Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their genomes, physiology and ecology

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ABSTRACT (≤200 words)

Fish represent the planet's most diverse group of vertebrates and they can be exposed to a wide range of pharmaceuticals. For practical reasons, extrapolation of the effects of chemicals, including pharmaceuticals, from 'model' species to other fish species is adopted in risk assessment. Here we critically assess this approach for pharmaceuticals. First we show that between 65-86% of human drug targets (459) are evolutionarily conserved in twelve diverse fish species. Focusing on nuclear steroid hormone receptors, we further show that the sequence of the ligand binding domain, that plays a key role in drug potency, varies between species but this does not explain entirely the observed differences in receptor-transactivation (2-10 times for the oestrogen receptor). Taking the synthetic oestrogen ethinyloestradiol (EE2) as a test case, and using life-table-response-experiments, we demonstrate significant reductions in population growth in fathead minnow and medaka, but not zebrafish, for environmentally-relevant exposures. This finding contrasts with zebrafish being ranked as more ecologically susceptible according to two independent life-history analyses. We conclude that whilst most drug targets are conserved in fish, evolutionary divergence in drug target activation, physiology, behaviour and ecological life-history make it extremely difficult to predict population-level effects, justifying the conventional use of a ×10 assessment factor in pharmaceutical risk assessment to account for differences in species susceptibility.

Key words:

Drug target, ortholog, physiology, population ecology, species, susceptibility

INTRODUCTION

Environmental risks associated with pharmaceuticals

Over 5000 human and veterinary pharmaceuticals are in use or in development and they target diverse physiological functions [1]. Many are highly potent, altering physiological processes at low therapeutic concentrations, between 0.05-100 μg/ml blood plasma in human/mammalian systems [2]. Furthermore, many drug targets are highly conserved across diverse vertebrate phyla [3-6]. Following the widespread detection of pharmaceuticals in the environment [7-9], concern has been raised over their potential impact on vertebrate wildlife health. The most notable example of an adverse effect in wildlife is for exposure to the non-steroidal anti-inflammatory drug diclofenac. This has been shown to cause population-level declines, and even localized extinctions, in three Asian vulture species (*Gyp* sp.*)* scavenging on the carcasses of treated cattle [10, Cuthbert et al., this issue 11]. In another case, the contraceptive oestrogen 17α-ethinyloestradiol (EE2) has been linked directly with population-level risks in wild fish, due to feminisation in males and reduced fertility in both sexes of several fish species [12-14, Kidd et al., this issue 15]. Generally, however, wildlife populations are exposed to relatively low-level environmental concentrations of pharmaceuticals and data confirming adverse effects are extremely limited. It is widely recognized that better insight and understanding of environmental risks are required concerning both newly developed drugs and older pharmaceuticals, some of which have been present in the environment for decades [16]. Due to the large number of compounds in use, several schemes have been proposed for prioritizing testing [4, 17, 18], which includes 'reading-across' plasma concentrations and therapeutic effects of human and veterinary pharmaceuticals to non-target organisms [19]. However, quantifying both inter-individual and inter-species variability in drug uptake and metabolism, and extrapolating between individual physiological responses and adverse population-level effects, represent major sources of uncertainty in pharmaceutical environmental risk assessment (ERA) [16]. Fish make up half of all vertebrate species, inhabiting virtually all aquatic environments [20] and exhibiting enormous diversity in morphology, physiology, behaviour, reproductive biology and ecology [21]. ERAs concerning the susceptibility of fish to pharmaceuticals, however, are based on studies on only a few model fish species and rarely extend to the quantification of population-level effects.

Assessing the potential susceptibility of fish to adverse effects from pharmaceuticals

The susceptibility of wildlife, including fish, to adverse population-level effects from chemicals and/or pharmaceuticals, depends on their exposure, physiological responsiveness and population resilience [22].

