# Patterns in Stress and Mortality in Small Ornamental Aquarium Fish and Interventions for Improving Health and Well-being

Submitted by Chloe Hannah Stevens to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences in September 2018.

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.

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# Abstract

The ornamental fish trade is an industry of significant size and scope, trading over 1.5 billion fishes each year, and worth approximately over 370 million USD. Ornamental fishes are kept world-wide, and are one of the most popular pets in UK households. The industry is currently experiencing steady growth, and has done since the FAO began keeping records in the 1970s. Despite this, the welfare of fishes within the industry remains one of the least-studied areas in the field of animal welfare. Mortality rates of fishes within the industry are debated, with estimates ranging from less than 2% to over 70%; however, a lack of clear data means that the accuracy of these figures is difficult to determine. Where mortality is believed to be high, stressors in the supply chain are thought to be a significant contributing factor. In this thesis, I explored some possible interventions designed to reduce the stress experienced by ornamental fishes.

Stress in fishes can be measured in a variety of ways, but the most common way is probably measurement of cortisol release rates. However, cortisol has often previously been measured in fishes by taking a blood sample – a technique which cannot be applied to many ornamental species as they are too small to obtain enough blood. Instead, cortisol released by small fishes can be measured in the fish holding water. I carried out a study to validate the use of this method in my study species and found that cortisol can be detected in the holding water of all three species, although I did not find clear differences between stressed fishes and controls. This highlighted the importance of using a variety of measures of stress, including behavioural measures, which are one of the most cost-effective ways to assess stress, and can easily be implemented in the ornamental fish supply chain.

Based on the literature, personal observations of industry practices, and the results of my analyses, a number of interventions intended to help reduce stress in ornamental species were developed. These involved training handlers to catch fish more effectively, providing neon tetras with environmental choices to allow them to select conditions which might promote welfare, and conditioning guppies to associate handling events with a reward or a predictable signal. I found fish which were not handled but were exposed to trained

handlers showed fewer behavioural signs of stress than those exposed to untrained handlers, and that neon tetras showed preferences for particular tank backgrounds over others. However, I did not find any evidence that trained handlers caused less stress in handled fish, or that conditioning led to lower stress in handled fish.

The results of this project suggest that there are a number of sources of stress and poor welfare in the ornamental fish industry which may be contributing to high mortality rates. However, many of these sources can be addressed, either through application of current best-practice guidelines or by introduction of training programmes which encourage understanding and empathy for fishes. Further work aimed at developing interventions including enrichment strategies, conditioning regimes, and other areas of research, will likely help to further reduce stress and mortality, and improve fish welfare.

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# Declaration

The five data chapters in this thesis were written separately in the form of manuscripts for publication; as a result, some information is repeated across chapters. I made substantial contributions to all chapters and am responsible for the work presented in this thesis. My supervisors Darren Croft, Charles Tyler, Gregory Paull, Ioanna Katsiadaki and Peter Carey all contributed to all of my chapters through discussion of the hypotheses and results; individual contributions are indicated below.

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Chapter 1 of this thesis (Introduction) has also previously been published in a modified format:

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# Chapter 1: General Introduction

## Abstract

The ornamental fish trade is estimated to handle up to 1.5 billion fishes annually and is worth between 800 million and 30 billion USD per year. Transportation and handling of fishes imposes a range of stressors that can result in mortality at rates of up to 73%. However, these rates vary hugely and can be as low as 2%, because they are generally estimated rather than based on experimental work. Given the numbers of ornamental fishes traded, any of the estimated mortality rates potentially incur significant financial losses and serious welfare issues. Industry bodies, such as the Ornamental Aquatic Trade Association (OATA), have established standards and codes of best practice for handling fishes, but little scientific research has been conducted to understand the links between stress, health and welfare in ornamental species. In aquaculture, many of the same stressors occur as those in the ornamental trade, including poor water quality, handling, transportation, confinement, poor social and physical environment, and disease, and in this sector directed research and some resulting interventions have resulted in improved welfare standards. This introduction considers the concept of 'welfare' in fishes and evaluates reported rates of mortality in the ornamental trade. It assesses how the stress response can be quantified and used as a welfare indicator in fishes. It then analyses whether lessons from aquaculture can be usefully applied to the ornamental fish industry to improve welfare. Finally, this analysis is used to suggest how future research might be directed to help improve welfare in the ornamental trade.

## 1.1 Introduction

Ornamental fishes are popular pets globally, with over 4500 freshwater species and 1450 marine species traded worldwide (Table 1.1) (Miller-Morgan, 2009). The trade in ornamental fishes is an important source of income for many countries, worth billions of USD annually (Saxby *et al.*, 2010; Ploeg, 2013). Accurate information on the numbers of fishes transported each year or the economic value of the industry, however, is lacking. Reflecting this some commonly presented figures on trading in the industry are 10 years or more out of date, e.g. FAO (1999) cited in Helfman *et al.* (2009) and Bartley (2000) cited in Saxby *et al.* (2010); Sloman *et al.* (2011). Estimates of the total value of the industry range between 800 million and 30 billion USD annually, and between

350 million and 1.5 billion live fishes are thought to be traded (Ploeg, 2007b; Saxby *et al.*, 2010). In contrast, the estimated value of the global aquaculture industry in 2012 was 137.7 billion USD (FAO, 2014). Mortality rates in the ornamental trade are most often based on estimates rather than empirical studies, and they vary considerably, ranging between 2% and 73% (Olivier, 2001; Ploeg, 2007a). This potentially represents both a major welfare issue and a significant economic cost to the industry. However, despite attention from the hobbyist literature (Butcher, 1992; Pasnik *et al.*, 2010) very limited scientific research has been conducted into mortality and welfare in ornamental fishes.

Table 1.1. The most common families of fishes sold within the ornamental fish industry (Olivier, 2003; Wabnitz *et al*, 2003; UNEP-WCMC, 2007).

| Habitat | Family |
|---|---|
| Marine | Pomacentridae (damselfish, clownfish) |
| | Pomacanthidae (angelfish) |
| | Acanthuridae (surgeonfish) |
| | Labridae (wrasses) |
| | Gobiidae (gobies) |
| | Chaetondontidae (butterflyfish) |
| | Syngnathidae (seahorses, pipefish, sea dragons) |
| Freshwater | Cyprinidae (cyprinids) |
| | Poeciliidae (livebearers) |
| | Cichlidae (cichlids) |
| | Callichthyidae (armored catfish) |
| | Characidae (characins) |
| | Gasteropelecidae (hatchetfish) |
| | Loricariidae (catfish) |

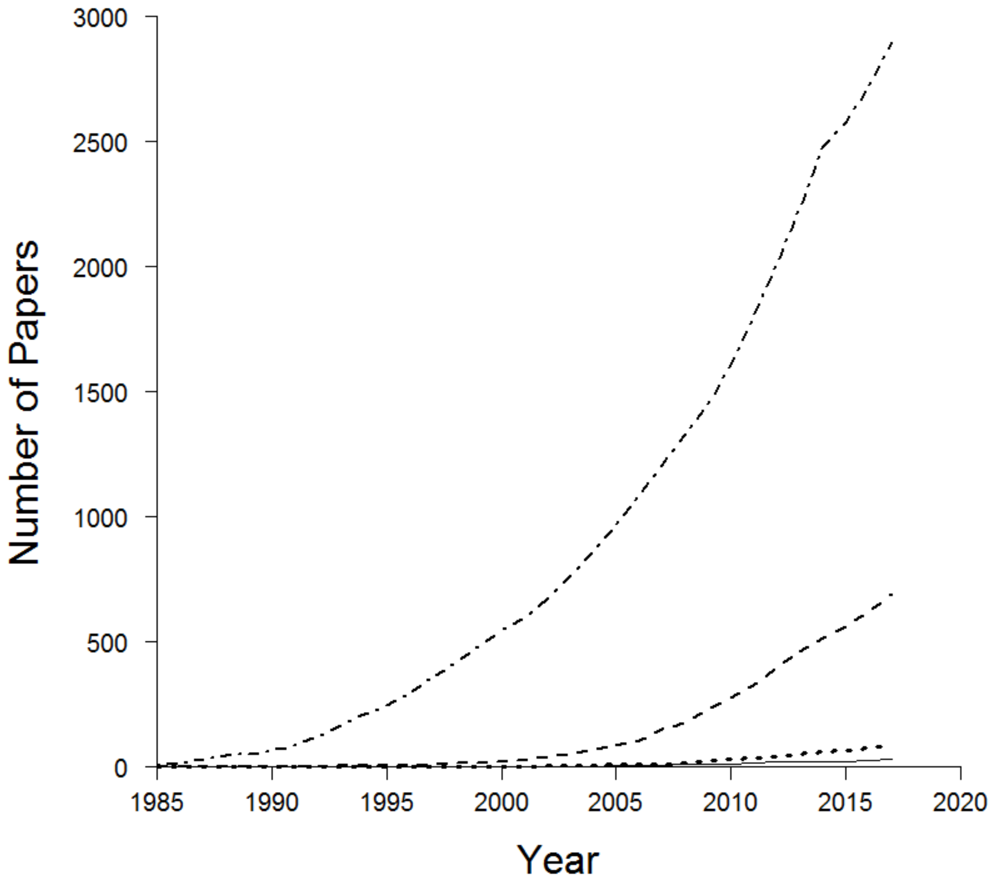

Figure 1.1. The cumulative number of papers related to stress and welfare in aquaculture and ornamental fish between 1985 and 2017. Number of papers were obtained from a literature search on the Web of Science database. Search terms were: aquaculture + fish + stress (· – · –), aquaculture + fish + welfare (– – –), ornamental + fish + stress (· · ·) and ornamental + fish + welfare (——).

Interest in fish welfare has increased considerably in recent years, shown by improved production standards, growing public interest, and a scientific debate over fishes' capacity for suffering. However, research into fish welfare has largely concentrated on species in aquaculture (Figure 1.1) (Huntingford *et al.*, 2006; Ashley, 2007; Braithwaite and Ebbesson, 2014). Ornamental fishes, like all captive animals, are exposed to various stressors which may contribute to poor welfare and mortality. Controversy remains over whether fishes feel pain or 'suffering' (Sneddon, 2009; Rose *et al.*, 2014), nevertheless, there is recognition of the detrimental and potentially lethal effects of stress and reducing mortality rates is a priority within the industry.

Efforts to reduce mortality rates and fish welfare concerns are reflected in directives designed to improve welfare in aquaculture (e.g. EU Directive 98/58/EC), and in scientific research (e.g. EU Directive 2010/63/EU). However, there is a lack of legislation addressing pet fishes. For example, in the UK fishes are covered by the Animal Welfare Act 2006, but this legislation provides no guidelines beyond prevention of suffering. For retailers, bodies such as Ornamental Fish International (OFI) and the Ornamental Aquatic Trade Association (OATA) exist and promote high welfare standards and set out codes of conduct, particularly for factors like water quality which is a key determinant of good fish health. They also provide training courses and advocate for improvements in fish handing and welfare (OATA, 2014). Membership of these organisations however, is not compulsory (OFI, 2014), and the global trade has no regulatory body. A greater understanding of mortality rates and welfare of ornamental fishes is urgently required to direct research efforts towards improving living conditions for fishes in the ornamental trade.

The introduction to this thesis first addresses the concept of welfare in fishes. It then reviews research into stress and welfare in fishes to understand whether lessons learnt from aquaculture can be usefully transferred to the ornamental fish industry, with a focus on the supply chain. From this analysis, key areas for future research are identified, which could help to better protect the health and welfare of fishes in the ornamental trade. More focussed research and improved integration between the ornamental and scientific communities should lead both to improved welfare standards and decreased mortality rates, and also economic gains in the ornamental fish trade.

## 1.2 Fish welfare

Animal welfare is difficult to both define and assess. Definitions include ‘quality of life’ (Duncan and Fraser, 1997), whether the animal has good health and can obtain ‘what it wants’ (Dawkins, 1998; 2006), or whether the animal has good health, can express natural behaviour and is not experiencing negative emotions (Farm Animal Welfare Council, 2009). The definition chosen for welfare therefore inevitably affects how welfare is assessed and measured. Welfare in fishes is further complicated by debates over whether fishes experience pain and suffering, or even experience consciousness.

Consciousness is currently poorly understood even in humans and the existence of consciousness in fish, as well as pain perception and suffering, is much debated within the literature (Sneddon, 2003b; Arlinghaus *et al.*, 2007; Sneddon, 2009; Rose *et al.*, 2014; Key, 2016; Sneddon *et al.*, 2018). It is argued that as fish lack a neocortex, known to play a role in human consciousness, they cannot be capable of consciousness (Rose *et al.*, 2014), although this kind of information may simply be processed though different neural pathways in fish (Braithwaite and Huntingford, 2004). It has also been suggested that the fish telencephalon is functionally homologous to the mammalian limbic system and thus involved in emotions, as they contain several counterpart structures (e.g. the mammalian amygdala and fish amygdaloid complex) (Portavella *et al.*, 2002; Chandroo *et al.*, 2004). Furthermore, nociceptors have been found in the head regions of rainbow trout *Oncorhynchus mykiss*, behind the operculum in goldfish *Carassius auratus*, and around the tail base in Atlantic salmon *Salmo salar*, indicating that fish can detect noxious stimuli, but not necessarily perceive pain (Sneddon *et al.*, 2003; Dunlop and Laming, 2005; Nordgreen *et al.*, 2007). For scientific research within the EU fishes are assumed to feel pain and accordingly are protected animals (EU Directive 2010/63/EU). Adopting the precautionary principle and assuming that fishes do feel pain is arguably the only ethically acceptable approach given this uncertainty. There is no question that fishes experience stress, which can be measured in a variety of ways, as illustrated below. Measures of stress therefore are a useful way of assessing welfare in fishes. It is known that stress has impacts on fish health and condition (Wendelaar-Bonga, 1997), which affects mortality, thus decreasing stress is likely to result in fewer economic losses due to lower mortality rates, better food conversion and growth rates, better reproductive rates and output, a more positive public perception of the industry, more attractive colouration, fins and body shape and improved disease control (Ashley, 2007).

### 1.3 The stress response

Stress is a coordinated suite of physiological and behavioural responses to any perceived challenge to homeostasis (or, more recently, allostasis) (Korte *et al.*, 2007; Braithwaite and Ebbesson, 2014). The stress response has three successive stages. The first stage involves two hormone secretory axes, the hypothalamic-sympathetic-chromaffin cell (HSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis. These axes produce various hormones, most importantly catecholamines – adrenaline and noradrenaline – from the HSC axis and cortisol from the HPI axis (Iwama, 1998; Flik *et al.*, 2006). Cortisol helps maintain homeostasis but also regulates the stress response, rising quickly after the detection of a stressor (Barton and Iwama, 1991; Wendelaar-Bonga, 1997; Mommsen *et al.*, 1999).

The secondary stage of the stress response involves the effects of these hormones on tissues. Catecholamines and cortisol can alter hydromineral balance, oxygen uptake and blood chemistry and mobilise energy sources, including plasma free fatty acids and liver glycogen, increasing plasma glucose (Johnson *et al.*, 1992). Some changes are closely linked with immune system function, including an anti-inflammatory effect of glucocorticoids on immune cells (Galhardo and Oliveira, 2009).

The tertiary stage of the stress response involves the integrative effects on the whole organism (Pasnik *et al.*, 2010). Costs associated with prolonged stress may cause the fish to reallocate energy resources, directing them into essential processes such as metabolism. Effects may include inhibited growth and reproduction, loss of body condition and compromised immune function, leaving the fish more vulnerable to pathogens. Tertiary responses also include behavioural and psychological changes based on the appraisal or perception of the stressor and the coping abilities of the individual (Barton, 2002; Galhardo and Oliveira, 2009). Fishes often cease feeding or movement, and may display the ‘fight-or-flight’ response – either by showing aggression to the stressor, or fleeing, hiding or shoaling (Galhardo and Oliveira, 2009). Fishes may go on to show erratic swimming patterns, reduced feeding, increased territoriality, social isolation, shelter-seeking and anti-predator behaviour (Pasnik *et al.*, 2010). Stereotypies may also manifest – repetitive behaviours that have been linked to poor welfare in other captive animals (Dawkins, 1998).

Whilst the acute stress response is usually adaptive, chronic, severe or repetitive stress can be damaging to health and welfare and result in poor growth, disease, and potentially death. For example, in Atlantic salmon, repeated handling stress over 4 weeks lead to impaired immune function as measured by macrophages isolated from stressed fish that, when exposed to pathogens, had lower survival than those from unstressed fish (Fast *et al.*, 2008). Similarly, repeated chasing and handling has been shown to cause immunosuppression in goldfish, rendering them more susceptible to disease (Eslamloo *et al.*, 2014). This is of particular relevance in the ornamental industry, where each step of the supply chain may inflict a new stressor on the fish, which may be brief but can have severe consequences. Whilst it is accepted that transport and handling will impose stress in fishes that cannot be avoided completely, reduction of chronic stress is likely to reduce mortalities and improve welfare.

### 1.4 Measures of stress

A wide variety of physiological and behavioural measures have been used to quantify stress in fishes, but the most common method is to measure cortisol. Cortisol measures can be obtained using a variety of body tissues and exudates including blood plasma, faeces, homogenates of the whole body, or by measuring the water in which the fish is held (Pottinger and Carrick, 2001; Turner *et al.*, 2003; Ellis *et al.*, 2004; Ramsay *et al.*, 2006). Measuring cortisol in the water has increased in popularity as it is non-invasive. Furthermore, in an enclosed tank, cortisol levels in the water have been shown to be well correlated with plasma cortisol concentrations (Ellis *et al.*, 2004). This method is particularly useful for small species where extraction of a blood sample is difficult, and it has been applied to various species, including ornamental species, e.g. rainbowfish *Melanotaenia duboulayi* (Zuberi *et al.*, 2011), zebrafish *Danio rerio* (Felix *et al.*, 2013) and guppies *Poecilia reticulata* (Fischer *et al.*, 2014). Recently, techniques that measure cortisol from gill biopsies (Gesto *et al.*, 2015) and fish scales have been developed (Aerts *et al.*, 2015). Different species have different natural baseline levels of cortisol, and differences in their sensitivity to stressors, so using cortisol as an endpoint measure for stress has to be developed for the species of interest (Barton, 2000). Other measures that have been used to assess stress and its effects include plasma glucose (Barton *et al.*, 2005), plasma lactate (Davis and Schreck, 1997), immunological status (Endo *et al.*, 2002), disease occurrence (Davis *et al.*, 2002), expression of genes related to the stress axis, growth and reproduction (Filby *et al.*, 2010), and mortality (Ramsay *et al.*, 2009c). Each of these methods and endpoints have their merits and shortfalls.

Behavioural measures of stress may be observed more quickly and/or more easily than physiological signs and without the requirement (for the most part) of invasive sampling. However, measures of behaviour are not necessarily easy to interpret. Whilst a physiological measure might fall outside acceptable limits, behaviours are subject to the interpretation of the viewer. Furthermore, most ‘normal’ fish behaviours have not been well characterised. However, there are some general features of fish behaviour that clearly signal for signs of stress, which include ‘flashing’ (scratching body surfaces on objects), gasping at the water surface, fin clamping, colour changes, changes in ventilation rate, social isolation, changes in activities such as feeding behaviour and neophobia (Pasnik *et al.*, 2010; Martins *et al.*, 2011). These behaviours are more easily measured compared with many physiological endpoints, and therefore are potentially especially useful for application in aquaculture and ornamental fishkeeping settings.

## 1.5 The aquaculture and ornamental industries

Among the major challenges that captive fishes may face within the supply chain are inappropriate holding conditions, poor water quality, handling, transport, confinement, crowding, poor diet or feeding methods, and disease (Huntingford *et al.*, 2006; Pasnik *et al.*, 2010). Some of the ways in which information on (and manipulation of) these factors could be used to improve welfare of ornamental fishes are discussed below.

### *1.5.1 Physical environment*

One of the most important environmental stressors for captive fishes is poor water quality. Water quality encompasses salinity, pH, temperature, ammonia, nitrate, nitrite, dissolved oxygen, hardness and carbon dioxide (Andrews *et al.*, 1988; Portz *et al.*, 2006). If any of these variables falls outside the normal range for a particular species it may cause stress, leading to a reallocation of the fish’s energy budget to facilitate metabolic processes to allow the fish to cope with increased physiological demand (Barton and Iwama, 1991). More specific effects depend on the stressor. For example, low dissolved oxygen alters respiratory and metabolic activity, blood chemistry and heart rate (Barton and

Taylor, 1996). However, there is no one-size-fits-all approach; a challenge for ensuring the appropriate water quality is that ranges, tolerances and adaptive capabilities are diverse and vary strongly among fish species. For example, normal pH for different freshwater fishes can range from 5.0 to over 10.0 (Portz *et al.*, 2006). Minimising stress from poor water quality must therefore be guided by knowledge of the 'natural' conditions for the species in question.

Monitoring water quality variables and ensuring that changes in physical conditions, such as temperature, occur gradually to avoid shock are essential for minimising stress. However, in the ornamental industry this is not always possible. During transportation, most fishes are kept in plastic bags with no filtration, in some instances for 12 hours or more (Portz *et al.*, 2006). The alternative of tank-based transport would make water quality variables easier to monitor, but this would greatly increase shipping weights and costs, therefore is unlikely to be adopted in the future (Berka, 1986; Lim *et al.*, 2003). Techniques such as adding slow-release oxygen tablets to water, or reducing the volume of water in the bag and filling the rest with pure oxygen, can help maintain water quality, which is particularly important for warm-water ornamental species as water holds less dissolved oxygen at higher temperatures (Portz *et al.*, 2006). To reduce waste build-up, fishes can be starved and water temperatures reduced: Piper *et al.* (1982) recommended starving fishes under 10cm in body length for up to 2 days, fishes over 10cm in body length for at least 48 hours and fishes over 20cm in body length for at least 72 hours to protect against ammonia build-up. Reducing fish biomass per bag in transport also reduces the risks of metabolic waste build-up. However, it is difficult to give general recommendations for optimal fish densities as many of the variables that make up water quality interact with each other. The status of the fish also complicates recommendations on what might constitute an optimised approach. For example, levels of ammonia excretion depend on the species, how recently the fish has been fed, the level of activity, and on ammonia levels that have already accumulated in the water (Randall and Tsui, 2002).

At the retailer or consumer some fishes are placed into 'community' tanks with other species. These different species may have different optimal ranges for water quality variables and thus members of one species may be exposed to mild but prolonged stress if the variables are unsuitable – they may survive, but not necessarily thrive. Mixed tank species should therefore be chosen carefully with these ranges in mind.

The appropriateness of the physical environment extends beyond water quality and may include lighting and photoperiod, tank size, substrate, or enrichment. Light intensity, duration or wavelength may provoke different physiological responses in different species, depending, for example, on the stage of development and natural light levels in that species' normal habitat (Boeuf and Le Bail, 1999). The ability of photoperiod to manipulate fish condition is known from aquaculture where artificial photoperiods are used to improve growth rates (e.g. for an extended photoperiod (20L:4D) in turbot, *Scophthalmus maximus* (Stefansson *et al.*, 2002)), control the phasing of sexual development, or induce spawning (Biswas *et al.*, 2010; Sarameh *et al.*, 2012). In some species altered photoperiod manipulation appears to induce little or no stress (Biswas *et al.*, 2006) but, in others, reduced growth rates, increased cortisol levels and immunosuppression have been shown to occur (Stefansson *et al.*, 2002; Leonardi and Klempau, 2003). The light spectrum may also have an effect on fish health. Head and Malison (2000) found that yellow perch *Perca flavescens* had better growth rates when reared under red or full-spectrum light compared with blue light. However, growth rates were also less affected by disturbance stress when perch were reared under blue light. Similar findings have been reported for rainbow trout, together suggesting red light might be useful in intensive rearing, where other stressors are minimal, and blue light may be useful in the ornamental trade for mitigating against the effects of stress (Karakatsouli *et al.*, 2008).

Tank shape and size may affect behaviour and welfare. As an example, male swordtails *Xiphophorus helleri* in tall, narrow tanks showed less intraspecific aggression than males in long, shallower tanks of the same volume. This effect may have come about because dominant individuals are less likely to view submissive individuals in the horizontal plane (Magellan *et al.*, 2012). Similarly, it is possible that tanks that are wider from front to back could provide fishes with more space to retreat from disturbances caused by fishkeepers or viewers, which might be less stressful than narrower tanks. Larger tanks are likely to be more stable in terms of the physicochemistry of the water.

Provision of substrates and structures that provide refuges for fishes are less common in aquaculture and laboratory settings compared with in ornamental fishkeeping, where environmental enrichment is common. There is, however, an increasing level of interest in the benefits that tank enrichment may offer for captive fish health and welfare (Näslund and Johnsson, 2016). Various laboratory studies have reported that fishes can show a strong preference for certain tank features. For example, in environmental choice experiments zebrafish show a strong preference for enriched rather than barren tanks (Schroeder et al. 2014). Kistler *et al.* (2011) found both zebrafish and checker barbs *Oliotius oligolepis* showed a significant preference for a structured tank compartment (one with plants and clay pots) compared with an empty compartment. Batzina *et al.* (2014) found that gilthead sea bream *Sparus aurata* reared in tanks with blue gravel had higher cortisol responses to stressors than those reared without gravel, but also were quicker to recover brain serotonergic activity, had higher growth rates and lower levels of aggression, indicating quicker recovery from stress and better overall performance.

### *1.5.2 Handling*

Fishes are stressed by handling and in some cases significantly so. This can occur frequently in the ornamental trade. Handling fishes for capture or husbandry reasons may mean removing them from water, and this elicits a strong stress response. Handling for as little as 30 seconds has been found to increase cortisol, plasma glucose, haemoglobin and haematocrit in a number of fish species, as well as increasing the fish's metabolic rate (Barton, 2000; Falahatkar *et al.*, 2009). Handling stressors also tend to be repetitive (Burnley *et al.*, 2012). Furthermore, handlers throughout the supply chain may vary greatly in their competency. Development of new techniques and training of handlers in these techniques could reduce the effects of handling as one of the most common stressors.

Techniques that can reduce stress include handling fishes with wet, gloved hands, using nets that are designed not to disrupt scales or the skin surface mucous layer, and ensuring that nets are not overcrowded when lifted from the water (Conte, 2004; Ashley, 2007; EFSA, 2009). In aquaculture, fish pumps are often used to avoid removing the fishes from water when moving large numbers, but this is less practical for small ornamental species in the pet trade. However, the type and nature of the apparatus used can have a bearing on how stressful the netting process is to fishes. For example, Brydges *et al.* (2009) found that using a small, darkened scoop to move three-spined sticklebacks *Gasterosteus aculeatus* and Panamanian bishops *Brachyrhaphis episcopi* reduced the stress response, measured as opercular beat rate. More severe handling events can be managed by sedating fishes, with anaesthetics such as clove oil or tricaine methanosulphonate (MS-222) as part of the procedure. Sedation methods however are not without their own inherent risks for the fishes as often appropriate dosing is not well established for the different species and repeated exposure to sedatives can become a stressor itself (Wagner *et al.*, 2003). These sedatives are now commonly used in the capture of wild ornamental fishes, having replaced the more stressful technique of cyanide fishing (Rubec *et al.*, 2000).

In addition to the use of sedatives, a number of water conditioning products are commercially available which may be used after handling or other stressful procedures. These products claim to have a variety of functions, including reducing stress, buffering water pH, removing unwanted substances such as heavy metals from water, maintaining electrolyte balance, and helping to maintain the fishes' mucus layer (Harmon, 2009; Harnish *et al.*, 2011; Vanderzwalmen *et al.*, 2018). Although the mode of action by which these products might directly reduce stress is unclear, they may nonetheless indirectly contribute to reduced stress by maintaining better water quality, or by reducing the possibility of fishes sustaining injuries or infections that are more likely to occur when the mucus layer is depleted. For example, Swanson *et al.* (1996) found that survival in transported delta smelt *Hypomesus transpacificus* was approximately 27% higher when NovAqua (Kordon LLC) was added to the transport water, and Snellgrove *et al.* (2007) found that Stress Coat (API Aquarium Pharmaceuticals Inc.) reduced the cortisol release rate in goldfish by 40% after a netting stressor. However, research into the efficacy of these products is limited, and more exploration of their effects would be useful to establish how they affect fish stress.

### *1.5.3 Stocking density*

For ornamental fishes, as with aquaculture species, densities of fishes need to be chosen with their social tendencies and tank size in mind, although little is known about the effects of long-term confinement (Huntingford *et al.*, 2006). Zebrafish, a popular pet and research species, have been shown to have significantly elevated cortisol in crowded conditions (Ramsay et al., 2006). Crowding of fishes can also result in reduced growth rates, which is an issue for some species where they might only be sold after reaching a certain size. Also, smaller fishes tend to be more vulnerable to stress. For instance, Jha (2010) found that rainbow sharks *Epalzeorhynchus frenatus* took significantly longer to reach a marketable size of 4g and experienced significantly greater mortalities and deformities stocked at 1.0 fishes $L^{-1}$ compared to at 0.3 fishes $L^{-1}$.

### *1.5.4 Exporting and importing*

For ornamental fishes, welfare issues are complicated by the organisational structure of the industry. Fishes are captured or bred, flown to their destination country in journeys that may have several legs and last many hours, and collected by a wholesaler or distributor before reaching the retailer (Wabnitz et al., 2003). 'Middlemen' may be involved at each stage, as generally occurs between the fish farmer and the exporter. The continual changing of hands for each shipment of fishes makes it difficult to know exactly what has happened to the fishes at each stage of the journey. During their transportation, fishes may be placed in poorly packed containers, experience sudden changes of temperature, or experience events such as being dropped in their shipping boxes or loss of water through damaged and leaking bags (Ploeg, 2007a). Unusually high mortalities (around 50%) of cardinal tetras *Paracheirodon axelrodi* have been observed in a small number of shipments from Brazil, and this was linked to rough handling during water changes and temperature changes (Tlusty *et al.*, 2005). While various national and international guidelines exist for shipping fishes (OATA, 2015), handlers often are not fish specialists and therefore fishes are not always handled with appropriate care (Walster, 2008).

### *1.5.5 Disease*

Chronically or repeatedly stressed fish experience immunosuppression and as a consequence are more at risk of disease (Wendelaar-Bonga, 1997). High stocking densities and transportation of fishes can exacerbate these risks by contributing to stress and facilitating disease spread. Many diseases of ornamental fishes are caused by pathogens that are extremely common, and tend to manifest opportunistically in times of stress (e.g. whitespot or ‘ich’, caused by the protozoan *Ichthyophthirius multifiliis*) (Noga, 2010). This increase in disease susceptibility of stressed fishes has been seen in both aquaculture (Iguchi *et al.*, 2003) and ornamental fishes (Ramsay *et al.*, 2009c).

