rate at between 10^{-8} and 10^{-9} per codon per generation, whereas the crossing-over frequency (recombination) between two existing mutations in acetylcholine esterase has been shown to reach 10^{-5} (in *Drosophila*; Nagoshi & Gelbart 1987).

The evolution of pesticide resistance requires a number of conditions to be satisfied and depends on the initial frequencies of the resistance allele(s), the dominance of the allele(s), the extent and duration of chemical or toxin exposure and the intensity of the chemical effect (Nacci & Hoffman 2008). This is likely also to be the case for other chemical toxicants. Furthermore, not all traits associated with selected resistant phenotypes are beneficial, and adaptive sweeps can drag deleterious alleles linked to the locus under positive selection (hitch-hiking), resulting in fitness costs unassociated with resistance. Nevertheless, significant life-history costs associated with pesticide resistance (Carrière *et al.* 1994) and chemical resistance in general (Van Straalen & Timmermans 2002) appear to be relatively rare. When they do occur, they may be due to negative gene interaction (pleiotrophy and epistasis) and not simply the diversion of energy budgets, as is often suggested (Taylor & Feyereisen 1996).

In addition to genotypes that confer susceptibility or resistance to chemicals with specific modes of action, there are a number of cases where particular sets of genes produce phenotypes resistant to general toxicity (so-called genes of 'major effect'), and these are also conserved across taxa (Hoffman & Parsons 2002). In many cases, genetic variation (polymorphism) at these loci confers significant ecological

Table 1. Physiological mechanisms and genetic basis of antibiotic and pesticide resistance.

compound/chemical class	resistant species	resistance mechanism and genetic basis	reference
antibiotics			
spectinomycin (antibiotic)	*Escherichia coli*	inhibition of peptidyl translocation via a base transition C/G to T/A at position 1192 of a 16S RNA gene	Sigmund *et al.* (1984)
streptogramin, lincosamide, macrolide (type B antibiotics)	*E. coli*	inhibition of peptidyl translocation via a base transversion A/T to T/A at position 2058 of a 23S RNA gene	Sigmund *et al.* (1984)
fungicides			
triadimenol (DMI fungicide)	grape powdery mildew fungus (*Uncinula necator*)	cytochrome P450-mediated resistance to demethylation and sterol biosynthesis inhibition linked to a phenylamine to tyrosine substitution mutation at codon 136	Delye *et al.* (1997)
fluconazole and itraconazole (azole fungicides)	*Candida albicans*	reduced azole affinity of cytochrome P450 14-α-demethylase *ERG11* (*CYP51*) gene via mutations at codon Y132H and 266–287.	Marichal *et al.* (1999)
cyclodiene insecticides			
dieldrin (cyclodiene)	fruitfly (*D. melanogaster*)	insensitivity of the $GABA_A$ receptor-chloride ion channel via an unknown mutation at the *Rdl* gene locus	ffrench-Constant *et al.* (1993)
dieldrin (cyclodiene)	house fly (*Musca domestica*) red flour beetle (*Tribolium castaneum*) American cockroach (*Periplaneta americana*)	insensitivity of the $GABA_A$ receptor-chloride ion channel via an alanine to serine substitution mutation at the *Rdl* (resistant to dieldrin) gene locus.	Thompson *et al.* (1993)
pyrethroid insecticides			
pyrethroid and DDT (organophosphate)	house fly (*M. domestica*)	nerve insensitivity via two point mutations in sodium-channel domain II: leucine to phenylamine at IIS6; methionine to threonine at IIS4-S5	Williamson *et al.* (1996)
α-cypermethrin (pyrethroid)	olive fruitfly (*Bactrocera oleae*)	cytochrome P450 mono-oxygenase upregulation via an iAChE *G4884* point mutation	Margaritopoulos *et al.* (2008)
organophosphate insecticides			
diazinon (organophosphate)	house fly (*M. domestica*)	conversion of carboxyl esterase to OP hydrolase via a *LcαE7* point mutation	Claudianos *et al.* (1999)
diazinon (organophosphate)	blow fly (*Lucilia cuprina*)	conversion of carboxyl esterase to OP hydrolase via *MdαE7* point mutation	Claudianos *et al.* (1999)
insecticide mixtures (*cross resistance*)			
organophosphates and carbamates (various)	lower insects e.g. *Myzus* sp., *Anopheles* sp., *Aphis* sp.	AChE receptor insensitivity in carbamates is greater than OPs owing to Pattern I resistance in *AChE-1* gene—several possible mutations at two sites (119 and 331)	Russell *et al.* (2004)
organophosphates and carbamates (various)	higher diptera e.g. *Musca* sp., *Bactrocera* sp., *Drosophila* sp.	AChE receptor insensitivity in carbamates is equal to OPs owing to Pattern II resistance in *AChE-2* gene—11 possible mutations at six sites	Russell *et al.* (2004)
permethrin (pyrethroid) carbaryl (carbamate) malathion (organophosphate)	*Drosophila simulans*	cross resistance owing to AChE receptor insensitivity. Resistant allele at the AChE locus, position 299 encoding a protein with at least one amino acid leucine is less than methionine substitution near the active site of AChE enzyme	Cochrane *et al.* (1998)

benefits in coping with a wide range of stressors and/or stress gradients. Initial chemical stress responses often involve metabolic regulation through various enzyme variants (allozymes) (Guttman 1994; Hoffman & Parsons 2002) and allozymes feature in a number of pathways initiated in general response to environmental stress. They include: (i) alcohol dehydrogenase (Adh) associated with the metabolism and detoxification of

environmental ethanol during the growth and germination of plants under variable climatic conditions (Brown *et al.* 1976), and also with the synthesis of heat-shock proteins conferring high-temperature resistance in insects (Alahiotis 1982); (ii) heat-shock protein genes (e.g. hsp70), which are essential for cellular survival in prokaryotes and eukaryotes, performing a multitude of house-keeping functions via their ability to interact with a wide range of proteins and peptides, a property that is shared by major histocompatibility complex (MHC) molecules (Srivastava 2002); (iii) glutamate pyruvate transaminase (Gpt) and leucine amino-peptidase (Lap94s) involved in the regulation of hyper-osmotic stress in the estuarine copepod *Tigriopus californicus* (Burton & Feldman 1983) and the blue mussel *Mytilus edulis* (Beaumont *et al.* 1988); (iv) glucose isomerase (Gpi) associated with resistance in mosquitofish and fathead minnows (*Pimephales promelas*) to multiple environmental contaminants, including metals (Heagler *et al.* 1993; Schlueter *et al.* 1997) and fluoranthene (Schlueter *et al.* 2000); (v) cytochrome P450 genes, which encode a super-family of enzymes in prokaryotes and eukaryotes involved in the oxidative, peroxidative and reductive metabolism of numerous endogenous compounds and a wide range of environmental chemicals (Nebert & Nelson 2006). Accordingly, the physiological genotype or 'physiotype' of an organism will determine its ability to regulate environmental, including chemical, stress (Depledge 1990; Guttman 1994).

3. INBREEDING, AND EVIDENCE OF INBREEDING DEPRESSION IN WILDLIFE AND ITS SIGNIFICANCE

Inbreeding can create problems for natural populations because it frequently leads to inbreeding depression. Moreover, traits closely related to fitness appear especially susceptible to inbreeding depression (e.g. Wright *et al.* 2008), and as a result, inbreeding can reduce effective population size, further increasing the likelihood of inbreeding and genetic drift (Lande 1976; Falconer 1989). Inbreeding depression occurs either because more homozygous individuals have increased expression of deleterious, recessive alleles (partial dominance hypothesis) and/or from the loss of heterozygote superiority (overdominance hypothesis) (Charlesworth & Charlesworth 1987). An inbreeding coefficient of $F = 0.33$ seems to represent a threshold marking the onset of significant inbreeding depression in populations in the laboratory (Frankham 1995*b*); however, inbreeding in the wild may progress more slowly, and hence selection may be able to purge the most deleterious mutations (Lande 1995; Brook *et al.* 2002; Keller & Waller 2002). In a changing environment, however, purging will be more limited (Bijlsma *et al.* 1999; Miller & Hedrick 2001) and in any event will not prevent erosion of heterozygosity. Furthermore, inbreeding depression may occur owing to large numbers of mildly deleterious alleles (Charlesworth & Charlesworth 1987), which may evade selection (Lande 1994; Lynch *et al.* 1995; see below).

(a) *Inbreeding in wildlife populations*

Most of our knowledge concerning inbreeding depression in animals has been derived from domestic populations and wildlife maintained in captivity. It has been argued that inbreeding depression is generally not a significant issue for wild-animal populations because inbreeding avoidance occurs via selective mate choice, multiple mating (Hosken & Blanckenhorn 1999), delayed maturation/reproductive suppression and dispersal (reviewed in Greenwood 1980; Pusey & Wolf 1996). However, there is a significant body of evidence to the contrary. Indeed, inbreeding can be pronounced in small wildlife populations that result following bottlenecks caused, for example, by a disease outbreak (O'Brien 1994) or pollution incident (Bickham *et al.* 2000). Inbreeding may also be promoted by specific behaviours, such as reproductive homing (philopatry), where animals, including some species of fish, amphibians and reptiles, return to their natal spawning grounds to breed (reviewed in Waldman & McKinnon 1993). In some small mammals (e.g. *Sorex araneus, Peromyscus leucopus*; Stockley *et al.* 1993), inbreeding occurs because individuals do not disperse. In fact, there is now extensive evidence of inbreeding and inbreeding depression in a wide range of wildlife populations (Wright 1984; Ralls *et al.* 1988; Frankham 1995*a*; Crnokrak & Roff 1999; Keller & Waller 2002; Frankham 2003). Crnokrak & Roff (1999), examining inbreeding depression in 137 traits in 35 wildlife species, found significant inbreeding depression, and in most cases, estimates were sufficiently high ($F > 0.1–0.33$) to be considered to have significant fitness implications (Frankham 1995*c*; Halverson *et al.* 2006).

Estimates for the minimum Ne for avoidance of the adverse consequences of genetic drift and inbreeding and for maintenance of a self sustaining, genetically viable population vary between 500 and 5000 (500–1000, Franklin & Frankham 1998; 1000–5000, Lande 1995; Lynch 1996; Lynch & Lande 1998) and depend on a range of demographic and environmental factors (Gilpin & Soulé 1986). Many wildlife populations do not meet this minimum Ne. As an example, many of the world's birds (ca. 1000 of the 9000+ species) have population sizes well below 1000 individuals. Two hundred bird species have census population sizes of less than 100 individuals (Green & Hirons 1991), and Ne may be considerably smaller. Some fish populations can also contain fewer than 200 individuals (e.g. sockeye salmon (*Oncorhynchus nerka*); Altukhov 1982), and reproductive homing adds further to the risk of inbreeding (FAO/UNEP 1981). It is recognized, however, that most genetic problems in endangered populations have accumulated over tens or hundreds of generations and a low Ne for several generations will not necessarily lead to irreversible genetic damage (Amos & Balmford 2001). Consequently World Conservation Union criteria (IUCN 2008) include demographic population structure, geographical distribution, temporal trends and generation time as well as breeding population numbers for defining endangered (Ne = 250–2500) and critically endangered species (Ne = 50–250).

(b) *Reduced genetic variation and health impacts in wildlife populations*

Reduced genetic variation that results from inbreeding and small population size has also been found to correlate with a range of defects, many of which are associated with reproductive traits. This has been demonstrated in African lion (*Panthera leo krugeri*) and Asiatic lion populations (*Panthera leo persica*), where males from small founder populations have reduced sperm count and motility, lower levels of male sex hormones and a greater proportion of abnormal sperm compared with larger populations having experienced less severe or no such bottlenecks (O'Brien 1994). In African cheetah (*Acinonyx jubatus*) populations too, low heterozygosity (at MHC loci and loci more widely) is associated with impaired immunocompetence and congenital defects including low sperm counts and viability in males (O'Brien 1994; Roldan & Gomendio 2009). A similar associated decline in genetic diversity and fitness has been shown in the Florida panther, a species on the brink of extinction with a remaining population of only 30 individuals (Roelke *et al.* 1993). In the case of the Florida panther, inbreeding may be responsible for several deleterious developmental and immunological impairments, including a high prevalence of cryptorchidism (undescended testes, now affecting over 90% of males) a significantly (fourfold) reduced sperm count, malformations in 90 per cent of the sperm and in many cases (20%) sterility, congenital cardiac defects and impaired immune system (Roelke *et al.* 1993). Similar findings have also been reported for rabbit populations (Gage *et al.* 2006). Examples of inbreeding depression in wild birds includes the song sparrow (*Melospiza melodia*) of Mandarte Island, British Columbia, and this has resulted in reduced life-time fecundity in females and reduced offspring survival (Keller 1998). Conversely, a male bias on reduced fitness, especially fertility, is displayed in the house mouse (*Mus domesticus*) (Meagher *et al.* 2000) and *Drosphila melanogaster* (Hughes 1995). Male fitness appears to be especially affected, possibly because male sexual fitness is under stronger selection than females' (there is greater variance in male reproductive success).

Most field or semi-field studies have focused on juvenile fitness and may have overlooked the effects of deleterious alleles expressed in later life (Meagher *et al.* 2000). It is likely that fitness differences will accumulate throughout an individual's life (Clutton-Brock 1988) and late-acting deleterious alleles are less likely to be removed by natural selection than early-acting mutations. This could lead to increased inbreeding depression in later life, and this is one explanation for why organisms senesce (Charlesworth & Hughes 1996). For example, in red deer, there is inbreeding depression in male breeding success (Slate *et al.* 2000), but none in neonatal survival or birth weight (Coulson *et al.* 1998), while in the house mouse there is significant inbreeding depression in adult male survivorship, paternity and male territoriality (Meagher *et al.* 2000).

Most studies that have shown inbreeding increases the risk of population extinction have been conducted on experimental populations (e.g. Wright *et al.* 2008), but there is also evidence that inbreeding can contribute significantly to population decline and extinction in the wild (Frankham 2003). This has been documented in mammals (e.g. Isle Royale wolf (Wayne *et al.* 1991); Florida panther (Roelke *et al.* 1993)), birds (e.g. heath hen; Simberloff 1988), reptiles (e.g. adder; Madsen *et al.* 1999), fish (e.g. topminnow; Vriejenhoek 1994) and invertebrates (e.g. colonial spiders; Riechert & Roeloffs 1993), although in each case genetic and non-genetic causes of extinction or local extirpation were not delineated. One of the most comprehensive examples of the impact of inbreeding and the importance of genetic diversity for population viability in the wild comes from studies on the Sonoran topminnow (*Poeciliopsis monacha*) (Vriejenhoek 1994). Here, a population decline coincided with reduced genetic diversity following a history of repeated bottlenecking and recolonization owing to alternating periods of drought and flooding. Following the translocation of individuals from a more genetically diverse upstream population, heterozygosity (allozyme heterozygosity at 4 loci (Idh-2, Ldh-1, Pgd, Ck-A) and fitness were reinstated and the population recovered. The initial fitness reduction was attributed to inbreeding depression in tolerance to physical extremes, in resistance to parasitism and in reproductive output (Vriejenhoek 1994). More recently, a single migrant wolf credited with introducing new allelic variation into an inbred population was found to coincide with a major population recovery (Vilà *et al.* 2003).

A major complication for studies of inbreeding in wild populations is that estimating inbreeding coefficients from heterozygosity estimates is problematic. This is demonstrated in a study in sheep of 138 microsatellite markers spread across all their 26 autosomes (Slate *et al.* 2004). Only a very weak association was found between heterozygosity and the inbreeding coefficient estimated from a known pedigree, and inbreeding depression was not predicted by multilocus heterozygosity (Slate *et al.* 2004). The low correlation between individual heterozygosity and inbreeding stems from the low variance of inbreeding in natural populations and the high stochasticity of individual genetic markers. Some gains could be achieved by estimating inbreeding coefficient rather than some of its proxies (see appendix A for the correct derivation).

4. COMBINED EFFECTS OF INBREEDING AND CHEMICAL TOXICITY

Given the widespread occurrence of inbreeding, the resultant loss of heterozygosity and subsequent inbreeding depression, inbreeding can be an important determinant of the vulnerability of populations to environmental stressors (Armbruster & Reed 2005). Indeed, several controlled laboratory studies have demonstrated interactive effects of inbreeding and chemical exposure. Miller (1994) showed that fitness was reduced in a laboratory strain of *D. melanogaster* when exposed to lead contamination after periods of inbreeding. In another study on *D. melanogaster*, where isolines were exposed to chemicals during eight consecutive generations of full-sib matings (generating a theoretical inbreeding coefficient of $F = 0.83$) more

inbred lines failed (became extinct) under chemical exposure (copper sulphate, 76% failure; methanol, 83% failure) than inbred controls (63%), while extinction rates for outbred controls with and without exposure ranged between only 3.1 and 4.4 per cent (Reed *et al.* 2002). Similarly, Nowak *et al.* (2007*a*) found amplified inbreeding depression in midges (*Chironomus riparius*) exposed to cadmium. Here there was a highly significant interaction between exposure concentration (0–0.72 mg kg^{-1} in sediment) and level of inbreeding ($F = 0$, 0.125 and 0.375) influencing development time and survival of homozygotes. The above laboratory studies generally indicate that environmentally realistic levels of inbreeding ($F = 0.1–0.33$) and chemical exposure can combine to severely reduce fitness.

Similar interactions between inbreeding and chemical stress are likely to occur in the wild but evidence for this is extremely limited. Correlative evidence for an interaction between inbreeding and chemical exposure in the wild comes from studies on the Florida panther. This species is now significantly inbred in Florida and many reproductive traits are impaired (described above). These animals also contain high levels of a range of EDCs, including mercury and PCBs, known to be able to cause reproductive impairment at the detected levels (Roelke *et al.* 1992; Facemire *et al.* 1995).

It is possible that combined effects of inbreeding and environmental chemical contamination could cause declines in some wildlife populations, and indeed may have already done so. However, until recently, this possibility has largely been ignored (Liao & Reed 2009). Interactive effects are arguably most likely for populations exposed to chemicals that cause reproductive impairment, as life-history characters related to reproductive output show most inbreeding depression. One example where this may have occurred is in North Sea seal populations. Here there is compelling evidence that exposure to a range of environmental chemicals including PCBs, DDT, polychlorinated dibenzofurans and polychlorinated dibenzo-*para*-dioxins led to reproductive failure and immunosuppression (Van Loveren *et al.* 2000; Vos *et al.* 2000), and ultimately contributed to mass mortality during a morbillivirus outbreak in the 1980s. The chemical effects were verified in a controlled field experiment in which common (harbour) seals (*Phoca vitulina*) fed with PCB-contaminated fish showed lower reproductive success than those fed with fish from a less polluted area of the Wadden Sea (Reijnders 1986). Inbreeding levels of harbour seal parents were not reported in that study; however, European populations of this species are apparently somewhat inbred, and inbreeding depression in pup survivorship is well documented (Coltman *et al.* 1998).

5. GENETIC VARIABILITY, INBREEDING AND CHEMICAL TESTING

Chemical Safety Assessment and ERA, set to protect humans and wildlife, respectively, require testing with laboratory animals. Testing guidelines have for several decades employed both inbred and outbred laboratory strains (here we define a strain as a stock or line derived from a closed population with distinct genetic characteristics that distinguish them from other groups within a species). In toxicology studies employed in human safety assessment, iso-genic strains of mice and rats—animals derived from at least 20 consecutive generations of full-sib matings—are often used (they are virtual clones) and are effectively purged of deleterious recessive alleles (Kacew 2001). The principle of using domesticated laboratory strains of animals in ecotoxicology studies supporting ERA, however, and their ability to predict and prevent adverse effects in wild animals has been questioned (Schaeffer & Beasley 1989; reviewed by Hill 1994). Thus, for ERA, strains of animals are often used that have been selectively bred to maintain heterozygosity with the intention that they are representative of wild populations. In the final section of this paper, we critically analyse the use of inbred versus outbred animals in ecotoxicology and for ERA.

(a) *Use of inbred animals for human safety assessment*

Two US Government agency white papers on toxicity testing (US EPA 2003; US FDA 2004) have recognized that the field of toxicology has been hampered by the wide variation in strains of animals used across different laboratories because strains can vary in their sensitivity to chemical effects. As an example of this, the sensitivity of outbred stains of rat to the model carcinogen TCDD has been shown to vary by almost 1000-fold (Kacew & Festing 1996). For human chemical safety assessments, the US National Academy of Science (NAS 2007) has now advocated the use of inbred isogenic strains in preference to outbred strains. This approach is also advocated in animal-based research in the UK (Festing *et al.* 2002). The compelling case presented is that side effects, such as carcinogenicity of a chemical, can be better evaluated by choosing a sensitive strain. Strains can also be genotyped accurately to both help inform on the intended mode of action of the chemical and authenticate the strains themselves. Phenotypic variation is also minimized, enabling increased statistical power based on fewer test organisms, with obvious financial and ethical benefits.

(b) *Inbred or outbred animals for use in environmental risk assessment?*

Ecotoxicology is a rather different process for the majority of chemicals under consideration. Here the process is a 'risk assessment', a balanced consideration of potential risk, as opposed to a 'safety assessment' that implies a higher level of knowledge. For the majority of chemicals entering the environment, little is known about the toxicology or potential adverse effects they may have at the concentrations that are present or predicted. In ecotoxicology, however, inbred strains may also have some merit, as they can have increased susceptibility to chemical stressors. This has been shown in the fruitfly *Drosophila buzzatti* for exposures to the organophosphorus insecticide dimethoate (Kristensen *et al.* 2003), in the midge (*C. riparius*) for cadmium exposure

(Nowak *et al.* 2007*a*) and in inbred strains of mice for sensitivity to oestrogen (Spearow *et al.* 1999; Spearow 2004). Conversely, there is the possibility of pre-existing, heritable resistance to certain chemicals in wild populations (Pasteur & Raymond 1996; Futuyma 1998) compared with inbred laboratory strains, as was shown for DDT resistance in a wild-type strain of *D. melanogaster* (Bijlsma *et al.* 1999).

In addition to offering increased biological sensitivity, the use of inbred, isogenic strains increases statistical power since variability in phenotypic response to chemical exposure is minimized. However, by the same token, they may fail to take into account the full spectrum of genetic variation and responses in natural populations. Therefore, although inbred strains may well be protective in many cases, they should not be considered wholly representative of wild populations. By definition, outbred strains should be more representative of wild populations. However, details of the origin and breeding history of strains are rarely reported for animals used in ecotoxicology and when they are, they are typically described as 'outbred' or 'genetically diverse'. Furthermore, this assumption is often untested and in cases where it has been, genetic variation has been shown to differ widely between strains and between populations derived from the same strain, as shown in laboratory rats (Kacew 2001) and zebrafish (Guryev *et al.* 2006; Coe *et al.* 2009). Genomic variation in zebrafish may be particularly high: there are an estimated 425 000 coding single-nucleotide polymorphisms, four times greater than in outbred strains of rat and higher than in any vertebrate genome sequenced to date (Guryev *et al.* 2006). Nevertheless, even the most outbred laboratory strains are likely to be significantly less genetically diverse than wild populations, as has been indicated by neutral markers of heterozygosity in trematode worms (Stohler *et al.* 2004), dipteran insects (Nowak *et al.* 2007*b*), amphipod crustaceans (Duan *et al.* 1997), rodents (Razzoli *et al.* 2003) and fish (Coe *et al.* 2009). It is likely that the comparatively lower genetic diversity in these outbred laboratory strains is because their outbreeding is normally limited to introgression with other laboratory strains or substrains and they lack the gene flow required to counterbalance genetic drift, resulting in the loss of alleles over time. Even the generation of new wild-type laboratory strains may be hampered at the outset by founder events or genetic bottlenecks in which the founders carry only a limited proportion of the initial allelic variation in the source populations (Nowak *et al.* 2007*b*).

There are also difficulties associated with characterizing outbred strains as highlighted by the US FDA (2004). Genome-wide characterization is not practical or useful because every individual will be different. Instead, a surrogate measure of genetic diversity, such as heterozygosity or allelic richness, for example, at highly variable microsatellite loci can indicate how representative a strain is of wild populations (Nowak *et al.* 2007*b*; Coe *et al.* 2009). Setting acceptance criteria for such measures, based on neutral markers of genetic diversity, however, needs careful consideration, since in some cases their correlation with fitness traits has been shown to be limited (Lynch 1996; Reed & Frankham 2003; Kohn *et al.* 2006). The targeted assessment of heterozygosity at quantitative trait loci (Falconer *et al.* 1996) may provide a better, practical solution. However, in the future, genomic measures of genetic diversity being advocated for conservation (Kohn *et al.* 2006), evolutionary biology (McKay & Stinchcombe 2008), pharmacology (Guryev *et al.* 2006) and ecotoxicology (Ankley *et al.* 2006) may also prove to be useful. Further evidence to support the contention that outbred strains are more representative of wild populations could be gained by comparing their respective levels of inbreeding, based on markers of heterozygosity (Goodnight & Queller 1999) or, better still, this in combination with pedigree information (Pemberton 2004). Alternatively, see appendix A.

(c) *Critical consideration on genetic variation and standard test guidelines in ecotoxicology*

Standard test guidelines for ecotoxicology studies including maximum tolerable limits for mortality in controls are probably not sufficient to prevent potential bias owing to genetic drift, mutational accumulation and inbreeding depression, which may affect other, perhaps more sensitive fitness components. These potential problems stem from limited genetic diversity and population numbers constituting laboratory strains. These problems are not easily overcome and, in practice, the use of robust outbred laboratory strains in ecotoxicology, which are closely representative of wild populations, is an ideal that is hard to achieve for the following reasons:

(i) Some control of genetic variability is required to maintain consistency within strains and therefore between tests and laboratories. Some degree of restricted breeding will be required to achieve this, and this may be justifiable since it occurs in a wide range of species in the wild. However, it is important to avoid repeated inbreeding as this could lead to inbreeding depression, the oversensitization of laboratory strains to chemical exposure and, ultimately, potentially over conservatism in ERA. Conversely, introgressions involving other outbred strains and wild-types will certainly lead to wider genotypic variation, potentially affecting phenotypic responses within and between treatments and studies. However, basic data quantifying phenotypic variation in apical endpoints concerning growth, development and reproduction in relation to genotypic variation in laboratory strains under control conditions is lacking.

(ii) In order to maintain genetic diversity in laboratory strains, sizeable breeding populations in excess of 1000 animals would ideally be required in a self-sustaining group-breeding situation. This may be impractical for some species; however, a more structured breeding programme (e.g. pair breeding) based on smaller numbers of individuals could possibly achieve similar results. Ethical and practical considerations will dictate the final solution, but the alternative solutions for maintaining genetic diversity in strains (suggested above) need to be tested first.

Before attempting to devise quality control guidelines with respect to genetic variation and inbreeding in laboratory strains, two key questions need to be addressed: (i) What is an acceptable level of genetic variation within a test organism/ batch of test organisms? (ii) What level of inbreeding significantly reduces fitness? For outbred strains traditionally used in ecotoxicology, there is no immediate answer to the first question, since fundamentally a universal, quantitative relationship between the erosion of genetic diversity, reduced adaptation and fitness is lacking (Nacci & Hoffman 2008) and establishing this relationship may be particularly challenging where novel chemical stressors with multiple modes of action are concerned. However, in answer to question (ii), the threshold inbreeding coefficient of $F = 0.33$ proposed by Frankham (1995*b*) provides a guideline maximum tolerable limit for inbreeding under laboratory conditions.

In this final section, we have raised the question of how useful or representative inbred laboratory strains are in ecotoxicology for the protection of wildlife. Although we present evidence that laboratory strains of animals can be conservative predictors of adverse effects, there may be instances in which they fail to take into account the full spectrum of genetic variation and responses in wildlife populations. Therefore, there is a possibility that they may sometimes be over-protective and at other times under-protective. As we move forward in ERA in our attempts to develop more realistic and intelligent testing strategies, we need to consider the fundamental assumption that our test subjects are representative of the wild populations that we aim to protect. It therefore would seem logical that effort is put into assessing the diversity of our laboratory animals and the populations that they are meant to represent.

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APPENDIX A

An individual can be homozygote at any given allele p_i, through identity by descent with probability $F(p_i)$, and through identity by state (IBS) with probability $(1 - F)(p_i^2)$, F being the inbreeding coefficient of an individual.

Summing over all alleles at a locus, the probability of being homozygote at any allele yields

$$P(\text{hom}) = F\left(\sum p_i\right) + (1 - F)\left(\sum p_i^2\right).$$

As $\sum p_i = 1$, this simplifies to

$$P(\text{hom}) = F + (1 - F)\left(\sum p_i^2\right).$$

This can be rearranged as

$$F = \frac{P(\text{hom}) - \sum p_i^2}{1 - \sum p_i^2}.$$

When considering a single individual, $p(\text{hom})$ can take only two values 0 and 1, so that

$$F = \frac{\text{hom}[1,0] - \sum p_i^2}{1 - \sum p_i^2}.$$

For a multi-locus estimator, each locus must be weighted by the term $1/\sum p_i^2$, as this represents the amount of information for each locus. Note that $\sum p_i^2$ is not an unbiased estimator of $E(\sum p_i^2)$, but the difference is trivial. One could also consider a weighting for the number of alleles rather than their frequencies.

A multi-locus inbreeding estimator at l loci should thus read

$$F = \sum_{l=1}^{\text{loci}} \left[\frac{1}{\sum_{i=1}^{\text{alleles}} p_{i,l}^2} \left(\frac{\text{hom}[1,0] - \sum_{i=1}^{\text{alleles}} p_{i,l}^2}{1 - \sum_{i=1}^{\text{alleles}} p_{i,l}^2} \right) \right].$$

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3 CHAPTER 3: MATERIALS AND METHODS

3.1 Gene Transcript Analysis

Methods for the extraction of messenger RNA and analysis of specific gene transcripts (targeted analysis) based on polymerised chain reaction (PCR) are set out below (Sections 3.1.1-6). Qualitative analysis of gene expression was achieved using Standard PCR (Eppendorf Mastercycler Gradient, Hamberg, Germany) and agarose gel electrophoresis (BioRad SubCell GT) to identify gene products based on molecular weight (3.1.5a). Quantitative analysis of gene expression was achieved using Real-Time Quantitative PCR (BioRad IQ5 qPCR, Bio-Rad Laboratories, Hercules, CA) (3.1.5b). These methods were used in Chapters 5 and 7.

3.1.1 General guidance for RNA sampling

RNA is highly susceptible to degradation by RNAse enzymes. RNAse enzymes are present in cells and tissues, and carried on hands, labware, and dust. They are very stable and difficult to inactivate. For the above reasons, it is important to follow best laboratory practices while preparing and handling RNA samples

- When harvesting total RNA, use a method that quickly disrupts tissue and isolates and stabilizes RNA
- Wear gloves and use sterile technique at all times
- Reserve a set of pipettes for RNA work. Use sterile RNAse-free filter pipette tips to prevent cross-contamination
- Use disposable plasticware that is certified to be RNAse-free, otherwise autoclave plasticware before use
- All reagents should be prepared from RNAse-free components, including ultra pure water
- Store RNA samples by freezing (-80°C). Avoid repeated re-freezing and extended pauses in the protocol until the RNA is in the form of double-stranded (ds) DNA
- Use a RNAse/DNAse decontamination solution or pure ethanol to decontaminate work surfaces and equipment prior to starting work

3.1.2 RNA Extraction and purification using Qiagen RNeasy™ Micro Kit

Purifies RNA (maximum 30 µg) from small amounts of tissues or cells

Qiagen RNeasy™ Micro Kit Contents (catalogue no. 74004)
(for x 50 samples)

- RNeasy™ MinElute Spin Columns (each in a 2 mL collection tube)
- Collection Tubes (1.5 mL)
- Collection Tubes (2 mL)
- Buffer RLT 45 mL
- Buffer RW1 45 mL
- Buffer RPE# (concentrate) 11 mL
- RNAse-free Water 3 x 10 mL

Add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Qiagen RNase-free DNase Kit Contents (catalogue no. 79254)

- RNAse-free DNAse set
- RNAse-free DNAse 1 (lyophilised) 1500 Units
- Buffer RDD 2 x 2 mL
- RNAse-free water 1.5 mL

Other Consumables & Equipment

- RNA later
- Ethanol (70 % and 96 – 100 %)
- Sterile, RNAse-free pipette tips
- Microcentrifuge
- Vortexer
- Disposable gloves
- Liquid nitrogen (for RNA stabilization prior to sample preparation)
- Hand held homogenizer & probes (sterile)
- RNAse-free 0.5 mL PCR tubes
- RNAse-free 1.5 mL microcentrifuge tubes

Frozen tissues were placed in an ice bucket prior to extraction.

All steps of the following extraction and purification procedure were conducted at room temperature (15 – 25 °C), working quickly and carefully.

All centrifugation steps were conducted between 20 – 25 °C in a Eppendorf microcentrifuge, ensuring the microcentrifuge did not cool below 20 °C.

I. Extraction of Nucleic Acids

1. **Add 10 µL of β-mercaptoethanol (β-ME) to 1 mL of Buffer RLT** (breaks di-sulphide bonds in proteins).

- **Remove tissue from RNAlater© solution and homogenise the tissue in 350 µL of Buffer RLT buffer with β-ME in a separate microcentrifuge tube.**
- **Note**: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy™ spin column. Typical time for homogenisation is 30 seconds. Compress the tissue with the pestle before using the homogeniser. Use a 200 gauge hyperdermic needle to sheer samples (x10).
- Do not use more than 5 mg (1.5 mm^3) tissue – RNeasy™ MicroKit, for more tissue (up to 30 mg) use RNeasy™ MiniKit.

2. Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new labelled microcentrifuge tube. Use only this supernatant (lysate) in subsequent steps.

- **Note**: In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

3. **Add 350 µL of 70% ethanol to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 4.**

- **Note**: The ethanol used should be molecular grade, dilute ethanol with RNase-free water (autoclaved water).
- Precipitates may be visible after addition of ethanol. This does not affect the procedure.

4. **Transfer the sample (up to 700 µl), including any precipitate that may have formed, to an RNeasy™ spin column placed in a 2 mL collection tube. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through.**

- **Note:** There may be more sample left from step 3. Centrifuge all of the sample to avoid loss before proceeding to step 5.
- Reuse the collection tube in step 5.

II. Removal of genomic DNA

5. **Add 350 µL Buffer RW1 to the RNeasy™ spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

- **Note:** Reuse the collection tube in step 8.

6. **Add 10 µL DNase I stock solution to 70 µL Buffer RDD. Mix by gently inverting the tube and centrifuge briefly to collect residual liquid from the sides of the tube. Make enough for all samples and excess.**

- **Note**: Prepare DNase I stock solution with RNase-free (DEPC) water.
- DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex!!!

7. **Add the DNase I incubation mix (80 µL) directly to the RNeasy™ spin column membrane, and place on the benchtop (20–30°C) for 15 min.**

- **Note**: Add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

8. **Add 350 µL Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm). Discard the flow-through and collection tube.**

III. Washing of the RNA

9. **Place the RNeasy™ spin column in a new 2 mL collection tube. Add 500 µL Buffer RPE to the RNeasy™ spin column. Close the lid gently, and centrifuge for 15 s at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

- **Note:** Buffer RPE is supplied as a concentrate. Ensure ethanol (molecular grade) is added to Buffer RPE before use.
- Reuse the collection tube in step 10.

10. **Add 500 µL 80% ethanol to the RNeasy™ spin column. Close the lid gently, and centrifuge for 2 min at 8000 x g (10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

- **Note**: After centrifugation, carefully remove the RNeasy™ spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

11. **Place RNeasy™ spin column and collection tube in centrifuge for 5 mins at max (e.g. 14,000 rpm) to dry the spin column membrane. Discard the flow-through and collection tube.**

- **Note:** The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions (e.g. reverse transcription).

12. **Place the RNeasy™ spin column in a new 1.5 mL collection tube. Add 15 µL RNase-free water (DEPC water) directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000 x g (10,000 rpm) to elute the RNA.**

13. **Re-elute sample through the same spin column and collection tube. Centrifuge for 1 min @ 8000 x g (10,000 rpm).**

- **Note:** Do not elute with less than 10 µL RNAse-free water as the spin column membrane will not be sufficiently hydrated.
- The dead volume of the RNeasy™ spin column is 2 µL

3.1.3 Quantification of RNA using Nanodrop™ spectrophotometer

14. Using Nanodrop™ spectrophotometer (Thermo Finnigan, Hemel Hempstead, UK). Select sample type RNA-40. Initialise with 1.5 µL of RNase-free water. Calculate a blank reading before measurement and after every 5-10 samples.

15. Pipette 1.5 µL of the RNA solution onto the pedestal of the Nanodrop instrument and quantify the amount of RNA in your sample. Record the concentration and quality of RNA in each sample:

..... ng / µL

..... 260/280

..... 260/230

- **Note:** 260/280 ratio is to assess purity of RNA, A ratio of ~ 2.0 is generally accepted as 'Pure' for RNA.
- 260/230 ratio is to assess nucleic acid purity, commonly ~2.0-2.2. If lower it may indicate the presence of contaminants e.g. ethanol

16. Aliquote RNA into smaller subsamples if necessary.

17. Store extracted RNA sample at -80 °C.

3.1.4a cDNA synthesis via Reverse Transcription: standard method

USE THIS METHOD OR VILO™ ON NEXT PAGE

This method is for **larger amounts of RNA**. For reverse transcription, **we need a total of 2ug of RNA in 10 µL i.e. conc. of RNA extract ≥200 ng/ml.** Total reaction volume = 20 µL.

Calculate how many microliters we need to use for the reaction (may require dilution).

2 ug of RNA = ... µL

If the concentration of the RNA extract is 200 ng/ml, we will need 10 µL to give 10 x 200 = 2000 ng or 2 µg. [Formua: 2/(conc/1000) : 2/0.2 = 10 µL].

1. Add the following components to a 0.5 mL PCR tube. Make up to a total of 12 µL:

..…... µL RNA (2 ug)

Master mix 1 (mix by pipetting)

0.5 µL random hexamers (10 pMol/ µL) – *can bind anywhere on DNA*

0.5 µL oligo (dT) (500 µg/mL) – *bind to poly-Adenylated tail only*

1 µL 10 mM dNPT mix – *de-oxynucleotide triphosphates (A, T, G, C)*

…..... µL RNase free H_20

12 µL

2. Select PCR programme named "RT"

3. Incubate sample and reagent mixture at 65˚C for 5 min in a PCR machine (melts secondary structure within template).

4. Cool at 4˚C in PCR machine for 2 mins (prevents secondary structure from reforming)

Make a 2nd master mix, enough for all samples and add 7 µL to each sample tube.

2 µL 0.1 M DTT – *prevents oxidation and dimerisation*

1 µL RNaseOUT Recombinant Ribonuclease Inhibitor

4 µL buffer - 5x first-strand buffer

5. Mix contents of the tube gently and incubate in PCR machine at 37°C for 2 mins.

6. Add 1 µL (200 units) of M-MLV RT, and mix by pipetting gently up and down.

NB - M-MLV RT is Monkey-Murine Leukemia Virus containing Reverse Transcriptase

7. Incubate tube at 25°C for 10 mins.

8. Incubate tube at 37°C for 50 mins.

9. Inactivate the reaction by heating at 70°C for 15 mins then hold at 10°C.
The cDNA can now be used as a template for amplification in normal PCR or qPCR.

3.1.4b cDNA synthesis via Reverse Transcription: "Vilo™ method

This method is for **smaller amounts of RNA**. For the reverse transcription, **we need a total of 10-100 ng of RNA**. Total reaction volume = 20 µL. Remaining RNA can be used for other purposes e.g. Illumina sequencing.

Calculate how many microliters we need to use for the reaction:

100 ng of RNA = ... µL (if conc. = 10 ng/µL, need 10 µL)

1. Add the following components to a 0.5 mL PCR tube. Make up to a total of 20 µL:

4 µl of 5X SuperScript VILO™ Reaction Mix (Invitrogen, catalogue no. 11754-050).

2 µl of 10X SuperScript® Enzyme Mix

x µl RNA (up to 100 ng)

.... µl DEPC-treated water

2. Gently mix tube contents (don't vortex)

3. Place tube(s) in PCR machine set to programme "RT1" or "VILO":

- 25°C for 10 minutes
- 42°C for 60 minutes (reverse transcription)
- 85°C at 5 minutes (inactivation)
- 10°C hold

4. Use diluted or undiluted cDNA in standard PCR or qPCR (see section 4), or store at –20°C until required.

3.1.5a PCR and Gel Electrophoresis

Standard PCR was performed using an Eppendorf MasterCycler Gradient (Eppendorf, Hamberg, Germany)

1. Each PCR reaction requires the mixture of components below.

Note: the primers will be different for each gene. Master mix can be made up without primers. These can be added with the cDNA template.

PCR reaction mix:	**1x**	**65x**
DMSO (inhibits 2ndry structures)	1 µL	65 µL
5x Go Taq™ flexi buffer (green top and green solution)	5 µL	325 µL
$MgCl_2$ solution, 25 mM (cofactor)	1 µL	65 µL
PCR nucleotide mix, 10 mM each (dNTPs)	0.5 µL	32.5 µL
GoTaq™DNA polymerase (5u/µL) (Green top, white solution)	0.5 µL	32.5 µL
Forward primer	1 µL	65 µL
Reverse primer	1 µL	65 µL
RNase-free water	14 µL	910 µL
Total Volume	**24 µL**	**1560 µL**

2. Add 1 µL of cDNA template to the PCR plate and mix with 24 µL of master mix (above).

Note: Place a PCR plate cover over the plate and seal to avoid evaporation.

3. PCR cycling program:

- 95°C for 5 mins (denaturing/melting – two DNA strands separate)
- 40 cycles of: denaturation at 95°C for 45 secs, annealing at 55°C for 45 secs (primers anneal to cDNA template strands) and elongation at 68°C for 90 secs (*Thermus aquaticus* (Taq) polymerase duplicates cDNA bounded by primers).
- 68°C for 15 mins (elongation)
- End-hold at 10°C, then place in fridge until needed for gel electrophoresis

NB annealing temperature will vary for different primers (see primer design 3.1.6)

Elongation temperature typically varies from 68-72°C – typlically at least 1 min of elongation time is required in each PCR cycle for products ≥ 1000 base pairs.

Gel Electrophoresis

4. Make 1% PCR gel (or can use 2% gel for small PCR products <1000 base pairs)
Weigh out 4 g of agrose and make up to 400 mL with 1 x Tris-acetate EDTA (TAE) in a duran bottle (Dilute TAE with distilled water if required). Place in microwave with loose fitting lid on a medium heat for 2 minutes.

5. Remove bottle and stir up the agrose. Place the bottle in the microwave for a further 2 minutes. Repeat this step until the agrose goes clear and is fully dissolved. Once fully dissolved remove from microwave and leave to cool until the bottle is cool enough to pick up (stir agrose as its cooling, to avoid patchy cooling).

6. 5 µL of ethidium bromide should be added to the 400 mL of liquid gel as a suitable visualisation reagent.

7. Clear and dry the casting tray and combs, to ensure there is no remaining dried agrose. Using suitable tape (autoclave tape) seal the end of the tray to ensure no leaks.

8. Pour the agrose into the casting tray and use a micro pipette-tip to remove any bubbles or push them to the bottom end of the tray.

9. Lay the combs to create the wells ensuring there are no bubbles introduced between the teeth of the combs. Leave the gel to set and go opaque (approximately 40 mins).

10. Once the gel has set, carefully remove the tape at either end of the tray. Ensure the gel is the right way round (the gel will run black to red i.e. towards the +ve, as DNA is –ve). Place the tray into the running tank and pour enough TAE to cover the whole gel.

11. Slowly remove the combs, ensuring one end is always removed before the other, to avoid introduction of bubbles into the wells. To visualise the individual wells and to aid loading, a small amount of loading dye can be run across the wells.

12. Using a small tip and changing tip for each sample, load samples into fill the well almost full (15-20 µL).

- **Note:** Be careful not to damage wells when loading.

13. Load a suitable DNA ladder in (100 or 1000 base pair ladder) the middle well of each row.

14. Once the samples are loaded, place the lid and ensure the contacts are made. Set a suitable voltage (75 V) and press run on the power pack.

- **Note:** Check the wire electrical nodes in the tank to make sure they are bubbling.

15. Run until the loading dye markers have run 2/3rds down the individual sections of the gel.

16. Once the gel has run take an image using gel doc system (UV).

17. Once the gel has been imaged, the gel can be disposed of and the casting tray, combs and tank (if necessary) should be washed with hot soapy water and rinsed in distilled water.

18. Confirm desired PCR product has been generated by comparing migration of product versus DNA ladder.

3.1.5b Real-time quantitative PCR

RT qPCR was performed using a BioRad IQ5 (BioRad Laboratories, Hercules, CA)

1. Prepare a super mix in the following proportions (amounts required per well of well plate):
Note samples should be in triplicate, so multiply these volumes x no. of samples & x3

- 8.5µl RNase free water
- 10µl IQ SYBR® Green absolute fluorescine dye (BioRad cat no. 170-8882)
- 0.5µl of each primer set (0.25µl forward and 0.25µl reverse)

2. Pipette 19 µl of supermix (above) into each well.

3. Pipette 1µl of cDNA template into each well.
Note for qPCR Using Fluorescent Primers or Probes:

If you started with ≤100 ng of total RNA, up to 10% of the qPCR reaction volume may be undiluted cDNA (e.g., for a 20µl qPCR, use up to 2 µl of undiluted cDNA).

If you started with >100 ng total RNA, dilute the cDNA prior to qPCR, because higher concentrations of cDNA will affect the signal baseline in SYBR® Green and (SYBR GreenER™) reactions. For example, if you started with 2 µg of total RNA, prepare a 20-fold dilution of the resulting cDNA to achieve the concentration equivalent of starting with 100 ng of RNA. Then use up to 2 µl of the diluted cDNA in a 20-µl qPCR (≤10% of qPCR volume).

4. Setup qPCR programme
a) Perform a temperature gradient with each primer set on a "wild-type" sample (containing pooled tissue samples 'same tissue type' from controls) to **confirm the optimum annealing temperature**. A typical gradient should span approximately 15°C (e.g. 53-68°C).

q-PCR analysis of target genes Target gene	Forward primer	Reverse primer	Annealing temp (°C)	Efficiency (%)
Ribosomal protein l8 (*rpl8*)	CCG AGA CCA AGA AAT CCA GAG	CCA GCA ACA ACA CCA ACA AC	59.5	2.07

Insulin-like growth factor (*igf1*)	GCA TAG CCA CTC TTC CTG TAA G	AAC GGT TTC TCT TGT CTC TCT C	60.5	1.83
Hyrdoxy-steroid dehydrogenanse (*hsd17b3*)	GCT CTT CTG TCC TCT TCC TG	TCT CTT GAT TTC TGC TGA TGA TG	59.5	1.95
17α-hydroxylase/ 17,20-lyase (*cyp17a1*)	CGA CAG TAA GAT TGG GAA AGA AAG	GAT GAG GAG CGG AGA ACC AG	60.5	1.96
Aromatase (*cyp19a1a*)	AGC CGT CCA GCC TCA G	ATC CAA AAG CAG AAG CAG TAG	61.5	1.89
14α-lanosterol demethylase (*cyp51*)	TTC GCC TAC ATC CCA TTC G	AAC ATC CGC AGC AGA GTC	61.5	1.93

b) **Determine the specificity of the primers** using a melt curve. A good melt curve must have only one sharp peak, any other peaks may indicate primer binding (forming primer dimers) or non-specific binding and formation of an undesired product.

c) **Assess primer efficiency** using a standard curve. To determine the detection range, linearity and real-time qPCR amplification efficiency (E; E = 10[−1/slope]) (Pfaffl, 2001) of each primer pair, real-time qPCR amplifications is run in triplicate on a 10-fold serial dilution series of zebrafish testis cDNA pooled from all samples. Standard curves are generated by the instrument (BioRad IQ5) based on the threshold cycle (Ct; the PCR cycle at which fluorescence increased above background levels) to the logarithm of the cDNA dilution. **Primer efficiency should be in the range 1.8-2.2**, where 2.0 represents doubling of DNA in each PCR amplification cycle. (See examples for the target genes below).

d) Run test samples in triplicate to identify errors in pipetting/ contamination in individual wells. As far as possible separate 96 well plates should be set up for each primer set, since different primer sets may require different annealing temperatures. This approach will also help to minimise or prevent inconsistencies which could arise if primers were to span different plate runs.

e) A basic 2-step qPCR cycling program is listed below, and will result in the amplification of the desired gene target(s):

Cycle	Repeat	Step	Temperature	Time
1	1	1 Denaturing	95°C	3 mins
2	40	1 Denaturing	95°C	10 secs
		2 Annealing	55°C	30 secs

f) Once the plate is setup with test samples and No-Template Controls (NTCs), continue to 'Run', and select 'Collect well factors' and begin.

5. Quantification of gene expression

a) Recap

During real-time PCR each cDNA sample is amplified in triplicate using 96-well optical plates in a 20-µl reaction volume using 1 µl cDNA, 10 µl 2× Absolute SYBR Green (Flourescein) Supermix (BioRad), 5 µM (0.5 µL) of the appropriate forward and reverse primers. Hot start Taq polymerase was activated by an initial denaturing step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at the primer-specified temperatures for 30 sec and, finally, melt curve analysis. No-template controls are run for each plate and all samples are run on the same plate ensuring consistent quantification of the expression of each target gene.

b) Relative expression levels are determined using the following calculation (using the IQ5 instrument or Microsoft exel):

$RE = (E\ ref)^{Ct\ ref} / (E\ target)^{Ct\ target}$

Where RE is relative gene expression, ref is the housekeeping gene, target is the gene of interest, E is PCR amplification efficiency and Ct is cycle threshold (number of temperature cycles yielding above background expression) for that particular gene.

c) Statistical differences in relative gene expression between experimental groups are assessed by one-way ANOVA of log-transformed data, followed by Dunn multiple pair-wise comparison test or Student t-test using Minitab. Differences are considered statistically significant at $p \leq 0.05$.

3.1.6 PCR primer design

Primers specific for the target mRNAs were designed with Beacon Designer 3.0 software (Premier Biosoft International, Palo Alto, CA, US) according to the manufacturer's guidelines and purchased from Invitrogen UK Limited. The target sequence was inputted in text format and a primer search was first conducted changing the following search parameters from the default settings:

NB Alternative primer design software tools e.g. "Primer3" (freeware) may be suitable for normal PCR, but NOT qPCR.

Under the 'Search Parameters' tab:

1. The **'Avoid Secondary Structure'** function was switched OFF
2. Under **'Search Range'**, the sequence region of the mRNA desired for primer design was specified. This must be a region highly specific to the target mRNA with low conservation with mRNAs related to the target mRNA, to ensure specificity of the primer pair (the 5'- or 3'-UTRs were chosen for primer design where possible). Leave open to whole mRNA sequence if possible though.
3. The **'Avoid Cross Homology'** function was switched on ON (homology of the primers to related genes was also checked manually using multiple sequence alignment techniques and by BLASTn search).

Under Primer Parameters:

1. **Target Annealing temperature (T_a)** was set to 55 ± 8 ^{o}C. A target T_a of 60 ^{o}C (approximated as T_m + 5 ^{o}C) was considered ideal. Primer sets with lower T_as (< 55 ^{o}C) were avoided wherever possible because the secondary structure of the target mRNA template is higher at lower temperatures, causing extension difficulties, and primer annealing is less specific at lower temperatures leading to multiple product and primer dimer formation.
2. **Primer Length Range** was set to 16-24 bp but 17/18 bp was considered optimal.
3. **Amplicon Length** was set to 80-160 bp but 100 bp was considered optimal.
4. **Alternate Primer Pairs per Sequence** was set to 50 to enable a wide choice of primer sets.

Under Primer Parameters Advanced:

1. **3'End Max ΔG** was set to -4.0 (the lowest permissible).
2. **Self Dimer Max ΔG** was set to -3.0.
3. **Run/Repeat Max** was set to 4 bases (a maximum of four identical bases were permitted in a run).

Under Primer Pairs Parameters Advanced:

1. **Maximum Primer Pair T_m Mismatch** was set at 3.0.
2. **Cross dimer Max ΔG** was set at -3.0.

The best two primer sets for each target mRNA were chosen from the selection given, using the following priorities for choice:

1. **Region of primer annealing** – must be entirely specific to the target mRNA (check by BLAST search of the primers after design).
2. **Self and Cross dimer ΔG** must be as close to 0 as possible (ideally no greater than -0.5).
3. **T_m** must be high – as close to 55^{o}C as possible (no lower than 50^{o}C) to give an approximate target T_a of 60^{o}C (no lower than 55^{o}C).

The chosen primer sets were purchased from Invitrogen UK Limited, resuspended to 100 pmol/µl with ice-cold molecular-grade water, and then aliquots were diluted 1:10 with ice-cold molecular-grade water to 10 pmol/µl in 1.5 ml microcentrifuge tubes and stored at -20^{o}C.

Primer specificity was confirmed by gel electrophoresis and/or melt curve analysis and automated fluorescence sequencing of PCR products using RT qPCR (3.1.5b).

3.2 Micro-Satellite Analysis

Methods for the extraction of DNA and qualitative analysis of selected micro-satellites (Short Tandem Repeats (STRs) of up to 10 bases e.g. ATATATAT) in zebrafish are described in 3.2.1-5. Analysis was performed on zebrafish embryos and adult fin clips in order to assess multi-locus heterogygosity and allelic richness (genetic diversity) (Chapters 4 and 5) and also male paternity (for breeding trials, Chapter 6).

3.2.1a DNA extraction from zebrafish embryos

Extraction and precipitation (via ammonium acetate) of DNA was performed according to Bruford et al. (1998).

1. Prepare extraction solution (DigSol 500 ml), mix the following reagents, make up to 500 mL with double distilled (HPLC) water and heat gently to disolve.

20 mL of 0.5M EDTA (1/25 dilution = 20mM)

57 mL of 1M NaCl (1/8.8 dilution = 114 mM)

25 mL of 1M Tris.HCl pH 8 (1/20 dilution = 50 mM)

25 mL of 20% sodium lauryl sulphate (SDS) ”soap” (1/20 dilution = 1%)

2. Remove individual embryos from -80°C freezer and place in a 1.5 mL autoclaved, labelled centrifuge tube using a ”cut-off” pipette. Squash embryo using side of pipette tip. Place tube in ice.

3. To each tube add 200 µL of Digsol (lyses cells and removes lipid) **and 10 µL Proteinase K** (removed protein) **(previously prepared 10 mg/mL).** Add this to the inside of the lid.

4. Place tubes in a water bath at 55°C for 3 hrs, vortex briefly every hour.

5. Switch centrifuge to 4°C and allow to cool.

6. Remove tubes from water bath and add 400 µL of 4M ammonium acetate to each one (solution goes cloudy as precipitate is formed from remaining protein)**. Vortex several times over 15 mins.**

7. Centrifuge for 10 mins at 13,000 rpm, at 4°C.

8. Add 1 mL of 100% ethanol (removes proteins, salts reagents) **to an autoclaved, labelled 1.5 mL centrifuge tube. Pipette 500 µL of supernatent into tube and invert tube gently several times.**

9. Centrifuge for 10 mins at 13,000 rpm, at 4°C. DNA pellet may be visible afterwards.

10. Pour off ethanol taking care not to dislodge the DNA pellet.

11. Add 500 µL of 70% ethanol to rinse the DNA pellet. If necessary, at this point the tube can be stored at -20°C.

12. Centrifuge for 50 mins at 13,00 rpm at room temperature. Pour off ethanol and stand tubes upside down on a clean tissue for approx 30 mins to dry.

13. When fully dry add 70 µL of HPLC water and flick tube to dislodge and disolve DNA pellet.

14. Place tubes in a water bath for 30 mins at 37°C. Flick tube every 10 mins.

15. Store tubes at -20°C long-term or 4°C short-term (couple of days).

3.2.1b DNA extraction from zebrafish adult fin clips

Equipment/reagents:

Glass plate, scalpel, forceps, autoclaved/labelled 1.5 mL micro-centrifuge tubes
HPLC water
100% Ethanol
ProteinaseK (200 mg/mL)
Chelex/beads (pre-heated to 80°C, stir on a hotplate)

1. Clean glass plate with 100% ethanol – mark grid using a pen, to avoid cross-contamination when cutting fin tissue. Clean scalpel and forceps after each use in HPLC water then 100% ethanol.

2. Put a small section of fin (incl. peduncle) into a clean tube and add 10 µL of ProteinaseK to the inside of the tube lid.

3. Add 500 µL of pre-heated (80°C Chelex to each tube with 1 mL pipette tip with the end cut off.

4. Close tube lid and vortex for a few secs.

5. Place tubes in a water bath at 55°C for 75 mins. Vortex briefly at 15 min intervals and ensure tube lids don't pop open.

6. Remove tubes from water bath and place in another one at 100°C for 15 mins – ensure lids a firmly closed. Tubes can be stored at -20°C after this point if necessary.
7. Use supernatent for PCR (chelex and other reagents/impurities collect at bottom of tube).

3.2.2 PCR multi-plexing

The extracted DNA (from embryos or fin clips) was PCR'd in order amplify PCR products for selected micro-satellites (using primer sets, each with distinct coloured, fluorescent forward primers).

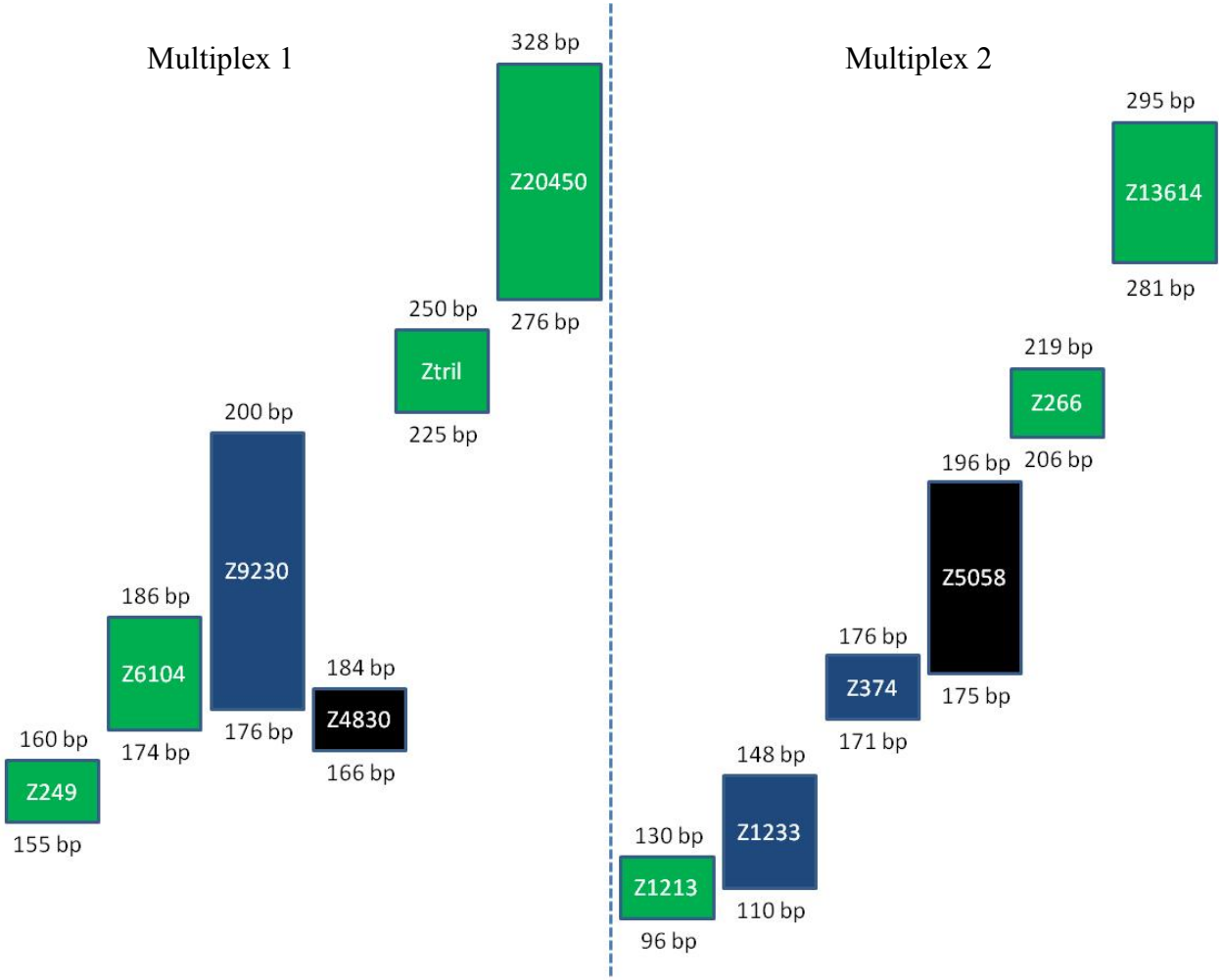
1. Prepare PCR mastermixes for each primer set in a 1.5 µL micro-centrifuge tube and keep on ice, add reagents in the following order:

Reagent	1x volume (µL)	105x volume (x96 + 9 extra)
Forward primer (fluorescent)	1	105
Reverse primer	0.5	52.5
Buffer	1	105
dNTPs	1	105
$MgCl_2$	0.3	31.5
DEPC water	1	105
Taq polymerase*	0.5	52.5

***Don't add red Taq polymerase until ready to start PCR!**

2. Combine 3 primer sets in each multi-plex eg. batch 1: Z249 (green); ZTril (green, but not overlapping in PCR product [amplicon] size) see Figure GSA.1 below); Z4830 (black).