Exposure of fish to pharmaceuticals is generally assumed to be via water, since the majority of pharmaceuticals partition to the water phase in waste water treatment and remain in solution following discharge to surface waters. In cases where pharmaceuticals partition to solids and or lipids, this may trigger specific studies, simulating benthic sediment exposure and/or potential bioaccumulation in the food chain [23]. There are few data on pharmaco -dynamics and -kinetics (drug absorption, distribution, metabolism and excretion) in fish [24], but there is a wealth of human/mammalian data, which offer the potential for "read-across" to fish [16]. More studies are needed to explore the extrapolation of external aqueous exposure

concentrations to internal drug concentrations in blood plasma [19, 25], to confirm whether expected 'therapeutic' effects occur in non-target organisms [Hutchinson et al., this issue 26]. Drug uptake across fish gills, may be controlled by a variety of membrane transporters, including multi-drug transporters [27] and/or more specific transporters, such as sex hormone binding globulin SHBG, which shows affinity across a wide dynamic range for both natural and synthetic steroids [28]. Predicting the metabolism and excretion of drugs in fish is even more challenging. Whilst several fish species possess enzyme systems responsible for metabolism and excretion of most drugs in human/mammalian systems, limited comparative biotransformation data are available and they indicate that read-across is not straightforward. For example, in mammals hepatic cytochrome P-450 enzymes (CYPs) are known to play a major role in xenobiotic metabolism and detoxification with CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 mediating the metabolism of approximately 70% of pharmaceuticals [29]. However, no significant biotransformation could be measured for the known substrates of human CYP2D6, CYP2C9, or CYP3A4 for seven drugs in a study on rainbow trout (*Oncorhynchus mykiss*) [30]. Furthermore, the pregnane X receptor (PXR nuclear receptor subfamily 1, group I, member 2; NR1I2), which regulates many of the CYP enzymes, along with phase II metabolic enzymes and drug transporter proteins in human/mammalian systems, appears to play a role in metabolism in some fish species, but not others. PXR is active in Common carp (*Cyprinus carpio*) [27], fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) [31], but appears to be absent in cod, (*Gadus morhua*), sea lamprey (*Petromyzon marinus*) and three-spined stickleback (*Gasterosteus aculeatus)*; based on the latest gene builds for these species in Ensembl, version 74 [32]). At this time therefore, it is very difficult to predict drug metabolism and bioconcentration in fish based on the systems established for mammals, or to extrapolate between different fish species.

Physiological responsiveness of fish to pharmaceutical exposure will depend, in part, on the level of conservation of high-affinity interactions with designated drug targets (proteins) that cause the intended pharmacological action in human patients and veterinary animals. Thus, wildlife species that express proteins that are orthologous, i.e. proteins that share a common evolutionary origin to human and veterinary drug targets, may be more responsive than species lacking orthologs. Fish underwent evolutionary divergence from other vertebrates 450 million years ago [21], but they nevertheless exhibit high evolutionary conservation of human and veterinary drug targets compared with other taxonomic groups used in aquatic ERA. For example, in zebrafish and three-spined stickleback, orthologs are predicted for 86% of human drug targets [3]. These (and other) fish species used in ecotoxicology belong to the last* of the following three super classes: i) Agnatha, jawless fish including lampreys and hagfish ii) Chondrichthyes, cartilaginous fish and; iii) Osteichthyes*, bony fish. The final class is the most diverse and comprises the Actinopterygii, ray-finned fishes and Sarcopterygii, a parphyletic class containing lobe-finned fishes, and all tetrapod vertebrates, including humans [21]. As Sarcopterygii are more directly related to tetrapods they may be expected to show greater conservation of human and veterinary drug targets compared to fish from other classes, particularly the more ancient super classes Chondrichthyes and Agnatha. The influence of phylogeny on drug target conservation in fish has not yet been examined and existing ortholog predictions of drug targets have focused on only a few model ray-finned fish species [3], and/or employed simple best-match approaches [4, 5]. Phylogenetically-based predictions can better account for evolutionary events, including speciation and gene duplications [33], the latter being most prevalent in the ray-finned fish linage [34]. The degree of protein sequence

similarity between the human drug target and the fish ortholog (especially within the ligand binding domain (LBD)) may enable better predictions regarding the species responsiveness to pharmaceutical exposure [6]. However, currently there are few experimental data comparing drug target responsiveness in fish, other than for the oestrogen receptor (ESR1) and these indicate that LBD sequence similarity is not always predictive of receptor activation and therefore physiological response [35].