Disease prevention procedures (e.g. injecting fishes with vaccines) and disease treatments can inflict stress on fishes, particularly where fishes are in mixed communities and infected individuals are not isolated from healthy ones (Sørum and Damsgård, 2004). There are trade-offs, but generally it is likely that the benefits of disease prevention and treatment will outweigh the stress inflicted, provided an appropriate treatment is chosen and applied correctly (Midtlyng, 1997). As an illustrative example, the application of chlorine dioxide to transport water of guppies to kill bacterial pathogens before shipping was found to decease mortality rates (Lim *et al.*, 2003). Selectively breeding fishes with higher levels of disease resistance has been attempted for some aquaculture species (Silverstein *et al.*, 2009), but has not been a focus for the ornamental fish trade. This is probably because implementing any program of this kind within the industry currently is unlikely to be either practical or cost-effective (Midtlyng *et al.*, 2002). Efforts to prevent disease through good husbandry and biosecurity and by minimising stress should be a priority, with research efforts focussed on increasing disease resistance through areas like improved nutrition.

### *1.5.6 Social environment*

Social factors such as bullying and social ranking can inflict stress (Huntingford *et al.*, 2006). For example, Filby *et al.* (2010) found that social ranking affected zebrafish stress, immune function and body condition after only 5 days. These effects may have been exacerbated by the experimental design, where fish were kept in pairs and it is possible that fish kept in larger groups would result in less marked negative effects. Ornamental species may face further complexity with regards to their social environment as, in some cases, fishes may be housed in community tanks by retailers or consumers. Fishes may find themselves in direct or visual contact with incompatible species, or even predators. In zebrafish this has been found to significantly increase stress as measured by whole-body cortisol (Barcellos *et al.*, 2007). For shoaling species, group size may play a role: Saxby *et al.* (2010) found that neon tetras *Paracheirodon innesi* and white cloud mountain minnows *Tanichthys albonubes* shoaled more, were less aggressive and performed less darting behaviour in larger groups. In contrast, angelfish *Pterophyllum scalare* and tiger barbs *Puntigrus tetrazona* showed greater aggression and more darting behaviour in larger groups, although they also had a greater tendency to shoal. The composition of mixed-species groups must also be considered: when angelfish were added to groups of neon tetras or white cloud mountain minnows, there were fewer interspecific interactions, which was suggested to indicate improved welfare (Sloman *et al.*, 2011). However, the authors noted that this interpretation should be considered with caution, as it is possible that the behavioural changes were the result of neon tetras and white cloud mountain minnows fearing the angelfish.

### *1.5.7 Genetics and domestication*

It is known that genotype can have a major bearing on stress resistance; however, little research has addressed this in ornamental species. Research into aquaculture species has shown that fishes may consistently differ in their stress response (Pottinger *et al.*, 1992; Sadoul *et al.*, 2015) and that these differences are heritable (Pottinger and Carrick, 1999). Research has also suggested that selective breeding or domestication can lead to improved stress resistance (Pottinger and Carrick, 1999; Lepage *et al.*, 2000; Øverli *et al.*, 2002; Douxfils *et al.*, 2011). Such breeding, however, can lead to maladaptive changes in behaviour and physiology (Pottinger and Carrick, 2001; Øverli *et al.*, 2005; Mayer *et al.*, 2011).

Zebrafish captured from the wild in Bangladesh and subsequently bred in the laboratory show reduced behaviours associated with stress on human disturbance over four successive generations (C. Tyler, 2014, pers. comm.). On the other hand, changes in stress resistance due to domestication are not always the result of heritability and may be developmental; first generation captive Atlantic cod *Gadus morhua* had larger hearts and smaller brains than their parental wild types, and were less robust in their ability to withstand stress (Mayer *et al.*, 2011). It was suggested that lack of environmental enrichment caused the reduction in brain size, while heart enlargement was attributed to increased swimming activity – commonly seen in captive fishes relative to wild fishes due to high stocking densities.

Little attention has been paid to breeding stress resistance in ornamental fishes. Some species have been domesticated by aquarists, and some have been selectively bred for attractive colouration and fin shapes (Balon, 2004). There is economic interest in domesticating new species for the ornamental trade (Nunez, 2009), and selective breeding may have great potential to improve stress resistance in ornamental fishes. However, the focus for selective breeding in the ornamental trade has been on appearance and, as yet, not for reducing stress.

### *1.5.8 Nutrition and feeding*

The diets provided for fishes influence biological functioning and can cause stress, or affect stress resistance of fishes, if they do not meet specific nutritional requirements, and this may vary according to life stage or species (Strange, 2009; Oliva-Teles, 2012). Dietary supplementation can be used to ensure the correct balance of nutrients is delivered for optimal welfare. For example, vitamins A, C and E have all been linked to improved immune system function in fishes (Sakai, 1999). When gilthead sea bream were fed diets low in vitamin E they had higher mortality rates when exposed to repetitive and chronic stress, which was thought to be due to the effect of a lack of vitamin E on several immune system functions (Montero *et al.*, 2001). Among ornamental species, vitamin C was found to enhance the ability of guppies to tolerate osmotic stress, as cumulative mortality rates were lower in groups fed a vitamin C-enriched diet (Lim *et al.*, 2002b). Diets can also be supplemented with prebiotics or probiotics, which may contribute to improved growth, survival and gut microbiome health; in mammals, processes carried out by symbionts in the gut microbiome have been linked to health and disease and similar interactions may occur in fishes (Kinross *et al.*, 2011). Ferguson *et al.* (2010) found that Nile tilapia *Oreochromis niloticus* fed a diet containing a probiotic, the bacterium *Pediococcus acidilactici*, had their gut colonised by the bacteria and had a stimulated nonspecific immune response and higher survival than the group not fed the probiotic. Supplementation with *Bacillus* spp. was found to improve growth, survival and immune function in olive flounder *Paralichthys olivaceus*

(Cha *et al.*, 2013) and in guppies, mollies *Poecilia sphenops*, common platies *Xiphophorus maculatus* and swordtails (Ghosh *et al.*, 2008).

Live feeds may also be beneficial for ornamental fishes, particularly for larvae; decapsulated brine shrimp cysts were found to increase resistance to osmotic stress in the fry of guppies, black neon tetras *Hyphessobrycon herbertaxelrodi*, swordtails and mollies (Lim *et al.*, 2002a). Fish larvae may also be more likely to eat live feeds than formulated diets, as live feeds are thought to be easier for larvae to see, more easily available to larvae as they swim in the water column rather than float or sink like many formulated diets, and may be easier for larvae to digest (Conceicao *et al.*, 2010).

It is important to be aware of the differences between feeding approaches in aquaculture and ornamental species – for example, the amount of food provided to fishes in aquaculture is often carefully calculated, motivated by a desire to attain optimal food conversion efficiency and growth rates, while the amount of food for ornamental species is usually at the discretion of the fish keeper (Oliva-Teles, 2012). However, it is possible that research into nutrition will provide multiple beneficial effects that can be applied to both industries.

The time and method of providing food can also affect stress levels in fishes. For example, Sánchez *et al.* (2009) found that gilthead sea bream fed on a schedule had lower plasma cortisol than those that were fed randomly. Endo *et al.* (2002) found that Nile tilapia fed with fish-activated feeders had lower plasma cortisol and improved immune systems compared to fish with a scheduled feeding. Some timid species may be reluctant to leave cover to feed; therefore the use of 'dither' fishes (bolder species which are included in tanks to encourage feeding and exploration in timid species), may reduce stress (Loiselle, 1979). Starvation is common before handling and transport procedures in order to reduce physiological stress levels by reducing metabolic demand (Barton and Iwama, 1991). However, Ramsay *et al.* (2009a) found that there was no difference in stress levels, measured as cortisol increase, in fed or fasted juvenile rainbow trout exposed to a crowding stressor.

### *1.5.9 Interactive and cumulative effects*

A final concern related to fish stress is the increased damage caused by cumulative effects of multiple or sequential stressors compared to individual stressors alone (Jarvi, 1990; Mugnier *et al.*, 1998). For example, Barton *et al.* (2005) found that gilthead sea bream subjected to chronic confinement stress had a reduced cortisol response to an acute handling stress. They suggested this was due to impairment of the stress response, which left individuals ill-equipped to deal with other acute stressors. Waring *et al.* (1997) found that recovery periods may negate this, as the cumulative effects of handling stress were removed when fish were given a 24 hour interval between each event. However, in some scenarios repeated stressful events are difficult to avoid. A potentially useful technique is conditioning. Various aquaculture species, including Chinook salmon *Oncorhynchus tshawytscha* (Schreck *et al.*, 1995), Atlantic salmon (Bratland *et al.*, 2010) and Atlantic cod (Nilsson *et al.*, 2012), have shown reductions in their response to stressors when they have learnt to associate them with positive events, like feeding. This could be an easy modification to make to current practices within the ornamental industry at various points in the supply chain and even in home aquaria, which may help to mediate any of a variety of stressors.

## 1.6 Conclusions

Welfare and mortality rates remain a major concern for the ornamental trade. Simultaneously, the study of welfare is hampered by continuous debate over the presence or absence of fish consciousness (Sneddon, 2009; Rose *et al.*, 2014). Whilst more research into the potential for fish pain perception and suffering may produce more solid conclusions, a stress-focussed approach may provide an easily measurable approach to studying welfare in the interim. This approach has already been used to great effect within the aquaculture industry as the result of a recent growing interest in welfare in that sector, and has been applied to a lesser extent within the ornamental fish industry. The supply chain within both industries involves multiple stressors which can affect fishes, resulting in poor welfare, increased disease prevalence and death. Many of the examples of interventions to improve ornamental fish welfare mentioned here have originated from the methods used in aquaculture, such as starving fishes to reduce metabolism before transportation or altering diets to enhance stress resistance. Other interventions to improve welfare that have been in place in the aquaculture industry for several decades have only partially been adopted within the ornamental industry. For example, live feeds have long been used during larviculture of food fishes, but were adopted only recently in the rearing of freshwater ornamental species. These can make a fundamental difference to larval survival rates. More of the available knowledge in the aquaculture sector on larval feeding applied to marine ornamental larviculture, could help significantly in the captive breeding of more marine ornamental species, reducing depletion of wild populations (Moorhead and Zeng, 2010). Other avenues to explore in using knowledge gained in the aquaculture sector for the ornamental industry include on handling techniques. It is now uncommon to remove aquaculture fishes from the water when moving them, but the majority of the handling of ornamental fishes, particularly small species, is still done using dip nets, which is a highly stressful experience. It has to be accepted however, that some procedures in the ornamental industry cannot be made stress-free and thus there is a need for explorations into selective breeding for more stress-resistant fishes, and for conditioning and habituation to decrease the impact of the stress response (Table 1.2).

Despite the size of the ornamental industry, there is a conspicuous lack of scientific literature that directly addresses ornamental fish welfare (Figure 1.1). However, there is a great deal of information available in the hobbyist literature that represents a huge knowledge base, e.g. textbooks, health guides and identification manuals (Andrews *et al.*, 1988; Butcher, 1992; Baensch and Fischer, 2007). A further wealth of knowledge is available online or in hobbyist magazines such as *Practical Fishkeeping.* This knowledge could be incorporated more into the scientific domain to provide directions for further experimental work.

Table 1.2. Suggested future interventions and research into welfare in the ornamental fish industry

| Stressor | Current Interventions | Future research |
|---|---|---|
| Physical environment | Provide structure and enrichment with plants, shelter, etc. | Impact of light levels, light spectrum and photoperiod on stress. |
|  | Provide substrate rather than | Impact of different types of substrate or background |

|  |  |  |
|---|---|---|
|  | bare tanks.<br>Monitor water quality parameters regularly.<br>Ensure any change in water conditions occurs gradually to avoid shock. | colour on long-term stress levels.<br>Impact of different types of tank enrichment on different species, e.g. comparing between plastic and real plants.<br>Establish optimal water quality parameters for different species. |
| Handling | Handle fish using wet, gloved hands.<br>Minimise number of handling events.<br>Add anaesthetic and/or water conditioners to water if necessary. | Effects of multiple handling events.<br>Developing techniques for handling fish without removing them from water.<br>Use of conditioning and habituation to reduce stress response of fish to handling stress. |
| Stocking density | Choose densities according to nature and social tendencies of the species. | Establish optimal densities for different species for both shipping and keeping in tanks. |
| Transportation | Starve fish before transporting.<br>Cool holding water before transporting.<br>Add anaesthetic and/or water conditioners to water if | Establish optimal water quality parameters for different species.<br>Establish optimal stocking densities for different species. |

|  |  |  |
|---|---|---|
|  | necessary.<br>Minimise density of fish per bag to limit metabolic waste build-up. |  |
| Social environment | Choose species assemblages according to social tendencies of species. | Appropriate species assemblages to decrease social stress, aggression and competition<br>Effects of tank conditions on social behaviour, e.g. tank dimensions or amount of structure available in the tank. |
| Nutrition | Select diets based on different species requirements.<br>Include live feeds in fish diets.<br>Ensure food is well-dispersed when provided to minimise competition. | Dietary requirements for different species.<br>Stress-reducing effects of dietary supplements and probiotics.<br>Role of gut microflora in health and stress. |
| Disease | Minimise stressors to minimise immunosuppression from stress.<br>Consider adding pre-emptive treatments to water if necessary.<br>Consider treating fish before transportation. | Increased understanding of biological processes underlying stress, immunosuppression and health in fish.<br>Selective breeding for higher disease resistance; also selective breeding for higher stress resistance |

| | | |
|---|---|---|
| | | may reduce opportunistic infections. |
| Interactive/ cumulative effects | Allow fish ‘rest’ periods between stressful events. | Use of conditioning and habituation to reduce response of fish to stressful events through positive associations or by improving predictability of stressors. |

A significant factor when considering the ornamental industry is the number and diversity of species that are traded. A ‘one-size-fits-all’ approach is unlikely to be useful. Moreover, the extent to which research into stress and welfare in one species of fish can be generalised to improve the welfare of another species is questionable (Huntingford et al., 2006). Increased knowledge of the biology and needs of individual species and their responses to stress is vital in future to identify appropriate interventions for different species. More welfare-focussed experimental techniques can supplement this information. For example, choice experiments can be used effectively to ascertain optimal conditions for welfare. More research into diseases and treatments, and specifically diseases that are common in ornamental fishes could help drastically reduce mortality rates. Furthermore, many physiological measures of welfare, such as cortisol levels in water, plasma or faeces are not currently easy to implement at most stages of the ornamental fish industry, as they require specialist knowledge and equipment. Therefore, a major goal in improving welfare and mortality must be to develop indicators that are usable by a non-specialist (e.g. features of behaviour) and can be responded to on a case-by-case basis in order to constantly monitor the welfare of fishes.

There is a second major issue to address to improve welfare in the ornamental industry and this is the structure of the current system of trade. Little regulation exists, and the supply chain can be long and complex. Therefore, even if knowledge of ornamental fishes can be expanded, the integration of new techniques into the system will present a significant challenge. Research must be conducted recognising that costly interventions that save only a few fishes each shipment are highly unlikely to be adopted. In contrast there is significant potential for cheaper interventions to be adopted, particularly if supported by retailers.

Although the figures often quoted regarding the ornamental industry are uncertain, the fact remains that millions of live animals are transported worldwide every year (Ploeg, 2007b). Estimates of mortality rates are strongly contested, ranging from between 2-3% (Ploeg, 2007a) up to 73% (Olivier, 2001), but even small percentage reductions in mortality would have considerable beneficial impact, in both animal welfare and economic terms. Furthermore, as interest in welfare increases, better standards will increase the marketability of the hobby, potentially translating into greater profits. There is a great need for more accurate statistics, improved husbandry standards and more extensive research to improve welfare standards within the industry.

## 1.7 Chapter overview/aims of thesis

The first overall aim of this thesis is to identify sources of stress and poor welfare within the ornamental fish trade. These sources of stress might be contributing to mortalities within the industry, so by targeting research to try and address these sources of stress, mortality may be reduced and welfare improved. Therefore, the second overall aim of this thesis is to develop and test some potential interventions that could be used within the ornamental fish supply chain to reduce stress and improve welfare in ornamental fishes.

Stress in fishes can be assessed and measured in a variety of ways, as discussed above. One of the most common ways to assess stress is to measure cortisol levels in the blood plasma, the faeces, the whole body of the fish, or in water in which the fish has been housed (Pottinger and Carrick, 1999; Ellis *et al.*, 2004; Ramsay *et al.*, 2006). The last of these methods has a number of benefits over other methods: it allows multiple samples to be taken over time without inducing a handling effect, is non-invasive and non-intrusive. However, a disadvantage of this method is that it requires validation when being applied to a new species – for example, it must be established that stressors induce a detectable rise in cortisol release rates as measured by this method, and the time at which cortisol release rates peak after the onset of a stressor must be identified (Scott *et al.*, 2008). Therefore, in Chapter 3, I present a study of the time-course of cortisol release rates in three popular ornamental fish species (the guppy, the molly and the neon tetra) after being exposed to a netting stressor.

One of the most commonly-experienced stressors for fish at any point in the supply chain is likely to be handling. Despite a move towards handling techniques which do not involve removing the fish from water in other fields, such as aquaculture or laboratory research, much handling in the ornamental fish industry is still done using dip-nets (Conte, 2004; Brydges *et al.*, 2009). In addition to this, workers throughout the supply chain may vary greatly in skill level and the level of training they have received in how best to handle fish. In Chapter 4, I present the results of two experiments in which inexperienced fish handlers were either presented with a short training video prior to handling guppies, or a control video. I then measured cortisol release rates and used behavioural measures to assess the stress levels of fish captured by handlers in these two groups.

Much research into the welfare of captive animals focusses on the role of environmental enrichment. Environmental enrichment, which refers to modifications made to an animal’s environment with the aim of improving that animal’s well-being, is often considered to promote good welfare (Young, 2013). However, some studies have found that modifications intended to provide enrichment were actually neutral or negative with respect to the animal’s welfare (Landin, 2012; Wilkes *et al.*, 2012). Modifications must therefore be tested before being introduced into the environments of captive animals. A popular experimental approach used to assess whether modifications might promote good welfare is the use of choice tests, which is based on the assumption that if animals spend more time in an area or interacting with an item than alternatives, that area or item is providing the animals with something they want. In Chapter 5, I use this approach to test the preferences of neon tetras for different environmental conditions.

Whilst not all stressors can be eliminated from the lives of captive animals, it is possible that the way an animal responds to a stressor can be modulated by changing the animal’s appraisal of the stressor. In Chapter 6, I test two interventions designed to change the way that guppies appraised a netting stressor using conditioning techniques. The first experiment aimed to train guppies to associate netting with a positive event in the form of a food reward; the second aimed to make netting more predictable by training guppies to recognise that netting happened after being exposed to a red light cue.

Finally, in Chapter 7, I present a general discussion of the results of Chapters 3-6, with specific emphasis on the interventions that have been developed and introduced into the supply chain as a result of this work. I also discuss potential future areas of research into the topics of ornamental fish stress and welfare.

# Chapter 3: Detection of water-borne cortisol in three ornamental fish species

## Abstract

Corticosteroid release rates are commonly measured as an indicator of stress in many taxa, including in teleost fishes. Previously, measurement of corticosteroids required collection of a blood sample from fish, or else required the fish to be sacrificed. However, methods have improved considerably in the last 15 years with the increasing popularity of the water-borne method – a non-invasive approach which allows steroid hormones to be quantified from samples of fish holding water. However, the water-borne method requires a number of validation steps to be carried out when it is applied to a new species to ensure that corticosteroids can be reliably detected, are biologically meaningful, and are sampled at the optimum time-point for the species in question (e.g. when cortisol release rates peak after the onset of a stressor). This study aimed to validate the water-borne method for cortisol detection in three popular ornamental fish species, the guppy *Poecilia reticulata*, the molly *Poecilia sphenops*, and the neon tetra *Paracheirodon innesi*. These three species are all highly popular aquarium fishes, making up approximately 15% of fish sales for a major UK pet retailer. Validating this method in these species therefore provides a useful tool for studying potential causes of stress, poor welfare and mortality. Water samples from the holding tanks of each of these species were collected over several hours after a handling event, or from control tanks, and the cortisol levels in the samples were quantified using a cortisol radioimmunoassay (RIA). Cortisol was detected in the holding water of all three species. Release rates were significantly higher over the first 30-minute interval in guppies and mollies (guppies: 2.46 ± 0.52 ng $g^{-1}$ $h^{-1}$; mollies: 1.46 ± 1.18 ng $g^{-1}$ $h^{-1}$), but had dropped to consistently lower levels 2 hours after the start of the sampling period (guppies: 0.44 ± 0.20 ng $g^{-1}$ $h^{-1}$; mollies: 0.13 ± 0.06 ng $g^{-1}$ $h^{-1}$). These results may indicate that these species showed a stress response. However, there were no significant differences between net-stressed and unstressed individuals in any species. This study therefore meets some of the objectives required in order to consider the water-borne cortisol measurement technique validated in guppies and mollies: there was no background activity in water provided to fishes, extracts and standards diluted parallel to each other, and cortisol release

rates were consistently detectable with the technique. The pattern of cortisol release rate was less clear in neon tetras as there were no significant differences in cortisol release rate over time or between treatment groups. Therefore, further work comparing the release rates of cortisol in stressed and unstressed guppies, mollies and neon tetras is required to consider this technique fully validated.

## 3.1 Introduction

Interest in fish welfare as a research topic has been growing consistently over the last 30 years, with the majority of studies focussing on fish in aquaculture (see Fig. 1.1, Chapter 1) (Ashley, 2007; Huntingford and Kadri, 2009; Braithwaite and Ebbesson, 2014). An increasing number of studies on stress and welfare in aquaculture species have helped to explore the range of stressors which may impact fish health and welfare, including transportation, handling, confinement, inappropriate stocking densities, etc., but we cannot always extrapolate from them to address questions in all fish species, or for all areas in which fish are used (e.g. ornamental fish, research, public aquaria or sport fishing) (Huntingford *et al.*, 2006). For example, inappropriate stocking densities can be a significant source of stress for fish, but appropriate stocking densities are highly species-dependent – Arctic charr *Salvelinus alpinus* show better growth and welfare at high stocking densities (≥60 kg $m^{-3}$) than low stocking density (15 kg $m^{-3}$) (Jorgensen *et al.*, 1993), whilst gilthead sea bream *Sparus aurata* showed a greater stress response at a low stocking density (approx. 10 kg $m^{-3}$) than a high density (approx. 40 kg $m^{-3}$) (Montero *et al.*, 1999). The huge diversity of species across all of these fields means that, in many cases, research into stress and welfare needs to be targeted to the species and/or field in question.

One such area that would benefit from more research is the ornamental fish industry. Although reported rates of mortality within the industry vary, some reported figures are high. These high mortality rates indicate that there are likely to be welfare issues within the trade that could be addressed to improve fish health with direct benefits for the ornamental fish trade as a consequence (Ellis *et al.*, 2012). However, the concept of ‘welfare’ is difficult to define and measure (see Chapter 1; Dawkins (2006); Carenzi and Verga (2009); Volpato (2009); Broom (2011)), and in the case of fish the discussion is hampered by debates over the abilities of fish to suffer and/or feel pain (Rose, 2002; Sneddon, 2003b; Braithwaite and Boulcott, 2007; Sneddon, 2015; Key, 2016). To circumvent this, many authors have instead focussed on the stress response as a welfare indicator (Conte, 2004; Arlinghaus *et al.*, 2007). Stress, a coordinated suite of behavioural and physiological responses to a challenge to homeostasis (Wendelaar-Bonga, 1997), is more easily measured than welfare, and is known to have potentially detrimental effects. For example, whilst the response to an acute stressor is often seen as adaptive, stress that is chronic, repetitive, or severe can lead to a decreased growth and reproductive rate and immunosuppression, which can in turn lead to higher mortality (Barton, 2002; Fast *et al.*, 2008; Eslamloo *et al.*, 2014).

There are a variety of ways in which stress in fishes can be measured, the most common of which is measurement of corticosteroid levels (Barton and Iwama, 1991; Barton, 2002). Corticosteroids can be measured in blood plasma, faeces, from gill biopsies, fish scales, homogenates of the whole body, or in the water the fish has been housed in (Pottinger and Carrick, 2001; Turner *et al.*, 2003; Ellis *et al.*, 2004; Ramsay *et al.*, 2006; Aerts *et al.*, 2015; Gesto *et al.*, 2015). The last of these involves using solid-phase extraction cartridges and organic solvents to extract and concentrate the steroids in the water sample, then using radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs) to quantify the amount of steroid present (Scott and Sorensen, 1994; Ellis *et al.*, 2004). It is an increasingly popular metric, as it is non-invasive, can be sampled without disturbing the fish (i.e. is non-intrusive) and it allows multiple measurements to be made over several time-points (Ellis *et al.*, 2013). It is particularly useful for small fish species, as many common model species (e.g. zebrafish *Danio rerio*) are too small to extract a blood sample and therefore are often sacrificed to obtain whole-body measures (Ramsay *et al.*, 2006; Felix *et al.*, 2013). However, before this method can be applied to a new species, it needs to be validated, as steroid release rates in different species can vary due to differences in biomass, metabolic rate, preferred holding temperature, steroid readsorption rate and gill surface area and permeability (Scott *et al.*, 2008; Felix *et al.*, 2013).

In this study, a method for detecting water-borne cortisol by extracting cortisol through solid-phase extraction cartridges and measuring amounts by radioimmunoassay was validated in three popular ornamental fish species: the guppy *Poecilia reticulata*, the molly *Poecilia sphenops* and the neon tetra *Paracheirodon innesi*. These three species were chosen as they are highly popular ornamental fish species, representing approximately 15% of all fish sales for a major UK pet retailer (Chapman *et al.*, 1997; Bailey & Burgess, 2004). Although this method has been validated and used with guppies, previous work has used wild-type guppies rather than domestic guppies (e.g. Kolluru *et al.* (2015)), or looked only at females (e.g. Eaton *et al.* (2015)). The latter case is important here because guppies in the ornamental fish trade tend to be sold separately by sex. To the best of the author's knowledge, this method has also not been validated in common mollies (although it has been applied to a congener – the sailfin molly *Poecilia latipinna* (Muraco *et al.*, 2014)), nor has it been validated in neon tetras. In order to validate this method in these species, a number of procedures suggested by Scott *et al.* (2008) were carried out. These were: measurement of background rates of cortisol in the water supplied to the fish tanks, assessing for parallelism between extracts and standards to ensure that there was no interference from other immunoreactive substances, assessing whether fishes mounted a cortisol response to a stressor (i.e. whether the results of this assay are biologically meaningful in these species) and quantifying when cortisol release rates peaked after the onset of a stressor to determine the optimum sampling time for measuring for a stress response after application of a stressor to that species.

## 3.2 Methods

### *3.2.1 Animals and maintenance*

Captive-bred adult guppies (60 males), mollies (20 males, 20 females) and neon tetras (40 males, 40 females) were purchased from a UK-based ornamental fish wholesaler. Fish were housed in a flow-through system in barren 8 L glass tanks (150 x 240 x 295 mm) (6 guppies/tank; 2 male and 2 female mollies/tank; 4 male and 4 female neon tetras/tank). These stocking densities were chosen as they were considered to represent the greatest number of individuals for each species which could be housed in tanks of this size without compromising fish welfare. Tanks were maintained at 25 ± 1°C with a photoperiod of 12L:12D hours and fed twice daily *ad libitum* with TetraMin fish flake and once daily with live *Artemia* (brine shrimp) nauplii. Fish were given a minimum of 1 week to acclimatise to their surroundings before sampling took place and all handling and feeding was carried out with gloves on to prevent cortisol from human skin from contaminating the water (Ellis *et al.*, 2013).

### *3.2.2 Water sample collection*

Prior to sampling, the water flow rate into the tank was measured by filling a 50 mL measuring cylinder; the exact volume of water and the time taken to collect that volume was used to calculate flow rate. Fish were not fed for approximately 18 hours prior to sampling as food intake can affect cortisol responses (Bry, 1982; Pickering and Pottinger, 1983). For each species, tanks were randomly assigned to either an ‘unstressed’ condition ($n$ = 5) or a ‘stressed’ condition ($n$ = 5) to see whether a netting stressor would elicit a detectable increase in cortisol release rate. A sample size of 5 tanks per condition was considered to be appropriate based on previous published studies – many previous studies of water-borne steroid hormones have studied 3 tanks of fish per condition (Ellis *et al.*, 2004; James *et al.*, 2004; Ellis *et al.*, 2007; Zahl *et al.*, 2010; Zuberi *et al.*, 2011). At the beginning of the experiment, an initial water sample of approximately 500 mL was collected from each tank in an acid-washed beaker from the outflow of the tank to avoid disturbing the fish. This sample was used to measure the amount of cortisol in each tank prior to the onset of a stressor as fish continually release cortisol into the water, this measurement provided the ‘baseline’ level of cortisol in each tank prior to the onset of a stressor, against which subsequent samples could be compared. Fish in the ‘stressed’ condition were then immediately exposed to a stressor by being chased for 30s with a net. A further water sample was collected from each tank in the same way at 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours and 6 hours after the start of the sampling period. A second measure of flow rate was then taken for each tank to check that it was consistent with the flow rate at the start of the sampling period. Each sampling period was started at approximately 9:30am (± 20 mins) to minimise potential variation caused by cortisol levels naturally fluctuating throughout the day (Wendelaar-Bonga, 1997). Four samples of the water (approximately 500 mL each) that was supplied to aquaria were also collected at the beginning of the sampling period to check for the presence of background rates of cortisol – i.e. this water was collected before it went into tanks to detect whether there was any cortisol present in the water before the water came into contact with the fish. All samples were processed immediately after collection.

### 3.2.3 Sample processing

Water samples were weighed before processing, and beakers were weighed after the sample had been processed so that sample volume could be determined gravimetrically. Samples were pumped through solid-phase extraction cartridges (C18 Classic, Waters Ltd, UK) at c. 15 mL $s^{-1}$ using a vacuum pump (Watson-Marlow 202 U/1). Before use cartridges were primed with two washes each of 5 mL methanol and 5 mL distilled water pushed through with a syringe. Once all the water sample had been pumped through the cartridges, these were rinsed with 5 mL distilled water, and 40 mL air was pushed through with a syringe to dry the cartridge. Cartridges were wrapped in Parafilm and frozen at -20°C (freezing has been shown not to affect the extraction of hormones (Ellis *et al.*, 2004)). When all samples had been collected, cartridges were defrosted and eluted with 4 ml of ethyl acetate into test tubes. Air was pumped through the cartridge into the tube to remove excess ethyl acetate. The eluted sample was dried under a stream of nitrogen, then the resulting pellet was re-suspended in 1 mL of 0.5 M RIA buffer (buffer solution made up of 8 g $L^{-1}$ NaCl, 5.8 g $L^{-1}$ $Na_2HPO_4$, 2 g $L^{-1}$ BSA, 1.3 g $L^{-1}$ $NaH_2PO_4 \cdot H_2O$, 0.3g $L^{-1}$ EDTA and 0.1g $L^{-1}$ sodium azide), vortexed and frozen at -20°C until samples could be assayed.