Figure 3.2.2: Positions in base pairs of PCR products (micro-satellite, short tandem repeat regions) targeted by primer sets



Note: 12 micro-satellite loci were targeted for amplification by PCR: Z249; Z266; Z374; Z1213; Z1233; Z4830; Z5058; Z6140; Z9230; Z13614; Z20450 (www.zfin.org) and Ztril (Coe et al. 2008).

3. Pipette 20 µL of DNA extract into separate wells of a 96 well plate, using a separate pipette tip for each sample. Lable tray ”DNA extracts”.

4. Using a multi-pipette transfer 3 x 5 µL of extracted DNA from each sample well from the ”DNA extract” plate into separate wells in a 96 well plate, one plate for each primer set. Lable plates according to ”Primer name” and keep on ice.

5. Pipette 3 x 4.5 µL of mastermix (for each primer set) into the appropriate 96 well plates.

6. Add a drop of oil to each well to prevent contents escaping during PCR.

7. PCR cycling programs for primer sets:

Multi-plex 1		**Multi-plex 2**	
PCR programme	**Primer sets**	**PCR programme**	**Primer sets**
”Z58”	Z249, Z4830, Z20450	”Touchdown”	Z374, Z1213, Z1233
”Touchdown”	Ztril, Z6104, Z9230	”Touchdown”	Z266, Z5058, Z13614

”Z58” PCR cycling program: 95°C denaturing for 90 s, followed by 30 cycles of 30 s dentauring at 95°C, 30 s annealing at 58°C and 60 s extension at 72°C and a final extension at 72°C for 10 min. ‘Touchdown’ PCR cycling program: as above, but with the annealing temperature decreasing every five cycles (62°C, 58°C, 55°C, 53°C, 51°C, 49°C, 47°C).

8. PCRs were run on an Applied Biosystems 2700 series Thermal Cycler. Reaction volume was set to 10 µL (though it is actually 24.5 µL)**.**

3.2.3 Gene sequencing of multi-plexes

The PCR products were sequenced in groups (multi-plexes) using a Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Essex, UK).
Multi-plex 1: Blue = Z9230, Z20450; Black = Z4830; Green = Z249, Z6149, Ztril.
Multi-plex 2: Blue = Z374, Z1233; Black = Z5058; Green = Z266, Z1213, Z13614.

1. For each multi-plex (1 and 2) use a multi-pipette to transfer sequentially the following volumes of PCR products into a new 96 well plate: Blue = 1 µL; Green = 1.2 µL; Black = 1.8 µL.

2. Fill pipette rinse tray with deionised water.

3. Add 4 µL of size standard to 350 µL sample loading solution (SLS) in a 500 µL micro-centrifuge tube and vortex to mix. NB 1 tube is required per row of the 96 well plate.

4. Using a multi-pipette, transfer 28 µL of SLS and size standard to each well in the first row of each "primer plate". Pipette up and down to mix, but do not introduce air bubbles! Rinse pipettes tips after each use. It is best to analyse only the first row to make sure the PCR has worked! Add 1 drop of Beckman oil to each well.

6. Prepare a separate "Buffer plate": fill equivalent rows to "Multi-plex plate" with each well ¾ full of separation buffer – keep at 4°C.

7. Select working directory on CEQ 8000 Genetic Analysis System. From main screen select "Sample set up" and fill in plate identifiers. Click on "Frag 3" and save as "file name".

8. Run analysis. Select direct control tab, click access plates and Start.

9. Open sample loading door when given the green light "Go". Fill capillary tray with de-ionised water. Insert "Multi-plex plate" and "Buffer plate" slide lid over plates and close loading door.

10. Click "load" and "run". Click on "sample plate" and select file just set up – check wells are OK.

3.2.4 Gene sequence data processing (micro-satellite peak tagging)

The analytical data from the CEQ 8000 Genetic Analysis System require manual confirmation and tagging of peaks representing micro-satellites.

1. Open CEQ 8000 data system, select ”database” icon, right-click on database folder and set as working directory.

2. Minimise screen, go to main menu and select ”fragments” icon.

3. Select ”raw data” – next – default analysis – analyse – finish

4. Click on ”data tab” at bottom of screen – select data – right click – re-analyse. Re-analyse data using ”additional/edited locus tags”.

5. Select allele identification type * STR (Short Tandem Repeat) and select locus tags for multi-plex: Multi-plex 1: Blue = Z9230, Z20450; Black = Z4830; Green = Z249, Z6149, Ztril. **Multi-plex 2:** Blue = Z374, Z1233; Black = Z5058; Green = Z266, Z1213, Z13614.

6. Go to ”results set” folder. Select data – right click – show stacked graphs.

7. Check peaks are tagged correctly, one primer colour set at a time. NB keep red set (reference standard) selected. Right click on graph and uncheck ”show sizes” box. Click on peak – include/exclude – edit annotation – finish and save file.

8. Exit from stacked graph fragment analysis and return to main menu.

9. Select ”fragment analysis” again. Open an existing study – click on data tab at the bottom. Select and right click ”fragments list” – open – select data by blocking in.

10. Go to ”exclusion filter set” – click on name and select ”allele identification”, click on ”operator” (=), click on ”value” enter nothing.

11. Click on each of the remaining colour primer sets at the top of the screen to perform exclusion filter i.e. to exclude all non-tagged peaks.

12. Right click on a line of data – ”export grid” to .CSV file.

3.2.5 Micro-satellite data analysis

The processed STR data from the CEQ 8000 Genetic Analysis System require formating in MicroSoft Excel ready for analysis using freeware micro-satellite statistical analysis tools: Microsatellite toolkit v3.1.1 (Park, 2001); Fstat v2.9.3.2 (Goudet, 2001); Genepop v4.0 (Rousset, 2008); Coancestry v8.0 (Wang, 2010). A suite of indices were calculated for both inbred and outbred zebrafish: observed and expected multi-locus heterozygosity (H_O and H_E); gene diversity (H_S); allelic richness (R'); effective inbreeding coefficient (F_{IS}). Additional indices were also determined: internal relatedness (IR) (Amos et al. 2001); homozygosity by loci (HL) (Aparicio et al. 2006).

1. Open .CSV file and remove all columns except: Allele ID,; locus name; RN; Comment. Save as .XLS file.

2. Sort by comment – make sure all primers are labelled consistently. Then sort by locus name – make sure all samples have a locus name. Then sort by RN, locus name, allele ID.

3. Start at cell F6 and enter all locus names in allele pairs i.e. Z1213, Z1213 Z1233, Z1233 horizontally accross the worksheet.

4. Obtain macro from existing Excel spreadsheet. Click on tools – macro- visual basic editor (VBE) – highlight and copy code (ctrl C).

5. Open working spreadsheet (make sure all other spreadsheets are closed) – click on tools – macro – VBE (Alt F11). Click on view code – paste – save and close VBE.

6. Return to spreadsheet view and click on tools – macro – macros – copy data and press run.

7. Check the spreadsheet. Colour in primer sets using text colour. Make sure there are no blank cells in the 1st column of each locus – if so add a zero to blank cells. Check to make sure allele pairs look OK and consistent down the worksheet. Check there are no extra alleles on the RHS of the table. Check allele bp numbers are consistent i.e. all even or all odd. Check allele pairs increase in bp from left to right. NB 2 bp base pair separation is the minimum for resolution of two alleles!

8. Combine both sets of multi-plex results into one data table in Excel.

9. Open Microsatellite Toolkit after downloading as an ”Excel add-in” – Go to ”tools” and select from drop-down list.

10. Select input data format from drop-down list – Diploid two column format – OK. This creates a number of worksheets (check different tick boxes first) that are compatible with other micro-satellite statistical analysis programmes e.g. GenePop, Fstat. NB Paste Excel data into Notebook and save before inputting to Fstat. Rename .out files as .txt files! When running analyses average all loci for each population – make sure names of each sample define ”PopName_SampleNo” in <10 characters!

3.3 Plasma 11-Ketotestosterone Analysis

The analytical method for the quantification of 11-ketotestosterone (11-KT) was taken from Scott et al. (1980). This analysis was performed in Chapter 5.

3.3.1 Sample collection and preparation

Blood was collected from the caudal vein after cutting the base of the tail fin using a narrow bore haematocryt capillary tube pre-coated with heparin, and was immediately stored on ice. Samples were then centrifuged in groups of 8 for 4 minutes at 3200G to separate the plasma. All samples were then frozen and stored at -20°C.

Plasma samples were subsequently defrosted on ice and a 1 µl aliquot was removed from each of the haematocryt tubes using a disposable capillary pipette tip and transferred to a 1.5 ml microcentrifuge tube.

3.3.2 Radio-immunoassay materials

11-ketotestosterone was purchased from Sigma Aldrich (Poole, Dorset, UK).
Tritiated 11-ketotestosterone was synthesised from 1, 2 -^{3}H Cortisone (TRK 237, specific activity 35.4 Ci/mmol) obtained from The Radiochemical Centre Ltd. (Amersham, Bucks, UK) following the method of Simpson and Wright (1977).

Anti-serum to 11-ketotestosterone was raised by 2-4 week injections of 4 androsten-11α, 17β-diol-3 one 11-hemisuccinate: BSA (Steraloids Ltd., Croydon, UK) mixed with Freund's complete adjuvent.

3.3.3 Radio-immunoassay equipment

Extraction tubes – conical sodalime glass centrifuge tubes (4 ml)
Assay tubes – Pyrex© borosilicate glass culture tubes (4 ml)
Evaporation tubes - Pyrex© borosilicate glass culture tubes (10 ml)
Scintiliation vials and scintillation counter

3.3.4 Radio-immunoassay procedure

11-KT was quantified from 1 µl of blood plasma using radio immunoassay (RIA) according to the method in Scott (1980). The minimum detection limit for the RIA was 1.22 ng ml^{-1}.

1) Add radio-labelled "antigen" [^{3}H]ketotestosterone (100 ct/min) in 50 µl of distilled water to each 1 µl plasma sample in a 1.5 ml microcentrifuge tube.

2) Incubate samples over night at 4°C (fridge).

3) Extract (all 11-KT, labelled and unlabelled) in 5 ml diethyl ether/heptane (4:1) for 1 min.

4) Centrifuge at 2000 rpm for 10 mins.

5) Freeze bottom phase in liquid nitrogen.

6) Pour off upper 'organic' phase into a clean evaporation tube.

7) Evaporate samples at 45°C under a stream of oxygen-free nitrogen.

8) Rinse sides of tube with 200 µl of methanol, collect in bottom of tube and evaporate in oxygen-free nitrogen.

9) Dissolve extract on bottom of tube in 50 µl of dichloromethane and spot onto a TLC plate.

10) TLC plates developed successively in 50:50 petroleum ether:ethylacetate (v/v); 98.5:1.5 dichloromethane:ethanol (v/v); 96.5:3.5 dichloromethane:ethanol (v/v) – separate 11-KT.

11) Oven dry plates at 100°C for 3 mins.

12) 45 min exposure to obtain photograph of each plate in a Radiochromatogram Spark Chamber.

13) Scrape off the silica gel from the plate where the location of 11-KT is indicated.

14) Elute 11-KT from silica gel with 1 ml methanol.

15) Centrifuge tubes at 2000 rpm for 2 mins.

16) Place 100 µl of supernatant into an assay tube (in duplicate). Place 250 µl of supernatant into a scintillation vial for recovery determination – see how much radio-labelled 11-KT is lost in waste plasma sample.

17) Evaporate methanol in assay tubes under a vacuum.

18) Add 100 µl of assay buffer (0.05 M phosphate, 0.1% gelatine, w/v) and incubate over night at 4°C.

19) Make separate standards to run alongside samples. Prepare standard solutions of 11-KT to give a series from 1 ng to 4 pg in 100 µl of buffer.

20) Add 100 µl of buffer containing antisera at 1/5000 dilution and add labelled 11-KT to give 5000 ct/min/tube.

21) Incubate over night at 4°C. (In each tube radio-labelled 11-KT and plasma 11-KT compete for binding to antibody (Figure 3.3.4).

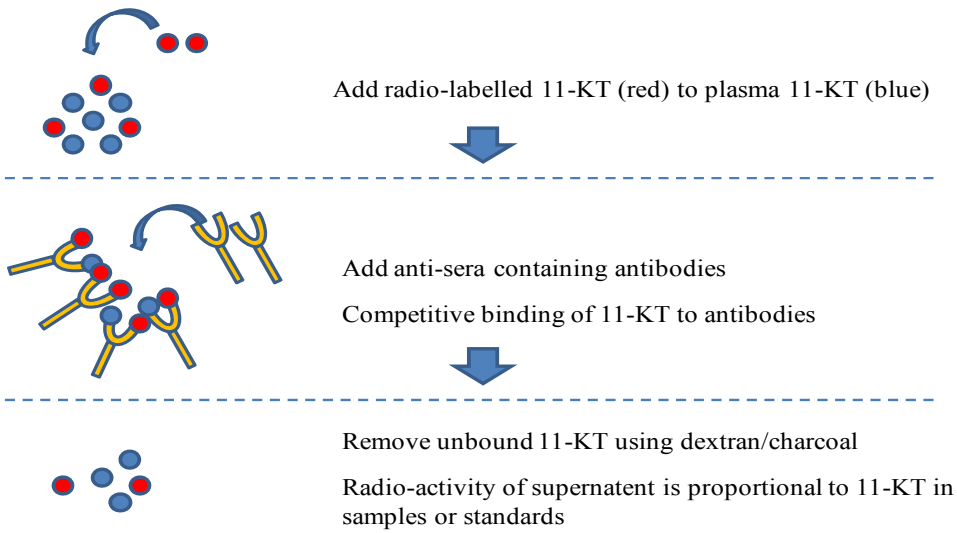
22) Separate unreacted 11-KT by adding 1 ml of dextran-coated charcoal suspension in buffer, keep on ice for 15 mins.

23) Centrifuge at 2000 rpm for 15 mins and pour off supernatant into scintillation vials.

24) Add 10 ml of scintillation fluid (toluene 1l, 4 g PPO, 100 mg POPOP).

25) Scintillation count each tube for 10 mins.

Figure 3.3.4 Principle steps in radioimmunoassay



3.4 Gonadal Histopathology

Gonadal histopathology was performed in Chapters 4, 5 and 7 according to the OECD draft guidance document for the diagnosis of endocrine-related histopathology of fish gonads (Johnson et al., 2009).

3.4.1 Tissue processing and wax embedding

1. Whole bodies were fixed with the visceral cavity closed after necropsy/removal of the liver and right gonad in Bouins solution (Sigma Aldrich, Dorset, UK). Bouins solution was replaced with 70% ethanol after 4-6 hours and this was replaced with fresh 70% ethanol after 24 hours prior to storage.

2. Heads were removed in front of the gill operculum and tails were removed behind the anus using a sharp scalpel. Heads and tails were retained in 70% Industrial Methylated Spirits (IMS). The trunks were placed in labelled tissue cassettes and progressively dehydrated in 70-100% IMS/Ethanol (Sigma Aldrich, Dorset, UK), Histoclear™ (National Diagnostics, Hessle, Humberside, UK) and embedded in paraffin wax (Paraplast™) using a Thermo Shandon Citadel 2000 tissue processor (Thermo Finnigan, Hemel Hempstead, UK):

Embedding stage	Reagent	Temperature (°C)	Time (min)
1	70% IMS	Ambient	60
2	70% IMS	Ambient	60
3	80% IMS	Ambient	60
4	90% IMS	Ambient	90
5	95% IMS	Ambient	90
6	100% IMS	Ambient	90
7	100% Ethanol	Ambient	90
8	Histoclear	Ambient	90
9	Histoclear	Ambient	90
10	Histoclear	Ambient	60
11	Paraffin (vacuum on)	60	60
12	Paraffin (vacuum on)	60	120

3. Trunks were then removed from the tissue cassettes and placed, tail facing downwards, in a wax mould, which was then filled with paraffin wax and the cassette base placed on the top. Moulds were placed on a cooling plate to set.

3.4.2 Microtomy and slide preparation

4. Wax blocks were microtomed using a Leica RM2145 Microtome (Leica Microsystems UK Limited, Milton Keynes, UK). Four ribbons of serial tranverse sections (5 μm) were cut at intervals of 500-1000 μm and placed in 40% ethanol and then in a water bath at 40°C, before being mounted onto glass microscope slides and dried on a warm plate at 35-40°C overnight.

5. Slides were then cover-slipped using Histomount™ (National Diagnostics, Hessle, Humberside, UK) to fix cover-slips.

3.4.3 Slide staining

6. Slides were then stained using Haematoxylin and Eosin as follows:

Station number	Reagent	Time (min)
1	Histoclear	5
2	Histoclear	5
3	100% IMS	2
4	90% IMS	2
5	80% IMS	2
6	Tap water running	2
7 Stains basophilic, nucleic material	Harris Haematoxylin (non acidified)	15
8	Tap water running	2
9	Acid alcohol (0.5% conc. HCl in 70% IMS)	5 seconds
10	Tap water running	30 seconds
11	70% IMS / ammoniated	30 seconds

	alcohol	
12	Tap water running	30 seconds
13 Stains cytoplasm, collagen, muscle (eosinophilic)	Eosin Y aqueous	15 seconds
14	Tap water running	30 seconds
15	80% IMS	30 seconds
16	90% IMS	1
17	95% IMS	1
18	100% IMS	2
19	100% Ethanol	2
20	Histoclear	2
21	Histoclear	3

3.4.4 Gonad staging and examination of gonadal pathologies

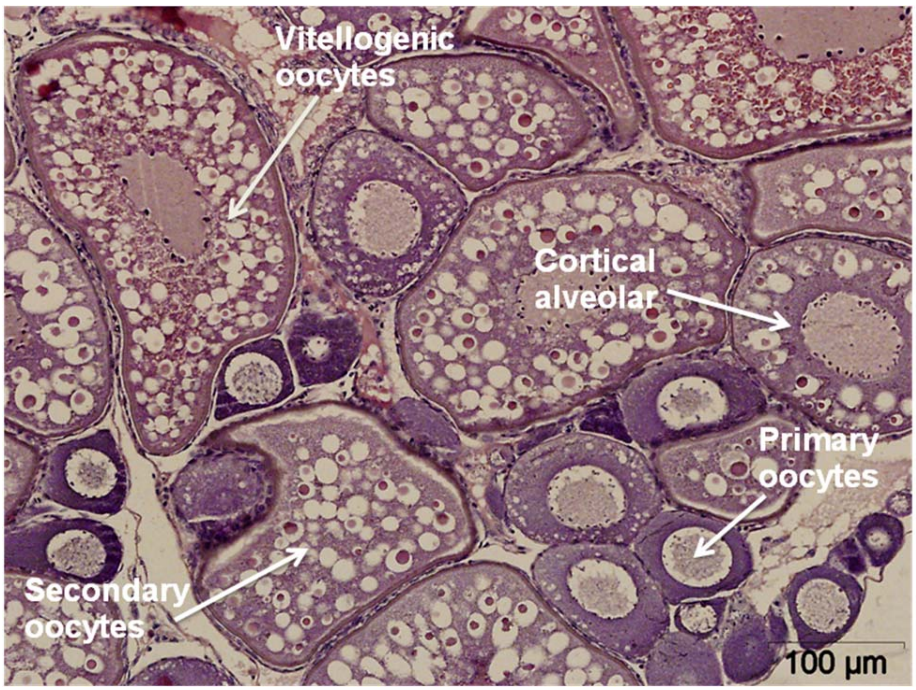
7. Slides were examined using a Leitz Diaplan light microscope (×10-100) magnification).

8. Gonadal germ cell progression was staged either at the most advanced stage in each of the transverse sections or as the % proportions of gonadal cell types in males: spermatogonia; spermatocytes; spermatids/ spermatozoa and in females: primary oocytes; cortical alveolar/2ndry oocytes; vitellogenic oocytes (Figure 3.4.1), from each experimental treatment. For proportional counts, the occurrence of each cell type was counted at intersections of a 10×10 (=100) 10 μm grid square overlaid on two section images per male.

9. The proliferation of interstitial Leydig and Sertoli cells were quantified according to a relative severity scale: 0 =unremarkable; 1=mild hyperplasia; 2=moderate hyperplasia (Johnson et al., 2009) (Figure 3.4.2)

Figure 3.4.1: Staging of germ cell progression

Female germ cell stages at x10 magnification:



Male germ cell stages at x40 magnification:

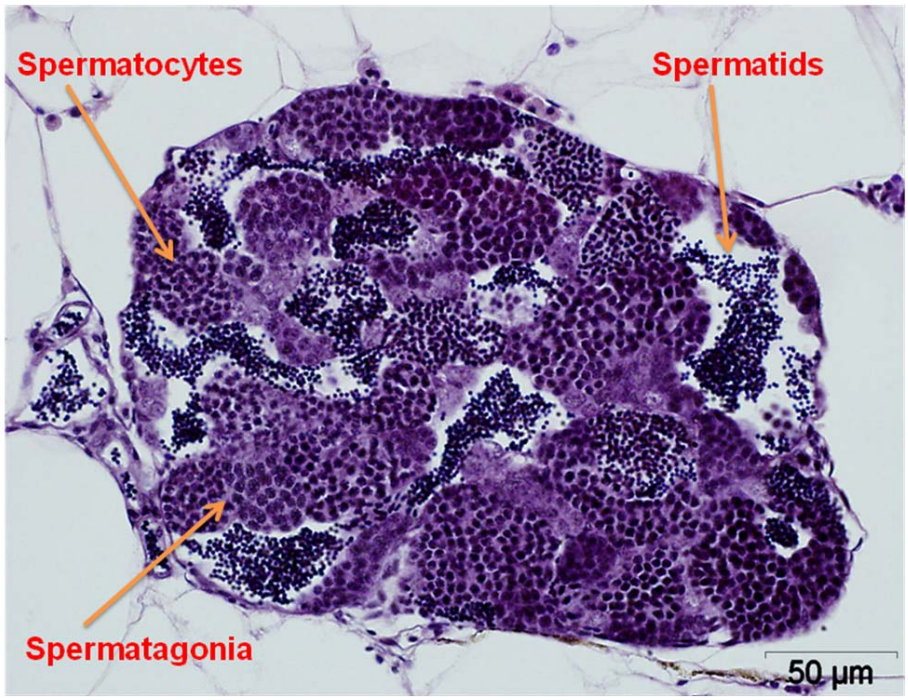
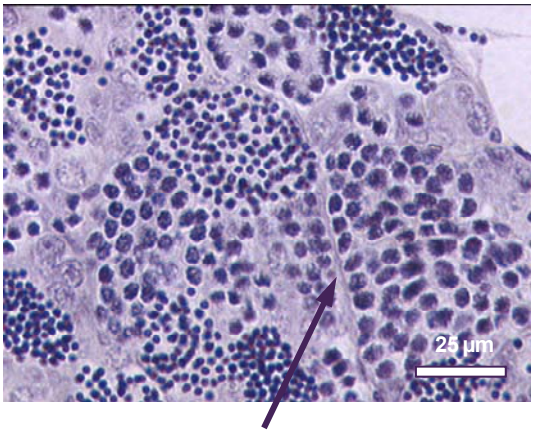


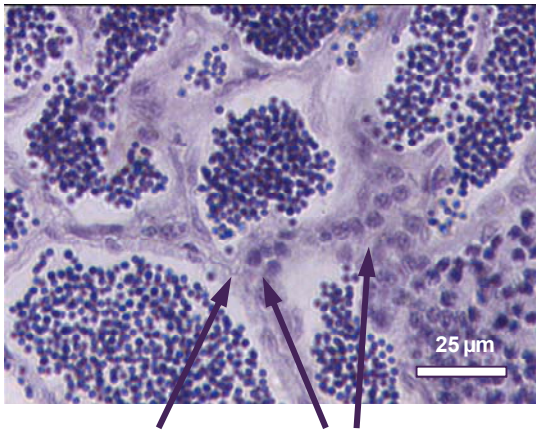
Figure 3.4.2: Leydig cell proliferation in testis of inbred males compared with oubred males following 96 days high-level exposure to clotrimazole (43.7 µg/L) (Chapter 5)

Testis – inbred male



Interstitium (no Leydig cells visible)

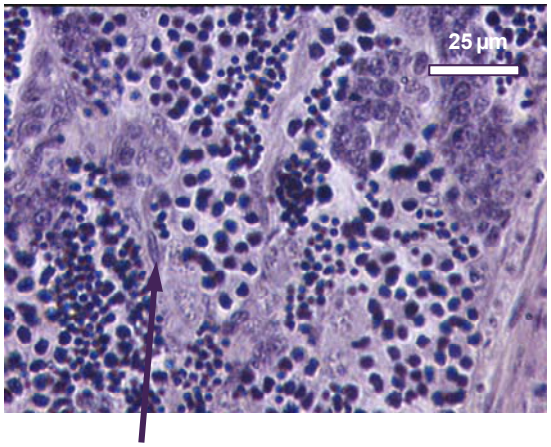
Testis – outbred male



Interstitium Leydig cells

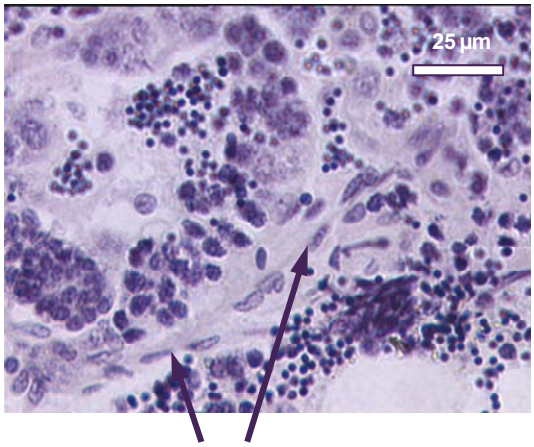
Sertoli cell proliferation in the testis of inbred males compared with oubred males

Testis – inbred male



Interstitial Sertoli cell

Testis – outbred male



Interstitial Sertoli cells (abundant)

3.5 Population Viability Analysis

The population viability analysis (PVA) model Vortex© (Lacy et al., 2005) was used in Chapter 8 to predict extinction probability (PE) , mean time to extinction (MTE) and per capita population growth rate (r) for wild zebrafish populations, based on ”control” life-history data from the available published literature and data from experimental studies (Chapter 7). Predictions were made with and without simulating inbreeding depression on age 0+ survivorship, assuming 50% to be caused by recessive lethal alleles (subject to purging) and 50% attributed to sub-lethal alleles (Simmons and Crow, 1977). It was for this latter capability in particular that I selected to use this model in my research.

3.5.1 Description of the Vortex PVA model

Vortex© is owned and copyrighted by the Chicago Zoological Society and is provided at no cost and without warranty. The current version of software is v9.99 (Lacy et al., 2005) and is available for download along with a user manual (Miller and Lacy, 2005) at http://www.vortex9.org/vortex.html. The model is a contribution of the IUCN/SSC Conservation Breeding Specialist Group to further conservation and science.

Vortex is a stochastic, individual-based simulation model for population viability analysis (PVA) with a Microsoft Windows-based graphical user interface. The program was developed in C++ programming language and is presented within an interface developed in MS Visual Basic. It will install and run properly on computers with Pentium (or newer) processors running Win95, Win98, WinXP, Win2000, or WinNT operating systems.

Vortex simulates a population by stepping through a series of sequential events (e.g., births, maturation, reproduction, deaths) that occur according to defined probabilities (e.g. standard deviations) and essentially describe the typical life-cycle of sexually reproducing diploid organisms. The program was written originally to model mammalian and avian populations, but its capabilities have improved so that it can now be used for modeling other groups including fish, invertebrates, or even plants—if they have relatively low fecundity or could be modeled as if they do (Table 3.5.1).

Table 3.5.1: Applicability of the individual-based PVA model Vortex compared to alternative models

***Vortex* is less appropriate**	***Vortex* is more appropriate**
High fecundity	Low fecundity
Short lifespan	Long lifespan
Polyploid	Diploid
Genetic effects of little interest	Changes in genetic variation of interest
Local population (N) > 500	Local population (N) < 500
> 20 populations modelled	< 20 populations modelled
Demographic rates not estimable (only population growth trajectories known)	Age-specific fecundity and survival rates estimable
Stage- or size-dependent demography	Age-dependent fecundity and survival rates
Demographic rate fluctuations not estimable	Fluctuations in rates can be estimated
No catastrophic events of interest	Catastrophic events modelled
Only polygamous breeding	Polygamous or monogamous breeding
Random breeding	Non-random distribution of fecundity (some adults excluded from breeding)

Key attributes relevant to my research are highlighted in blue.

3.5.2 PVA model parameterisation

Parameters quantifying mean demographic rates/population parameters and standard deviations were input to Vortex under the ”Simulation Input” tab using a series of input screens (Figure PVA.2):

1. Scenario Settings - enter

Scenario name e.g. ”Inbred low clotrimazole 33”, no. of iterations = 100, no. of years = 100

Extinction definition = only one sex remains

No. of populations = 1

2. Species description - enter

Inbreeding depression – check box, Lethal equivalents = 5, % due to recessive lethal = 50

EV (environmental variation) concordance of reproduction and survival – check box

No. of catastrophes = 0

3. Labels and state variables – leave

4. Dispersal – not available since only one population simulated

5. Reproductive system – enter

Mating system = polygynous

Age of first offspring for males and females = 1 (i.e. age 1+)

Maximum age of reproduction = 1 (then = 2 in additional simulations during sensitivity analysis)

Maximum number of broods per year = 6 (=60/10, NB no. of offspring per female per brood* increased by a factor of 10 – see step 6 Reproductive rates)

Sex ratio at birth (% males) = 42.1-97.2%

6. Reproductive rates - enter

% adult females breeding = 100

EV in % breeding = 5 (then = 50 in additional simulations during sensitivity analysis)

Distribution of broods per year: 0 broods = 0, 1 broods = 16.666%, 2 broods = 16.666%, 3 broods = 16.666%, 4 broods = 16.666%, 5 broods = 16.666%, 6 broods = 16.666%

Distribution of no. of offspring per female per brood*: mean = 500; SD = 200 (i.e. 50 ± 20 (x10))

7. Mortality rates - enter

Mortality of males and females (%)

Inbreds: Age 0-1 = 96, SD = 3; Annual mortality after age 1 = 10, SD = 3

Alternatively

Outbreds: Age 0-1 = 91, SD = 3; Annual mortality after age 1 = 10, SD = 3

8. Mate monopolisation – enter

% Males in the breeding pool

(Following are calculated assuming stable age distributions: % Males successfully siring offspring).

9. Initial population size - enter

Stable age distribution - check box

Initial population size = 4000 (Distribution of males and females calculated from stable age distribution and sex ratio)

10. Carrying capacity - enter

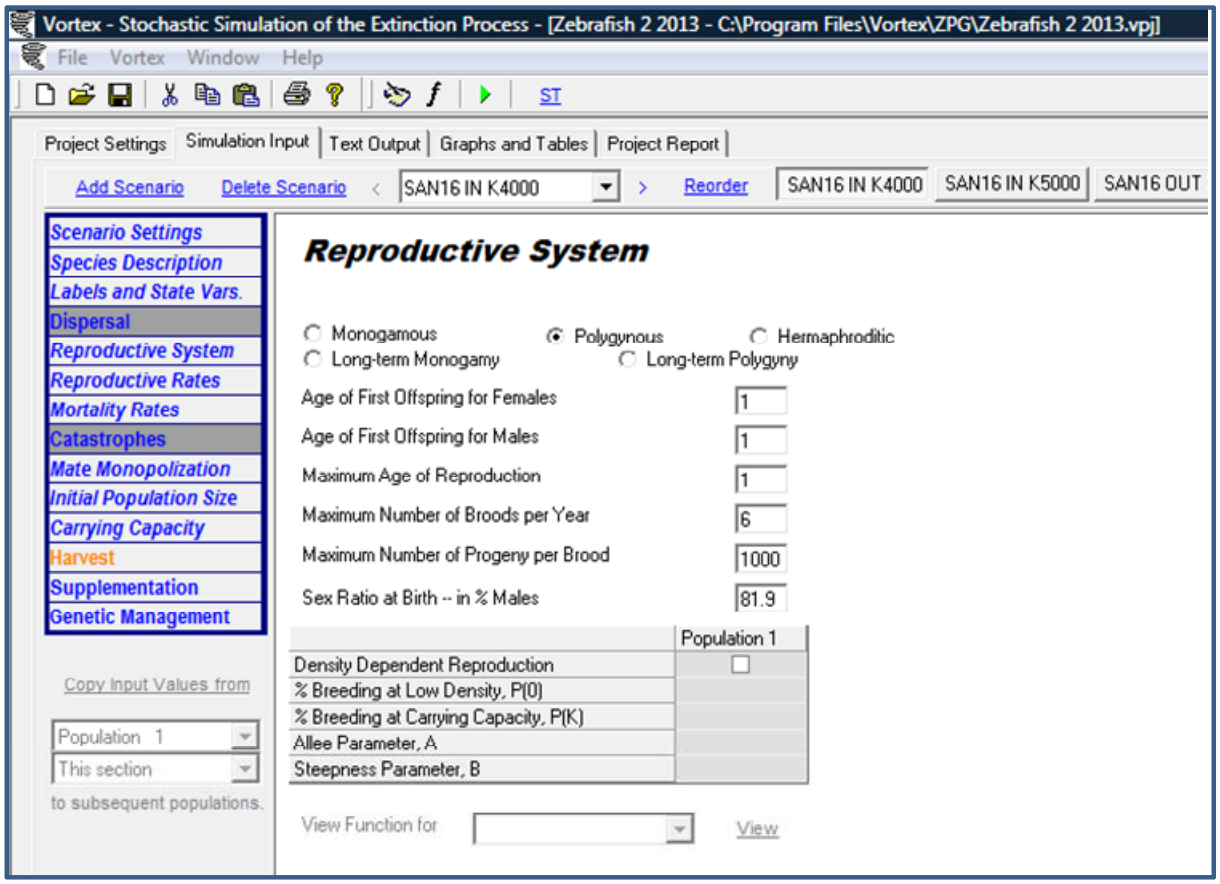
Carrying capacity = 5000, SD = 1000 (initial simulation)

Future change in carrying capacity – leave unchecked

11. Harvest, 12. Supplementation, 13. Genetic management – un check all options

After entering all input parameters – press run (Green arrow icon). Select **Text output** tab.

Figure 3.5.2: Vortex model input screen showing step 5 – reproductive system



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4 CHAPTER 4: DIFFERENCES IN SEXUAL DEVELOPMENT IN INBRED AND OUTBRED ZEBRAFISH AND IMPLICATIONS FOR CHEMICAL TESTING

Citation

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Differences in sexual development in inbred and outbred zebrafish (*Danio rerio*) and implications for chemical testing

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ABSTRACT

Outbred laboratory animal strains used in ecotoxicology are intended to represent wild populations. However, breeding history may vary considerably between strains, driving differences in genetic variation and phenotypes used for assessing effects of chemical exposure. We compared a range of phenotypic endpoints in zebrafish from four different "breeding treatments" comprising a Wild Indian Karyotype (WIK) zebrafish strain and a WIK/Wild strain with three levels of inbreeding ($F_{IT} = n$, $n + 0.25$, $n + 0.375$) in a new Fish Sexual Development Test (FSDT). There were no differences between treatments in terms of egg viability, hatch success or fry survival. However, compared with WIKs, WIK/Wild hybrids were significantly larger in size, with more advanced gonadal (germ cell) development at the end of the test (63 days post fertilisation). Increasing the levels of inbreeding in the related WIK/Wild lines did not affect body size, but there was a significant male-bias (72%) in the most inbred line ($F_{IT} = n + 0.375$). Conversely, in the reference WIK strain there was a significant female-bias in the population (80% females).

Overall, our results support the use of outbred zebrafish strains in the FSDT, where one of the core endpoints is sex ratio. Despite increased variance (and reduced statistical power) for some endpoints, WIK/Wild outbreds ($F_{IT} = n$) met all acceptance criteria for controls in this test, whereas WIKs failed to comply with tolerance limits for sex ratio (30–70% females). Sexual development was also more advanced in WIK/Wild outbreds (cf. WIKs), providing greater scope for detection of developmental reproductive toxicity following chemical exposure.

1. Introduction

1.1. The interaction of genetic and environmental factors and their influence on phenotype

The ability to detect biological effects of chemical exposure is dependent on the level of the response induced, as well as the consistency of the targeted phenotype. Consistency in phenotypic traits in laboratory animals therefore has major relevance for chemical testing, hazard identification and risk assessment. Consequently the use of isogenic lines (virtual clones) is advocated in mammalian toxicology, thus reducing both the level of statistical replication and numbers of animals required, in turn having both financial and ethical benefits (NAS, 2007). However, in ecotoxicology, arguably laboratory test animals with phenotypic variation and underlying genotypic variation representative of wildlife populations are more appropriate for protecting those populations (Evenden and Depledge, 1997; Brown et al., 2009). If susceptibility to chemical exposure is influenced strongly by genetic factors, isogenic lines or clones may have limited value in predicting toxicity to wildlife populations (Forbes and Depledge, 1992). Indeed standing genetic variation in wildlife populations is correlated directly with population fitness (Reed and Frankham, 2003) and adaptability to environmental change (Hoffman and Parsons, 1991; Lande and Shannon, 1996; Reed et al., 2003), including novel chemical exposure (reviewed in Brown et al., 2009). This is because rare alleles linked with novel stress resistance are more likely to occur within larger populations containing more genetically diverse individuals (Hoffman and Parsons, 1991). The interaction between genotypes and environmental factors can also lead to even greater variation in phenotypes within populations (Pigliucci et al., 1996), and this in turn can facilitate better utilisation of ecological resources in changeable environments (Snorrason and Skúlason, 2004). The variable expression of individual phenotypes (determined by a given genotype) under different environmental conditions is termed phenotypic plasticity (Bradshaw, 1965). One form of phenotypic plasticity is developmental conversion

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(Smith-Gill, 1983), a threshold response in which an environmental factor, when at a critical level, triggers developmental change. A prime example is sex determination and differentiation in fish. In the majority of fish species studied so far, sex appears to be determined primarily by genotype, but can be subject to genotype × environment interaction (Ospina-Alvarez and Piferrer, 2008). Water temperature appears to be a key environmental factor co-determining sex in fish (Delvin and Nagahama, 2002; Baroiller et al., 2009). Elevated temperatures consistently have been shown to promote the development of male fish (Ospina-Alvarez and Piferrer, 2008). In zebrafish (*Danio rerio*) this may occur via apoptosis of primordial germ cells (Baroiller et al., 2009), apoptosis of immature (presumptive) oocytes and localised inhibition of gonadal aromatase ≥35 °C (Uchida et al., 2004). Sexual phenotype in fish may also be influenced by dissolved oxygen, pH, salinity, nutrition, crowding and social interaction (reviewed in Delvin and Nagahama, 2002; Godwin et al., 2003; Uchida et al., 2004; Baroiller et al., 2009). So far no definitive genetic sex marker has been identified in zebrafish, instead the potential involvement of multiple genes in sex determination (von Hofsten and Olsson, 2005; Bradley et al., 2011) provides scope for multiple genotype × environment interactions. This could lead to the evolution of adaptive variation in sex determination in populations experiencing different environmental conditions, as shown in the Atlantic silverside (*Menidia menidia*) in response to geographical variations in temperature (Conover and Heins, 1987). Consequently different populations could yield different sex ratios under the same conditions.

1.2. Consequences of inbreeding and interaction with environmental factors

The fastest acting and most significant biological mechanism contributing to the loss of genetic variation within wildlife populations is inbreeding, leading to inbreeding depression (Brook et al., 2002), the depression in fitness traits following the increased frequency of homozygotes and the expression of deleterious, recessive alleles and/or the loss of heterozygote superiority (Charlesworth and Willis, 2009). Interactions between inbreeding and environmental (including chemical) stress can amplify depression in fitness traits and increase phenotypic variance (Crnokrak and Roff, 1999; Armbruster and Reed, 2005). The latter appears to be a more universal symptom of inbreeding × environment interaction (Armbruster and Reed, 2005) and is another consequence of the emergent expression of recessive alleles, due to additive genetic variance (Falconer and Mackay, 1996). Due to practical constraints limiting effective population sizes (i.e. number of breeding individuals), laboratory animal strains are generally more inbred, less genetically diverse and more susceptible to genetic drift than their wild counterparts, resulting in noticeable differences between them, as illustrated in the zebrafish (Coe et al., 2009; Whiteley et al., 2011). Studies associating genetic and environmental variation with variation in developmental phenotypes including growth, sexual differentiation and gonadal development are generally lacking in the zebrafish. There is evidence of some phenotypes (body size) being more variable amongst inbred zebrafish compared to outbreds, while other phenotypes (gonad size) have been show to be less variable or non-responsive following exposure to the aromatase inhibitor clotrimazole (Brown et al., 2011). "Environmental" factors including increased nutrition and reduced stocking densities have been shown to lead to enhanced growth rates and female development in zebrafish (Lawrence et al., 2008), which is consistent with adaptive sex allocation affording a greater pay-off for larger size in females (Parker, 1992). Genetic out-crossing of different zebrafish strains has also been shown to promote female-biased sex ratios compared with pure bred strains held under the same environmental conditions (Lawrence et al., 2008).

1.3. Study outline

Here we compared a range of developmental phenotypic endpoints and their variation in four zebrafish "breeding treatments" under standardised control conditions in a new Fish Sexual Development Test (FSDT) (Holbech et al., 2006; OECD, 2011). Breeding treatments included a WIK zebrafish strain (used as a standard reference) and a WIK/Wild hybrid zebrafish strain consisting of three related family lines with different levels of inbreeding–outbreeding. We hypothesised that growth (body size) would be depressed and variability increased with increasing levels of inbreeding in the related WIK/Wild lines due to inbreeding depression (Charlesworth and Willis, 2009) and additive genetic variance (Falconer and Mackay, 1996), respectively (Section 1.2). In accordance with growth-related sex allocation (Parker, 1992) we also predicted an increasing male-bias in sex ratios in the more inbred lines. We consider the implications of our findings in terms of the need to further standardise the regulatory testing of chemicals, by characterising (by pedigree) and/or qualifying (by genotyping) the animal strains used.

2. Materials and methods

2.1. Study design and test conditions

The study aimed to examine a range of developmental phenotypic endpoints and their variation in four zebrafish "breeding treatments" with different levels of inbreeding, under standardised control conditions. The study design followed the OECD Guideline 234: Fish Sexual Development Test (OECD, 2011).

The initial study set up (see Table 1) incorporated four families of zebrafish representing each of the four breeding treatments (Section 2.2). Thirty viable embryos were selected randomly from each family for use in the study. At 1 day post fertilisation (dpf) viable embryos were transferred from petri dishes containing sterilised, aerated laboratory water to a dynamic (flow-through) test system consisting of glass beakers with a 1 l working volume and a nominal flow rate of the dilution water of 4.8 ml min^{-1} (equivalent to an exchange of 6.9 working volumes of water per day). At 28 dpf, juvenile fish were transferred to 9.5 l glass, flow-through aquaria with a nominal flow rate of the dilution water of 42 ml min^{-1} (6.3 working volumes per day). From 4 to 15 dpf fish were fed to excess twice a day (am and pm) with ZM000 grade food (Zebrafish Management Ltd., Winchester, UK). Excess waste food was removed prior to feeding each morning. Between 16 and 63 dpf ZM000 was replaced by ZM100 at a rate of feeding of 3% body weight per day. From 9 dpf, freshly hatched *Artemia salinus* nauplii were provided as additional food in the morning and afternoon at a feeding rate of 5% body weight per day.

The following test conditions were maintained throughout the study: water temperature 26.0–27.4 °C; dissolved oxygen 6.2–7.8 mg l^{-1} (>60% saturation); pH 7.23–7.90; hardness 40–69 mg l^{-1}; alkalinity 25–35 mg l^{-1}; ammonia <2 μg l^{-1}; chlorine <2 μg l^{-1}; suspended solids <3.00 mg l^{-1}; DOC 0.41–0.56 mg l^{-1}; COD <12 mg l^{-1}; inorganic and organic analytes <predicted no effect concentrations. The photoperiod was set at 12 h:12 h light:dark with 20 min sunrise/sunset.

2.2. Zebrafish breeding treatments

The Wild Indian Karyotype (WIK) zebrafish strain, derived originally from a single pair mating of second generation wild-caught Indian zebrafish (WIK11) (Rauch et al., 1997), was sourced via Zfin

Table 1
Test design that was based on the Fish Sexual Development Test (OECD Guideline 234).

Breeding treatment	Inbreeding coefficient	Family/tank	No. viable embryos[a]
WIK/Wild outbred	($F_{IT} = n$)	$W/W_{outbred}$ 1	30
		$W/W_{outbred}$ 2	30
		$W/W_{outbred}$ 3	30
		$W/W_{outbred}$ 4	30
WIK/Wild inbred	($F_{IT} = n + 0.25$)	W/W_{inbred} 1	30
		W/W_{inbred} 2	30
		W/W_{inbred} 3	30
		W/W_{inbred} 4	30
WIK/Wild inbred × 2	($F_{IT} = n + 0.375$)	$W/W_{inbredx2}$ 1	30
		$W/W_{inbredx2}$ 2	30
		$W/W_{inbredx2}$ 3	30
		$W/W_{inbredx2}$ 4	30
WIK	(F_{IT} unknown)	WIK 1	30
		WIK 2	30
		WIK 3	30
		WIK 4	30

Note: embryos in replicate WIK families W1–W3 were pooled from three distinct families, whereas WIK family W4 comprised embryos from one of these three families. All WIK/Wild families were distinct, comprised of embryos derived from four separate parental pairs per breading treatment (see Fig. 1 and Supplementary Table S1 for further details of breeding design).

[a] Number of viable embryos per family/tank at the start of the study.

(www.zfin.org) from the Zebrafish International Resource Centre in Eugene, Oregon, USA. The Zfin WIK zebrafish were maintained in our laboratory in two breeding populations, each with 100–200 fish, which were crossed via pair-breeding using a minimum of 20 pairs per generation (F_0–F_3) to minimise inbreeding. Three pairs were subsequently used to produce F_4 fish for this study (Fig. 1a). Three replicate, pooled families were derived from the mixing of eggs from the three parental pairs (according to the FSDT guideline) and one distinct family was preserved by partitioning some eggs from one of the three parental pairs (Fig. 1a).

The WIK/Wild hybrid zebrafish strain used in the study was derived initially from WIK laboratory females and wild Bangladesh males, using 20 pairs per generation (F_0–F_3), see Brown et al. (2011) for further details. These WIK/Wild hybrids were subsequently used to generate F_4 related (within population) inbred progeny resembling laboratory strains and outbred progeny resembling wild populations, which were clearly differentiated in terms of their heterozygosity: $WIK/Wild_{outbred}$ ($F_{IT} = n$); $WIK/Wild_{inbred}$ ($F_{IT} = n + 0.25$); $WIK/Wild_{inbredx2}$ ($F_{IT} = n + 0.375$), the "*n*" notation reflecting the unknown pedigree of the F_0 parents (Fig. 1b). Each line of the WIK/Wild strain consisted of four families obtained as separate batches of eggs from four distinct parental pairs (Fig. 1b).

The breeding programmes for the WIK strain and the three lines of the WIK/Wild hybrid strain are detailed further in Supplementary Table S1. The use of F_4 generation fish (after hybridisation at F_0) in our study guarded against outbreeding depression in the hybrid WIK/Wild lines. The symptoms of outbreeding depression may be confused with inbreeding depression, however they occur as a result of initial outbreeding and the breakdown of co-adapted gene complexes in genetically divergent populations (e.g. between WIK and Wild zebrafish populations). It has been shown that these complexes are generally restored or reconfigured by the F_3 generation (Templeton, 1986; Lynch, 1991; Monson and Sadler, 2010).

2.3. Genetic variation in each breeding treatment

Genetic variation was assessed in eight randomly collected embryos from each of the pooled WIK "family replicates" and the distinct WIK family, as well as from each of the WIK/Wild lines and corresponding families. These were additional embryos to those reared from 0 to 63 dpf in the treatment assessment study. These embryos were sampled at 1–2 dpf, before hatching and fixed in 90% (v/v) ethanol at −20 °C. DNA was extracted according Bruford et al. (1998) and genetic variation was assessed using 11 microsatellite loci: Z249; Z266; Z374; Z1233; Z4830; Z5058; Z6140; Z9230; Z13614; Z20450 (www.zfin.org) and Ztril (Coe et al., 2008) as described previously in Brown et al. (2011). Results were

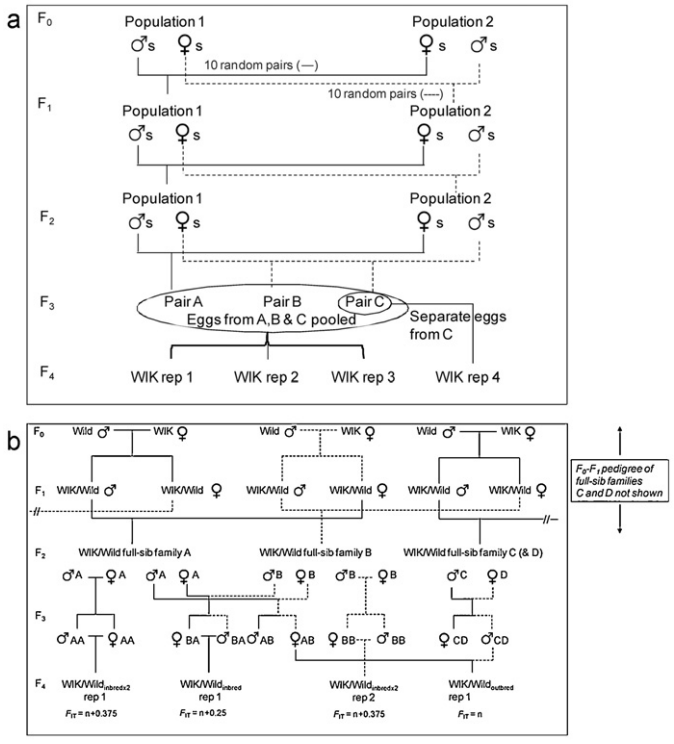

Fig. 1. Pair-breeding designs used to generate zebrafish (F_4 generations) used in this study. (a) WIK zebrafish strain. The WIK strain was maintained in two breeding populations, that were cross bred for F_0–F_4 generations. The F_4 generation fish used in the study consisted of three pooled replicates, in which eggs from three parental pairs were pooled and sampled three times (OECD, 2011) and one distinct family replicate derived from one of the three pairs. (b) WIK/Wild zebrafish strain. The WIK/Wild strain consisted of 16 full-sibling families (A–P) at the F_2 generation (families E–P are not shown). The F_4 generation fish used in the study consisted of three lines (outbred, inbred, inbred × 2), each represented by four families obtained as separate batches of eggs from four distinct parental pairs. F_{IT} is theoretical inbreeding coefficient, the "*n*" notation reflecting the unknown pedigree of the F_0 wild males and WIK females.

interpreted using various statistical software: Microsatellite toolkit v3.1.1 (Park, 2001); Fstat v2.9.3.2 (Goudet, 2001); IRN4 (Amos et al., 2001); Genepop v4.0.10 (Rousset, 2008); Coancestry v8.0 (Wang, 2010). A suite of indices were calculated for each of the four zebrafish strains: observed and expected multi-locus heterozygosity (H_O and H_E); standardised allelic richness (R'); effective inbreeding coefficient (F_{IS}). Additional indices were also determined: internal relatedness (IR) (Amos et al., 2001); homozygosity by loci (HL) (Aparicio et al., 2006).

2.4. Sampling and analysis of phenotypic endpoints

Embryo development was assessed at 2, 6 and 24 hours post fertilisation (hpf) with the aid of a binocular microscope (10× magnification). After 24 hpf, the number of viable embryos were counted and nominally 30 from each parental cross were obtained and assigned randomly to separate flow-through beakers, in which the numbers of hatched fry were counted each morning between 2 and 5 dpf. Subsequent fry survival, feeding and swimming behaviour and the emergence of any morphological abnormalities were monitored on a daily basis until the end of the test. At 28 dpf the numbers of surviving fry were counted before being transferred to separate aquaria (for each family), providing a definitive check against in situ counts made during the initial test phase. At 63 dpf individual fish were removed from each of the aquaria and anaesthetised terminally using 500 mg l^{-1} benzocaine, followed by destruction of the brain. Standard length and wet weight were determined and any morphological abnormalities recorded. Whole fish, with the visceral cavity opened, were placed in Bouins fixative (Sigma–Aldrich) for a maximum of 6 h and then washed twice in 70% industrial methylated spirits (IMS), at 12 h and 24 h post sampling. Fish were progressively dehydrated in IMS up to 100% and embedded in paraffin wax. Four serial transverse sections (5 μm) per individual were cut at intervals of 500–1000 μm and placed on glass slides. Sections were then stained using haematoxylin and eosin and analysed using a light microscope (10–100× magnification). Gonadal differentiation was recorded as either male testis, female ovary, unknown (gonad not identified or found) or undifferentiated gonad. In zebrafish the initial undifferentiated gonad becomes female-like (Maack and Segner, 2003) and subsequently differentiates fully as female: primary oocyte – cortical alveolar – secondary oocyte – vitellogenic oocyte, or male: spermatogonia – spermatocyte – spermatid – spermatozoa. The stage of gonadal development was recorded according to the most mature germ cells present (Supplementary Fig. S1). Gonadal staging was treatment blinded and was verified by a second, experienced histopathologist.

2.5. Statistical analysis

Embryo viability, hatching success and rate, and post-hatch fry survival were expressed as a percentage of the starting number in each of the four families in each of the four zebrafish breeding treatments. These data were transformed (arcsin $\sqrt{\text{precentage}/100}$) to promote normality and equality of variances. Data for individual length and weight measurements, numbers of fish differentiated as males, females or remaining undifferentiated, and numbers of fish in each gonadal development stage within each treatment replicate at the end of the study were not transformed. In all cases, normality was assessed using the Anderson–Darling test and data were then assessed for equality of variances between each zebrafish breeding treatment using Bartlett's or Levene's test prior to statistical analysis. Data satisfying these tests were analysed using parametric tests, e.g. ANOVA, while non-conforming data were subject to non-parametric tests, e.g. Kruskal–Wallis. Subsequent statistical tests concerning each endpoint are stipulated in the appropriate section of the results. Principal Components Analysis (PCA) was used to assess the combined variance in sex (male or female) and gonadal development stage (three stages in males: spermatogonia; spermatocytes; spermatids/spermatozoa and three stages in females: primary oocytes; cortical alveolar/secondary oocytes; vitellogenic oocytes) see Supplementary Figs. S1 and S4. All statistical analyses were run in Minitab 15 (Minitab, Coventry, UK) and differences were accepted as significant when $p \leq 0.05$. Values are quoted as mean values and ranges as standard error of the mean.

3. Results

3.1. Genetic variation in each breeding treatment

The genetic variation within zebrafish breeding treatments, in terms of mean number of alleles per microsatellite locus, standardised allelic richness (R'), observed heterozygosity (H_O) and expected heterozygosity (H_E) decreased in the following order: WIK/$\text{Wild}_{\text{outbred}}$ > WIK/$\text{Wild}_{\text{inbred}}$ > WIK/$\text{Wild}_{\text{inbred}\times 2}$ > WIK. Multi-locus (microsatellite) estimates of inbreeding coefficients (F_{IS}, IR and HL) all showed the opposite trend and exceeded coefficients based on known pedigree (F_{IT}) (Table 2). Multi-locus heterozygosity for the WIK strain ($H_O = 0.37 \pm 0.01$, $H_E = 0.56 \pm 0.02$) was comparable to that reported for other reported laboratory zebrafish strains ($H_O = 0.40$–0.62, $H_E = 0.39$–0.68), while the WIK/$\text{Wild}_{\text{outbred}}$ line ($H_O = 0.60 \pm 0.01$, $H_E = 0.69 \pm 0.02$) was intermediate between these laboratory strains and wild zebrafish populations ($H_O = 0.71 \pm 0.08$, $H_E = 0.86 \pm 0.02$) (Coe et al., 2009). Pair-wise comparisons of sample means and standard deviations for H_O [using a 2-sample t-test, without assuming equal variances] showed that the WIK strain and the outbred line of the WIK/Wild strain were significantly different from each other and significantly different from the inbred WIK/Wild lines ($p < 0.0001$).

3.2. Phenotypic endpoints

3.2.1. Embryo viability and development (0–1 dpf)

Three out of four family replicates in the WIK/$\text{Wild}_{\text{inbred}\times 2}$ line showed early cleavage within the first 2 h. Otherwise, embryo development progressed normally from the initial blastocyst stage to the morula stage at 2 hours post fertilisation (hpf). All viable embryos were observed to be tail-free by 24 hpf. At this time, the proportion of viable embryos in each breeding treatment (mean range 61–94%) were not significantly different ($F_{(3,12)} = 2.01$, $p = 0.167$) [one-way ANOVA], indicating no detectable effect due to pedigree or inbreeding on embryo viability (Fig. 2a). There are no stipulated acceptance criteria regarding the proportion of viable embryos in controls in the OECD guidelines (OECD, 2011).

3.2.2. Embryo hatching success (3–5 dpf)

The majority of viable embryos in each breeding treatment (mean range 77–92%) hatched at 3 dpf (nominal hatching time) and there were no statistically significant differences between strains ($F_{(3,12)} = 0.46$, $p = 0.716$) [one-way ANOVA]. All surviving embryos from each strain had hatched by 4 or 5 dpf and therefore the latter time point (5 dpf) was used to determine hatching success. The total mean proportion of viable embryos hatching as sac-fry, up to 5 dpf, was ≥94% in each strain (Fig. 2b), which exceeds the OECD guideline requirement of >80% hatching success for controls (OECD, 2011) and there were no significant differences between breeding treatments ($F_{(3,12)} = 3.09$, $p = 0.068$) [one-way ANOVA].