Population resilience (versus susceptibility) to environmental stressors is governed by life-history traits relating to reproductive strategy, longevity, dispersal, niche specificity, demographics and dynamic stock-recruitment [22, 36-37]. A key challenge in ERA is determining whether or not physiological effects in individuals translate to adverse impacts on wild populations [16, 38]. This extrapolation between effect-levels and between species has been attempted using population dynamics models, which project forward the life-histories of wild populations, with and without superimposing chemical effects measured in surrogate, laboratory-exposed populations. Some models have indicated that short-lived, asynchronous spawning fish, such as the fathead minnow, may be more susceptible to population decline compared to longer-lived, seasonal spawning brook trout (*Salvelinus fontinalis*), following multi-generation exposures to endocrine active chemicals [39]. These findings are consistent with studies on an experimental lake dosed for five years with the synthetic oestrogen EE2, which resulted in faster population decline in fathead minnows compared to longer-lived, demersal white sucker (*Catostomus commersonii*) (Kidd et al., this issue [15]). There is also some indication of increasing, heritable susceptibility to EE2 over generations, according to mesocosm studies on fathead minnows [40], and this is supported by laboratory life-cycle studies on fathead minnows and zebrafish [41-42]. Other small fish species, such as the Chinese rare minnow (*Gobiocypris rarus*) may be even more susceptible to EE2 [43]. It is also possible that longer-term exposures to chemicals, including pharmaceuticals, may eventually become more problematic for longer-lived species with relatively low life-time fecundity and inflexible life-history strategies [36-37] and/or fish species occurring higher in the food chain (Kidd et al., this issue [15]).

With a view to aiding the environmental risk assessment of pharmaceuticals, we investigate a range of risk "predictors" spanning drug target conservation in model species to ecological life-histories of these and other species. We assess the ability to extrapolate between species and biological effect-levels. This work encompasses assessments of exposure potential, physiological responsiveness and population resilience.

METHODOLOGICAL APPROACH

We first examined variation in potential susceptibility in fish by describing the presence or absence of orthologs to 459 human/mammalian drug targets in twelve diverse species with fully-sequenced genomes and complete gene builds [32]. Then, prioritising a subset of 45 active pharmaceutical ingredients (APIs) with potential to have direct effects on reproduction, we assessed the inter-species variation in target sequence similarity compared to the human target. The majority of the prioritised APIs mediate their pharmacological action via one or several steroid receptors and the sequence similarity of the ligand binding domain (LBD) of these receptors were assessed. Using experimental data we then compared differences in ligand- and species-specificity of the oestrogen receptor (ESR1). In the next stage of our

analysis we evaluated fish life-history traits influencing environmental exposure to pharmaceuticals and population resilience e.g. dispersal, reproductive strategy, generation time. Finally, a case study analysis was conducted for the highly potent steroidal oestrogen EE2, in order to investigate linkages between individual and population effect-levels, enabling an overall comparative assessment of risk for three model species commonly used in ERA. All fish species included in the study are listed in the Supplementary material: Table S1.

Assessment of physiological responsiveness

Orthologs in fish for human/mammalian drug targets

Genomes were studied for twelve fully-sequenced fish species with complete gene builds held in Ensembl Compara [44] (version 74, accessed January 2014): cod (*Gadus morhua*), coelacanth (*Latimeria chalumnae*), fugu (*Taikfugu rubripes*), medaka (*Oryzias latipes*), Mexican cavefish (*Astyanax mexicanus*), Nile tilapia (*Oreochromis niloticus*), sea lamprey (*Petromyzon marinus*), southern platyfish (*Xiphophorus maculates*), spotted gar (*Lepisosteus oculatus*), three-spined stickleback (*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridis*) and zebrafish (*Danio rerio*).

Information regarding human drugs and their targets were downloaded from DrugBank v3.0 [1]. Only drugs annotated in DrugBank as “small molecule”, “approved” and with “humans and other mammals, as affected organisms” were considered. Drug targets with “unknown pharmacological action” were excluded from our analyses. In total, information on 978 active pharmaceutical ingredients (API) associated with 459 unique drug targets was downloaded. DrugBank previously listed over 1000 drug targets, including metabolizing enzymes and transporters, but now shows that only 459 have specific pharmacological action. These drug targets were mapped to the Ensembl database (version 74, accessed January 2014) [32] using protein sequences from Uniprot [45]. Drug target orthologs in the twelve fully sequenced fish species and in the tree frog (*Xenopus tropicalis),* included as a tetrapod outgroup, were then calculated (Supplementary material: Table S2) based on the phylogenetic gene tree predictions in Ensembl Compara (version 74, accessed January 2014) [44]. A drug target was considered to be conserved in a species it if had at least one human ortholog. The associated taxonomic information was retrieved from the NCBI Taxonomy database (accessed February 2014) [46].