### 3.2.4 Cortisol radioimmunoassay

Radiolabelled cortisol was purchased from Perkin Elmer, UK, cortisol antibody was purchased from abcam, UK, and standard cortisol was purchased from Sigma Aldrich, UK. The amount of cortisol in each sampled was quantified by radioimmunoassay (RIA), following the protocol of Ellis *et al.* (2004). 100 µL of sample were added to tubes in duplicate, and a standard curve was created, also in duplicate, using a known concentration of cortisol. The standard curve ranged from 2 to 500 pg 100 $\mu L^{-1}$. A cocktail containing approximately 6000 dpm of radiolabelled cortisol, enough antibody to bind 50% radiolabelled cortisol in the absence of sample cortisol, and RIA buffer was added to the tubes, which were left overnight to bind (approx. 18 hours, 4°C). 1 mL dextran-coated charcoal suspension was then added to tubes to separate out unbound steroid for 30 minutes on ice. Tubes were centrifuged at 2500 rpm for 12 minutes, and the supernatant decanted into a scintillation vial. 7 mL of scintillation cocktail (Ultima Gold, Perkin Elmer, UK) was added to each vial, and the tubes were read in a Beckman-Coulter LS6500 liquid scintillation counter for 5 minutes each. The standard curve was used to determine the amount of cortisol in each tube, and measures were averaged across duplicate tubes. The biomass of the fish was not recorded due to experimenter oversight, therefore the average biomass of each species from a pilot experiment was used as an estimate for further calculations. Cortisol release rate was calculated using the following equation from Ellis *et al.* (2004):

$$\text{Release rate} = ((V(C_t - C_0 e^{-kt})kt)/(1 - e^{-kt}))/W$$

where $V$ = tank volume (L), $C_0$ and $C_t$ = concentration of cortisol at the start and end of the sampling period, respectively (ng $L^{-1}$), $k$ = instantaneous rate of loss due to dilution (flow rate (L $h^{-1}$)/$V$), $t$ = time interval and $W$ = mass of fish. This gives release rates in ng of cortisol per gram of fish body mass per hour (ng $g^{-1}$ $h^{-1}$), a standard unit used when reporting cortisol release rates to best allow comparisons between different species (Ellis *et al.*, 2004; Sebire *et al.*, 2007; Scott *et al.*, 2008; Zuberi *et al.*, 2011; Felix *et al.*, 2013).

To assess whether extracts and standards diluted in a parallel manner, aliquots of a number of randomly-selected samples for each species were pooled. A dilution series of each of these pools (neat, 1:1, 1:2, 1:4, 1:8) was then created in the same manner as the dilution of the standards and radioimmunoassayed as described above.

### *3.2.5 Data analysis*

Repeated-measures ANOVAs were used to examine the effect of netting stress and time on cortisol release rate for each species. To assess whether the sample extracts analysed diluted parallel with the standards, an ANCOVA was used. To assess the intra-assay variation, the coefficient of variation was calculated for the duplicates of all tube in each assay, and the mean of these coefficients was calculated. To assess the inter-assay variation, a number of randomly-chosen samples were run in different assays. The coefficient of variation was calculated for the results of these samples, and the mean of these coefficients was calculated. All analyses were completed in R ver. 3.4.1 (R Development Core Team, 2015).

### *3.2.6 Ethics*

This study was conducted in accordance with Home Office guidelines under project license PPL 3002868. Fish were monitored daily for signs of stress or poor welfare. After the study was complete, the fish were health checked by the named veterinary surgeon and when certified as disease free and in good general health were rehomed into the ornamental fish trade.

## 3.3 Results

The average intra-assay variation in the study was 9.31%. The average inter-assay variation was 7.79%. No cortisol was detected in the water supplied to experimental aquaria.

The peak cortisol release rates found in this study were 2.46 ± 0.52 ng $g^{-1}$ $h^{-1}$ in guppies, 1.46 ± 1.18 ng $g^{-1}$ $h^{-1}$ in mollies, and 2.66 ± 1.82 ng $g^{-1}$ $h^{-1}$ in neon tetras. There was a significant effect of sampling time point on cortisol release rate in guppies ($F_{1, 59} = 12.461$, $p < 0.001$), but stressed and unstressed fish did not differ in cortisol release rate ($F_{1, 59} = 0.171$, $p = 0.680$) (Fig. 3.1). There was also a significant effect of sampling time point on cortisol release rate in mollies ($F_{1, 60} = 6.685$, $p = 0.012$), but no overall effect of treatment (stressed vs unstressed) ($F_{1, 60} = 2.968$, $p = 0.090$) (Fig. 3.2). Mean cortisol release rates did appear to be higher in unstressed neon tetras than stressed neon tetras; however, neither treatment group nor time point had a significant effect (treatment: $F_{1, 61} = 0.537$, $p = 0.467$; time point: $F_{1, 61} = 0.003$, $p = 0.958$) (Fig. 3.3). Examination of the raw data revealed that means appeared to be higher in unstressed fish mainly due to unusually high cortisol release rates at some time points in two tanks of ‘unstressed’ fish, but it was unclear whether these high rates were due to an unexpected stressor affecting the fish, or possible contamination of the samples. However, when this tank was removed from the analysis, there was still no significant effect of treatment group or sampling point on cortisol release rates (treatment: $F_{1, 48} = 0.004$, $p = 0.952$; time point: $F_{1, 48} = 0.120$, $p = 0.731$) (Fig. 3.4).

There were no significant differences between the slopes of the dilution curves for standard cortisol and cortisol released by guppies, mollies or neon tetras ($F_{3, 12} = 0.679$, $p = 0.582$), indicating that that extracts and standards diluted in a parallel manner (Fig. 3.5).

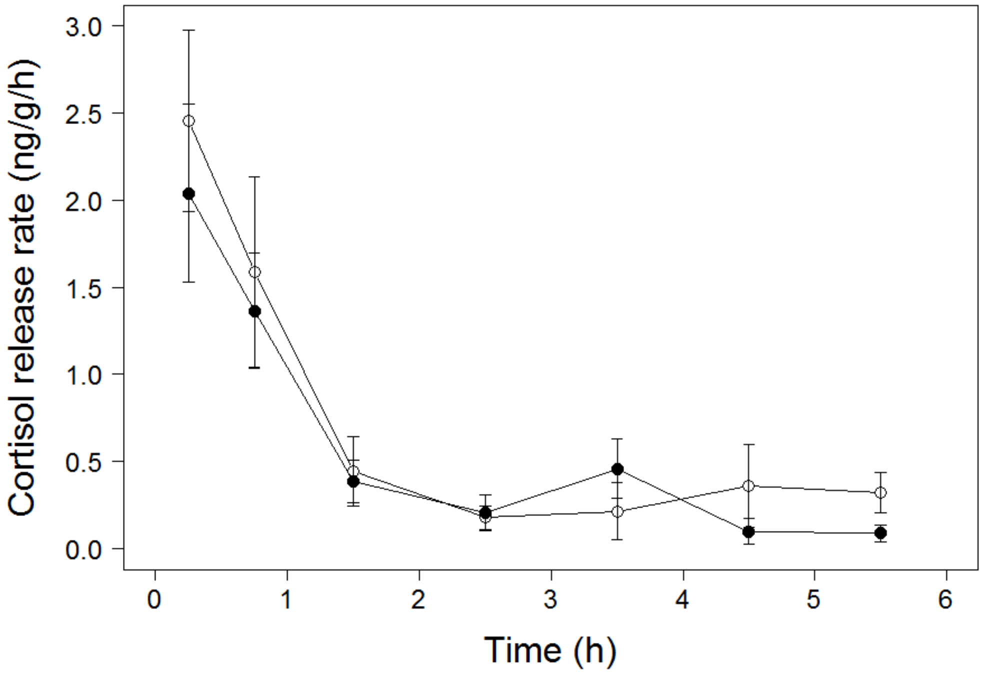

Figure 3.1. Mean ± S.E. release rates of cortisol into the water in guppy tanks ($n = 5$ per treatment) after exposure to a handling stressor at 0h (open circles) or no stress (filled circles). Values are plotted at the midpoint of the time interval that release rates were measured over.

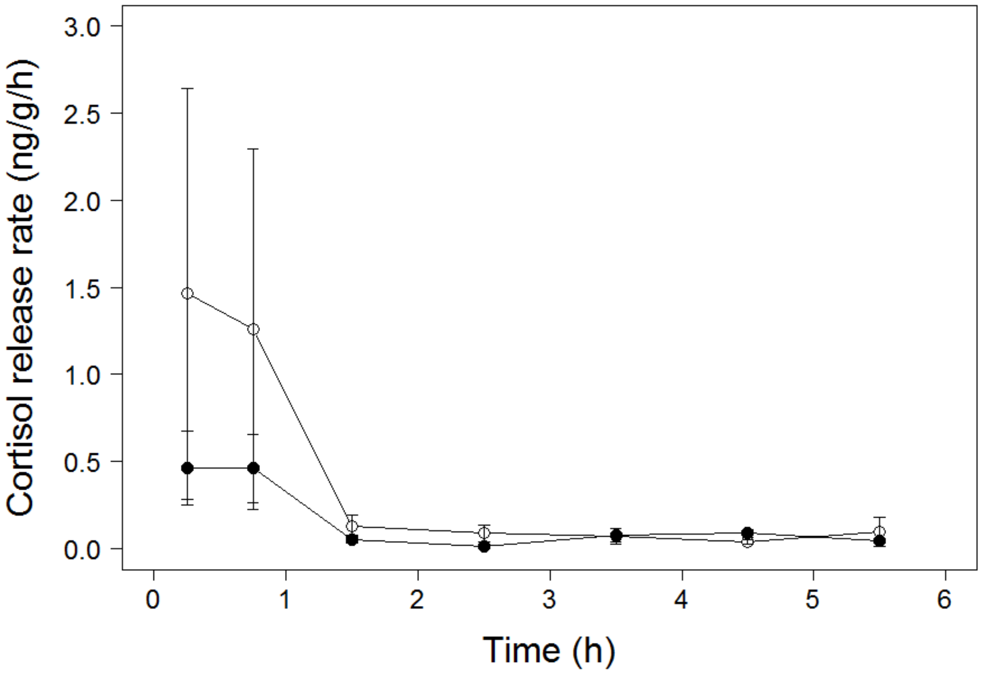

Figure 3.2. Mean ± S.E. release rates of cortisol into the water in molly tanks (*n* = 5 per treatment) after exposure to a handling stressor at 0h (open circles) or no stress (filled circles). Values are plotted at the midpoint of the time interval that release rates were measured over.

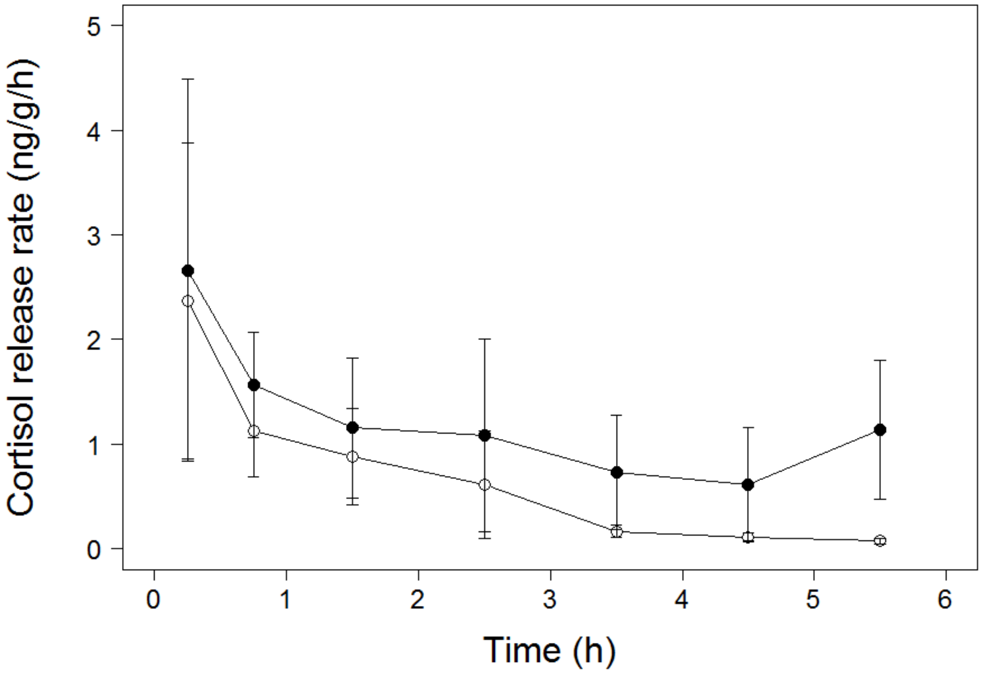

Figure 3.3. Mean ± S.E. release rates of cortisol into the water in neon tetra tanks (*n* = 5 per treatment) after exposure to a handling stressor at 0h (open circles) or no stress (filled circles). Values are plotted at the midpoint of the time interval that release rates were measured over.

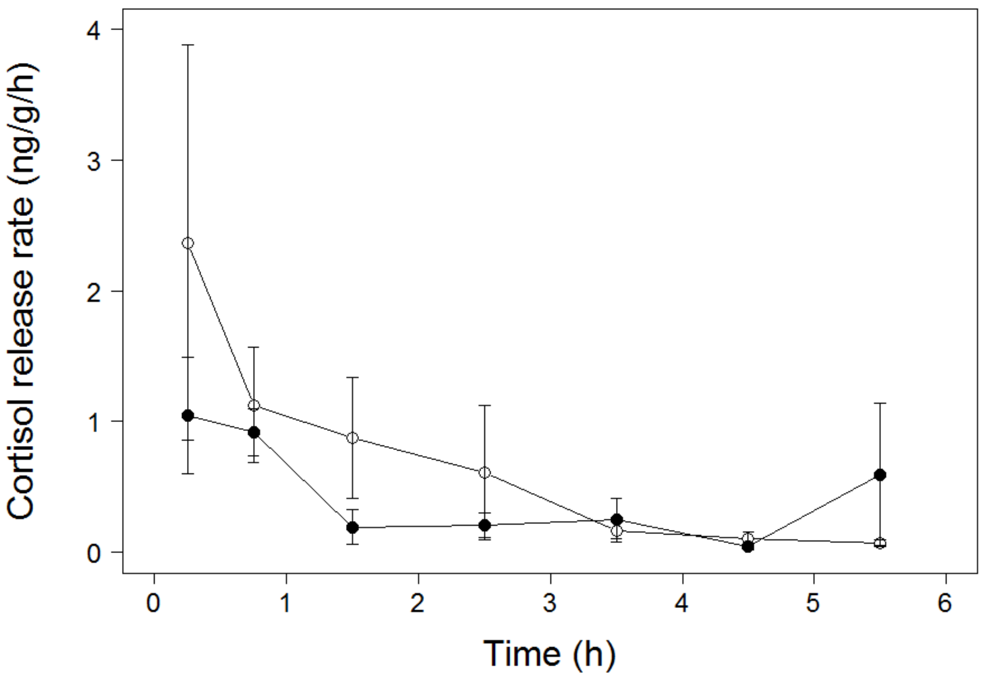

Figure 3.4. Mean ± S.E. release rates (without extreme values) of cortisol into the water in neon tetra tanks after exposure to a handling stressor at 0h (open circles) (*n* = 5) or no stress (filled circles) (*n* = 3). Values are plotted at the midpoint of the time interval that release rates were measured over.

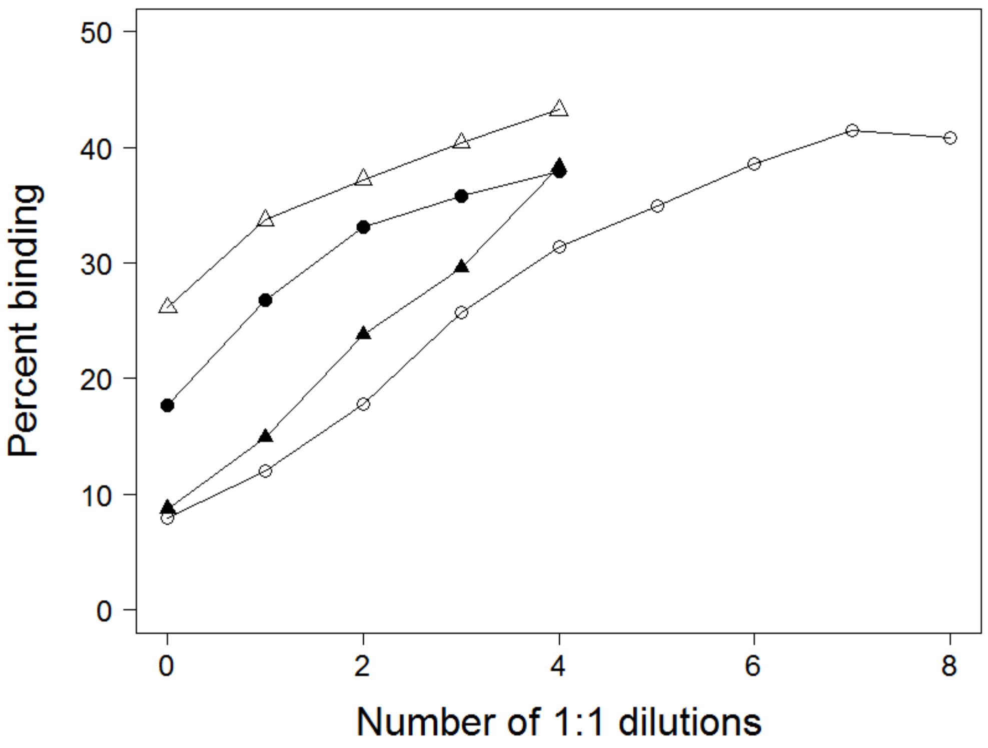

Figure 3.5. Parallelism between cortisol standard curve (open circles) and serial 1:1 dilutions of cortisol extracts from guppies (filled triangles), mollies (filled circles) and neon tetras (open triangles).

## 3.4 Discussion

The concentration of cortisol in water samples collected from tanks containing fish was consistently above the assay's lower detection limit of 2 pg 100 $\mu L^{-1}$, but no background activity (i.e. cortisol present in the water before coming into contact with fish) was detected. The slopes of the cortisol standard curve and the dilution curves of samples from water containing guppies, mollies or neon tetras diluted parallel, indicating little interference from other immunoreactive substances (Felix *et al.*, 2013). Demonstrating a lack of background activity, presence of detectable levels of cortisol, and parallelism between extracts and standards are all considered key objectives when validating this method according to Scott *et al.* (2008).

Both guppies and mollies in this study showed a clear peak in cortisol release rate relative to the end of the sampling period within 30 minutes of being exposed to a netting stressor, with release rates levelling out approximately 1.5 hours after the beginning of sampling. However, no significant differences were found between net-handled and unstressed groups in either species. There are two possible explanations for this: either the group which was exposed to the net stressor did not experience an increase in cortisol release rate, and the measured rates simply reflect diurnal changes in cortisol release rate, or the group which was not exposed to the net stressor experienced some other form of stress during sampling and thus also showed an increase in cortisol release rate. Of these two explanations, the second is more likely: cortisol release rates over the first 30 minutes of sampling were markedly higher than release rates between 2 and 6 hours after the beginning of the sampling period for both guppies and mollies. This may suggest that release rates peaked in response to stress, but that fish in 'unstressed' tanks were responding to a different form of stressor. Whilst efforts were made to ensure that fish in 'unstressed' tanks were not in visual contact with other tanks or with the experimenter during the sampling procedure by placing barriers between tanks and in front of tanks, it is possible that gaps in the barriers were present, allowing the unstressed fish to view either stressed conspecifics or the experimenter. Fish in unstressed tanks may also have responded to vibrations or noise disturbance occurring during sampling, although attempts were made to keep these to a minimum by only allowing the experimenter to enter the room where fish were housed during sampling and moving quietly whilst in the room. Furthermore, personal observations of fish exposed to the netting stressor indicated that fish showed behavioural signs of stress, such as darting and freezing, and it is unlikely that these behavioural signs of stress would not be reflected in cortisol release rates.

No significant differences in cortisol release rates were found between treatment groups in neon tetras. Although this was initially thought to be due to the presence of a number of samples with an unusually high concentration of cortisol, removal of these extreme samples did not change this result. It is possible that, as with the guppies and mollies, fish in the ‘unstressed’ condition were responding to an unintended source of stress; however, it is more difficult to establish whether this was the case as there was also no significant effect of sampling time point. It is therefore not clear whether cortisol release rates peaked soon after exposure to the netting stressor relative to the end of the sampling period. Taken together, the results of this study go some way towards validating the water-borne cortisol measurement technique in domestic guppies and mollies, but further work would be needed to consider this technique validated in neon tetras.

A substantial amount of variation in cortisol release rates, especially in the ‘stressed’ groups, was seen in all three species in this experiment. Although the sample size used here was higher than the sample size of 3 used in many previously published studies (Ellis *et al.*, 2004; James *et al.*, 2004; Zahl *et al.*, 2010; Zuberi *et al.*, 2011), larger sample sizes may have helped to clarify the results. In particular, although the difference between ‘stressed’ and ‘unstressed’ mollies was not significant ($p$ = 0.09), the pattern of release rates shown in Figure 3.2 are similar to those that would be predicted if ‘stressed’ mollies had shown a response to a netting stressor.

The cortisol release rates found in this study are roughly within the scope of release rates in stressed fish reported by other studies (e.g. 9.6 ng $g^{-1}$ $h^{-1}$ in zebrafish (Gronquist and Berges, 2013), 0.628 ng $g^{-1}$ $h^{-1}$ in rainbowfish *Melanotaenia duboulayi* (Zuberi *et al.*, 2011), 1.5 ng $g^{-1}$ $h^{-1}$ in sticklebacks *Gasterosteus aculeatus* (Fürtbauer *et al.*, 2015), 0.13 pg $g^{-1}$ $h^{-1}$ in Panamanian bishops *Brachyrhaphis episcopi* (Archard *et al.*, 2012) and approximately 1.5 ng

$g^{-1}$ $h^{-1}$ in rainbow trout *Oncorhynchus mykiss* (Ellis *et al.*, 2004). However, direct comparisons of release rates between studies are difficult due to variation in the intensity of stressors, differences in units reported (e.g. some papers report ng standard length$^{-1}$ $h^{-1}$ or ng fish$^{-1}$ $h^{-1}$) and species differences. The higher release rates seen in guppies in this study might suggest that the guppies experienced a stronger stress response to the netting stressor than mollies did. However, mortality tends to be more associated with the presence of chronic stress, and this study focussed on acute stressors. The species in this study might have different levels of responsiveness to chronic or repetitive stress. In all species, the highest cortisol release rates were seen in the first 30 minutes after the stressor, suggesting that the optimum time to sample these species after the onset of a stressor is within the first 30 minutes. This peak occurs faster than has been reported for various other species, except for zebrafish, which also showed a cortisol peak in the first 30 minutes post-stressor (Felix *et al.*, 2013). In other species, peak cortisol release rate has been reached between 30 minutes and 1 hour after stress in rainbow trout (Ellis *et al.*, 2004), between 1 and 1.5 hours after stress in sticklebacks (Sebire *et al.*, 2007), and carp *Cyprinus carpio* (Lower *et al.*, 2005), approximately 2 hours after stress in wild-caught rainbowfish, (Zuberi *et al.*, 2011), between 2 and 4 hours after stress in roach, *Rutilus rutilus* (Lower *et al.*, 2005), or after more than 4 hours in captive-bred rainbowfish (Zuberi *et al.*, 2011). This variation in release rates may reflect differences in metabolic rate, which is affected by factors such as fish body size and temperature (Barton, 2002). The variation in peak cortisol release rate across these different species also highlights the necessity of conducting time-course studies in order to determine when best to sample fish.

Whilst the results of this study can help to increase the number of techniques and approaches to studying stress in ornamental fish, there are a number of considerations that must be made. As mentioned above, this study examined the effect of a single, acute stressor on cortisol release rates. However, immunosuppression and mortality are more usually thought to be caused by chronic or repetitive stressors (Wendelaar-Bonga, 1997). Measuring cortisol in holding water may therefore be of use when looking at the response of fish to single, stressful events, such as transportation, but less so when considering the effects of chronic stress, such as that caused by bullying.

Indeed, some studies have shown that whole-body or plasma cortisol levels were not significantly different from controls after chronic stress, an effect which is likely to be mirrored in the water-borne cortisol release rates (O'Connor *et al.*, 2011; Aerts *et al.*, 2015). Alternative approaches may therefore be needed to examine the effects of chronic stress in fishes, such as cortisol content in in fish scales (Aerts *et al.*, 2015), or the presence of or changes in certain protein biomarkers (Alves *et al.*, 2010). Unfortunately, none of these approaches are likely to be useful in the day-to-day monitoring of stress and welfare within the supply chain, as they require specialist knowledge and equipment. Therefore, behavioural measures of stress are also very useful when studying stress in ornamental fish. Behavioural measures also have the advantage of being able to indicate the valence of a response – an increase in cortisol release rate may indicate arousal in response to a non-negative stimulus, such as anticipation of feeding (Sánchez *et al.*, 2009). Utilising a range of techniques appropriate to the type of stressor being studied is therefore likely to be the best approach to reducing stress in ornamental fish.

In conclusion, this study has shown that guppies, mollies and neon tetras all show detectable levels of cortisol release after being exposed to a stressor, that these extracts diluted parallel to standards, and that there was no background activity in the water provided to fishes. Whilst the study did not find clear differences between stressed and unstressed fish in cortisol release rate, it is possible that the higher cortisol release rates in guppies and mollies in the first 30 minutes of the sampling period reflects a stress response to a stressor. We can therefore consider these results as helping to validate the water-borne cortisol measurement technique in these species, although further work is needed to assess release rates in stressed and unstressed fish. The results were less clear for neon tetras as there was no clear peak in cortisol release rate or clear difference in release rate between treatment groups, suggesting that further work would need to be done before this procedure could be considered validated in neon tetras. These results help to strengthen the range of techniques that can be used to study stress in fishes, and may be of particular use in expanding research into stress and mortality in the ornamental fish trade.

# Chapter 4: The effect of a simple training instruction exercise on induced handling stress in the guppy, *Poecilia reticulata*

## Abstract

Fishes in the ornamental industry are frequently exposed to handling events. These handling events, which can involve chasing, confinement, and removal from water, can be deeply stressful, and it is possible that the stress response to these events may be exacerbated if the handler is untrained or unskilled. This study aimed to find whether a simple training procedure could improve handlers' skills enough to reduce the stress experienced by handled guppies *Poecilia reticulata*. Two experiments were conducted with slightly varying protocols. In both experiments, participants with no experience of handling fish were asked to watch either a short training video, which detailed best practice for capturing and handling a fish, or a control video, which provided no information on how best to capture the fish. Participants were then asked to catch fish from a tank. Cortisol release rates and behavioural measures of stress, including duration of freezing behaviour and latency to leave a refuge area, were measured in captured fish. In the first experiment, there was no significant difference in cortisol release rates. There were also no significant differences in stress-related behaviours in fish handled by trained or untrained handlers. In the second experiment there were no significant differences in behaviour of fish handled by a trained or untrained handler and due to a technical difficulty cortisol samples could not be processed. However, fish which were present in the test tank, but were not handled themselves, showed significant differences in stress-related behaviours between training conditions. These results suggest that handling technique may not have a major impact on stress in handled fish, but may be important for the fish remaining in the tank. This may have implications for fish in the ornamental industry, which are often housed at high stocking densities but usually sold in small groups, and therefore may be exposed to multiple handling events a day without being captured.

## 4.1 Introduction

Fishes in the ornamental industry are frequently exposed to handling events (Huntingford *et al.*, 2006; Pasnik *et al.*, 2010). Handling occurs as part of many routine husbandry procedures in the ornamental supply chain such as cleaning, sorting, and packing for transportation between breeders, exporters, wholesalers, retailers and consumers (Wabnitz *et al.*, 2003; Huntingford *et al.*, 2006; Prang, 2007). Handling is also necessary for less common practices, such as isolating a fish for medical treatment, or removing a fish from a group where bullying is occurring. However, handling events, which can involve chasing, confinement, capture, and removal from water, can be highly stressful to fish (Barton, 2000; Biswas *et al.*, 2006; Ramsay *et al.*, 2009b; Sneddon *et al.*, 2016).

Stress, a series of physiological, behavioural and psychological changes experienced by a fish in response to a challenge to homeostasis (a stressor) (Wendelaar-Bonga, 1997; Barton, 2002), is usually considered to be adaptive. However, fishes may experience an acute behavioural or psychological response (anxiety or fear-like responses) to a stressor that contributes to reduced welfare (Schreck *et al.*, 1997; Nilsson *et al.*, 2012; Thompson *et al.*, 2016), and when stressors are particularly severe, repetitive, or long-lasting, adverse physiological consequences such as reductions in growth and reproductive rate, immunosuppression, and even death, due to decreased disease resistance (McCormick *et al.*, 1998; Davis *et al.*, 2002; Iguchi *et al.*, 2003). Handling can induce both an acute, severe stress response to traumatic handling events, and a chronic stress response due to the repetitiveness of handling in fish husbandry (Pickering *et al.*, 1982; Schreck *et al.*, 2001; Falahatkar *et al.*, 2009; Ramsay *et al.*, 2009b). These responses may be exacerbated if the handler is untrained or unskilled. Handling may also cause physical damage to the fish, including disruption of the scales or mucous layer which can increase the likelihood of contracting infectious diseases (Conte, 2004).

To reduce these detrimental effects, moving fish under containment in water has been suggested to best support fish welfare, and this approach is adopted in some aquaculture practices, moving fishes though the use of pump systems and transfer piping (Farm Animal Welfare Council, 1996; Ashley, 2007; Robb, 2008). These techniques do not involve chasing and limit the amount of confinement experienced by the fish. However, handling with dip-nets is still commonplace for small ornamental species and fish in laboratory settings, as it is an easy, cheap and convenient way to handle and move fishes. Therefore, there is a need to develop interventions to reduce the stress associated with handling to improve the health and welfare of fishes used in the ornamental trade and in research.