3.2.3. Post-hatch fry survival (5–63 dpf)

Post-hatch fry survival in each breeding treatment was 77–83% for the period 5–28 dpf and 76–82% for the period 5–63 dpf (mean

Table 2
Genetic diversity indices and inbreeding coefficients for zebrafish derived from different breeding treatments.

Statistics		Breeding treatment			
		WIK/Wild outbred $F_{IT} = n$	WIK/Wild inbred $F_{IT} = n + 0.25$	WIK/Wild inbred × 2 $F_{IT} = n + 0.375$	WIK F_{IT} unknown
Sample size	No. of individuals sampled per breading treatment	24	24	32	16
Loci	No. loci typed	11	11	11	11
Allelic richness[a]	No. of alleles per locus ± SD	5.55 ± 1.97	5.09 ± 1.14	4.36 ± 1.29	3.09 ± 1.58
	Standardised allelic richness (R') ± SD	4.71 ± 1.10	4.18 ± 1.00	3.74 ± 1.57	2.87 ± 1.12
Allelic heterozygosity[b]	Observed heterozygosity (H_O) ± SD	0.60 ± 0.03	0.46 ± 0.03	0.46 ± 0.03	0.37 ± 0.04
	Expected heterozygosity (H_E) ± SD	0.69 ± 0.05	0.61 ± 0.06	0.62 ± 0.03	0.56 ± 0.05
Allelic homozygosity[c]	Homozygosity by loci (HL)	0.37	0.51	0.53	0.61
Internal relatedness[d]	Internal relatedness (IR)	0.17	0.32	0.33	0.53
Effective inbreeding coefficient (F_{IS})[e]	Wright's within-population estimate of inbreeding (from Fstat)[1]	0.14	0.25	0.26	0.34
	Wright's within-population estimate of inbreeding (from Genepop)[2]	0.16	0.21	0.27	0.32
	Ritland's moment estimate of inbreeding (from Coancestry)[3]	0.14	0.22	0.28	0.60
	Lynch and Ritland's moment estimate of inbreeding (from Coancestry)[4]	0.12	0.28	0.27	0.50
	Wang's likelihood estimate of inbreeding (from Coancestry)[5]	0.21	0.30	0.36	0.53
	Anderson and Weir's likelihood estimate of inbreeding (from Coancestry)[6]	0.22	0.33	0.38	0.54

Treatments: WIK/Wild Bangaldesh strain (3 lines outbred, inbred, inbred × 2) and WIK strain (single line).

[a] Allelic richness: mean number of alleles per locus. Standardised measure derived using the rarefaction method (El Mousadik and Petit, 1996) in Fstat (Goudet, 2001) http://www2.unil.ch/popgen/softwares/fstat.htm.

[b] Allelic heterozygosity: proportion of heterozygotes in each treatment meaned across all sampled loci. Expected heterozygosity (H_E) assumes Hardy Weinberg equilibrium in diploid allele frequencies. Fstat (Goudet, 2001).

[c] Allelic homozygosity (Aparicio et al., 2006). From IRmacroN4.xls (http://www.zoo.cam.ac.uk/zoostaff/meg/amos.htm).

[d] Internal relatedness: mean of all individuals in each treatment (Amos et al., 2001). From IRmacroN4.xls.

[e] Effective inbreeding coefficient: mean of all individuals in each treatment: (1) from Fstat using (Nei, 1977); (2) from Genepop (Rousset, 2008) http://genepop.curtin.edu.au/using (Weir and Cockerham, 1984); (3) from Coancestry (Wang, 2010) http://www.zsl.org/science/research/software/coancestry,1360,AR.html using (Ritland, 1996); (4) from Coancestry using (Lynch and Ritland, 1999); (5) from Coancestry using (Wang, 2007); (6) from Coancestry using (Anderson and Weir, 2007). Based on the most recently developed indices (5 and 6), the effective inbreeding coefficients of the WIK/Wild$_{inbred\times2}$ strain were similar to the theoretical inbreeding coefficient (F_{IT} = 0.375) but were also close to estimates for the WIK/Wild$_{inbred}$ strain (F_{IS} = 0.3–0.33). This relatively poor discrimination may be due to our limited sample size and considerable allelic variation (polymorphism) at the microsatellite loci sampled (Slate et al., 2004; Szulkin et al., 2010).

ranges) (Fig. 2c). This exceeded the OECD guideline of ≥70% survival for controls (OECD, 2011). There were no statistically significant differences between breeding treatments: (5–28 dpf, $F_{(3,11)} = 0.18$, $p = 0.91$); (5–63 dpf, $F_{(3,11)} = 0.07$, $p = 0.97$) [one-way ANOVAs]. In addition, the rate of mortalities and the resulting stocking densities in each treatment replicate (tank) were consistent across each of the treatments. No abnormal behaviour was observed in any of the surviving fish.

3.2.4. Body size (63 dpf)

There were significant differences in length and weight between breeding treatments at 63 dpf: mean standard length ($H = 19.83$, DF = 3, $p < 0.0001$); mean wet weight ($H = 13.21$, DF = 3, $p = 0.004$) [Kruskal–Wallis]. These results reflected true differences in body size, since there were no physical abnormalities in any of the breeding treatments. Pair-wise comparisons confirmed that mean standard lengths for each of the WIK/Wild lines: WIK/Wild$_{inbred\times2}$ (20.05 ± 0.32 mm, $p < 0.001$); WIK/Wild$_{inbred}$ (20.03 ± 0.33, $p < 0.001$); WIK/Wild$_{outbred}$ (19.72 ± 0.17, $p = 0.001$) were not significantly different from one another, but were greater than for the reference WIK strain (18.93 ± 0.17) [Mann–Whitney] (Fig. 3a). Pair-wise comparisons of mean wet weight also showed that WIK/Wild$_{inbred\times2}$ (141.0 ± 6.5 mg, $p = 0.002$) and WIK/Wild$_{inbred}$ (142.2 ± 6.8, $p = 0.007$), but not WIK/Wild$_{outbred}$ (127.8 ± 3.2, $p = 0.052$), were heavier than the WIK strain (116.8 ± 3.1) [Mann–Whitney] (Fig. 3b). Nevertheless, in each breading treatment body size exceeded the minimum acceptance criteria for controls: 14 mm standard length; 75 mg blotted wet weight (OECD, 2011).

3.2.5. Sexual differentiation and development (63 dpf)

At the end of the study, a proportion of fish in each breeding treatment, ranging from 7 to 16%, had not undergone sexual differentiation (and/or the sex could not be identified). Analysis of the entire sample sets (including both differentiated and undifferentiated fish) indicated that there were significant differences in sex ratio between treatments at 63 dpf (Fig. 4) ($\chi^2_{(6,N=332)} = 82.5$, $p < 0.001$) [Chi-square contingency table]. The proportion of females in the WIK strain (74/93 = 80%) was significantly higher than expected (i.e. 45% rather than 50%, given the proportion of fish that were not sexually differentiated) [χ^2 contribution = 24.9], whereas the proportion of males in the WIK/Wild$_{inbred\times2}$ line (58/81 = 72%) was significantly higher than expected (45%) [χ^2 contribution = 12.5].

Excluding the undifferentiated individuals, there were significant differences in both female germ cell development ($\chi^2_{(6,N=149)} = 78.1$, $p < 0.001$) and male germ cell development ($\chi^2_{(6,N=150)} =$

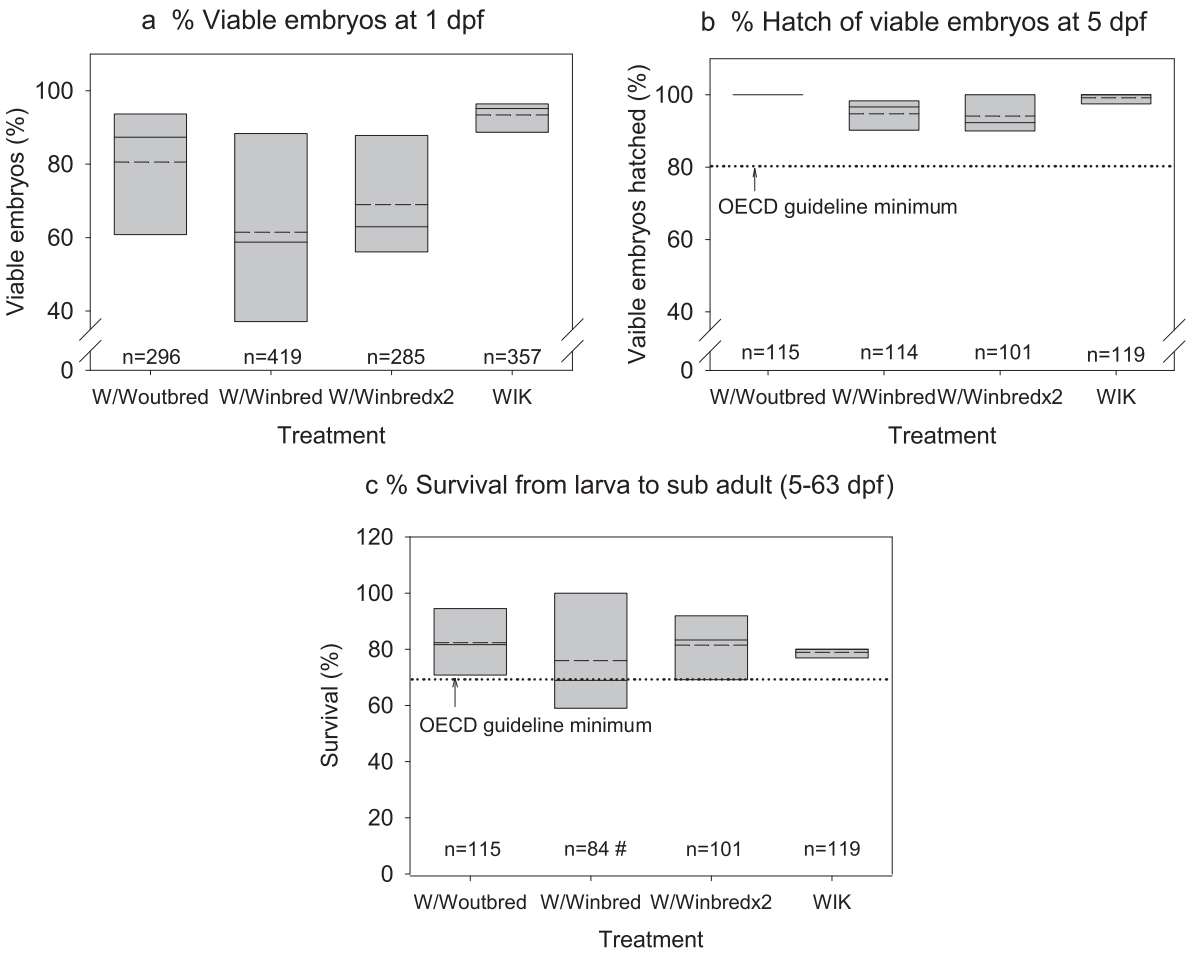

Fig. 2. Embryo viability, hatchability and subsequent survival to 63 days post fertilisation (dpf) in zebrafish derived from different breeding treatments. Treatments: WIK/Wild Bangladesh strain (W/W – 3 lines outbred, inbred and inbred × 2) and WIK strain (single line). Tank-level replication: 4 family "replicates" for each treatment = 16 tanks. (a) n = total no. of individuals spawned at 0 dpf per strain. (b and c) n = total number of viable embryos sampled at 1 dpf per strain (nominally 4 × 30 = 120). # 15 fry escaped between 5 and 6 dpf from 1 tank replicate, leaving only 3 viable tank replicates for the WIK/$Wild_{inbred}$ strain. Boxes show inter-quartile range, dashed line is the mean, solid line is the median.

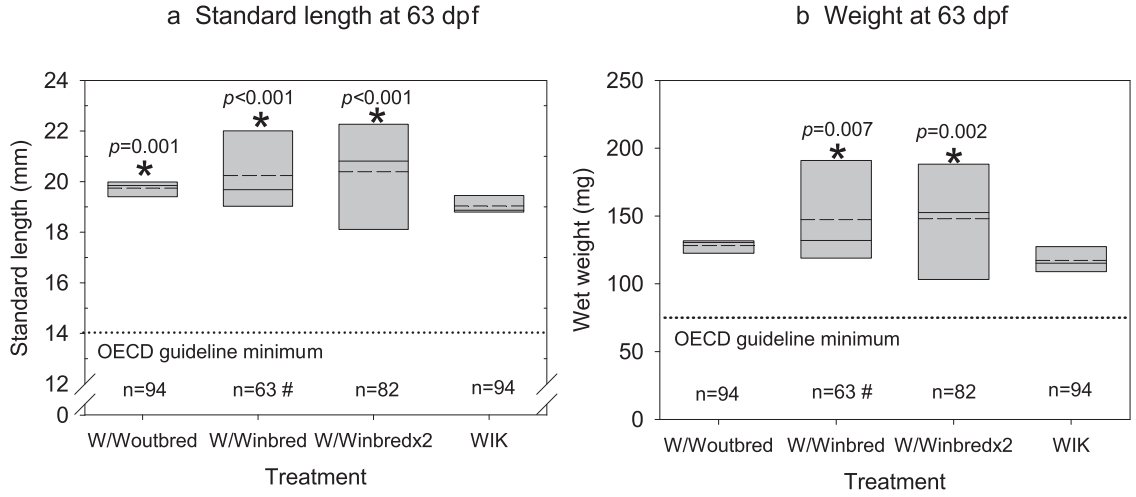

Fig. 3. Body length and weight in zebrafish derived from different breeding treatments. Treatments: WIK/Wild Bangladesh strain (W/W – 3 lines outbred, inbred and inbred × 2) and WIK strain (single line). Tank-level replication: 4 family "replicates" for each treatment = 16 tanks. # 15 fry escaped between 5 and 6 dpf from 1 tank replicate, leaving only 3 viable tank replicates for the WIK/$Wild_{inbred}$ strain. *Significantly different from the WIK strain according to Kruskal–Wallis non-parametric analysis of individual wet weight or standard length data, using strain as a predictor, followed by Mann–Whitney pair-wise comparisons. Boxes show inter-quartile range, dashed line is the mean, solid line is the median.

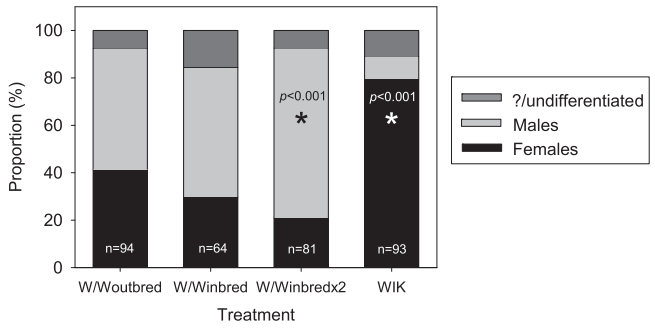

Fig. 4. Sexual differentiation in zebrafish derived from different breeding treatments (63 dpf). Treatments: WIK/Wild Bangladesh strain (W/W – 3 lines outbred, inbred and inbred × 2) and WIK strain (single line). Tank-level replication: 4 family "replicates" for each treatment = 16 tanks. ? indicates gonad was not identified or not found. *Significant difference from expected sex ratio according to a chi-square contingency table – treatments and genders highlighted contributed most to chi-square.

50.0, $p < 0.001$) between breeding treatments [Chi-square contingency table]. The proportion of WIK females with primary oocytes (71/74 = 96%) was significantly higher than expected (52%) [χ^2 contribution = 11.7], as was the proportion of WIK males with spermatogonia (7/9 = 78%) [χ^2 contribution = 22.9]. Female germ cell development in each line of the WIK/Wild strain was more advanced compared with the WIK strain (46–74% of females with cortical alveolar – vitellogenic oocytes compared to 4% in the WIK strain). Male germ cell development was also more advanced in each line of the WIK/Wild strain, with 54–85% of males containing spermatids – spermatozoa versus 0% in the WIK strain (Fig. 5). However, the proportion of mature male germ cells did not significantly exceed expected chi-square proportions.

Sexual differentiation (male versus female) and gonadal development (germ cell development stage) were related to body size within each breeding treatment [Two-way ANOVA of size (standard length and wet weight) with sex and stage as predictors] (Figs. 6 and 7). As expected, female phenotype corresponded with larger body size in each breeding treatment: WIK/Wild inbred lines standard length ($F_{(1,123)} = 17.25$, $p < 0.001$) and wet weight ($F_{(1,123)} = 24.15$, $p < 0.001$); WIK/Wild outbred line standard length ($F_{(1,83)} = 9.85$, $p = 0.002$) and wet weight ($F_{(1,83)} = 9.82$, $p = 0.002$); WIK strain standard length ($F_{(1,79)} = 10.83$, $p = 0.001$) and wet weight ($F_{(1,79)} = 11.70$, $p = 0.001$). Advancement in germ cell development in both sexes also corresponded with larger body size within each line of the WIK/Wild strain: WIK/Wild inbred lines standard length ($F_{(2,123)} = 19.18$, $p < 0.001$) and wet weight ($F_{(2,123)} = 20.51$, $p < 0.001$); WIK/Wild outbred line standard length ($F_{(2,83)} = 6.81$, $p = 0.002$) and wet weight ($F_{(2,83)} = 7.69$, $p = 0.001$). However, there was no significant relationship between body size and germ cell developmental stage in the WIK strain.

3.2.6. Variance in phenotypic endpoints

Variances (between tank replicates) in embryo viability and fry survival in each line of the WIK/Wild strain were over 14× and 34× greater, respectively, compared to the WIK strain (Fig. 2a and c). Variances in hatch success, growth and sexual development were similar in the WIK strain and WIK/Wild outbreds, while they were ≥3-fold greater in both inbred WIK/Wild lines (Figs. 2b and 3a and b). Further examination of body size and sexual development in the related WIK/Wild lines showed that phenotypic variances in WIK/Wild inbreds exceeded expectations based on levels of inbreeding (Supplementary Figs. S2–S4).

4. Discussion

Our results highlight significant differences in mean trait values and ranges for body size (wet weight and standard length) and sexual phenotype (gonadal sex and development stage) and sex ratios in inbred versus outbred WIK/Wild zebrafish lines maintained under standardised control conditions in a new Fish Sexual Development Test (FSDT). There were also noticeable differences between these WIK/Wild lines and our pure bred WIK zebrafish strain (used as a reference). This work supports existing concerns over the genetic and phenotypic consistency of laboratory animal

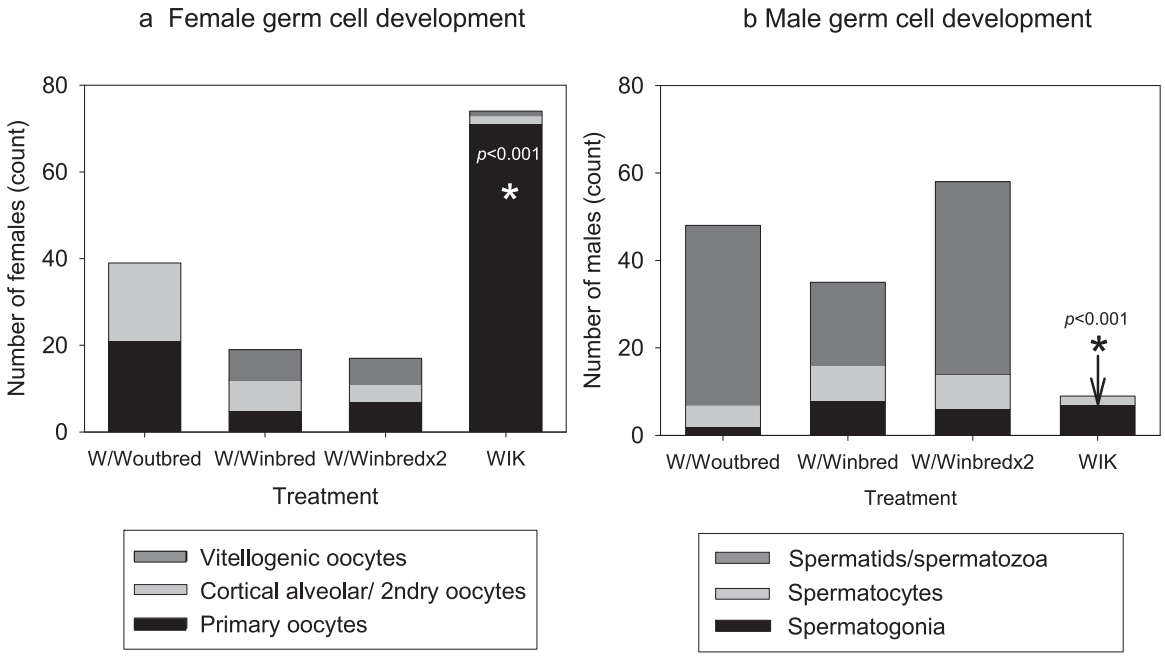

Fig. 5. Germ cell development in zebrafish derived from different breeding treatments (63 dpf). Treatments: WIK/Wild Bangladesh strain (W/W – 3 lines outbred, inbred and inbred × 2) and WIK strain (single line). Tank-level replication: 4 family "replicates" for each treatment = 16 tanks. The most advanced germ cell development stage was recorded in each individual. *Significant difference from expected proportion of germ cells according to a chi-square contingency table – treatments and germ cell development stages highlighted contributed most to chi-square.

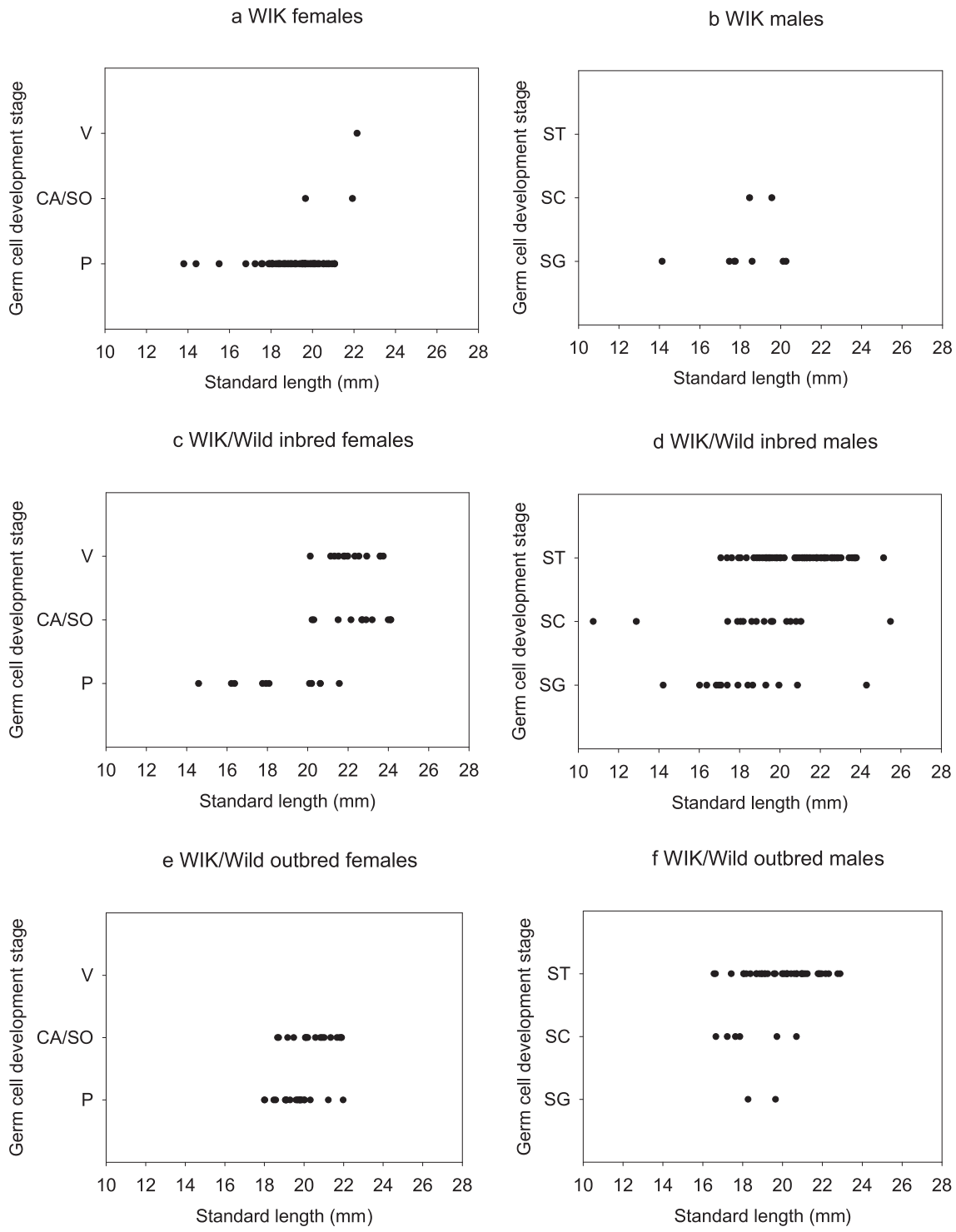

Fig. 6. Standard length compared with the most advanced stage of germ cell development in zebrafish derived from different breeding treatments (63 dpf). Points represent the standard lengths and the corresponding most advanced germ cell development stage for individual fish. Females: P – primary oocytes, CA/SO – cortical alveolar/secondary oocytes, V - vitellogenic oocytes. Males: SG – spermatogonia, SC – spermatocytes, ST – spermatids. (c and d) WIK/Wild inbreds consist of both WIK/$Wild_{inbred}$ and WIK/$Wild_{inbred\times2}$ lines.

strains/lines and consequently how repeatable (Nowak et al., 2007), statistically sensitive (NAS, 2007; Spence et al., 2008) and representative (Brown et al., 2009) ecotoxicological studies are for predicting effects on wildlife populations.

Inbreeding often occurs in laboratory animal populations and may lead to inbreeding depression and/or allelic loss (genetic impoverishment). Inbreeding depression has been identified as an important factor impacting on zebrafish survival and growth (Mrakovcic and Haley, 1979), especially in wild-sourced (McCune et al., 2004) and recently domesticated zebrafish strains (Piron, 1978). However, mortality leads to purging of lethal alleles and consequently smaller, already inbred populations, including laboratory animal populations, may be less prone to further inbreeding depression (Swindell and Bouzat, 2006). The effects of allelic loss may be less acute, but longer lasting, leading to inconsistencies between laboratory animal strains and therefore between studies

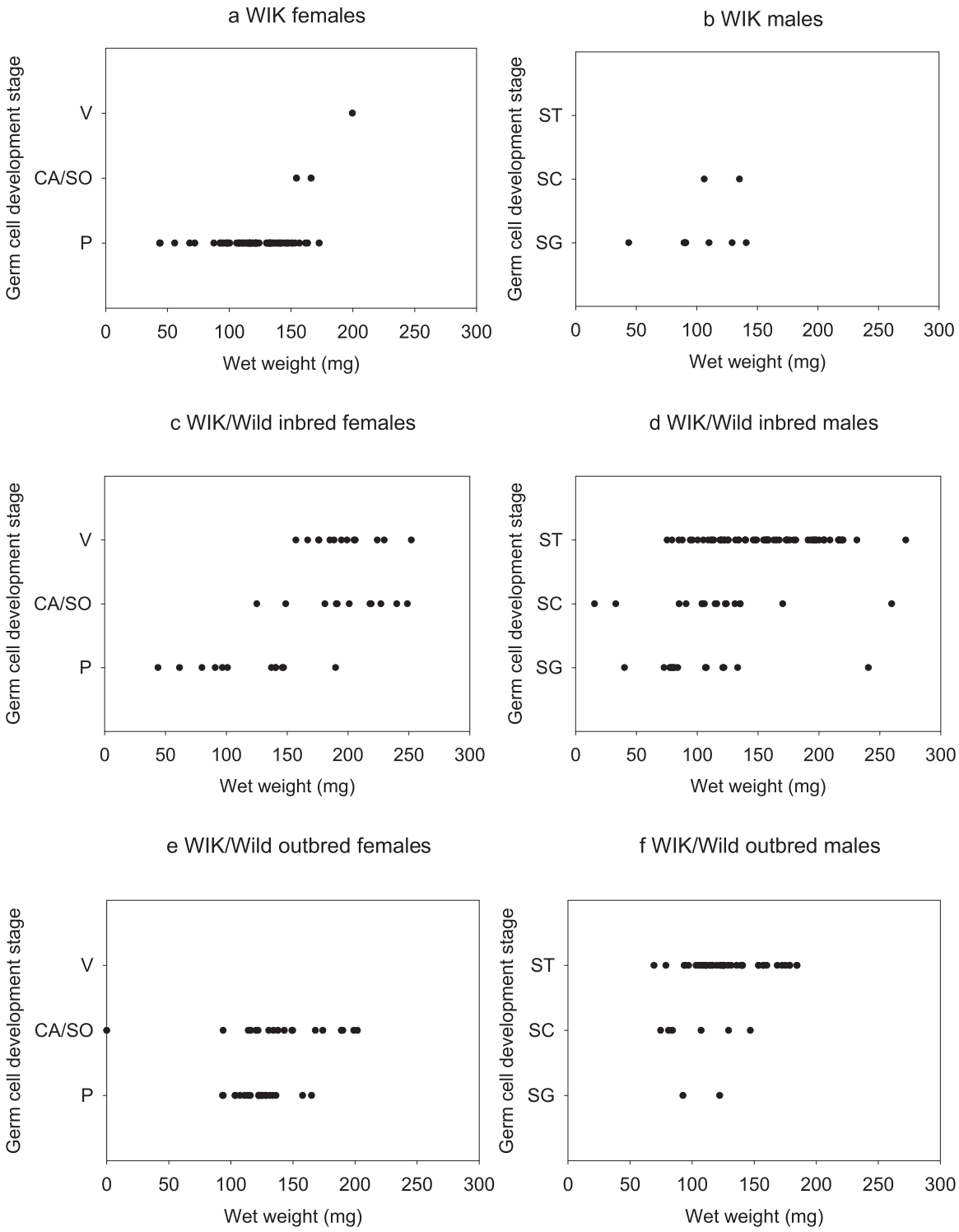

Fig. 7. Wet body weight versus most advanced stage of germ cell development in zebrafish derived from different breeding treatments (63 dpf). Points represent the standard lengths and the corresponding most advanced germ cell development stage for individual fish. Females: P – primary oocytes, CA/SO – cortical alveolar/secondary oocytes, V – vitellogenic oocytes. Males: SG – spermatogonia, SC – spermatocytes, ST – spermatids. (c and d) WIK/Wild inbreds consist of both WIK/Wild$_{inbred}$ and WIK/Wild$_{inbred\times2}$ lines.

(Nowak et al., 2007). We observed no indication of inbreeding depression on embryo viability, hatch success, fry survival (Fig. 2) or body size (Fig. 3): there were no significant differences between inbred and outbred WIK/Wild lines, and all values were within the recommended acceptance criteria for these endpoints for controls in the FSDT (OECD, 2011). In addition there were no signs of physical deformity in the inbred or outbred lines.

Inbreeding has also been shown to affect sexual development (without affecting growth) leading to male-biased sex ratios in pure bred (inbred) zebrafish strains compared with outcrossed strains (Lawrence et al., 2008). Similarly our results showed that increasing levels of inbreeding in the related WIK/Wild lines, although not affecting body size (Fig. 3), led to increasing proportions of male fish (Fig. 4) and a significant male-bias (72%) in

the WIK/Wild$_{inbred\times2}$ line (at the end of the study, 63 dpf). Conversely our reference WIK strain was significantly female-biased, consisting of 80% females, and suggesting alternative phenotypic plasticities in our WIK versus our WIK/Wild strains. Since zebrafish are juvenile hermaphrodites and the initial gonad is female-like (Maack and Segner, 2003), differentiation of the testis may be defined with more certainty than differentiation of the ovary in sub-adults (63 dpf). When studying different strains of zebrafish, Maack et al. (2003) found that a proportion of individuals in a heterozygous WIK strain and a more homozygous inbred WIK strain (ranging in total length from 12–23 mm and 13–22 mm respectively) did not complete sexual differentiation (transforming from presumptive ovary to testis) until 74–77 dpf. A proportion of individuals (ranging between 7 and 16% across breeding treatments) were sexually undifferentiated or their phenotypic sex could not be identified histologically at the end of our study (63 dpf). The subset of our fish that were confirmed as undifferentiated (3–8%) ranged in size between 14 and 20 mm standard length (from mouth to dorsal peduncle) and were therefore similar in size to those aged 74–77 dpf in the study reported on by Maack et al. (2003). This finding provides further confidence that sexual differentiation was complete in those fish that we were able to assign sex, since growth is a more reliable indicator of ontogeny of sexual development in fish compared to age (Paull et al., 2008; Lawrence et al., 2008). Females were associated with larger body size in the WIK strain, but there was no size-related effect on germ cell development in either sex in this strain. Female sex was also associated with larger body size in each line of the WIK/Wild strain. Advancement in germ cell development in both sexes also corresponded with larger body size within each line of the WIK/Wild strain. Furthermore, regardless of sex and level of inbreeding, WIK/Wild zebrafish were consistently larger in body size and their gonadal development more advanced than WIK zebrafish (Figs. 3 and 5). This may be attributed to hybrid vigour (heterosis), which has been associated with enhanced growth following outbreeding in various species of fish (McClelland and Naish, 2007).

Differences in embryo viability, hatchability and survival (serving as "control validation" endpoints in the FSDT) highlighted some differences in phenotypic variance between related WIK/Wild lines. Most notably variance in embryo hatchability in both inbred lines (90 ± 3–4%) was greater compared to the WIK/Wild$_{outbred}$ line (100 ± 0%). With the exception of embryo hatchability in the WIK/Wild$_{outbred}$ line, variances in these endpoints in the WIK/Wild lines were considerably greater than for the reference WIK strain (Fig. 2). Variance in body size (standard length and weight) constituting a more "focal" endpoint in the FSDT (related to sexual development) increased progressively with higher levels of inbreeding in the related WIK/Wild lines (Fig. 3). However, variance in body size was higher than predicted by inbreeding coefficient and the anticipated frequency of homozygote recessives (Falconer and Mackay, 1996). This phenomenon following inbreeding has been attributed elsewhere to the loss of heterosis and the associated breakdown of gene interactions (epistasis), which would otherwise control gene expression (Chevalet and Gillois, 1978). Variance in body size also mirrored variance in sexual phenotype (based on sex and stage of gonadal development) (Supplementary Figs. S2–S4). Variances in body size and sexual phenotype were greater in both inbred lines of the WIK/Wild strain (but not the outbred line) compared to the reference WIK strain. Low phenotypic variance for the majority of endpoints measured in the WIK strain may have been due, at least in part, to the use of three pooled family replicates and one individual family, as opposed to four discrete family replicates for each line of the WIK/Wild strain. Nevertheless, there was very low variation in embryo viability, hatchability and survival between the pooled and individual WIK family replicates and the results are consistent with greater genetic uniformity amongst the WIKs (Table 2).

All phenotypic endpoints measured in our study complied with tolerance limits recommended for zebrafish controls in the FSDT guideline (OECD, 2011), except for sex ratio (30–70% females, or vice versa 70–30% males). Only the WIK/Wild$_{outbred}$ line satisfied this criterion. Male-biased sex ratios observed in the WIK/Wild inbred lines compared with a female-bias in the WIK strain are likely to be due to plasticity in sex determination and the action of multiple genes (von Hofsten and Olsson, 2005; Bradley et al., 2011). Plasticity in sex is acknowledged in the FSDT guideline, which sets strict guidelines for water temperature, oxygen saturation and food ration, amongst other things, but also allows a degree of latitude in sex ratios in controls (as mentioned above). Development of the male phenotype in zebrafish has been demonstrated to be affected (induced) in response to inbreeding, reduced food ration and growth (Lawrence et al., 2008), elevated temperature ($\geq$35 °C) (Uchida et al., 2004) and hypoxia (0.8 mg/L) (Shang et al., 2006). This directional response to these stressors may be considered to be ecologically advantageous in terms of reproduction in fish, since reproduction in males is generally less metabolically demanding than for comparatively larger females (Conover and Kynard, 1981; Parker, 1992). In other words, in a fish population in which sex is determined environmentally and females are generally larger than males, an individual fish that is below the size of an average female is likely to be better off becoming an above-average sized male, since that individual is likely to perform better as a male (have greater reproductive fitness) than it would as a small female (adapted from Charnov and Bull, 1987). The bias towards males in the inbred WIK/Wild zebrafish lines under standardised control conditions is not entirely consistent with this theory since growth was not impaired by inbreeding. Nevertheless, the male-bias mirrors previously reported data showing a more direct effect in purebred (inbred) zebrafish strains (Lawrence et al., 2008). The opposing bias towards females in the reference WIK strain is intriguing, especially since the WIK fish were smaller in size and arguably more inbred compared with the WIK/Wild fish. A possible explanation is that, as plasticity in sexual phenotype is subject to adaptive variation via genotype $\times$ environment interaction, e.g. genotype $\times$ temperature (Ospina-Alvarez and Piferrer, 2008), our results reflect different breeding/evolutionary histories (encompassing different levels of inbreeding, selection and random genetic drift) between our WIK and WIK/Wild zebrafish strains/lines. These contrasting genetic backgrounds favour female development in WIKs versus male-biased development in the inbred lines of the WIK/Wild strain and more balanced sex ratios in the outbred WIK/Wild line under standardised control conditions. This conclusion is supported by preliminary studies performed during the development of the FSDT guideline, which showed some considerable variation in zebrafish sex ratios in control treatments (44–67% females) (Holbech et al., 2006), but with less bias in outbred zebrafish strains (Holbech, personal communication).

5. Conclusions

Overall, our results support the use of outbred zebrafish strains in the FSDT, in which one of the core endpoints is sex ratio. Despite increased variance in some endpoints, notably embryo viability and fry survival (Fig. 2, also see Supplementary Figs. S2–S4), the WIK/Wild outbred strain ($F_{IT} = n$) met all acceptance criteria stipulated for controls, whereas the WIK strain failed to comply with tolerance limits for sex ratio (30–70% females). Although our results for the WIK strain provided clear evidence of a bias in sex ratio, with a minimum of 80% confirmed females, it is important to note that a proportion of fish in each breeding treatment were undifferentiated

at the end of the study (63 dpf) and we may therefore have underestimated the bias.

Our main conclusions and recommendations from these findings are as follows: (i) Uncertainties surrounding developmental rates and resulting sex ratios in different zebrafish strains could be removed by extending the test by a further 2 weeks; (ii) Further standardisation of experimental test conditions, including setting a maximum and minimum daily ration would also be beneficial, given the influence of growth rate on ontogeny and sex determination in fish (Paull et al., 2008), including zebrafish (Lawrence et al., 2008); (iii) Similar attention should be paid to the nature (genetics) of laboratory animals as is applied currently to experimental (environmental) conditions. Pedigree and/or genetic information confirming the use of outbred strains (often advocated but rarely demonstrated in ecotoxicology) would also aid standardisation and promote the repeatability of studies (Nowak et al., 2007; Brown et al., 2009, 2011); (iv) A distinct advantage of using outbred strains highlighted by our WIK/Wild outbreds is that more advanced sexual development is attained within the stipulated time-frame of the FSDT, providing greater scope for the detection of developmental toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2012.01.017.

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4.1 Chapter 4 Supporting Information

TABLES

FIGURES

Supplementary Table S1
1a: Details of parental pair breeding conducted to generate the F_4 WIK zebrafish family lines used in the study

Breeding Treatment	Family (F_4 generation)	Paternal family (F_3 generation)	Maternal family (F_3 generation)
WIK (F_{IT}=?)	WIK 1	Pool of 3 (A,B, C)	Pool of 3 (A,B,C)
	WIK 2	Pool of 3 (A,B, C)	Pool of 3 (A,B,C)
	WIK 3	Pool of 3 (A,B, C)	Pool of 3 (A,B,C)
	WIK 4	C	C

The WIK strain consisted of three pooled replicates (WIK 1-3), in which eggs from three parental F_3 pairs (A,B,C) were pooled (according to the FSDT guideline) and three sets of 30 viable, fertilized eggs were sampled randomly from the pool. There was also a distinct family replicate (WIK 4) consisting of eggs from one of the three F_3 pairs (C). See also see Figure 1a in main article.

1b: Details of parental pair breeding conducted to generate the F_4 WIK/Wild inbred and outbred zebrafish family lines used in the study

Breeding Treatment	Family (F_4 generation)	Paternal family (F_3 generation)	Maternal family (F_3 generation)
WIK/$Wild_{outbred}$ (F_{IT}=n)	W/$W_{outbred}$ 1	O1	O2
	W/$W_{outbred}$ 2	O6	O7
	W/$W_{outbred}$ 3	O7	O8
	W/$W_{outbred}$ 4	O8	O9
WIK/$Wild_{inbred}$ (F_{IT}=n+0.25)	W/W_{inbred} 1	O15	O15
	W/W_{inbred} 2	O17	O17
	W/W_{inbred} 3	O18	O18
	W/W_{inbred} 4	O20	O20
WIK/$Wild_{inbredx2}$ (F_{IT}=n+0.375)	W/$W_{inbredx2}$ 1	I2	I2
	W/$W_{inbredx2}$ 2	I5	I5
	W/$W_{inbredx2}$ 3	I11	I11
	W/$W_{inbredx2}$ 4	I12	I12

F_3 generation fish consisted of inbred "I" (F_{IT}=n+0.25) and outbred "O" (F_{IT}=n) family lines, whose pedigrees have been described previously (Brown et al., 2011). See also Figure 1b in main article. The "n" notation reflects the unknown pedigree of the original (F_0) wild males and WIK females.

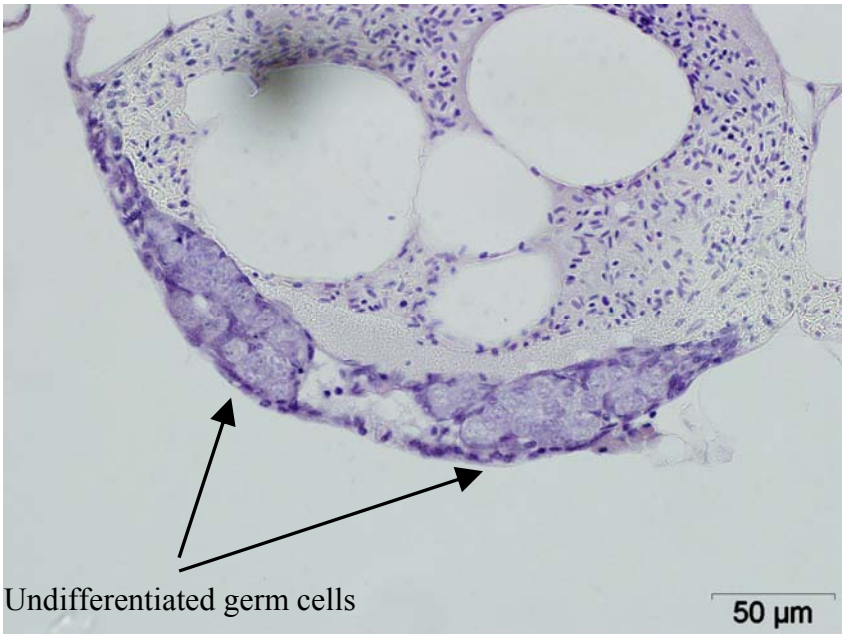
Supplementary Table S2
Variation in sex ratio per tank per breeding treatment

Breeding Treatment	Family (F_4 generation)	Number of females	Number of males	Sex ratio (% females)	Sex ratio range (%)	Variance (trans)
WIK/Wild$_{outbred}$ (F_{IT}=n)	W/W$_{outbred}$ 1	16	8	67	34	0.02
	W/W$_{outbred}$ 2	6	10	38		
	W/W$_{outbred}$ 3	7	14	33		
	W/W$_{outbred}$ 4	10	16	38		
WIK/Wild$_{inbred}$ (F_{IT}=n+0.25)	W/W$_{inbred}$ 1	3	17	15	56	0.1
	W/W$_{inbred}$ 2	4	13	24		
	W/W$_{inbred}$ 3	12	5	71		
	W/W$_{inbred}$ 4	-	-	-		
WIK/Wild$_{inbredx2}$ (F_{IT}=n+0.375)	W/W$_{inbredx2}$ 1	12	11	52	52	0.11
	W/W$_{inbredx2}$ 2	2	18	10		
	W/W$_{inbredx2}$ 3	0	14	0		
	W/W$_{inbredx2}$ 4	3	15	17		
WIK (F_{IT}=?)	WIK 1	19	0	100	26	0.08
	WIK 2	17	6	74		
	WIK 3	21	0	100		
	WIK 4	17	3	85		

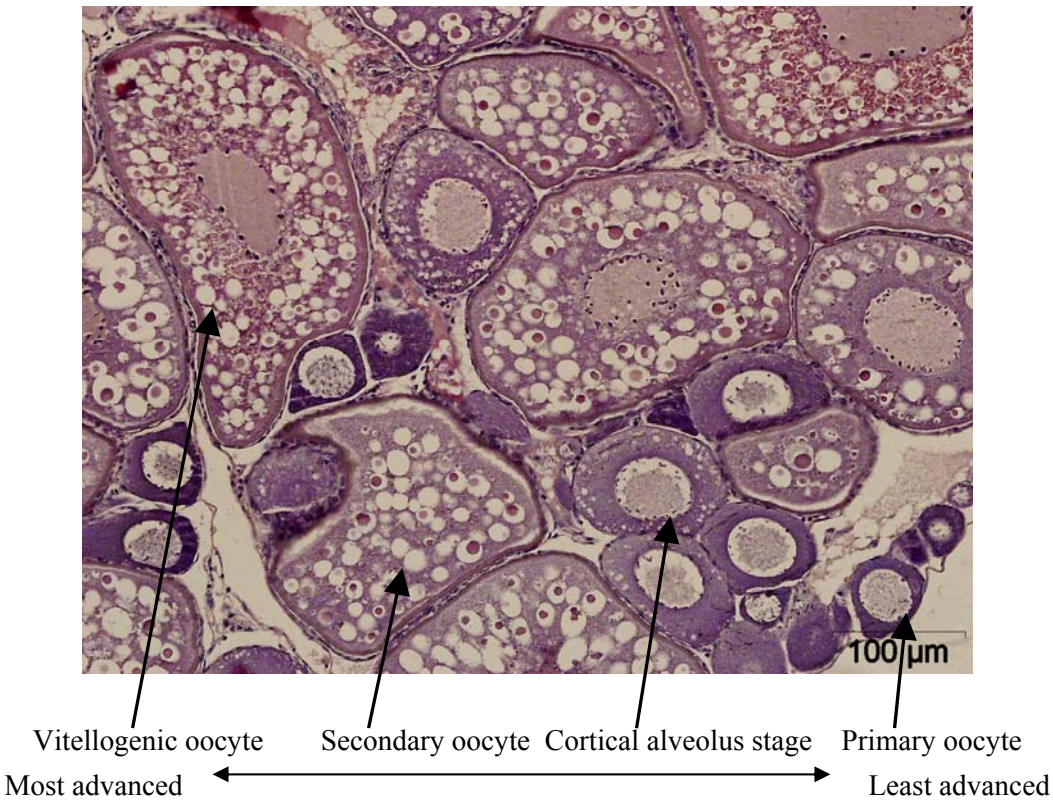
Notes: Sex ratio (% females) is the proportion 'percentage' of definitive females (over the sum of definitive males and females x 100).
Variance (trans) is determined from the arcsin square root transformed sex ratio (i.e. % females / 100) in each family/replicate per treatment.

Supplementary Figure S1
Sexual differentiation and development in zebrafish

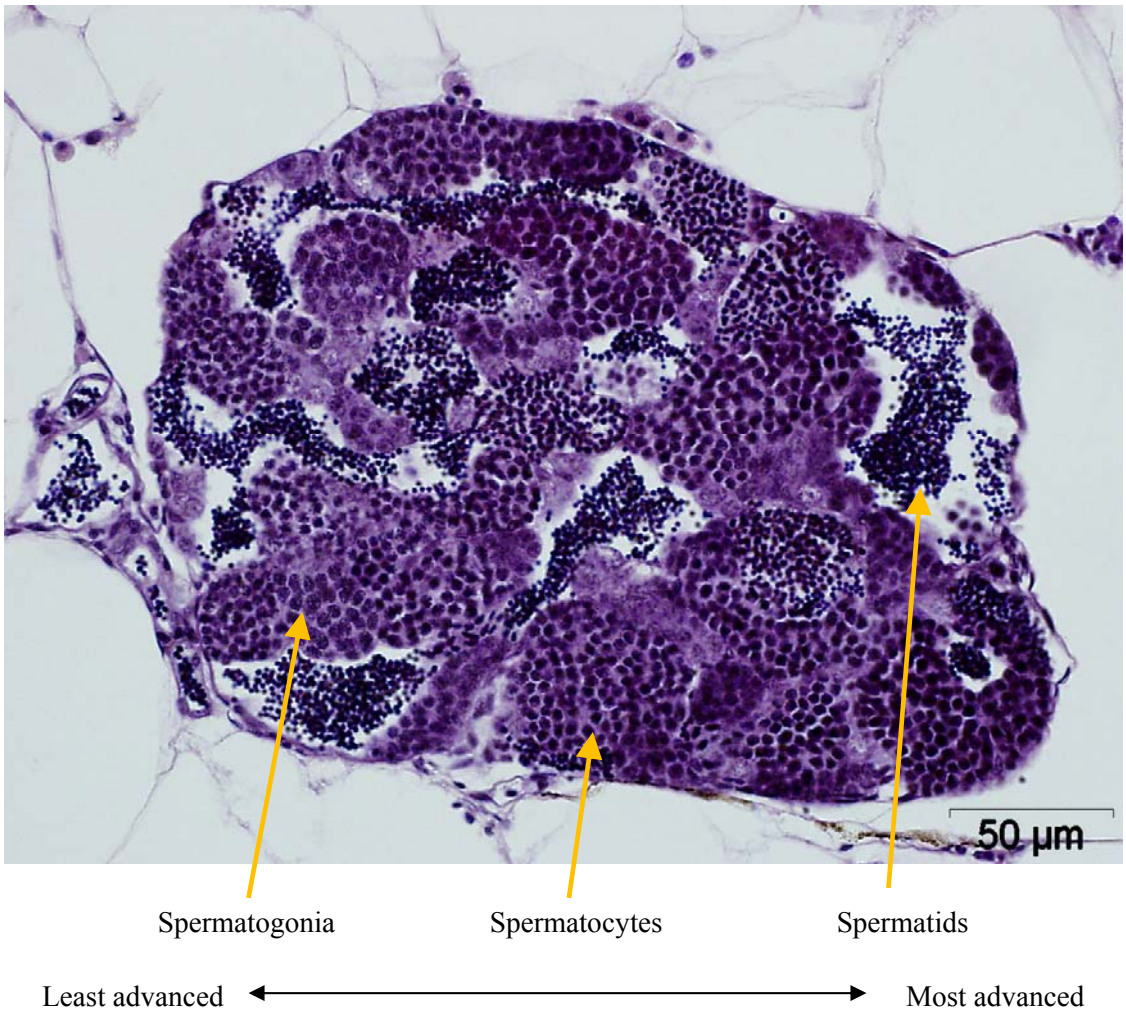
1a: Undifferentiated germ cells



1b: Female gonadal cell types

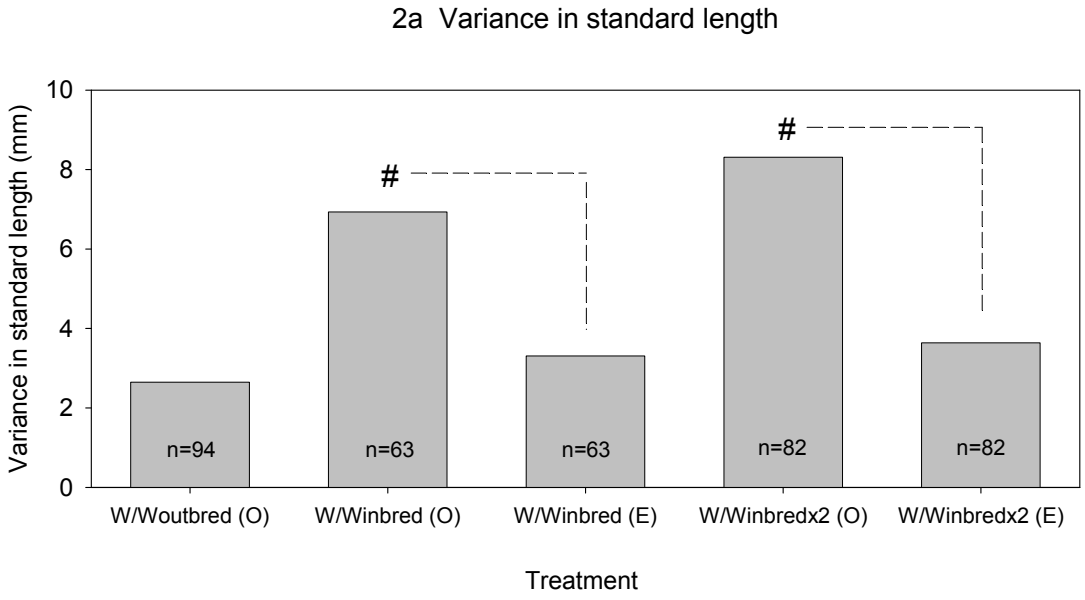


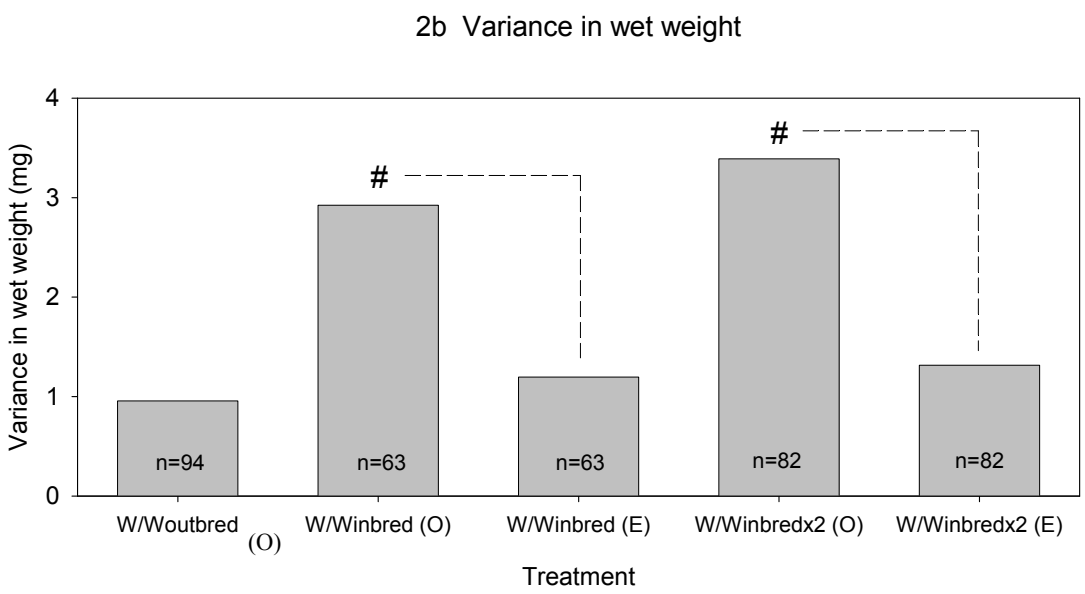
1c: Male gonadal cell types



Gonadal development was assigned based on the presence of the most advanced stage of gonadal cells present: e.g. in Figure 1a – undifferentiated germ cells; Figure 1b – vitellogenic oocytes; Figure1c – Spermatids.

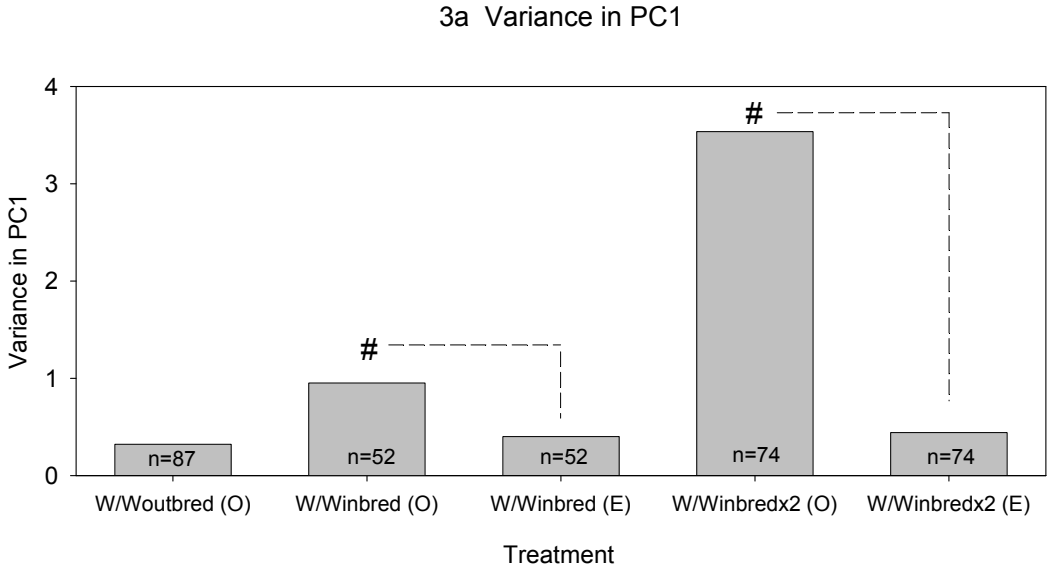
Supplementary Figure S2
Variance in body size in different lines of WIK/Wild zebrafish (63 dpf)

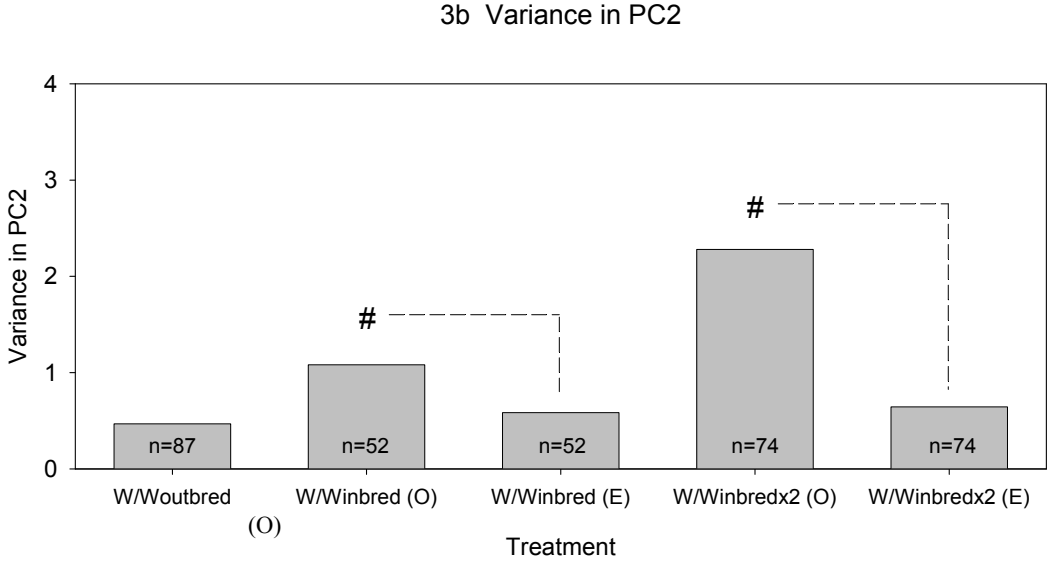




Treatments: WIK/Wild Bangladesh strain (W/W - 3 lines outbred, inbred and inbred×2). Tank-level replication: 4 family "replicates" for each treatment = 3 × 4 = 12 tanks.
>2-fold difference between observed (O) and expected (E) variance.
The expected variance for W/W_{inbred} and $W/W_{inbredx2}$ fish ($E = \sigma^2_I$) was calculated from the variance of the W/Woutbred fish (σ^2_O), as it is expected to increase in proportion with inbreeding coefficient (F) according to the equation: $\sigma^2_I = \sigma^2_O (1+F)$ (Falconer and Mackay, 1996). Note: theoretical inbreeding coefficient F_{IT} was used in this calculation since this approximates to the measured value F_{IS} (see Table 1 in main manuscript).