Assessment of sequence similarities

Sequence similarities were calculated for drug targets (21) associated with APIs (45) with Anatomical Therapeutic Chemical (ATC) classification codes suggesting direct effects on reproduction (prostaglandins (A02BB), oxytocics/uterus-contracting agents (G02A), contraceptives for topical use (G02B), sex hormones and modulators of the genital system (G03), and endocrine agents used in the treatment of neoplastic diseases (L02)). The sequence similarity was estimated from the multiple alignments available in Ensembl (version 74, accessed January 2014) [32]. To reduce the effects of erroneously aligned gene regions, produced by the large evolutionary distance between the species, only aligned amino acids were considered in the estimates. All sequence similarities are presented in Supplementary material: Table S3. Ligand binding domains (LBDs) were annotated using the position-specific

scoring matrices from the Conserved Domain Database [47]. The sequence similarities of LBDs were calculated analogously as for the complete drug targets.

Assessment of amino acid sequence alignments with the LDB for human ESR1

Multiple alignment of amino acid sequences of the LBD of ESR1 were assessed with the following sequences: human ENSP00000405330; tree frog ENSXETG00000012364; cavefish ENSAMXG00000006267; cod ENSGMOG00000014898; common carp BAF99812; fathead minnow AAV41373; fugu ENSTRUG00000018219; medaka ENSORLG00000014514; platyfish ENSXMAG00000003084; rainbow trout P16058; roach BAD91035; stickleback ENSGACG00000008711; tetraodon ENSTNIG00000012264; tilapia ENSONIG00000013354; sea lamprey ENSPMAG00000005727; zebrafish ENSDARG00000004111. The amino acid residues in the human ESR1 that have been shown to have direct contact with the co-crystallized ligands 17β-oestradiol (E2) and diethylstilbestrol (DES) according to pocketome.org [48] (accessed in April 2014) were highlighted. The alignment is presented in Supplementary material: Table S4. The ESR1 in the coelacanth was found to be erroneous and was therefore excluded from the alignment analysis.

Assessment of interactions of pharmaceutical oestrogens with ESR1

Interactions with the oestrogen receptor ESR1 of oestrogenic pharmaceuticals 17β-oestradiol, (E2), 17α-ethinyloestradiol (EE2), the equine oestrogen equilin (used in hormone replacement therapy) and diethylstilbestrol (DES) were compared across six different fish species (common carp, fathead minnow, medaka, roach (*Rutilus rutilus*), three-spined stickleback and zebrafish). Full-coding regions for ESR1 in each species were cloned and transfected into separate HEK293 cell lines and ESR1 receptor transactivation assays were conducted as described in [35].

Assessment of exposure and population resilience

Life-history trait analysis

Using the databases FishBase (version 12/2013) [49] and FishTraits (version 2) [50], and the available scientific literature, life-history data were obtained for a broad selection of fish species in which physiological responsiveness to pharmaceuticals had been assessed. Rainbow trout and the Chinese rare minnow were also included as an alternative model species used in ecotoxicology and ERA. Searches focused on the compilation of qualitative and quantitative trait data (Supplementary material: Table S5) required to calculate two alternative indices incorporating spawning frequency, parental care, lifespan, recruitment, niche specificity etc: Population Survivorship Index [36]; Ecological Vulnerability Index [37].

Life-table response experiments integrating life-cycle assessments of individual and population-level effects of EE2

Published life-cycle data (F_1 embryo-adult to F_2 embryo) quantifying the effects of the oestrogen receptor (ESR1, ESR2) agonist EE2 in three model freshwater fish species, fathead minnow, medaka and zebrafish under standard flow-through conditions and test temperatures

25-28°C, were compiled and scrutinised according to established quality/reliability criteria [51]. Those studies incorporating measurement of a range of effect-levels (molecular, physiological, behavioural, population-relevant effects) and confirmation of exposure concentrations by chemical analysis were used to determine the lowest observed effect concentration (LOEC) for population-relevant endpoints in each species: zebrafish LOEC = 0.5 - 1 ng/L EE2 [52-53]; fathead minnow and medaka LOEC = 1 ng/L EE2 [41, 54-55]). Endpoint values (vital rates: proportion of viable fertilized eggs; proportion of females; female fecundity (eggs per female per day); survival) were tabulated as mean values ± standard error of the mean (Supplementary material: Table S6). A lower LOEC of 0.2 ng/L EE2 has been reported for the Chinese rare minnow [43], however insufficient life-table data were available to permit LTREs for this species.