One way in which induced handling stress might be reduced is through improved training of staff. The ornamental fish supply chain may involve many different stages, and the handling conducted at each stage may be done by workers who lack training or appropriate fish handling experience and understanding (Wabnitz *et al.*, 2003; Prang, 2007). This study therefore aimed to find whether a short instructional video would be an appropriate training tool for handlers in order to reduce stress in net-handled fishes. To investigate this, participants with no previous experience of handling fishes were asked to catch guppies *Poecilia reticulata* from a tank after viewing either a training video, or a control video. Guppies were used in the experiment as they are a highly popular ornamental species (P Carey, 2014, pers. comm.), but are also widely used in laboratory research (Croft *et al.*, 2003; Magurran, 2005; Fraser *et al.*, 2011; Zandonà *et al.*, 2017). It was predicted that fish that interacted with a trained handler would show lower stress levels than fish exposed to an untrained handler. Following netting by the handler I recorded both behavioural and physiological signs of stress using a non-invasive measure of cortisol release and direct behavioural observations of the test fish when exploring a novel arena. In addition, it is possible that the remaining fish in the tank that were not netted but were exposed to the netting event may experience increased stress due to chasing with the net. Therefore, to investigate this, behavioural measures of stress for fish that were left in the experimental tank after the handling event took place were also taken (non-handled fish). A number of behavioural measures were used in both experiments as, unlike physiological measures, behaviours can provide information about the valence of a response. For example, although food anticipatory behaviour is not necessarily considered an indicator of poor welfare, it can lead to increased cortisol levels (Sánchez *et al.*, 2009). The behavioural measures used in this experiment included refuge use, as increased latency to leave a refuge and greater use of a refuge is associated with less risk-taking and more anti-predator behaviour, and therefore may indicate higher stress levels (Templeton and Shriner, 2004; Brydges *et al.*, 2009; Saxby *et al.*, 2010). Freezing behaviour, general activity levels and number of erratic movements performed were all recorded, as both lethargy and hyperactivity (which often involves erratic, darting movements) are associated with higher stress levels in fish (Pasnik *et al.*, 2010). Thigmotaxis, or the tendency of the fish to remain close to the walls of the tank, was recorded as it is associated with searching for shelter or escape routes, so a greater duration of thigmotaxis was thought to indicate higher stress levels (Champagne *et al.*, 2010). Finally, the latency of fish to feed after food was introduced into the tank was recorded, as greater latencies to feed may indicate a greater perception of risk, and thus, higher stress levels (Moretz *et al.*, 2007).

## 4.2 Methods

Two experiments were conducted. In the first experiment (Experiment 1), handlers watched either a training video detailing best practice for catching and handling fish, or a control video which provided no information on how to best capture the fish. Participants then captured three fish from a tank. The first of these fish was placed into a novel arena for behavioural testing, the second was placed into a beaker of water for cortisol collection, and the third was returned to its home tank. In the second experiment (Experiment 2), the same approach was adopted as the first, but with a number of modifications to the methodology.

Firstly, in Experiment 1, the fish had to be moved to a different room from where they had been housed for the experimental testing, which was likely to have been stressful. It was possible that the stress of transporting the fish might mask the stress response caused by handling, therefore in Experiment 2, all trials were run in same room in which the fish were housed.

Secondly, in Experiment 1, three fish were captured from the experimental tank so that the effects of the repetitive handling procedure on the fish which remained in the experimental tank could be measured. However, as the third captured fish was returned to the experimental tank, it was possible that its behavioural response to handling might have impacted the behavioural responses of the non-handled fish. It was therefore decided that two netting events would still cause non-handled fish to experience exposure to repeated handling events, but would avoid causing undue stress to the third captured fish. This would also avoid the behaviour of a handled fish influencing the non-handled fishes’ behaviour.

Thirdly, new recordings of the training videos were produced for Experiment 2. This was done due to concerns over the clarity of the explanation of how to catch fish in Experiment 1. The voiceovers in Experiment 1 were also recorded in the aquarium in which the fish were housed, therefore background noise from aquarium pumps was present. The voiceovers for the videos in Experiment 2 were recorded in a different room with no background noise, as there were concerns that this noise may have been distracting to viewers.

Finally, behavioural observations in Experiment 1 were carried out for three minutes for handled fish, three minutes for non-handled fish, and for three minutes after introducing food to the non-handled fish. However, there were concerns that this length of observation time would be too short to observe differences in stress-related behaviours between fish in the different training groups. For example, if fish in the different groups showed the same initial behavioural responses to handling, but one group showed faster recovery, three minutes might not be enough to capture these differences. Therefore, in Experiment 2 the observation periods were extended to ten minutes for handled fish, and five minutes for non-handled fish followed by five minutes after the introduction of food for non-handled fish.

### *4.2.1 Experiment 1*

#### *4.2.1.1 Study species*

The fish used were laboratory-bred guppies, the mixed-generation descendants of wild-caught guppies collected in the lower part of the Aripo River on the island of Trinidad (N 10°39’03’’; W 61°13’40’’). A total of 204 fish were size-matched by eye and sorted into mixed-sex groups of 6 (3 males, 3 females per group; 34 groups). Groups of fish were randomly allocated to either a ‘trained’ condition (*n* = 17), in which participants handling the fish first watched a training video or a ‘not trained’ condition (*n* = 17), in which participants watched a control video. Groups of fish were housed in aquaria measuring 230 x 160 x 175 mm at approximately 25°C, 12L:12D, and fed flake food and *Artemia* nauplii *ad libitum* twice a day. They were allowed at least 7 days in these groups to acclimatise to their new environment and each other before being used in the experiment.

#### *4.2.1.2 Training videos*

Two training videos were created, each 1.5 mins in length. The control video showed a tank of six fish swimming freely, with a voiceover describing basic aspects of guppy biology and behaviour. The experimental video showed the same tank of six fish with a handler catching each fish in a net, one at a time. The voiceover for the experimental video described best practice for catching fish, such as using slow, calm movements and avoiding sudden movements.

#### *4.2.1.3 Participants and procedure*

Participants were recruited voluntarily from staff and students within the department of Psychology, University of Exeter. The majority (26/34) were first-year undergraduates, the remainder were postgraduate students and members of staff. Participants were not informed of the aims of the experiment but were recruited with the stipulation that they should not have owned pet fish, or have similar experience in handling fish (e.g. no experience working in a pet store or working with fish as research animals).

Prior to the start of each trial, groups of guppies were moved from their housing tank to an experimental tank (395 x 240 x 310 mm) containing a layer of gravel and a small plastic plant. These conditions were chosen to mimic the conditions that might be present in a pet shop fish tank, and also to present enough of a challenge to participants that the fish would not be instantly caught. The tank was marked with a grid dividing the tank into 15 equally-sized zones to measure activity of the fish after each trial. Each group of fish was allowed a minimum of ten minutes to acclimatise to the experimental tank before they were handled – acclimatisation periods of two minutes have previously been used in studies of guppies (Jacquin *et al.*, 2016; Cattelan *et al.*, 2017) and 5 minutes has been recommended for zebrafish and other small teleosts (Wright and Krause, 2006), so a minimum period of ten minutes was thought to be appropriate.

Participants were shown either the control video or the experimental video. They were then presented with the experimental tank and asked to catch three fish with a dip-net. One of these fish was then placed into a glass beaker containing filtered water for cortisol collection, one was placed into a tank for behavioural observations, and the third was returned to its home tank.

#### *4.2.1.4 Cortisol collection and analysis*

Cortisol release rates of fish were measured non-invasively using the technique described by Ellis *et al.* (2004). Briefly, this involved placing individual fish in acid-washed glass beakers containing approximately 200 mL filtered water for 30 minutes (± 3 minutes). The fish were kept in visual contact with other fish at all times to minimise additional stress caused by isolation. After 30 minutes, the fish were removed from the water and returned to their home tanks.

Cortisol was extracted from water samples using Sep-pak C18 solid phase extraction cartridges. Cartridges were primed with a wash of 5 mL methanol, followed by 5 mL distilled water. Samples were then pumped through the cartridges using a Watson-Marlow vacuum pump at an approximate rate of 10 mL min$^{-1}$. After pumping, cartridges were washed with 5 mL distilled water, then 40 mL of air was pushed through with a syringe to remove excess moisture. Cartridges were wrapped in Parafilm and frozen at –80°C until they could be eluted. To elute cortisol from the cartridges, they were thawed, then rinsed with 4 mL ethyl acetate into a 4 mL silanized glass vial. The eluent was dried under a nitrogen stream and the resulting residue was resuspended with 1 mL RIA buffer (0.5 M phosphate buffer with added sodium chloride, EDTA, bovine serum albumin and sodium azide).

The amount of cortisol in each sampled was determined by radioimmunoassays (RIA), following the protocol of Ellis *et al.* (2004). 100 µL of sample were added to tubes in duplicate, and a standard curve was created using a known concentration of cortisol. The standard curve ranged from 2 to 500 pg 100 µL$^{-1}$. A cocktail containing approximately 6000 dpm of radiolabelled cortisol, enough antibody to bind 50% radiolabelled cortisol in the absence of sample cortisol, and RIA buffer was added to the tubes, which were left overnight to bind. The next day, 1 mL dextran-coated charcoal suspension was added to tubes to separate out unbound steroid for 30 minutes, then tubes were centrifuged and the supernatant decanted into a scintillation vial. 7 mL of scintillation cocktail was added to each vial, and the tubes were read in a Beckman-Coulter LS6500 liquid scintillation counter for 5 minutes each. The standard curve was used to determine the amount of cortisol in each tube, and measures were averaged across duplicate tubes. The exact duration of each sampling period was then used to convert these measures to the amount of cortisol released in ng fish$^{-1}$ hr$^{-1}$.

#### *4.2.1.5 Behavioural observations*

Fish that had been captured (handled fish) were moved to an open-field tank (285 x 460 x 140 mm) in which one corner (142.5 x 115 mm) contained gravel and a plastic plant to serve as a shelter area. The tank was marked with a grid to divide it into 16 equally-sized zones to measure activity, and a line was marked 20 mm from the edge of the tank to delimit the edge zone. Fish were released into the shelter area and observed for three minutes (Figure 4.1).

Fish that were left in the experimental tank (non-handled fish) were observed for three minutes after the end of the netting period, then were provided with a small amount of aquarium flake food before being observed for a further three minutes.

Videos of each trial were recorded and Solomon Coder software (available at https://solomoncoder.com/) was used to extract several different behavioural measures from the videos (Table 4.1). All behavioural measures were chosen on the basis that they were used in other experiments on small fishes to record anxiety-like or antipredator behaviours, or as measures of boldness. For observations of non-handled fish, one of the three guppies remaining in the experimental tank was selected at random and designated the focal fish; behavioural measures were taken from observations of this focal fish.

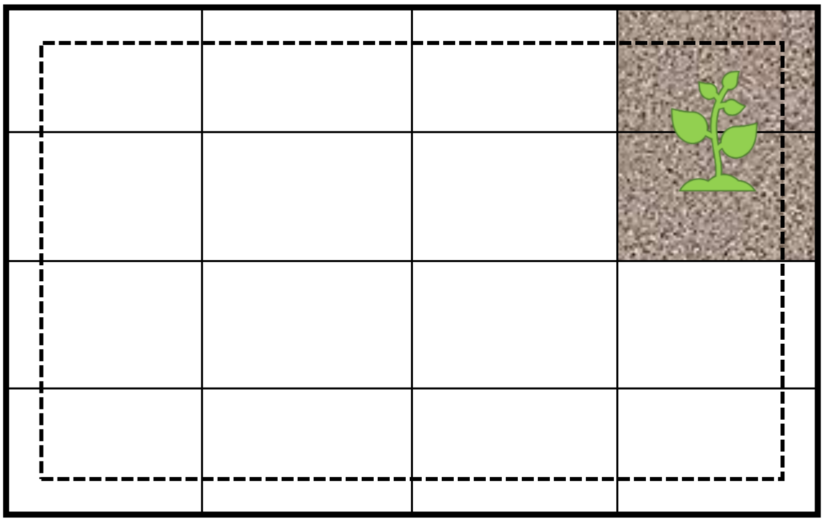

Figure 4.1. Diagram of novel test arena for measuring stress-related behaviours. Shaded area with plant indicates the refuge area. Dotted line was used to measure thigmotaxis. Gridlines were used to measure activity levels.

Table 4.1. Behavioural measures of stress recorded for handled and non-handled fish.

| Measure | Description |
|---|---|
| **Handled fish** | |
| Latency to leave refuge | The length of time between the fish being introduced into the arena and the fish leaving the refuge area. Higher latencies to leave a refuge are thought to indicate lower boldness (i.e. lower propensity to engage in risky behaviour), which may be linked to higher stress levels (Brydges *et al.*, 2009; Jacquin *et al.*, 2016). |
| Time spent in refuge | The total amount of time the fish spent in the refuge area. Shelter use has been shown to be an anti-predator behaviour in guppies (Templeton and Shriner, 2004), therefore greater time spent in the refuge is thought to indicate higher stress levels. Also, this area was the only enriched part of the tank – environmental enrichment use may be context-dependent, but as fish were introduced to the tank here, may indicate a decreased tendency to explore the tank, which also may be indicative of stress (Saxby *et al.*, 2010). |
| Duration of freezing bouts | The length of time the fish spent without moving except for gills (Egan *et al.*, 2009; Archard *et al.*, 2012). Freezing behaviour is an anti-predator response shown by guppies (Templeton and Shriner, 2004) and has been shown to decrease in Endler’s guppies *Poecilia wingei* in response to exposure to citalopram, an anxiolytic (Olsén *et al.*, 2014). Greater durations of freezing bouts were therefore thought to be indicative of higher levels of stress. |
| Thigmotaxis | The length of time the fish spent engaging in thigmotaxis (the tendency to hug the walls of the |

| | |
|---|---|
| | tank), measured as the amount of time the fish spent in the edge zone (Figure 4.1). Thigmotaxis is associated with searching for shelter or escape routes, therefore greater durations of thigmotaxis are thought to indicate higher levels of stress (Champagne *et al.*, 2010). |
| Activity | The number of gridlines crossed by the fish during the trial (Figure 4.1). Reduced activity levels are thought to indicate higher levels of stress – for example, in Panamanian bishops *Brachyrhaphis episcopi*, fish from higher-predation populations appeared to be less reactive to stress, as they had lower cortisol release rates and were more active than those from low-predation populations (Archard *et al.*, 2012). |
| Erratic movements | The number of movements made by the fish that showed a rapid change in direction or swimming speed (Egan *et al.*, 2009). This type of darting movement may be associated with being chased or avoiding a predator, and therefore is thought to indicate higher levels of stress (Saxby *et al.*, 2010). |
| **Non-handled fish** | |
| Activity | As above. |
| Duration of freezing bouts | As above. |
| Latency to feed | The length of time between food being introduced into tank and the focal fish beginning to feed. Increased latency to feed may indicate lower boldness and/or increased perception of risk (Moretz *et al.*, 2007; Saxby *et al.*, 2010). Increased latency to feed was therefore thought to indicate higher levels of stress. |

### *4.2.2 Experiment 2*

#### *4.2.2.1 Study species*

The fish used in this experiment were from the same population of laboratory-bred descendants of wild-type fish as used in Experiment 1. A total of 250 fish were size-matched by eye and sorted into mixed-sex groups of 5 (2 males, 3 females; 50 groups). Groups of fish were randomly allocated to either a ‘trained’ condition (*n* = 25) or a ‘not trained’ condition (*n* = 25), as above. Fish were housed and fed according to the same procedures as for Experiment 1.

#### *4.2.2.2 Procedure*

The training videos used in this experiment were slightly shortened versions of the videos used in Experiment 1 (1 min in length). Participants were also only asked to catch two fish from the tank, rather than three (see above).

Behaviours of handled fish were observed for ten minutes, and behaviours of non-handled fish were observed for five minutes, before flake food was introduced and the fish observed for a further five minutes. All other procedures were the same as in Experiment 1. Water samples for cortisol collection were also collected.

### *4.2.3 Data analysis for both experiments*

The difference in the time taken to catch the fish between trained and untrained handlers in each experiment was analysed with an independent samples Student’s t-test. Cortisol release rates were analysed with a generalized linear model (GLM) assuming a Gamma distribution and log link for the response variable as the response variable was continuous but zero-bounded (Thomas, 2015).

All behavioural measures were analysed using a multivariate approach to avoid increased risk of making a type I error (Field *et al.*, 2012). Multivariate analyses were therefore carried out on the effects of training group on behavioural measures in handled fish in Experiment 1, behavioural measures in non-handled fish in Experiment 1, behavioural measures in handled fish in Experiment 2, and behavioural measures in non-handled fish in Experiment 2. The time taken for the handler to net the fish was included in each model, and terms were added sequentially with netting time included first, therefore the results can be interpreted as the effect of training group after controlling for netting time. In all cases, the data did not meet the assumptions of multivariate normality and homogeneity of covariance matrices, therefore permutational multivariate analyses of variance (PERMANOVA) were performed using the ‘adonis’ function in the ‘vegan’ package in R – this form of analysis can be viewed as a non-parametric MANOVA (Anderson, 2001; McArdle and Anderson, 2001; Oksanen *et al.*, 2018). Where the results of the PERMANOVA were significant, post-hoc univariate permutational ANOVAs were conducted in the ‘adonis’ package to see the effect of training group on individual response variables. All analyses were completed in R ver. 3.4.1 (R Development Core Team, 2015).

### 4.2.4 Ethics

These experiments were approved by the University of Exeter College of Life and Environmental Sciences Ethics Committee. Human participants were not informed of the purpose of the study beforehand, but were fully debriefed once the study was completed and were told of their right to withdraw at any time. An experimenter remained close by whilst participants completed the study to step in if the participant showed any signs of distress, but this was not necessary for either experiment. Any trials in which netting took more than ten minutes were terminated to avoid causing undue distress to fish – this occurred once for both Experiments 1 and 2. Throughout this study, the ASAB/ABS Guidelines for the treatment of animals in behavioural research were followed. Fish were monitored daily for signs of stress or poor welfare.

## 4.3 Results

Netting time was not found to be a significant predictor of stress-related behaviours in any model ($p > 0.05$). Trained handlers did take significantly longer (212.7 s) than untrained handlers (54.1 s) to catch fish in Experiment 1 ($t = 3.716$, df = 18.306, $p = 0.002$)(Fig. 4.2). However, in Experiment 2, there were no significant differences between trained and untrained handlers (trained: 109.4 s, untrained: 77.5; $t = 1.351$, df = 37.479, $p = 0.185$).

### *4.3.1 Experiment 1*

There was no significant effect of training group (GLM: $F_{1, 18} = 0.346$, $p = 0.582$; Fig. 4.3) on cortisol release rate. There was no effect of training group on behavioural measures of stress in either handled fish (PERMANOVA: $F_{1, 27}$ = 1.212, $p$ = 0.320; Fig. 4.4) or fish that had not been handled ($F_{1, 19}$ = 0.129, $p$ = 0.847; Fig. 4.5).

### *4.3.2 Experiment 2*

The cortisol samples that were collected for this experiment could not be analysed due to a freezer malfunction, so only behavioural measures are presented here.

There was no effect of training group ($F_{1, 46}$ = 0.245, $p$ = 0.808; Fig. 4.3) on behavioural measures of stress in handled fish. However, there was a significant effect of training group on behavioural measures of stress in fish that had not been handled ($F_{1, 41}$ = 3.253, $p$ = 0.039; Fig. 4.5). Separate univariate permutational ANOVAs on the response variables showed that there was a trend towards an effect of training group on activity levels in non-handled fish ($F_{1, 41}$ = 3.585, $p$ = 0.064; Fig. 4.5a). There was a significant effect of training group on total duration of time spent frozen in non-handled fish ($F_{1, 41}$ = 4.150, $p$ = 0.042; Fig. 4.5b), but no significant effect of training group ($F_{1, 41}$ = 1.330, $p$ = 0.250; Fig. 4.5c) on the latency to feed after food was introduced into the tank.

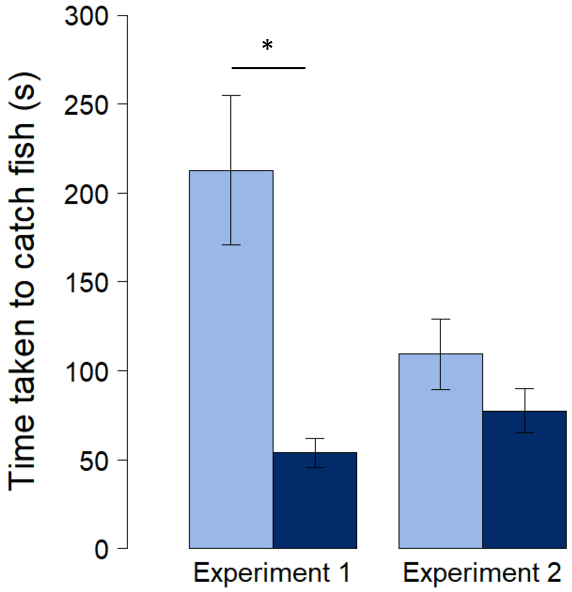

Figure 4.2. Time taken for handlers to catch fish in Experiments 1 and 2. Light blue bars show time taken for trained handlers (Experiment 1: *n* = 17; Experiment 2: *n* = 25), dark blue bars show time taken for untrained handlers (Experiment 1: *n* = 17; Experiment 2: *n* = 25). Bars represent means, error bars represent ± 1 S.E.

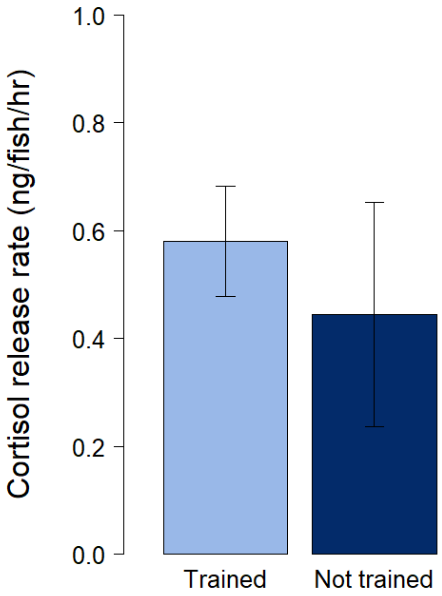

Figure 4.3. Cortisol release rate of fish handled by trained (light blue, *n* = 17) and untrained (dark blue, *n* = 17) handlers. Differences between bars are not significant at *p* = 0.05. Bars represent means; error bars represent ± 1 S.E.

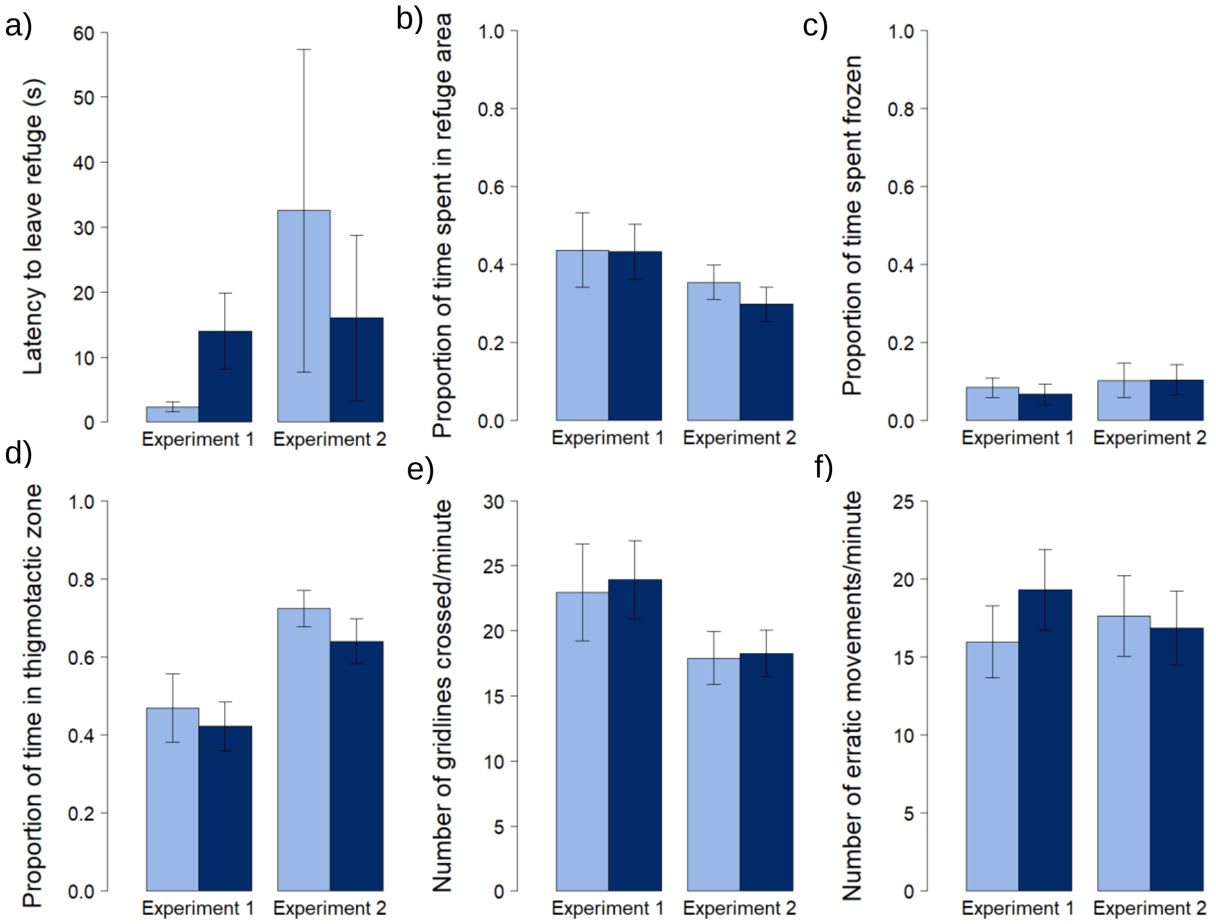

Figure 4.4. Behavioural measures of stress in handled fish in Experiments 1 and 2. Light blue bars show fish caught by trained handlers (Experiment 1: *n* = 17; Experiment 2: *n* = 25), dark blue bars show fish caught by untrained handlers (Experiment 1: *n* = 17; Experiment 2: *n* = 25). Latency to leave the refuge (a) is presented as raw data as it was not affected by trial length. The amount of time spent in the refuge area (b), the amount of time spent frozen (c), and the amount of time in the thigmotactic area (d) are presented as proportions of total trial time. The number of gridlines crossed (activity; e) and the number of erratic movements (f) were divided by the length of the trial to give measures per minute. Bars represent means, error bars represent ± 1 S.E.

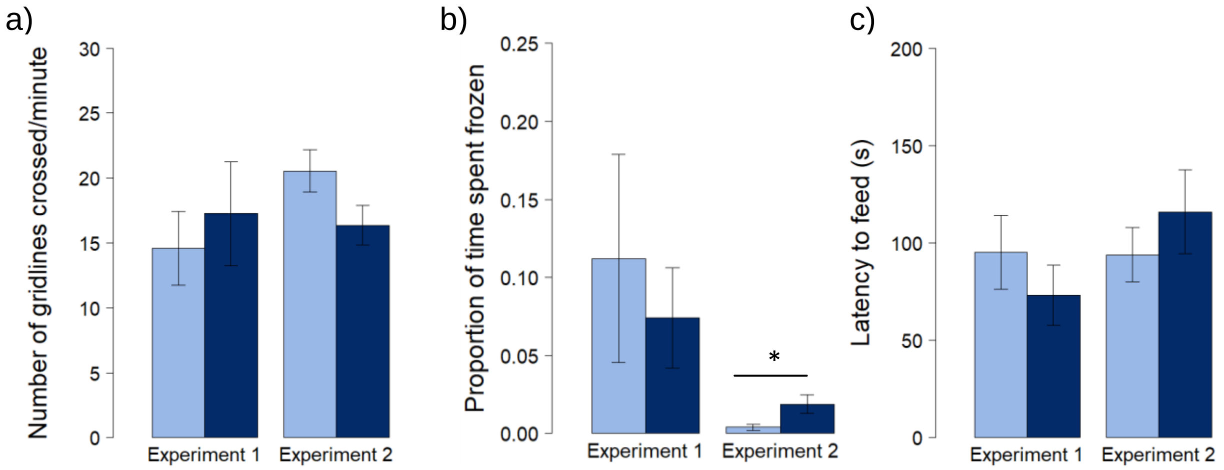

Figure 4.5. Behavioural measures of stress in non-handled fish in Experiments 1 and 2. Light blue bars show fish exposed to trained handlers (Experiment 1: *n* = 17; Experiment 2: *n* = 25), dark blue bars show fish exposed to untrained handlers (Experiment 1: *n* = 17; Experiment 2: *n* = 25). The number of gridlines crossed (activity; a) was divided by the trial length to give the number of gridlines crossed per minute, and the amount of time spent frozen (b) is presented as proportion of total trial time. Latency to feed (c) is presented as raw data as trial length did not affect this measure. Bars represent means, error bars represent ± 1 S.E.

### *4.3.3 Comparing Experiments 1 and 2*

As a significant effect of training group was found for non-handled fish in Experiment 2, but not in Experiment 1, it was useful to compare these data. The number of gridlines crossed in each experiment was divided by the length of the trial, and the amount of time the fish spent frozen was converted to a proportion of the total trial time for both experiments in order to allow direct comparisons between the two experiments. The latency of the fish to feed was not converted as this was not affected by the length of the trial. A further PERMANOVA on behavioural measures of stress in non-handled fish in Experiments 1 and 2 was then carried out.

There was a trend for behavioural measures of stress to differ between Experiments 1 and 2 for non-handled fish; however, this did not reach statistical significance ($F_{1, 63} = 2.755$, $p = 0.057$; Fig. 4.5).

## 4.4 Discussion

In Experiment 1, there were no differences in cortisol release rate between fish handled by trained or untrained handlers, and no differences in behavioural measures of stress for either handled or non-handled fish. In contrast, in Experiment 2, there were no differences in behaviour of handled fish between the training conditions, but there were differences in the non-handled fish – fish exposed to trained handlers showed a trend toward higher levels of activity, and a lower amount of time spent frozen than fish exposed to untrained handlers.

Cortisol release rates did not differ significantly between fish that had been netted by trained or untrained handlers. This may be because handling, which involves capture, confinement, and removal of the fish from water, generally is highly stressful, therefore any effect of method of capture may be outweighed (Davis and Schreck, 1997; Biswas *et al.*, 2006; Falahatkar *et al.*, 2009; Ramsay *et al.*, 2009b). These results are in agreement with those seen in Chapter 3, which did not find significant differences between stressed and unstressed groups of fish in cortisol release rate. This also appears to be in agreement with the behavioural measures of stress in handled fish – in both experiments, there were no differences in behaviour of fish handled by trained or untrained handlers, suggesting that handling is stressful regardless of technique. However, this finding was unexpected – for example, in Panamanian bishops, fish that experienced longer handling times had higher cortisol release rates, which might suggest that handling technique can affect stress levels (Archard *et al.*, 2012). Furthermore, handling time did not predict behavioural signs of stress in the same fish, indicating that the relationship between hormones and behaviour is not always clear-cut.

Handling time did significantly differ between training groups in Experiment 1, showing that the videos did result in a behavioural change in the human participants and thus may be an effective training technique. However, this also may affect a retailer’s willingness to introduce this procedure if there might be concerns over efficiency. There were no significant differences in handling time between training groups in Experiment 2, but as some differences in fish behaviour were found between the training groups, this lack of significant difference is more likely to be due to differences in how participants perceived the instructions in the video, rather than being due to a lack of behavioural change. This is positive when considering the likelihood of retailers adopting this training technique, as it shows that using this method of handling need not result in slower transactions during sales. Furthermore, although data on the techniques used by handlers was not collected, such as the number of times handlers chased the fish, I did note a tendency for trained handlers to use slower movements and to refrain from splashing, chasing and sudden movements, further supporting that the training videos resulted in behavioural changes in the human participants.