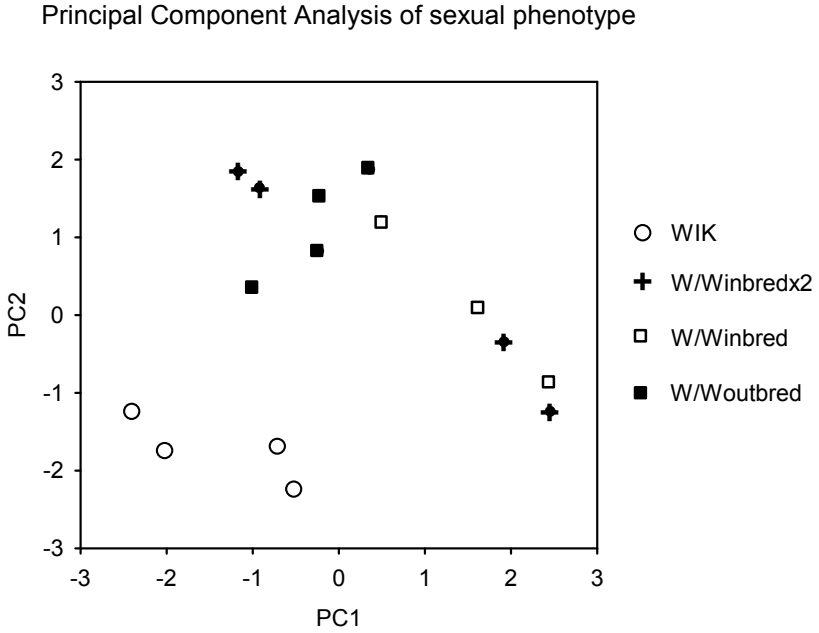
Supplementary Figure S3
Variance in sexual development in different lines of WIK/Wild zebrafish (63 dpf)





Treatments: WIK/Wild Bangladesh strain (W/W - 3 lines outbred, inbred and inbred×2). Tank-level replication: 4 family "replicates" for each treatment = 3 × 4 = 12 tanks.
A proportion of individuals in each strain (7-16%) were undifferentiated, or their gonadal tissues were unidentifiable. These fish were excluded from this analysis.
PC1 and 2 are principal components (see Supplementary figure S4).
PC1 accounted for 33% of variation in sexual development and principally represents sex.
PC2 accounted for 30% of variation in sexual development and principally represents gonadal stage.
>2-fold difference between observed (O) and expected (E) variance.
The expected variance for W/W_{inbred} and $W/W_{inbredx2}$ fish ($E = \sigma^2_I$) was calculated from the variance of the W/Woutbred fish (σ^2_O), as it is expected to increase in proportion with inbreeding coefficient (F) according to the equation: $\sigma^2_I = \sigma^2_O (1+F)$ (Falconer and Mackay, 1996). Note: theoretical inbreeding coefficient F_{IT} was used in this calculation since this approximates to the measured value F_{IS} (see Table 1 in main manuscript).

Supplementary Figure S4
Principal Components Analysis of sexual phenotype in WIK and WIK/Wild zebrafish family lines (63 dpf)



Sexual phenotype for individual fish was assessed in terms of sex (male/female) and gonadal development stage (1/2/3, see main paper).
A proportion of individuals in each strain (7-16%) were undifferentiated, or their gonadal tissues were unidentifiable. These fish were excluded from this analysis.

Principal Component (PC1) represents 33% of total variation in sexual phenotype, principally sex. Principal Component (PC2) represents 30% of total variation in sexual phenotype, principally gondal cell development stage.

Strain replicate	PC1	PC2
WIK	-2.03029	-1.74062
WIK	-0.55506	-2.23635
WIK	-2.38122	-1.25864
WIK	-0.71749	-1.69005
mean	-1.42102	-1.73142
variance	0.84601	0.160091
W/Winbredx2	2.454137	-1.23937
W/Winbredx2	1.919269	-0.34566
W/Winbredx2	-1.16626	1.844625
W/Winbredx2	-0.92123	1.637527
mean	0.571477	0.47428
variance	3.536292	2.279963

Strain replicate	PC1	PC2
W/Winbred	2.433836	-0.86921
W/Winbred	1.600453	0.090038
W/Winbred	0.489624	1.209033
mean	1.507971	0.143285
variance	0.951405	1.081905
W/Woutbred	-1.02295	0.361423
W/Woutbred	0.358041	1.874004
W/Woutbred	-0.22674	1.540467
W/Woutbred	-0.23411	0.822793
mean	-0.28144	1.149672
variance	0.321336	0.468521

4.2 References (Chapter 4 Supporting Information)

Brown A R, Bickley L K, Le Page G, Hosken D J, Paull G C, Hamilton P B, Owen S F, Robinson J, Sharpe A D and Tyler C R (2011). Are toxicological responses in laboratory (inbred) zebrafish representative of those in outbred (wild) populations? A case study with an endocrine disrupting chemical. Environmental Science and Technology 45:4166–4172.

Falconer D S, Mackay T F C (1996). Introduction to Quantitative Genetics, 4th ed. Longman, Harlow, UK.

5 CHAPTER 5: ARE TOXICOLOGICAL RESPONSES IN LABORATORY (INBRED) ZEBRAFISH REPRESENTATIVE OF THOSE IN OUTBRED (WILD) POPULATIONS? – A CASE STUDY WITH AN ENDOCRINE DISRUPTING CHEMICAL

Citation

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Are Toxicological Responses in Laboratory (Inbred) Zebrafish Representative of Those in Outbred (Wild) Populations? — A Case Study with an Endocrine Disrupting Chemical

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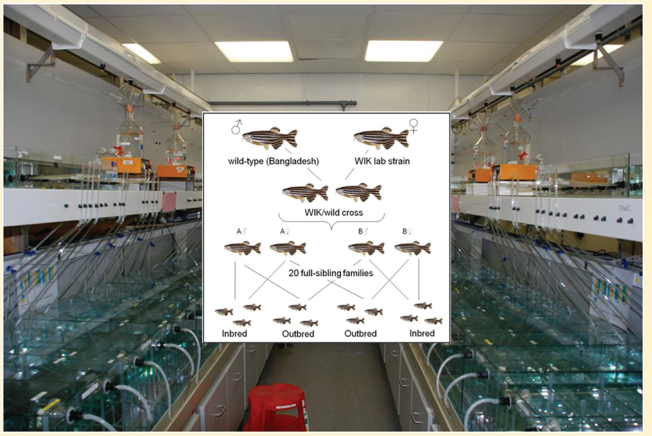
Supporting Information

ABSTRACT: Laboratory animals tend to be more inbred and less genetically diverse than wild populations, and thus may differ in their susceptibility to chemical stressors. We tested this hypothesis by comparing the responses of related inbred (theoretical inbreeding $F_{IT} = n + 0.25$) and outbred ($F_{IT} = n$) zebrafish (*Danio rerio*) WIK/Wild family lines to an endocrine disrupting chemical, clotrimazole. Exposure of inbred and outbred zebrafish to 2.9 μg clotrimazole/L had no effect on survival, growth, or gonadal development. Exposure of both lines to 43.7 μg clotrimazole/L led to male-biased sex ratios compared with controls (87% versus 55% and 92% vs 64%, for inbred and outbred males, respectively), advanced germ cell development, and reduced plasma 11-ketotestosterone concentrations in males. However, outbred males (but not inbred males) developed testis that were more than twice the weight of controls corresponding with a proliferation of Leydig cells and maintenance of the expression (rather than down-regulation occurring in inbreds) of gonadal aromatase (*cyp19a1a*) and insulin-like growth factor (*igf1*). Our results illustrate that the effects of an endocrine disrupting chemical (clotrimazole) on some end points (here testis development) can differ between inbred and outbred zebrafish. This highlights the need for reporting pedigree/genetic information and consistency in the responses of laboratory animals (e.g., by using model compounds as positive controls).



INTRODUCTION

Low genetic variation in wildlife populations is generally associated with reduced ability to adapt to changes in environmental conditions, including chemical exposure.[1] Relatively small and isolated populations are particularly at risk as a result of inbreeding leading to inbreeding depression/loss of heterosis[2] and allelic loss.[3] Allelic loss due to inbreeding has been associated with increased susceptibility to chemical exposure in a number of laboratory animals: fruit fly (*Drosophila melanogaster*) exposed to DDT,[4] *Drosophila buzzatti* exposed to dimethoate,[5] midge (*Chironomus riparius*) exposed to cadmium,[6] and mouse (*Mus musculus*) exposed to 17β-estradiol.[7] However, in some instances inbred laboratory animals may be less sensitive to certain stressors, due to the purging of deleterious alleles during inbreeding, which can be amplified under stable artificial selection,[8] whereas purging is less likely in the wild.[9] From another perspective, inbred animals are considered advantageous in mammalian toxicology as they tend to be less variable in their responses to chemicals, thus reducing the number of animals required.[10,11] Mammalian toxicologists therefore generally use highly inbred laboratory strains (>20 generations of full sibling mating) that are essentially isogenic.[12] Contrasting with this, in ecotoxicology outbred strains may better represent wild populations that favor or benefit from outbreeding, as shown in fish,[13] birds, and mammals.[14]

Sexual reproduction in vertebrates is mediated by steroid hormones, and numerous endocrine disrupting chemicals (EDCs) discharged into the environment have been shown to interfere with hormone signaling via several mechanisms, in a range of animal taxa.[15−20] In fish, sex determination and differentiation are particularly susceptible to endocrine disruption[21,22] and exposures to various synthetic and natural estrogens have been

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associated with significant effects on reproductive development and function in wild fish populations.[23,24]

An unresolved question is whether inbreeding affects susceptibility of fish to EDCs. Zebrafish (*Danio rerio*) are used extensively in ecotoxicology and while laboratory strains (genetically distinct laboratory populations) are relatively genetically diverse compared to other laboratory animals,[25,26] they are substantially less diverse than wild zebrafish populations.[27] In this study, we used hybrid WIK laboratory strain/Wild Bangladesh zebrafish to test the hypothesis that inbred zebrafish differ in their susceptibility to chemical effects compared with outbred zebrafish (representing wild populations). These fish were exposed to clotrimazole, a priority hazardous substance [28] belonging to a group of chemicals (imidazole fungicides), known to disrupt steroidogenesis in fish.[19,29] Effects were quantified on somatic growth (specific growth rate and condition), sexual differentiation (sex ratio), and sexual development (gonadal status, hormone levels, and steroidogenic gene expression).

MATERIALS AND METHODS

Test Substance. Clotrimazole (CAS number 23593−75−1) was obtained from Sigma-Aldrich Ltd., Dorset, UK (98% pure). Its intended mode of action is based on the inhibition of cytochrome P450 (CYP)51, 14α-lanosterol demethylase, resulting in impaired ergosterol synthesis and increased cell permeability in fungi.[30] (CYP)51[31] and several other CYP enzymes also inhibited by azole compounds, including (CYP)19 aromatase,[32] play a key role in steroidogenesis in many animal phyla.

Nominal clotrimazole exposure concentrations 0, 5, and 50 μg/L were selected to represent a range capable of inducing chronic biological effects in exposed fish,[33,34] with 5 μg/L approaching levels predicted in the most polluted aquatic environments.[28]

Genetic Variation and Relatedness of Individuals within Inbred and Outbred Family Lines. Our zebrafish consisted of 19 inbred (1 family failed to reproduce) and 20 outbred family lines with theoretical inbreeding coefficients of $F_{IT} = n + 0.25$ (due to full-sibling mating) and $F_{IT} = n$, respectively. These were third generation (F_3) fish derived from an F_0 cross between individual WIK laboratory females (http://zfin.org/) and wild Bangladesh males, the *n* notation reflecting the unknown pedigree of these males. The F_3 fish (Figure S1, details in Table S1, of the Supporting Information) produced from the hybrid WIK/Wild strain were intended to represent inbred and outbred wild fish populations, which differed in their levels of heterozygosity. To confirm this, genetic variation was analyzed at 12 microsatellite markers in the inbred and outbred lines (Table S2 of the Supporting Information).

Chemical Dosing and Analysis. Chemical dosing was conducted using a continual flow-through test system (Figure S2 of the Supporting Information). Flow rates equating to ×2 tank volume changes per day were used to provide nominal clotrimazole exposure concentrations of 0, 5, and 50 μg/L. Clotrimazole was quantified throughout the study using tandem liquid chromatography and mass spectrometry (LC-MS) (Table S5 of the Supporting Information).

Test Design and Test Conditions. Zebrafish were exposed to clotrimazole over a 96 day period between 37 and 133 days post hatch (dph), incorporating periods of exponential somatic growth, sexual differentiation, and sexual maturation.[35] Thirty exposure tanks provided a total of 240, 7.5 L compartments, giving 80 compartments for each of 3 chemical treatments: 0, 5, and 50 μg/L (nominal clotrimazole concentrations), 2 × 20 for inbred and 2 × 20 for outbred fish, which were separated to prevent pheromonal cues. Each compartment was stocked initially with 8 fish and subsequent sampling was conducted to maintain consistent stocking densities. The exposure conditions, including a range of water quality parameters, were monitored throughout the study (Figure S2 of the Supporting Information).

Sampling and Analysis of Developmental End Points. Mortalities and abnormalities (physical or behavioral) were recorded throughout the exposure period. A sampling schedule (sample sizes and sampling times) was defined in advance for the developmental end points (Table S3 of the Supporting Information).

Somatic Growth. In vivo growth measurements were determined as wet weight [±10 mg] in water and standard length [±1 mm] via scaled photography on exposure days 0, 12, 26, 48, and 96 using three randomly selected fish from each compartment. Direct growth measurements, determined as wet weight [±1 mg] and standard length [±0.1 mm] using vernier callipers, were also obtained following terminal sampling on exposure days 0, 48, and 96. In each case, male and female fish were not discriminated. These terminal measurements, and those from a preliminary growth study involving the F_2 parental generation, were used to calibrate the in vivo measurements (Figure S3 of the Supporting Information). In vivo measurements were converted to equivalent ex vivo measurements and these data were used to derive specific growth rates (SGR) [36] for length (SGR_{length}) and weight (SGR_{weight}) for defined study intervals (exposure days 0−12, 12−26, 26−48, 48−96). Condition index (K, weight/length^3) was determined at the end of each period.

Sexual Differentiation and Gonadal Development. Sexual differentiation was assessed histologically in all fish sampled terminally on exposure days 48 and 96. Whole bodies were fixed (maximum 6 h) in Bouins solution (Sigma Aldrich, Dorset, UK), progressively dehydrated in 70−100% IMS and embedded in paraffin wax. Serial tranverse sections (5 μm) on glass slides were then stained using hematoxylin and eosin and examined using a Leitz Diaplan light microscope [×(10−100) magnification]. The proportions of gonadal cell types (spermatogonia, spermatocytes, spermatids/spermatozoa, interstitial cells) were quantified in randomly selected males (n = 10) representing different inbred and outbred families from controls and high-level clotrimazole exposure treatments on exposure days 48 and 96. The occurrence of each cell type was counted at intersections of a 10 × 10 (=100) 10 μm grid square overlaid on two section images per male. The proliferation of interstitial Leydig and Sertoli cells were also quantified according to a relative severity scale: 0 = unremarkable, 1 = mild, 2 = moderate.[37]

Gene Expression. On exposure day 96, the left gonad of male fish selected for histological analysis were removed prior to fixing, weighed, snap frozen, and stored at −80 °C for transcript analysis. Total RNA was subsequently extracted and cDNA synthesized (details in Table S4 of the Supporting Information).

Oligo-nucleotide primers were designed for the following target genes using Beacon Designer 3.0 (Premier Biosoft International, Palo Alto, CA): hydroxy-steroid dehydrogenase (*hsd17b3*), 17α-hydroxylase/17,20-lyase (*cyp17a1*), aromatase (*cyp19a1a*), 14α-lanosterol demethylase (*cyp51*), insulin-like growth factor (*igf1*) (Table S4 of the Supporting Information). Selection of these target genes was based on the anticipated mode of action of clotrimazole, that is inhibition of genes coding for key enzymes in sex hormone steroidogenesis[31,32] and prior analysis of developmental (phenotypic) end points. Real-time quantitative PCR was

performed in triplicate for each sample using a BioRad IQ5 PCR system (Bio-Rad Laboratories, Hercules, CA). Target gene expression was efficiency-corrected and quantified relative to the housekeeping gene, ribosomal protein l8 (*rpl8*) (details in Table S4 of the Supporting Information).

11-Ketotestosterone. Blood plasma concentrations of 11-ketotestosterone (11-KT) were measured in male fish (confirmed via gonadal histology) terminally sampled on exposure days 48 and 96. Blood was collected from the caudal vein into a 100 μm diameter hematocryt tube coated in heparin. Samples were immediately stored on ice and then centrifuged for 4 min at 3200 g to separate the plasma. All samples were then frozen and stored at −20 °C. 11-KT was quantified from 1 μL of blood plasma using a radio-immunoassay[38] with a detection limit of 1.22 ng/mL in this study.

Statistical Analysis. Statistical tests were performed to confirm that representative sampling had taken place across the size spectra of inbred and outbred lines. Accordingly, the weights of fish sampled terminally and those that were retained (for a subsequent breeding study) were compared using one-way analysis of variance and no significant differences were found ($p \leq 0.05$).

The various developmental end points were expressed as family means and compared between $n = 19$ inbred and $n = 20$ outbred families (unless stated otherwise) across all treatments. Data were first assessed for normality using the Anderson-Darling test. Data failing this test (plasma 11-KT concentrations, gonad weight and relative gene expression) were $\log_{10}$ transformed. Percentage data representing the proportion of gonadal germ cells were transformed by arcsine $(percentage/100)^{1/2}$. Normality was confirmed as before and data were then assessed for equality of variances between family groups (inbred and outbred) and between chemical exposure treatment groups (control, low, high exposure concentration) using Bartlett's or Levene's test prior to statistical analysis. All statistical analyses were run in Minitab 15 (Minitab, Coventry, UK) and differences were accepted as significant when $p \leq 0.05$. Values are quoted as mean values and ranges as standard error of the mean, unless stated otherwise.

RESULTS

Genetic Variation and Relatedness of Individuals within Inbred and Outbred Family Lines. One of the F_2 inbred families failed to spawn, leaving 19 inbred and 20 outbred F_3 WIK/Wild family lines. The mean observed and expected heterozygosity ($H_O = 0.669$, $H_E = 0.745$) of our inbred zebrafish lines were lower than that of our outbred lines ($H_O = 0.753$, $H_E = 0.765$),based on variation at 12 microsatellite markers (Table S2 of the Supporting Information). However, both lines were more heterozygous than commonly used strains of zebrafish.[27] Inbreeding coefficients were consistently higher for inbreds compared to outbreds, by $F \approx 0.1$ (Table S2 of the Supporting Information).

Clotrimazole Exposure Concentrations. Measured clotrimazole concentrations were consistent within each treatment regime over the exposure period. The geometric mean measured concentrations of clotrimazole and 95% confidence intervals were 43.7 ± 6.14 μg/L for the nominal of 50 μg/L (87% of nominal) and 2.9 ± 0.57 μg/L (58%) for the nominal of 5 μg/L (Table S5 of the Supporting Information).

Effects Related to Clotrimazole Exposure and/or Inbreeding. *Mortality and Physical Deformity.* Mortality was below 2% in all treatment groups during the exposure study (37−133 dph). Physical deformities were prevalent in only two of the 19 inbred families: 4/16 individuals in one family had a shortened and bloated body-form, 8/16 individuals in another family showed dorso-ventral spinal curvature (lordosis). Similar deformities were encountered in two other inbred families and in 6 outbred families but frequencies were substantially below those given above (<5%). During the evaluation of somatic growth (below), we sampled 12 deformed fish from the two families showing highest incidence of deformity in our study. However, by taking mean replicate growth measurements by family and then by breeding/chemical exposure treatment, this minimized the effect of physical deformity on length and weight, and there was no detectable bias in the results.

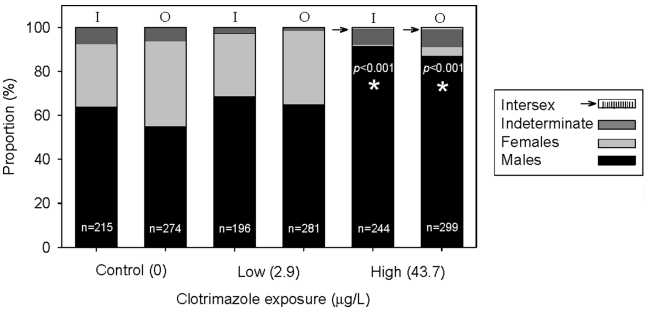

Figure 1. Sex ratio in inbred and outbred zebrafish sampled after 96 days (133 dph) exposure to clotrimazole. Replication *n* represents number of individuals per treatment, *I* = Inbred, and *O* = Outbred. *Significant difference in the proportion of males from the control according to a Chi-square goodness of fit test of transformed data (arcsine $\sqrt{(percentage/100)}$). → Two intersex fish were found in the high-level exposure treatments in both inbred and outbred fish.

Somatic Growth. At the start of the exposure (37 dph), outbred fish were significantly larger (12.3 ± 0.09 mm, standard length, and 37.5 ± 2.3 mg, wet weight) compared with inbred fish (12.0 ± 0.11 mm and 33.8 ± 2.4 mg) (ANOVAs: standard length $F_{(1,113)} = 13.77$, $p < 0.001$; wet weight $F_{(1,113)} = 3.89$, $p = 0.05$). Therefore, length and weight data were standardized via the calculation of mean SGR (rate of change in growth) between each sampling time point. Mean condition index K was also determined at each sampling time point. The necessary assumptions for K were satisfied (Figure S4 of the Supporting Information). There was a significant effect due to clotrimazole exposure on SGR_{weight} between exposure days 26−48 (63−85 dph) ($F_{(2,109)} = 4.60$, $p = 0.012$) and K on day 48 ($F_{(2,109)} = 3.50$, $p = 0.034$) but no effect from inbreeding [two-way ANOVAs]. Outbreds exposed to high-level clotrimazole grew at a slower rate and had a lower K (SGR_{weight} 1.30 ± 0.12, $p = 0.014$; K 2.52 ± 0.05, $p = 0.023$) compared with outbred controls (SGR_{weight} 1.83 ± 0.16, K 2.75 ± 0.06 [posthoc Dunnett's] (Figure S4 of the Supporting Information). However, no significant effects on growth were observed in the equivalent inbred treatment, or in either breeding treatment at any other time point.

Sexual Differentiation and Gonadal Development. On exposure day 96 (133 dph) 64% and 55% of fish were males in the inbred and outbred control groups, respectively. There were no significant differences in sex ratios between these controls (Chi-square goodness of fit test). These sex ratios are consistent with findings for other zebrafish studies.[39−41] There were also no significant effects on sex ratio in either the inbreds or outbreds, relative to their respective controls for low-level clotrimazole

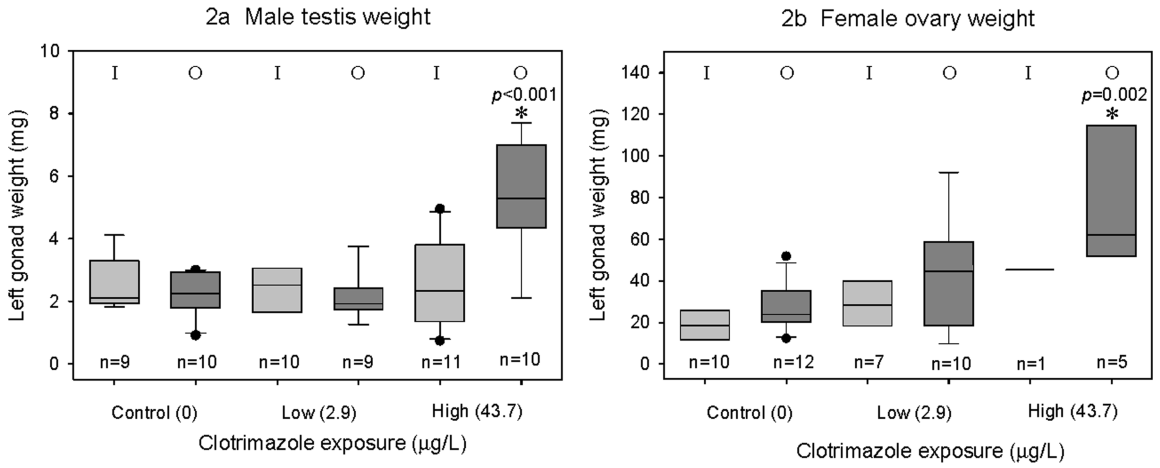

Figure 2. Gonad weight in inbred and outbred zebrafish sampled after 96 days (133dph) exposure to clotrimazole. Replication *n* represents number of individuals per treatment, each from different families. *I* = Inbred (light gray) and *O* = Outbred (dark gray). Boxes show interquartile range and median, whiskers show full range excluding outliers '•'. * Significantly different from control according to ANCOVAs of log10 gonad weight, using breeding treatment and clotrimazole exposure as predictors (also significant interaction $p = 0.002$) and $\log_{10}$ body weight as a covariate, followed by Dunnett's test.

exposure (Figure 1). High-level exposure to clotrimazole (43.7 μg/L), however, induced a significant skew in sex ratio toward males in both inbred and outbred fish: 92% in the inbreds ($\chi^2_{(2, N=244)} = 96.3$, $p < 0.001$) and 87% in the outbreds ($\chi^2_{(2, N=299)} = 152.1$, $p < 0.001$) compared with the respective controls (Figure 1). Histological analyses found germ cell development was significantly more advanced based on the proportions of spermatids and spermatozoa compared with spermatocytes and spermatogonia, in both the inbred and outbred males following high-level clotrimazole exposure, compared with their respective controls on exposure day 48 ($F_{(1,86)} = 10.28$, $p = 0.002$) and on day 96 ($F_{(1,35)} = 3.98$, $p = 0.05$) [two-way ANOVA] (Figure S5 of the Supporting Information).

There was a significant effect on testis weight due to clotrimazole exposure ($F_{(2,49)} = 6.75$, $p = 0.003$) and a significant interaction between breeding and clotrimazole exposure ($F_{(2,49)} = 7.12$, $p = 0.002$) [ANCOVA of $\log_{10}$ testis weight, with breeding treatment and clotrimazole exposure as predictors and $\log_{10}$ body weight as a covariate]. Following high-level clotrimazole exposure (43.7 μg/L) mean testis weight in inbred males was not significantly different to that in inbred controls, whereas mean testis weight in outbred males (5.3 $\pm$ 0.62 mg, $n = 10$ families) was more than twice that in outbred control males (2.2 $\pm$ 0.2 mg, $n = 10$ families, $p < 0.001$) (part a of Figure 2). Clotrimazole exposure also resulted in a greater ovary weight in females ($F_{(2,35)} = 3.27$, $p = 0.05$) [ANCOVA $\log_{10}$ ovary weight], but there was no interaction between breeding and clotrimazole exposure. Mean ovary weights in both inbred females (45.4 mg, $n = 1$ family) and outbred females (57.9 $\pm$ 4.7 mg, $n = 5$ families) under high-level clotrimazole exposure were more than twice that in their respective controls (inbred 18.1 $\pm$ 3.3 mg, $n = 10$ families; outbred 27.4 $\pm$ 3.4 mg, $n = 12$ families, $p = 0.002$) (Figure 2b).

Differences in the effect of high-level (43.7 μg/L) clotrimazole exposure on testis weight in inbred compared to outbred males were not related to the status of gonad maturation [two-way ANOVA on the proportions of mature male germ cells (spermatids and spermatozoa) with breeding treatment and clotrimazole exposure as predictors] (Figure S5 of the Supporting Information). However the relative abundance of Leydig cells (Figure S6, Table S6 of the Supporting Information) was shown to be significantly higher ($p < 0.0001$) [log-likelihood ratio for contingency tables] in the testis of outbred males under high-level clotrimazole exposure (0, 1, and 8 individuals with zero, mild and moderate hyperplasia respectively) compared with control outbred males (4, 5, and 0 individuals with zero, mild, and moderate hyperplasia respectively). There was no proliferation of Leydig cells in the testis and no significant difference between exposure treatments for inbred males.

11-Ketotestosterone (11-KT). There was a significant effect of inbreeding on plasma 11-KT concentration ($F_{(1,103)} = 4.8$, $p = 0.003$) [two-way ANOVA of $\log_{10}$ plasma 11-KT concentration, with breeding treatment and clotrimazole exposure as predictors] after 48 days of exposure (85 dph): inbred males had significantly higher plasma 11-KT concentrations compared with outbred males. There was also a significant difference in plasma 11-KT concentrations in the inbred males, between control fish (5.22 $\pm$ 0.69 ng/mL) and both low (3.62 $\pm$ 0.44 ng/mL, $p = 0.033$) and high-level (3.46 $\pm$ 0.21 ng/mL, $p = 0.024$) clotrimazole exposures [post hoc Dunnett's tests performed within each breeding treatment]. However, there was no effect of clotrimazole exposure on plasma 11-KT concentrations in the outbred zebrafish (part a of Figure S7 of the Supporting Information).

There was a significant effect of clotrimazole exposure on plasma 11-KT concentrations within the outbred treatments ($F_{(2,24)} = 10.93$, $p < 0.001$) but not the inbred treatments on day 96 (133 dph) [one-way ANOVA of $\log_{10}$ plasma 11-KT concentration, with clotrimazole exposure as predictor]. 11-KT concentrations in outbred males under high-level clotrimazole exposure (1.46 $\pm$ 0.38 ng/mL) were significantly lower than in outbred control males (4.78 $\pm$ 1.11 ng/mL, $p = 0.0002$) [post hoc Dunnett's] (part b of Figure S7 of the Supporting Information).

Variance in Phenotypic End Points. There was higher variance in body size in inbred compared to outbred zebrafish following high-level (43.7 μg/L) clotrimazole exposure (Figure S8 of the Supporting Information). Inbred phenotypic variance (V_I) in SGR_{weight} (63−85 dph) was 54% greater, SGR_{length} (63−85 dph)

Target genes		Inbred testis	Outbred testis
17a-hydroxylase/17,20-lyase	*cyp17a1*		
aromatase	*cyp19a1a*	#	
14a-lanosterol demethylase	*cyp51*		
hydroxy-steroid dehydrogenase	*hsd17b3*		
insulin-like growth factor	*igf1*		

Figure 3. Expression of target genes in testis of inbred and outbred male zebrafish exposed to highlevel clotrimazole (43.7 g/L) relative controls (0 g/L) at 96 days (133dph). No difference from control (dark hatched). Significant down regulation (light green), ǂ indicates significant interaction between breeding and exposure treatment ($p < 0.01$), according to two-way analysis of variance of transformed data (log10).

was 143% greater, and condition (85 dph) was 220% greater than expected due to the increased frequency of homozygote recessives (additive genetic variance). According to Falconer and Mackay,[42] expected $V_I = V_O(1 + F)$, where (V_O) is phenotypic variance in outbreds and (F) is inbreeding coefficient. Furthermore, a power analysis on the growth parameter showing the least variation (SGR_{weight}), showed that almost twice the number of inbred fish ($n = 37$ families) would be required compared with outbred fish ($n = 20$ families) to detect an equivalent level of effect (e.g., ± 30%) with standard power β=0.8.

Variance in the remaining phenotypic end points (gonad weight and plasma 11-KT concentration) did not follow the same trend as body size. In each exposure treatment, observed variance in outbreds was greater than or equal to the observed variance in inbreds.

Expression of Genes Involved in Gonadal Sex Steroid Biosynthesis and Testis Growth. There was a significant effect of clotrimazole exposure on the expression of four (4/5) of the target genes, expressed relative to the housekeeping gene *rpl8*, in male testis sampled on exposure day 96 (*cyp19a1a* $F_{(1,22)} = 6.60$, $p = 0.018$; *cyp51* $F_{(1,23)} = 10.55$, $p = 0.004$; *hsd17b3* $F_{(1,22)} = 6.85$, $p = 0.016$; *igf1* $F_{(1,23)} = 5.99$, $p = 0.022$) [two-way ANOVA]. High-level clotrimazole exposure (43.7 μg/L) resulted in a significant reduction in expression of four (4/5) target genes in inbred male zebrafish testis and only two (2/5) target genes (underlined above) in outbred male testis compared to their respective controls [Dunnett's] (Figure 3). There was also a significant interaction between breeding and clotrimazole exposure for *cyp19a1a* $F_{(1,22)} = 8.27$, $p = 0.009$ [two-way ANOVA]. *Cyp19a1a* expression in inbred controls (0.012 ± 0.005 relative to *rpl8* expression) were significantly different/higher compared to outbred controls (0.0027 ± 0.0007, $p = 0.032$) and compared to the inbred, high-level clotrimazole exposure group (0.0025 ± 0.0006, $p = 0.013$) [post hoc Dunnett's] (Figure S9 of the Supporting Information).

DISCUSSION

This study tested whether inbred and outbred fish differed in their response to an EDC. Clotrimazole exposure at 2.9 μg/L had no adverse effects on survival, growth or sexual development in related inbred and outbred zebrafish lines, and given the predicted environmental concentration (PEC_{local}) for clotrimazole is 0.19 μg/L,[28] this chemical is unlikely to cause adverse environmental health effects in this species. However, high-level clotrimazole exposure (43.7 μg/L), induced a number of effects on steroidogenesis and gonadal growth and development, some of which were distinctly different in inbred male zebrafish compared with outbred male zebrafish. Whereas genotypic differences between strains have previously highlighted inbreeding in laboratory zebrafish,[27] this is the first study to show that phenotypic end points used in (eco)toxicology may be affected by inbreeding in this species.

The masculinization effect of high-level clotrimazole exposure (43.7 μg/L), which led to male-biased sex ratios in both inbred and outbred zebrafish in our study was probably due to direct inhibition of steroidogenic enzymes, rather than an effect mediated by reduced somatic growth.[43] At the start of our study, inbred zebrafish were significantly smaller than outbreds, but subsequent measures of specific growth rate and relative body condition showed that they did not differ significantly in inbred compared outbred controls. High-level clotrimazole exposure resulted in a reduction in body condition and SGR_{weight} in outbred lines between 63 and 85 dph but this was after the critical period of sexual differentiation in zebrafish (≤42 dph) [35,44] and therefore would not be expected to impact on sexual phenotype. Furthermore, despite size differences in early life, sex ratios were skewed similarly in inbreds and outbreds (Figure 1). The direct effect of clotrimazole on sexual differentiation was indicated by the inhibitory responses seen on the steroidogenic enzyme transcripts *cyp51*, *hsd17b3*, and *cyp19a1a*. The latter gene encodes aromatase, responsible for the conversion of testosterone to estradiol, which prevents oocyte apoptosis during a juvenile hermaphrodytic stage in zebrafish, thus leading to ovarian (rather than testis) development.[45] Dietary exposure to another imidazole compound, fadrozole (≥10 mg/g of food) has similarly been shown to result in oocyte apoptosis and male-biased zebrafish populations.[46]

In our study, we observed significant enlargement of the gonads in outbred male and female zebrafish, albeit from a minimal nominal sample size ($n = 10$, Figure 2), following 96 days exposure to clotrimazole (43.7 μg/L). Similarly, exposure of fathead minnows (*Pimephales promelas*) to (≥100 μg/L) ketoconazole, another imidazole compound, resulted in a doubling in ovary and testis size. The latter was associated with a compensatory proliferation of interstitial Leydig cells, which in turn was associated with maintained testis androgen concentrations.[19] In our study, in outbred male zebrafish, we observed a proliferation of Leydig cells following 96 days exposure to 43.7 μg clotrimazole/L, and plasma androgen (11-KT) concentrations were maintained at levels equivalent to controls until sexual maturity (exposure day 48), and subsequently declined. In contrast, there were no increases in testis weight or Leydig cell proliferation, and plasma 11-KT concentrations were not maintained in the equivalent inbred males. The expression levels of *cyp19a1a* and *igf1* in the testis of outbred males did not differ from the untreated controls, but in inbred males, following high-level clotrimazole exposure there was significant down-regulation in the expression of these genes (Figure 3, and Figure S9 of the Supporting Information). The production of estrogen (17β-estradiol) by aromatase (*cyp19a1a*) and insulin-like growth factor (*igf1*) are essential for normal testicular function, and synthesis of both takes place in the Leydig cells.[47,48] Estrogen limits the growth and development of Leydig cells, but prolonged exposure to estrogen can also lead to up-regulation of *igf1*, promoting testis growth and spermatogenesis in the presence of androgens.[48] Therefore, maintenance of *cyp19a1a* (estrogen production) and *igf1* expression and 11-KT (until sexual maturity) in outbred male zebrafish is probably related to the enhanced testis growth and Leydig cell proliferation in response to clotrimazole exposure. In contrast, the down-regulation of these genes in the equivalent inbred treatment is likely to

contribute to the lack of gonad growth and Leydig cell proliferation. Polymorphism has been shown to be widespread in the zebrafish genome,[49] including the loci targeted in this study. Therefore inbreeding-associated allelic loss at, or interacting epistatically with, *cyp19a1a* and *igf1* is plausible.

Phenotypic variances in outbred zebrafish concerning male/female gonad size and male 11-KT hormone concentrations were greater than, or equal to, variances observed in inbreds in each exposure treatment. Consistently lower variance in gonad size in inbreds may indicate allelic loss as a possible causal mechanism affecting this trait. Conversely, with respect to body size, there was higher variance in inbreds compared to outbreds following high-level (43.7 μg/L) clotrimazole exposure. At first glance the latter results seem counterintuitive. However, in nonisogenic inbred lines (like ours), increased phenotypic variance commonly results from the unmasking of recessive alleles in homozygotes (additive variance), and, as occurs in inbreeding depression, the effect may be amplified under environmental stress.[50,51] Variance for SGR and condition index in our inbred zebrafish was 54−220% greater than expected due to additive variance. Elsewhere, this phenomenon has been attributed to dominance variance, following the loss of heterosis and epistatic control of gene expression.[52] Power analysis on the growth parameter showing the least variation (SGR_{weight}) demonstrated that almost twice the number of inbred fish (n = 37 families) would be required compared with the outbred fish (n = 20 families) to detect an equivalent level of effect (e.g., ± 30%).

It has frequently been shown that inbreeding (in laboratory animals) leads to increased susceptibility to chemical exposure,[4−7] thus giving a conservative indication of hazard. However, this may not always be the case, and in our study exposure to 43.7 μg clotrimazole/L led to a significant increase in testis growth in outbred male zebrafish but not inbred males. Which of these alternative responses (if either) represents an adverse outcome is not known. Importantly, our results highlight that interactive effects between inbreeding and chemical exposure can lead to both increased, as well as reduced, phenotypic variation, as illustrated by body size and gonad size, respectively. Regardless of the direction of response/effect (increased or reduced) or the underlying mechanism (inbreeding depression or allelic loss), there is a distinct possibility that inbreeding could lead to major inconsistencies in responses within (over generations) and between laboratory lines and strains.[1,53] Furthermore, the occurrence of inbreeding × chemical interactions, here in our study affecting gonad growth and *cyp19a1a* expression, could not be predicted or prevented by adopting performance criteria for negative (no chemical) controls.

Regular, structured out-breeding, spanning hybridization between closely related lines or strains to the introgression of distantly related individuals from wild populations, have been advocated in order to maintain fitness[54] and/or environmental relevance[27,53] of laboratory animals used in ecotoxicology but this information is rarely reported. Our study highlights the need for reporting of this pedigree/genetic information. The issue of consistency in responses of laboratory test animals has been addressed in mammalian toxicology by standardizing the use of model compounds as positive controls and this could be applied in ecotoxicology to better harmonize chemical testing.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Methods and data concerning chemical exposure and analysis, pedigree and genetic diversity of zebrafish family lines, phenotypic end points not detailed in the main manuscript. This material is available free of charge via the Internet at http://pubs.acs.org.

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5.1 Chapter 5 Supporting Information

TABLES

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S9 - Relative expression of target genes (versus housekeeping gene *rpl8*) in inbred and outbred zebrafish sampled after 96 days (133dph) exposure to clotrimazole (43.7 µg/L).

Table S1
Details of the parental pair breeding for generating the F_3 inbred and outbred lines

Inbred family (F_3 gen)	Maternal family (F_2 gen)	Paternal family (F_2 gen)	Outbred family (F_3 gen)	Maternal family (F_2 gen)	Paternal family (F_2 gen)
I1	T	T	O1	H	D
I2	M	M	O2	K	P
I3	O	O	O3	T	K
I4	S	S	O4	G	S
I5	C	C	05	Q	F
I6	E	E	O6	O	L
I7	P	P	O7	L	T
I8	G	G	O8	F	O
I9	D	D	O9	A	C
I10	L	L	O10	P	H
I11	B	B	O11	B	N
I12	A	A	O12	E	R
I13	I	I	O13	D	I
I14	Q	Q	O14	M	J
I15	J	J	O15	R	E
I16	F	F	O16	S	M
I17	H	H	O17	I	Q
I18	R	R	O18	J	G
I19	N	N	O19	N	A
I20	K	K	O20	C	B

F_3 families denoted 1-20 (for inbreds I and outbreds O)

F_2 families denoted (A-T rather than 1-20)

The family-level replication and the degree and rate of inbreeding (one generation of full-sibling mating) were consistent with those frequently used in studies assessing for inbreeding effects (Lynch 1988; Keller and Waller 2002; Armbruster and Reed, 2005). Our fish were the great grandchildren of wild (Bangladesh origin) male fish and female fish (F_0) of a Wild Indian Karyotype (WIK) laboratory strain female fish pair spawned at the University of Exeter (see main Figure 1). The approach used is highly relevant since the practice of outbreeding between strains is performed routinely in animal husbandry (Sadler and Monson, 2010) and the introgression of individuals from wild populations has also been advocated in order to maintain representative outbred stocks for use in ecotoxicology (Nowak et al. 2007b; Coe et al., 2009). Such practices can sometimes lead to outbreeding depression in F_1 and/or F_2 generations (Wu & Palopoli, 1994; Lynch & Walsh, 1998) due to a break-up of favourable epistatic interactions in the parental lines, or phenotype-environment interaction (Thornhill, 1993; Lynch & Walsh, 1998; Wade & Goodnight, 1998). The use of F_3 generation hybrids in our study minimised the possibility of outbreeding depression.

Table S2
Genetic diversity indices and inbreeding coefficients based on 12 microsatellite marker loci

Strain/ Statistic		**Inbred males (WIK/wild) F_{IT} =0.25**	**Outbred males (WIK/wild) F_{IT}=0**	**Outred females (WIK/wild) F_{IT}=0**
Sample size	No. individuals sampled per strain	20	20	19
Loci	No. loci typed	12	12	12
Allelic richness[1]	Mean no. of alleles per locus ± SD	6.17 ± 1.47	7.25 ± 1.54	7.33 ± 1.61
	Standardised allelic richness (R′) ± SD (from Fstat)	5.98 ± 1.38	6.85 ± 1.38	6.95 ± 1.52
Allelic heterozygosity[2]	Observed heterozygosity (H_O) ± SD	0.669 ± 0.032	0.767 ± 0.028	0.740 ± 0.030
	Expected heterozygosity (H_E) ± SD	0.745 ± 0.024	0.763 ± 0.029	0.766 ± 0.029
Allelic homozygosity[3]	Homozygosity by loci (HL)	0.328	0.222	0.254
Internal relatedness[4]	Internal relatedness (IR)	0.122	-0.014	0.030
Effective inbreeding coefficient[5]	Wright's within-population inbreeding (F_{IS}) (from Fstat)[5a]	0.105	-0.005	0.035
	Wright's within-population inbreeding (F_{IS}) (from Genepop)[5b]	0.107	-0.005	0.034
	Ritland's moment estimate of inbreeding (from Coancestry)[5c]	0.104	0.036	0.070
	Lynch and Ritland's moment estimate of inbreeding (from Coancestry)[5d]	0.119	0.024	0.059
	Wang's likelihood estimate of inbreeding (from Coancestry)[5e]	0.167	0.079	0.094
	Anderson and Weir's likelihood estimate of inbreeding (from Coancestry)[5f]	0.176	0.080	0.099

[1] Allelic richness: mean number of alleles per locus. Standardised measure compensates for the influence of sample size when estimating the expected mean number of alleles in a population, derived using the rarefaction method (El Mousadik and Petit, 1996) in Fstat (Goudet, 2001).

[2] Heterozygosity: proportion of heterozygotes in each strain averaged across all sampled loci. Expected heterozygosity (H_E) assumes Hardy Weinberg equilibrium in diploid allele frequencies.

[3] Allelic homozygosity (Aparicio et al., 2006).

[4] Internal relatedness (Amos et al. 2001).

[5] Effective inbreeding coefficient: [5a] Derived by Fstat using (Nei, 1977); [5b] Derived by Genepop (Raymond and Rousset, 1995; Rousset, 2008) using (Weir and Cockerham, 1984); [5c] Derived by Coancestry (Wang, 2010) using (Ritland, 1996); [5d] Derived by Coancestry using (Lynch and Ritland, 1999); [5e] Derived by Coancestry using (Wang, 2007); [5f] Derived by Coancestry using (Anderson and Weir, 2007). The effective inbreeding coefficients of inbreds were F≈0.1 greater than outbreds i.e. less than the theoretical difference in inbreeding coefficient (F_{IT}=0.25). This is likely due to our limited sample size and considerable allelic variation (polymorphism) at the microsatellite loci sampled (Slate et al., 2004; Szulkin et al., 2010).

Method

Microsatellites were analysed in DNA extracted from fin clips from a sample of 19 inbred male fish, 20 outbred males and 20 outbred female fish, representing a cross section of inbred and outbred family lines. Extraction and amplification of DNA was performed according to Bruford et al. (1998) using 12 disperse micro-satellite loci: Z249; Z266; Z374; Z1213; Z1233; Z4830; Z5058; Z6140; Z9230; Z13614; Z20450 (www.zfin.org) and Ztril (Coe et al. 2008). Genetic (allelic) variation due to Simple Sequence Repeat Length Polymorphisms (SSRLPs) at the pre-selected microsatellite loci was measured using a Beckman-Coulter CEQ 8000 gene sequencer. Results were interpreted using various statistical software: Microsatellite toolkit v3.1.1 (Park, 2001); Fstat v2.9.3.2 (Goudet, 2001); Genepop v4.0 (Rousset, 2008). A suite of indices were calculated for both inbred and outbred zebrafish: observed and expected multi-locus heterozygosity (H_O and H_E); gene diversity (H_S); allelic richness (R'); effective inbreeding coefficient (F_{IS}). Additional indices were also determined: internal relatedness (IR) (Amos et al. 2001); homozygosity by loci (HL) (Aparicio et al. 2006).

Table S3
Developmental effects endpoints, sample sizes per treatment and sampling time points

Endpoint (not sex specific unless stated)	**Method**	**Sample sizes (N families x n individuals) at time points: exposure day/dph**					
		0/37	**7/44**	**12/49**	**26/63**	**48/85**	**96/133**
1) SGR & condition - *in vivo*	Std length, wet weight	20 x 6	-	20 x 6	20 x 6	20 x 6	20 x 6
2) SGR & condition - terminal	Std length, wet weight	10 x 1	-	-	-	20 x 8	20 x 1
3) Sexual differentiation	Histology	10 x 1	20 x 1	-	-	20 x 8*	20 x 4*
4) Gonad weight	Right gonad	-	-	-	-	-	20 x 1
5) Gonad staging	Histology – right testis	-	-	-	-	10 x 1	10 x 1#
6) Gonad transcript	qPCR – left testis	-	-	-	-	-	10 x 1#
7) Plasma 11-KT	Radio-immunoassay	-	-	-	-	20 x 2	20 x 1

Endpoints 1-4 were not sex specific (males and females not discriminated)
Endpoints 5-7 were assessed in males only
* Denotes fish used to determine sex ratio
# Denotes same fish used for gonad staging and transcript analysis

SGR – specific growth rate
qPCR - quantitative polymerised chain reaction
11-KT – 11-ketotestosterone

Table S4
q-PCR analysis of target genes

Target gene	Forward primer	Reverse primer	Annealing temp (°C)	Efficiency (%)
Ribosomal protein l8 (*rpl8*)	CCG AGA CCA AGA AAT CCA GAG	CCA GCA ACA ACA CCA ACA AC	59.5	2.07
Insulin-like growth factor (*igf1*)	GCA TAG CCA CTC TTC CTG TAA G	AAC GGT TTC TCT TGT CTC TCT C	60.5	1.83
Hyrdoxy-steroid dehydrogenanse (*hsd17b3*)	GCT CTT CTG TCC TCT TCC TG	TCT CTT GAT TTC TGC TGA TGA TG	59.5	1.95
17α-hydroxylase/ 17,20-lyase (*cyp17a1*)	CGA CAG TAA GAT TGG GAA AGA AAG	GAT GAG GAG CGG AGA ACC AG	60.5	1.96
Aromatase (*cyp19a1a*)	AGC CGT CCA GCC TCA G	ATC CAA AAG CAG AAG CAG TAG	61.5	1.89
14α-lanosterol demethylase (*cyp51*)	TTC GCC TAC ATC CCA TTC G	AAC ATC CGC AGC AGA GTC	61.5	1.93

Note: Oligonucleotide primer sequences read from 5' to 3'

Total RNA was extracted from each gonad tissue sample using trizol/chloroform (Sigma Aldrich) and precipitated with isopropanol overnight at –20°C. Total RNA concentration was estimated from absorbance at 260 nm using a Nanodrop spectrophotomer (Thermo Finnigan, Hemel Hempstead, UK) and RNA quality was verified by absorbance ratios 260 nm/280 nm >1.8. Following DNase enzyme treatment, cDNA was reverse transcribed from the pure RNA extracts using "Superscript Vilo®" (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

Oligonucleotide primer pairs (forward and reverse) were designed using Beacon Designer 3.0 software (Premier Biosoft International, Palo Alto, CA) and purchased from Invitrogen. Primer-pair annealing temperatures were optimized for real-time PCR on a temperature-gradient program. Primer specificity was confirmed by gel electrophoresis and/or melt curve analysis and automated fluorescence sequencing of PCR products. To determine the detection range, linearity and real-time PCR amplification efficiency (E; E = 10[−1/slope]) (Pfaffl, 2001) of each primer pair, real-time PCR amplifications were run in triplicate on a 10-fold serial dilution series of zebrafish testis cDNA pooled from all samples, and standard curves were calculated referring the threshold cycle (Ct; the PCR cycle at which fluorescence increased above background levels) to the logarithm of the cDNA dilution.
During real-time PCR each cDNA sample was amplified in triplicate using 96-well optical plates in a 20-µl reaction volume using 1 µl cDNA, 10 µl 2× Absolute SYBR Green (Flourescein) Supermix (BioRad), 5 µM of the appropriate forward and reverse primers. Hot start Taq polymerase was activated by an initial denaturation step at 95°C for 15 min followed by 40 cycles of denaturation at

95°C for 10 sec and annealing at the primer-specified temperatures for 20 sec and, finally, melt curve analysis. No-template controls were run for each plate and all samples were run on the same plate ensuring consistent quantification of the expression of each target gene.

Relative expression levels were determined using the following calculation:

$$RE = (E\ ref)^{Ct\ ref} / (E\ target)^{Ct\ target}$$

Where RE is relative gene expression, ref is the housekeeping gene, target is the gene of interest, E is PCR amplification efficiency and Ct is cycle threshold (number of temperature cycles yielding above background expression) for that particular gene.

Statistical differences in relative gene expression between experimental groups were assessed by one-way ANOVA of log-transformed data, followed by Dunn multiple pair-wise comparison test or Student t-test. All statistical analyses were performed using Minitab TM version 15. All experimental data are shown as the mean ± 95% confidence interval. Differences were considered statistically significant at $p \leq 0.05$.

Table S5
Confirmation of clotrimazole concentrations (µg/L) throughout the *in vivo* exposure study

Exposure day	Control (0 µg/L nominal)	Low-level clotrimazole exposure (5 µg/L nominal)	High-level clotrimazole exposure (50 µg/L nominal)
-1	0	-	46.5
1	0	2.93	57.3
5	0	2.7	67.8
8	0	3.05	50.8
14	0	2.5	47.3
21	0	3.35	49.3
30	0.6*	1.8	39
41	0	2.48	48
54	0	3.18	46
68	0	2.45	30.5
82	0	5.57	31
96	0	2.8	34
Arithmetic mean	0	2.98	45.6
Geometric mean	0	2.87	43.7
95% CI	0.1	0.57	6.14
Limit of detection	0.5	0.5	0.5

Note * Analytical sample was contaminated – repeat analysis on the following day did not detect clotrimazole in the control.

Quantitation by LC-MS: initial chromatographic separation of clotrimazole was carried out on an Xbridge RP-18 column (50 × 1 mm, 3.5 µm, Waters, Ireland). The column was fitted with a pre-filter (0.5 µm, Supelco, USA) maintained at 50^{o}C and the flow rate was 150 µl/min. The elution gradient of eluent A) 0.1% ammonia in water and eluent B) 0.1 % ammonia in methanol (T(min)/ % A was 0/90→6/0→8/0→8.01/90→10/90). A Quadrupole Ion Trap (Thermo-Finnigan LCQ) mass spectrometer with electrospray ionisation was used with the following parameters: sheath gas flow 40 arbitrary units, auxiliary gas flow 10 arbitrary units, spray voltage 4.5 kV, capillary temperature 150^{o}C, capillary voltage 46 V, tube lens offset 20 V. Positive ionization with selected ion monitoring (SIM) was used for all analyses. The analyte (clotrimazole) corresponded to a predominant ion mass of 277 Da and the limit of determination was 0.5 µg/L.

Table S6
Relative proliferation of Leydig and Sertoli cells in testis of inbred and outbred male zebrafish on exposure day 96 (133 dph)

Exposure treatment	Fish sample number	Family I = Inbred O = Outbred	Leydig cell proliferation score	Sertoli cells noticeable/ abundant	Other comments
High exposure (43.7 µg l^{-1} clotrimazole)	18	I1	1		
	19	I4	1		
	20	I12	1		
	21	I15	2		Interstitial fibrosis
	23	I3	1		
	24	I18	2		
	25	I13	1		
	26	I15	0		
	27	I6	1		
	28	I8	0		
	30	I9	1	Y	
	40	016	2		
	44	03	2		Interstitial fibrosis
	45	012	2		
	46	08	2		Interstitial fibrosis
	47	01	2		
	48	018	2		Interstitial fibrosis
	49	016	2	Y	
	50	015	2		
	52	04	1		
Control (no clotrimazole)	89	I9	1	Y	
	93	I17	1		
	95	I1	1	Y	Interstitial fibrosis
	96	I12	2		
	97	I16	1		
	98	I10	1		
	100	I2	1		
	111	O19	0		
	112	O4	0		
	114	O13	1	Y	
	116	O16	1	Y	
	117	O3	1		
	118	O12	1		
	120	O20	1		
	121	O17	0		
	122	O7	0		

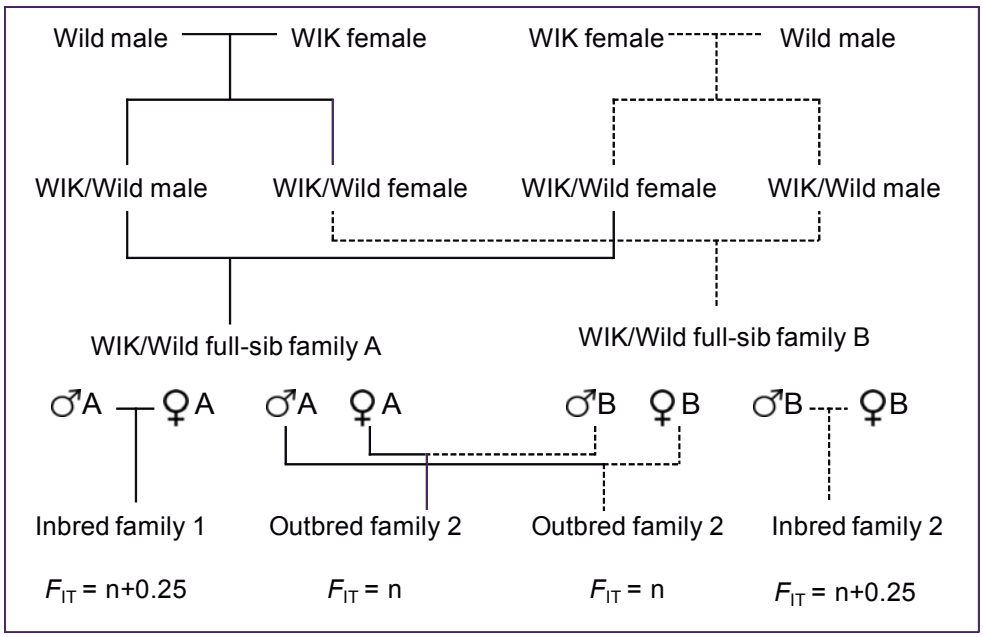
Leydig cell proliferation scores (Johnson et al., 2009):

0 = non remarkable, 1 = minimal severity, 2 = mild severity, 3 = moderate severity

Scores were based on cell number, cell aggregation, nuclei size and density, cytoplasm density

Figure S1

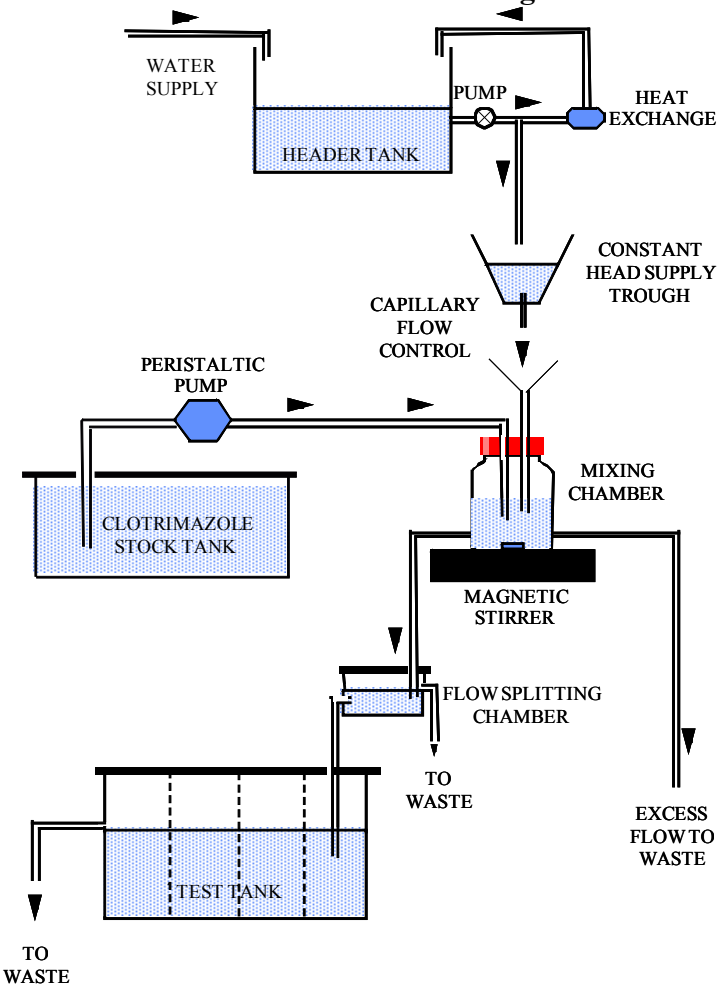
Pair breeding design for generation of inbred and outbred lines (F_3 generation) of zebrafish.