Species-specific vital rates for control (non-exposed) and EE2 (1 ng/L) exposed fish were then input with specific life-table data for wild populations of fathead minnow, medaka, zebrafish (Supplementary material: Table S7) to stage-based Leslie matrix population models (Supplementary material: Figure S1), constructed for each species using PopTools (version 3.2.5) [56]. Population projections were based on females, since female fecundity limits population numbers. Populations were assumed to be closed with no immigration or emigration, and spawning was assumed to be asynchronous and protracted in all three species [57-59]. Finite, geometric population growth rate (λ) was then projected from n=100 replicate life-table response experiments (LTREs) [60], for each species, by re-sampling vital rates from their means and standard deviations, assuming normal distributions for each vital rate [58]. The statistical significance of the effect of EE2 (1 ng/L) exposure on projected population growth rate (λE) versus control population growth rate (λC) was assessed on ranked projections using the non-parametric Kruskal-Wallis test, due to inequality of variances between exposed and control populations (according to Levene's Test). These stochastic projections of treatment effects at the population-level enabled an integrated assessment of ecotoxicological and ecological population susceptibility. Determining the mean sensitivities (Control + Treatment/2) of each vital rate in each LTRE also enabled decomposition analysis to quantify the relative contribution and importance of these vital rates (performed according to [60]).

RESULTS AND DISCUSSION

Assessment of physiological responsiveness

Conservation of human drug targets

More than 80% of the 459 human drug targets had orthologs in all the investigated bony fish, while the sea lamprey (a jawless fish), had orthologs to 65% of the targets. These results suggest that responses in sea lamprey may differ frequently and substantially to those in bony fish (Figure 1). Information regarding the APIs, the drug targets and their orthologs and co-orthologs in each species is presented in Supplementary material: Table S2.

Athough the vast majority of the APIs had at least one orthologous drug target in fish, this does not necessarily mean all these APIs will have a functional drug interaction and invoke a physiological response in fish, or that this response will resemble that occurring in humans.

Even between mammalian species (human-mouse) there are numerous examples of orthologs that have diverged functionally [61]. The general sequence similarity between orthologous proteins may potentially give more information, but the data should be interpreted with caution, since orthologous proteins with similar function can have significant domains that are missing or differ substantially. The ligand binding site and especially the specific amino acids that interact with the ligands are, however, generally highly conserved and could provide additional information regarding potential drug interactions [6].

Variation in sequence similarity

Alignments of the full protein sequence and LBD of the nuclear steroid hormone receptors, specifically, estrogen receptor α (ESR1), estrogen receptor β (ESR2), progesterone receptor (PGR), androgen receptor (AR), glucocorticoid receptor (NR3C1) and mineralocorticoid receptor (NR3C2), showed that the LBDs had higher sequence similarity to the corresponding human drug target than the full proteins (Figure 2). Furthermore, in line with the established phylogenetics of fish evolution, the lobe-finned fish had the highest sequence similarities (75-81%) with the LBDs of the human steroid receptors, whereas the more ancient sea lamprey had the lowest LBD sequence similarities (52-63%) (Figure 2). This finding however, does not necessarily mean that sea lamprey is less susceptible to drugs targeting these receptors, since it is also important to consider gene duplication and function. As an illustration of this, the human glucocorticoid- and mineralocorticoid- receptors are co-orthologous to a single corticoid receptor "CR", and the human androgen and progesterone receptor are co-orthologous to a receptor annotated as "PGR", in sea lamprey (Supplementary material: Table S2). The CR in sea lamprey appears to be promiscuous for binding corticosteroids [62], but there are only limited experimental data assessing the responsiveness of this receptor to drugs designed to affect the human glucocorticoid- and mineralocorticoid- receptors. There are also very few data concerning the promiscuity, responsiveness and function of the PGR, ESR1 and ESR2 in sea lamprey. Nevertheless, plasma progesterone and E2 concentrations have been shown to vary between the sexes and reproductive stages, suggesting links with sexual function in this species [63]. Here we show that sea lamprey has orthologs to both the human ESR1 and ESR2 and their LBDs are conserved to a similar degree in sea lamprey as in the ray-finned fishes (63-71%).