There were no differences in behavioural measures of stress for non-handled fish exposed to trained or untrained handlers in Experiment 1, but differences were found in Experiment 2. When these behaviours were examined in univariate analyses, fish exposed to trained handlers were found to spend less time showing freezing behaviour than those exposed to untrained handlers, and there was a trend towards fish exposed to trained handlers having higher activity, although this did not reach statistical significance. Freezing behaviour is an anti-predator response in guppies (Templeton and Shriner, 2004) and has been found to decrease in the presence of citalopram, an anxiolytic drug (Olsén *et al.*, 2014). Freezing behaviour, along with cortisol response, has also been shown to be lower in fish that have been trained to be able to predict a stressor based on visual cues compared with untrained fish (Galhardo *et al.*, 2011), and freezing behaviour and/or low activity are often considered behavioural indicators of stress in fish (Pottinger, 2008; Pasnik *et al.*, 2010). A longer duration of freezing behaviour in this experiment was therefore interpreted as indicating higher levels of stress. This result suggests that handling technique can influence stress levels in fish even if they are not themselves handled. This may be because some fish may be stressed if chased by the handler, even if they manage to avoid being captured. Other fish in the tank might, in turn, respond to behavioural cues from these fish, resulting in higher stress levels (Giacomini *et al.*, 2015). Stress may also be caused to non-handled fish if chased or captured fish secrete chemicals into the water when stressed which are detectable by other individuals (Barcellos *et al.*, 2011; Giacomini *et al.*, 2015).

The effect size of differences in behaviour of non-handled fish in this study was relatively small, but it is worth noting that this response was found after exposure to only two handling events. In the ornamental fish industry, especially in pet shops, fish may be housed at high stocking densities but may only be sold in relatively small groups; a fish may therefore be exposed to a high number of handling events before being captured themselves. The cumulative effects of these events may be more severe than those seen in this study, and so adoption of handling techniques which minimise stress to non-handled fish are likely to be of use within the ornamental fish industry.

The differences in the behaviour of non-handled fish between Experiments 1 and 2 may be explained by the differences in pre-experimental procedures; fish in Experiment 1 had to be moved from the aquarium in which they were housed to the room in which trials took place, which was probably a stressful experience, whereas fish in Experiment 2 were tested in the aquarium in which they had been housed. Repeated stressors have been shown to have a cumulative physiological effect in, for example, Chinook salmon *Oncorhynchus tshawytscha* and rainbow trout (Barton *et al.*, 1986; Ellis *et al.*, 2004), and a cumulative effect on behaviour in Chinook salmon (Sigismondi and Weber, 1988). It is therefore possible that the additional stress experienced by the fish in Experiment 1 masked any stress caused by exposure to handling for non-handled fish. When comparing the results of Experiments 1 and 2, which were adjusted according to trial length, there was a trend for non-handled fish to differ in their behaviour. Although this trend did not reach statistical significance, it indicates some support for differences in stress, as fish in Experiment 1 tended to spend more time showing freezing behaviour than fish in Experiment 2. This may also suggest that the prior experience of the fish is important – the impacts of milder stressors may only be noticed if the fish has not already experienced a more severe stressor, although when there are multiple more severe stressors, the effect may be cumulative (Jarvi, 1990).

The results of this study may be of use in informing the development of interventions to reduce stress in ornamental fish. As no evidence was found to support the hypothesis that a training procedure can reduce stress in handled fish, interventions which target some of the most stressful parts of handling, such removal of the fish from water, need to be developed. For example, a potential intervention that has been investigated is the use of scoops rather than nets to ensure fish are not removed from water during handling – this technique was found to cause lower ventilation rates in sticklebacks *Gasterosteus aculeatus* and Panamanian bishops but not in rainbow trout (Brydges *et al.*, 2009). This study has also shown that training may be of use in the ornamental fish industry, as only a minor training procedure was needed to cause differences in the behaviour of non-handled fish. Giving workers in the industry more extensive and targeted training, could therefore have marked impacts and help to reduce stress levels experienced by captive fish. Furthermore, handling is a repetitive procedure, used at every stage of the supply chain – interventions based around training workers throughout the supply chain could therefore have strong cumulative effects on reducing stress and improving welfare. Overall, the results of these studies suggest that training handlers before they catch fish can lead to fewer behavioural indicators of stress in non-handled fish, although the impacts of handling technique on fish may depend on the fish's prior stress levels.

# Chapter 5: Environmental preferences in neon tetras *Paracheirodon innesi*

## Abstract

Environmental enrichment is a broad term which covers any modifications made to the environment of a captive animal with the aim of improving that animal's welfare. Examples include provision of toys, a more structurally complex environment, addition of social partners, or temporal changes to the environment. It is widely recommended for animals in zoos, laboratories, and other animal keeping institutions in order to promote good welfare, and has been shown to reduce maladaptive behaviours, such as stereotypies, and stress. However, some studies have shown that modifications intended to be enriching can actually have neutral or negative effects on the animals. It is therefore necessary to test whether a given set of conditions results in behavioural or physiological indicators that suggest that those conditions are beneficial to health or welfare. Fishes within the ornamental industry supply chain may be held in barren tanks, or in tanks containing items selected to appeal aesthetically to consumers rather than because they benefit the fish's welfare, therefore the identification and introduction of appropriate enrichment may be an effective way to improve welfare. To identify conditions which may promote good welfare, a study of the environmental preferences of neon tetras *Paracheirodon innesi*, a popular ornamental species, was carried out. I hypothesised that fish would show preferences when presented with choices of environmental conditions. Groups of fish were presented with a series of preference tests; these consisted of two-chambered tanks containing different substrates, backgrounds or refuges. The location of the fish in each tank was recorded at different points over several days and used to calculate a preference index for condition within a pair. Neon tetras showed a preference for a tank background with an image of stones over either a plain blue background or a black background with images of plants, but did not show any preferences for any tank substrate, including different types of gravel and different colours, or any tank refuge, including a plastic plant, a pipe, and a shelter. These results suggest that neon tetras do show preferences for certain conditions over others, which might then inform future decisions about which modifications should be included in tests of welfare benefits.

## 5.1 Introduction

The presence of features considered to provide enrichment in the environments of captive animals is widely recommended to promote good welfare (Shepherdson, 2003; Young, 2013). Environmental enrichment can broadly be defined as any modifications made to an animal's environment with the overall aim of improving that animal's well-being (Newberry, 1995; Carlstead and Shepherdson, 2000; Näslund and Johnsson, 2016). These modifications will depend on the species or taxon of interest, but may include increasing the environmental complexity, adding shelters or other structural interest, provision of toys, different food options, sensory stimuli, addition of social partners, or changes to the environment over time.

The addition of enrichment to animals' environments can benefit welfare in a number of ways, including reduction of negative behaviours (i.e. behaviours associated with reduced welfare, or behaviours that may be maladaptive), reduction of stress or increased ability to cope with stress, or promotion of positive behaviours, such as increased space use in enclosures or a greater number of non-aggressive social interactions (Masefield, 1999; Carlstead and Shepherdson, 2000; Kells *et al.*, 2001). These benefits have been observed in many taxa, particularly birds and mammals (Young, 2013), but have increasingly also been observed in fish species. For instance, increasing habitat complexity by adding plants or woody debris has been associated with fewer aggressive interactions in zebrafish *Danio rerio* (Basquill and Grant, 1998) and brown trout *Salmo trutta* (Sundbaum and Näslund, 1998). Gilthead sea bream *Sparus aurata* showed less aggression as well as better growth performance and faster recovery from elevated serotonin, which is linked to acute stress, when housed in tanks with blue gravel compared with those housed with no gravel (Batzina *et al.*, 2014). Lower physiological and behavioural signs of stress have been observed in zebrafish provided with auditory enrichment (classical music) (Barcellos *et al.*, 2018), in Atlantic salmon *Salmo salar* provided with shelter and plastic substrate, and in rainbow trout *Oncorhynchus mykiss* housed with plants, gravel and shelter (Pounder *et al.*, 2016). Furthermore, benefits to captive animals from enrichment may go beyond welfare: enrichment can help promote growth, cognitive ability and post-release survival (Näslund and Johnsson, 2016).

Although there are definite welfare benefits that can be achieved through environmental enrichment, there are a number of potential problems associated with environmental modifications. Some studies have found that modifications intended to provide enrichment did not cause any change in behaviour, or led to behaviours considered to be negative for welfare. For example, zebrafish housed in tanks containing glass rods showed no differences in whole-body cortisol, physical activity or shoaling behaviour compared with fish housed without the rods (Wilkes *et al.*, 2012). Female juvenile rainbow trout provided with semi-transparent shelters showed greater signs of chronic stress than those housed without shelters (Landin, 2012). Other results found in enrichment studies which were not beneficial to the fish housed include higher stress and mortality in zebrafish housed with air stones compared to those without (Wilkes, 2011), and smaller brain sizes in Eastern mosquitofish *Gambusia holbrooki* housed in spatially enriched tanks as opposed to barren tanks (Turschwell and White, 2016). In mice *Mus musculus*, some cases of intended enrichment have been found to promote territorial or aggressive behaviours, possibly due to the introduction of a desirable resource (Nevison *et al.*, 1999; Howerton *et al.*, 2008), and similar effects have been observed in salmonid fish (I. Katsiadaki, 2015, pers. comm.) and ornamental species (Nijman and Heuts, 2000). More broadly, modifications such as the presence of gravel substrate in fish tanks can cause skin abrasions, harbour parasites or disease, or promote poorer water quality by making tanks more difficult to clean (Williams *et al.*, 2009). These potential problems highlight the importance of establishing whether environmental modifications actually have a positive impact on animals.

Despite the possibility that inappropriate environmental modifications can have negative effects, and the large numbers of fishes used in the ornamental fish trade, aquaculture and scientific research, enrichment for fishes has only been explored by a few studies, and little is currently known about the needs of individual species. Few studies have addressed enrichment for ornamental fishes; modifications made to tanks in pet stores or in home aquaria are likely to be chosen primarily to appeal aesthetically to the customer or the owner, rather than being based on evidence that they improve welfare. One approach that can be used to try and identify tank conditions that might promote welfare is the use of preference tests. These tests provide fish with access to two or more different forms of enrichment – for example, different colours of gravel – and allow the experimenter to see where fish preferentially spend most of their time. If fish more frequently occupy an area containing a particular type of enrichment, it can be assumed that this condition promotes better welfare than the alternative conditions available to them (Volpato *et al.*, 2007; Volpato, 2009). This approach is in accordance with the second of Dawkins' (2008) questions to help improve animal welfare: will it give the animals something they want?

To try and identify conditions that would promote good welfare in a common ornamental fish species, a series of preference tests were conducted on neon tetras *Paracheirodon innesi*. Neon tetras were chosen for this study as they are one of the most popular aquarium species, as they are relatively robust, non-aggressive, appropriate for community tanks and have attractive colouration (Chapman *et al.*, 1998; Roberts, 2010). Groups of fish were housed in two-chambered choice tanks, each chamber of which contained a different environmental condition. Fish were allowed to swim freely between the chambers, and their location in the tanks over several days was recorded. The conditions used were chosen on the basis of discussions with the retailer, in order to ensure that they would be relevant to fish in the ornamental fish trade. It was predicted that chambers containing a form of substrate, refuge, or naturalistic background (on the sides on the tank) would be preferable to barren chambers, or chambers with brightly-coloured walls.

## 5.2 Method

### 5.2.1 Study species

Forty-eight mixed-sex neon tetras were purchased from an ornamental fish and aquatics store and quarantined for 2 weeks prior to the start of the experiment. The fish were then sorted into groups of six fish (3 males, 3 females per group; $n = 8$) and moved into experimental tanks. Each experimental tank (395 x 240 x 310 mm) contained a translucent plastic divider with four circular holes (Ø = 58 mm) to divide the tank into two equal-sized chambers (Fig. 5.1). Groups were housed in these tanks for 1 week without experimental conditions being presented in order for them to acclimatise to the tanks. No evidence of a bias for a particular side of each tank was noted during this time based on experimental observations and observing fish during feeding. All tanks were maintained at 25 ± 1°C, 12L:12D. Fish were fed commercial flake food twice a day, with equal amounts of flake introduced simultaneously to each side of the divided tank to avoid influencing fish preferences.

### *5.2.2 Experimental conditions*

The experimental conditions presented to fish consisted of three different vinyl tank backgrounds, five different substrates, and three types of structural enrichment (Fig. 5.2). These conditions were chosen based on discussions with the retailer over the forms of enrichment used in their stores. The tank backgrounds consisted of a bright blue background which has previously been used as the standard backdrop for fish tanks at retailer stores, a background with a black base and pictures of plants which had been proposed as an alternative background for introduction into stores, and a naturalistic background depicting stones and rocks. The substrates consisted of white gravel, brown gravel, a gravel image, white plastic and black plastic – these were chosen as gravel is commonly used in retailer stores, but makes tanks more difficult to clean. The refuges chosen were a pipe, a rock-shaped shelter and a plastic plant as these are all forms of refuge seen in retailer stores. They were presented as a choice alongside a barren chamber, and also as choices between the different types of refuge, with the exception of the rock-shaped shelter, which could only be used in one set of trials (shelter vs pipe) as it was broken, and a similar alternative could not be found.

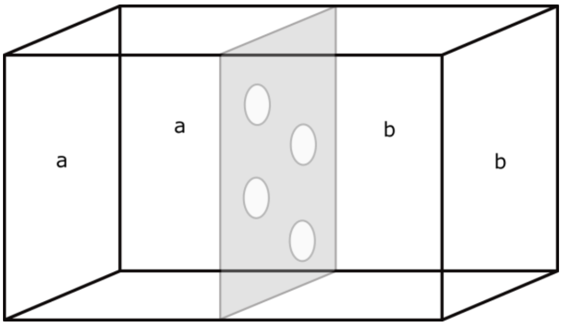

Figure 5.1. Diagram of the experimental tanks. Each tank was divided into two chambers with a semi-transparent plastic divider. The divider had four holes in it to allow free passage between the chambers. Fish were presented with pairs of conditions to choose from, one in each chamber. In the case of the tank backgrounds, these were attached to the walls labelled (a) and (b).

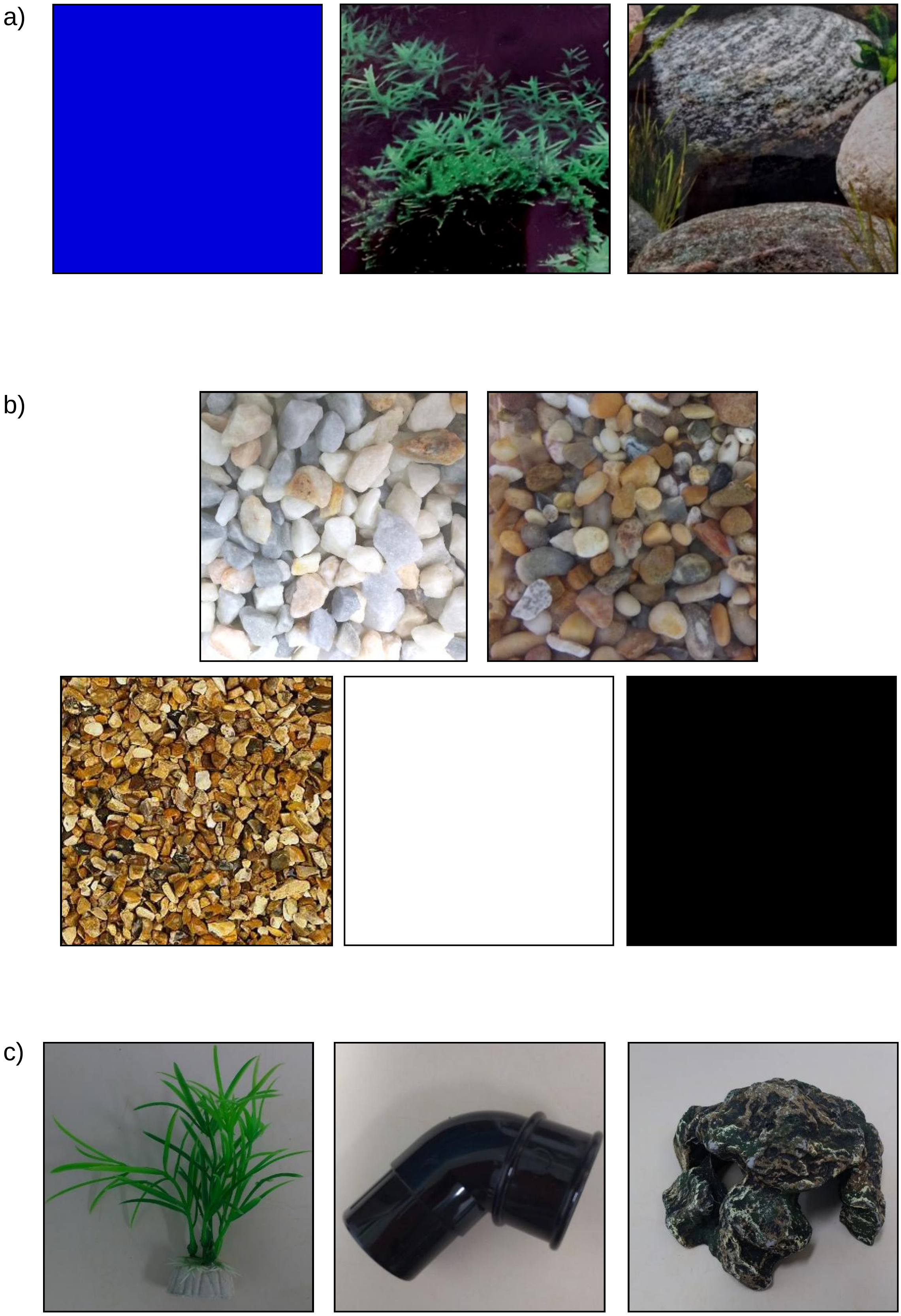

Figure 5.2. Pictures of environmental conditions presented in preference tests: a) backgrounds – plain blue, black planted and stone background; b) substrates – white gravel, brown gravel, gravel image, white plastic and black plastic; c) refuges – plastic plant, pipe, shelter.

The pairs of conditions used in the experiment are presented in table 5.1. Each group of fish experienced each set of conditions in a randomised order to avoid any bias generated by possible ‘carryover effects’ of previous tests on the behaviour, and the side of the tank containing condition 1 or condition 2 for each pair was changed between groups of fish to account for any side bias.

Table 5.1. Pairs of environmental conditions presented to fish in choice tanks.

| Condition 1 | Condition 2 |
|---|---|
| *Backgrounds* | |
| Blue background | Black planted background |
| Blue background | Stone background |
| Black planted background | Stone background |
| *Substrates* | |
| White gravel | Brown gravel |
| Brown gravel | Gravel image |
| Gravel image | White gravel |
| Gravel image | Black plastic |
| Brown gravel | White plastic |
| *Refuges* | |
| Plastic plant | Nothing |
| Pipe | Nothing |
| Pipe | Plastic plant |
| Shelter | Pipe |

### *5.2.3 Procedure*

Each group of fish was removed from its experimental tank and placed in a temporary holding tank (210 x 130 x 130 mm) whilst the experimental conditions were added to the tank. Fish were then returned to their tanks, with three fish re-introduced on each side of the divider. Groups were allowed two days to acclimate before being observed. Observations took place over the following four consecutive days, at 10.00 am and 4.00 pm (a total of eight sampling periods). During the sampling period, the number of fish on each side of the divider was recorded every 15 seconds for a total of 5 minutes.

### *5.2.4 Data analysis*

To calculate the preference of one group of fish for a particular chamber, the proportion of fish in that chamber was calculated for each time point across all eight sampling periods. The mean proportion of fish in that chamber across all sampling periods was then calculated, and these values were used to calculate Jacobs' preference index (Jacobs, 1974), using the formula:

$$J = \frac{(r - p)}{[(r + p) - 2rp]}$$

where $r$ = proportion of fish in a chamber and $p$ = proportion of space available represented by that chamber (in this case, 0.5). Preference scores were calculated in this way for each group of fish for each set of conditions. Preference index scores can take a value between -1 and +1, with 0 indicating no preference, therefore one-sample t-tests were performed on index scores to detect significant differences from 0.

### *5.2.5 Ethics*

Throughout this study, the ASAB/ABS Guidelines for the treatment of animals in behavioural research were followed. Fish were monitored daily for signs of stress or poor welfare. One group of fish was removed from the experiment whilst experiencing their final set of conditions due to presence of whitespot (*Ichthyophthirius multifiliis*). After the study was complete, the fish were health checked by the named veterinary surgeon and when certified as disease free and in good general health were rehomed into the ornamental fish trade.

## 5.3 Results

Neon tetras showed a significant preference for the background depicting stones over either the bright blue background ($t$ = 3.338, d.f. = 8, $p$ = 0.010) or the black planted background ($t$ = 3.434, d.f. = 7, $p$ = 0.011; Fig. 5.3). They did not show a significant preference for either the blue or the black planted background when these were compared against one another ($t$ = 0.682, d.f. = 7, $p$ = 0.517).

Neon tetras did not show any other significant preferences for any substrates or refuge ($p$ > 0.05; Fig. 5.4, Fig. 5.5).

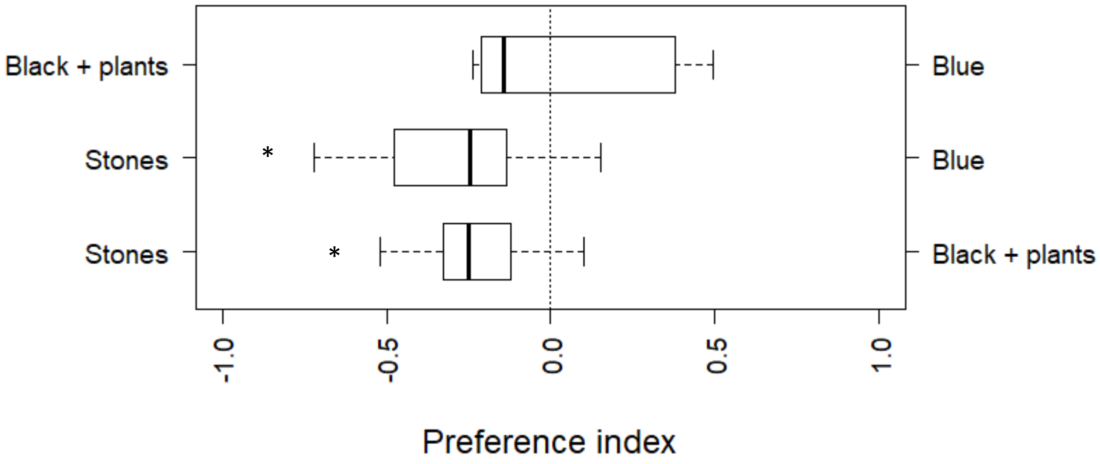

Figure 5.3. Jacobs' preference index for the use of different choice chambers containing different backgrounds (*n* = 7 for the comparison between the 'black+plants' background and the 'stones' background; *n* = 8 for the other pairs of conditions). Boxes represent the 25th and 75th percentile range and contain the median line. Whiskers represent the most extreme values. The dashed line at 0 indicates no preference. Boxes which do not overlap this line indicate that preferences were significantly different from 0 at $p < 0.05$.

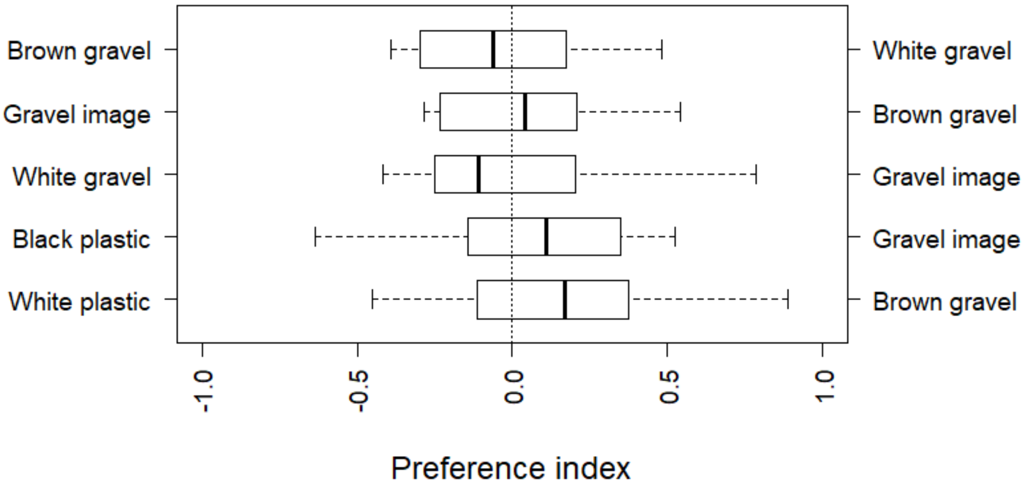

Figure 5.4. Jacobs' preference index for the use of different choice chambers containing different substrata (*n* = 8 for each pair of conditions). Boxes represent the 25th and 75th percentile range and contain the median line. Whiskers represent the most extreme values. The dashed line at 0 indicates no preference.

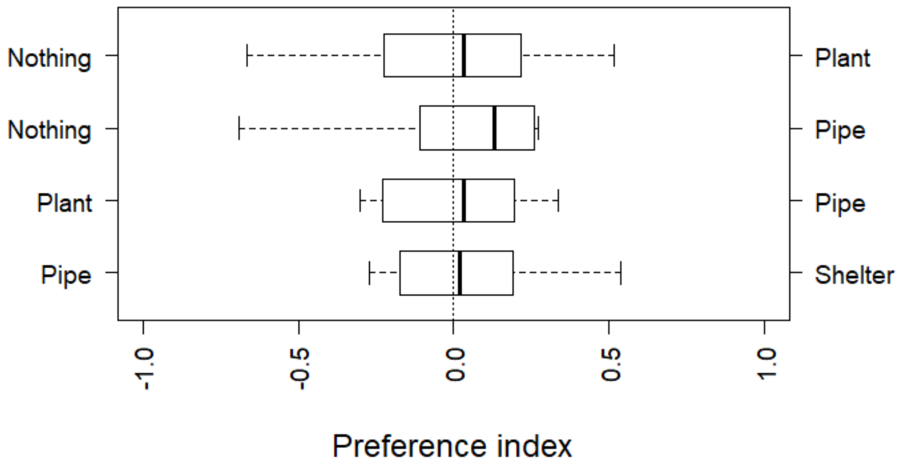

Figure 5.5. Jacobs' preference index for the use of different choice chambers containing different refuges (*n* = 8 for each pair of conditions). Boxes represent the 25th and 75th percentile range and contain the median line. Whiskers represent the most extreme values. The dashed line at 0 indicates no preference.

## 5.4 Discussion

In this study, I found that neon tetras showed a preference for a tank background with an image of stones over a plain blue background or a black background with images of plants. However, they did not show any preferences between the blue and the black planted background. Fish showed no preferences for any of the refuge conditions, even when the different refuges were compared against an empty chamber, nor did they show a preference for any substrate over another. Two questions therefore need to be considered here: why did the fish prefer one background over the others? And why did they show a preference for a background but not for other the forms of enrichment applied?

There are a number of possible explanations for the fish’s preference for the stone background. One explanation may be that the fish were less likely to spend time near the blue or black planted backgrounds because they were more conspicuous there. To the human eye, neon tetras were least easy to see against the stone background, and were more visible against the blue and black planted backgrounds, therefore the fish may have perceived the stone background as being a ‘safer’ environment. This explanation would appear to be at odds with the presence of colouration on the sides of neon tetras – an iridescent blue stripe and a bright red stripe – but a suggested function of this colouration is to confuse aquatic predators by reflecting a mirror image onto the surface of the water (Ikeda and Kohshima, 2009). It is therefore possible that neon tetras are subject to predation from aquatic predators in the wild, and therefore might seek environments where they are less conspicuous. Furthermore, this study found that neon tetras changed the intensity of their colouration according to background colour and lighting, with the least intense colours being produced when housed with a well-lit white background. This may further support the idea that the preference for the stone background was driven by a perception of this environment as making the fish less conspicuous

In contrast with the possibility that background preferences are influenced by anti-predator behaviour, no preference for any refuge or substrate was seen. If predator avoidance influences environment choice, I might have expected to see fish choose a chamber containing a refuge over an empty one, as the plant, pipe or shelter might have been perceived as providing safety. Similarly, although neon tetras have duller colouration when viewed from above than from the side, which was inconspicuous against the brown gravel substrate, fish did not show any preference for brown gravel, or indeed any aversion to white gravel or the white plastic base. Unfortunately, the predators of neon tetras in their natural environment have not been characterised, therefore the proportion of attacks from aquatic vs aerial predators is not known, and so this suggestion is purely speculative. An alternative explanation for the lack of preference for the refuges may relate to the social dynamics of the group: the addition of structures has been suggested to reduce aggression in groups of fish as they limit the amount of visual contact that takes place (Basquill and Grant, 1998; Wilkes *et al.*, 2012). In shoaling species, such as the neon tetra, a reverse effect may occur: refuges may be less preferred as they may limit contact with the rest of the social group.

Despite the possibility that the preference for the stone background expressed by neon tetras was influenced by their natural environment, it is also possible that these fish did not show the same kind of preferences as would be shown by wild fish due to domestication effects. Over 95% of neon tetras in the ornamental fish trade are bred in captivity (Chapman *et al.*, 1997), and they are considered to be a domesticated species (Balon, 2004). It is quite possible therefore that some preferences based on natural habitat have been lost or altered throughout the domestication process. Evidence that this occurs has been found in zebrafish: laboratory-reared zebrafish shared a preference for gravel as a spawning site with wild-caught zebrafish, but also showed a preference for vegetation which was not shown in the wild-caught fish (Spence *et al.*, 2007). However, looking at the natural history of species may provide a good starting point when considering potential forms of enrichment.

The preferences expressed by fish in this study may also have been influenced by their rearing environment. Early-life experiences have been shown to affect social preferences in later life in a number of fish species, including zebrafish (Spence and Smith, 2007), rainbowfish *Melanotaenia duboulayi* (Kydd and Brown, 2009) and Arctic charr *Salvelinus alpinus* (Winberg and Olsén, 1992), and can affect other behavioural, physiological and cognitive factors, including foraging behaviour, spatial navigation, behavioural flexibility, cerebellar growth and overall brain size (Brown *et al.*, 2003; Braithwaite and Salvanes, 2005; Kihslinger and Nevitt, 2006; Brown *et al.*, 2007; Burns *et al.*, 2009). Furthermore, observations in the laboratory have shown that guppies find a white tank base more stressful than a brown gravel substrate, unless reared from birth with the white base (D. Croft, 2015, pers. comm.). It is therefore possible that rearing experiences can influence environmental preferences in later life. If the fish in this study were reared in conditions which bore more resemblance to the stone background than either the blue or black planted backgrounds, this may explain why they showed a preference for the stone background. This may also explain why fish did not show preferences for particular substrates or refuges – if these were absent in their rearing environment, they may not have gained a preference for these items. This is likely to be highly relevant when considering the impact of potential interventions being introduced into stores based on this study. For example, had the fish been purchased from one of the wholesalers which supplies the retailer involved in this study, the fish would likely have experienced blue backgrounds before, as these same backgrounds are used in the wholesalers' tanks. This might have affected their preferences or aversions to particular backgrounds.