The notation "n" in the inbreeding coefficients of the F_3 generation reflects their unknown pedigree, relating to their wild great grandfathers (F_0 generation). One of the 20 inbred F_3 family lines failed.

Figure S2

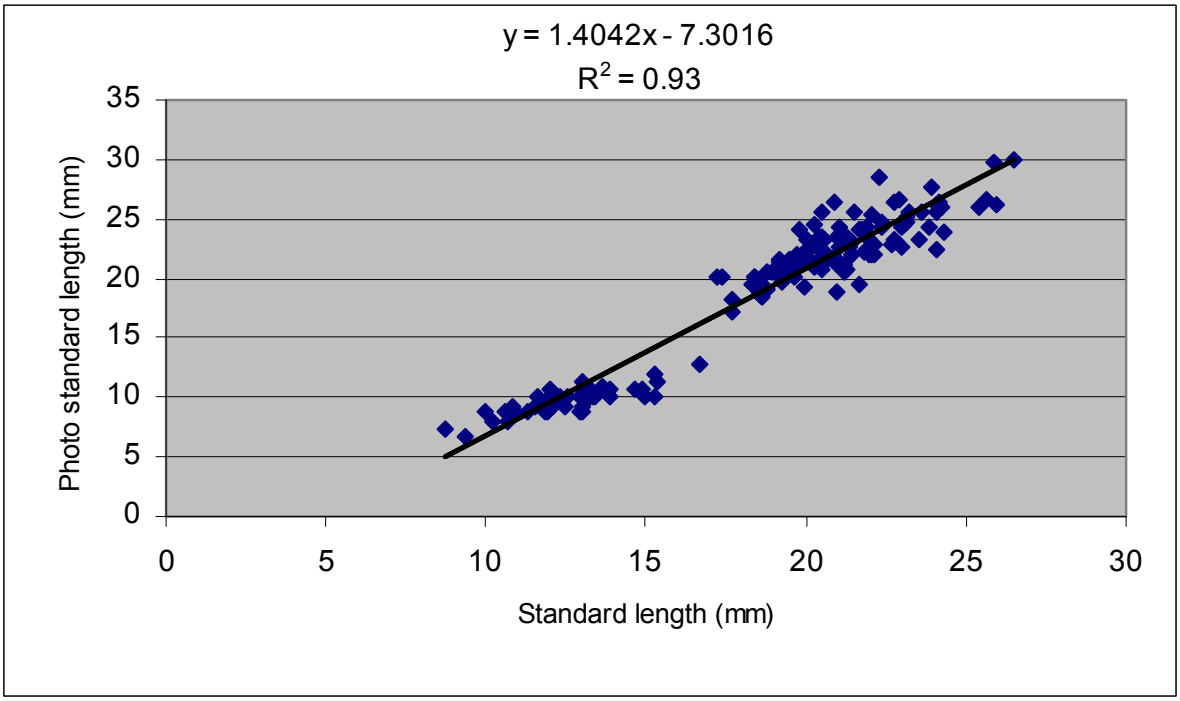
Details of chemical dosing



Two sets of two 120 litre saturated stock solutions (580 µg clotrimazole l^{-1}) were prepared alternately every 24 hours by mixing a ×2 excess of clotrimazole solid powder in dilution water (dechlorinated, 5µm filtered, UV treated mains water) at 28°C, aided through sonication for 15 minutes, followed by 12 hours mixing (using turbo pumps) and 12 hours settling. This final stage allowed the deposition of any un-dissolved test material at the meniscus or on the sides of the stock tanks. The saturated stock solution was then pumped from the middle of the stock tanks to separate mixing chambers, gravity fed with dilution control water, giving a ×10 or ×100 dilution for the nominal 5 and 50 µg l^{-1} exposure concentrations respectively. Test solutions were delivered to 60 litre glass exposure tanks with a longitudinal solid glass partition - each side of each tank (divided further into 4 interconnected sub-compartments) received flow rate of 42 ml min^{-1}, equating to ×2 tank volume changes per day.

The following exposure conditions were maintained throughout the study: water temperature 28±1°C; dissolved oxygen > 60% saturation; pH 7.14-8.16; hardness 40-69 mg/L; alkalinity 22-35 mg/L; chlorine <2 µg/L; ammonia <2 µg/L. The photoperiod was set at 12:12 light:dark with 20 mins sunrise/sunset and fish were fed daily on a fixed excess ration [> 4% body weight per day, comprising one meal of 300 pellet (Special Diets Services) @ 3% body weight in the afternoon, plus an excess of ≥24 hour old *Artemia* nauplii in the morning (and afternoon after exposure day 72, 109 dph)].

Figure S3
3a Calibration of *in vivo* (via photography) versus direct measurements for standard length



3b Calibration of *in vivo* (*in situ*) versus direct measurements of wet weight

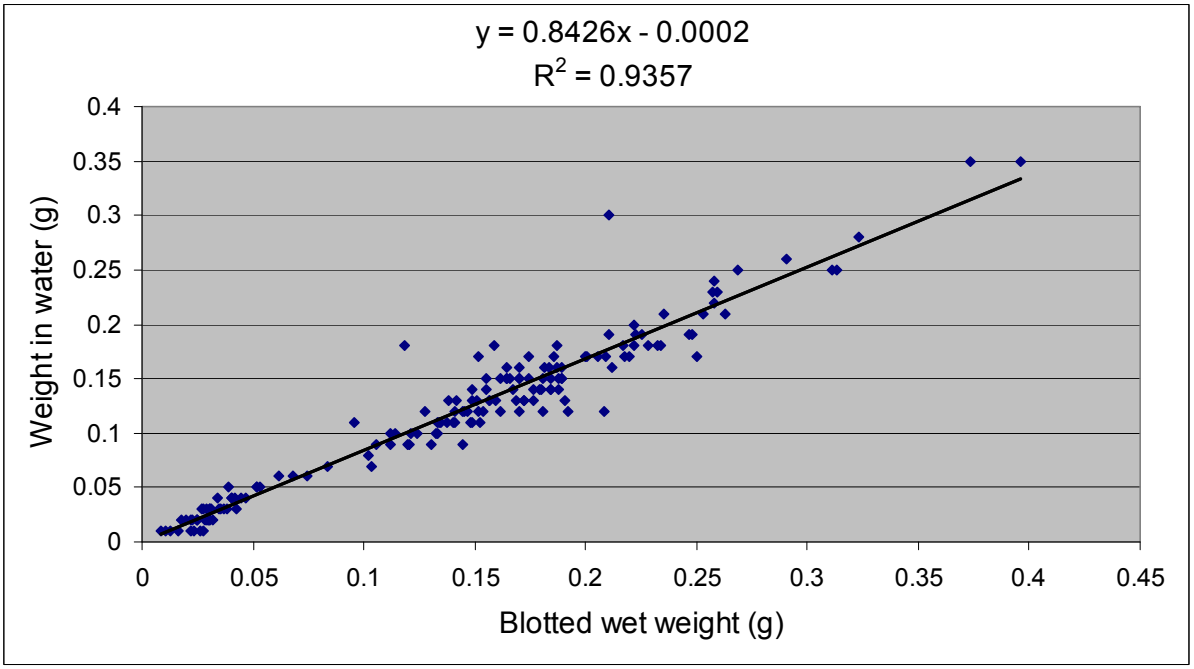
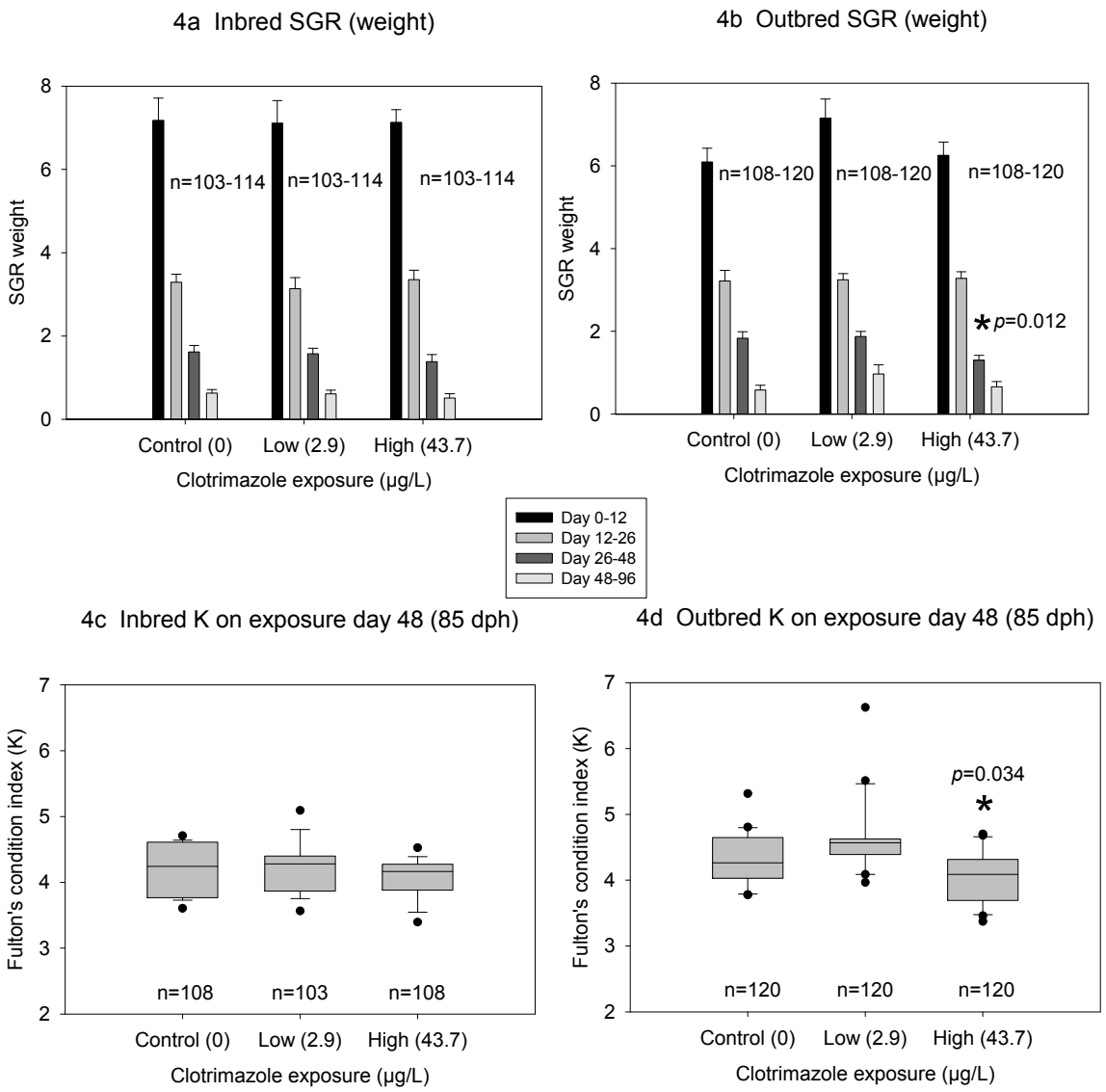


Figure S4
Variation in growth - SGR_{weight} and condition index (K) in inbred and outbred zebrafish



Replication 'n' represents number of individuals per treatment, representing up to 19 inbred and 20 oubred families (males and females not discriminated).

Boxes show inter-quartile range and median, whiskers show full range excluding outliers '•'.

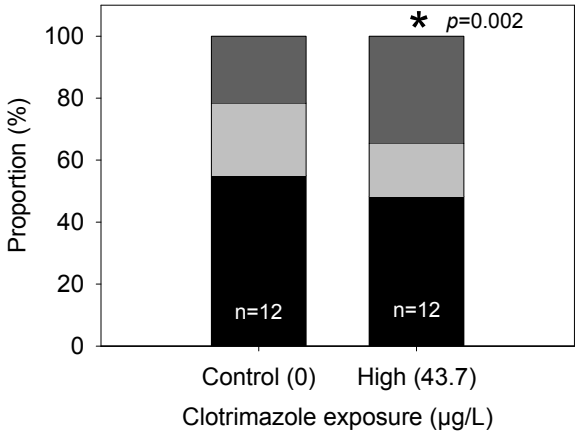
* Indicates a significant difference from the control according to two-way analysis of variance of untransformed data, followed by Dunnett's test.

The assumptions for condition index (K) were met. The slope of the regression of the natural logs of weight on length approximated to 3 (e.g. on day 48 [ln weight] = 2.46 [ln length] – 8.91 (r=0.74, p=0.000). Furthermore, analysis of covariance of condition index, using breeding treatment and clotrimazole exposure as predictors and standard length as a covariate, revealed a significant effect on condition due to clotrimazole exposure ($F_{(2,106)}$=6.95, p=0.001). Subsequent regression analysis of condition versus standard length for different exposure treatments showed that, in each case, slopes were not significantly different from zero.

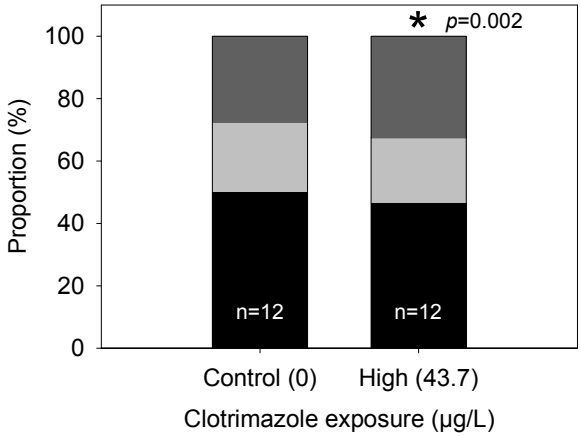
Figure S5
Proportion of gonadal cell types in inbred and outbred male zebrafish

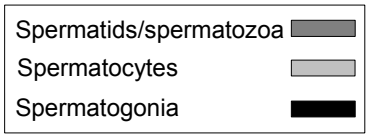
Exposure day 48 (85 dph)

5a Proportion of germ cells in inbred testis



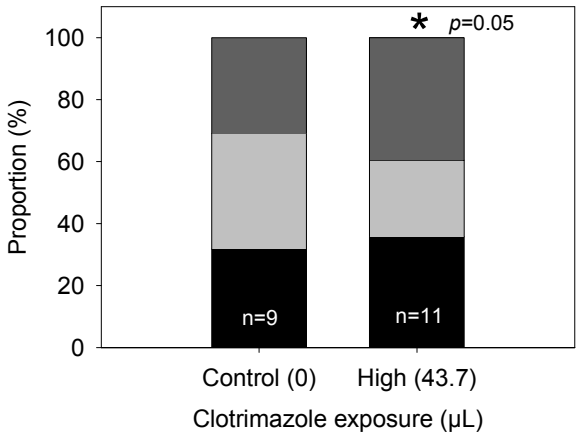
5b Proportion of germ cells in outbred testis



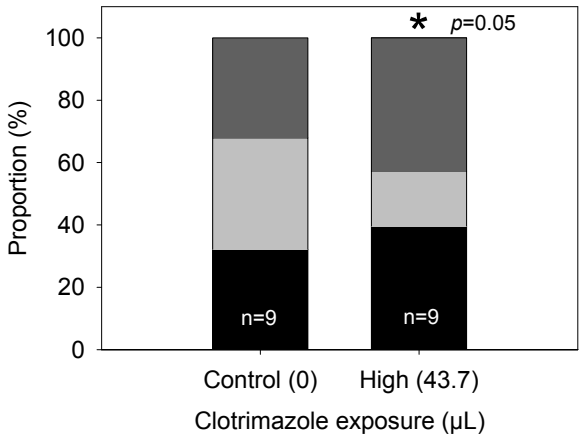


Exposure day 96 (133 dph)

5c Proportion of germ cells in inbred testis



5d Proportion of germ cells in outbred testis

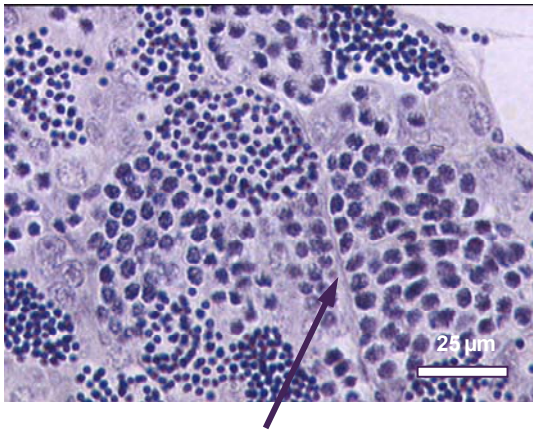


Replication 'n' represents number of individuals per treatment, each representing different inbred and outbred families.

* Indicates a significant difference in the proportion of spermatids/spermatozoa from the controls according to two-way analysis of variance of transformed data (arcsin $\sqrt{percentage/100}$), followed by Dunnett's test.

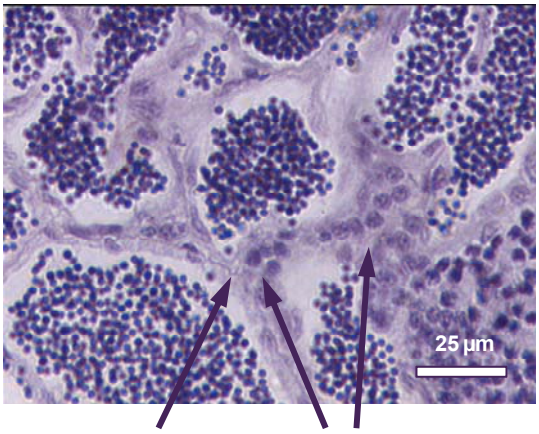
Figure S6
Leydig cell proliferation in the testis of inbred males compared with oubred males following 96 days high-level exposure to clotrimazole (43.7 µg/L)

6a Testis – inbred male



Interstitium (no Leydig cells visible)

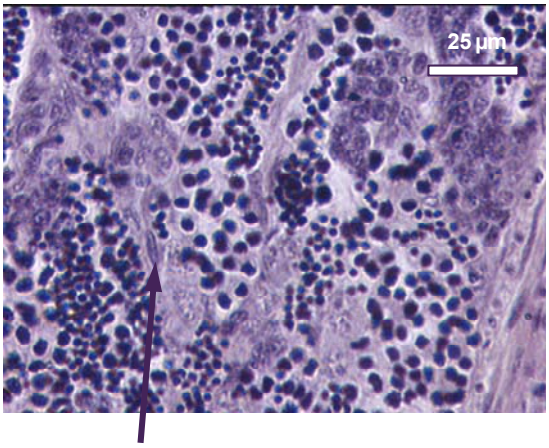
6b Testis – outbred male



Interstitium Leydig cells

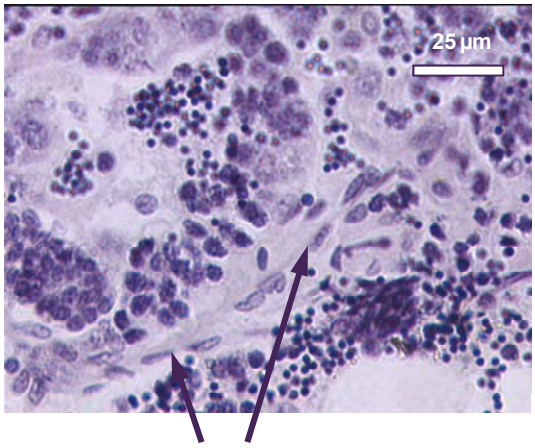
Sertoli cell proliferation in the testis of inbred males compared with oubred males

6c Testis – inbred male



Interstitial Sertoli cell

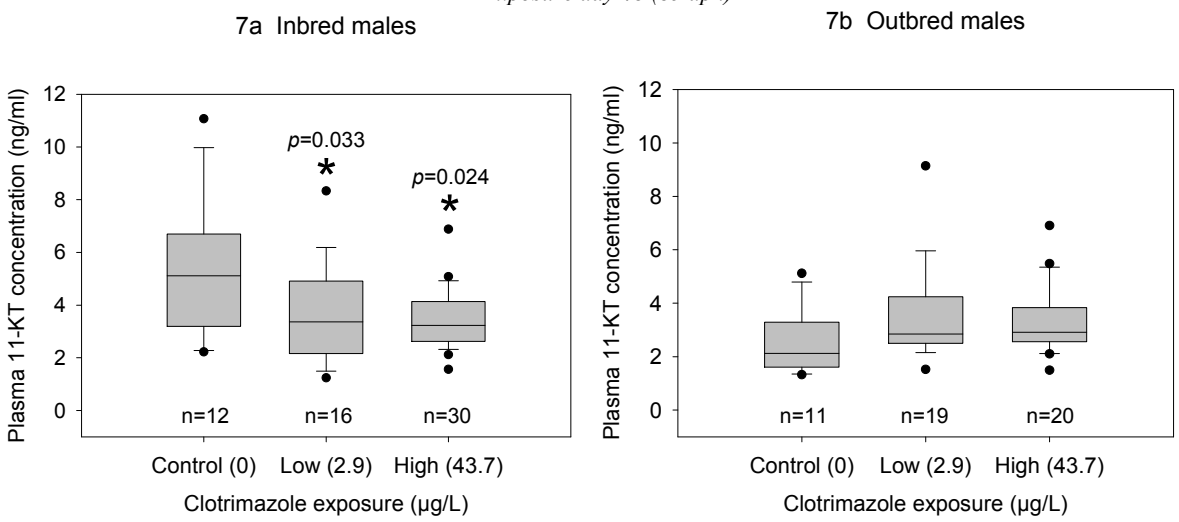
6d Testis – outbred male



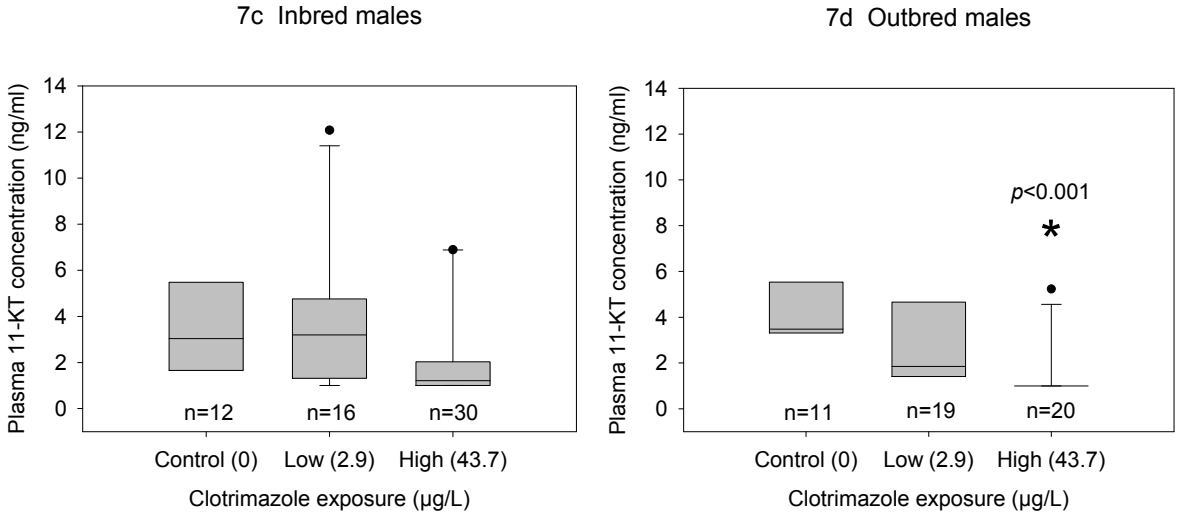
Interstitial Sertoli cells (abundant)

Figure S7
Plasma 11-KT concentrations in inbred and outbred zebrafish exposed to clotrimazole

Exposure day 48 (85 dph)



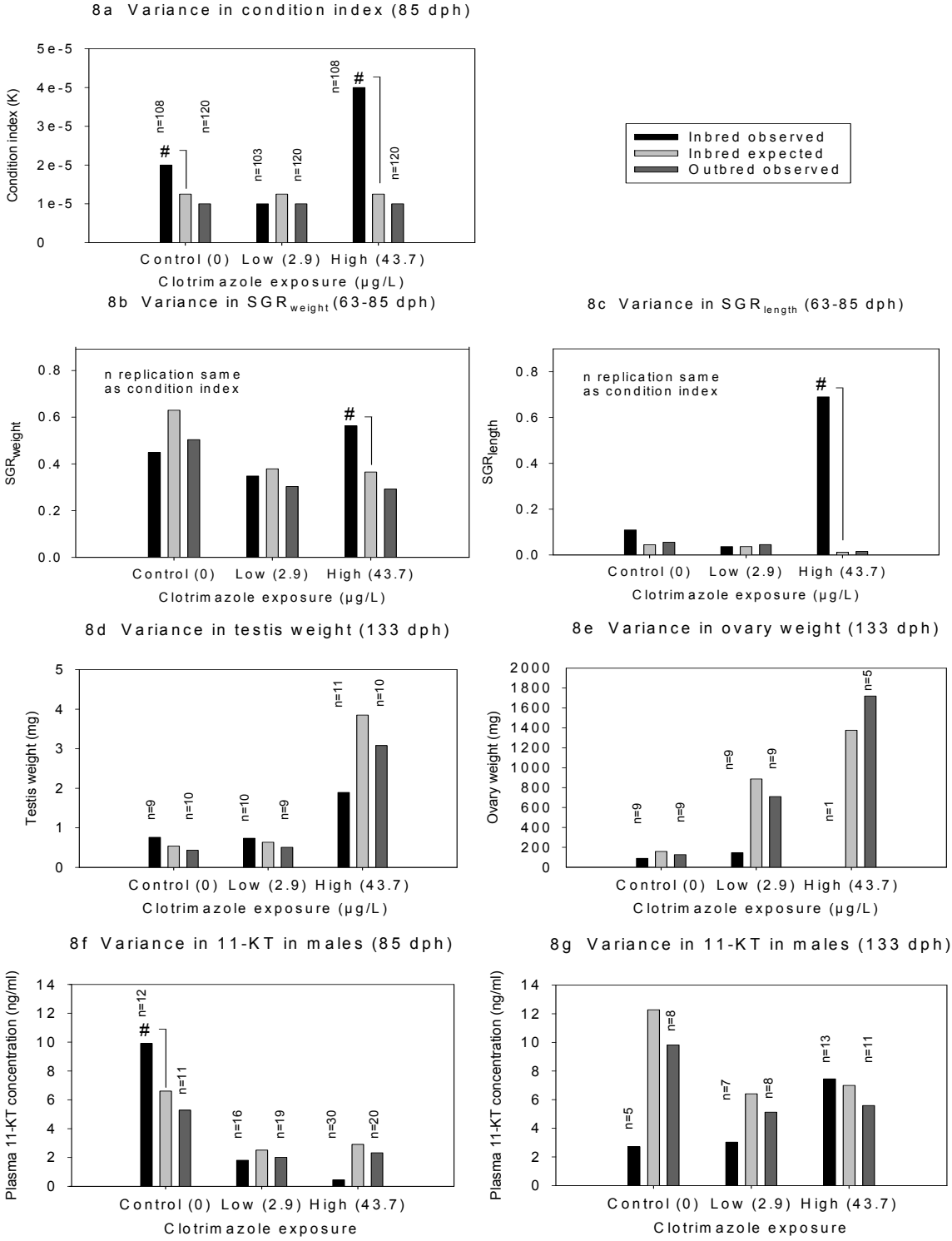
Exposure day 96 (133 dph)



Replication 'n' represents number of individuals per treatment, representing up to 19 inbred and 20 oubred families. Boxes show inter-quartile range and median, whiskers show full range excluding outliers '•'.

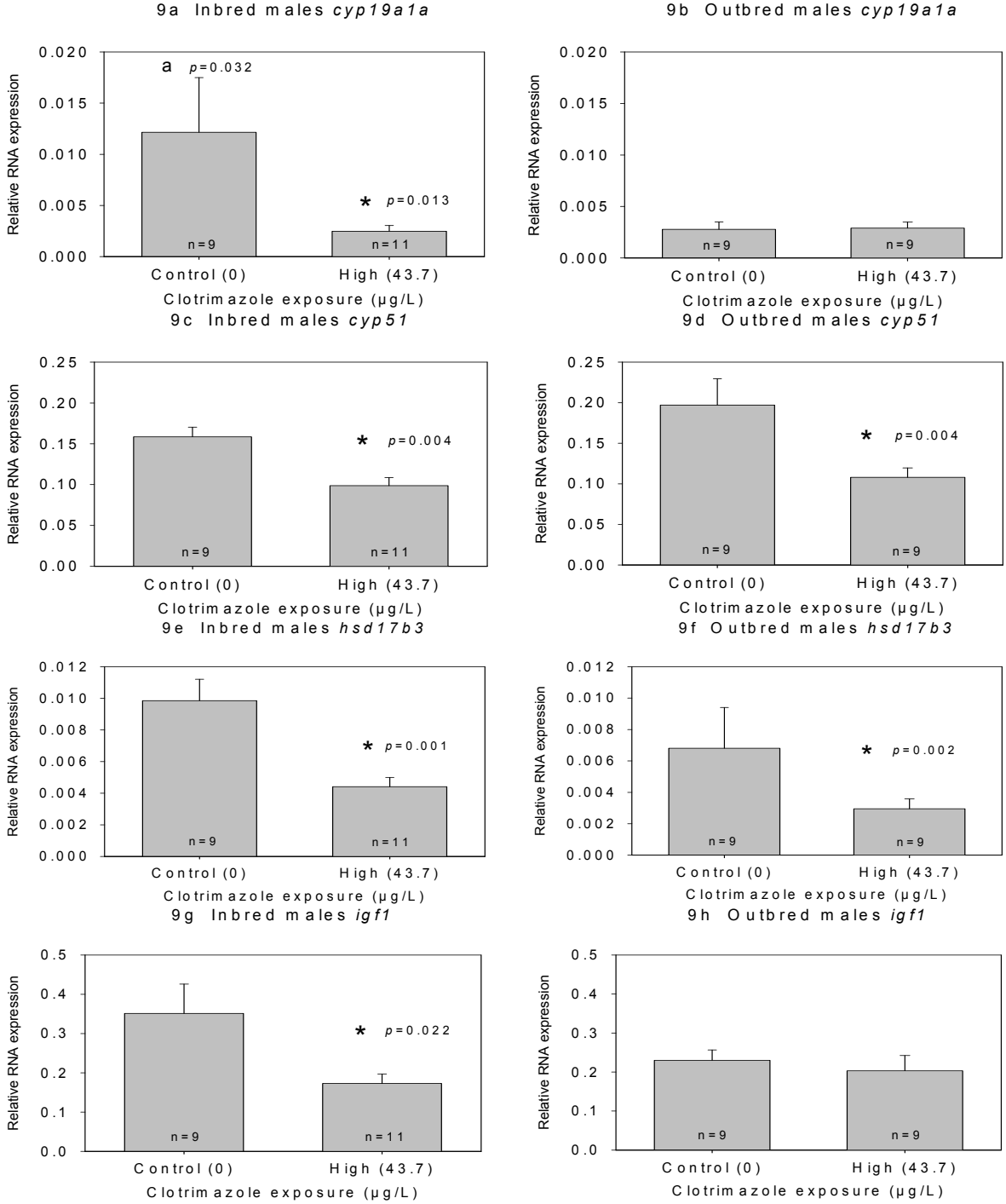
* Indicates a significant difference from the control, according to two-way analysis of variance of transformed data (log_{10}), followed by Dunnett's test.

Figure S8
Variance in phenotypic endpoints



Replication 'n' represents number of individuals per treatment, each representing different inbred, outbred families. Expected phenotypic variance in inbreds [V_I] = observed phenotypic variance in outbreds [V_O] × (1- inbreeding coefficient [*F*]). # Observed variance > expected variance in inbreds.

Figure S9
Relative expression of target genes (versus housekeeping gene *rpl8*) in inbred and outbred zebrafish sampled after 96 days (133dph) exposure to clotrimazole (43.7 µg/L)



Replication 'n' represents number of individuals per treatment, each from different families.

*** Significantly different from control, [a] significantly different from outbred control, according to two-way analysis of variance of transformed data (log_{10}), followed by Dunnett's test. Error bars represent standard error of the mean (SEM).**

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6 CHAPTER 6: INTERACTIVE EFFECTS OF INBREEDING AND ENDOCRINE DISRUPTION ON REPRODUCTION IN A MODEL LABORATORY FISH

Citation

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ORIGINAL ARTICLE

Interactive effects of inbreeding and endocrine disruption on reproduction in a model laboratory fish

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ecotoxicology, fitness, fungicide, zebrafish, reproduction

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Abstract

Inbreeding depression is expected to be more severe in stressful environments. However, the extent to which inbreeding affects the vulnerability of populations to environmental stressors, such as chemical exposure, remains unresolved. Here we report on the combined impacts of inbreeding and exposure to an endocrine disrupting chemical (the fungicide clotrimazole) on zebrafish (*Danio rerio*). We show that whilst inbreeding can negatively affect reproductive traits, not all traits are affected equally. Inbreeding depression frequently only became apparent when fish were additionally stressed by chemical exposure. Embryo viability was significantly reduced in inbred exposed fish and there was a tendency for inbred males to sire fewer offspring when in direct competition with outbred individuals. Levels of plasma 11-ketotestosterone, a key male sex hormone, showed substantial inbreeding depression that was unaffected by addition of the fungicide. In contrast, there was no effect of inbreeding or clotrimazole exposure on egg production. Overall, our data provide evidence that stress may amplify the effects of inbreeding on key reproductive traits, particularly those associated with male fitness. This may have important implications when considering the consequences of exposure to chemical pollutants on the fitness of wild populations.

Introduction

As anthropogenic pressures on the environment increase, wildlife populations are becoming more fragmented. Smaller and more isolated populations are not only more vulnerable to external environmental changes and chance fluctuations in local survival (Keller and Waller 2002) but inbreeding, or mating between closely related individuals, may become more prevalent (Höglund 2009).

One significant stressor of wildlife populations is exposure to chemicals discharged as a consequence of anthropogenic activities. The presence of novel, man-made chemicals in the environment is a relatively new pressure faced by wildlife populations, with potential evolutionary implications. Environmental perturbations such as toxicant exposure may lead to particularly strong novel selection pressures, which have the potential to cause the evolution of heritable traits over relatively few generations (contemporary evolution) (Stockwell et al. 2003). Some species or populations have been shown to adapt to environmental disturbances (for example, mosquitofish (*Gambusia affinis*, Baird and Girard) exposed to pesticides (Andreasen 1985), killifish (*Fundulus heteroclitus*, Linnaeus) exposed to various hydrocarbons (Nacci et al. 1999), and tomcod (*Microgadus tomcod*, Walbaum) exposed to polychlorinated biphenyls (PCBs) (Wirgin et al. 2011), and genome scans have shown that adaptation to such habitats can involve multiple genomic regions (Williams and Oleksiak 2008). However, most forms of adaptation result in a selective loss of genetic variation as the frequency of genotypes that improve fitness increase (Stockwell et al. 2003). Furthermore, small populations are often considered to have limited potential for such adaptive evolution because of reduced genetic diversity (Franklin and Frankham 1998). Therefore, reductions in population size as well as the

increased isolation of populations through habitat degradation are not only likely to impact on inbreeding but also on the complex interactions between inbreeding, reduced genetic diversity and the ability of populations to respond to current and future environmental disturbances.

The vast array of chemicals present in the environment means wildlife may be exposed to multiple chemicals with the potential to cause adverse effects via various physiological routes. One particular group causing widespread concern are endocrine disrupting chemicals (EDCs). These affect the hormonal systems of animals through both receptor- and non-receptor-mediated pathways, and exposure to EDCs is associated with deleterious impacts on reproduction and other aspects of health in a wide range of wildlife taxa, including invertebrates, fish, amphibians, reptiles, birds and mammals (reviewed in Tyler and Goodhead 2010). Exposure to some EDCs may also negatively impact on population dynamics in wildlife (Bryan et al. 1986; Fry 1995; Kidd et al. 2007; Harris et al. 2011) and as a consequence, considerable efforts are being directed at testing chemicals for endocrine disrupting properties.

Chemical toxicity testing in mammals typically employs inbred isogenic laboratory animals. This is because they show less phenotypic variation (NAS 2007), thereby reducing the numbers of animals needed in testing programs. Many of the laboratory strains used in ecotoxicological evaluations tend to be less genetically diverse than their wild counterparts (Woods et al. 1989; Nowak et al. 2007; including zebrafish (*Danio rerio*, Hamilton), Coe et al. 2009; Whiteley et al. 2011), although this is rarely quantified or reported. However, a fundamental aim of ecotoxicity testing is to protect and prevent adverse effects in wild animals, and hence the use of outbred strains has been argued to be more appropriate, as they may better represent wild populations (Brown et al. 2009). The few reported studies on combined inbreeding and chemical exposure effects (all of which are on invertebrates) generally show that inbreeding increases the impacts of chemical exposure in laboratory-maintained animals. For example, inbred midges (*Chironomus riparius*, Meigen) showed a greater reduction in fitness when exposed to cadmium (Nowak et al. 2007), and inbreeding depression, a reduction in trait values because of inbreeding, was greater in the fruit fly (*Drosophila melanogaster*, Meigen) under environmental stress conditions, including exposure to DDT (Bijlsma et al. 1999). To our knowledge, there have been no reported investigations into the combined effects of inbreeding and chemical exposure on the reproduction of fish under controlled experimental conditions. Therefore, evidence to support the use of either inbred or more genetically variable outbred fish strains in ecotoxicological tests is currently lacking.

In addition to potential negative synergies between inbreeding and chemical exposure (or other stressors), inbreeding itself can also have negative fitness consequences because it can lead to inbreeding depression. Inbreeding depression has been documented for birth weight, survival, reproduction and resistance to environmental stressors (reviewed in Keller and Waller 2002). Many male characters closely related to fitness seem especially susceptible to inbreeding depression, presumably because of their history of directional selection (Michalczyk et al. 2010; Prokop et al. 2010; Okada et al. 2011). It might be predicted therefore that inbreeding depression for male traits linked to fitness is more severe under chemical stress (Meagher et al. 2000; Armbruster and Reed 2005). Again this has not been tested in fish.

Here, we investigated potential interactions between inbreeding and chemical exposure on the reproductive success of the zebrafish. The chosen chemical for this work, clotrimazole, is used in agriculture and in veterinary and human medicine. Clotrimazole is an imidazole fungicide and inhibits cytochrome P450 14α-lanosterol demethylase (CYP51; Lupetti et al. 2002). CYP51 is the most widely distributed P450 gene family, being found in animals, plants, fungi, yeast, lower eukaryotes and bacteria (Lepesheva and Waterman 2004). It plays a key role in cholesterol biosynthesis (Debeljak et al. 2003) and in the production of meiosis-activating sterols (Zarn et al. 2003). Azole compounds, like clotrimazole, have also been shown to inhibit several other P450 enzymes (Zhang et al. 2002), including aromatase (Zarn et al. 2003) an important enzyme involved in steroidogenesis and converting androgens to estrogens, and have been shown to disrupt steroidogenesis in fish (Ankley et al. 2007; Brown et al. 2011). Clotrimazole is designated as a priority hazardous substance by the European Union (OSPAR 2005).

We exposed inbred and outbred zebrafish to clotrimazole, and measures of reproductive output (number of eggs spawned and embryo viability) and competitive siring success were determined, the latter via paternity assessments of the offspring using DNA microsatellite genotyping. The key objectives were to determine whether exposure to an EDC affected reproductive output to a greater extent in inbred fish compared to outbred fish and whether inbred males were less reproductively successful compared to outbred males in competitive breeding trials. This information is not only important for a better understanding of the efficacy of ecotoxicology studies, but also for our understanding of how chemicals can impact endangered species, which, almost by definition, are found in small inbred populations. Furthermore, inbreeding potentially alters phenotypic and genetic variances (Falconer 1989). This in turn influences fitness landscapes and evolution, potentially facilitating population divergence (Whitlock 1995),

especially when inbred and outbred populations respond differently to similar selection.

Materials and methods

Generation of inbred and outbred fish

The study was performed using replicate inbred and outbred lines generated through controlled zebrafish matings at the University of Exeter. Briefly, second-generation wild male zebrafish (of Bangladesh origin) were mated with Wild Indian Karyotype (WIK) females to generate hybrid WIK/wild strain fish (F_1, Fig. 1), from which 20 full-sibling families were produced (F_2, Fig. 1). From these, pairwise crosses were performed randomly to generate 20 outbred and 19 (one family failed to reproduce) inbred (via full-sibling mating) family lines (F_3, Fig. 1). The theoretical inbreeding coefficients of F_3 inbred and outbred fish used as parents in the breeding study were $F_{IT} = n + 0.25$ and $F_{IT} = n$, respectively, n representing the unknown inbreeding coefficients of WIK and wild zebrafish. Genetic variation at 12 microsatellite markers for both inbred and outbred zebrafish is reported in Brown et al. (2011).

Exposure regime

At 37 days post-hatch (dph), F_3 fish were either exposed to clotrimazole (CAS no. 23593-75-1) at a single nominal concentration of 5 μg L^{-1} or maintained in water without chemical treatment for 123 days (until 160 dph). Clotrimazole has a predicted environmental concentration (PEClocal) of 0.2 μg L^{-1} (OSPAR 2005) and higher concentrations (43.7 μg L^{-1}) have been shown to cause significant (>90%) skew in sex ratio towards males (Brown et al. 2011). The concentration of clotrimazole was measured in the water throughout the study using tandem liquid chromatography and mass spectrometry as described in Brown et al. (2011), further details are reported in Table S1 of the supporting information. During the *in vivo* exposure, fish were maintained in 60 L (working volume) glass aquaria divided into eight separate compartments, each holding eight fish (of the same family group). Further details of the exposure regime can be found in Brown et al. (2011). Inbred and outbred fish were maintained separately, preventing any olfactory (pheromonal) contact. Details of the experimental conditions are provided (Table S1 of the supporting information).

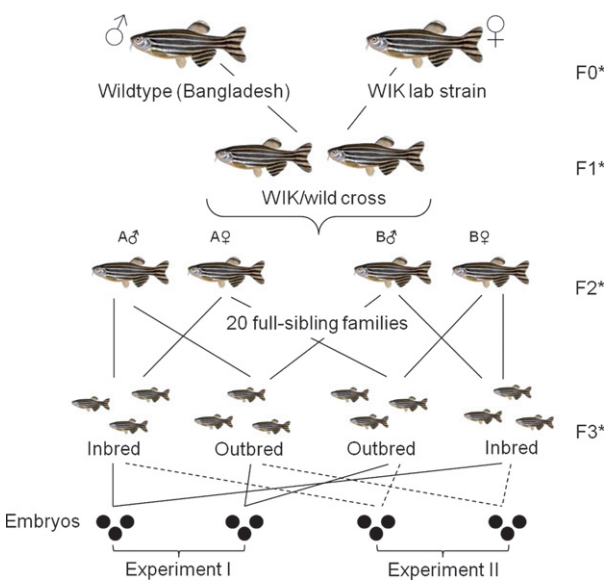

Figure 1 Generation of inbred and outbred zebrafish lines through controlled pairwise crosses. F0–F3* shows annotation corresponding to Brown et al. 2011.

Two experiments were conducted to investigate the combined effects of inbreeding and exposure to clotrimazole on the reproductive success of zebrafish. In the first experiment, spawning groups were established in which all fish in each group were either inbred or outbred (i.e. inbred and outbred fish did not directly compete) and had been either exposed to clotrimazole or maintained under control conditions (a fully factorial design, Fig. 2 Experiment I). Reproductive output was assessed in terms of number of eggs spawned and the proportion of live embryos. In the second experiment, the effect of clotrimazole exposure was investigated in a competitive breeding scenario, with inbred and outbred males directly competing for either an inbred or outbred female. As in the first experiment, all fish in each group had either been previously maintained under control conditions or exposed to clotrimazole (again a fully factorial design, Fig. 2 Experiment II). In addition to assessing reproductive output, paternity was also determined using DNA microsatellites. Because of cost constraints, we restricted our assessment to triads that contained an outbred female only, as previous (non-fish) studies indicate that male fitness is more susceptible to inbreeding (Saccheri et al. 1996).

Experimental design I: reproductive output assessed in inbred and in outbred fish

At 160 dph, clotrimazole exposure was terminated and fish of breeding condition from all treatment groups were transferred into the breeding aquaria. Fish were maintained in control water conditions as described earlier (Table S1). Forty separate spawning groups were established (20 controls and 20 exposed; see Fig. 2 Experiment I) and each group, comprising one female and two males (either all inbred or all outbred), were randomly assigned to breeding aquaria. All fish within each spawning group were from different families, having previously been maintained in

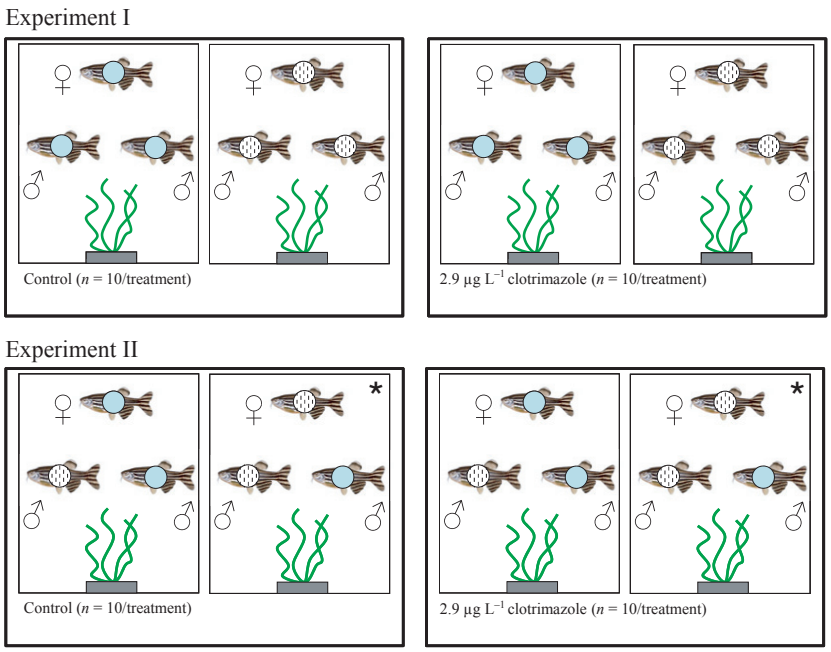

Figure 2 Experiment I: all fish in each spawning group were either inbred (●) or outbred (○) and had either been previously maintained under control conditions or exposed to clotrimazole; Experiment II: spawning groups contained one female (either inbred or outbred) and two males (one inbred and one outbred), and all fish in each spawning group had either been previously maintained under control conditions or exposed to clotrimazole. *denotes treatment used for subsequent parentage analysis.

separate compartments and were sized matched using wet weights. The mean standard length, wet weight and average size difference between fish in each group are provided in Table S2, the supporting information.

The fish were acclimated to test conditions for 2 days before the experiment commenced. For 10 consecutive days, reproductive output was determined for all 40 spawning groups. Eggs were collected daily, 2–3 h after the dawn transition, by removing the spawning tray from each aquarium. The eggs were cleaned in aquarium water and counted, and unfertilized/fungal-infected eggs were discarded. Fertilized eggs were incubated in aerated aquarium water at 28 ± 1°C, and fertilization rates were measured at 2, 8 and 24 h post-fertilization (hpf).

Experimental design II: reproductive output assessed incorporating direct competition between inbred and outbred males

Fish were allocated to 40 spawning groups (20 controls and 20 exposed), as described in Fig. 2 Experiment II. Each group contained one female (either inbred or outbred) and two males (one inbred and one outbred). As above, all fish within each spawning group were from different families and were sized matched using wet weights. The mean standard length, wet weight and average size difference between fish in each group are provided in Table S2. Reproductive output was recorded for 10 consecutive days, and fertilization rates measured at 2, 8 and 24 hpf as described earlier. Fertilized eggs were incubated until approximately 32 h prior to storage in 100% ethanol for subsequent paternity analysis.

At the end of the breeding studies, all fish were sacrificed by terminal anaesthesia (in 500 mg benzocaine, followed by destruction of the brain) in accordance with the UK Animals (Scientific Procedures) Act 1986. Fish were measured (standard length and wet weight) and a fin clip was taken and stored in 100% ethanol for subsequent paternity analysis. Blood was collected from each fish using heparinized capillary tubes, centrifuged and the plasma stored at −20°C for analysis of 11-ketotestosterone levels.

11-ketotestosterone quantification

11-Ketotestosterone (11-kt) is the most active androgen in male fish; it stimulates secondary sexual characteristics, spermatogenesis and reproductive behaviour, and the correlation between 11-kt concentrations, behavioural dominance and breeding performance has been widely documented in fish (reviewed in Borg 1994). 11-kt was quantified at the Centre for Environment, Fisheries and Aquaculture Science (CEFAS, Weymouth, UK) from 1-μL plasma using radioimmunoassay according to the method

described by Scott et al. (1984). The detection limit of the assay was 1.22 ng 11-kt mL^{-1}.

Paternity analysis

Paternity analysis was conducted on twenty fertilized eggs randomly taken from each triad across the experimental period: the number of eggs sampled per day was in direct proportion to the total number of eggs spawned on that day. In total, 378 eggs were genotyped. All adult fish (n = 20 inbred males, 20 outbred males and 20 outbred females) were genotyped from fin clips collected at the termination of the study.

DNA was extracted from parental fins and fertilized eggs using ammonium acetate precipitation (adapted from Bruford et al. 1998). Six microsatellite loci [Z249, Z6104, Z9230, Z20450, Z4830 (www.zfin.org) and Ztri1 (Coe et al. 2008)] were used to assign paternity following Coe et al. (2008). 98.5% of all embryos tested could be assigned to a single parental pair. Fertilized eggs unable to be unequivocally assigned to sires were not included in the analyses.

Data analysis

All statistical analyses were carried out using SigmaStat 3.1 (Systat Software Inc., Chicago, IL, USA) unless otherwise stated. Throughout the article, data are presented as mean ± one standard error of the mean. Data were assessed for normality and homogeneity of variances using the Kolmogorov–Smirnov and Levene's median tests, respectively. If parametric assumptions were met, data were analysed using analysis of variance (ANOVA) with *post hoc* assessment of differences made using the Holm–Sidak multiple comparison method. When normality assumptions were not met, Kruskal–Wallis one-way ANOVA was used to compare medians and *post hoc* Dunn's multiple comparison procedure applied as appropriate. Data for proportion of offspring sired by inbred and outbred males were analysed using a generalized linear model (GLM), with binomial errors and permutation tests (100 000), using the software R 2.8.1 (R: A Language and Environment for Statistical Computing, R Core Team, Vienna, Austria). Inbreeding depression was estimated for a number of traits (embryo viability, number of eggs produced, siring success and plasma levels of 11-kt) by calculating the coefficient of inbreeding depression (δ):

$$\delta = 1 - (X_I/X_O)$$

where X_I is the mean trait value for inbred progeny and X_O is the mean trait value for outbred progeny. Positive δ values indicate that trait values of outbreds exceed those of inbreds, whereas negative δ values indicate the opposite.

Results

Clotrimazole exposure concentrations

The geometric mean measured clotrimazole concentration was 2.9 μg L^{-1} (58% of the nominal 5 μg L^{-1}; Table S1).

Experiment I: reproductive output assessed in inbred and in outbred fish

Reproductive output

The mean numbers of eggs produced per spawning group per day (batch size) were as follows: control outbred fish, 26 ± 3; control inbred fish, 24 ± 2; exposed outbred fish, 26 ± 4, and exposed inbred fish, 27 ± 4 (Fig. 3 Experiment I). Whilst there was variation in the number of eggs produced on each study day (two-way repeated measures analysis of variance (ANOVA), $F_{9,324}$ = 17.23, P = <0.001), there was no overall difference in the number of eggs spawned between inbred and outbred females (P = 0.992), exposed or non-exposed (P = 0.336), and there was no statistical interaction between study day and treatment groups (P = 0.470). Embryo viability declined across all treatment groups over the 24 h period after egg collection (two-way ANOVA with Holm–Sidak multiple comparison procedure, $F_{2,108}$ = 52.0, P = <0.001, Fig. 4 Experiment I). However, at 24 hpf, viability was significantly reduced in inbred fish exposed to clotrimazole (39.0 ± 4.9%) compared to all other treatment groups (68.8 ± 6.0%, 62.9 ± 5.6% and 62.2 ± 7.1% in outbred control, inbred control and outbred exposed, respectively) ($F_{3,108}$ = 8.44, P = <0.001, Fig. 4 Experiment I). There was no statistically significant interaction between different treatment groups and the time of analysis ($F_{6,108}$ = 1.6, P = 0.151).

Experiment II: reproductive output assessed incorporating direct competition between inbred and outbred males

Reproductive output

The mean number of eggs produced per spawning group per day was as follows: control fish with outbred female, 12 ± 4; control fish with inbred female, 15 ± 1; exposed fish with outbred female, 16 ± 2; and exposed fish with inbred female, 13 ± 1. Similar to Experiment I, the daily number of eggs produced showed significant variation across the experimental period (two-way repeated measures ANOVA, $F_{9,324}$ = 2.62, P = 0.006; Fig. 3 Experiment II), but no differences were seen between inbred and outbred (P = 0.948), or exposed and non-exposed females (P = 0.766), and there was no statistical interaction between study day and treatment groups (P = 0.309). In this experiment, whilst there was a decline in embryo viability across all treatment groups over time (two-way ANOVA with Holm–Sidak multiple comparison procedure, $F_{2,107}$ = 24.9, P = <0.001, Fig. 4 Experiment II), there was

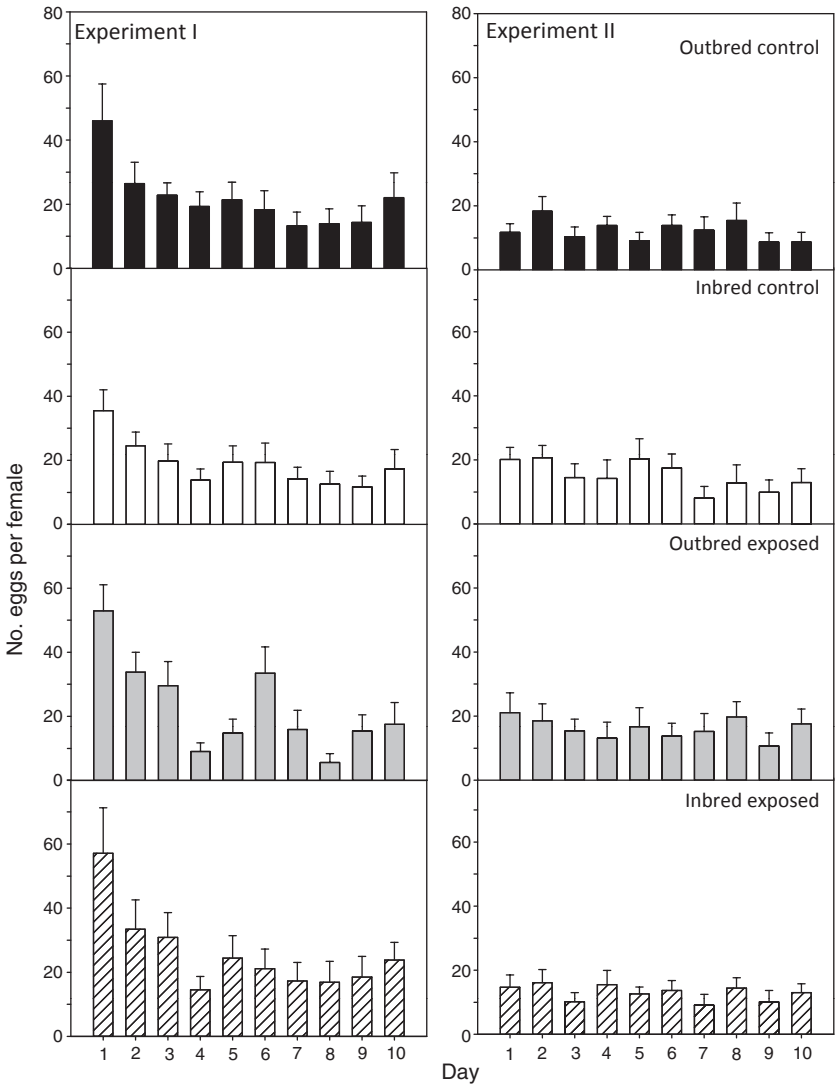

Figure 3 The effect of inbreeding and clotrimazole exposure on number of eggs spawned per female, over the 10-day study period of Experiment (I) and Experiment (II). Data presented are mean ± SE, $n = 10$. Each spawning group contained one female and two males. In Experiment I, all fish were either outbred or inbred and had either been previously maintained under control conditions or exposed to clotrimazole, as indicated. In Experiment II each spawning group contained one female (either inbred or outbred, as indicated) and two males (one inbred and one outbred), and all fish had either been previously maintained under control conditions or exposed to clotrimazole, again as indicated.

no significant differences between treatment groups ($F_{3,107} = 1.61$, $P = 0.191$; Fig. 4 Experiment II). There was also no statistically significant interaction between treatment groups and time of analysis ($F_{6,107} = 0.35$, $P = 0.91$). Embryo viability at 24 hpf in each treatment group was as follows: 89.6% ± 2.2 for outbred control, 91.1% ± 2.2 for outbred exposed, 84.7% ± 2.5 for inbred control and 89.8% ± 2.2 for inbred exposed, respectively. The lack of significant differences in embryo viability (which were seen in Experiment I) could be because outbred males sired more offspring in the fertilization competition between inbred and outbred males (see Paternity analysis and 11-kt) and therefore 'rescued' embryo viability.

Paternity analysis

Paternity analysis revealed that in non-exposed triads, outbred males sired on average 47% (±0.07) of the offspring (Fig. 5). Following exposure to clotrimazole, this proportion increased to 63% (±0.06) of the offspring. Using a two-tailed statistical test, this difference was not significant at the 5% level (GLM with binomial error; $F_{1,18} = 0.6221$; $P = 0.058$; Fig. 5). Arguably the use of a one-tailed statistical test is appropriate here because the prediction is

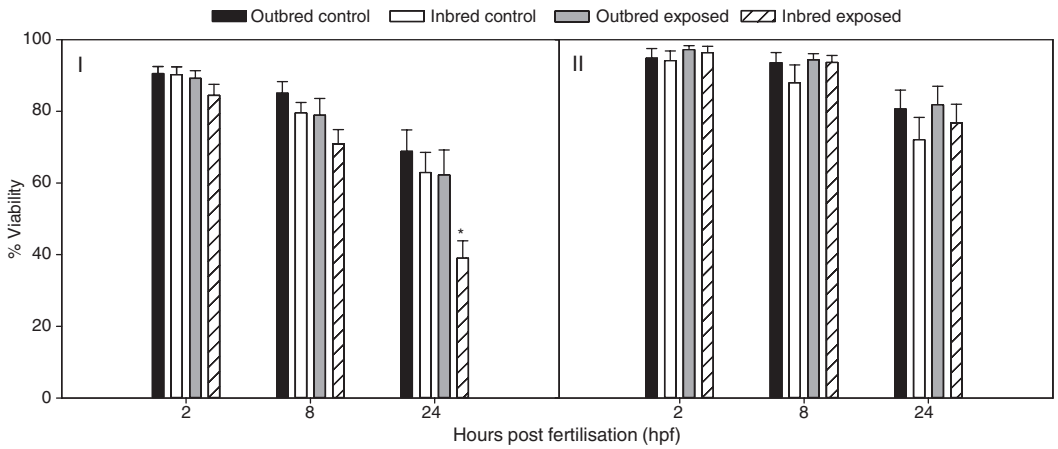

Figure 4 The effect of clotrimazole exposure on embryo viability at 2, 8 and 24 h post-fertilization (hpf), across the 10-day study period of Experiment (I) and Experiment (II). Data presented are mean ± SE, $n = 10$. * denotes significantly different to all other treatment groups ($P = <0.001$). In Experiment I, all fish were either outbred or inbred and had either been previously maintained under control conditions or exposed to clotrimazole, as indicated. In Experiment II each spawning group contained one female (either inbred or outbred, as indicated) and two males (one inbred and one outbred), and all fish had either been previously maintained under control conditions or exposed to clotrimazole, again as indicated.

directional (inbreeding and exposure should reduce reproductive success), in which case these results would be statistically significant ($P = 0.029$). *Post hoc* analysis revealed that a sample size of 75 (versus $n = 20$ used here) would have been great enough to achieve significance using a two-tailed test, assuming that the observed trend was maintained.