Variation in target-ligand binding and activation of ESR1 compared with amino acid sequence alignment

Assessment of pharmaceutical oestrogen interactions with fish ESR1 in trans-activation assays showed that DES was the most potent ligand and 10 times more potent than E2. EE2 was approximately twice the potency of E2, and equilin was around 10 times less potent (in all species) compared to E2. EE2 was approximately twice the potency of E2, and equilin was around 10 times less potent (in all species) compared with E2. The effective concentration corresponding with ESR1 receptor trans-activation in 50% of replicate transfected cell lines (EC50) showed little variation for E2 between the different fish species, ranging 2.4-fold between 0.18 and 0.43 nM (zebrafish and stickleback, respectively, Figure 3). Similarly for EE2, the EC50 ranged 4.3-fold between 0.07 and 0.3 nM ((zebrafish and stickleback, respectively, Figure 3). For equilin, the EC50 varied within the range 3.02 to 10.67 nM (roach and carp, respectively) and for DES, the EC50 spanned 0.024 to 0.077 nM (medaka and zebrafish, respectively). There were also differences in the fold activation for ESR1 between the different

fish species in these transactivation assays for all the oestrogens analysed. For E2 this difference across the species was only around 2–fold, and for EE2 around 2.5-fold, but for DES and equilin these differences were up to 4- and 6- fold, respectively. Zebrafish and fathead minnow showed a tendency for lower levels of fold-activation by E2 and EE2 compared with roach and medaka. Conversely, medaka showed lowest levels of activation by DES and equilin, followed by zebrafish and fathead minnow, while roach again showed highest fold-activation.

Although there were differences between the fish species studied for some of the interactions of pharmaceutical oestrogens with ESR1, far greater inter-species variation has been shown for other less potent environmental oestrogens. For example, ESR1 trans-activation EC50s for 4-nonylphenol and 4-octylphenol are 30 times and 23 times lower, respectively in common carp compared with medaka [33]. The much smaller (<2-fold) difference in the EC50 for E2 (and EE2) between the fish species studied, likely reflects the crucial roles of this natural oestrogen (and synthetic mimic) in a wide range of tissues functions and processes in fish [64-65]. Indeed, fourteen out of the fifteen amino acid residues of the human ESR1 known to have direct contact with E2 or DES were identical in all bony fish (Supplementary material: Table S4). The greater inter-species variation in EC50s for DES could thus not easily be explained by the variations in sequence similarity in the LBD (Supplementary material: Table S4). Instead, the influence of co-factors, differences in promoter sequences and amino acid residues in other domains, such as the DNA binding domain [35, 66-67], may be responsible for the observed differences in ESR1 activation.

Sequence alignments and molecular docking experiments, for example using pocketome, that can provide predictions of possible receptor-ligand interactions, could potentially better guide understanding on the likelihood for drug interactions with receptors. This *in silico* technique is being used in drug discovery to identify suitable ligands for ESR1 in breast cancer treatment [68]. In ERA, molecular docking of the drug target cyclooxygenase 2 (COX2) indicates that the orthologous COX2 proteins in rainbow trout, salmon (*Salmo salar*) and zebrafish are all likely to bind the drugs diclofenac and ibuprofen, while these drugs are not likely to bind the COX2 homolog in the water flea (*Daphnia pulex*) [69]. The limited number of protein structures (preferably co-crystalized with relevant ligands/drug) is however, still a limiting factor for such analysis for the majority of drug targets.

Variation in exposure susceptibility and population resilience

Life-history trait analysis

According to two alternative susceptibility indices, based on a range of life-history traits, species with longer generation times and lower spawning frequency, fecundity and recruitment (coelacanth, sea lamprey and fugu) were ranked as most susceptible to environmental stressors (Figure 4). The higher ranking of these species is consistent with their higher conservation status, compared to other species analysed (Supplementary material: Table S1). The rapidly reproducing southern platyfish was ranked as the least susceptible to environmental stressors (Figure 4). The 'Population survivorship index', specifically tailored for fish and amphibians [36], provided greater differentiation between the most and least susceptible species, indicating a 3-fold difference, compared to a 1.6-fold difference indicated by the more general 'Ecological vulnerability index' [37]. Both of these ranges are within the x10 assessment factor traditionally

used in ERA to account for variation in species susceptibility [23, 70]. Nevertheless, it is interesting to note that zebrafish may be more susceptible to population decline compared with the rare minnow, fathead minnow and medaka, which is due principally to broadcast spawning behaviour and lack of parental care in zebrafish. Since the life-history strategies of many fish are highly plastic, however, enabling adaptation to their specific environments, our results should be used only as a general guide [36, 71]. Furthermore, the general life-history data analysis we adopted does not account species-specific effects of pharmaceuticals. The inclusion of specific effects data in susceptibility indices requires some degree of weighting and/or expert judgement [37].