Although it is difficult to establish exactly why the fish in this study expressed particular preferences, the fact that some preferences existed indicates that there are particular environmental conditions that may promote better welfare in neon tetras. This highlights that the technique of presenting animals with a choice can be an effective way of helping to identify these conditions. Furthermore, conditions for which fish expressed no preference can also be informative and useful – for example, fish in this study expressed no preference for gravel over other substrates, despite the fact that gravel is often present in pet store tanks. Gravel can make tanks harder to clean, and may harbour faeces, uneaten food, disease or parasites, therefore the fact that it does not appear to be necessary for good welfare in this species means it need not be present in pet store tanks (Williams *et al.*, 2009; Lidster *et al.*, 2017). The overall welfare of the fish may then be improved by not including gravel, by helping to maintain higher water quality and reducing the risk of disease.

Whilst preference tests may be an effective tool for helping to improve animal welfare, some considerations must be taken into account when using them. Firstly, it is important to note that when animals are presented with a selection of choices, only the preferred condition out of those available can be chosen, and while that condition may represent the best of those on offer, it does not necessarily represent the best overall condition for welfare (Young, 2013). Preference tests would therefore be more effective if comparing a wider range of conditions. Secondly, the exemplars used for each condition must be carefully considered: a limitation of the study presented here is that only one exemplar was used for each condition. It is therefore not possible to be certain that preferences or aversions displayed in this study were not a response to a specific feature of each exemplar (i.e. a particular characteristic which may not be representative of the condition the experimenter intends to test). Future studies employing this approach should therefore use several exemplars within each condition to ensure that any preferences/aversions displayed by the test animals are, in fact, for the conditions being presented by the experimenter, and not for some idiosyncrasy of a single exemplar. Thirdly, the results of preference tests, and the forms of enrichment that produce a welfare benefit, are likely to be species-specific, depending on the natural history of the species. Care should therefore be taken when attempting to extrapolate from one species to another. Furthermore, although allowing animals to make a choice and assuming this promotes good welfare is a widely used approach, it does not actually test for differences in welfare when animals are housed under preferred and non-preferred conditions. This approach would therefore be strengthened if a range of other welfare measures, such as behavioural indicators of stress, or stress hormones, were measured after establishing preferred conditions. Finally, simple choice tests as were applied in this study cannot clearly indicate the relative value animals place on a particular condition. Future work should therefore emphasise looking at a fish's motivation to make a particular choice. Studies looking at motivation for preferences in fish are rare in the literature, but have been carried out – for example, Sullivan *et al.* (2016) used increasingly strong water currents to test the degree of preference of goldfish *Carassius auratus* for real or artificial plants.

In conclusion, this study has shown that some modifications made to captive fish's environments are seen as preferable, and therefore can be considered to promote better welfare. It has also shown that some modifications that might be expected to be valued by neon tetras are not preferred, and therefore do not need to be included in tanks to promote good welfare. This lack of inclusion may be more beneficial to the fish overall for practical reasons, such as maintaining good water quality. Not all modifications provided to captive animals should therefore be referred to as enrichment, and modifications should not be assumed to be beneficial unless this has previously been demonstrated.

# Chapter 6: Conditioning techniques do not reduce signs of behavioural stress in guppies *Poecilia reticulata* in response to husbandry procedures

## Abstract

The response of a fish to a stressor depends not only on that stressor's properties (nature, intensity, duration), but on the fish's appraisal of the stressor and its own coping abilities. Many transportation and husbandry procedures for ornamental fish will inevitably involve some stress, but interventions based on altering the fish's appraisal of the stressor may be able to reduce the degree of stress the fish experiences. This study consisted of two experiments which aimed to alter a fish's appraisal of a stressor through conditioning techniques. The first experiment aimed to see whether guppies *Poecilia reticulata* could be trained to associate a stressful event, such as handling, with a positive event in the form of a food reward in order to reduce the stress experienced by the fish. Groups of guppies were subjected to a handling event three times a day over eight days, which was immediately followed by presentation of a bloodworm reward. Control groups also experienced handling, but were presented with bloodworm up to an hour before or after the handling event. The second experiment aimed to see whether guppies would be less stressed by handling if they learned to associate the handling with a signal (a red light) before the handling event happened. Groups of fish were exposed to the red light signal and then immediately handled three times a day for eight days; control groups were also handled but were exposed to the red light at unrelated times of day. After the training period, behavioural observations were used to assess the stress levels of fish immediately following a handling event. Neither the food reward nor the light signal had any effect on behavioural measures of stress in handled fish, suggesting that more research into training regimes is needed before any can be applied in the ornamental fish industry.

## 6.1 Introduction

The stress response, a coordinated suite of physiological, behavioural and psychological changes resulting from a challenge to homeostasis, is often considered adaptive when the challenge, or stressor, is acute (Wendelaar-Bonga, 1997). However, when stress is chronic, repetitive, or severe, it can cause reduced reproduction and growth, suppression of the immune system, and poor welfare (Moberg, 2000). The use of training techniques as a means of reducing stress, improving welfare, and increasing the ease of carrying out certain procedures has been applied to many animals within zoos and laboratories. In particular, these techniques have been applied with mammals – training is used with various species to reduce stereotypic and stress-related behaviours (O'Brien *et al.*, 2008; Pomerantz and Terkel, 2009; Coleman and Maier, 2010), to encourage individuals to move into different areas of an enclosure of their own volition (Bloomsmith *et al.*, 1998; Bloomsmith *et al.*, 2003), to promote positive social interactions and reduce aggression (Schapiro *et al.*, 2001; Bloomsmith *et al.*, 2003) or to encourage individuals to tolerate medical procedures such as blood sampling (Grandin *et al.*, 1995; Coleman *et al.*, 2008). There are yet few examples of training being used with captive fishes, but there is some evidence to suggest that these techniques can be an effective means of reducing stress and improving welfare. For example, Schreck *et al.* (1995) found that Chinook salmon *Oncorhynchus tshawytscha* could be trained to respond less to transportation stress, which also resulted in less susceptibility to disease and greater survival. Mozambique tilapia *Oreochromis mossambicus* also showed a less pronounced stress response to confinement when trained to associate it with a visual signal (Galhardo *et al.*, 2011).

There are a number of reasons why training may help reduce the impacts of stressors. Firstly, more frequent presentation of a stressful stimulus may result in habituation to the stressor. For example, oxygen consumption in Atlantic salmon *Salmo salar* exposed to either a weak or strong stressor (15 seconds or 5 minutes of chasing) returned to baseline levels more quickly as their experience of the stressor increased (Madaro *et al.*, 2016). However, repeated exposure to stressors can also have negative effects – gilthead sea bream *Sparus aurata*, Atlantic salmon and goldfish *Carassius auratus* have been shown to experience immunosuppression in response to repeated acute stressors (Sunyer *et al.*, 1995; Fast *et al.*, 2008; Eslamloo *et al.*, 2014). Training may also help reduce the impacts of stress through physical conditioning; that is, making the individual more physiologically capable of responding to the stressor. Training regimes involving forced swimming as a means of providing exercise have been shown to lead to lower circulating cortisol levels and faster post-stress recovery of baseline cortisol in rainbow trout *Oncorhynchus mykiss*

(Woodward and Smith, 1985), Atlantic salmon (Boesgaard *et al.*, 1993) and striped bass *Morone saxatilis* (Young and Cech Jr, 1993; 1994), and can have a range of other benefits, such as increased growth and food conversion efficiency (Davison, 1997). However, this type of training may only be beneficial in the case of certain stressors which may be physiologically challenging to the fish, such as chasing or removal from water.

A third way in which training may help to reduce an individual's response to a stressor is by changing the psychological component of the stress response (Galhardo and Oliveira, 2009). Despite continuing debate on the subject of whether fishes have some form of subjective internal experience, or 'consciousness' (Sneddon, 2003b; Arlinghaus *et al.*, 2007; Braithwaite, 2010; Rose *et al.*, 2014; Sneddon, 2015; Key, 2016), there is much evidence to suggest that fishes do experience some form of consciousness. For example, fishes possess homologous brain structures to the mammalian limbic system, which plays a role in memory, spatial and associative learning, fear and emotional processing (Chandroo *et al.*, 2004), can learn to avoid areas where they have had negative experiences (Csanyi and Doka, 1993), learn by observing others (Schuster *et al.*, 2006), transitively infer information (McGregor *et al.*, 2001; Swaney *et al.*, 2001), and some may even be able to pass the 'mirror test' for self-awareness (Ari and D'Agostino, 2016). This suggests that fishes may therefore have some ability to psychologically appraise a stressor. The stress response begins with the perception of a potentially threatening stimulus, and this perception affects the rest of the stress response, regardless of whether the stimulus is actually dangerous (Moberg, 2000; Schreck and Tort, 2016). If an individual's perception of a stressor, or their own ability to cope with that stressor can be changed, it may be beneficial to that individual's stress levels and welfare.

An individual's perception of a stressor, and how threatening it is, depends on the properties of the stressor itself (nature, intensity, duration), but also on the individual's experience of the stressor, their assessment of their own coping abilities, and modulators such as the predictability of the stressor (Galhardo and Oliveira, 2009). For example, Höglund *et al.* (2005) found that crucian carp *Carassius carassius* housed in tanks without shelters had higher serotonin activity and less efficient avoidance behaviour in response to exposure to skin extracts, and suggested it is possible that fish perceived that they had more control over stressors due to the ability to hide when there was a shelter present in the environment. Schreck *et al.* (1995) showed that Atlantic salmon conditioned to associate a confinement stressor with food had lower physiological responses to the stressor, recovered more quickly, and seemed better able to cope with a different challenge (transportation) later on. This could not be explained by habituation or physical conditioning alone, as fish exposed to the same pattern of training without the reward did not perform as well. Mozambique tilapia trained to associate a confinement stressor with a visual cue (a patterned piece of card) being presented 5 minutes before the stressor commenced, showed lower cortisol levels than those not trained to the visual cue (Galhardo *et al.*, 2011). Interventions focussed on changing an individual's perception of the stressor may therefore be an effective way to reduce stress and improve welfare of fishes in a variety of captive environments.

In this chapter I test the effectiveness of training interventions to reduce stress in ornamental fishes by modifying the perception of the stressful event using two experiments. In the first experiment, guppies *Poecilia reticulata* underwent a training procedure in which they were handled and immediately presented with a food reward, whilst in the second, a red light was turned on to signal to guppies before being handled. After the training phase, fish were placed into behavioural arenas to examine behavioural signs of stress. Stress was assessed using behavioural measures rather than physiological measures because, unlike many physiological measures, behaviours can tell us about the valence of a reaction. For example, fishes might show increased cortisol release rates as part of food anticipatory behaviour prior to being fed, but this behaviour is not necessarily associated with higher stress or reduced welfare (Sánchez *et al.*, 2009; Galhardo *et al.*, 2011). The measures of stress chosen were the same as those in Chapter 4 – the latency to leave a refuge and amount of time spent in the refuge, freezing behaviour, general activity, thigmotactic behaviour, and erratic movements (see Table 4.1 for details). These measures were chosen as they can all be interpreted as being indicative of stress – for example, in guppies, shelter use is known to be an anti-predator behaviour (Templeton and Shriner, 2004), therefore greater reluctance to move away from a shelter, and more time spent in a shelter area, are interpreted as being indicative of less propensity to engage in risky behaviour and higher levels of stress. Guppies were chosen as the study species as they are one of the most popular species in the ornamental fish industry (Chapman *et al.*, 1997; P. Carey, 2014, pers. comm.). Bloodworm was used as the food reward as guppies in our laboratory have been observed to show high motivation to eat bloodworm when presented, which I interpret as suggesting they have a preference for this food. A red light cue was used as orange/red colourations are known to be ecologically important to wild guppies, therefore it was hoped that this colour cue would be notable to the guppies in the experiment (Sandkam *et al.*, 2015).The interventions tested in this study were predicted to reduce signs of stress in handled fish by either increasing positive associations with the handling event, or by increasing the predictability of the handling event and therefore increasing the perceived degree of control the fish had over the event.

## 6.2 Methods

### *6.2.1 The effect of a post-handling food reward on stress-related behaviours*

#### *6.2.1.1 Study animals*

The experimental fish were mixed-strain male domestic guppies *Poecilia reticulata* obtained from a local pet shop. Guppies were sorted into groups of five (*n* = 25 per experimental condition; total fish = 50) and housed in 10L aquaria (300 x 195 x 205mm) containing gravel and a plastic plant. Aquaria were maintained at approximately 25°C, 12L:12D. Fish were fed flake food *ad libitum* in the afternoons after all training sessions had been completed in order to ensure they were motivated to feed before training began. All fish were observed consuming flake food on all days of the experiment, suggesting they were not fully satiated by the end of training.

#### *6.2.1.2 Training phase*

The training phase of the experiment lasted for eight consecutive days, with three handling events occurring each day (a total of 24 training events). At least three hours were left between each handling event to allow fish to recover from the previous event. In the ‘rewarded’ group, all fish in each tank were captured together in a dip net and held out of the water for ten seconds. They were then returned to the tank and immediately received a few drops of chopped bloodworm. Control fish received the same handling procedure as the rewarded fish, but received their bloodworm up to an hour before or after the handling event. The duration of the training period was chosen as there is evidence that guppies are capable of learning a task within this time period or within this number of trials – guppies have been trained to locate a food patch when trained three times a day for 6 days (Swaney *et al.*, 2001), to forage from within a plastic ring within 20 training events (Dugatkin and Alfieri, 2003) and to complete a plus-maze task in 20 training events (Eaton *et al.*, 2015).

### *6.2.2 The effect of a pre-handling light signal on stress-related behaviours*

#### *6.2.2.1. Study animals*

The experimental fish were mixed-strain male domestic guppies obtained and housed as in Experiment 1. Fish were fed flake food in the morning and *Artemia ad libitum* in the afternoon.

#### *6.2.2.2 Training phase*

As in Experiment 1, the training phase of the experiment lasted for eight consecutive days, with three handling events occurring each day and a minimum of three hours between handling events. In the ‘signalled’ group, a red light was turned on above the tank 5 minutes before handling, and remained on until the beginning of the handling procedure. Fish were then handled as in Experiment 1. Control fish received the same handling procedure as the rewarded fish, but the red light signal was turned on up to an hour before or after the handling event.

### *6.2.3. Test phase*

On day 9 in both experiments, fish received the same handling procedure as described above, before being individually transferred to test tanks to examine behavioural indicators of stress. Fish were moved into new tanks rather than observed in their home tanks to more closely mimic the type of procedure that might happen in the ornamental fish trade. The test tanks used, and behavioural measures recorded were the same as detailed in Chapter 4 (see Figure 4.1 and Table 4.1). Briefly, this involved placing the fish in an open-field tank (285 x 460 x 140 mm) in which one corner (142.5 x 115 mm) contained gravel and a plastic plant to serve as a shelter area. The tank was divided into 16 equally-sized zones and was marked with a line 20 mm from the edge to aid behavioural measurements. Fish were released into the shelter area and observed for ten minutes. Videos of each trial were recorded and analysed using Solomon Coder software (available at https://solomoncoder.com/) to record the latency of the fish to leave a refuge area, the amount of time spent in the refuge, the duration of freezing bouts, overall activity, time spent showing thigmotactic behaviour, and the number of erratic movements performed.

### *6.2.4 Statistical analysis*

Multivariate analyses were used to avoid increased risk of making a type I error (Field *et al.*, 2012). In both experiments, results did not meet the assumptions of multivariate normality and homogeneity of covariance matrices, therefore a permutational multivariate analysis of variance (PERMANOVA) was performed using the ‘adonis’ function in the ‘vegan’ package in R – this form of analysis can be viewed as a non-parametric MANOVA (Anderson, 2001; McArdle and Anderson, 2001; Oksanen *et al.*, 2018). All analyses were completed in R ver. 3.4.1 (R Development Core Team, 2015).

### *6.2.5 Ethics*

Throughout this study, the ASAB/ABS Guidelines for the treatment of animals in behavioural research were followed. Fish were monitored daily for signs of stress or poor welfare. After the study was complete, the fish were health checked by the named veterinary surgeon and when certified as disease free and in good general health were rehomed into the ornamental fish trade.

## 6.3 Results

### *6.3.1 The effect of a post-handling food reward on stress-related behaviours*

There were no significant differences in stress-related behaviours between fish trained to associate handling with a reward and fish that received rewards separately from handling events ($F_{1, 45} = 0.333$, $p = 0.821$; Fig. 6.1).

### *6.3.2 The effect of a pre-handling light signal on stress-related behaviours*

There were no significant differences in stress-related behaviours between fish trained to associate a light signal with handling and those that received the signal at random times of day ($F_{1, 47} = 1.273$, $p = 0.276$; Fig. 6.2). Although examination of graphs 6.2a and 6.2c suggested that trained and untrained groups of fish might differ in their latency to leave a refuge area and time spent frozen, neither of these measures was significant in subsequent univariate analyses (latency to leave refuge: $F_{1,\,47} = 1.698$, $p = 0.235$; time spent frozen: $F_{1,\,47} = 3.198$, $p = 0.079$).

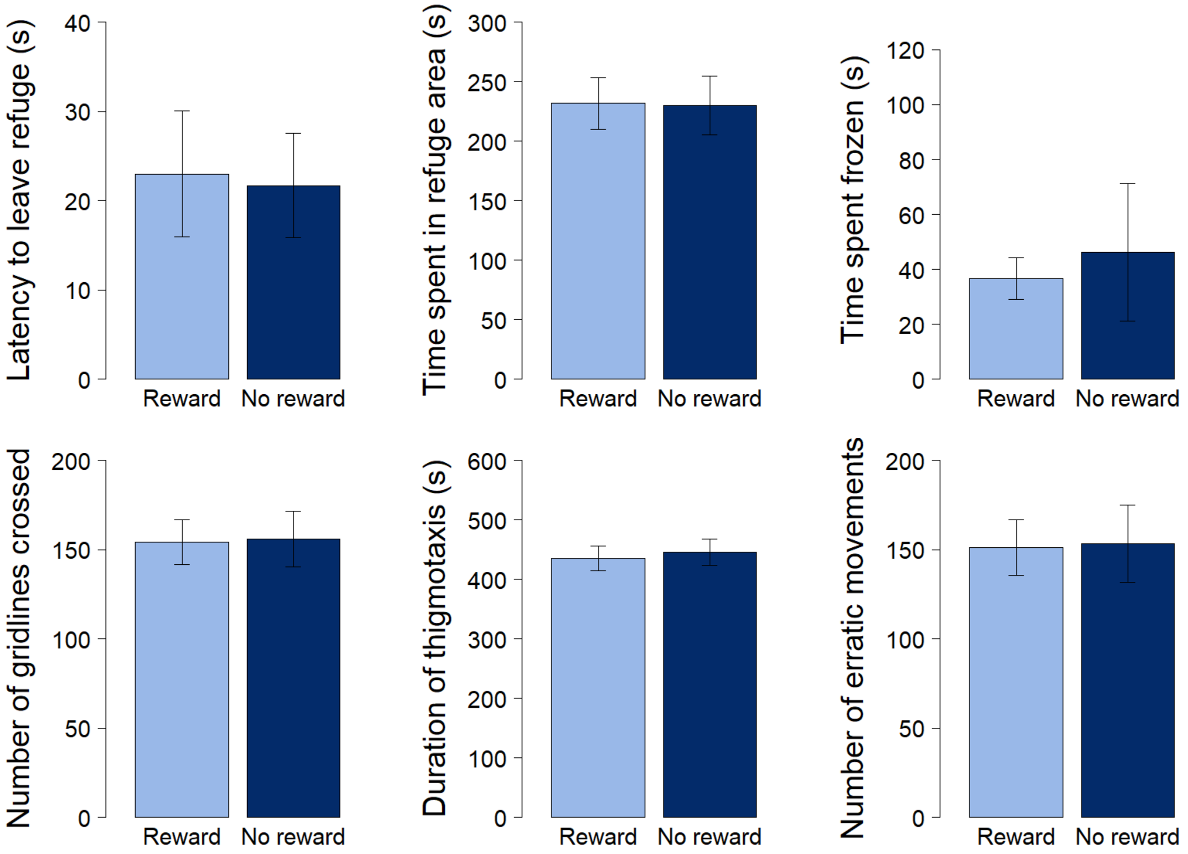

Figure 6.1. Behavioural measures of stress in fish trained to associate handling with a food reward (light blue, $n = 25$) and fish that did not receive a reward after being handled (dark blue, $n = 25$). Bars represent means; error bars represent ±1 S.E.

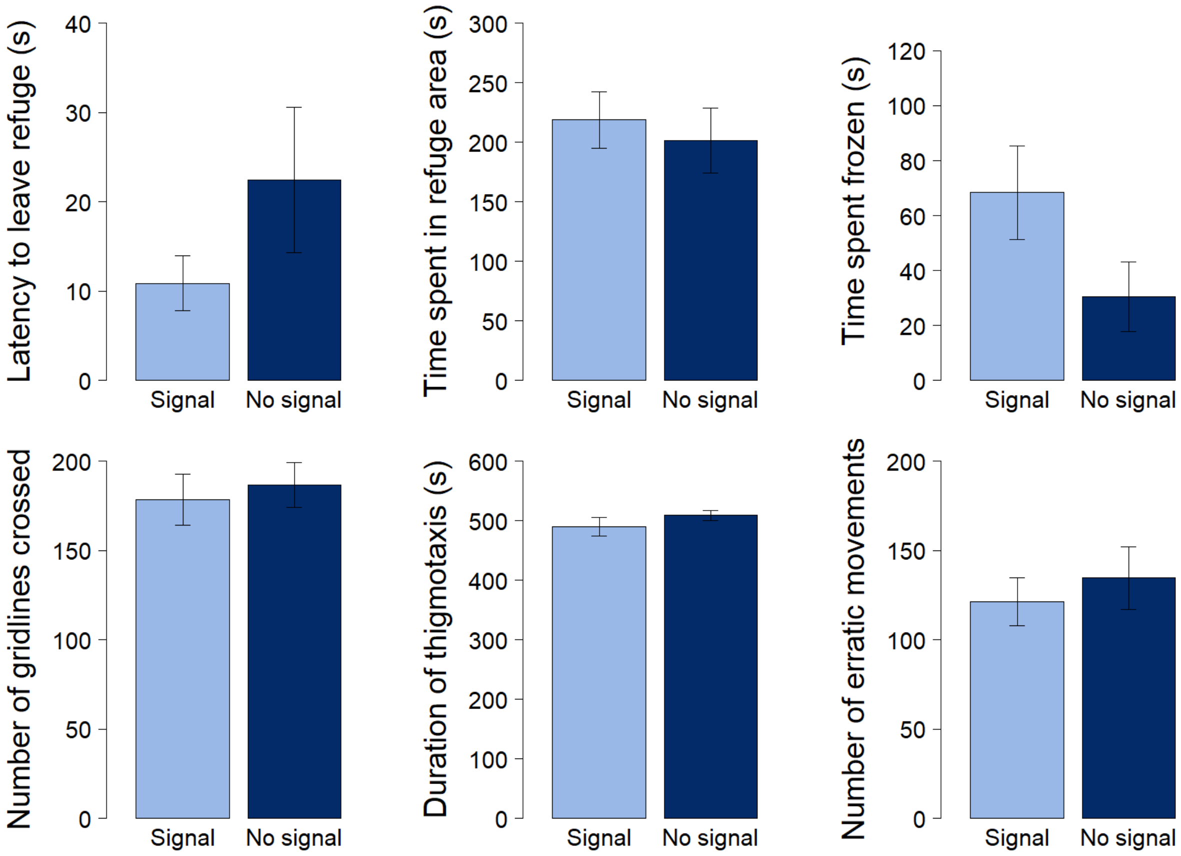

Figure 6.2. Behavioural measures of stress in fish trained to associate handling with a light signal (light blue, *n* = 25) and fish that did not receive a signal before being handled (dark blue, *n* = 25). Bars represent means; error bars represent ±1 S.E.

## 6.4 Discussion

Fish conditioned to receive a food reward after handling did not show any differences in the signs of behavioural stress measured compared with fish not conditioned to receive a reward. Fish conditioned to expect an imminent handling event after the onset of a red light signal also did not show any differences from controls in behavioural signs of stress.

There are several possible explanations as to why neither the food reward nor the light signal appeared to reduce stress in handled fish. These explanations include the stressors present in the study, the stimuli the fish were conditioned to associate with handling, or the learning capabilities of the fish themselves. In the first instance, it is possible that the severity of the stressors, or presence of stressors other than those the fish were specifically trained with, may have caused any reduction in stress from conditioning to be outweighed.

For example, removing the fish from water as part of handling can be highly stressful to fish and can elicit what has been termed a ‘maximal’ stress response (Biswas *et al.*, 2006; Brydges *et al.*, 2009; Ramsay *et al.*, 2009b). The stressor in this study may, therefore, have exerted a strong enough physiological challenge on the fish to mask any effects of psychological modulators of stress. Similarly, as fish were conditioned only to handling, and not to being placed in the novel test tanks used, the stress of being exposed to a novel environment may have outweighed any potential reductions in stress. Social isolation can be a cause of stress or anxiety-like behaviours in social fishes (Earley *et al.*, 2006; Galhardo and Oliveira, 2014; Shams *et al.*, 2015), therefore the fish may have been reacting to this rather than to the handling stressor, regardless of experimental group. However, social isolation can also help lead to a reduced cortisol response to stress – Giacomini *et al.* (2015) found that zebrafish exposed to an acute stressor had lower cortisol responses in isolation than in groups, which may be due to the fact that isolated fishes could not be affected or influenced by the responses of conspecifics. If this effect was present in the fish used in the current study, it may further have obscured any effect of conditioning.

Another explanation for the lack of differences between conditioned and control groups in this study might be that, even if the fish did learn to associate stimuli with the handling stress, the impacts of these stimuli were not enough to reduce the effects of stress. For example, if the value the fish placed on the bloodworm was low, presentation of this may not have been rewarding enough (i.e. enough of a positive experience) to lead to reduced levels of stress. In the case of the light signal, it is possible that there was insufficient information conveyed by this signal for these fish – predictability cues depend on factors such as the duration between the onset of the signal and onset of the stressful event, and the duration selected for this experiment may not have been appropriate for these fish (Galhardo and Oliveira, 2009). The light signal may even have caused anticipation of the stressful event and therefore increased the stress levels of the conditioned fish – Nile tilapia *Oreochromis niloticus* conditioned to associate a light cue with handling showed an increased cortisol response when presented with only the light cue (Moreira and Volpato, 2004).

Finally, the lack of differences in stress-related behaviours in fish in the different experimental groups may be explained by a failure of the fish to learn to associate the food reward or the light signal with the handling stressor. If this were the case the fish would neither perceive the bloodworm as a reward, nor would they be able to predict the onset of the stressor after being presented with the light signal. However, this explanation is unlikely: guppies in this experiment were trained three times a day for a period of eight days – 24 trials in total. Guppies have been shown in a number of experiments that they are capable of learning tasks in fewer trials than this – for example, guppies have been trained to find a food patch in 18 trials (Swaney *et al.*, 2001), to forage from within a plastic ring in 20 trials (Dugatkin and Alfieri, 2003), and to solve a plus-maze task in 20 trials (Eaton *et al.*, 2015). Fewer trials have also been needed for associative learning experiments in other fish species – Panamanian bishops *Brachyrhaphis episcopi* learned to associate a light being turned on with food being presented in 14 trials (DePasquale *et al.*, 2014), rainbow trout *Oncorhynchus mykiss* learned to forage from a feeding ring in an average of 5 trials for bold fish and 15 trials for shy fish (Sneddon, 2003a), and Atlantic salmon became conditioned to associate food with strong light flashes after about 19 days (Bratland *et al.*, 2010). The training period used here was therefore likely to be enough for fish to learn the association.

It is possible that the behavioural measures of stress selected were not, in fact, good measures of stress for guppies and therefore any stress response was not captured by the measures used - for. However, I suggest this is unlikely. For example, Templeton and Shriner (2004) found that guppies used shelter in the presence of predators, which is a stressful experience, therefore it is reasonable to suggest that stressful experiences would lead to greater shelter use. Furthermore, higher latencies to leave a shelter are thought to indicate lower boldness (i.e. lower propensity to engage in risky behaviour), which may be linked to higher stress levels (Brydges *et al.*, 2009; Jacquin *et al.*, 2016). Although some doubt may be cast on this interpretation as neon tetras in Chapter 5 were shown to not have a preference for shelter over any other structural enrichment, this may have been because the neon tetras were not exposed to a stressor and therefore may not have needed to use a shelter. Furthermore, guppies held in the lab have been observed making use of refugia, especially if startled, therefore it seems unlikely that this was not a good measure of stress.

The results of this study may indicate that the potential of conditioning techniques to improve welfare within the ornamental fish industry is limited. If fishes cannot be conditioned to perceive stressors differently and therefore show reduced stress-related behaviour because stressors are too severe, this may limit how conditioning techniques may be applied. However, it does suggest that conditioning techniques may be useful when stressors are less severe and more consistent from event to event, such as with tank cleaning. On the other hand, if the lack of differences between experimental groups is best explained by a lack of value or information provided by the reward stimulus or the light signal, conditioning may yet be a promising area, but a great deal of work would need to be done to find stimuli that provide the right level of perceived reward or information to the fish. If the fish simply did not learn in the timespan of this experiment, it suggests that particular care would need to be taken when implementing training regimes. In the pet trade, once the fishes have left the breeder, they might be moved through the supply chain too quickly for a training regime to be effectively started, therefore training regimes would need to start at the breeder, and ideally, be maintained throughout the supply chain.

In conclusion, this experiment demonstrated that guppies did not show reduced behavioural signs of stress when conditioned to associate a handling stressor with either a food reward or a light signal. This suggests that, while conditioning techniques may still be useful in reducing stress in ornamental fishes, more work first needs to be done exploring which stressors they may be effective for, and which stimuli are the most effective for eliciting differences in the fish stress response.

# Chapter 7: General discussion

The ornamental fish industry has shown steady growth since the 1970s (when the FAO began keeping records), and continues to do so, yet the stress and welfare of the fishes traded remains understudied (Leal *et al.*, 2016). The overall aims of this thesis were to explore some of the potential causes of stress and poor welfare in ornamental fishes to develop interventions to reduce stress, improve welfare and decrease mortality rates. To address these aims, I conducted experiments using both physiological and behavioural measures of stress to explore how stress might be reduced in ornamental fishes, which may help to lead to lower mortality rates. In this chapter, I present a critical analysis of the main findings of this thesis, with discussion of their implications. I then suggest some future directions for scientific research, and also for areas which would benefit from development within the ornamental fish industry.