Quantification of 11-kt in male plasma

Plasma 11-kt concentrations in male fish in Experiment II were as follows: 6.2 ± 0.42 ng mL^{-1} in control outbreds; 3.24 ± 0.23 ng mL^{-1} in control inbreds; 6.29 ± 0.44 ng mL^{-1} in exposed outbreds; and 3.40 ± 0.27 ng mL^{-1} in exposed inbreds. Three samples that recorded below the limit of detection were assigned a value of half of that of the detection limit (0.61 ng mL^{-1}). There were no overall differences in 11-kt levels between control and exposed treatment groups (three-way ANOVA with Holm–Sidak multiple comparison procedure, $F_{1,29} = 3.33$, $P = 0.078$, Fig. 6A). However, in both control and clotrimazole exposed males, levels were significantly higher in outbred fish compared with inbred fish ($F_{1,29} = 19.43$, $P = <0.001$, Fig. 6a). There was no statistically significant interaction between treatments and the degree of inbreeding ($F_{1,29} = 0.01$, $P = 0.92$).

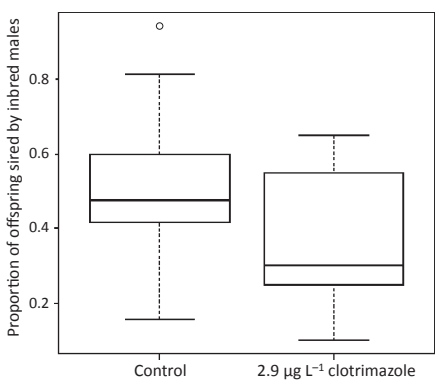

Figure 5 Proportion of offspring sired by inbred males in competition with outbred males in control and exposed treatment groups (GLM with binomial error; $F = 0.6221$; df = 1, 18; $P = 0.058$). Data analysed for spawning groups containing outbred females only. The central line within the box is the median. The box represents the upper and lower quartiles, and the whiskers the 95% confidence intervals. Open circles are outliers beyond the 95% confidence intervals. $n = 9$.

Levels of 11-kt were also analysed based on the proportion of embryos sired by each male. In the control group, there were no significant differences in plasma 11-kt concentrations between male fish siring the highest proportion of offspring (the most reproductively successful males; 3.75 ± 0.56 ng mL^{-1}), and the male fish siring the lowest proportion of offspring (the least reproductively successful males; 4.66 ± 0.77 ng mL^{-1}), irrespective of the degree of inbreeding. However, in the clotrimazole treatment group, the most reproductively successful outbred males had significantly higher plasma 11-kt levels (8.713 ± 0.86 ng mL^{-1}) compared to the least successful males (4.559 ± 1.40 ng mL^{-1}; three-way ANOVA with Holm–Sidak multiple comparison procedure, $F_{1,29} = 6.899$, $P = 0.014$, Fig. 6B). This trend was not observed in inbred fish, and there was no overall statistically significant interaction between the degree of inbreeding, exposure to clotrimazole and the proportion of offspring sired ($F_{1,29} = 2.129$, $P = 0.087$).

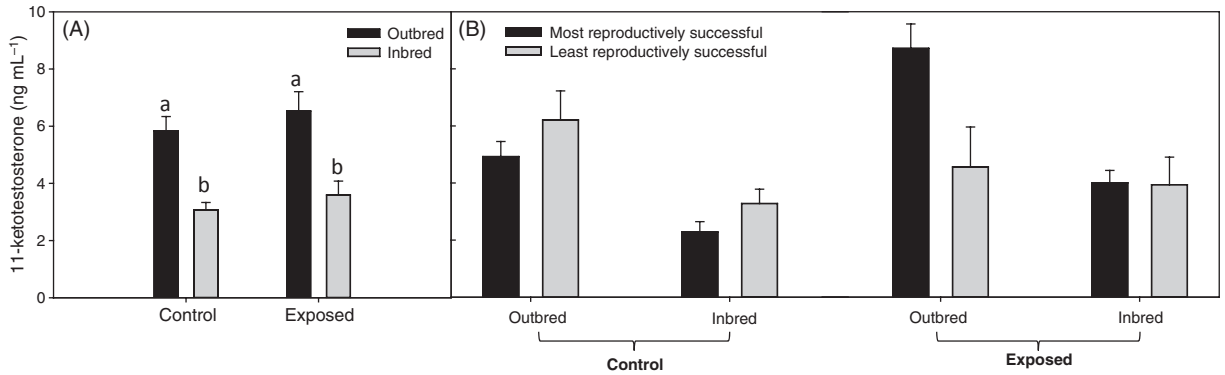

Figure 6 Comparison of plasma 11-kt concentrations in male zebrafish from Experiment II between (A) control and exposed, outbred and inbred fish (n = 20), and (B) the most and least reproductively successful male zebrafish, split by treatment group and degree of inbreeding (n = 9). Reproductive success was determined by the number of offspring sired by each male within each spawning group. Different letters denote significant differences between treatment groups.

Inbreeding depression

Inbreeding depression coefficient values (δ) were calculated for key reproductive traits. In Experiment I, where fish in each spawning group were either all inbred or all outbred, estimates of δ values ($\times 100$ for ease of comparison) in control and clotrimazole exposed fish were -1.4 and 24.0 for embryo viability, and 9.9 and -0.5 for the number of eggs produced, respectively. Thus, the addition of chemical stress increased inbreeding depression for embryo viability, but had no great effect on egg production. In Experiment II, we calculated inbreeding depression for siring success in control and exposed fish. We found no evidence for reduced siring success when fish were inbred and not exposed to clotrimazole ($\delta = -12.8$: i.e. inbred males were more successful than outbred males), but with exposure, inbreeding depression in siring success was recorded (δ = 21.3). Thus, as with embryo viability, additional stress caused by clotrimazole exposure amplified the effects of inbreeding on a key male fitness component. Plasma 11-kt concentrations measured in fish in Experiment II showed δ values of 48.0 and 43.5 for control and exposed fish, respectively. Therefore, for this parameter, we found substantial inbreeding, which was not amplified by environmental stress.

Discussion

In this study, we found that inbreeding depression in a range of critical fitness determinants only became apparent when fish were additionally stressed by exposure to an EDC, the fungicide clotrimazole. Overall, we found that the additional stress caused by clotrimazole exposure increased inbreeding depression in half of the traits we assessed. Thus, our results are consistent with an increasing body of evidence suggesting stress can at least sometimes amplify inbreeding depression. In a meta-analysis of published experimental data, stress significantly increased inbreeding depression in 48% of cases reviewed (Armbruster and Reed 2005). Inbred individuals are expected to be less fit than outbred individuals because of increased homozygosity at genetic loci influencing fitness (Slate et al. 2000), and populations with low genetic variation appear less able to adapt to changes in environmental conditions, including exposure to different physical and chemical stressors (Brown et al. 2009). However, in line with the variability observed in our results, the impact of additional stress on inbreeding depression appears to vary across populations (Keller and Waller 2002) and trait types (Roff 1997).

To our knowledge, this is the first study examining the combined effects of inbreeding and exposure to an EDC in fish. Previous studies have shown inbreeding in fish can cause significant perturbations in life-history traits. For example, inbreeding has been shown to increase fry abnormalities, as well as reduce feeding efficiency, growth rate and fry survival in rainbow trout (*Oncorhynchus mykiss,* Walbaum) (Aulstad and Kittelsen 1971; Kincaid 1976; Gallardo and Neira 2005) and inbreeding lowers fertilization rates and egg hatching success in three spined sticklebacks (*Gasterosteus aculeatus,* Linnaeus) (Frommen et al. 2008). Similarly, in zebrafish, inbreeding leads to a significant reduction in fry survival and growth to 30 days post-hatch (Mrakovčič and Haley 1979).

Our results show that embryo viability significantly declines under the combined effects of exposure and inbreeding. However, inbreeding alone (theoretical F = 0.25) had no effect on reproductive output. Lack of inbreeding effects on egg production is consistent with inbreeding studies using other strains of zebrafish (Piron 1978; Mrakovčič and Haley 1979). Furthermore, the lack of

effects of clotrimazole exposure on fecundity compare well with those of Ankley et al. (2007) following exposure of fathead minnow (*Pimephales promelas,* Rafinesque) to ketoconazole (7 μg L^{-1}), another azole fungicide, and with other studies indicating an apparent resilience of zebrafish egg output to perturbations through chemical exposure. The decline in egg production over the two study periods is likely related to the higher levels and frequency of aggressive behaviours typical of fish maintained in small breeding groups (Paull et al. 2008), which may impact on the ability to maintain high rates of egg production over prolonged periods.

Paternity analysis in the non-exposed spawning triads revealed inbreeding alone (one generation of full-sibling inbreeding) did not affect male reproductive success. This is surprising given the apparent sensitivity of male fertility to inbreeding in other species (Okada et al. 2011). However, in spawning triads previously exposed to clotrimazole, there was a strong trend for outbred males to have superior siring success compared to inbred males. This apparent difference in paternity share could partly explain why embryo viability was decreased in the inbred spawning triads in Experiment I but not in Experiment II (where inbred females were teamed with both inbred and outbred males). The magnitude of inbreeding depression for siring success following exposure was about 21%, suggesting the additional stress caused by the fungicide was significant for this key male fitness component. This estimate of inbreeding depression is similar to those reported for other male life-history traits [e.g. 19% for male fertility in *Drosophila simulans* (Okada et al. 2011) and 18% for longevity in *D. melanogaster* (Hughes 1995)]. Inbreeding depression has also been found to affect other aspects of male reproduction, particularly male attractiveness, including copulation latency in *D. simulans* (Okada et al. 2011), sperm number and ornamental traits in guppies *Poecilia reticulate* (Peters) (Zajitschek and Brooks 2010), and the courtship calls of male crickets (Drayton et al. 2010).

The reduced (50%) plasma 11-kt concentrations seen in inbred fish cannot be attributed to size, as male zebrafish within each spawning triad were sized matched before the breeding studies commenced. We found substantial inbreeding depression in this trait, which was not affected by exposure to clotrimazole. High 11-kt levels in male fish have been correlated with behavioural dominance (Borg 1994) and increased reproductive success (Coe et al. 2008). We found no difference in plasma 11-kt concentrations between the most and least reproductively successful males in the control fish. However, in this study, outbred exposed males had high 11-kt levels and increased reproductive success, corresponding with an increase in paternity share of offspring. Although disruption to plasma 11-kt levels is consistent with the mode of action of clotrimazole (aromatase inhibition), it is unclear why this response would differ between inbred and outbred fish.

On balance, our data suggest inbreeding combined with clotrimazole exposure at a concentration marginally exceeding maximum predicted environmental concentrations [PEC_{local} = 0.2 μg L^{-1} (OSPAR 2005)] can reduce the reproductive competitiveness of male zebrafish. However, a larger scale study (adopting a similar competitive breeding design) would be needed to demonstrate this unequivocally. An alternative to this might be to simulate increased competition, for example, between four males as opposed to only two, to better discern chemical effects, as has been shown for 17α-ethinyloestradiol (Coe et al. 2009). In addition, maintaining chemical exposure during the breeding trials could allow a direct assessment of the chronic effects of clotrimazole exposure and inbreeding on adult spawning, as well as combined acute toxicity effects on embryonic life stages.

In conclusion, our data have shown that inbreeding can impact on fitness-related reproductive traits. However, not all traits are affected equally, and in some cases, inbreeding depression only becomes apparent with the additional stress of chemical exposure. This is one of the first studies reporting that effects of exposure to an EDC on reproductive success can differ between outbred and inbred animal strains. Consequently, EDCs may potentially affect inbred wild populations differently to outbred wild populations, and this is likely to be important when considering how populations might respond evolutionary to pollutants. Our data indicate the importance of better understanding interactions between pollutants and inbreeding. This is particularly pertinent when considering that both inbreeding and adaptive selection because of toxicant exposure may impact on the maintenance of genetic diversity and potential for future evolutionary change in wild populations, as well as the response of laboratory-maintained fish in ecotoxicology tests. Furthermore, this study also provides useful insights into the environmental relevance of using laboratory strains in ecotoxicology when addressing the consequences of exposure to chemical pollutants on the fitness of wild populations. An option for future ecotoxicology testing studies may be to measure genetic diversity of the animal models used. However, this may not be practical in many cases. When considering that genetically impoverished populations are generally more sensitive to the effects of chemical exposure, including results from the current study, inbred laboratory strains may provide adequate protection for the wild populations they are designed to protect. Additional approaches that may benefit the interpretation of ecotoxicology data in this respect include reporting of pedigree records, routinely checking the sensitivity of laboratory strains using standard toxicants, or the use of positive controls in standardized tests.

Data archiving statement

Data for this study are available at Dryad – doi:10.5061/dryad.6h822.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Experimental Conditions.

Table S2. Fish Size.

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6.1 Chapter 6 Supporting Information

TABLES

Table S1 - Experimental Conditions: Details of experimental set up conditions and fish husbandry

Table S2 - Fish Size: Standard length and wet weight for zebrafish in each treatment group

Table S1

Experimental Conditions: Details of experimental set up conditions and fish husbandry regime

Parameter	Experimental Conditions
Water Temperature	28 ± 1°C
Dissolved Oxygen	≥ 60 % saturation
pH	6.5 – 8.5
Photoperiod	12h:12h light:dark 07:00 – 19:00 with an artificial dawn/dusk transition of 20 minutes
Feeding Regime	2x/day • freshly hatched *Artemia nauplii* (4 % body weight per day; ZM Premium Grade *Artemia*, ZM, Hampshire, UK) • pelleted food (
Aquaria	450 x 350 x 300 mm in dimension 12L working volume and a flow-through of 6 tank volume changes per day. Contained a central spawning site, consisting of a metal tray (150 x 100 x 20 mm) filled with glass marbles and an artificial weed placed in the middle. Opaque dividers were placed between aquaria to prevent visual interactions between neighbouring spawning groups.
Analysis of clotrimazole concentrations (µg L^{-1}) (for more information, refer to Brown *et al*., 2011)	Chemical analysis was undertaken on Exposure Days -1, 1, 5, 8, 14, 21, 30, 41, 54, 68, 82 and 96, in control and exposure tanks. Control (0 µg L^{-1} nominal) • Arithmetic mean 2.98 µg L^{-1} • Geometric mean 2.87 µg L^{-1} • 95 % CI 0.57 • Limit of Detection 0.5 µg L^{-1} Clotrimazole (5µg L^{-1} nominal) • Arithmetic mean 0 µg L^{-1} • Geometric mean 0 µg L^{-1} • 95 % CI 0.1 • Limit of Detection 0.5 µg L^{-1}
Fish Health and General Condition	All fish responded well to feeding and were checked twice daily for any visible signs of ill health or abnormal swimming behaviour. Visual observations to confirm normality and apparent health of internal organs were made during necropsy.

Table S2

Fish Size: Standard length and wet weight for zebrafish in each treatment group

Experiment I: average size difference between male fish in spawning groups 0.017 ± 0.002g [mean ± standard error]; average size difference between female and male fish in spawning groups 0.092 ± 0.010g

Experiment II: average size difference between male fish in spawning groups 0.014 ± 0.003g; average size difference between female and male fish in spawning groups 0.090 ± 0.007g

Fish Size		**Control**		**Clotrimazole Exposed ($2.9\ \mu g\ L^{-1}$)**	
		Outbred	**Inbred**	**Outbred**	**Inbred**
Female	Weight (g)	0.321 ± 0.02	0.315 ± 0.03	0.315 ± 0.02	0.331 ± 0.02
	Standard Length (mm)	25.98 ± 8.2	26.54 ± 8.4	26.31 ± 8.3	26.31 ± 8.3
Male	Weight (g)	0.252 ± 0.01	0.241 ± 0.01	0.242 ± 0.01	0.247 ± 0.01
	Standard Length (mm)	25.73 ± 5.8	25.72 ± 5.8	25.88 ± 5.8	25.19 ± 5.6

7 CHAPTER 7: SKEWS IN SEX RATIO INDUCED BY ELEVATED TEMPERATURE COMBINED WITH AN ENDOCRINE DISRUPTING CHEMICAL ARE COMPOUNDED BY INBREEDING IN ZEBRAFISH

Skews in sex ratio induced by elevated temperature combined with an endocrine disrupting chemical are compounded by inbreeding in zebrafish

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7.1 Abstract

Endocrine disrupting chemicals (EDCs) can cause reproductive impairment in individuals and/or skew sex ratios in fish populations. These effects are potentially exacerbated by elevated environmental temperature, which can also influence gondal sex in fish and effective (breeding) population size. Small, inbred wildlife populations may be particularly at risk due to their already limited genetic variation, reduced adaptive capacity and increased susceptibility to inbreeding depression affecting survival, growth and reproduction.

Here we investigated effects on sexual differentiation and development in inbred (F_{IT} = n+0.25) and outbred (F_{IT} = n) zebrafish (*Danio rerio*), following combined exposure to an endocrine disrupting chemical, the aromatase inhibitor clotrimazole (0, 1.7 or 8 μg l^{-1}) and elevated environmental temperature (33°C versus 28°C). Inbred and outbred zebrafish family lines (n=20) were derived (at F_0) from the out-breeding of two wild populations sourced from Bangladesh and the F_3 generation were introduced to the exposure study from 40-100 days post fertilisation (dpf). Sex ratios in both inbred and outbred fish were skewed significantly towards males following high-level (8 μg l^{-1}) clotrimazole exposure and/or elevated temperature (33°C). The male-skew was greater in inbred treatments compared with

equivalent outbred treatments. There was also significant male-skew in inbreds (but not outbreds) following low-level clotrimazole exposure (1.7 μg l^{-1}), in combination with elevated temperature (33°C). Expression of *cyp19a1a* encoding aromatase, which plays a pivotal role in sex determination by converting androgens to oestrogens, exhibited no down-regulation (inhibition), but there was significant up-regulation (compensation) in the few remaining females in the inbred, high clotrimazole exposure treatments. Collectively our results indicate that inbred zebrafish are more susceptible to masculinisation from combinational exposure to an aromatase inhibiting chemical and elevated temperatures compared to outbred zebrafish. This highlights the potential vulnerability of inbred populations and therefore the importance of considering biological (genetic), as well as physical and chemical interactions in cumulative environmental risk assessment (ERA). However, by the same token, the conventional use of "relatively inbred" animals in ecotoxicology and ERA is likely to provide a degree of conservatism for the protection of wild fish populations in general.

Keywords: EDC, inbreeding, temperature, sex ratio

7.2 Introduction

Conserving global biological diversity (biodiversity) is essential for the maintenance of earth's support systems and the supply natural goods and services on which humans depend (UNEP, 2005). Major pressures on biodiversity include habitat fragmentation, rapid climate change and environmental pollution, which may impact on the migration (gene flow), adaptation and reproduction of wildlife populations (UNEP, 2012). Environmental risk assessment (ERA) (US EPA, 1992; EC, 2009; EFSA, 2010) serves to protect wildlife populations against the effects of chemical pollutants. This process relies ultimately on the detection of adverse "apical", population-relevant effects, including survival, growth and reproduction in laboratory tests on model organisms, which are intended to be representative of wildlife. However, these model organisms are often highly inbred and are reared and tested under optimal (control) laboratory conditions. Therefore the extent to which derived effects reflect

the situation in the wild is uncertain (Flint and Mackay, 2009; Nawy, 2012) and it has been argued that the measured endpoints are far removed from the ecological features that ERA aims to protect (Forbes et al., 2008). Currently safety factors are applied, where required, in ERA to account for uncertainties in the sensitivity of test endpoints, model test species and the potential biomagnification of chemicals up the food chain.

Effects of multiple/cumulative chemical exposures can potentially be additive, particularly for compounds with similar modes of action (Boobis et al., 2011). Understanding the interactive effects of chemicals with different modes of action and their effects in combination with other environmental stressors has recieved little study and has been identified as a research priority by the EU (EC, 2012). This is especially true for aquatic wildlife given predicted global climate change and associated pressures on water quantity and quality (Bates et al., 2008). Wildlife with temperature-dependent sex-determination, including some species of fish and reptiles, may be particularly susceptible to changes in climate. With the exception of tuatara (*Sphenodon guntheri*) (Mitchell et al., 2010), warmer temperatures during incubation induce female development in reptiles e.g. turtles (Kamel and Mrosovsky, 2006; Hawkes et al., 2007), whereas temperature elevation has been shown consistently to induce male development in fish (Ospina-Alvarez and Piferrer, 2008). Male-biased sex ratios could have a dramatic impact on fish population numbers, since the loss of females limits reproduction more than the loss of males (males only gain fitness through females) (Helfman et al. 1997; Parker, 1980; Warner 1997). Male-biased sex ratios have been demonstrated in fish in response to numerous other environmental changes too (reviewed by Delvin and Nagahama, 2002; Godwin et al., 2003; Uchida et al., 2004; Baroiller et al., 2009), including reduced dissolved oxygen, reduced nutrition, overcrowding, acidic pH<7 and salinity change, most of which are likely to accompany increased global temperature. Sexual differentiation and development in fish may also be strongly influenced by environmental chemicals that disrupt key endocrine signalling pathways by (i) binding with hormone receptors and acting as receptor agonists or antagonists (Andersen et al., 2006), (ii) altering hormone receptor expression, amplifying or reducing the effects of endogenous hormones (Chen et al. 2008) or (iii) interfering with the biosynthesis (Uchida et al. 2004), metabolism and transport (Hegelund et al. 2004) of endogenous hormones.

The zebrafish (*Danio rerio*, Hamilton) is a model species used widely in biomedical research (Rubinstein, 2003; Amsterdam and Hopkins, 2006), human toxicology (Spitzbergen and Kent, 2003; Rubinstein, 2006; and ecotoxicology (Scholtz et al., 2008). Like many other fish, gonadal sex in zebrafish is both genetically and environmentally determined (Ospina-Alvarez and Piferrer, 2008), involving the interaction of multiple autosomal genes, whose inlfuence may vary between different zebrafish strains (encompassing inbred laboratory and outbred wild strains) (Whitely et al., 2011; Anderson et al., 2012) and under different environmental conditions (Anderson et al., 2012). Sexual differentiation takes place from 45-77 days post fertilsation (dpf) under laboratory conditions (Maack and Segner, 2003; Maack et al., 2003). Ultimately differentiation of the testes or ovaries is under hormonal control, making the species particularly sensitive to endocrine disrupting chemicals (EDCs) (Maack and Segner 2004). The steroidogenic enzyme cytochrome P450 aromatase plays a pivotal role in sexual differentiation in zebrafish, as in other fish species, being solely responsible for the conversion of male hormones (androgens) to female hormones (estrogens) (Guiguen et al., 2010). Exposures of zebrafish to azole (aromatase inhibiting) compounds from early life (0-40-60 dpf: Uchida et al. 2004; Kinnerberg et al., 2007) or delayed exposures spanning pubescence/adolescence (45->70 dph) (Fenske and Segner, 2004; Brown et al., 2011) have been shown to lead to male development in zebrafish.

In this study we investigated the combined effects of an aromatase inhibitor, clotrimazole (at aqueous concentrations of 1.7 or 8 μg l^{-1}) and elevated temperature (33°C versus 28°C) on sexual differentiation and development in both inbred (F_{IT} = n+0.25) and outbred (F_{IT} = n) zebrafish, exposed in laboratory aquaria from 40-100 dpf. Inbreeding was achieved by full-sibling mating in the F_2 generation and although this represents a high-level, similar levels could be achieved in the wild over several generations, with similar or more severe consequences in terms of inbreeding depression (Crnokrak and Roff, 1999; Miller and Hedrick 2001). The relevance of the selected test temperatures is supported by extensive meteorological measurements and predictions across India and Bangladesh. These indicate that mean air temperatures are currently 27.5°C during the summer monsoon, coinciding with the zebrafish breeding season (Spence et al., 2008), potentially rising 3-5°C by the end of the

century under IPCC Scenario A2 (Rupa Kumar et al., 2006). We hypothesised that sex ratios would be increasingly skewed towards males with increasing concentrations of clotrimazole and with elevated temperature, and that the effects of these environmental factors would be additive (Boobis et al., 2011) due to a common, principal mode of action (here assumed to be aromatase inhibition). We also hypothesised that inbred zebrafish would be more susceptible to masculinisation due to inbreeding depression and loss of heterozygosity (including hybrid vigour) at the aromatase (*cyp19a1a*) locus and other sex-related loci. Implications of the interactive effects of the mixture of stressors applied (chemical, physical and biological (genetic) are considered for cumulative ERA.

7.3 Methods

7.3.1 Test substance

Clotrimazole (CAS number 23593-75-1) was obtained from Sigma-Aldrich Ltd., Dorset, UK (98% pure). Clotrimazole is a potent ligand and inhibitor of P450(CYP)19 aromatase, which catalyses the conversion of androgens to estrogens in mammals (Trösken et al., 2004) and in other vertebrates, including fish (Noaksson et al. 2003; Hinfray et al. 2006). This property is exploited in the treatment of estrogen-responsive human breast cancer (Trösken et al., 2004).

Nominal aqueous exposure concentrations of 2 and 10 µg clotrimazole l^{-1} (versus a predicted environmental concentration (PEC_{local}) of 0.2 µg l^{-1} (OSPAR, 2005), represent a range capable of inducing chronic effects in fish (Bruns, 2003), including male-biased sex ratios (Brown et al. 2011) and reduced fitness (Bickley et al., 2012) in zebrafish.

7.3.2 Breeding of inbred and outbred zebrafish family lines

Inbred and outbred zebrafish family lines (n=20) were derived in the laboratory at F_0 from >20 reciprocal pairings of individual males and females selected randomly from two distinct wild Bangladeshi populations: a) Mozahadi and b) Kechuri Beel (Supporting Information:

Figure S1; Table S1a). Brood stock were maintained in separate full-sibling families under standard laboratory conditions: 28°C; 12h:12h light:dark photoperiod with a 20 min dawn-dusk transition and fed *ad libitum*. At each subsequent generation, pair-breeding was performed using a random-stratified approach: outcrosses between families were structured in order to maintain distinct lineages, but individual males and females were selected at random from each pre-determined family (Supporting Information: Table S1b-c). This resulted in 20 outbred (F_3) family lines with theoretical inbreeding coefficient F_{IT} = n, where n is the background inbreeding coefficient in the wild source populations. Full-sibling mating was also performed using randomly selected brothers and sisters from each of the F_2 brood stock family lines, resulting in 18 inbred (F_3) family lines with theoretical inbreeding coefficient F_{IT} = n+0.25 (Supporting Information: Table S1d). In order to characterise inbreeding, inbreeding depression (δ) was quantified according to Equation 1 (Falconer and Mackay, 1996) and the number of lethal equivalent alleles per diploid genome was estimated using Equation 2 (Morton et al., 1956).

Equation 1

$$\delta = (1 - (X_I/X_O)) / F$$

Equation 2

$$LE = - 2/F * \ln (X_I/X_O)$$

Where X_I is mean inbred survivorship, X_O is mean outbred survivorship, F is inbreeding coefficient = 0.25

The use of F_3 generation fish in our study guarded against outbreeding depression in the outbred wild zebrafish family lines. The symptoms of outbreeding depression may be confused with inbreeding depression, however, they occur as a result of initial outbreeding and the breakdown of co-adapted gene complexes in genetically divergent populations (e.g. wild zebrafish populations a and b). It has been shown that these complexes are generally restored or reconfigured by the F_3 generation (Templeton, 1986; Lynch, 1991; Monson and Sadler, 2010).

7.3.3 Experimental design and exposure treatments

Inbred and outbred zebrafish were subject to constant, combined exposures to clotrimazole (0, 2 or 10 µg l^{-1} nominal concentrations) and temperature (28 or 33°C) in a continual flow-through test system (Supporting Information: Figure S2) from exposure days 0-60 (40-100 dpf). Fish were introduced to the exposure system in three main groups, as determined by the breeding programme, and the exposure study ran for 180 (3×60) days. Twenty five sub-divided exposure tanks provided a total of 200, 7.5 litre compartments, giving 40 compartments for each of the combined treatments (partial factorial design, Table 1), 20 compartments for inbred and 20 for outbred families, which were separated to prevent exchange of pheromonal/olfactory cues. Treatments were set up in blocks, and block and tank positions (within blocks) were randomized. Each tank compartment was stocked with 4 fish selected at random from a given family.

Table1: Experimental treatments (partial factorial design)

Clotrimazole nominal exposure conc.s (µg l^{-1})	Inbred zebrafish (F_{IT}=n + 0.25)		Outbred zebrafish (F_{IT}=n)	
	28°C	33°C	28°C	33°C
0	✓	✓	✓	✓
2		✓		✓
10	✓	✓	✓	✓

Clotrimazole exposure concentrations were quantified in water samples on study days -4, 0 4, 9, 15, 17, 35, 56, 77, 119, 161, 178 using tandem liquid chromatography and mass spectrometry (LC-MS). Initial chromatographic separation of clotrimazole was carried out on a Gemini-NX C18 column (50 x 2 mm, 3.0 µm, Phenomenex, Torrance, CA). The column was fitted with a pre-filter (0.5 µm, Supelco, USA) maintained at 50°C and the flow rate was 500 µl min^{-1}. The elution gradient of eluent A) 0.1% ammonium hydroxide in water and

eluent B) LCMS grade methanol (T(min)/ % A was 0/90→3/0→5/0→5.1/90→6/90). A Quadrupole Ion Trap (Thermo-Finnigan TSQ Quantum Access) mass spectrometer with electrospray ionisation was used with the following parameters: sheath gas flow 60 arbitrary units, auxiliary gas flow 50 arbitrary units, spray voltage 3.0 kV, capillary temperature 300°C, capillary offset voltage 39 V, tube lens offset tuned. Positive ionization with selected reaction monitoring (SRM) was used for all analyses. The analyte (clotrimazole) corresponded to a product ion mass of 169 Da and the limit of quantitation was 0.2 $\mu g\ l^{-1}$.

Water temperature was measured hourly by an automated monitoring system (Facility Monitoring Systems, Malvern, UK), calibrated weekly by taking thermometer readings in each exposure tank. In the 33°C temperature treatments, water temperature was increased gradually from 28°C to 33°C over 48 hours (from Day 0 to the end of Day1). The following water quality parameters were monitored throughout the exposure study (see Supporting Information: Figure S2) and measurements were within guideline limits for fish sexual development tests (OECD, 2011): dissolved oxygen 70-100% saturation); pH 7.1-8.2; total ammonia-N <2 $\mu g\ l^{-1}$; chlorine <2 $\mu g\ l^{-1}$; hardness 41-69 mg l^{-1}; alkalinity 21-39 mg l^{-1}; suspended solids <3.00 mg l^{-1}; TOC <1-2.5 mg l^{-1}; COD <10 mg l^{-1}; inorganic and organic analytes <predicted no effect concentrations.

7.3.4 Sampling and analysis of developmental endpoints

Mortality and developmental abnormalities (physical or behavioural) were recorded throughout the exposure period.

Body size was determined in all fish via *in vivo* measurements on exposure days 0/0, 14/15, 30/30, 34/40 and 51/60 for 28/33°C exposures respectively, underlined time points representing equivalent degree-day exposure periods (Neuheimer and Taggart, 2007). The first period represented exponential growth leading up to the critical period of sexual differentiation in zebrafish (40-55 dpf), the second spanned the latter part of this critical period (55-80 dpf), and the final period represented pubescence/adolescence under standard

28°C laboratory conditions (Maack and Segner, 2003). Wet weight [±10 mg] was measured using a chemical balance and standard length [±1 mm] was measured from the mouth to the base of the tail fin via scaled photography (Nikon D90, Nikon Imaging, Tokyo, Japan), followed by image analysis (ImageJ: Schneider et al., 2012). Male and female fish were not discriminated for these *in vivo* measurements. Rather than comparing absolute length and weight, mean specific growth rate (SGR) was calculated (Bolger and Connolly, 1989) per tank compartment (family) for each of the degree day growth periods.

All fish were sampled terminally on exposure day 60 and *ex vivo* measurements of blotted wet weight [±1 mg] and standard length [±0.1 mm] were made for each fish. Necropsy was then performed and the right gonad was excised, weighed [±0.05 mg], snap frozen in liquid nitrogen and stored at –80°C for subsequent molecular analysis. The left gonad was left *in situ* for histological processing. Whole bodies were fixed (maximum 6 hours) in Bouin's solution (Sigma Aldrich, Dorset, UK), progressively dehydrated in 70-100% industrial methylated spirits and embedded in paraffin wax. Serial transverse body sections (replicate 5 µm sections, obtained at four 500-1000 µm intervals) were mounted on glass slides, stained using haematoxylin and eosin and examined using a Leitz Diaplan (Leica Microsystems GmbH, Wetzlar, Germany) light microscope (×10-100) magnification). The most mature germ cell stages were recorded in individual male gonads (stages: i spermatogonia; ii spermatocytes; iii spermatids/spermatozoa) and individual female gonads (stages: i primary oocytes; ii cortical alveolar/secondary oocytes; iii vitellogenic oocytes) as a measure of gonadal development.

7.3.5 Gene expression analysis

Total RNA was extracted from individual male and female gonad tissue samples (n=6 per treatment) and cDNA was synthesised using Superscript Vilo© (Invitrogen, Paisley, UK) (Supporting Information: Table S2). Oligo-nucleotide primers were designed for the following target gene using Beacon Designer 3.0 (Premier Biosoft International, Palo Alto, CA): aromatase (*cyp19a1a*: NCBI accession NM_00131154.2). Real-time quantitative PCR

was performed in triplicate for each sample using a BioRad IQ5 PCR system (Bio-Rad Laboratories, Hercules, CA). Target gene expression was efficiency-corrected and quantified relative to the housekeeping gene, ribosomal protein l8 (*rpl8*: NCBI accession NM_200713.1) (Supporting Information: Table S2).

7.3.6 Statistical analysis

Raw data were first assessed for normality using the Anderson-Darling test. Data failing this test (gonad weight and relative gene expression) were $\log_{10}$ transformed. All proportional data including % fertilization, % hatching, % survival (survivorship) and the proportion of females per tank per treatment were transformed by arcsine square root. Normality was confirmed as before and data were then assessed for equality of variances between treatment groups using Bartlett's or Levene's test prior to statistical analysis. One-way ANOVA was used to compare mean fecundity, fertilization, hatching and survivorship in inbred versus outbred families before the commencement of the exposure study (0-30 dpf). The size spectra (standard length and wet weight) of inbred and outbred families were also compared using one-way ANOVA at the start of the study (40 dpf). The above routine tests were run in Minitab 16 (Minitab, Coventry, UK) and differences were accepted as significant when $p \leq 0.05$.

The various developmental endpoints measured during and at the end of the exposure study were expressed as family means and compared between n=18 inbred and n=20 outbred families across all treatments (unless stated otherwise). Ranges were expressed as standard error of the mean (SEM). The data for each endpoint/response were analysed using linear mixed effects (lme) models with family as a random effect (R-statistics version 2.15.2, R Foundation for Statistical Computing). Specific lme models are defined below. Statistical significance of fixed effects was determined by ANOVA comparisons of successively simpler models, with improved Akaike Information Criterion (AIC) scores.

Specific growth rate

Mean specific growth rates (SGR_{weight} and SGR_{length}) per tank compartment (family), per treatment were compared for each of the degree day growth periods (Days 0-14/15, 14/15-34/40 and 34/40-51/60). Therefore fixed effects were period, breeding, clotrimazole exposure and temperature. The initial lme model included interactions between period, breeding and clotrimazole exposure and between breeding and temperature. The final best fit models were found to be additive models: SGR_{weight} ~ period + temperature (AIC = 2760); SGR_{length} ~ period + breeding + clotrimazole exposure + temperature (AIC = 1173) (Supporting Information: Table S4a and S4b).

Sex ratio

Sex ratios were quantified at the end of the exposure study as the proportion of females in each tank compartment (family), per treatment, since this takes into account the effect of social interaction on sex determination (Delvin and Nagahama, 2002). Proportions were transformed (arcsine square root) before lme modelling. Fixed effects were breeding, clotrimazole exposure and temperature. Interactions were considered initially between breeding and clotrimazole exposure and between breeding and temperature, but not between the two environmental factors, since their principal modes of action on sex determination (inhibition of aromatase) are the same and were therefore assumed to be additive (Boobis et al., 2011). The final best fit model (AIC = 218) was found to be an additive model including all three fixed effects: Arcsine square root proportion of females ~ breeding + clotrimazole exposure + temperature (Supporting Information: Table S5).

Gonadal germ cell progression

Gonadal development stage was defined categorically in terms of germ cell progression for each individual (stage i, ii or iii) per sex, per tank compartment (family), per treatment. Fixed effects were gonadal sex, breeding, clotrimazole exposure and temperature. Inidividual was included as a random effect, in addition to family. The final best fit model (AIC = 993) was an additive model ~ gonadal sex + breeding. (Supporting Information: Table S6).

Gonad weight

Gonad weights (log_{10} transformed right gonad weights) were compared for each individual, per sex, per germ cell development stage, per tank compartment (family), per treatment using log_{10} body weight as a covariate. Therefore fixed effects were log_{10} body weight, gonadal sex, gonadal stage, breeding, clotrimazole exposure and temperature. Inidividual was again included as a random effect, in addition to family. The final best fit model (AIC = 570) was: log_{10} gonad weight ~ log_{10} body weight * gonadal sex * gonadal stage * breeding * clotrimazole exposure + temperature (Supporting Information: Table S7).

Aromatase expression

Aromatase expression (log_{10} transformed *cyp19a1a* expression relative to the control gene *rpl8*) was compared in n=6 females and n=6 males from different families in each treatment. All fish of each sex were at the same, most common gonadal germ cell development stage: i primary oocytes in females; iii spermatids/spermatozoa in males. Fixed effects were breeding, clotrimazole exposure and temperature. The initial and best fit model (AIC = 111) was: log_{10} aromatase expression ~ breeding * clotrimazole exposure + temperature (Supporting Information: Table S8).

7.4 Results

7.4.1 Breeding of inbred and outbred family lines

Fecundity (eggs per female per day), % fertilisation (0 dpf), % embryo hatch (3 dpf) and juvenile survivorship (% survival to 30 dpf) were measured in each of the inbred and outbred family lines prior to the start of the exposure study at 40 dpf. Mean fecundity, % fertilsation and % hatch were not significantly different between inbred and outbred family lines: fecundity 80.1±9 versus 92.1±7 eggs ANOVA $F_{(1,38)}$=0.08, p=0.78; % fertilisation 80.4±2% versus 83.9±2% ANOVA $F_{(1,38)}$=2.03, p=0.16; % hatch 69.5±6% versus 61.9±7% ANOVA

$F_{(1,38)}$=0.38, p=0.54. However, two of the inbred lines (2 and 15) failed to recruit beyond swim-up and exogenous feeding (5 -10 dpf) and comparing mean survivorship at 1, 3, 5, 10, 15, 20 and 30 dpf for inbreds versus oubreds revealed significant differences, with both the inclusion $F_{(1,169)}$=27.52, p<0.001 and exclusion $F_{(1,163)}$=16.62, p<0.001 of inbred families 2 and 15 (Figure 1). Inbreeding depression on survivorship from 0-30 dpf amounted to a value of δ=4.0 in inbred families 2 and 15 and a mean value of δ=2.7 for all inbred families, equating to LE=12.05 and LE=4.99 lethal equivalent alleles per diploid genome respectively.

During the exposure study (40-100 dpf) mortality was below 2% across all treatments. Physical deformities were rare, with dorso-ventral spinal curvature (lordosis) or jaw deformity occurring in 4 individuals (1%) amongst inbred families and 2 individuals (0.5%) amongst outbred families. Therefore the effect of physical deformity on treatment mean length and weight measurements was considered negligable.

7.4.2 Test exposure conditions

Measured clotrimazole concentrations were consistent within each treatment regime over the exposure period. The geometric mean measured concentrations of clotrimazole were 1.7±0.2 μg l^{-1} (85%) for the nominal of 2 μg l^{-1} and 8.0±0.8 μg l^{-1} (80%) for the nominal of 10 μg l^{-1} (Supporting Information: Table S3). Temperature was maintained at 28±1.5°C or 33±1.5°C throughout the exposure study.

Figure 1: Survivorship (0-30 dpf) for inbred versus oubred family lines

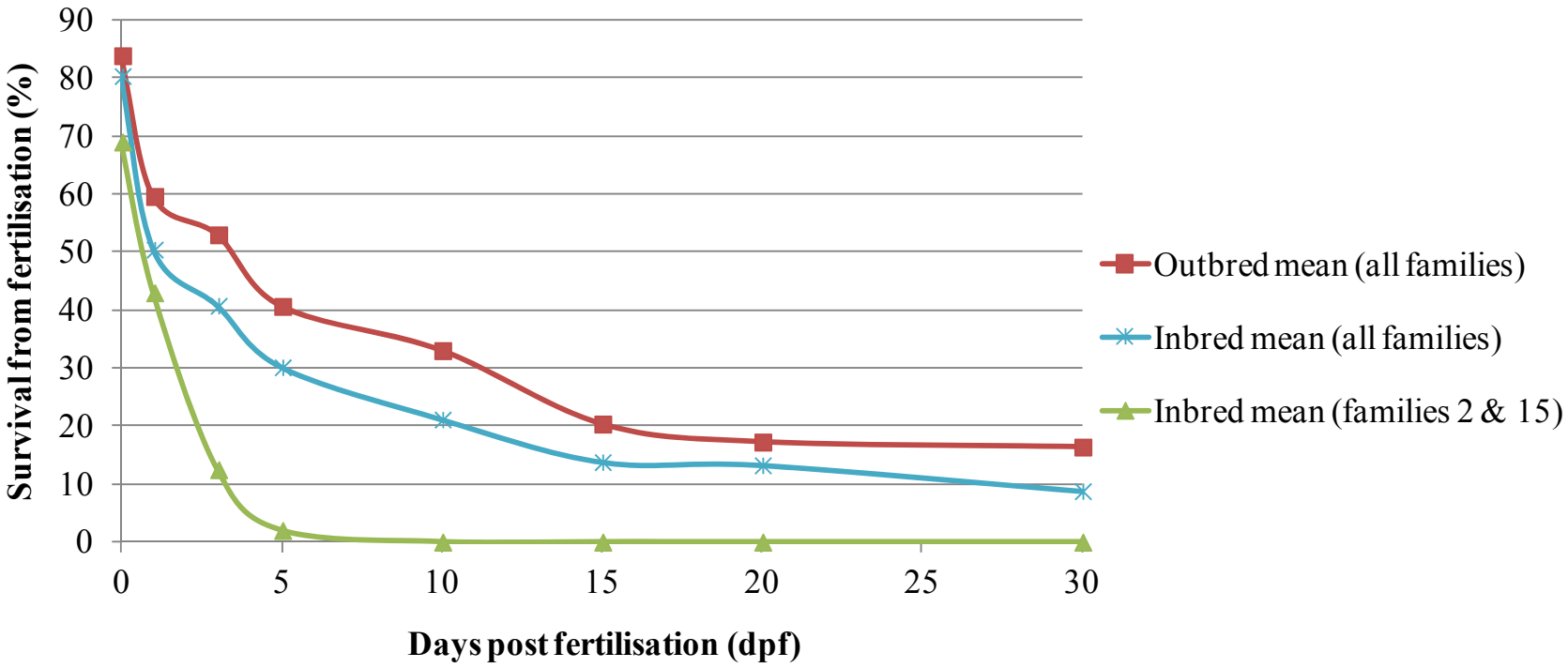
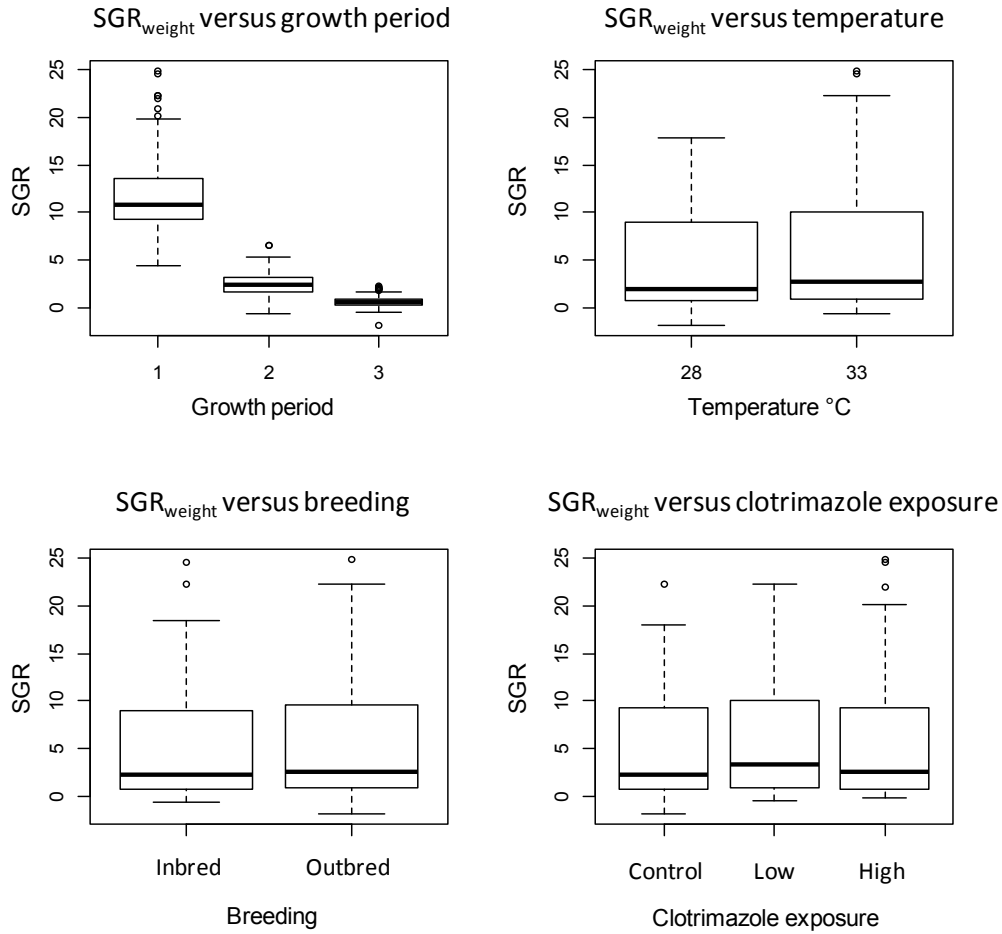


Figure 2: Specific growth rates versus exposure period, temperature, breeding and clotrimazole exposure treatment

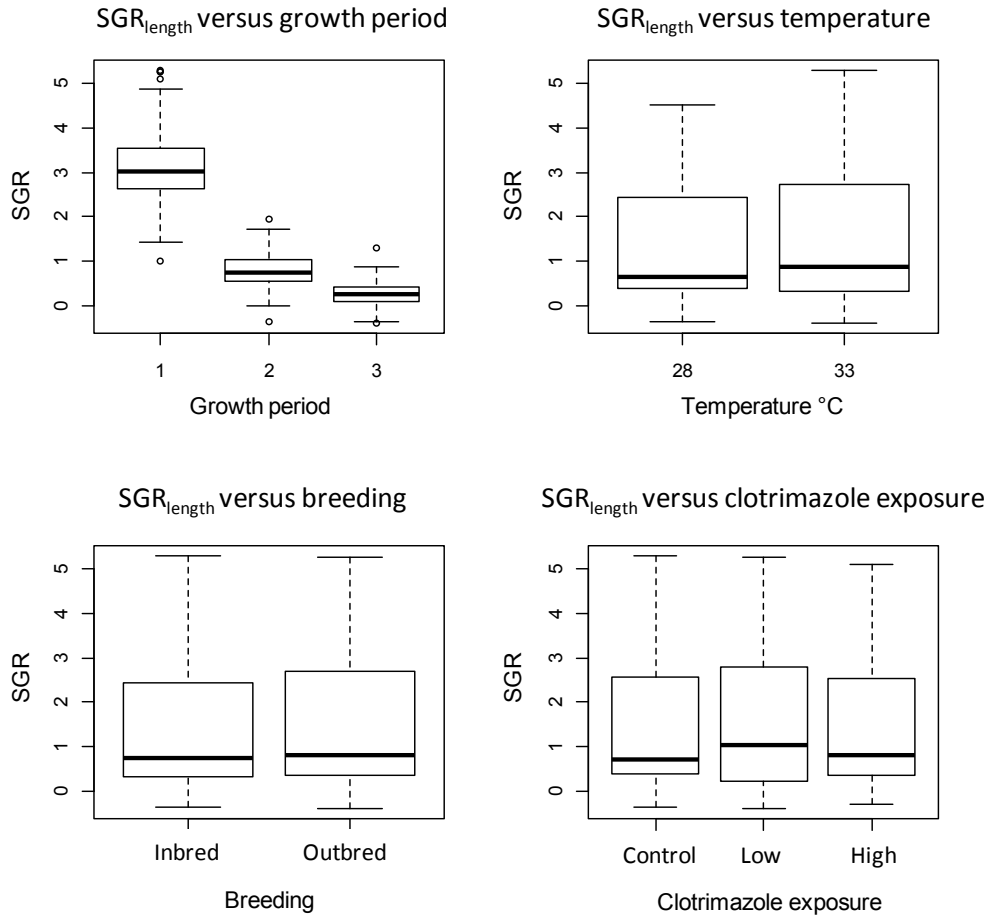
2a: Specific growth rate based on wet weight



Growth periods: 1 = 0-14/15 days; 2 = 14/15-35/40 days; 3 = 35/40-51/60 days
Boxes represent interquartile range and median (line), whiskers show full range excluding outliers (o)

Figure 2: Specific growth rates versus exposure period, temperature, breeding and clotrimazole exposure treatment

2b: Specific growth rate based on standard length



Growth periods: 1 = 0-14/15 days; 2 = 14/15-35/40 days; 3 = 35/40-51/60 days
Boxes represent interquartile range and median (line), whiskers show full range excluding outliers (o)

7.4.3 Effects of clotrimazole exposure and/or temperature on inbred versus outbred family lines

Somatic growth

At the start of the exposure (40 dpf), the standard lengths of inbred (10.16±0.13 mm) and outbred fish (10.09±0.14 mm) did not significantly differ, but inbreds were significantly heavier (14.10±0.73 mg) than outbreds (12.01±0.59 mg) [ANOVA wet weight $F_{(1,187)}$=5.05, p=0.026]. Therefore rather than comparing absolute length and weight data, mean specific growth rates (SGR) were calculated per tank compartment (family) for each of the degree day growth periods (Days 0-14/15, 14/15-34/40 and 34/40-51/60) during the study. According to the best fit lme model, Supporting Information: Table S4a and S4b), the fixed effects of growth period and temperature were additive and contributed significantly (p<0.015) towards SGR, which declined over time and increased with temperature (Figure 2) (SGR_{weight} 28/33°C: period 1 = 10.3/12.5; 2 = 2.0/2.9; 3 = 0.68/0.70) (SGR_{length} 28/33°C: period 1 = 2.8/3.2; 2 = 0.64/0.88; 3 = 0.20/0.34). This was despite defining equivalent degree day growth periods for the two alternative temperature treatments.

Sexual differentiation

On exposure day 60 (100 dpf) gonadal sex was confirmed histologically in the majority of fish (91-100%) in each of the experimental treatments (Table 2). The remainder had either not undergone sexual differentiation or a gonad was not identifiable.

The proportion of females in each tank compartment (family) per treatment, were not significantly different from overall sex ratios in each treatment group [Chi-square goodness of fit test: proportions (%) ($\chi^2_{(9,\ N=325)}$=0.184, p=1.0]. According to the best fit lme model for arcsine square root transformed data (AIC = 218), Supporting Information: Table S5), the fixed effects of breeding, clotrimazole exposure and temperature were additive and contributed significantly (p<0.0007) towards reductions in the proportion of females per compartment (family) per treatment, leading to greatest skew (3% females : 97% males) in the

combined inbred, high clotrimazole exposure (8 μg l^{-1}), elevated temperature (33°C) treatment (Figure 3).

Table 2: Treatment sex ratios

Treatment (breeding,clotrimazole,temp)	Overall proportion of males (%)	Overall proportion of females (%)	Proportion of females per tank compartment per treatment (%)
Inbred,control,28°C	55.4	41.5	40.7
Outbred,control,28°C	42.9	57.1	57.9
Inbred,high,28°C	78.5	18.5	17.6
Outbred,high,28°C	55.1	39.7	40.0
Inbred,control,33°C	66.0	27.0	28.2
Outbred,control,33°C	44.2	51.9	51.7
Inbred,low,33°C	78.0	18.0	18.1
Outbred,low,33°C	42.6	51.5	51.3
Inbred,high,33°C	87.5	3.1	2.8
Outbred,high,33°C	75.3	17.8	17.1

Clotrimazole treatment mean measured concentrations: control (0 μg l^{-1}); low (1.7 μg l^{-1}); high (8 μg l^{-1}).

Gonadal germ cell progression

According to the best fit lme model (AIC = 993) fixed effects influencing germ cell progression were determined to be breeding + gonadal sex (Supporting Information: Table S6). These factors were additive and contributed significantly ($p \leq 0.0017$) towards greater germ cell progression in outbred males (91±0.4% spermatids) versus inbred males (79±0.8% spermatids) but not in outbred females (44±2.8% ≥cortical alveolar stage) compared to inbred females (42±3.4% ≥cortical alveolar stage) (Figure 4).