Life-table response experiments

Available life-table data and full life-cycle studies for EE2 enabled integrated assessments of physiological susceptibility and population resilience for three model freshwater species commonly used in pharmaceutical ERA, specifically, fathead minnow, medaka and zebrafish.

Beginning with an analysis of life-cycle effects data, the lowest observed adverse effect concentrations (LOECs) for EE2 were: zebrafish LOEC = 0.5 ng/L [52]; fathead minnow and medaka LOEC = 1 ng/L [41, 53-55]). Whilst there were no effects on survival at these concentrations, there were significant effects on other population-relevant endpoints (vital rates), and their magnitudes of effect differed between species. There was a female-biased sex ratio in both zebrafish (57%) [53] and fathead minnow (65%) [41], but there was no female-bias in medaka (50%) [54-55]. Plasticity in sexual differentiation is typical in some species, such as zebrafish, that are sometimes referred to as juvenile hermaphrodites, whereas in other species, gonochorists, such as fathead minnow and medaka, sex is determined around fertilisation and feminization of males typically results in ovo-testes, as occurs for exposure to 1 ng EE2/L [41, 54]. Female-bias appeared to be 'compensated' for by reductions in fecundity and fertilization success in fathead minnows. Greater reduction in fertilization success occurred in zebrafish, which may be linked to their broadcast spawning strategy, reduced likelihood of fertilisation and oophagy, as opposed to substrate spawning and egg guarding displayed by fathead minnows. Fertilisation success was affected least in medaka, where females produced fewer eggs. EE2 exposure concentrations between 0.5 and 2 ng/L resulted in no significant alteration in male courtship behaviour in zebrafish [52, 72]. In contrast, male courtship was reduced in medaka [51-52]. Overall, there were reductions in fertilization/hatching success in all three species compared with controls: 25$\pm$5% in medaka [55]; 35$\pm$30% in fathead minnows [41]; 54.5$\pm$15% in zebrafish [42]. Female fecundity also showed declining (but non-significant) trends for all three species. Reciprocal pair-breeding of control (non-exposed) and EE2 exposed fish revealed that reproductive impairment occurred in both sexes, but was generally greater in males in both zebrafish [52] and medaka [55].

The integration of life-cyle effects data and ecological life-history data for fathead minnow, medaka and zebrafish, in separate life-table response experiments, showed that finite population growth (λE) following exposure to 1 ng EE2/L was more variable than in controls (λC), but was reduced in fathead minnow (λC=1.12, λE=0.90, -20%; Kruskal-Wallis H=64.82, DF=1, p<0.001) and medaka (λC=3.59, λE=2.97, -17%; Kruskal-Wallis H=43.55, DF=1, p<0.001), but not in zebrafish (λC=2.30, λE=2.17, -6%; Kruskal-Wallis H=2.88, DF=1, p=0.089) (Figure 5). Proportional reductions in population growth rate (fathead minnow > medaka > zebrafish)

contrasted with the susceptibility indices derived from ecological life-history data for these species. Absolute population growth rate for fathead minnows was also projected to fall below λ=1 under EE2 exposure, indicating population decline.

Decomposition analysis revealed that reduction in age 0+ fecundity (fecundity in the first year of life), although highly variable, was most influential on reducing population growth in all three model species under EE2 exposure. These results highlight potential drawbacks of traditional statistical evaluation of individual endpoints such as fecundity, which are often shown to be highly variable and "statistically insignificant" [41, 42, 55]. Whereas integrative modelling approaches can utilise stochastic variation in multiple endpoints and, by extrapolating population-level effects, can indicate "ecological significance". The second most influential parameter affecting population growth was age 0 viability (proportion of viable female eggs × proportion fertilised), in which the proportion of eggs fertilised is influenced by the effects of EE2 on reducing male fertility. Reduction in fertilisation success, that can act directly to reduce population growth, was greatest in zebrafish. This was nevertheless, compensated for by female-biased sex ratios in this species. Alternative modelling approaches including mechanistic, individual-based models [38, 59] may be better able to discern effects of EE2 on male reproductive fitness, including those relating to effects on behaviour.

CONCLUSIONS

In this review, we identify factors for consideration when extrapolating between fish species and endpoints in pharmaceutical ERA. This approach could be applied to help identify which biological effect levels are most likely to account for inter-specific variation.