## 7.1 Summary of main findings

### *7.1.2 Cortisol produced by ornamental fishes is detectable in holding water but differences in stress responses to challenges are unclear*

Measuring cortisol levels in fishes is a common and useful approach for assessing stress levels (Pottinger and Carrick, 2001; Turner *et al.*, 2003; Ellis *et al.*, 2004; Ramsay *et al.*, 2006; Felix *et al.*, 2013). Although most popular ornamental fish species are too small to take a blood sample from to measure plasma cortisol levels, the water-borne cortisol measurement technique allows cortisol in fish holding water to be measured non-invasively and non-intrusively. This technique should be validated before being applied to a new species by establishing that cortisol levels in water are detectable, that they increase in response to a stressor (i.e. that cortisol increases are biologically meaningful), that water provided to fish contains little or no background activity and that extracts dilute parallel to standard cortisol (Scott *et al.*, 2008). The study presented in chapter 3 met a number of these objectives for guppies, mollies and neon tetras – for all three species, cortisol levels in water samples were consistently above the lower detection limit of the assay, background levels of cortisol were negligible, and parallelism was found between extracts and standards. However, higher cortisol release rates in response to a netting stressor were not found for any of the study species, although differences in release rates over time were found for guppies and mollies. The lack of differences between net-stressed fishes and controls in this study also means it was difficult to establish exactly when cortisol release rates peak in these species after the onset of a stressor, although the significantly higher cortisol release rates in the first 30 minutes of the study for guppies and mollies suggest that this is the optimum time to measure release rates.

The results of this study suggest that the water-borne cortisol measurement technique can be a useful tool when evaluating the effects of stressors on ornamental fish, although more work is needed to clarify the effects of stressors on cortisol release rates. A modified methodology, using fish sampled in static containers, was therefore used to assess the effects of different handling techniques on fish in Chapter 4. In both chapters, no differences were found in cortisol release rates in different treatment groups, highlighting the need to integrate measures of stress, as cortisol measurements are still limited when it comes to assessing stress and welfare. For example, cortisol release rates may rise in response to an acute stressor, but may not do so, or may not be maintained at high rates in response to repetitive or chronic stressors (Aerts *et al.*, 2015). As chronic and repetitive stressors are most likely to be linked to negative effects such as suppression of growth, reproduction and immune system function, measurements of biomarkers that indicate stress under chronic conditions are extremely important. Some attempts have been made to develop methods for detecting long-term cortisol levels, such as detecting cortisol content in fish scales (Aerts *et al.*, 2015), but a wider range of tools would be useful here. Furthermore, cortisol release rates as a measure of stress and/or decreased welfare status may be misleading, as cortisol cannot tell us about the valence of an animal’s experience. Cortisol release rates rise in response to arousal, and the experiences which lead to arousal (e.g. food anticipatory behaviour) may not be negative for welfare (Sánchez *et al.*, 2009). For this reason, behavioural measures of stress were gathered in Chapters 4 and 6.

### *7.1.3 Poor handling technique causes stress to non-handled fish*

Handling with dip nets is an inevitable part of the life of an ornamental fish, and often elicits a strong stress response (Barton, 2000; Brydges *et al.*, 2009; Ramsay *et al.*, 2009b). Whilst attempts have been made in the aquaculture industry, and in scientific research to introduce methods of handling fish which do not involve capture, chasing, or emersion, such as the use of pump systems (Ashley, 2007), or removing fish from tanks once they have swum into removable containers (personal observations), these methods may simply not be practical within the ornamental fish supply chain. Research that focuses on refining the techniques already used is therefore crucial to help reduce handling stress in ornamental fish.

Whilst the study in Chapter 4 found no evidence that different handling techniques affect the stress and welfare of captured fish, it did find evidence that handling technique affects the fish which remain in the tank after the handling event. This result was found after exposure to only two nettings, whereas fish in a pet store tank may experience many more nettings than this before they are captured and sold. This highlights the importance of having properly trained workers in the supply chain, and making workers aware of the potential impacts on poor handling on the fish. The handling training video used in Chapter 4 has been provided to the industry partner to be incorporated into training programmes for new employees that have fish handling responsibilities. Monitoring the effectiveness of this video at reducing stress and mortality in the commercial context provides an exciting opportunity for future work.

An area that was not explored by this study, and thus provides another opportunity for future work, was the effects of the training videos on handlers' behaviour over time. Although there was evidence that the handlers did change their behaviour in response to watching the training videos, it is important to note that handlers then caught fish immediately after viewing – had they watched a training video some hours or days before being asked to catch a fish, as might well happen if an employee was being trained to work in a store, the effects might have been different. For example, if more time had elapsed between watching the video, participants might have been more likely to forget some of the instructions and revert to chasing the fish, causing more stress. However, it is also possible that allowing some time between watching the video and being asked to catch the fish might allow handlers to retain the key messages of the video without becoming too fixated on copying the technique exactly – as such, handlers might have displayed a more relaxed technique which might even have reduced the stress of the fish yet more.

Beyond the question of the effects of time between viewing the training video and first catching a fish, there is also an effect of both time and experience to be considered as handlers become more used to catching fish. Whilst one might expect the adherence to the technique demonstrated to decrease over time, as handlers become more experienced in catching fish, they may develop their own techniques. It is unclear, however, whether the techniques displayed by experienced handlers might be better for the fish's stress levels, or might merely reflect a pattern of behaviour most comfortable to the handler. It would therefore be interesting to explore the relationship between these two factors – for example, to find out whether the individual techniques developed by handlers which have never been trained are comparable to those developed by those who have been trained first.

### *7.1.4 Neon tetras express preferences for certain environmental conditions*

A choice test, where animals are allowed to choose freely between two or more environmental conditions, is a widely-used experimental approach in the animal welfare literature, but remains little-used with fish (Van de Weerd *et al.*, 1998; Dawkins, 1999; Duncan, 2005). However, it provides an opportunity to try and identify conditions which may promote better welfare. This study allowed the preferences of fish for various conditions, some of which were requested to be included by the retailer, to be tested. The results of the experiment presented in Chapter 5 indicate that neon tetras showed a preference for a tank background with a print of rocks over either a plain blue background or a black background with a print of plants. It is possible that fish perceived themselves to be less conspicuous against the rock background than the other backgrounds, which may explain the preference. However, fish expressed no preferences for different tank substrates or tank ornamentation, which might be expected if anti-predator behaviour was influencing fish's choices. It is therefore possibly more likely that preferences were influenced by rearing environment. The results of this study will be of use in decision-making regarding how best to house fishes in store, as it supports the decision of the retailer not to include gravel in tanks. It also supports the retailer's decision to alter the plain blue background which was previously used in all stores, but may indicate that the decision to introduce the black background with plants may need to be reconsidered.

The possibility that rearing environment influences preferences in later life provides an interesting potential avenue of research, as it may be possible to promote fish welfare by creating more consistency in tank conditions between different stages of the supply chain. Furthermore, in other taxa, certain forms of enrichment and environmental modification have been found to help increase recovery rates after stressful events (Batzina *et al.*, 2014). If interventions, such as those tested in Chapters 4 and 6, cannot directly reduce the stress associated with certain events, such as handling or husbandry, it may be possible to design tanks according to fish preferences in order to better promote recovery from stressful procedures.

Whilst preferences were found in this experiment, two important questions were not answered: how much did the fish value their preferred conditions? And does housing fish in conditions they have previously expressed a preference for lead to lower stress levels? Future research should answer these questions when using choice tests to identify better environmental conditions in which to house fish. In the case of the first question, the amount of value an animal places on a chosen condition is usually tested by making the chosen condition harder to reach (Jensen and Pedersen, 2008). This type of motivation test may be difficult to design for fish, but one proposed solution is to present fish with increasingly strong water currents to swim against to reach their preferred condition (Sullivan *et al.*, 2016). Another option required fish to push against transparent hinged doors to reach their condition of choice (Maia *et al.*, 2017). The second question – the degree to which an environmental modification reduces stress – is key to establishing whether a modification can be considered enrichment and whether it is beneficial for welfare. Aiming to address both of these points also raises the interesting question of whether fishes make adaptive choices for their welfare – i.e. are those conditions that most promote welfare and reduce stress the ones that are most valued by the fish?

A final important consideration for this experiment, which may also lead to future work, is the role of context and its effects on the preferences expressed. The fish in this experiment were housed in a quiet area of the lab, were observed from a distance when data was collected, and had been allowed time to acclimatise to their surroundings. This may be in contrast with many of the conditions that fishes may experience in a pet store, where there may be noise, disturbances from customers, and little time to settle into their new tank. As such, the preferences fish may express under these conditions might be very different – for example, fish did not express a preference for any refuge in this experiment, but under more stressful conditions they might show a preference for the refuges. Future work could therefore explore how preferences are influenced by context, and whether the preferences expressed differ when fish are stressed or unstressed.

### *7.1.5 Guppies do not show less stress when handled, even if handling is rewarded or predictable*

Some events in the life of a captive animal are inherently stressful, and there is probably no way to avoid this. However, the impact of stressors on that captive animal may be lessened if the way the animal appraises the stressor, and its own coping abilities, are altered. In Chapter 6, I presented the results of two experiments aimed at reducing the stress experienced by handled guppies – one in which handling was rewarded, and one in which handling was preceded by a signal to make handling events predictable. Neither experiment found any significant differences in behavioural signs of stress in handled fish. Whilst it is possible that guppies were not trained for long enough to learn the associations between the food reward and handling, or the light cue and handling, this seems unlikely, as the number of training events the fish received was within the scope of other published studies (Swaney *et al.*, 2001; Dugatkin and Alfieri, 2003; Eaton *et al.*, 2015). Instead, it may be that handling is too stressful an experience for training interventions to reduce the amount of stress experienced by a handled guppy. This may explain why fish in Chapter 4 did not show differences in stress levels when handled by untrained handlers. However, other studies have found evidence to suggest that conditioning techniques may be of use in altering the stress levels experienced by captive fish (Galhardo *et al.*, 2011), therefore this may be a useful avenue of research to continue to explore. That being said, the results of Chapters 4 and 6 both suggest that handling is stressful, as fish showed behavioural signs of stress regardless of treatment group. Pursuit of solutions aiming at altering the appraisal of a stressor should not, therefore, be done at the expense of research into improving techniques and practices, even if it may be possible to completely eliminate all stress associated with that technique or practice.

As well as considering the degree of stress that fish are exposed to in future experiments of this nature, we must also consider the influence of the stimuli themselves. As mentioned in Chapter 6, although the bloodworm reward was chosen as guppies show high feeding anticipation, suggesting high motivation to eat, and the red light signal was chosen to be ecologically relevant, the true value which guppies might have placed on these stimuli is unknown. As shown in Chapter 5, at least some ornamental fish species are capable of expressing preferences for certain conditions. It may therefore be possible to conduct some form of preference tests for the types of stimuli that might be used in training, in order to ascertain which conditions – e.g. which food types – are most preferred by the fish being studied. Identification of preferred stimuli would be likely to improve the efficacy of training procedures.

## 7.2 Implications and future directions

Although this thesis has primarily discussed stress and welfare, this has been done with the broad aim of helping to reduce mortality rates of fish in the ornamental fish trade. Efforts to quantify mortality in the past have been hampered by a lack of clear and accurate data; it is therefore imperative that more monitoring of the numbers of fish traded, and the numbers of fish lost, is collected in order to better understand mortality rates and the possible causes of mortality. Collection of this type of data would continue to help provide direction for future research aimed at developing interventions to reduce mortality.

As mentioned above, some interventions that may be introduced to reduce stress and improve welfare reflect recommendations for best practice within the industry. Ensuring that shipping bags and tanks are not over-stocked, providing measures to ensure transported fish are adequately insulated, and training workers in the supply chain to handle fish with care and empathy all represent best practice, and are all relatively straightforward interventions to introduce. In contrast, some interventions to reduce stress are less straightforward to develop, either because the primary causes of stress and mortality are unclear, or because potential interventions may conflict with practicality. For example, handling, especially when fish are removed from water, is often considered to be a severe stressor, and some of the results in this thesis may reflect this: in Chapters 4 and 6, no significant differences in stress levels were found in handled fish regardless of experimental condition, which may indicate that handling is a stressful enough experience to outweigh any possible benefits of the interventions tested. Further interventions that may be developed from the results of this thesis would require some more work before they could be introduced, but provide some possibilities – using choice tests to establish environmental conditions most preferred by ornamental fish may allow identification of conditions which help to reduce stress and promote welfare, especially if those conditions are consistently introduced along the supply chain. Combining these choice tests with tests of motivation and stress assays would be an effective approach to improving welfare. Similarly, there may be potential for interventions based around conditioning fish to predict stressors or associate stressors with positive events, but a great deal more work would be needed to identify species, stressors, cues and rewards that this may work for. Furthermore, as the evidence to support this approach to improving welfare is limited, it should not be interpreted as a way to avoid trying to improve practices and procedures which are known to be stressful.

The lack of current research into stress, welfare and mortality in ornamental fish means that there is a great deal of scope for future research to explore. At a fundamental level, more information on the basic biology of the different species and their responses to common stressors would greatly help develop this field. Even within the existing literature on fish stress and welfare, over 30,000 fish species are represented by relatively few model systems. Given the huge diversity in fish species, it is likely that what suits one species will not be applicable to another, therefore more studies exploring the welfare needs and behaviours of ornamental fish are needed. Beyond this, some particular research areas may be particularly interesting to explore.

The first of these areas is the impact of early-life effects, including maternal stress and rearing environment. Early-life effects can have long-term impacts on physiology and behaviour – for example, maternal stress has been shown to negatively impact offspring survival in wild sockeye salmon *Oncorhynchus nerka* (Sopinka *et al.*, 2014), whilst a structurally complex or variable early rearing environment can affect brain development in rainbow trout *Oncorhynchus mykiss*, behavioural flexibility in cod *Gadus morhua*, and neural plasticity and cognitive ability in Atlantic salmon (Braithwaite and Salvanes, 2005; Kihslinger and Nevitt, 2006). Effects of early-life experience can be seen even when these experiences are relatively brief – for example, juvenile rainbow trout reared in physically enriched tanks for two months showed greater agility in a swimming test than those reared in barren conditions (Bergendahl *et al.*, 2016). Similarly, zebrafish *Danio rerio* exposed to varying structural enrichment or daily net chasing for three months as juveniles showed better learning capacity and less anxiety than controls, and these effects persisted approximately 9 months later even though fish were not exposed to enrichment or chasing after 3 months of age (DePasquale *et al.*, 2016). In contrast to this, sea bass *Dicentrarchus labrax* exposed to more husbandry-related stressors at early life stages showed poorer survival and disease resistance as juveniles (Varsamos *et al.*, 2006). Stressors therefore appear to have a variety of potential effects on resilience in later life. Exploring the impact of early-life factors on captive-bred ornamental fish may allow identification of conditions which may promote more resilience to stress, better disease resistance, and lower mortality rates.

A second area which could be explored is the potential role of nutrition or supplementation as a means of increasing resilience to stress in ornamental fish. Provision of diets rich in vitamins C and E has been shown to enhance stress tolerance in gilthead sea bream *Sparus aurata* (Montero *et al.*, 2001) and guppies (Lim *et al.*, 2002b). Furthermore, Nile tilapia *Oreochromis niloticus* fed a diet containing a probiotic showed greater survival than those not fed the probiotic (Ferguson *et al.*, 2010). Given that some stressors may be unavoidable for captive animals, greater exploration of diets which may promote stress resistance in fishes may therefore lead to lower mortality rates. This may be particularly important for ornamental fish, as they are highly diverse, but are usually fed flake or pellet diets designed to be generally appropriate for broadly-defined groups of fish (e.g. ‘tropical fish’, ‘bottom-feeders’).

Finally, an area which could be explored to benefit the welfare of ornamental fish is the impacts of the social environment. Many popular varieties of ornamental fish are social, and many ornamental fishes will go on to live in community tanks containing multiple species (Saxby *et al.*, 2010). It is therefore possible that the social environment of these fishes may be highly stressful, as fishes may be exposed to bullying, low social rankings, and high social variability. For example, social ranking has been found to impact zebrafish stress and immune function after only 5 days (Filby *et al.*, 2010). Social stress can impact metabolic rate (Sloman *et al.*, 2000; Nadler *et al.*, 2016), cortisol response (Jeffrey *et al.*, 2014) and immune system function (Peters *et al.*, 1991). Besides this, fish behaviour has been shown to be impacted by the presence or absence of familiar individuals – many species of fish have been shown to recognise and show a preference for familiar individuals over unfamiliar ones which can lead, for example, to decreased aggression in groups (Ward and Hart, 2003). Taken together, these two areas raise a potentially interesting area of research – can the way in which social environments are impacted by the supply chain be modified to reduce stress and improve welfare in ornamental fish? In other taxa, disruption of an animal's social environment has been shown to lead to decreased welfare, e.g. in laboratory rodents and non-human primates (Olsson and Westlund, 2007). It is therefore possible that the frequent changes in social environment might have a similar effect in fish. Studies combining social network analysis with welfare assays would be a way to explore the effects of this disrupted social environment.

## 7.3 Final conclusions

This thesis aimed to identify sources of stress and poor welfare within the ornamental fish trade, and to develop and test some potential interventions to reduce stress and improve welfare in ornamental fishes. Whilst both of these aims have been met, this thesis has also highlighted an important message: not enough is known about the ornamental fish industry, in terms of numbers of fish sold, numbers of fish dying, basic biology of the species and most potent causes of stress.

Despite this message, the future perspective is positive. The growing amount of attention being paid to the welfare of captive fish, including ornamental varieties, is encouraging, and has helped to expand the types of assays that can be used to assess fish welfare. Also, the ongoing debate over the existence of consciousness and the capacity for pain and suffering in fish, although it may never be won by either side, aids progress. Some areas which may become useful avenues of research to improve welfare in ornamental fish might not have been considered as being relevant to fish had this debate not taken place – for example, the potential for the development of psychological interventions aimed at altering the appraisal of stressors. Continuing these discussions may help to open up a range of possibilities that can be further explored to improve fish welfare.

# References

Adámek, Z., Linhart, O., Kratochvíl, M., Flajšhans, M., Randak, T., Policar, T., Masojídek, J. & Kozák, P. (2012). Aquaculture in the Czech Republic in 2012: modern European prosperous sector based on thousand-year history of pond culture. In *World Aquaculture*, pp. 20-27.

Aerts, J., Metz, J. R., Ampe, B., Decostere, A., Flik, G. & De Saeger, S. (2015). Scales Tell a Story on the Stress History of Fish. *PLoS ONE* **10**, e0123411.

Alves, R. N., Cordeiro, O., Silva, T. S., Richard, N., de Vareilles, M., Marino, G., Di Marco, P., Rodrigues, P. M. & Conceição, L. E. C. (2010). Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics. *Aquaculture* **299**, 57-66.

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26**, 32-46.

Andrews, C., Exell, A. & Carrington, N. (1988). *The Interpet Guide to Fish Health*. Surrey, UK: Salamander Books.

Angelidis, P., Baudin-Laurencin, F. & Youinou, P. (1987). Stress in rainbow trout, *Salmo gairdneri*: effects upon phagocyte chemiluminescence, circulating leucocytes and susceptibility to *Aeromonas salmonicida*. *Journal of Fish Biology* **31**, 113-122.

Animal Welfare Act (2006). Available at: http://www.legislation.gov.uk/ukpga/2006/45/pdfs/ukpga_20060045_en.pdf (last accessed 19 August 2016).

Archard, G. A., Earley, R. L., Hanninen, A. F. & Braithwaite, V. A. (2012). Correlated behaviour and stress physiology in fish exposed to different levels of predation pressure. *Functional Ecology* **26**, 637-645.

Ari, C. & D'Agostino, D. P. (2016). Contingency checking and self-directed behaviors in giant manta rays: Do elasmobranchs have self-awareness? *Journal of Ethology* **34**, 167-174.

Arlinghaus, R., Cooke, S. J., Schwab, A. & Cowx, I. G. (2007). Fish welfare: a challenge to the feelings-based approach, with implications for recreational fishing. *Fish and Fisheries* **8**, 57-71.

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science* **104**, 199-235.

Baensch, H. A. & Fischer, G. W. (2007). *Aquarium Atlas*. Melle, Germany: Mergus.

Bailey, M. & Burgess, P. (2004). *Tropical Fishlopaedia: A Complete Guide to Fish Care*. Gloucestershire, UK: Ringpress Books.

Balon, E. K. (2004). About the oldest domesticates among fishes. *Journal of Fish Biology* **65**, 1-27.

Barcellos, H. H. A., Koakoski, G., Chaulet, F., Kirsten, K. S., Kreutz, L. C., Kalueff, A. V. & Barcellos, L. J. G. (2018). The effects of auditory enrichment on zebrafish behavior and physiology. *PeerJ* **6**, e5162.

Barcellos, L. J. G., Kreutz, L. C., Koakoski, G., Oliveira, T. A., da Rosa, J. G. S. & Fagundes, M. (2012). Fish age, instead of weight and size, as a determining factor for time course differences in cortisol response to stress. *Physiology & Behavior* **107**, 397-400.

Barcellos, L. J. G., Ritter, F., Kreutz, L. C., Quevedo, R. M., da Silva, L. B., Bedin, A. C., Finco, J. & Cericato, L. (2007). Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, *Danio rerio*. *Aquaculture* **272**, 774-778.

Barcellos, L. J. G., Volpato, G. L., Barreto, R. E., Coldebella, I. & Ferreira, D. (2011). Chemical communication of handling stress in fish. *Physiology & Behavior* **103**, 372-375.

Bartley, D. (2000). Responsible Ornamental Fisheries. In *FAO Aquaculture Newsletter*, pp. 10-14: FAO.

Barton, B. A. (2000). Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. *North American Journal of Aquaculture* **62**, 12-18.

Barton, B. A. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology* **42**, 517-525.

Barton, B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1**, 3-26.

Barton, B. A., Ribas, L., Acerete, L. & Tort, L. (2005). Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. *Aquaculture Research* **36**, 172-179.

Barton, B. A., Schreck, C. B. & Sigismondi, L. A. (1986). Multiple acute disturbances evoke cumulative physiological stress responses in juvenile Chinook salmon. *Transactions of the American Fisheries Society* **115**, 245-251.

Barton, B. A. & Taylor, B. R. (1996). Oxygen requirements of fishes in northern Alberta Rivers with a general review of the adverse effects of low dissolved oxygen. *Water Quality Research Journal of Canada* **31**, 361-409.

Basquill, S. P. & Grant, J. W. A. (1998). An increase in habitat complexity reduces aggression and monopolization of food by zebra fish (*Danio rerio*). *Canadian Journal of Zoology* **76**, 770-772.

Batzina, A., Kalogiannis, D., Dalla, C., Papadopoulou-Daifoti, Z., Chadio, S. & Karakatsouli, N. (2014). Blue substrate modifies the time course of stress response in gilthead seabream *Sparus aurata*. *Aquaculture* **420**, 247-253.

Benhaim, D., Begout, M.-L., Pean, S., Manca, M., Prunet, P. & Chatain, B. (2013a). Impact of a plant-based diet on behavioural and physiological traits in sea bass (*Dicentrarchus labrax*). *Aquatic Living Resources* **26**, 121-131.

Benhaim, D., Guyomard, R., Chatain, B., Quillet, E. & Begout, M.-L. (2013b). Genetic differences for behaviour in juveniles from two strains of brown trout suggest an effect of domestication history. *Applied Animal Behaviour Science* **147**, 235-242.

Bergendahl, I. A., Miller, S., Depasquale, C., Giralico, L. & Braithwaite, V. (2016). Becoming a better swimmer: structural complexity enhances agility in a captive-reared fish: structural complexity enhances agility. *Journal of Fish Biology* **90**, 1112-1117.

Berka, R. (1986). The transport of live fish: A review. In *European Inland Fisheries Advisory Committee Technical Paper*, p. 52. Rome: FAO.

Betancur-R, R., Wiley, E. O., Arratia, G., Acero, A., Bailly, N., Miya, M., Lecointre, G. & Orti, G. (2017). Phylogenetic classification of bony fishes. *BMC Evolutionary Biology* **17**, 162.

Biswas, A., Kazushige, I. & Takii, K. (2010). Feeding interval and photoperiod influence the growth performance of striped knifejaw, *Oplegnathus fasciatus*. *Aquaculture Research* **41**, e517-e523.

Biswas, A. K., Seoka, M., Takii, K., Maita, M. & Kumai, H. (2006). Stress response of red sea bream *Pagrus major* to acute handling and chronic photoperiod manipulation. *Aquaculture* **252**, 566-572.

Bloomsmith, M. A., Jones, M. L., Snyder, R. J., Singer, R. A., Gardner, W. A., Liu, S. & Maple, T. L. (2003). Positive reinforcement training to elicit voluntary movement of two giant pandas throughout their enclosure. *Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association* **22**, 323-334.

Bloomsmith, M. A., Stone, A. M. & Laule, G. E. (1998). Positive reinforcement training to enhance the voluntary movement of group-housed chimpanzees within their enclosures. *Zoo Biology* **17**, 333-341.

Boesgaard, L., Nielsen, M. E. & Rosenkilde, P. (1993). Moderate exercise decreases plasma cortisol levels in Atlantic salmon (*Salmo salar*). *Comparative Biochemistry and Physiology Part A: Physiology* **106**, 641-643.

Boeuf, G. & Le Bail, P. Y. (1999). Does light have an influence on fish growth? *Aquaculture* **177**, 129-152.

Braithwaite, V. A. (2010). *Do Fish Feel Pain?* Oxford: Oxford University Press.

Braithwaite, V. A. & Boulcott, P. (2007). Pain perception, aversion and fear in fish. *Diseases of Aquatic Organisms* **75**, 131-138.

Braithwaite, V. A. & Ebbesson, L. O. E. (2014). Pain and stress responses in farmed fish. *Revue Scientifique Et Technique-Office International Des Epizooties* **33**, 245-253.

Braithwaite, V. A. & Huntingford, F. A. (2004). Fish and welfare: do fish have the capacity for pain perception and suffering? *Animal Welfare* **13**, S87-S92.

Braithwaite, V. A. & Salvanes, A. G. (2005). Environmental variability in the early rearing environment generates behaviourally flexible cod: implications for rehabilitating wild populations. *Proceedings of the Royal Society of London B: Biological Sciences* **272**, 1107-1113.

Bratland, S., Stien, L. H., Braithwaite, V. A., Juell, J.-E., Folkedal, O., Nilsson, J., Oppedal, F., Fosseidengen, J. E. & Kristiansen, T. S. (2010). From fright to anticipation: using aversive light stimuli to investigate reward conditioning in large groups of Atlantic salmon (*Salmo salar*). *Aquaculture International* **18**, 991-1001.

Broom, D. M. (2011). Animal welfare: concepts, study methods and indicators. *Revista Colombiana De Ciencias Pecuarias* **24**, 306-321.

Brown, A. A., Spetch, M. L. & Hurd, P. L. (2007). Growing in circles: Rearing environment alters spatial navigation in fish. *Psychological Science* **18**, 569-573.

Brown, C., Davidson, T. & Laland, K. (2003). Environmental enrichment and prior experience of live prey improve foraging behaviour in hatchery-reared Atlantic salmon. *Journal of Fish Biology* **63**, 187-196.

Bry, C. (1982). Daily variations in plasma cortisol levels of individual female rainbow trout *Salmo gairdneri*: Evidence for a post-feeding peak in well-adapted fish. *General and Comparative Endocrinology* **48**, 462-468.

Brydges, N. M., Boulcott, P., Ellis, T. & Braithwaite, V. A. (2009). Quantifying stress responses induced by different handling methods in three species of fish. *Applied Animal Behaviour Science* **116**, 295-301.

Burnley, T., Stryhn, H. & Hammell, K. L. (2012). Post-handling mortality during controlled field trials with marine grow-out Atlantic salmon, *Salmo salar* L. *Aquaculture* **368**, 55-60.

Burns, J. G., Saravanan, A. & Helen Rodd, F. (2009). Rearing Environment Affects the Brain Size of Guppies: Lab-Reared Guppies have Smaller Brains than Wild-Caught Guppies. *Ethology* **115**, 122-133.

Butcher, R. L. (1992). *BSAVA Manual of Ornamental Fish*. Cheltenham, United Kingdom: BSAVA.

Carenzi, C. & Verga, M. (2009). Animal welfare: review of the scientific concept and definition. *Italian Journal of Animal Science* **8**, 21-30.

Carlstead, K. & Shepherdson, D. (2000). Alleviating stress in zoo animals with environmental enrichment. In *The Biology of Aimal Stress: Basic Principles and Implications for Animal Welfare*, pp. 337-354. UK: CABI Publishing.

Cattelan, S., Lucon-Xiccato, T., Pilastro, A. & Griggio, M. (2017). Is the mirror test a valid measure of fish sociability? *Animal Behaviour* **127**, 109-116.

Cha, J. H., Rahimnejad, S., Yang, S. Y., Kim, K. W. & Lee, K. J. (2013). Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. *Aquaculture* **402**, 50-57.

Champagne, D. L., Hoefnagels, C. C. M., de Kloet, R. E. & Richardson, M. K. (2010). Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): Relevance for stress research. *Behavioural Brain Research* **214**, 332-342.

Chandroo, K. P., Duncan, I. J. H. & Moccia, R. D. (2004). Can fish suffer?: perspectives on sentience, pain, fear and stress. *Applied Animal Behaviour Science* **86**, 225-250.

Chapman, F. A., Colle, D. E., Rottmann, R. W. & Shireman, J. V. (1998). Controlled Spawning of the Neon Tetra. *The Progressive Fish-Culturist* **60**, 32-37.

Chapman, F. A., Fitz-Coy, S. A., Thunberg, E. M. & Adams, C. M. (1997). United States of America Trade in Ornamental Fish. *Journal of the World Aquaculture Society* **28**, 1-10.

Coleman, K. & Maier, A. (2010). The use of positive reinforcement training to reduce stereotypic behavior in rhesus macaques. *Applied Animal Behaviour Science* **124**, 142-148.

Coleman, K., Pranger, L., Maier, A., Lambeth, S. P., Perlman, J. E., Thiele, E. & Schapiro, S. J. (2008). Training rhesus macaques for venipuncture using positive reinforcement techniques: a comparison with chimpanzees. *Journal of the American Association for Laboratory Animal Science* **47**, 37-41.

Conceicao, L. E. C., Yufera, M., Makridis, P., Morais, S. & Dinis, M. T. (2010). Live feeds for early stages of fish rearing. *Aquaculture Research* **41**, 613-640.

Conte, F. S. (2004). Stress and the welfare of cultured fish. *Applied Animal Behaviour Science* **86**, 205-223.

Council of the European Union (1998). Council Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes

Croft, D. P., Arrowsmith, B., Bielby, J., Skinner, K., White, E., Couzin, I. D., Magurran, A. E., Ramnarine, I. & Krause, J. (2003). Mechanisms underlying shoal composition in the Trinidadian guppy, *Poecilia reticulata*. *Oikos* **100**, 429-438.

Csanyi, V. & Doka, A. (1993). Learning interactions between prey and predator fish. *Marine Behaviour and Physiology* **23**, 63-78.

Davenport, K. (1996). Characteristics of the current international trade in ornamental fish, with special reference to the European Union. *Revue Scientifique Et Technique-Office International Des Epizooties* **15**, 435-441.