Figure 3: Sex ratios in the different treatments

3a: Overall sex ratios in the different treatments

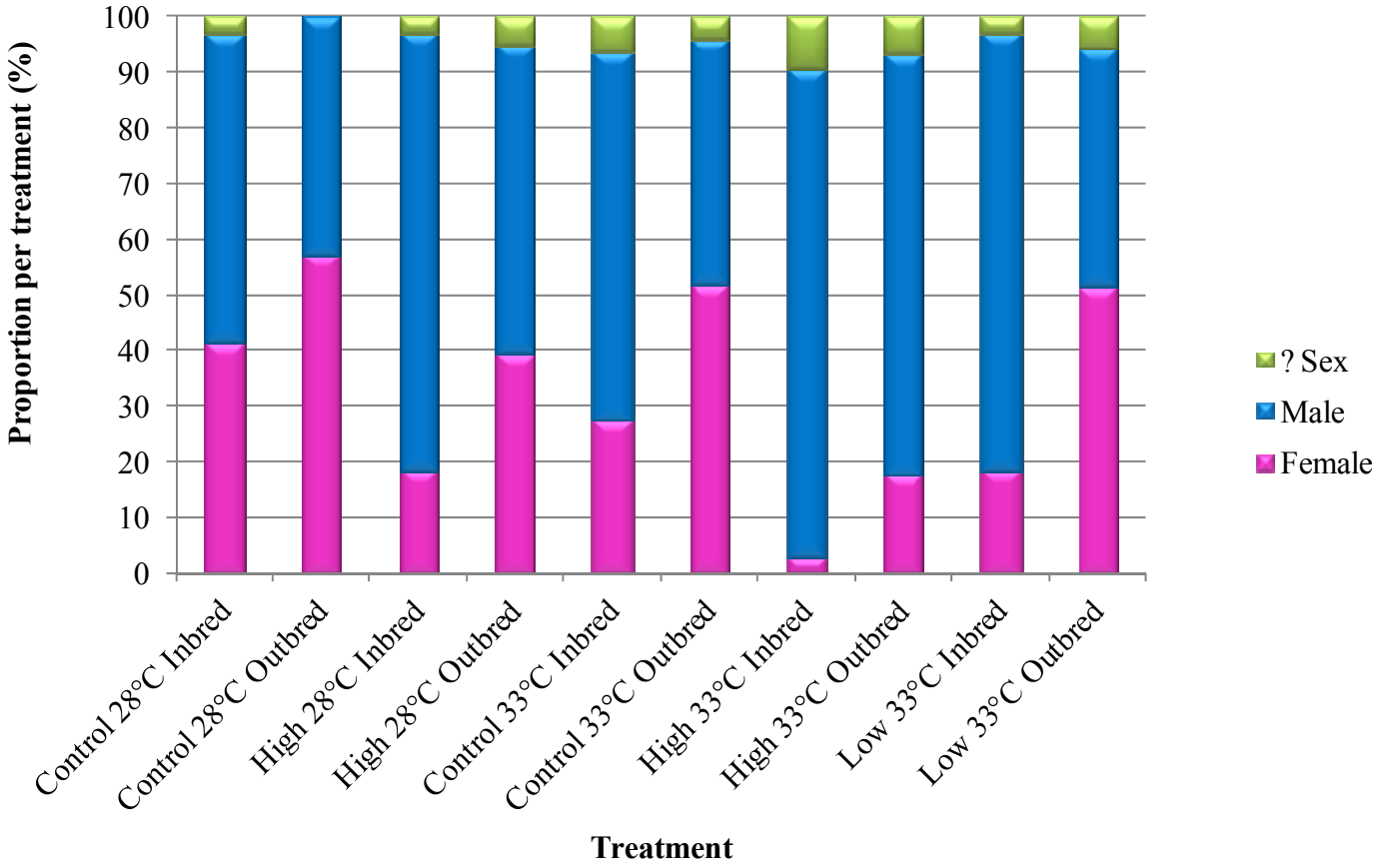
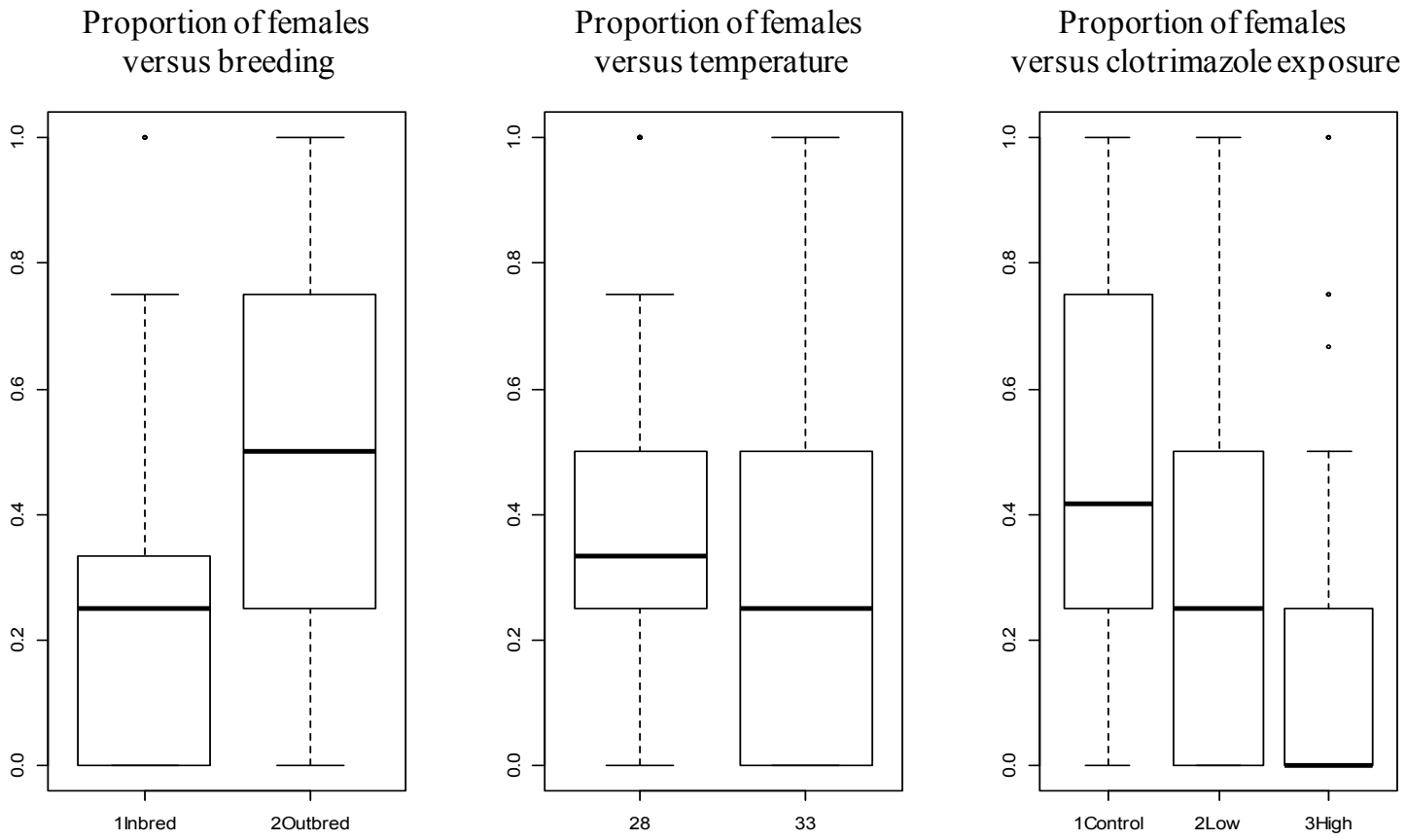


Figure 3: Sex ratios in the different treatments

3b: Proportion of females per family per treatment



Boxes represent interquartile range and median (line), whiskers show full range excluding outliers (o)

Figure 4: Germ cell progression in males and females: Individuals were classified based on their most advanced developmental stage

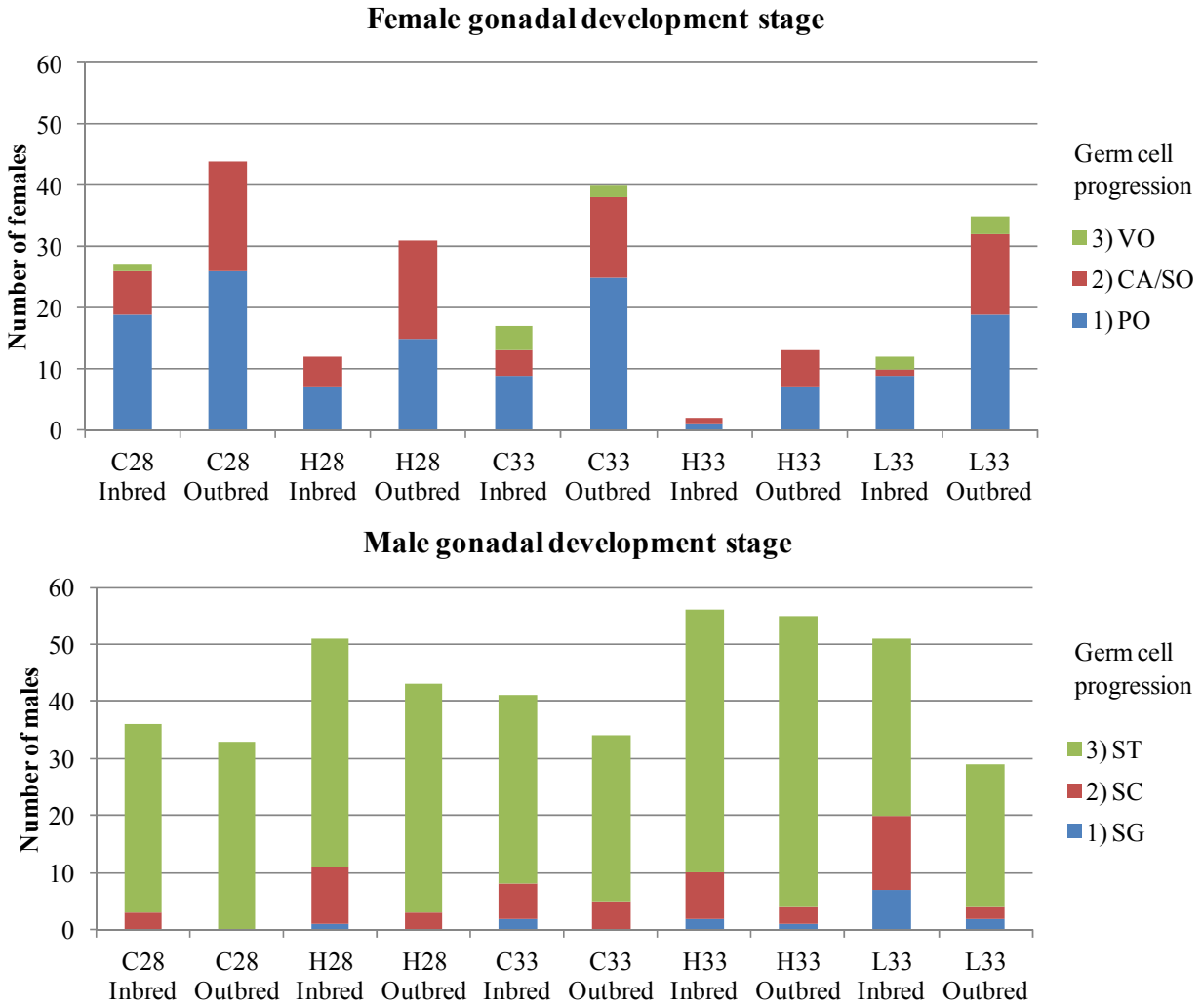
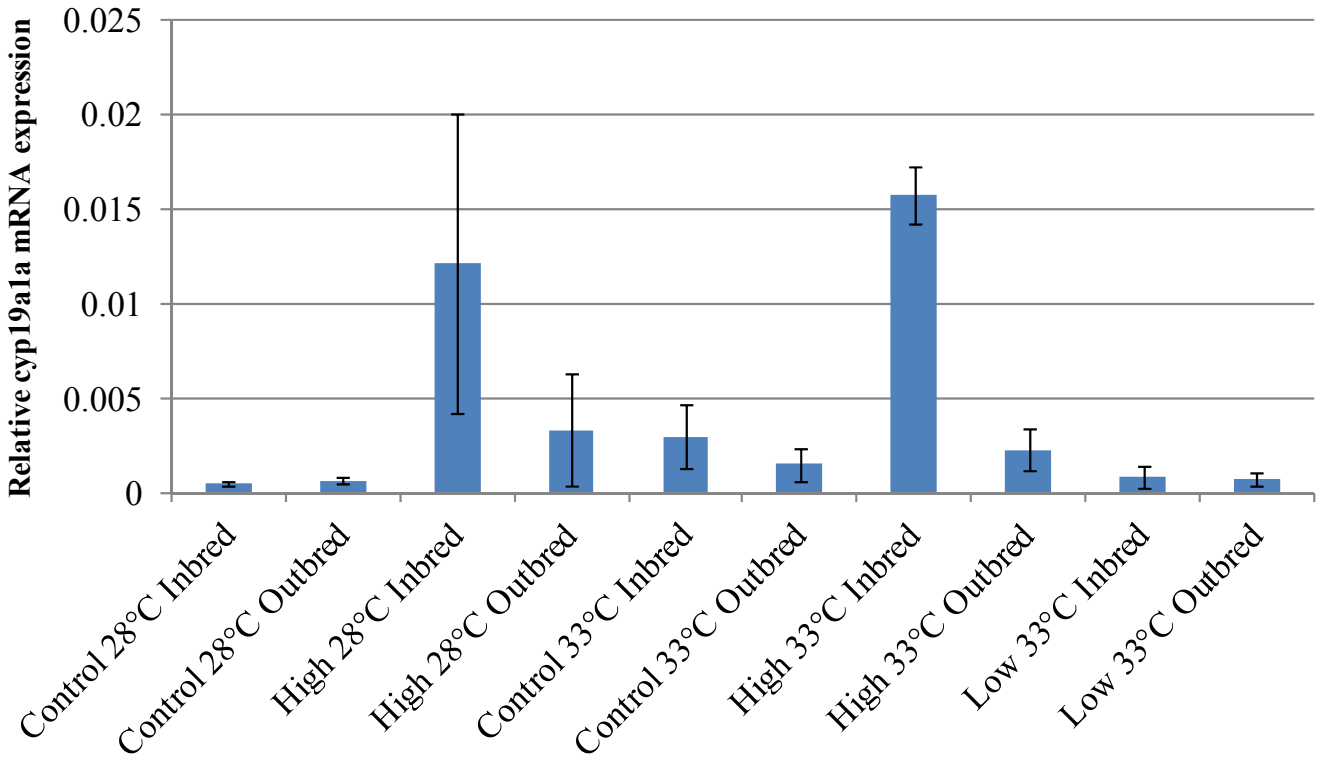


Figure 5: Relative aromatase (*cyp19a1a*) expression in female ovaries (primary oocyte stage)

5a: Relative expression across all treatments

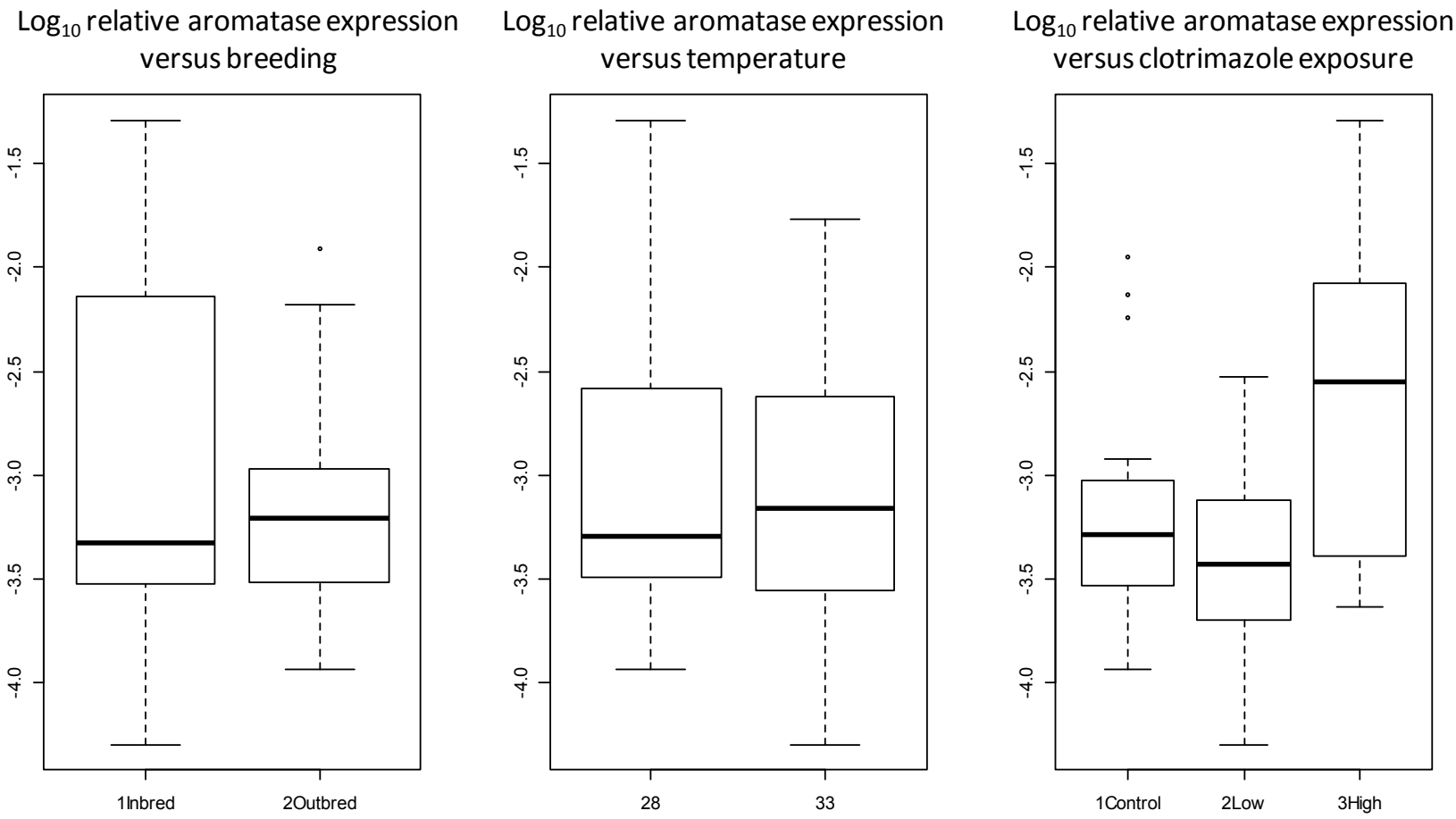


Relative expression = $(E\ ref)^{Ct\ ref}$ /$(E\ target)^{Ct\ target}$

Where ref is the housekeeping gene (*rpl8*), target is the gene of interest (*cyp19a1a*), E is PCR amplification efficiency and Ct is cycle threshold (number of temperature cycles yielding above background expression) for that particular gene.

Figure 5: Relative aromatase (*cyp19a1a*) expression in female ovaries (primary oocyte stage)

5b Log_{10} relative expression compared with breeding, temperature and clotrimazole exposure



Boxes represent interquartile range and median (line), whiskers show full range excluding outliers (o)

Gonad weight

There was no significant effect from breeding on gonad weight, although it was required in the minimum best fit lme model (AIC = 570) (Supporting Information: Table S7). According to this model, female sex was a significant predictor of larger gonadal size (p=0.01) (Supporting Information: Figure S3) and there were significant interactions between gonadal sex * stage * $\log_{10}$ body weight ($p \leq 0.0148$).

Aromatase expression

There were no discernable treament effects on the level of expression of *rpl8* in testicular or ovarian tissue samples, as expected for our reference gene. The relative expression of *cyp19a1a* (versus *rpl8*) in male testes was significantly lower than in female ovaries [ANOVA $\log_{10}$ relative aromatase expression $F_{(1,68)}$=4.05, p=0.01]. There was also a significant treatment effect from breeding and clotrimazole exposure on relative *cyp19a1a* expression in ovaries. According to the best fit lme model (AIC = 111), high-level clotrimazole exposure (8 μg l^{-1}) was shown to be a reliable predictor (p=0.0003) of elevated ovarian aromatase expression (Supporting Information: Table S8) and there was a significant interaction with inbreeding (p=0.0147) (Figure 5).

7.5 Discussion

The need for cumulative environmental risk assessment

Examining the interactions between environmental temperature and chemical contaminants is critically important to be able to gauge their potential combined impact on wildlife populations, given climate change predictions and associated pressures on water quality and quantity (Bates et al., 2008). Global trends indicating habitat loss leading to the increasing isolation, contraction and inbreeding of wildlife populations (UNEP, 2012) is another important factor, which needs to be taken into consideration. Here we examined the

combined effects of elevated temperature and exposure to the aromatase inhibitor clotrimazole in inbred versus outbred zebrafish. Consistent with their shared mode of action, laboratory exposure to high-level (8 μg l^{-1}) clotrimazole and/or elevated temperature (33°C) skewed sex ratios significantly towards males and their effects were shown to be additive. Male-skew was significantly greater in inbred treatments compared with equivalent outbred treatments and reached 97% males in the inbred, high-level clotrimazole exposure, elevated temperature treatment. Sex ratios were also skewed significantly towards males (80%) in inbreds (but not outbreds) following low-level clotrimazole exposure (1.7 μg l^{-1}), in combination with elevated temperature. Our results illustrate the importance of considering biological (genetic), as well as physical and chemical interactions in cumulative ERA.

Relevance of exposure simulations

Although the lowest observed effect concentration was an order of magnitude above the predicted environmental concentration (PEC_{local}) for clotrimazole (OSPAR, 2005), it is conceivable that combined environmental exposures to similarly acting chemicals (e.g. other azole compounds used in crop protection, veterinary and human medicine) could produce similar effects to those we observed. The selected test temperatures are also representative of current and future situations in the native environment of our wild-sourced zebrafish (Rupa Kumar et al., 2006). Furthermore, numerous other environmental stressors including reduced dissolved oxygen, reduced nutrition, overcrowding and acidification are likely to accompany elevated water temperatures, with projected climate change, and all are capabale of inducing masculinisation in fish (Delvin and Nagahama, 2002; Baroiller et al., 2009). The common directional effects of all these factors adds further plausibility to our experimental findings. However, it could be argued that other chemicals or agents with opposing modes of action could be present in the environment and ameliorate or counter-act to some extent the masculinising effects we observed (Hoffman and Kloas, 2012).

Relevance of selected test organisms

The zebrafish used in this study were three generations removed from the wild (Bangladesh). In generating the inbred families we observed inbreeding depression for juvenile survivorship between 0-30 dpf, which equated to 5 lethal equivalent (LE) alleles per individual (diploid) genome. Similar estimates of LE have been made for other wild-caught zebrafish from the Ganges river basin near Calcutta, India, based on embryo-larval and juvenile survival up to 48 dpf (McCune et al., 2002; McCune et al., 2004). The inbred and outbred zebrafish used in our study were intended to be representative of a spectrum of wild populations (small and large). Although the rate of inbreeding simulated (full-sibling mating) is extreme, it was only imposed for one generation. Slower rates of inbreeding, combined with variable selection in the wild may well lead to greater inbreeding depression for reproductive characters and overall fitness (Miller and Hedrick 2001), especially as environmental stress can amplify inbreeding depression (Crnokrak and Roff, 1999). Our results nevertheless demonstrate that male sex-ratio skews are likely to be greater in smaller, more inbred populations and can increase under ”low-level” clotrimazole exposure, in combination with elevated temperature.

Possible mechanisms underlying greater sensitivity in inbred zebrafish

The mechanisms underlying the interactive effects of genetic and environmental factors on sex determination and differentiation in zebrafish have yet to be fully elucidated (Pifferer et al., 2012). The same can be said for inbreeding×environment interactions in zebrafish. In the fruit fly *Drosphila melanogaster*, molecular effect pathways associated with temperature and chemical-induced physiological stress have been shown to resemble inbreeding effects on metabolism and homestasis (Kristensen et al., 2005) and inbreeding×environmental temperature interactions have been shown to depress quantitative fitness traits (Joubert and Bijlsma, 2010). Our data show that male-biased sex ratio skews in zebrafish were not mediated by reduced growth (an integrative measure of metabolism). Although there is minimal sexual dimorphism in terms of body size in zebrafish (Pyron, 2003), males generally weigh less than females and therefore the observed increase in SGR_{weight} in the elevated temperature treatments could be expected to favour female development (Parker, 1992).

However this is contrary to what we observed. Instead male-biased sex determination in the elevated temperature and clotrimazole exposure treatments is likely to be due to the inhibition of CYP450 aromatase (Brown et al., 2011; Baudiffier et al., 2013). This is supported in this study by the elevation of *cyp19a1a* expression in the few females (n= 12 (18%) and n=2 (3%)) remaining in the inbred, high-level (8 µg l^{-1}) clotrimazole exposures, held at 28°C and 33°C respectively. These elevated transcripts appear to be a compensatory response to the direct inhibition of the aromatase enzyme by clotrimazole and may have contributed to the resistance of the female phenotype in these few inbred individuals. Assessment of allelic variation at the *cyp19a1a* locus and its promoter region could help to elucidate the inbreeding×environment interactions underlying the different levels of masculination induced by our exposure treatments. However, there is considerable scope for other genotype×environment interactions to affect sex determination and differentiation in zebrafish and numerous other candidate genes may be involved (von Hofsten and Olsson, 2005; Bradley et al., 2011; Anderson et al., 2012; Pifferer et al., 2012). In any case, an increase in homozygosity would be consistent with inbreeding depression and/or loss of heterosis.

7.6 Conclusions

Inbreeding in zebrafish has previously been shown to skew sex ratios towards males under laboratory control conditions (Brown et al., 2012) and skews have been shown to be exacerbated following high-level clotrimazole exposure (43.7 µg l^{-1}) during maturation (Brown et al., 2011). Here we show that similar sex skews (>80%) can be induced at substantially lower clotrimazole exposure concentrations (1.7 µg l^{-1}), in combination with elevated temperature, simulating predicted mean temperature rises in the zebrafish's native India and Bangladesh by the end of the century (Rupa Kumar, 2006). It is not yet clear what level of male sex skew would ensure an adverse impact on zebrafish populations in the wild. Population viability analysis for reptiles exhibiting female to male temperature dependent sex determination (*Sphenodon guntheri*), predicts population extinction once hatchling sex ratios reach 80% males, or 65% males if inbreeding is simulated at realistic levels via depression of juvenile survivorship (Mitchell et al., 2010). However, when attempting to predict extinction

risk in other taxa, many other factors need to be taken into account in addition to inbreeding, including population size, fecundity, generation time, and dispersal/gene flow. Further work is needed to assess the risks for zebrafish populations.

Overall our work highlights the potential vulnerability of inbred populations and therefore the importance of considering biological (genetic), as well as physical and chemical interactions in cumulative environmental risk assessment (ERA). However, by the same token, the conventional use of "relatively inbred" animals in ecotoxicology and ERA is likely to provide a degree of conservatism for the protection of wild fish populations in general.

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7.8 Chapter 7 Supporting Information

Pages

TABLES

FIGURES

Table S1
Details of the parental pair breeding for generating the F_3 inbred and outbred lines

Table S1a: Outbreeding to generate F_1 fish (grandparents of fish to be used in final study)

Outbred population/ individual male	Outbred population/ individual female	F_1 family ID
1/1	2/1	1
2/2	1/2	2
1/3	2/3	3
2/4	1/4	4
1/5	2/5	NE
2/6	1/6	6
1/7	2/7	7
2/8	1/8	8
1/9	2/9	9
2/10	1/10	10
1/11	2/11	11
2/12	1/12	12
1/13	2/13	13
2/14	1/14	NE
1/15	2/15	15
2/16	1/16	16
1/17	2/17	17
2/18	1/18	18
1/19	2/19	19
2/20	1/20	20
1/21	2/21	21
2/22	1/22	22
1/23	2/23	NE
2/24	1/24	24

Population 1 = Mozahadi, Gastala Bazar, Tarakanda, 10 km north of Mymensingh and the Brahmaputra River (Lat 24.8710109 Long 90.4148744). Population 2 = Kechuri Beel, Badai Barera, Kotwali, Mymensingh, adjacent to the Brahmaputra River (Lat 23.4067115, Long 88.4979698). All individual individuals were sampled randomly from each population (n=100).
NE = No embryos (from non viable or non compatible pair)

Table S1b: Outbreeding to generate F_2 fish (parents of fish to be used in final study)

Outbred Family[#] (males)	Outbred Family[#] (females)	F_2 family ID
1	2	A
3	4	B
6	7	C
8	9	D
10	11	E

12	13	F
15	16	G
17	18	H
19	20	I
21	22	J
2	3	K
4	6	L
7	8	M
24	10	N
11	12	O
13	15	P
16	17	Q
18	19	R
20	21	S
22	1	T

[#] Families are full-sibling families and correspond to family IDs in Table S1.1

Table S1c: Outbreeding to generate F_3 test fish (via reciprocal crossing)

Outbred Family[†] (males)	Outbred Family[†] (females)	Outbred F_3 family ID
A	B	OP1
B	A	OP2
C	D	OP3
D	C	OP4
E	F	OP5
F	E	OP6
G	H	OP7
H	G	OP8
I	J	OP9
J	I	OP10
K	L	OP11
L	K	OP12
M	N	OP13
N	M	OP14
O	P	OP15
P	O	OP16
Q	R	OP17
R	Q	OP18
S	T	OP19
T	S	OP20

Note [†] Families are full sibling families and correspond to family IDs in Table S1.2

Table S1d: Inbreeding to generate F3 test fish

Outbred Family* (males)	Outbred Family* (females)	Inbred F_3 family ID
A	A	IP1
B	B	IP2
C	C	IP3
D	D	IP4
E	E	IP5
F	F	IP6
G	G	IP7
H	H	IP8
I	I	IP9
J	J	IP10
K	K	IP11
L	L	IP12
M	M	IP13
N	N	IP14
O	O	IP15
P	P	IP16
Q	Q	IP17
R	R	IP18
S	S	IP19
T	T	IP20

* Families are full-sibling families and correspond to family IDs in Table S1.2

F_3 families denoted 1-20 (for inbreds I and outbreds O)

F_2 families denoted (A-T rather than 1-20)

The family-level replication and the degree and rate of inbreeding (one generation of full-sibling mating) were consistent with those frequently used in studies assessing for inbreeding effects (Lynch 1988; Keller and Waller 2002; Armbruster and Reed, 2005). Our fish were the great grandchildren of wild (Bangladesh origin) male fish and female fish (F_0) of a Wild Indian Karyotype (WIK) laboratory strain female fish pair spawned at the University of Exeter (see main Figure 1). The approach used is highly relevant since the practice of outbreeding between strains is performed routinely in animal husbandry (Sadler and Monson, 2010) and the introgression of individuals from wild populations has also been advocated in order to maintain representative outbred stocks for use in ecotoxicology (Nowak et al. 2007b; Coe et al., 2009). Such practices can sometimes lead to outbreeding depression in F_1 and/or F_2 generations (Wu & Palopoli, 1994; Lynch & Walsh, 1998) due to a break-up of favourable epistatic interactions in the parental lines, or phenotype-environment interaction (Thornhill, 1993; Lynch & Walsh, 1998; Wade & Goodnight, 1998). The use of F_3 generation hybrids in our study minimised the possibility of outbreeding depression.

Table S2
q-PCR analysis of target genes

Target gene	Forward primer	Reverse primer	Annealing temp (°C)	Efficiency (%)
Ribosomal protein l8 (*rpl8*)	CCG AGA CCA AGA AAT CCA GAG	CCA GCA ACA ACA CCA ACA AC	59.5	2.07
Aromatase (*cyp19a1a*)	AGC CGT CCA GCC TCA G	ATC CAA AAG CAG AAG CAG TAG	61.5	1.89

Note: Oligonucleotide primer sequences read from 5' to 3'
Oligonucleotide primer pairs (forward and reverse) were designed using Beacon Designer 3.0 software (Premier Biosoft International, Palo Alto, CA) and purchased from Invitrogen. Primer-pair annealing temperatures were optimized for real-time PCR on a temperature-gradient program. Primer specificity was confirmed by gel electrophoresis and/or melt curve analysis and automated fluorescence sequencing of PCR products. To determine the detection range, linearity and real-time PCR amplification efficiency (E; E = 10[−1/slope]) (Pfaffl, 2001) of each primer pair, real-time PCR amplifications were run in triplicate on a 10-fold serial dilution series of zebrafish testis cDNA pooled from all samples, and standard curves were calculated referring the threshold cycle (Ct; the PCR cycle at which fluorescence increased above background levels) to the logarithm of the cDNA dilution.
During real-time PCR each cDNA sample was amplified in triplicate using 96-well optical plates in a 20-µl reaction volume using 1 µl cDNA, 10 µl 2× Absolute SYBR Green (Flourescein) Supermix (BioRad), 5 µM of the appropriate forward and reverse primers. Hot start Taq polymerase was activated by an initial denaturation step at 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at the primer-specified temperatures for 20 sec and, finally, melt curve analysis. No-template controls were run for each plate and all samples were run on the same plate ensuring consistent quantification of the expression of each target gene.

Relative expression levels were determined using the following calculation:
$RE = (E\ ref)^{Ct\ ref} / (E\ target)^{Ct\ target}$
Where RE is relative gene expression, ref is the housekeeping gene, target is the gene of interest, E is PCR amplification efficiency and Ct is cycle threshold (number of temperature cycles yielding above background expression) for that particular gene.

Statistical differences in relative gene expression between experimental groups were assessed by one-way ANOVA of log-transformed data, followed by Dunn multiple pair-wise comparison test or Student t-test. All statistical analyses were performed using Minitab TM version 15. All experimental data are shown as the mean ± 95% confidence interval. Differences were considered statistically significant at $p \leq 0.05$.

Table S3
Confirmation of clotrimazole concentrations (µg/L) throughout the *in vivo* exposure study

Exposure day	Control (0 µg/L nominal)	Low-level clotrimazole exposure (2 µg/L nominal)	High-level clotrimazole exposure (10 µg/L nominal)
-4	0	0.47	2.74
0	0	0.71	3.2
4	0	1.2	5.9
9	0	2.13	6.0
15	0	1.65	7.24
17	0	2.03	11.5
35	0	1.53	9.33
56	0	2.6	8.35
77	0	2.13	9.45
119	0	1.6	10.9
161	0	3.4	11.4
178	0	1.5	9.2
Arithmetic mean	**0**	**1.9**	**8.4**
Geometric mean	**0**	**1.73**	**7.95**
95% CI	0	0.42	1.54
SEM	0	0.22	0.79
Limit of detection	0.2	0.2	0.2

Quantification by LC-MS: Initial chromatographic separation of clotrimazole was carried out on an Gemini-NX C18 column (50 x 2 mm, 3.0 µm, Phenomenex, Torrance, CA). The column was fitted with a pre-filter (0.5 µm, Supelco, USA) maintained at 50°C and the flow rate was 500 µl min^{-1}. The elution gradient of eluent A) 0.1% ammonium hydroxide in water and eluent B) LCMS grade methanol (T(min)/ % A was 0/90→3/0→5/0→5.1/90→6/90). A Quadrupole Ion Trap (Thermo-Finnigan TSQ Quantum Access) mass spectrometer with electrospray ionisation was used with the following parameters: sheath gas flow 60 arbitrary units, auxiliary gas flow 50 arbitrary units, spray voltage 3.0 kV, capillary temperature 300°C, capillary offset voltage 39 V, tube lens offset tuned. Positive ionization with selected reaction monitoring (SRM) was used for all analyses. The analyte (clotrimazole) corresponded to a product ion mass of 169 Da, the limit of quantitation was 0.2 µg l^{-1}.

Table S4

Table S4a: Linear mixed model (lme) of the influence of period, breeding, clotrimazole exposure and temperature on specific growth rate based on wet weight (SGR_{weight})

Parameter	Initial model (AIC = 2763)			Best fit model (AIC = 2760)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	516	18.740842	0.0000	525	44.21295	0.0000
Period (1/2/3)	516	-14.949220	**0.0000**	525	-39.47455	**0.0000**
Breeding (in/out)	36	2.198038	0.0345			
Clotrimazole (low/control)	516	2.571851	**0.0104**			
Clotrimazole (high/control)	516	1.522590	0.1285			
Temperature (28/33°C)	516	3.696080	**0.0002**	525	4.45007	**0.0000**
Period×breeding	516	-1.786088	0.0747			
Period×low clotrimazole	516	-2.191622	0.0289			
Period×high clotrimazole	516	-1.184986	0.2366			
Breeding×low clotrimazole	516	-0.942814	0.3462			
Breeding×high clotrimazole	516	-0.268396	0.7885			
Period×breeding×low clotrimazole	516	0.827256	0.4085			
Period×breeding×high clotrimazole	516	0.288040	0.7734			

Significant effects in **bold**

Initial model: fixed effects on SGR_{wt} ~ period * breeding * dose + temperature; random effect = family.

Best fit model: fixed effects: SGR_{wt} ~ period + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S4b: Linear mixed model (lme) of the influence of period, breeding, clotrimazole exposure and temperature on specific growth rate based on standard length (SGR_{length})

Parameter	Initial model (AIC = 1189)			Best fit model (AIC = 1173)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	516	22.124920	0.0000	522	33.68445	0.0000
Period (1/2/3)	516	-16.841512	**0.0000**	522	-28.26626	**0.0000**
Breeding (in/out)	36	1.955093	0.0584	36	1.58791	0.1211
Clotrimazole (low/control)	516	2.994716	**0.0029**	522	0.83895	0.4019
Clotrimazole (high/control)	516	0.576241	0.5647	522	0.42345	0.6721
Temperature (28/33°C)	516	2.449853	**0.0146**	522	2.44249	**0.0149**
Period×breeding	516	-1.526349	0.1275	522	-1.15786	0.2475
Period×low clotrimazole	516	-2.688911	0.0074			
Period×high clotrimazole	516	-0.401908	0.6879			
Breeding×low clotrimazole	516	-1.586510	0.1132			
Breeding×high clotrimazole	516	-0.658711	0.5104			
Period×breeding×low clotrimazole	516	1.288006	0.1983			
Period×breeding×high clotrimazole	516	0.573846	0.5663			

Significant effects in **bold**

Initial model: fixed effects on SGR_{lth} ~ period * breeding * dose + temperature; random effect = family.

Best fit model: fixed effects on SGR_{lth} ~ period * breeding + dose + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S5

Linear mixed model (lme) of the influence of breeding, clotrimazole exposure and temperature on sex ratio (arcsine square root proportion of females per tank compartment per treatment)

Parameter	Initial model (AIC = 224)			Best fit model (AIC = 218)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	146	8.616649	0.0000	148	9.416995	0.0000
Breeding (in/out)	36	3.038948	**0.0044**	36	4.477125	**0.0001**
Clotrimazole (low/control)	146	-1.182211	0.2390	148	-0.413375	0.6799
Clotrimazole (high/control)	146	-4.192633	**0.0000**	148	-6.276231	**0.0000**
Temperature (28/33°C)	146	-3.680676	**0.0003**	148	-3.676509	**0.0003**
Breeding×low clotrimazole	146	1.282888	0.2016			
Breeding×high clotrimazole	146	-0.181759	0.8560			

Initial model: fixed effects on arcsine sqrt proportion of females ~ breeding * clotrimazole exposure + temperature; random effect = family.

Best fit model: fixed effects on arcsine sqrt proportion of females ~ breeding + clotrimazole exposure + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S6

Linear mixed model (lme) of the influence of gonadal sex, breeding, clotrimazole exposure and temperature on germ cell progression

Parameter	Initial model (AIC = 1010)			Best fit model (AIC = 993)		
	df	t-value	*p*-value	df	t-value	*p*-value
(Intercept)	500	16.712741	0.0000	509	21.38003	0.0000
Sex (M/F)	500	14.379486	**0.0000**	509	32.28709	**0.0000**
Breeding (in/out)	36	-0.406454	0.6868	36	3.14891	**0.0017**
Clotrimazole (low/control)	500	-0.296678	0.7668			
Clotrimazole (high/control)	500	-0.242949	0.8081			
Temperature (28/33°C)	500	-0.334590	0.7381	509	-1.56436	0.1184
Sex×breeding	500	1.052747	0.2930			
Sex×low clotrimazole	500	-1.741223	0.0823			
Sex×high clotrimazole	500	-0.127810	0.8948			
Breeding×low clotrimazole	500	0.832651	0.4045			
Breeding×high clotrimazole	500	0.482947	0.6293			
Sex×breeding×low clotrimazole	500	0.381902	0.7027			
Sex×breeding×high clotrimazole	500	-0.040515	0.9677			

Significant effects in **bold**

Initial model: fixed effects on germ cell progression ~ gonadal sex * breeding * clotrimazole exposure + temperature; random effects = family and individual.

Best fit model: fixed effects on germ cell progression ~ gonadal sex + breeding, random effects = family and individual.

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

All fixed effects are categorical variables. Random effects are family (inbred 1-18, outbred 1-20) and individual (1-4).

Table S7

Linear mixed model (lme) of the influence of log_{10} body weight, gonadal sex, stage, breeding, clotrimazole exposure and temperature on log_{10} gonad weight

Parameter	Initial and best fit model (AIC = 570)		
	df	t-value	*p*-value
(Intercept)	297	-2.7159275	0.0078
Log_body_weight	297	-0.3254790	0.7450
Sex (M/F)	297	2.5887624	**0.0101**
Stage (1/2/3)	297	1.4120575	0.1590
Breeding (in/out)	35	0.5561966	0.5816
Clotrimazole (low/control)	297	0.3156156	0.7525
Clotrimazole (high/control)	297	-0.0982313	0.9218
Temperature (28/33°C)	297	0.0063095	0.9950
Log_body_weight:sex	297	2.4795349	**0.0137**
Log_body_weight:stage	297	1.2584813	0.2092
Sex:stage	297	-2.6512701	**0.0084**
Log_body_weight:breeding	297	0.6595118	0.5101
Sex:breeding	297	-0.0556987	0.9556
Stage:breeding	297	-0.3243966	0.7459
Log_body_weight:low clot	297	0.3798591	0.7043
Log_body_weight:high clot	297	-0.0575429	0.9542
Sex:low clotrimazole	297	-0.9670904	0.3343
Sex:high clotrimazole	297	-0.6122394	0.5408
Stage:low clotrimazole	297	-0.8748545	0.3824
Stage:high clotrimazole	297	0.0371107	0.9704
Breeding:low clotrimazole	297	-0.0945798	0.9247
Breeding:high clotrimazole	297	1.1255811	0.2613
Log_bodywt:sex:stage	297	-2.4506156	**0.0148**
Log_bodywt:sex:breeding	297	-0.0154394	0.9877
Log_bodywt:stage:breeding	297	-0.4869563	0.6266
Sex:stage:breeding	297	-0.0642231	0.9488
Log_bodywt:sex:low clot	297	-0.9686878	0.3335
Log_bodywt:sex:high clot	297	-0.6088119	0.5431

Log_bodywt:stage:low clot	297	-1.0077234	0.3144
Log_bodywt:stage:high clot	297	-0.1124103	0.9106
Sex:stage:low clot	297	1.4991316	0.1349
Sex:stage:high clot	297	0.4491426	0.6537
Log_bodywt:breed:low clot	297	-0.1732852	0.8625
Log_bodywt:bred:high clot	297	1.0757006	0.2829
Sex:breed:low clotrimazole	297	-0.3640634	0.7161
Sex:breed:high clotrimazole	297	0.0743347	0.9408
Stage:breed:low clotrimazole	297	0.1563683	0.8758
Stage:breed:high clotrimazole	297	-1.1824296	0.2380
Log_bodywt:sex:stage:breed	297	-0.0653231	0.9480
Log_bodywt:sex:stage:high clot	297	1.5788938	0.1154
Log_bodywt:sex:stage:high clot	297	0.5405424	0.5892
Log_bodywt:sex:breed:low clot	297	-0.3703319	0.7114
Log_bodywt:sex:breed:high clot	297	-0.1692549	0.8657
Log_bodywt:stage:breed:low clot	297	0.2780639	0.7812
Log_bodywt:stage:breed:high clot	297	-1.0205510	0.3083
Sex:stage:breeding:low clot	297	0.2381292	0.8119
Sex:stage:breeding:low clot	297	0.3433032	0.7316
Log_bodywt:sex:stage:breed:low clot	297	0.1841107	0.8541
Log_bodywt:sex:stage:breed:high clot	297	0.4933085	0.6222

Significant effects in **bold**

Initial and best fit model: fixed effects on log_gonad_weight ~ log_body_weight * gonad_sex * gonad_stage* breeding * dose + temperature; random effects = family and individual.

All fixed effects are categorical variables except the covariate log_body_weight. Random effects are family (inbred 1-18, outbred 1-20) and individual (1-4).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Table S8

Linear mixed model (lme) of the influence of breeding, clotrimazole exposure and temperature on ovarian aromatase expression (log_{10} transformed)

Parameter	Initial and best fit model (AIC = 111)		
	df	t-value	*p*-value
(Intercept)	25	-4.505143	0.0001
Breeding (in/out)	21	0.004159	0.9967
Clotrimazole (low/control)	25	-1.308236	0.2027
Clotrimazole (high/control)	25	4.202001	**0.0003**
Temperature (28/33°C)	25	1.543240	0.1353
Breeding×low clotrimazole	25	0.251999	0.8031
Breeding×high clotrimazole	25	-2.620412	**0.0147**

Significant effects in **bold**

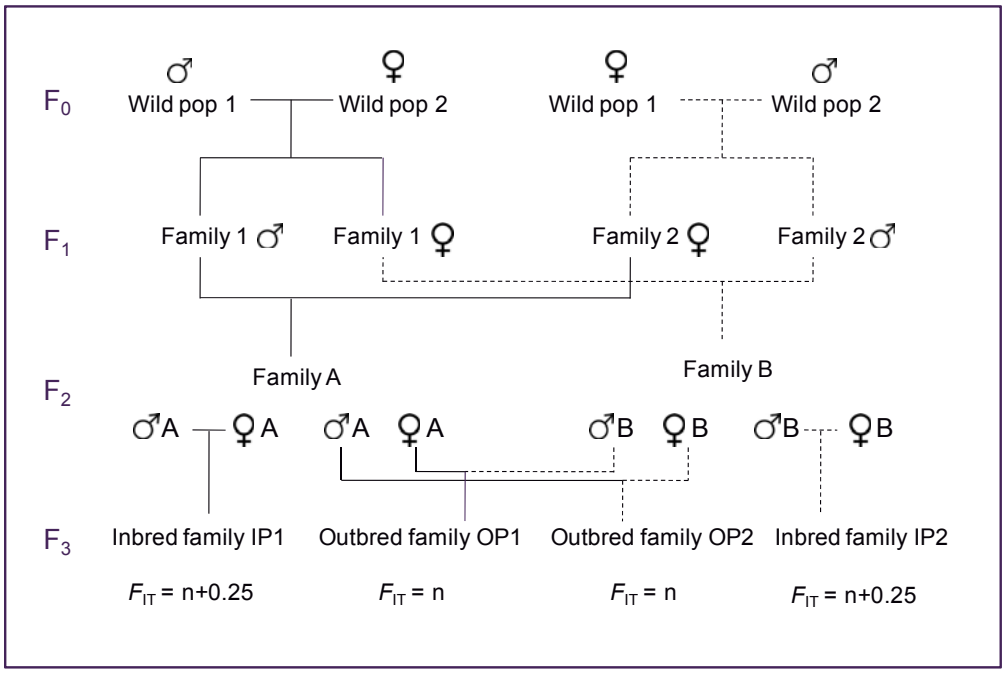
Initial and best fit model: fixed effects on log_{10} aromatase expression ~ breeding * clotrimazole exposure + temperature; random effect = family.

All fixed effects are categorical variables. Random effect is family (inbred 1-18, outbred 1-20).

AIC is the Akaike Information Criterion (smaller values indicate better model fit).

Figure S1

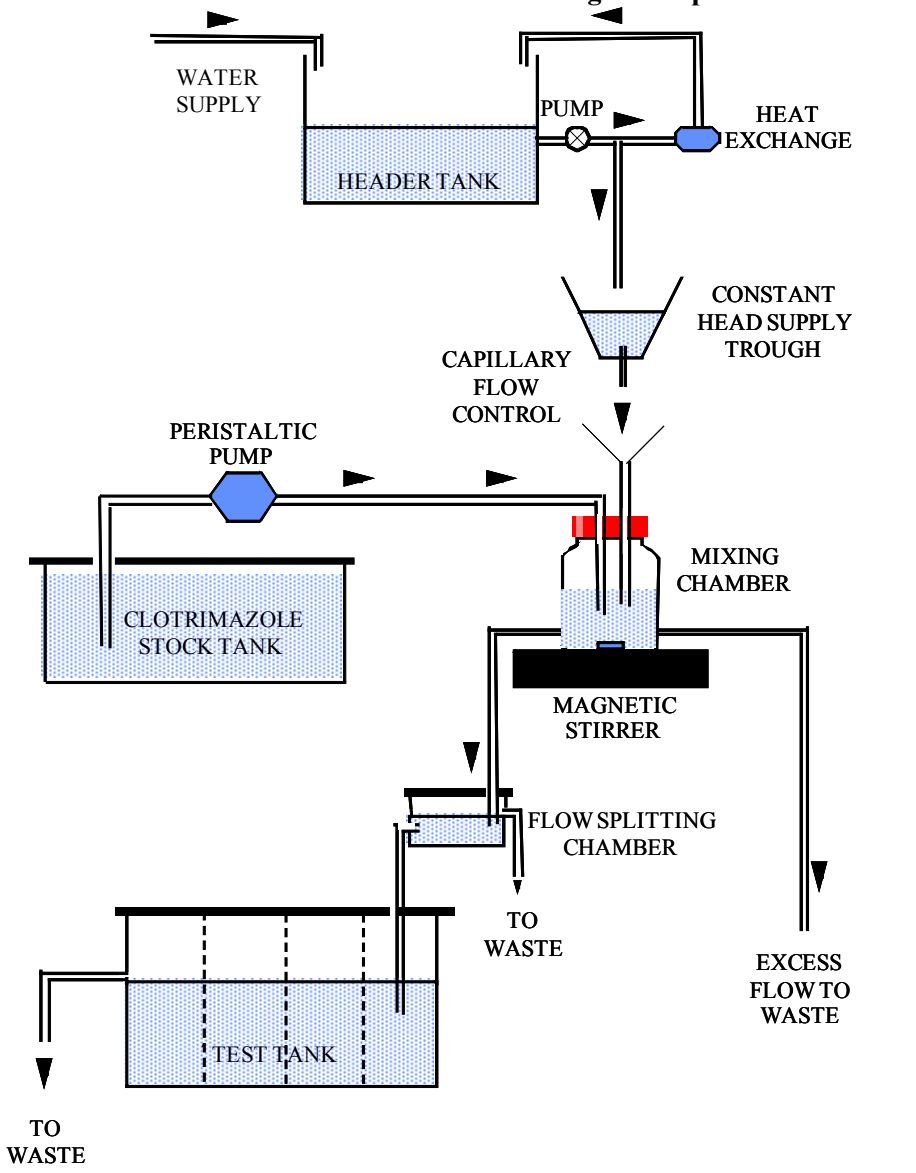
Pair breeding design for generation of inbred and outbred lines (F_3 generation) of zebrafish



The notation "n" in the inbreeding coefficients of the F_3 generation reflects their unknown pedigree, relating to their wild great grandfathers (F_0 generation). Two of the 20 inbred F_3 family lines failed to recruit.

Figure S2

Details of chemical dosing and exposure conditions

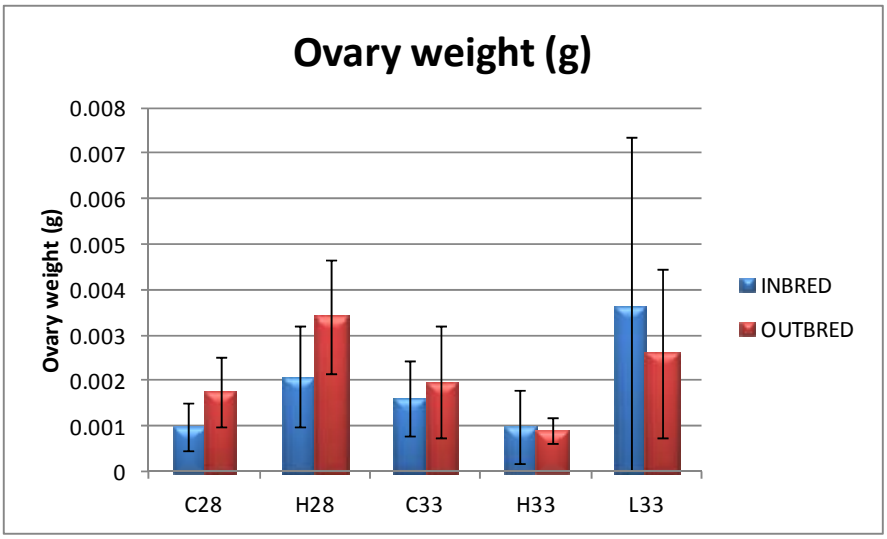


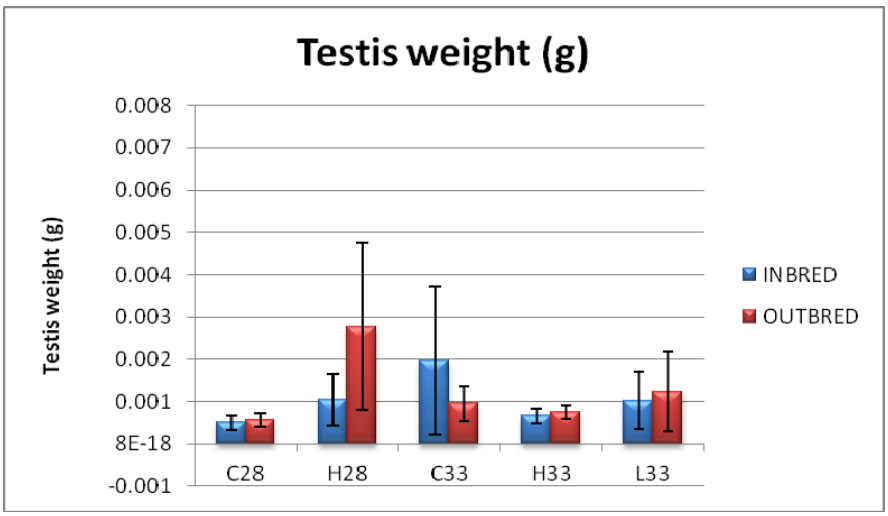
Two sets of two 120 litre saturated stock solutions (580 µg clotrimazole l^{-1}) were prepared alternately every 24 hours by mixing a ×2 excess of clotrimazole solid powder in dilution water (dechlorinated, 5µm filtered, UV treated mains water) at 28°C, aided through sonication for 15 minutes, followed by 12 hours mixing (using turbo pumps) and 12 hours settling. This final stage allowed the deposition of any un-dissolved test material at the meniscus or on the sides of the stock tanks. The saturated stock solution was then pumped from the middle of the stock tanks to separate mixing chambers, gravity fed with dilution control water, giving a ×10 or ×100 dilution for the nominal 5 and 50 µg l^{-1} exposure concentrations respectively. Test solutions were delivered to 60 litre glass exposure tanks with a

longitudinal solid glass partition - each side of each tank (divided further into 4 interconnected sub-compartments) received flow rate of 42 ml min^{-1}, equating to ×2 tank volume changes per day.
The following exposure conditions were monitored and maintained throughout the study: water temperature 28±1°C; dissolved oxygen > 60% saturation; pH 7.14-8.16; hardness 40-69 mg/L; alkalinity 22-35 mg/L; chlorine <2 µg/L; ammonia <2 µg/L. The photoperiod was set at 12:12 light:dark with 20 mins sunrise/sunset and fish were fed daily on a fixed excess ration [> 4% body weight per day, comprising one meal of 300 pellet (Special Diets Services) @ 3% body weight in the afternoon, plus an excess of ≥24 hour old *Artemia* nauplii in the morning and afternoon.

Figure S3

Gonad weights at the end of the exposure study (100 dpf)





Weights of right side gonds presented

7.9 References (Chapter 7 Supporting Information)

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8 CHAPTER 8: POPULATION LEVEL CONSEQUENCES OF MASCULINISATION IN ZEBRAFISH INDUCED BY THE COMBINATION OF INBREEDING CHEMICAL EXPOSURE AND ELEVATED CLIMATIC TEMPERATURE

Population-level consequences of masculinisation in zebrafish induced by the combination of inbreeding chemical exposure and elevated climatic temperature

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8.1 Abstract

The need to assess the cumulative environmental risk of chemicals in combination with other environmental pressures is emphasised by a changing global climate. Species in which environmental temperatures and chemicals can combine to influence sex determination/ differentiation and significantly skew sex ratios are likely to be among the most susceptible to demographic collapse. Furthermore, such impacts are more likely in small inbred populations, which are subject to stochastic variation in numbers and sex ratios, and in which genetic diversity and adaptive capacity are limited or declining.

Here stochastic population viability analysis was applied to assess the risks of exposure to the aromatase inhibitor clotrimazole, in combination with elevated temperature and inbreeding in the zebrafish (*Danio rerio*). Empirical data from laboratory-based exposure experiments were integrated with comprehensive ecological life-history data to develop predictions on per capita population growth rate (r) and probability of extinction (PE) over a simulation period of 100 years. Sensitivity analysis was used to assess the relative importance of sex ratio skews compared with other demographic/population input parameters in inbred versus outbred populations. PE was unaffected by maximum recorded sex ratio skews of >97% males. Nevertheless, skews of >80% males in inbred populations and >90% males in outbred populations represented thresholds below which r declined sharply from 2.4 to 0.7 and from

2.4 to 1.4 respectively. The inclusion of compounding (emergent) inbreeding depression on age 0+ survivorship further reduced r to 0.19 in all simulations, regardless of sex ratio or initial (in)breeding status. Sensitivity analysis further demonstrated the greater influence of age 0+ survivorship on PE and r, compared to male-skews in sex ratio and/or reduced female fecundity, which we attribute to surplus fecundity in this r-strategist. PE was also highly sensitive to carrying capacity; a ceiling of 2000±1000 total individuals (100 breeding adults, with <20 females) represented a point of convergence where all populations, regardless of initial level of inbreeding or age structure went extinct. This work highlights the need to define "appropriate assessment populations" in environmental risk assessment, in terms of their size, demographics, gene flow, inbreeding and the stochastic variation in each factor. High fecundity in r-strategists such as zebrafish may offer a buffer against imbalances in sex ratio, but inbreeding depression on age 0+ survivorship may override this, particularly in small, isolated populations.

8.2 Introduction

Rapid growth in the human population, leading to extensive loss of natural habitats, greater levels of environmental chemical pollution and accelerated climate change, is placing increasing pressure on wildlife populations (UNEP, 2005; UNEP, 2012). The combined action of multiple physical, chemical and biological factors complicates the environmental risk assessment (ERA) of chemicals, which fundamentally aims to protect populations that, in turn, sustain ecological communities and ecosystems (EC, 2012). Traditionally ERA relies on laboratory tests quantifying adverse effects of individual chemicals in a relatively small number of 'model' organisms, and safety factors are applied to account for potential variation in sensitivity between biological effects endpoints, test species and the potential for biomagnification of chemicals up the food chain (ECHA, 2008). Extrapolation from individual responses to population-level effects can be estimated using population dynamics models. Outputs from these models, such as predicted per capita population growth rate (r) have been reported to be more relevant measures of environmental risk than ecotoxicological endpoints measured in individuals (Forbes and Calow, 1999). This is because population

growth rate provides an integrative measure of effects on survival and fecundity and other associated demographic parameters: age structure; development; generation time; sex ratio. In some cases, effects on these individual components will counter act one another, whist in others the effects may be additive or multiplicative (Akçakaya et al., 2008).

Population modelling is most frequently used in conservation management of species and populations threatened with extinction (Akçakaya et al., 2008). A prioritised assessment of 65,000 species (less than 5% of the world's described species) has shown that 40,429 species are currently threatened with extinction (IUCN, 2012). These assessments of extinction risk are based on a range of factors including geographical distribution, dispersal, demographic population structure, and generation time, as well as temporal trends and fluctuations in breeding population numbers in the wild (IUCN 2012). These factors are integrated in population viability analysis (PVA) models, which predict the fate of threatened populations by projecting life-histories forward via stochastic computer simulations (Akçakaya et al., 2008). Adult breeding population size or effective population size (N_e) is often most highly correlated (inversely) with extinction risk (Brook et al., 2002; O'Grady et al., 2004). This is because larger population size buffers against stochastic variation in environmental carrying capacity and demographic rates and also genetic stochasticity, the loss of genetic variation due to genetic drift and inbreeding (Gilpin and Soule, 1986). In the broadest sense, standing genetic variation in wildlife populations is correlated directly with population fitness (Reed and Frankham, 2003) and adaptability to environmental change (Hoffman and Parsons, 1991; Lande and Shannon 1996; Reed et al., 2003a), including novel chemical exposure (reviewed in Brown et al., 2009). Numerous chemicals have been shown to cause genetic bottlenecks, impair gene flow (Van Straalen and Timmermans, 2002) and erode reproductive fitness (Bickham et al., 2000; Bickham, 2011). Chemicals, for example endocrine disrupting chemicals, may also affect evolutionary processes indirectly, without selection on adaptive traits, by skewing sex ratios, reducing effective population size (N_e) or by reinforcing the reproductive isolation of exposed populations (Nacci and Hoffman 2008).

The impact of sex ratio skews on the viability of sexually reproducing populations depends on the direction of the skew, the size of the population and population growth rate. Female-

skews pose little risk provided that males are produced periodically and the breeding system is polygynous (Wapstra et al., 2009). This is because per capita population growth rate (r) is enhanced by a greater abundance of females, despite an overall reduction in N_e (Wedekind, 2002). Conversely, male-skews may contribute significantly to population extinction risk if the resulting scarcity of females leads to a significant reduction in r (Mitchell and Janzen, 2010). Male-skews are likely to present greater problems for small populations with reduced population growth rates, because even small variations in r and N_e may lead to declines below minimum viable population thresholds (Miller and Lacy, 2005). In addition, populations with unequal numbers of males and females will lose heterozygosity at a greater rate than the same sized population with a balanced sex ratio, and this effect is exacerbated if the skew is more extreme (Allendorf and Luikart, 2007). Imbalances in sex ratio and N_e can increase genetic drift and inbreeding, leading to further loss of genetic variation (heterozygosity) and fitness due to the emergence of deleterious recessive alleles, fuelling a self-perpetuating decline or "extinction Vortex" (Gilpin and Soulé 1986). For these numerous reasons, species existing in small populations, exhibiting male-skews in sex ratios induced by elevated temperatures could be particularly at risk, given future climate predictions. Whilst the phenomenon of female to male temperature dependent sex determination (FM TSD) is rare in reptiles (Mitchell and Janzen, 2010) it has been demonstrated in several fish species, and many more fish show a combination of genetic and environmental sex determination (Ospina-Alvarez and Piferrer, 2008). It may be argued that species with true TSD are at the greatest risk from rapid global warming. However, the adaptability of species to rapid climate change will depend on the extent to which heritable traits are preserved or otherwise overridden by environmental effects (Hulin et al., 2009).

The zebrafish (*Danio rerio,* Hamilton) is a model species used widely in ecotoxicology (Scholtz et al., 2008). Both genetic and environmental factors influence sex determination in this species (Ospina-Alvarez and Piferrer, 2008; Pifferer et al., 2012). Wild populations of zebrafish are widely distributed, often in shallow surface waters, across the Indian subcontinent, from the foot of the Himalayas to the Cape of Comorin. Whilst the ecological life-history of the species is generally well described, data concerning population sex ratios in the wild are limited and they are generally assumed to be 50:50 (Spence et al., 2007). In this

study we examined the long-term impact of male-skewed sex ratios compared with effects on other demographic parameters (survivorship and fecundity) in the zebrafish, following exposure to the aromatase inhibitor clotrimazole, in combination with elevated temperature and inbreeding. Effects data from a partial factorial laboratory exposure study (Chapter 6; Brown et al., 2013) were input to the PVA model Vortex, previously parameterised using control data from the exposure study and relevant, background life-history data reported elsewhere (Spence et al., 2008; Hazlerigg et al., 2013). We discuss our results in the context of cumulative environmental risk assessment and evaluate the importance of environmental, demographic and genetic (inbreeding) factors on population viability in zebrafish exposed to chemical (clotrimazole) and physical (temperature) stressors.

8.3 Methods

8.3.1 Population viability analysis model

Population Viability Analysis (PVA) to assess the future trajectory of populations based on life-history data (Akçakaya et al., 2008) incorporates modelling stochasticity, including environmental and demographic stochasticity and genetic drift (Soule and Gilpin, 1986), and inbreeding depression, which is correlated with genetic drift (Miller and Lacy, 2005). The simulation of emergent inbreeding depression, combined with the loss (purging) of genetic variation over repeated generations (≥40) is essential to assess the viability of threatened populations. This includes small populations and those with high initial population growth rate (like zebrafish), which are prone to rapid inbreeding (Brook et al., 2002).