We illustrate that although fish generally show high conservation of human drug targets across diverse taxonomic groups there can be significant inter-species variation in drug target ligand binding domains. Considerable (up to 6-fold) variation was also identified in physiological responsiveness of different fish species to drugs targeting reproductive hormone receptors. Drug bioavailability and biotransformation in fish is likely to be a further source for interspecies variation in responsiveness to drugs, however, data here are lacking currently, which is a major knowledge gap.

Variation in ecological life-history strategies, was shown to generate three-fold differences in the susceptibility of different species to population decline. Furthermore, we showed distinct differences in population-level effects of the synthetic reproductive hormone EE2 when comparing fathead minnow, medaka and zebrafish, that were due to differences in their reproductive strategies and the variable contribution of individual vital rates to population growth rate. Without question extrapolating from individual effects in model fish to population effects in wild fish is challenging. Variation in life-history strategies will affect dynamic stock-recruitment and potentially drive the need for spatially-explicit, rather than generic, ERA. Whilst small fish models offer enormous utility in ecotoxicology, they cannot be representative and protective of all fish, due to wide ranging evolutionary divergence of physiologies, behaviours and ecological life-histories, which collectively define species, populations and population-level risk. Nevertheless, based on our analyses on the data available, the traditional use of at least a $\times$10 assessment factor, to account for uncertainties in species extrapolation in pharmaceutical ERA.

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Figure legends

Figure 1: Species tree illustrating the taxonomic relationships (based on NCBI taxonomy) between all species included in this study. The total number of conserved human drug targets for each of the twelve fish species with fully sequenced genomes and complete gene builds is presented, as well as the number of active pharmaceutical ingredients (API) with at least one drug target ortholog.

Figure 2: Sequence similarities (%) between six human nuclear steroid hormone receptors and their corresponding orthologs in 13 species. Sequence similarities of the full sequences are presented to the left and similarities of the ligand binding domains are presented to the right. AR – Androgen Receptor; PGR – Progesterone Receptor; ESR1 – Estrogen Receptor 1 (α); ESR2 – Estrogen Receptor 2 (β); NR3C1 - Nuclear Receptor subfamily 3, group C, member 1 (glucocorticoid receptor); NR3C2 - Nuclear Receptor subfamily 3, group C, member 2 (mineralocorticoid receptor). % similarities are emphasized using a coloured heat-map. Empty cells indicate that evidence for an ortholog is lacking in current genome versions and gene builds.*Missing value due to errors in current gene build. Online version in colour.

Figure 3: Fish oestrogen receptor (ESR1) responses in trans-activation assays induced by oestrogenic pharmaceuticals
a) 17β-oestradiol (E2), b) 17α-ethinyloestradiol (EE2), c) diethylstilbestrol (DES), d) equilin
Receptor transactivation is measured in terms of fold activation and EC50. Fold activation represents the difference in promoter activity (pre versus post pharmaceutical exposure) of chimera ESR1 from six selected fish species cloned into human HEK293 cells [32]. Promoter activity was quantified using a luciferase fluorescence reporter assay. EC50 is the effective concentration corresponding with ESR1 receptor trans-activation in 50% of replicate transfected cell lines. Data are presented as mean ± SEM from three independent assays, each consisting of three technical replicates per concentration tested. Online version in colour.

Figure 4: Potential susceptibility of fish populations to environmental (chemical) stress
Population susceptibility index (1-Population survivorship index, Spromberg and Birge, 2005) was calculated using a scoring system based on spawning frequency, parental care, lifespan, recruitment, niche specificity. Ecological vulnerability index (De Lange et al., 2009) was based on broader life-history data. Data were obtained from www.fish.base.org/ and www.fishtraits.info/ (see Supplementary material: Table S5)

Figure 5:
5a) Mean (SEM) projected finite population growth in model fish species following life-time exposure (embryo to adult) to 1 ng/L ethinylestradiol (EE2) compared with populations with no-chemical exposure
5b) Contribution of each vital rate to species specific treatment-induced reduction in finite population growth rate …*far too wordy – needs revising……*
Mean of n=100 stochastic matrix projections and standard error of the mean (SEM) shown
** $p<0.001$ according to the Kruskal-Wallis test comparing ranked projections of finite population growth in EE2 exposed populations versus non-exposed (control) populations of model fish species. Online version in colour.

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