We selected to use the PVA model Vortex, version 9.99 (Lacy et al., 2005) based on its ability to model the population impact of sex ratio skews (accounting for mating system) and emergent inbreeding depression (on age 0+ survivorship) alongside "standard" population input parameters e.g. age-specific survivorship and fecundity and their standard deviations. The construction and operation of Vortex is described in detail by Miller and Lacy (2005).

8.3.2 Control scenarios

Two control models were set up to account for potential differences in age 0+ survivorship due to initial breeding condition (before simulation of emergent inbreeding depression over future generations): high survivorship in outbreds (0.09); low survivorship in inbreds (0.04) (Chapter 6; Brown et al., 2013). Sex ratios were 50:50 and all other parameters represented conservative values and ranges (standard deviations) obtained from published literature (reviewed in Spence et al., 2008; Uusi-Heikkilä et al., 2010, 2011, 2012a, 2012b; Hazlerigg et al., 2013) and were consistent between each of the control models (Table 1).

Populations were assumed to be "closed", consisting initially of 4000 individuals divided between two age classes (*circa* 3800-3940 age 0+ sub-adults and 60-200 age 1+ adults) consistent with stable age distributions, taking into account the range of sex ratios in the experimental control treatments and observed adult population counts in natural ponds (Hazlerigg pers. comm.). There was assumed to be no difference in male and female survivorship and this was based on lack of sexual size dimorphism in zebrafish (Pyron, 2003a). Breeding was limited initially to 60 spawning events per adult female in their second year (age 1+), simulating a mean inter-spawning interval of 2 days throughout the 120 day monsoon season (June to beginning of October). Mating was assumed to be polygynous with a degree of mate monopolisation based on female-preference for larger males (Pyron, 2003b; Uusi-Heikkilä, 2010). Male breeding success was assumed to follow a Poisson distribution (Miller and Lacy, 2005) simulating (without the need for growth data) increased monopolisation by larger males when the proportion of females was reduced. Maturation takes up to 150 days in wild zebrafish (Uusi-Heikkilä et al., 2011; Uusi-Heikkilä et al., 2012b), which is longer than the monsoon breeding season, therefore generations were assumed to be non-overlapping (preventing exponential population growth) and generation time was assumed to be one year. Asymptotic population growth was modelled using a logistic model (Miller and Lacy, 2005), adopting a ceiling carrying capacity e.g. K = 5000±1000 total individuals, rather than by employing functional forms of density dependence. Given the zebrafish's short generation time of 1 year, the duration of simulations was limited to 100 years. Each simulation was run in stochastic mode, with input parameter

values being selected at random from a standard distribution (standard deviation) of values (Table 1). Simulations were repeated 100 times for each scenario enabling probability of extinction (PE) and mean time to extinction (MTE) to be determined, as well as mean per capita population growth rate (r).

Table 1: Parameterisation of the VORTEX PVA model for zebrafish

Parameter (data source)	Reference values (control model)	Alternative values (incl. *effects model)
Population structure: (Field observation)	Single, isolated population (no immigration or emigration)	
	One adult age class (age 1+)[a]	Two adult age classes (1+, 2+)
Maximum population size (Field observation)	Carrying capacity 'K' K= 5000 ± 1000 based on 60-100 adult census[b]	K= 2000 ± 1000 to 10000 ± 2000
Inbreeding (This lab study)	Inbreeding depression of age 0+ survival based on 5 lethal equivalents (LE)	Inbreeding depression of age 0+ survival based on 10 LE Inbreeding depression of growth, fecundity and reproductive success not accounted for
Survivorship (l_x) (Laboratory studies) (Field observation)	Ref l_x (age 0+) = 0.07 ± 0.03[c] Ref l_x (age 1+) = 0.9 ± 0.03[c]	Low l_x (age 0+) = 0.04 ± 0.03 High l_x (age 0+) = 0.09 ± 0.03 l_x (age 1+) = 0.25-0.75 ± 0.1
Growth	Not modelled	
Sex ratio (Laboratory studies and field observation)	Balanced sex ratio[a, c] 50 ± 5% breeding females 100% of males available for breeding but success is determined by proportion of females*	Skews in sex ratios due to exposure treatments = 2.8 - 58 ± 5 - 40% breeding females (See Table 2 for sex ratios simulated in effects models)
Fecundity (Laboratory studies)	Fecundity (m_x) = 50 ± 20 eggs per female[c]	Fecundity (m_x) = 20 - 40 ± 20 eggs per female[c]
Spawning	Alternate days[c] during 120 day spawning season[d]	Alternate days[c] all year round[a]

Values represent means ± standard deviations.
Table references: a Spence et al., 2008; b Hazlerigg pers comm.; c Hazlerigg et al., 2013; d Spence et al., 2007).

8.3.3 Exposure scenarios

Effects data concerning sex ratio skews were obtained from a laboratory exposure study of the combined effects of exposure to the aromatase inhibitor clotrimazole, elevated temperature and inbreeding on sexual differentiation and development in inbred (F_{IT} = n+0.25) and outbred (F_{IT} = n) zebrafish (Chapter 6, Brown et al., 2013). Sex ratios were input accordingly, otherwise all model input parameters were the same as the inbred and outbred control models. A total of 20 basic model scenarios covering a range of sex ratio skews were simulated, based on 10 experimental treatments (Table 2).

Clotrimazole is used globally in veterinary and human medicine as an anti-fungal (anti-mycotic) and anti-cancer (cytostatic) treatment. Clotrimazole is a potent ligand and inhibitor of (CYP)19 aromatase, which catalyses the conversion of androgens to estrogens in mammals (Trösken et al., 2004) and fish (Noaksson et al. 2003; Hinfray et al. 2006), including zebrafish (Brown et al., 2011). The chemical is capable of producing male-sex skews in zebrafish and skews have been shown to be greater in inbred compared to outbred lineages (Chapter 6; Brown et al., 2013).

Elevated temperature Water temperature of 28°C and 33°C are intended to simulate current and future (2100) mean temperatures across India and Bangladesh during the monsoon season, coinciding with the main spawning season for zebrafish (Spence et al., 2007). This temperature elevation is based on worst-case predictions according to IPCC Scenario A2 for the Indian subcontinent (Rupa Kumar et al., 2006).

Emergent inbreeding depression was modelled over successive generations as a reduction in age 0+ survivorship, based on an estimated mean of 5 lethal equivalent (LE) recessive alleles per diploid genome (Morton, 1956, Miller and Lacy, 2005) in our zebrafish (Chapter 6, Brown et al., 2013). Half of the inbreeding depression was assumed to be caused by recessive lethal alleles and was subject to purging, while the remaining 50% was attributed to sub-lethal alleles (Simmons and Crow, 1977).

Table 2: Model scenarios based on sex ratio skews in assorted/combined clotrimazole exposure, temperature and breeding treatments

No.	Emergent inbreeding depression	Scenario description	Age 0+ survivorship	Proportion of females (%)
1 2	✓ ×	Inbred, zero clotrimazole, 28°C	low	40.7
3 4	✓ ×	Outbred, zero clotrimazole, 28°C	high	57.9
5 6	✓ ×	Inbred, zero clotrimazole, 33°C	low	17.6
7 8	✓ ×	Outbred, zero clotrimazole, 33°C	high	40.0
9 10	✓ ×	Inbred, high clotrimazole, 28°C	low	28.2
11 12	✓ ×	Outbred, high clotrimazole, 28°C	high	51.7
13 14	✓ ×	Inbred, low clotrimazole, 33°C	low	18.1
15 16	✓ ×	Outbred, low clotrimazole, 33°C	high	51.3
17 18	✓ ×	Inbred, high clotrimazole, 33°C	low	2.8
19 20	✓ ×	Outbred, high clotrimazole, 33°C	high	17.1

Scenarios represent treatments in a controlled exposure study (Chapter 6, Brown et al., 2013). Low clotrimazole represents aqueous exposure concentrations of 1.7 $\mu g\ l^{-1}$. High clotrimazole represents aqueous exposure concentrations of 8 $\mu g\ l^{-1}$. Inbred treatment effects on sex ratio were input to the inbred, low survivorship control model. Outbred treatment effects were input to the outbred, high survivorship control model.

8.3.4 Sensitivity analysis

Following the initial model simulations, sensitivity analysis was performed on inbred and outbred populations (defined by age 0+ survivorship). Population sex ratio was fixed at 18.1% females representing a threshold below which r declines sharply (Section 8.4). The remaining demographic rates/input parameters were varied in turn within specified ranges of their mean reference values (Table 3). Scenarios generating probabilities of extinction PE>0.05 were re-run on populations with: increased carrying capacity (10000±2000 total individuals); two adult age classes; year-round spawning. Other recorded model outputs included mean time to extinction (MTE), mean population number, per capita population growth rate (r) and associated standard deviations.

Table 3: Sensitivity analysis design

Parameter	Inbred population		Outbred population	
	Mean	SD	Mean	SD
Survivorship l_x (age 0+)	0.04	0.03	0.09	0.03
Survivorship l_x (age 1+, 2+)	0.25, 0.5, 0.75, 0.9	0.03, 0.1	0.25, 0.5, 0.75, 0.9	0.03, 0.1
Fecundity m_x (age 1+, 2+)	20, 30, 40, 50	5, 10, 20	20, 30, 40, 50	5, 10, 20
Carrying capacity K	2000, 3000, 5000, 10000	1000, 2000	2000, 3000, 5000, 10000	1000, 2000
Lethal equivalents LE	5, 7, 10		5, 7, 10	
Sex ratio*	18% females : 82% males			

Reference values highlighted in blue.

* Sex ratio was not varied in the sensitivity analysis – reference represents inbred, low clotrimazole, 33°C treatment scenario (no. 13, 14: Table 2).

SD is standard deviation.

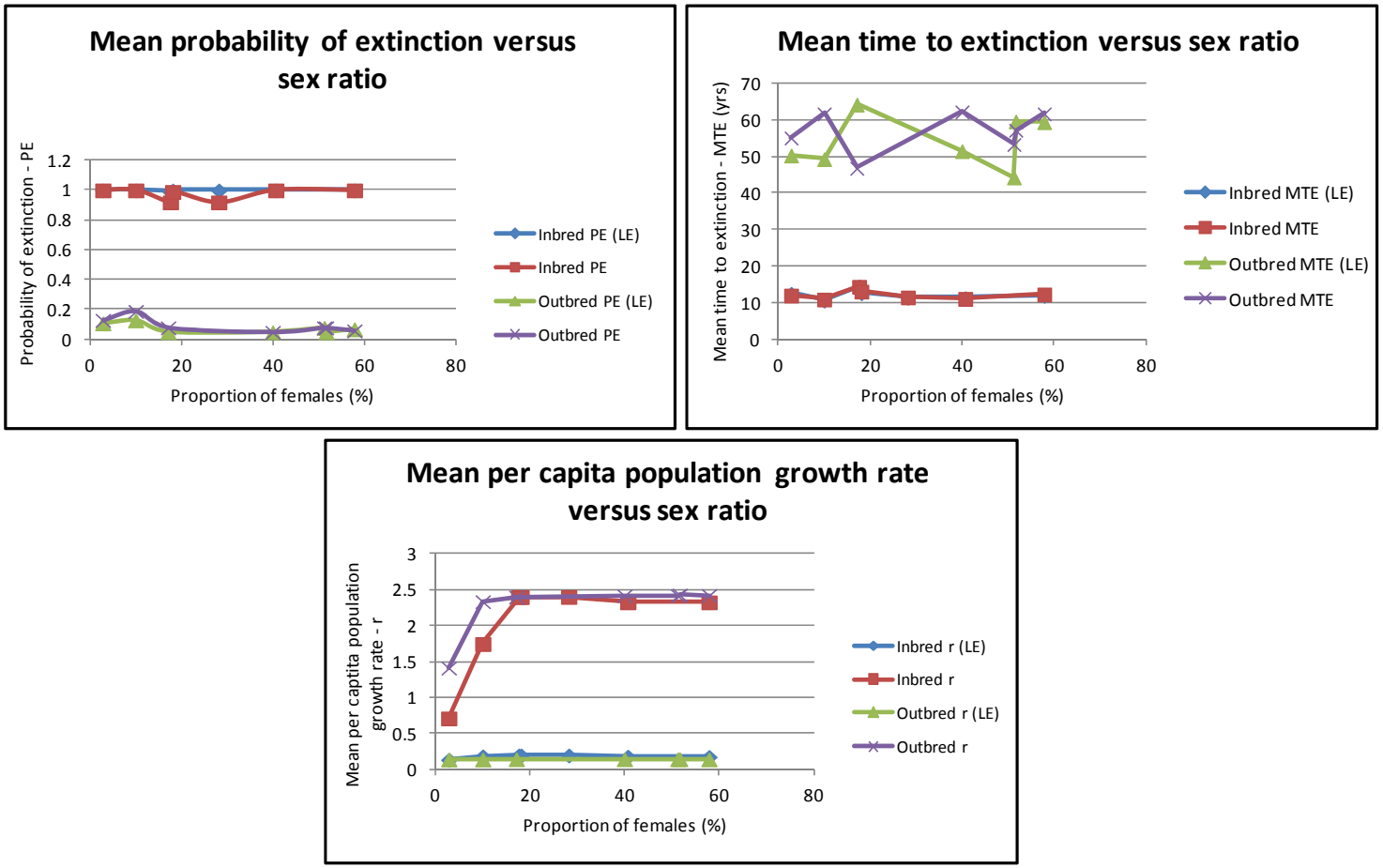
Additional sensitivity analysis was performed by varying age 0+ survivorship between inbred (low) and outbred (high) reference values.

8.4 Results

Initial simulations (Figure 1) generated significant probabilities of extinction (PE ≥0.05) for all scenarios (Table 2). PE was 0.05-0.08 and MTE was 44–62 years in outbreds, whereas PE was higher at 0.92-1 and MTE more rapid at 11–14.6 years in inbred populations, with lower age 0+ survivorship (0.04 ± 0.03) and generally more male-biased sex ratios (2.8% to 40% females). Plotting the model results showed that inbred treatments were clearly distinguishable from outbred treatments but that PE and MTE did not vary with sex ratio (Figure1). Reducing the proportion of females from 18.1% to 2.8% and from 10% to 2.8% corresponded with a reduction in mean per capita population growth rate from a baseline of r = 2.4 to 0.72 in inbreds and from r = 2.4 to 1.4 in outbred populations respectively. The additional simulation of inbreeding depression on age 0+ survivorship, based on 5 lethal equivalents (LE) per diploid genome did not affect PE or MTE, but further reduced mean per capita population growth rate from r = 2.4 to 0.19 in inbreds and outbreds, irrespective of sex ratio. The results generated by simulations based on 10 lethal equivalents were virtually the same (r = 0.17).

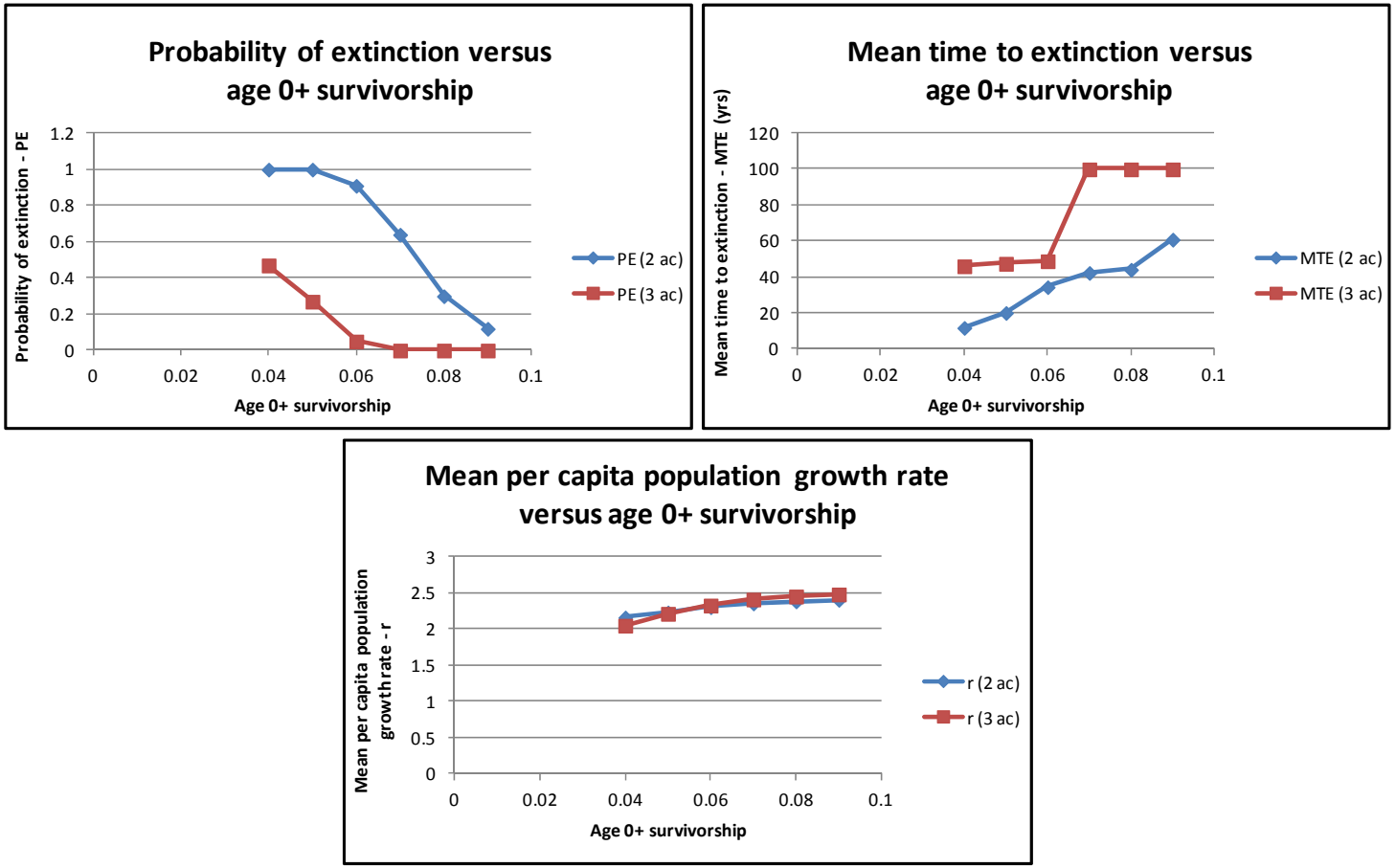
Results of the sensitivity analysis are shown in Figures 2a-c.

Figure 1: Probability of extinction, mean time to extinction and per capita population growth rate versus proportion of females in inbred versus outbred zebrafish populations



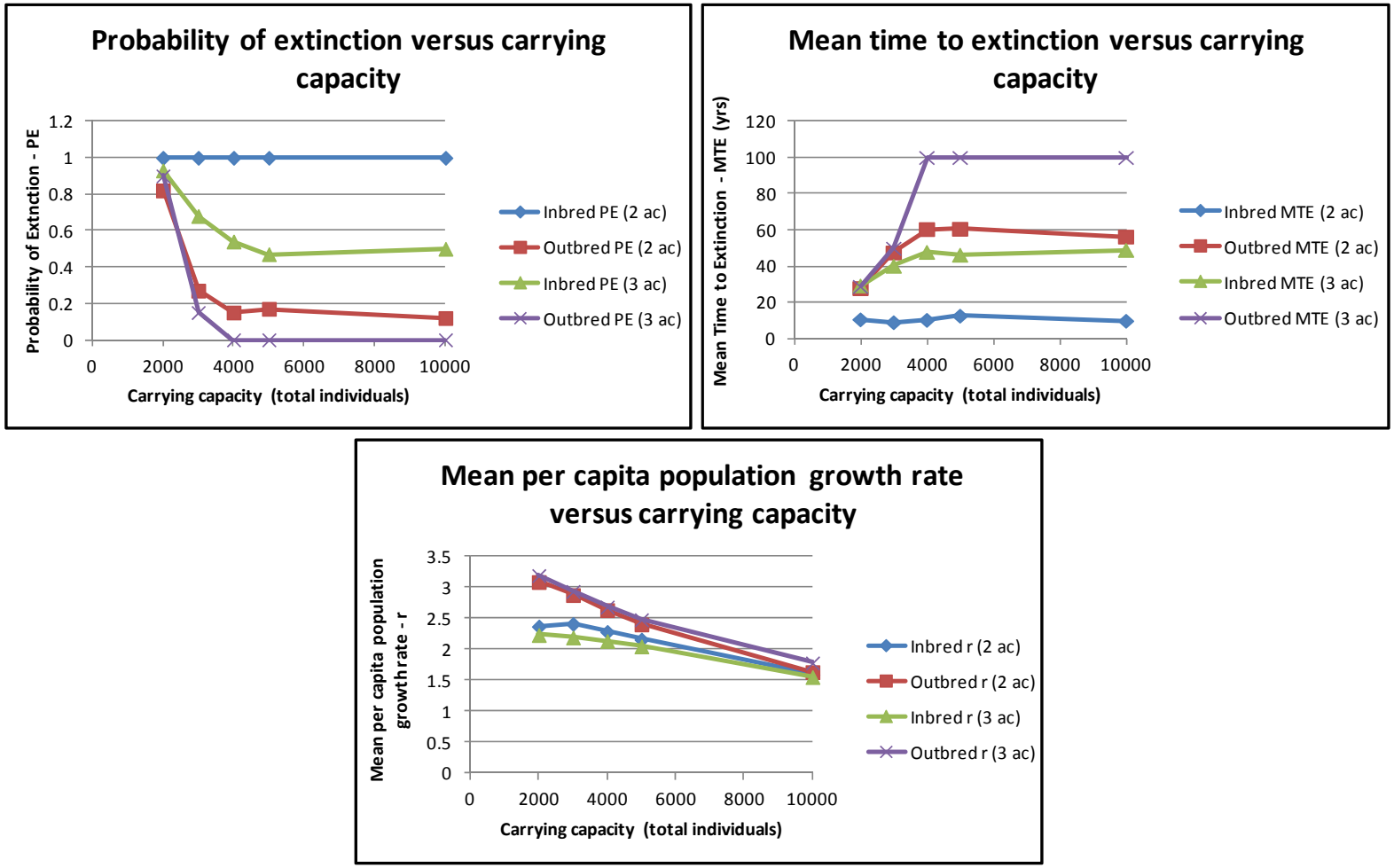
All scenarios based on a maximum carrying capacity of 5000±1000 total individuals including *circa* 100-200 age 1+ adults capable of breeding on alternate days during the 120 day monsoon spawning season. Fecundity was 50±20 eggs per female.

Figure 2a: Sensitivity in probability of extinction, mean time to extinction and per capita population growth rate to model input parameters other than sex ratio (age 0+ survivorship)



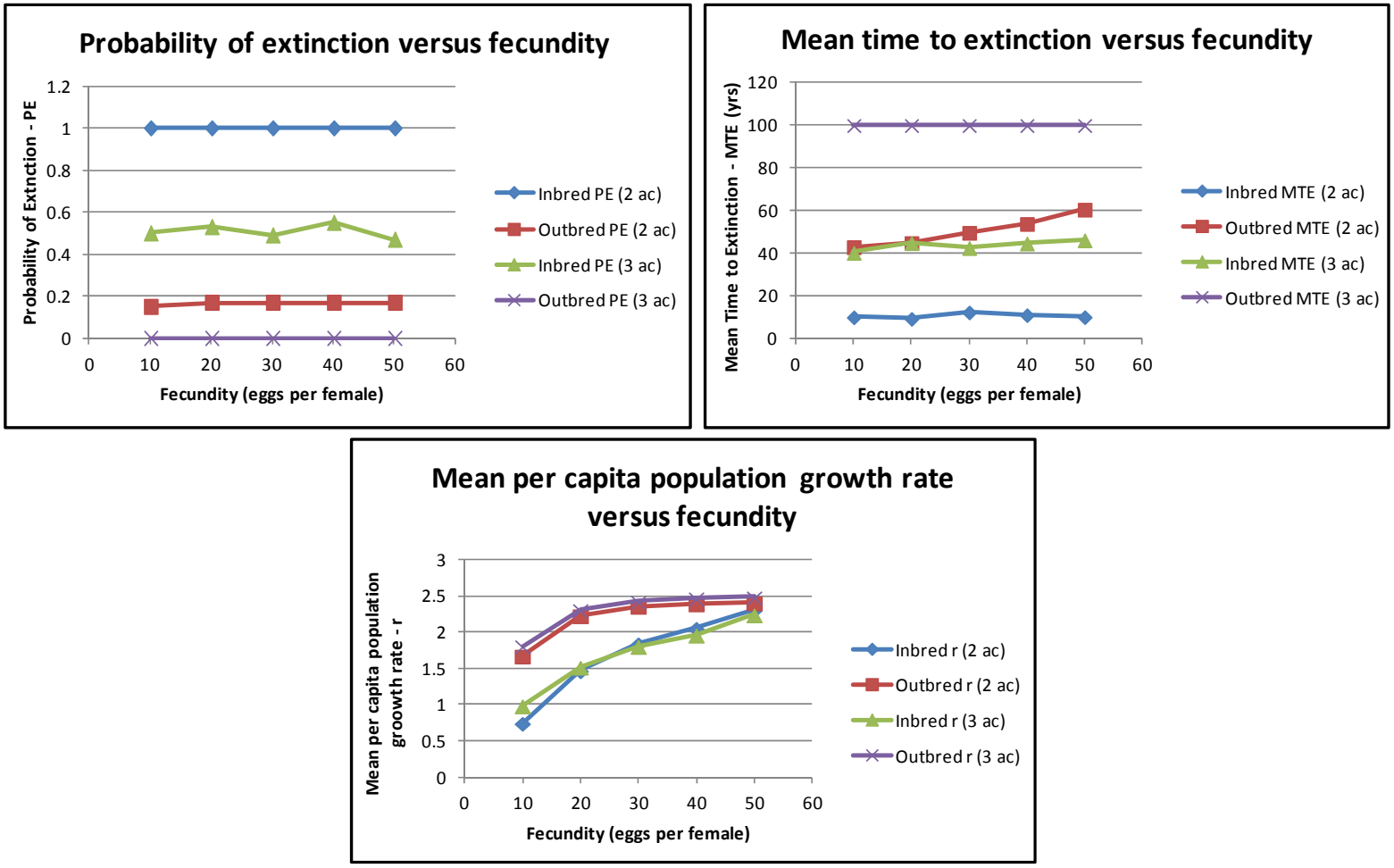
All scenarios simulate breeding on alternate days during the 120 day monsoon spawning season. Simulations in blue represent two age classes (2 ac), in which reproduction is restricted to age 1+ adults. Simulations in red represent three age classes (3 ac), in which reproduction can occur in age 1+ and age 2+ adults. Emergent inbreeding depression (based on LE) was not included in sensitivity analysis simulations.
Total carrying capacity = 5000±1000, fecundity = 50±20 viable eggs, age 1+ survivorship = 0.9±0.1 (see Table 3 for other input parameter reference values and ranges).

Figure 2b: Sensitivity in probability of extinction, mean time to extinction and per capita population growth rate to model input parameters other than sex ratio (carrying capacity)



All scenarios simulate breeding on alternate days during the 120 day monsoon spawning season. Inbreds and outbreds were distinguished by age 0+ survivorship (0.04±0.03 and 0.09±0.03 respectively), total carrying capacity = 5000±1000, fecundity = 50±20 viable eggs, age 1+ survivorship = 0.9±0.1 (see Table 3 for other input parameter reference values and ranges).

Figure 2c: Sensitivity in probability of extinction, mean time to extinction and per capita population growth rate to model input parameters other than sex ratio (fecundity)



All scenarios simulate breeding on alternate days during the 120 day monsoon spawning season. Inbreds and outbreds were distinguished by age 0+ survivorship (0.04±0.03 and 0.09±0.03 respectively), total carrying capacity = 5000±1000, fecundity = 50±20 viable eggs, age 1+ survivorship = 0.9±0.1 (see Table 3 for other input parameter reference values and ranges).

Sensitivity analysis showed that the 2.25-fold increase in age 0+ survivorship between inbreds and outbreds (0.04-0.09) corresponded with a near 10-fold, largely monotonic reduction in PE (from 1-0.12) and a six-fold increase in MTE (from 12-61 years). Model simulations based on three age classes, including a second adult age class (age 2+), also generated considerably lower PEs and higher MTEs than those for two age classes (Figure 2a). Similarly, a 2.5-fold increase in carrying capacity (from 2000±1000 to 5000±1000 total individuals) led to a five-fold decrease in PE (from 0.82-0.17) and a two-fold increase in MTE (from 28-60 yrs) in outbred populations with age 0+ survivorship = 0.09, while inbred populations with age 0+ survivorship = 0.04 remained extinct. However, a further two-fold increase in environmental carrying capacity from the reference value of 5000±1000 to 10000±2000 total individuals had no effect on PE and MTE (Figure 2b). There was also no noticeable population effect from simulating year-round spawning, as opposed to seasonal monsoon spawning. Varying age 1+ survivorship, fecundity and LE within ranges reported for zebrafish (Table 3) also had no effect on PE or MTE (results not shown).

Despite no effect on PE or MTE , a 5-fold reduction in fecundity, from the reference value of 50±20 to 10±10 eggs per female, resulted in a 2.4-fold reduction in mean per capita population growth rate in inbreds (from r = 2.4 to <1) and a 1.33-fold reduction in outbreds (from r = 2.4 to <1.8) ((Figure 2c). This change in r was comparable with that caused by reducing the proportion of females to from 18% to 2.8% (Figure 1).

8.5 Discussion

Population viability analysis was used to assess the population impacts of sex ratio skews generated in controlled laboratory exposures of inbred and outbred zebrafish to the aromatase inhibitor clotrimazole and/or elevated temperature (Chapter 6, Brown et al., 2013). Initial simulations based on two age classes showed that inbred zebrafish populations had considerably higher probability and rate of extinction (PE = 0.92-1, MTE = 11–14.6 years) compared to outbred populations (PE = 0.05-0.08, MTE = 44-62 years) regardless of sex ratio. Sensitivity analysis showed that PE and MTE were influenced by differential age 0+

survivorship in inbreds and outbreds (0.04 and 0.09 respectively), whereas varying female fecundity and sex ratio within ranges reported for zebrafish had no effect on these metrics (Figure 1). Excess fecundity, typical of broadcast spawning fish (r-strategists that rely on large numbers of progeny to offset low survivorship) appears to buffer the impact of male-biased sex ratios in zebrafish populations. Nevertheless, skews of >80% males in inbred populations and >90% males in outbred populations represented thresholds below which per capita population growth rate (r) declined sharlply. Our experimental results show that such skews in sex ratio may be induced in inbred zebrafish populations exposed during sexual development in the laboratory to high concentrations of the aromatase inhibitor clotrimazole (8 $\mu g\ l^{-1}$) or lower concentrations (1.7 $\mu g\ l^{-1}$), when exposure is combined with elevated temperature (33°C) (Chapter 6, Brown et al., 2013). Whilst the lower exposure concentration is a factor of 10 above predicted environmental concentrations (OSPAR, 2005) this work highlights the need to consider the cumulative risk of chemicals in combination with other environmental factors with similar modes of action.

Sex ratio skews may also occur due to stochasticity in small populations (Miller and Lacy, 2005) and the effects on population growth may be compounded by inbreeding and genetic drift (Gilpin and Soulé 1986). The additional simulation of inbreeding depression on age 0+ survivorship, based on 5 lethal equivalents (LE) per diploid genome did not affect PE or MTE, but further reduced mean per capita population growth rate from r = 2.4 to 0.19 in inbreds and outbreds, irrespective of sex ratio. A similar number of LEs have been estimated based on embryonic and juvenile survival in other wild-caught zebrafish (McCune et al., 2002; McCune et al., 2004). However, simulating inbreeding depression based on 5 LE may represent a significant underestimate, since the impact of inbreeding has been shown to be more than three times greater when fitness components other than juvenile survival are taken in account over the full life-cycle, including adult survival, fecundity and mating success (Frankel and Soulé, 1981). Furthermore, inbreeding depression may be up to seven times greater in the wild compared to the laboratory (Crnokrak and Roff, 1999). Therefore additional simulations based on a more conservative value of 10 LE were performed, but the result (r = 0.17) was virtually the same as for 5 LE (r = 0.19).

According to an established model for vertebrates including for fish (Reed et al., 2003), reduced per generation population growth rates (ln R_0) equate to larger minimum viable population sizes (MVP_A). Based on a generation time of one year for zebrafish, r equates to ln R_0 and the above model predicts MVP_A to be ≈500 adults when r = 2.4 and ≈10000 adults when r = 0.19. Our results therefore indicate that inbreeding depression can build up rapidly over successive generations, leading to compounding reductions in baseline population growth rate (r = 2.4) and threatening the viability of zebrafish populations with fewer than 500 adults (as modelled here). It is not possible to obtain an accurate census of entire fish populations due to practical difficulties in sampling small juveniles. Nevertheless, observed counts of only *circa* 100 adults in natural ponds (Hazlerigg pers.comm.) seem common, equating to 4000-5000 total individuals, assuming populations follow a stable age distribution (Miller and Lacy, 2005). This suggests that substantial gene flow from immigration/emigration is required between neighbouring populations to increase effective meta-population size, in order to minimise inbreeding depression and assure population viability. This level of gene flow may occur in low-lying areas central to river flood plains, but populations inhabiting more remote or higher-lying areas could be vulnerable. The vulnerability of smaller, more isolated populations was demonstrated during sensitivity analysis by adjustment of carrying capacity (from a reference value of 5000 ± 1000 down to 2000 ± 1000 total individuals) (Figure 2b). Population vulnerability may be reduced if ≥50% of adults are able to survive and breed in successive second (age 1+) and third years (age 2+). Model scenarios based on three age classes (3ac = age 0+ to 2+) and the reference carrying capacity showed that probability of extinction was reduced substantially in outbred populations (PE_{3ac} = 0; PE_{2ac} = 0.18) and inbred populations (PE_{3ac} = 0.43; PE_{2ac} = 1).

8.6 Conclusions

With the aid of stochastic population modelling we have shown that small reductions in 0+ survivorship are considerably more influential on PE and MTE than large male-skews in zebrafish sex ratios and/or reduced female fecundity and we attribute this to high ('surplus')

fecundity in this r-strategist. Nevertheless, male sex skews >80% generated in our combined exposure study (Chapter 6, Brown et al., 2013) and/or emergent inbreeding depression can depress per capita population growth rate (r), requiring larger numbers of breeding adults (MVP_A) than are commonly found in individual natural ponds in the flood plains of Bangladesh to maintain viable zebrafish populations. Therefore remote populations with low immigration may be at risk of population growth spiralling downwards, leaving them increasingly vulnerable to stochastic factors and inbreeding (Brook et al., 2002; Keller and Waller, 2002; O'Grady et al., 2004). This vulnerability was clearly illustrated in our simulations based on low carrying capacity (2000±1000 total individuals). This represented a point of convergence where all populations, regardless of initial level of inbreeding or age structure, yielded extinction probabilities approximating to PE = 1 (Figure 2b). PE was also highly sensitive to increased environmental stochasticty in carrying capacity (5000±2000 individuals). This work highlights the need to define "appropriate assessment populations" in environmental risk assessment (Akçakaya et al., 2008) in terms of their size, demographics, gene flow, inbreeding and the stochastic variation in each factor (Shaffer, 1981).

It is possible that other ecological factors could act in combination with those we have simulated to induce male-skews in wild fish populations. For example, dissolved oxygen stress, reduced nutrition and overcrowding, are all capable of promoting male-skewed sex ratios in fish (Delvin and Nagahama, 2002; Baroiller et al., 2009) and are likely to accompany projected climate change in combination with rising temperatures. The impact of these changes will depend on the adaptability of populations governed by changes in dispersal patterns (Perry et al., 2005), mutational adaptation and the direction of evolutionary selection involving either frequency-dependent selection of the more abundant sex (Tucker et al., 2008) or conversely the less abundant sex (Ospina-Álvarez and Piferrer, 2008) in sex-skewed populations. Species with shorter generation times are likely to adapt more quickly, and high fecundity in r-strategists such as zebrafish may offer a buffer against imbalances in sex ratio. However, these 'attributes' can also lead to rapid inbreeding in isolated populations with limited carrying capacity, and depression for age 0+ survivorship may become the overriding factor, as we have shown.

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9 CHAPTER 9: CONCLUSIONS

9.1 Relevance of research

This thesis examines the potential interactions between inbreeding in the zebrafish (*Danio rerio*, Hamilton) and environmental stressors clotrimazole and temperature. Treatment effects are assessed across a spectrum of biological responses, from the molecular level to the population level, in terms of the direction, magnitude and variability of these responses.

The work presented is pertinent to the environmental risk assessment (ERA) of chemicals, since the process relies on the confirmation of adverse responses/effects in often highly inbred, genetically impoverished model organisms, which are intended to be representative and protective of wild populations. However, potential differences between laboratory and field populations stem from the founding of laboratory strains based on a limited gene pool, sometimes using as a few as two individuals, as in the case of the widely used Wild Indian Karyotype (WIK) zebrafish strain (Rauch et al., 1997). Genetic diversity and "quality" are subsequently eroded by successive generations of inbreeding amongst the founders' progeny and by genetic drift, due to the random segregation of genes in gametes and unequal reproduction among individuals.

9.2 Occurrence of inbreeding and consequences for wildlife and laboratory populations

A critical review of the published literature (Chapters 1 and 2) revealed that inbred laboratory strains are generally more sensitive to environmental stressors than outbred strains and this tallies with observations in wild populations, where inbreeding and loss of genetic diversity correlate directly with reduction in population fitness (Reed and Frankham, 2003) and adaptability to environmental change (Reed et al., 2003). Although mating systems in sexually reproducing organisms generally guard against inbreeding and inbreeding depression (Pusey and Wolf, 1996), inbreeding does happen in the wild, threatening the viability of small or geographically isolated populations (Keller and Waller, 2002) and some migratory species returning each year to the same breeding ground (Waldman and McKinnon, 1993). Therefore it could be argued that using inbred organisms in ecotoxicology is both representative and

protective of wild populations. However, rates of inbreeding and the expression of recessive, deleterious alleles in homozygotes (contributing to inbreeding depression), versus the rates of purging of these alleles may be very different in the laboratory versus the wild (Miller and Hedrick 2001). Higher purging rates in the laboratory may propagate lower genetic diversity in laboratory strains. Furthermore, some recessive, deleterious alleles are cryptic in inbred animals under laboratory control conditions and may only be expressed under stressful environmental conditions (Crnokrak and Roff, 1999), as simulated in ecotoxicity tests. Adverse phenotypic effects may be exacerbated in multi-generational studies due to rigid experimental design and forced inbreeding within treatments, rather than being due solely to chemical exposure. In the longer-term, loss of heterozygosity in laboratory cultures can lead to significant differences between strains and between studies, which again may be hard to identify. The unveiling of recessive characters in homozygotes also increases variability in biological response in inbred test organisms and the perceived advantages of reduced variation, improved "signal to noise" and increased statistical power, are not guaranteed.

9.3 Phenotypic variance and plasticity

Increased phenotypic variance appears to be a common symptom of inbreeding due to the unveiling of recessive characters (Armbruster and Reed, 2005). The phenomenon was illustrated for a number of apical effects endpoints (hatch success, growth and sexual development) under control conditions in Chapter 4. A further increase (amplification) in phenotypic variance was observed for specific growth rate in inbred zebrafish under high-level clotrimazole exposure (Chapter 5). Power analysis indicated that almost twice the number of inbreds would be required compared to outbreds to detect an equivalent level of effect on specific growth rate. Developmental phenotypes can show high variability in their responses to environmental factors due to inbreeding×environment interactions (c.f. genotype×environment interactions), leading to phenotypic plasticity. This variability is due to compounding changes over time (ontogeny) and the interdependency of developing characters (allometry) (Pigliucci et al., 1996). Phenotypic plasticity may be continuous, illustrated for example by an increase body size with temperature, or discontinuous, as is the

case for phenotypic sex. Some of the most striking results in this thesis were directional skews in sex ratio in response to clotrimazole (Chapter 5) and elevated temperature exposure (Chapter 7). However, a study assessing sexual development in different zebrafish strains under laboratory control conditions (Chapter 4) also illustrated the effect on sex ratio of both i) inbreeding: with 55% males in WIK/Wild outbreds, increasing to 72% males in inbred WIK/Wild zebrafish and ii) strain: with 20% males in genetically distinct WIK zebrafish. The effect of inbreeding on sexual development supports a prior study showing male-biased sex ratios in pure-bred lab strains (Lawrence et al., 2008), whereas the effect of genetic strain has been reported in another more recent study (Liew et al., 2012). Some of the skews observed exceed acceptance limits for zebrafish in experimental controls (30-70% males) in partial life-cycle studies (OECD, 2011). Therefore, unless strains are properly characterized beforehand, it is easily conceivable that biases may emerge at the end of a study, which then render it unsuitable for regulatory submission. This would have direct financial implications, requiring the study to be repeated, but potential delays in product registration could be far more costly.

9.4 Plasticity in sexual development in inbred versus outbred zebrafish and impact of the endocrine disrupting chemical clotrimazole

The process of sexual development in zebrafish is highly plastic and numerous physical, chemical and biological stress factors have been shown to direct male development, including elevated temperature, reduced dissolved oxygen, overcrowding, reduced food ration and inbreeding. This plasticity may be due to polygenic sex determination (Whitely et al., 2011; Anderson et al., 2012; Liew et al., 2012). However, ultimately during sexual differentiation in zebrafish, *cyp19a1* plays a pivotal role encoding aromatase, the only steroidogenic enzyme responsible for the conversion of male hormones (androgens) to female hormones (estrogens) (Guiguen et al., 2010). Therefore *cyp19a1* is undoubtedly a gene of "major effect" defining male or female development in this species. As juvenile hermaphrodytes, commitment to sexual differentiation in zebrafish is delayed until around 45 days post fertilization (dpf) in the laboratory, extending up to 77 dpf in some cases (Maack and Segner, 2003). This presents a relatively wide window for environmental factors, including endocrine disrupting chemicals

(EDCs), to redirect sexual differentiation. It has even been proposed that sex reversal may be permitted throughout the life of the zebrafish via *cyp19a1* (Le Page et al., 2010).

In a partial life-cycle study (40-136 dpf), high-level exposure to the EDC (aromatase inhibitor) clotrimazole (43.7 μg l^{-1}) induced significantly male-biased sex ratios (87 and 92% males) in WIK/Wild inbred and outbred zebrafish respectively (Chapter 5). It was confirmed that male-biased sex determination was not due to sexual size dimorphism and the observed sex ratios therefore appeared to be indicative of aromatase inhibition by clotrimazole. Although no additional bias towards male phenotype was found in inbreds, analysis of gonadal transcripts revealed significant inhibition of aromatase (*cyp19a1a*) and insulin-like growth factor (*igf1*) in inbred males at the end of the exposure study, but this did not occur in outbred males. A series of additional compensatory responses were observed in the physiological and gonadal development of outbred males, contributing to increased/prolonged testosterone production (as indicated by plasma concentrations of 11-ketotestosterone). Testosterone is converted to 17α-estradiol (E2) by aromatase and both E2 and insulin-like growth factor (igf1) are essential for normal testicular growth and function (Abney, 1999; Reinecke, 2010). Similar compensatory responses have been observed in adult fathead minnows exposed to ketoconazole (Ankley et al., 2007), but have not previously been noted in developmental studies involving the exposure of zebrafish to other azole compounds from early life. In the case of fadrozole, permanent testicular development appeared to be induced by exposure from 35-71 dpf to 500 μg fadrozole g^{-1} food, persisting for up to 90 days following cessation of exposure (Fenske and Segner, 2004).

9.5 Consequent effects on male fitness in inbred versus outbred zebrafish following exposure to clotrimazole

Although we did not investigate the permanency of sexual differentiation in zebrafish following clotrimazole exposure, we did examine persistent effects on reproductive fitness. Four weeks after cessation of exposure to low-level clotrimazole (2.9 μg l^{-1}), breeding trials

were performed and indicated that there was a tendency for inbred males to sire fewer offspring when in direct competition with similarly sized, outbred males (Chapter 6). Plasma concentrations of 11-kt also showed substantial inbreeding depression and this is likely to have promoted sub-ordinance in inbreds versus outbred males (Coe et al., 2008). Whilst there was no additional effect on 11-kt following prior clotrimazole exposure, plasma concentrations correlated with siring success in outbred, exposed males. There was no effect of inbreeding or clotrimazole exposure on female fecundity, but embryo viability was significantly reduced in the inbred low-level clotrimazole treatment. Overall, these data provided evidence that exposure to clotrimazole can interact with inbreeding to bias sexual development towards males in zebrafish and can subsequently impact on reproductive traits, particularly those associated with male fitness. This is consistent with increased susceptibility of male fitness components to inbreeding depression, as observed in other species (Michalczyk et al. 2010; Okada et al. 2011), and the amplification of these inbreeding effects under chemical stress (Armbruster & Reed, 2005; Meagher et al. 2000).

9.6 Interactive effects of environmental stressors on wild zebrafish populations

Combined exposure of inbred and outbred wild-type zebrafish to clotrimazole (at 8 $\mu g\ l^{-1}$) and elevated temperature (33°C c.f. 28°C) from 40-100 dpf generated significantly male-skewed sex ratios in both inbred and outbred zebrafish. Male-skew was greater in inbred treatments compared with equivalent outbred treatments and was also significant in inbreds (but not outbreds) following low-level clotrimazole exposure (1.7 $\mu g\ l^{-1}$), in combination with elevated temperature. The masculinising effects of clotrimazole and elevated temperature exposure were found to be additive, which is consistent with the general model for chemical mixtures with similar modes of action (Boobis, 2011). These data support the contention that the effects of chemicals may be exacerbated by other environmental stressors, further justifying the need for cumulative environmental risk assessment (EC, 2012). The exacerbation of water quality problems by climate change has also been highlighted by the Intergovernmental Panel on Climate Change (Bates et al., 2008). The further additive effect of inbreeding (exemplified

under low clotrimazole exposure and elevated temperature) is noteworthy, given global trends indicating habitat loss (UNEP, 2012) combined with increasing scarcity of freshwater (Bates et al., 2008) and the potential isolation and contraction of wildlife populations. Although the lowest observed effect concentration (1.7 μg l^{-1}) is an order of magnitude above the predicted environmental concentration (PEC_{local}) for clotrimazole (OSPAR, 2005), our results illustrate the importance of considering biological (genetic) as well as physical and chemical interactions in cumulative ERA.

Some species and populations are expected to be more susceptible than others to cumulative environmental changes, including those in which sex determination is partially or entirely temperature/environmentally dependent (Ospina-Alvarez and Piferrer, 2008) and small populations with low population growth rates, or high growth rates, leading to rapid inbreeding (Brook et al., 2002). Sex ratios skews present greater risks when population numbers are low because numbers of the least abundant sex are more susceptible to stochastic fluctuation and chance extinction. However, reducing effective population size (N_e) also potentiates genetic drift, inbreeding and the loss of heterozygosity and fitness. Thus a combination of stochastic factors (environmental, demographic and genetic) can generate a self-perpetuating decline or "extinction Vortex" (Gilpin and Soulé 1986). According to the stochastic population viability analysis (Chapter 8) model Vortex© (Lacy et al., 2005), male-skews >80%, induced by clotrimazole in combination with elevated temperature and inbreeding, represented a threshold above which per capita population growth rate (r) in zebrafish declines sharply. This was due to the proportional lack of females. However, small reductions in juvenile (age 0+) survivorship (including simulated inbreeding depression) were more influential on r and extinction probability than large sex ratio skews (80-97%) in zebrafish and we attributed this to "surplus" fecundity in this "r-strategist". Populations of less fecund species, or so-called "k-strategists" may be more at risk from sex ratio imbalances.

It is possible that other ecological factors could act in combination with those we have simulated to induce similar or even more extreme male-skewed sex ratios in wild zebrafish populations. For example, dissolved oxygen stress, reduced nutrition and overcrowding, are all capable of promoting male-skewed sex ratios in fish (Delvin and Nagahama, 2002;

Baroiller et al., 2009) and are likely to accompany projected climate change in combination with rising temperatures. However, it could be argued that other chemicals or agents with opposing modes of action could be present in the environment and counter-act to some extent the masculinising effects we observed (Hoffman and Kloas, 2012). The impact of these various environmental factors will depend ultimately on adaptive/evolutionary responses in the populations affected (Ospina-Álvarez and Piferrer, 2008). Of course inbreeding can potentially impact on these adaptive processes too, but this work is beyond the scope of this thesis.

9.7 Practical implications of inbreeding and recommendations for (eco)toxicology

Increased sensitivity in inbred laboratory strains to environmental stressors potentially offers additional protection in ecotoxicology and ERA. However, this may be counteracted by increased variance in responses, impacting on the statistical power of studies. The long-term effects of inbreeding depression on reproductive fitness and skewing of sex ratios may also have serious practical implications for stock maintenance. These problems can be mitigated by structured breeding and periodic outbreeding with other healthy populations (US EPA, 1987; Monson and Sadler, 2010). However, outbreeding between genetically distinct strains can also lead to fitness depression in offspring (outbreeding depression), due to the dilution of adaptive genetic variation and/or the breakdown of beneficial (pleiotropic or epistatic) gene×gene interactions. It has been shown that these complexes are generally restored or reconfigured by the F_3 generation (Monson and Sadler, 2010). Therefore details of the origin, breeding history and/or genetics of laboratory strains are important, but they are rarely given the same amount of attention as the qualification of environmental exposure conditions employed in ecotoxicology studies. Claims that strains are “outbred” are often not supported and, in cases where they are, evidence is often based on heterozygosity measured at neutral loci, which may (Szulkin et al., 2010) or may not reflect heterozygosity at QTLs or provide reliable estimates of inbreeding levels (Slate et al. 2004). Genome-wide characterisation of

animal strains may be feasible and economically viable in the future, but interpreting and comparing these data will present a major challenge.

Throughout the experimental studies conducted in this thesis, attention was given to controlled breeding and the avoidance of outbreeding depression. All test subjects were at least F_3 generation and inbreeding (F_{IT} = n+0.25 or n+0.375) was controlled by pair breeding between full-siblings. In Chapters 4 and 5 we verified inbreeding coefficients using 11-12 micro-satellite markers and found these to be capable of differentiating between breeding treatments in terms of comparative level of inbreeding. However, there is no substitute for reliable pedigree information and maintaining accurate breeding records is recommended. Periodic verification of genetic diversity of breeding stock should be implemented as part of the husbandry of animals used in (eco)toxicology and, as we have shown, assessment of multi-locus heterozygosity using micro-satellite markers is adequate for this and not too onerous. Similarly micro-satellite analysis could be used to confirm pedigree in situations where this is not clear (Pemberton 2004), in the same way we used them to confirm the paternity of progeny in our breeding trials (Chapter 6). This level of effort would be commensurate to that currently devoted to the standardisation of environmental exposure conditions in regulatory guidelines for (eco)toxicology studies. A final check to ensure the consistency of response of laboratory strains (between and within laboratories over time), would be to employ model compounds as 'positive controls', as used routinely in mammalian toxicology studies. These controls should be employed in life-cycle and multi-generation studies in which inbreeding is guaranteed within treatments, due to rigid statistical design. However, more regular screening for potential inbreeding effects would be advisable, in order to detect problems before they arise in an expensive and/or urgently required study.

9.8 Further work

Although the zebrafish is a well-studied animal model (Chapter 1), it may not be representative and protective of all fish species. Its sensitivity to environmental stressors relates to high levels of plasticity in sex determination and differentiation, combined with a

short-life cycle and therefore a "flat" population structure, offering a limited number of age classes for buffering poor cohort recruitment. However, these attributes are compensated for by relatively high levels of fecundity and it is possible that less fecund species or so-called "k-strategists" may be more susceptible to disruption of sexual development, sex ratio skews and overall reproductive fitness? Unfortunately such species tend to be longer-lived, much slower to mature and consequently do not lend themselves to laboratory life-cycle or partial life-cycle/developmental testing. The lack of studies on inbreeding×chemical interactions in fish, due to practical limitations presented by their longer generation times compared to many invertebrates, was highlighted in a critical review of the available literature (Chapters 1 and 2). Despite the lack of empirical data, computational population modeling can provide some insights concerning the comparative sensitivity of r versus k-strategist fish species (Brown et al., 2003).

There are still several gaps in our understanding of the zebrafish's basic biology, particularly sexual development. Several genes may be involved in sex determination and these may differ between strains and some may be more important under different environmental conditions. Clearly there is some work still to do in order to understand genotype×environment interactions in zebrafish and this is likely to provide insights into the evolution of sex determining mechanisms in other fish, with wider potential benefits for environmental science, predictive (eco)toxicology (read across) and aquaculture (Pifferer et al., 2012). Benefits to aquaculture relate to species showing sexual size dimorphism, and the skewing of sex ratios towards the larger sex. However, male and female zebrafish display minimal size differences (Pyron, 2003a) and the benefits of larger size in males for attracting females and defending spawning territories (Pyron, 2003b; Uusi-Heikkilä et al., 2012) conflict with the proposal that slower growth mediates male sexual differentiation and development (Lawrence et al., 2008). We did not observe any growth-mediated effects on sexual development in inbred or outbred zebrafish.

As stated earlier, *cyp19a1* encoding aromatase plays a pivotal role in sexual differentiation (Guiguen et al., 2010). A potential causal mechanism behind different levels of masculination induced by the different exposure treatments in the experimental studies in this thesis is the

variable inhibition of the steroidogenic enzyme aromatase, and this may be due in part to allelic variation at the aromatase locus. This was evidenced by contrasting mean levels of expression *cyp19a1a* in the gonads of inbred versus outbred zebrafish: down-regulation in inbred males, but not outbreds, under high-level clotrimazole exposure (43.7 µg l^{-1}) (Chapter 5); up-regulation in inbred females, but not outbreds, under moderate-level clotrimazole exposure (8 µg l^{-1}) and elevated temperature (33°C) (Chapter 7). Consistently lower variance in *cyp19a1a* expression in inbreds indicated allelic loss (loss of heterozygote advantage) as a possible causal mechanism explaining the greater susceptibility of inbreds to male sex skews (Chapters 5 and 7). Assessment of allelic variation using targeted sequencing and frequency analysis of single nucleotide polymorphisms (SNPs) at the aromatase locus and its promoter region would help to elucidate the inbreeding×environment interactions observed. However, there is scope for many other genotype×environment interactions to affect sex determination and differentiation in zebrafish and numerous other candidate genes may be involved (von Hofsten and Olsson, 2005; Bradley et al., 2011; Anderson et al., 2012; Pifferer et al., 2012).

There is also some uncertainty concerning some basic ecological life-history parameters for wild zebrafish: duration of the spawning season (Spence et al., 2008); rates of juvenile (age 0+) survival (Hazlerigg et al., 2013); population sex ratios; effective (breeding) population sizes (N_e). Based on the sensitivity analysis conducted using PVA modelling, juvenile survival and effective population size were shown to be critical factors determining extinction risk, and of course sex ratio is also linked to effective population size. Data on juvenile survival are notoriously difficult to obtain in the wild due to practical constraints in sampling small fish and obtaining accurate census sizes. Nevertheless, valuable data could be provided by sampling small pond enclosures or microcosms within natural ponds during the spawning season. Ideally data on adult breeding population sizes and sex ratios should be gathered over a wider geographical area in native Bangladesh and India, including central floodplain areas and remote, higher-lying areas. At the same time a representative of number of males and females should be obtained from each sampling point and analysed using micro-satellites or similar tools to assess gene diversity and gene-flow between sub-populations, in order to quantify immigration/emigration and ultimately meta-population sizes, which determine population viability. The importance of quantifying/modelling spatial as well as temporal

dynamics in wildlife populations has been highlighted in pesticide risk assessment (Van den Brink et al., 2007), but the principle applies to ERA in general.

9.9 Impact of research

The work from this thesis has been published in a number of high impact journals (Chapters 2-5), and one of the earlier papers (2009) has been cited over 20 times according to NCBI and Google Scholar. A total of 10 external presentations have been given at various international workshops and conferences, including two invited platform presentations hosted by the European Federation for Pharmaceutical Sciences (EUFEPS) and the Royal Society, London. Another 8 presentations have also been given at AstraZeneca's biannual SHE Research Forum and the internal seminar series at the University of Exeter.

External presentations

Presentation title and presenters	**Venue and date**
Assessing the combined effects of inbreeding and EDC exposure in the zebrafish *Poster by:* ***Brown AR, Bickley LK****, Le Page G, Hosken D, Paull GC , Owen SF, Wilga J, Hetheridge MJ, Tyler CR*	Zoological Society of London Symposium: The impacts of environmental change on reproduction and development in wildlife.' 15 - 16 Oct **2009, ZSL, London**
Combinational effects of inbreeding and EDC exposure in the zebrafish *Poster by:* ***Brown AR,*** *Bickley LK, Le Page G, Hosken D, Paull GC , Owen SF, Wilga J, Hetheridge MJ, Tyler CR*	SETAC Europe 19th Annual Meeting, 31 May-4 June **2009, Göteborg, Sweden**
Investigating the combined effects of inbreeding and chemical exposure on reproductive success in zebrafish *Poster by:* ***Bickley LK****, Brown AR, Le Page G, Hosken DJ, Hamilton PB, Paull GC, Owen SF, Tyler CR*	SETAC Europe 20th Annual meeting. 23 - 27 May **2010, Seville, Spain**
Combined effects of inbreeding & endocrine disrupting chemical exposure on sexual development and	SETAC Europe 20th Annual meeting. 23 - 27 May **2010, Seville, Spain**

reproductive fitness in the zebrafish *Platform by: **Brown AR**, Owen SF Wilga J, Bickley LK, Le Page G, Paull GC, Hosken DJ, Tyler CR*	
Environmental effects causally related to pharmaceutical exposure *Invited platform by: **Brown AR**, Owen SF, Murray-Smith RJ, Snape JR*	EUFEPS PharmaSciFair Conference: Pharmaceuticals in the Environment 17 June **2011, Prague, Czech Republic**
Interactive effects of inbreeding and exposure to an endocrine disrupting chemical on life history traits in fish *Poster by: **Bickley LK**, Brown AR, Le Page G, Hosken DJ, Hamilton PB, Paull GC, Owen SF, Tyler CR*	9th International Symposium on Reproductive Physiology of Fish, 9-14 August, **2011, Cochin, India**
Is there a need to better standardise test organisms in ecotoxicology? *Platform by **Brown AR**, Ryan TA, Sharpe AD, Owen SF, Bickley LK, Hamilton PB, Paull GC, Tyler CR*	SETAC World Congress and Europe 22th Annual meeting. 20 - 24 May **2012, Berlin, Germany**
Environmental risk assessment of pharmaceuticals: extrapolation from lab to field, from inbred to wild-type organisms *Invited platform by: **Brown AR**, Owen SF, Le Page G, Ryan TA, Peters J, Bickley LK, Paull GC, Hamilton PB, Tyler CR*	Royal Society Workshop: Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems 14-16 April **2013, Milton Keynes, UK**
Skewed sexual differentiation induced by combined effects of temperature and an endocrine disrupter is greater for inbred compared with outbred fish *Poster by: **Brown AR**, Peters J, Paull GC, Hamilton PB, Owen SF, Hosken DJ, Tyler CR*	SETAC Europe 23rd Annual meeting. 12 - 16 May **2013, Glasgow, UK**

9.10 References (Chapter 9)

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10 FINAL NOTE

Despite the trials and tribulations I have enjoyed completing this thesis, not least getting the pagination right! Its a good job I had this extra page.