Davis, K. B., Griffin, B. R. & Gray, W. L. (2002). Effect of handling stress on susceptibility of channel catfish *Ictalurus punctatus* to *Icthyophthirius multifiliis* and channel catfish virus infection. *Aquaculture* **214**, 55-66.

Davis, L. E. & Schreck, C. B. (1997). The energetic response to handling stress in juvenile coho salmon. *Transactions of the American Fisheries Society* **126**, 248-258.

Davison, W. (1997). The Effects of Exercise Training on Teleost Fish, a Review of Recent Literature. *Comparative Biochemistry and Physiology Part A: Physiology* **117**, 67-75.

Dawkins, M. S. (1998). Evolution and animal welfare. *Quarterly Review of Biology* **73**, 305-328.

Dawkins, M. S. (1999). The role of behaviour in the assessment of poultry welfare. *Worlds Poultry Science Journal* **55**, 295-303.

Dawkins, M. S. (2006). A user's guide to animal welfare science. *Trends in Ecology & Evolution* **21**, 77-82.

Dawkins, M. S. (2008). The science of animal suffering. *Ethology* **114**, 937-945.

de Villemereuil, P. & Nakagawa, S. (2014). General quantitative genetic methods for comparative biology. In *Modern phylogenetic comparative methods and their application in evolutionary biology* (Garamszegi, L. Z., ed.), pp. 287-303. Heidelberg, Germany: Springer.

DePasquale, C., Neuberger, T., M. Hirrlinger, A. & Braithwaite, V. (2016). The influence of complex and threatening environments in early life on brain size and behaviour. *Proceedings of the Royal Society B: Biological Sciences* **283**, 2-8.

DePasquale, C., Wagner, T., Archard, G. A., Ferguson, B. & Braithwaite, V. A. (2014). Learning rate and temperament in a high predation risk environment. *Oecologia* **176**, 661-667.

Douxfils, J., Mandiki, S. N. M., Marotte, G., Wang, N., Silvestre, F., Milla, S., Henrotte, E., Vandecan, M., Rougeot, C., Melard, C. & Kestemont, P. (2011).

Does domestication process affect stress response in juvenile Eurasian perch *Perca fluviatilis*? *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **159**, 92-99.

Dowd, S. (2003). Observations on the cardinal tetra (*Paracheirodon axelrodi*) ornamental fishery with an emphasis on assessments of stress. In *Institute of Aquaculture*, p. 102. Stirling: University of Stirling.

Dugatkin, L. A. & Alfieri, M. S. (2003). Boldness, behavioral inhibition and learning. *Ethology Ecology & Evolution* **15**, 43-49.

Duncan, I. (2005). Science-based assessment of animal welfare: farm animals. *Revue Scientifique Et Technique-Office International Des Epizooties* **24**, 483.

Duncan, I. J. H. & Fraser, D. (1997). Understanding animal welfare. In *Animal Welfare* (Appleby, M. C. & Hughes, B. O., eds.), pp. 19-31. Wallingford: CAB International.

Dunlop, R. & Laming, P. (2005). Mechanoreceptive and nociceptive responses in the central nervous system of goldfish (*Carassius auratus*) and trout (*Oncorhynchus mykiss*). *Journal of Pain* **6**, 561-568.

Earley, R. L., Edwards, J. T., Aseem, O., Felton, K., Blumer, L. S., Karom, M. & Grober, M. S. (2006). Social interactions tune aggression and stress responsiveness in a territorial cichlid fish (*Archocentrus nigrofasciatus*). *Physiology & Behavior* **88**, 353-363.

Eaton, L., Edmonds, E. J., Henry, T. B., Snellgrove, D. L. & Sloman, K. A. (2015). Mild maternal stress disrupts associative learning and increases aggression in offspring. *Hormones and Behavior* **71**, 10-15.

EFSA (2009). Species-specific welfare aspects of the main systems of stunning and killing of farmed seabass and seabream. *The EFSA Journal* **1010**, 1-52.

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., Elkhayat, S. I., Bartels, B. K., Tien, A. K., Tien, D. H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z. & Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research* **205**, 38-44.

Ellis, T., Berrill, I., Lines, J., Turnbull, J. F. & Knowles, T. G. (2012). Mortality and fish welfare. *Fish Physiology and Biochemistry* **38**, 189-199.

Ellis, T., James, J. D., Stewart, C. & Scott, A. P. (2004). A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. *Journal of Fish Biology* **65**, 1233-1252.

Ellis, T., James, J. D., Sundh, H., Fridell, F., Sundell, K. & Scott, A. P. (2007). Non-invasive measurement of cortisol and melatonin in tanks stocked with seawater Atlantic salmon. *Aquaculture* **272**, 698-706.

Ellis, T., Sanders, M. B. & Scott, A. P. (2013). Non-invasive monitoring of steroids in fishes. *Wiener Tierarztliche Monatsschrift* **100**, 255-269.

Endo, M., Kumahara, C., Yoshida, T. & Tabata, M. (2002). Reduced stress and increased immune responses in Nile tilapia kept under self-feeding conditions. *Fisheries Science* **68**, 253-257.

Eslamloo, K., Akhavan, S. R., Fallah, F. J. & Henry, M. A. (2014). Variations of physiological and innate immunological responses in goldfish (*Carassius auratus*) subjected to recurrent acute stress. *Fish & Shellfish Immunology* **37**, 147-153.

Falahatkar, B., Poursaeid, S., Shakoorian, M. & Barton, B. (2009). Responses to handling and confinement stressors in juvenile great sturgeon *Huso huso*. *Journal of Fish Biology* **75**, 784-796.

FAO (1999). Ornamental aquatic life: what's FAO got to do with it? Available at: http://www.fao.org/News/1999/990901-e.htm (last accessed 13/11/2014).

FAO (2014). Year Book of Fishery Statistics 2012. Available at: http://www.fao.org/3/a-i3740t.pdf (last accessed 10/08/16).

FAO (2017). Fish industry recognizing ornamental fish trade at the 2nd International Ornamental Fish Trade and Technical Conference. Available at: http://www.fao.org/in-action/globefish/news-events/details-news/en/c/469648/ (last accessed 13/08/2018).

Farm Animal Welfare Council (1996). Report on the Welfare of Farmed Fish. Surbiton, Surrey.

Farm Animal Welfare Council (2009). FAWC Report on Farm Animal Welfare in Great Britain: Past, Present and Future. In *FAWC advice to government*: Department for Environment, Food and Rural Affairs (DEFRA).

Fast, M. D., Hosoya, S., Johnson, S. C. & Afonso, L. O. B. (2008). Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress. *Fish & Shellfish Immunology* **24**, 194-204.

Felix, A. S., Faustino, A. I., Cabral, E. M. & Oliveira, R. F. (2013). Noninvasive Measurement of Steroid Hormones in Zebrafish Holding-Water. *Zebrafish* **10**, 110-115.

Felsenstein, J. (1985). Phylogenies and the Comparative Method. *The American Naturalist* **125**, 1-15.

Ferguson, R. M. W., Merrifield, D. L., Harper, G. M., Rawling, M. D., Mustafa, S., Picchietti, S., Balcazar, L. & Davies, S. J. (2010). The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). *Journal of Applied Microbiology* **109**, 851-862.

Fevolden, S.-E., Røed, K. H. & Fjalestad, K. (2003). A combined salt and confinement stress enhances mortality in rainbow trout (*Oncorhynchus mykiss*) selected for high stress responsiveness. *Aquaculture* **216**, 67-76.

Field, A., Miles, J. & Field, Z. (2012). *Discovering Statistics Using R*. UK: SAGE Publications Ltd.

Filby, A. L., Paull, G. C., Bartlett, E. J., Van Look, K. J. W. & Tyler, C. R. (2010). Physiological and health consequences of social status in zebrafish (*Danio rerio*). *Physiology & Behavior* **101**, 576-587.

Fischer, E. K., Harris, R. M., Hofmann, H. A. & Hoke, K. L. (2014). Predator exposure alters stress physiology in guppies across timescales. *Hormones and Behavior* **65**, 165-172.

Flik, G., Klaren, P. H. M., Van den Burg, E. H., Metz, J. R. & Huising, M. O. (2006). CRF and stress in fish. *General and Comparative Endocrinology* **146**, 36-44.

Fosså, S. A. (2004). Man-made fish: domesticated fishes and their place in the aquatic trade and hobby. *OFI Journal* **44**, 1-23.

Fraser, B. A., Weadick, C. J., Janowitz, I., Rodd, F. H. & Hughes, K. A. (2011). Sequencing and characterization of the guppy (*Poecilia reticulata*) transcriptome. *BMC Genomics* **12**, 202.

Freckleton, R. P., Harvey, P. H. & Pagel, M. (2002). Phylogenetic Analysis and Comparative Data: A Test and Review of Evidence. *The American Naturalist* **160**, 712-726.

Fürtbauer, I., Pond, A., Heistermann, M. & King, A. J. (2015). Personality, plasticity and predation: linking endocrine and behavioural reaction norms in stickleback fish. *Functional Ecology* **29**, 931-940.

Galhardo, L. & Oliveira, R. F. (2009). Psychological stress and welfare in fish. *Annual Review of Biomedical Sciences* **11**, 1-20.

Galhardo, L. & Oliveira, R. F. (2014). The effects of social isolation on steroid hormone levels are modulated by previous social status and context in a cichlid fish. *Hormones and Behavior* **65**, 1-5.

Galhardo, L., Vital, J. & Oliveira, R. F. (2011). The role of predictability in the stress response of a cichlid fish. *Physiology & Behavior* **102**, 367-372.

Garamszegi, L. Z. (2014). *Modern phylogenetic comparative methods and their application in evolutionary biology: concepts and practice*. Heidelberg, Germany: Springer.

Gelman, A. & Rubin, D. B. (1992). Inference from iterative simulation using multiple sequences. *Statistical Science* **7**, 457-472.

Gesto, M., Hernandez, J., Lopez-Patino, M. A., Soengas, J. L. & Miguez, J. M. (2015). Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **188**, 65-69.

Ghosh, S., Sinha, A. & Sahu, C. (2008). Dietary probiotic supplementation in growth and health of live-bearing ornamental fishes. *Aquaculture Nutrition* **14**, 289-299.

Giacomini, A. C. V. V., de Abreu, M. S., Koakoski, G., Idalêncio, R., Kalichak, F., Oliveira, T. A., da Rosa, J. G. S., Gusso, D., Piato, A. L. & Barcellos, L. J. G. (2015). My stress, our stress: Blunted cortisol response to stress in isolated housed zebrafish. *Physiology & Behavior* **139**, 182-187.

Gomes, L. C., Roubach, R., Araujo-Lima, C. A., Chippari-Gomes, A. R., Lopes, N. P. & Urbinati, E. C. (2003). Effect of fish density during transportation on stress and mortality of juvenile tambaqui Colossoma macropomum. *Journal of the World Aquaculture Society* **34**, 76-84.

Grandin, T., Rooney, M. B., Phillips, M., Cambre, R. C., Irlbeck, N. A. & Graffam, W. (1995). Conditioning of nyala (*Tragelaphus angasi*) to blood sampling in a crate with positive reinforcement. *Zoo Biology* **14**, 261-273.

Gronquist, D. & Berges, J. A. (2013). Effects of Aquarium-Related Stressors on the Zebrafish: A Comparison of Behavioral, Physiological, and Biochemical Indicators. *Journal of Aquatic Animal Health* **25**, 53-65.

Hadfield, J. D. (2010). MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *Journal of Statistical Software* **33**, 1-22.

Hadfield, J. D. & Nakagawa, S. (2010). General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. *Journal of Evolutionary Biology* **23**, 494-508.

Harmon, T. S. (2009). Methods for reducing stressors and maintaining water quality associated with live fish transport in tanks: a review of the basics. *Reviews in Aquaculture* **1**, 58-66.

Harnish, R. A., Colotelo, A. H., Brown, R. S. J. R. i. F. B. & Fisheries (2011). A review of polymer-based water conditioners for reduction of handling-related injury. *Reviews in Fish Biology and Fisheries* **21**, 43-49.

Head, A. B. & Malison, J. A. (2000). Effects of lighting spectrum and disturbance level on the growth and stress responses of yellow perch *Perca flavescens*. *Journal of the World Aquaculture Society* **31**, 73-80.

Helfman, G., Collette, B., Facey, D. & Bowen, B. (2009). *The Diversity of Fishes: Biology, Evolution, and Ecology*. UK: Wiley-Blackwell.

Höglund, E., Weltzien, F.-A., Schjolden, J., Winberg, S., Ursin, H. & Døving, K. B. (2005). Avoidance behavior and brain monoamines in fish. *Brain Research* **1032**, 104-110.

Housworth, E. A., Martins, E. P. & Lynch, M. (2004). The Phylogenetic Mixed Model. *The American Naturalist* **163**, 84-96.

Howerton, C. L., Garner, J. P. & Mench, J. A. (2008). Effects of a running wheel-igloo enrichment on aggression, hierarchy linearity, and stereotypy in group-housed male CD-1 (ICR) mice. *Applied Animal Behaviour Science* **115**, 90-103.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Sandoe, P. & Turnbull, J. F. (2006). Current issues in fish welfare. *Journal of Fish Biology* **68**, 332-372.

Huntingford, F. A. & Kadri, S. (2009). Taking account of fish welfare: lessons from aquaculture. *Journal of Fish Biology* **75**, 2862-2867.

Iguchi, K., Ogawa, K., Nagae, M. & Ito, F. (2003). The influence of rearing density on stress response and disease susceptibility of ayu (*Plecoglossus altivelis*). *Aquaculture* **220**, 515-523.

Ikeda, T. & Kohshima, S. (2009). Why is the neon tetra so bright? Coloration for mirror-image projection to confuse predators? “Mirror-image decoy” hypothesis. *Environmental Biology of Fishes* **86**, 427-441.

Iwama, G. K. (1998). Stress in fish. *Annals of the New York Academy of Sciences* **851**, 304-310.

Jacobs, J. (1974). Quantitative measurement of food selection. *Oecologia* **14**, 413-417.

Jacquin, L., Reader, S. M., Boniface, A., Mateluna, J., Patalas, I., Pérez-Jvostov, F. & Hendry, A. P. (2016). Parallel and nonparallel behavioural evolution in response to parasitism and predation in Trinidadian guppies. *Journal of Evolutionary Biology* **29**, 1406-1422.

James, J. D., Ellis, T. & Scott, A. P. (2004). Water-based measurement of rainbow trout melatonin. *Journal of Fish Biology* **65**, 1298-1304.

Jarvi, T. (1990). Cumulative acute physiological stress in Atlantic salmon smolts - the effect of osmotic imbalance and the presence of predators. *Aquaculture* **89**, 337-350.

Jeffrey, J. D., Gollock, M. J. & Gilmour, K. M. (2014). Social stress modulates the cortisol response to an acute stressor in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology* **196**, 8-16.

Jensen, M. B. & Pedersen, L. J. (2008). Using motivation tests to assess ethological needs and preferences. *Applied Animal Behaviour Science* **113**, 340-356.

Jha, P. (2010). Exogenous plankton as food for intensive rearing of ornamental cyprinid, *Epalzeorhynchus frenatus*. *Archivos de Zootecnia* **59**, 11-20.

Jha, P. & Barat, S. (2005). The Effect of Stocking Density on Growth, Survival Rate, and Number of Marketable Fish Produced of Koi Carps, *Cyprinus carpio* vr. koi in Concrete Tanks. *Journal of Applied Aquaculture* **17**, 89-102.

Johnson, E. O., Kamilaris, T. C., Chrousos, G. P. & Gold, P. W. (1992). Mechanisms of stress - a dynamic overview of hormonal and behavioural homeostasis. *Neuroscience and Biobehavioral Reviews* **16**, 115-130.

Jorgensen, E. H., Christiansen, J. S. & Jobling, M. (1993). Effects of stocking density on food-intake, growth performance and oxygen consumption in Arctic charr (*Salvelinus alpinus*). *Aquaculture* **110**, 191-204.

Jun, L. J., Jeong, J. B., Kim, J. H., Nam, J. H., Shin, K. W., Kim, J. K., Kang, J. C. & Jeong, H. D. (2009). Influence of temperature shifts on the onset and development of red sea bream iridoviral disease in rock bream *Oplegnathus fasciatus*. *Diseases of Aquatic Organisms* **84**, 201-208.

Kamilar, J. M. & Cooper, N. (2013). Phylogenetic signal in primate behaviour, ecology and life history. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368**.

Karakatsouli, N., Papoutsoglou, S. E., Panopoulos, G., Papoutsoglou, E. S., Chadio, S. & Kalogiannis, D. (2008). Effects of light spectrum on growth and stress response of rainbow trout *Oncorhynchus mykiss* reared under recirculating system conditions. *Aquacultural Engineering* **38**, 36-42.

Kells, A., Dawkins, M. S. & Borja, M. C. (2001). The Effect of a 'Freedom Food' Enrichment on the Behaviour of Broilers on Commercial Farms. *Animal Welfare* **10**, 347-356.

Key, B. (2016). Why fish do not feel pain. *Animal Sentience* **2016.003**, 1-34.

Kihslinger, R. L. & Nevitt, G. A. (2006). Early rearing environment impacts cerebellar growth in juvenile salmon. *Journal of Experimental Biology* **209**, 504-509.

Kinross, J. M., Darzi, A. W. & Nicholson, J. K. (2011). Gut microbiome-host interactions in health and disease. *Genome Medicine*, 3-14.

Kistler, C., Hegglin, D., Wurbel, H. & Konig, B. (2011). Preference for structured environment in zebrafish (*Danio rerio*) and checker barbs (*Puntius oligolepis*). *Applied Animal Behaviour Science* **135**, 318-327.

Koakoski, G., Oliveira, T. A., da Rosa, J. G. S., Fagundes, M., Kreutz, L. C. & Barcellos, L. J. G. (2012). Divergent time course of cortisol response to stress in fish of different ages. *Physiology & Behavior* **106**, 129-132.

Kolluru, G. R., Walz, J., Hanninen, A. F., Downey, K., Kalbach, B., Gupta, S. & Earley, R. L. (2015). Exploring behavioral and hormonal flexibility across light environments in guppies from low-predation populations. *Behaviour* **152**, 963-993.

Korte, S. M., Olivier, B. & Koolhaas, J. M. (2007). A new animal welfare concept based on allostasis. *Physiology & Behavior* **92**, 422-428.

Kruschke, J. (2014). *Doing Bayesian data analysis: A tutorial with R, JAGS, and Stan*. London: Academic Press.

Kubokawa, K., Watanabe, T., Yoshioka, M. & Iwata, M. (1999). Effects of acute stress on plasma cortisol, sex steroid hormone and glucose levels in male and female sockeye salmon during the breeding season. *Aquaculture* **172**, 335-349.

Kydd, E. & Brown, C. (2009). Loss of shoaling preference for familiar individuals in captive-reared crimson spotted rainbowfish *Melanotaenia duboulayi*. *Journal of Fish Biology* **74**, 2187-2195.

Landin, J. (2012). Using physiology and behaviour to assess enrichment strategies for the welfare of rainbow trout. In *Department of Biosciences*: University of Exeter.

Leal, M. C., Vaz, M. C. M., Puga, J., Rocha, R. J. M., Brown, C., Rosa, R. & Calado, R. (2016). Marine ornamental fish imports in the European Union: an economic perspective. *Fish and Fisheries* **17**, 459-468.

Leonardi, M. O. & Klempau, A. E. (2003). Artificial photoperiod influence on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) in the Southern Hemisphere. *Aquaculture* **221**, 581-591.

Lepage, O., Øverli, Ø., Petersson, E., Järvi, T. & Winberg, S. (2000). Differential Stress Coping in Wild and Domesticated Sea Trout. *Brain, Behavior and Evolution* **56**, 259-268.

Lidster, K., Readman, G., Prescott, M. & Owen, S. (2017). International survey on the use and welfare of zebrafish *Danio rerio* in research. *Journal of Fish Biology* **90**, 1891-1905.

Lim, L. C., Cho, Y. L., Dhert, P., Wong, C. C., Nelis, H. & Sorgeloos, P. (2002a). Use of decapsulated *Artemia* cysts in ornamental fish culture. *Aquaculture Research* **33**, 575-589.

Lim, L. C., Dhert, P., Chew, W. Y., Dermaux, V., Nelis, H. & Sorgeloos, P. (2002b). Enhancement of stress resistance of the guppy *Poecilia reticulata* through feeding with vitamin C supplement. *Journal of the World Aquaculture Society* **33**, 32-40.

Lim, L. C., Dhert, P. & Sorgeloos, P. (2003). Recent developments and improvements in ornamental fish packaging systems for air transport. *Aquaculture Research* **34**, 923-935.

Loiselle, P. V. (1979). On dither fish. In *Freshwater and Marine Aquarium Magazine*, pp. 30-34, 76-79.

Lower, N., Moore, A., Scott, A. P., Ellis, T., James, J. D. & Russell, I. C. (2005). A non-invasive method to assess the impact of electronic tag insertion on stress levels in fishes. *Journal of Fish Biology* **67**, 1202-1212.

Madaro, A., Fernö, A., Kristiansen, T. S., Olsen, R. E., Gorissen, M., Flik, G. & Nilsson, J. (2016). Effect of predictability on the stress response to chasing in Atlantic salmon (*Salmo salar* L.) parr. *Physiology & Behavior* **153**, 1-6.

Magellan, K., Johnson, A., Williamson, L., Richardson, M., Watt, W. & Kaiser, H. (2012). Alteration of tank dimensions reduces male aggression in the swordtail. *Journal of Applied Ichthyology* **28**, 91-94.

Magurran, A. E. (2005). *Evolutionary ecology: the Trinidadian guppy*. UK: Oxford University Press.

Maia, C. M., Ferguson, B., Volpato, G. L. & Braithwaite, V. A. (2017). Physical and psychological motivation tests of individual preferences in rainbow trout. *Journal of Zoology* **302**, 108-118.

Manuel, R., Boerrigter, J., Roques, J., van der Heul, J., van den Bos, R., Flik, G. & van de Vis, H. (2014). Stress in African catfish (*Clarias gariepinus*) following overland transportation. *Fish Physiology and Biochemistry* **40**, 33-44.

Martins, C. I. M., Eding, E. H. & Verreth, J. A. J. (2011). Stressing fish in Recirculating Aquaculture Systems (RAS): does stress induced in one group of fish affect the feeding motivation of other fish sharing the same RAS? *Aquaculture Research* **42**, 1378-1384.

Masefield, W. (1999). Forage preferences and enrichment in a group of captive Livingstone's fruit bats *Pteropus livingstonii*. *Dodo* **35**, 48-56.

Mayer, I., Meager, J., Skjaeraasen, J. E., Rodewald, P., Sverdrup, G. & Ferno, A. (2011). Domestication causes rapid changes in heart and brain morphology in Atlantic cod (*Gadus morhua*). *Environmental Biology of Fishes* **92**, 181-186.

McArdle, B. & Anderson, M. (2001). Fitting Multivariate Models to Community Data: A Comment on Distance-Based Redundancy Analysis. *Ecology* **82**, 290-297.

McCormick, S. D., Shrimpton, J. M., Carey, J. B., O'Dea, M. F., Sloan, K. E., Moriyama, S. & Bjornsson, B. T. (1998). Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. *Aquaculture* **168**, 221-235.

McGregor, P. K., Peake, T. M. & Lampe, H. M. (2001). Fighting fish *Betta splendens* extract relative information from apparent interactions: what happens when what you see is not what you get. *Animal Behaviour* **62**, 1059-1065.

Midtlyng, P. J. (1997). Vaccinated fish welfare: Protection versus side-effects. *Developments in Biological Standardization* **90**, 371-379.

Midtlyng, P. J., Storset, A., Michel, C., Slierendrecht, W. J. & Okamoto, N. (2002). Breeding for disease resistance in fish. *Bulletin of the European Association of Fish Pathologists* **22**, 166-172.

Miller-Morgan, T. (2009). A Brief Overview of the Ornamental Fish Trade and Hobby. In *Fundamentals of Ornamental Fish Health* (Roberts, H. E., ed.), pp. 25-32. Malaysia: Wiley-Blackwell.

Moberg, G. P. (2000). Biological response to stress: implications for animal welfare. In *The Biology of Aimal Stress: Basic Principles and Implications for*

*Animal Welfare* (Moberg, G. P. & Mench, J. A., eds.), pp. 1-21. UK: CABI Publishing.

Mommsen, T. P., Vijayan, M. M. & Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9**, 211-268.

Montero, D., Izquierdo, M. S., Tort, L., Robaina, L. & Vergara, J. M. (1999). High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiology and Biochemistry* **20**, 53-60.

Montero, D., Tort, L., Robaina, L., Vergara, J. M. & Izquierdo, M. S. (2001). Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. *Fish & Shellfish Immunology* **11**, 473-490.

Monticini, P. (2010). Production and Commerce of Ornamental Fish: technical-managerial and legislative aspects. In *GLOBEFISH Research Programme*, p. 134. Rome: FAO.

Moreira, P. S. A. & Volpato, G. L. (2004). Conditioning of stress in Nile tilapia. *Journal of Fish Biology* **64**, 961-969.

Moretz, J. A., Martins, E. P. & Robison, B. D. (2007). Behavioral syndromes and the evolution of correlated behavior in zebrafish. *Behavioral Ecology* **18**, 556-562.

Mugnier, C., Fostier, A., Guezou, S., Gaignon, J. L. & Quemener, L. (1998). Effect of some repetitive factors on turbot stress response. *Aquaculture International* **6**, 33-45.

Muraco, J. J. J., Aspbury, A. S. & Gabor, C. R. (2014). Does male behavioral type correlate with species recognition and stress? *Behavioral Ecology* **25**, 200-205.

Nadler, L. E., Killen, S. S., McClure, E. C., Munday, P. L. & McCormick, M. I. (2016). Shoaling reduces metabolic rate in a gregarious coral reef fish species. *The Journal of Experimental Biology* **219**, 2802-2805.

Näslund, J. & Johnsson, J. I. (2016). Environmental enrichment for fish in captive environments: effects of physical structures and substrates. *Fish and Fisheries* **17**, 1-30.

Nevison, C. M., Hurst, J. L. & Barnard, C. J. (1999). Strain-Specific Effects of Cage Enrichment in Male Laboratory Mice (*Mus musculus*). *Animal Welfare* **8**, 361-379.

Newberry, R. C. (1995). Environmental enrichment: Increasing the biological relevance of captive environments. *Applied Animal Behaviour Science* **44**, 229-243.

Nijman, V. & Heuts, B. A. (2000). Effect of environmental enrichment upon resource holding power in fish in prior residence situations. *Behavioural Processes* **49**, 77-83.

Nilsson, J., Stien, L. H., Fosseidengen, J. E., Olsen, R. E. & Kristiansen, T. S. (2012). From fright to anticipation: Reward conditioning versus habituation to a moving dip net in farmed Atlantic cod (*Gadus morhua*). *Applied Animal Behaviour Science* **138**, 118-124.

Noga, E. J. (2010). *Fish Disease: Diagnosis and Treatment*. Singapore: Wiley-Blackwell.

Nordgreen, J., Horsberg, T. E., Ranheim, B. & Chen, A. C. N. (2007). Somatosensory evoked potentials in the telencephalon of Atlantic salmon (*Salmo salar*) following galvanic stimulation of the tail. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **193**, 1235-1242.

Nunez, J. (2009). Domestication of new economically important Amazonian fish species. *Cahiers Agricultures* **18**, 136-143.

O'Brien, J., Heffernan, S., Thomson, P. & McGreevy, P. (2008). Effect of positive reinforcement training on physiological and behavioural stress responses in the hamadryas baboon (*Papio hamadryas*). *Animal Welfare* **17**, 125-138.

O’Connor, E. A., Pottinger, T. G. & Sneddon, L. U. (2011). The effects of acute and chronic hypoxia on cortisol, glucose and lactate concentrations in different populations of three-spined stickleback. *Fish Physiology and Biochemistry* **37**, 461-469.

OATA (2014). OATA Annual Review 2013/2014. Available at: http://www.ornamentalfish.org/wp-content/uploads/OATA-AnnRep-13-14-single-WEB.pdf (last accessed 31/10/14).

OATA (2015). Code of Conduct. Available at: https://ornamentalfish.org/wp-content/uploads/2015/10/CODE-OF-CONDUCT-FINAL-OCT-2015.pdf (last accessed 13/08/2018).

OFI (2014). Code of Ethics. Available at: http://www.ofish.org/about/code-of-ethics (last accessed 14 November 2014).

OFI (2015). Ornamental fish industry data. Available at: http://www.ofish.org/ornamental-fish-industry-data (last accessed 13 August 2018).

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, B., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E. & Wagner, H. (2018). vegan: Community Ecology package.

Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. *Journal of Fish Diseases* **35**, 83-108.

Olivier, K. (2001). The Ornamental Fish Market. In *GLOBEFISH Research Programme*. Rome: FAO.

Olivier, K. (2003). World Trade in Ornamental Species. In *Marine Ornamental Species: Collection, Culture and Conservation* (Cato, J., Brown, CL, ed.), pp. 49-64. Iowa: Iowa State Press.

Olsén, K. H., Ask, K., Olsén, H., Porsch-Hällström, I. & Hallgren, S. (2014). Effects of the SSRI citalopram on behaviours connected to stress and reproduction in Endler guppy, *Poecilia wingei*. *Aquatic Toxicology* **151**, 97-104.

Olsson, I. A. S. & Westlund, K. (2007). More than numbers matter: The effect of social factors on behaviour and welfare of laboratory rodents and non-human primates. *Applied Animal Behaviour Science* **103**, 229-254.

Øverli, Ø., Pottinger, T. G., Carrick, T. R., Øverli, E. & Winberg, S. (2002). Differences in behaviour between rainbow trout selected for high- and low-stress responsiveness. *Journal of Experimental Biology* **205**, 391-395.

Øverli, Ø., Sørensen, C. & Nilsson, G. E. (2006). Behavioral indicators of stress-coping style in rainbow trout: Do males and females react differently to novelty? *Physiology & Behavior* **87**, 506-512.

Øverli, Ø., Winberg, S. & Pottinger, T. G. (2005). Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout - a review. *Integrative and Comparative Biology* **45**, 463-474.

Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature* **401**, 877.

Pasnik, D. J., Evans, J. J. & Klesius, K. H. (2010). Stress in Fish. In *Fundamentals of Ornamental Fish Health* (Roberts, H. E., ed.), pp. 33-38. Malaysia: Wiley-Blackwell.

Peters, G., Nüßgen, A., Raabe, A. & Möck, A. (1991). Social stress induces structural and functional alterations of phagocytes in rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology* **1**, 17-31.

Pickering, A. D. & Pottinger, T. G. (1983). Seasonal and diel changes in plasma cortisol levels of the brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* **49**, 232-239.

Pickering, A. D., Pottinger, T. G. & Christie, P. (1982). Recovery of the brown trout, *Salmo trutta* L., from acute handling stress: a time-course study. *Journal of Fish Biology* **20**, 229-244.

Piper, R., McElwain, I., Orme, L., McCraren, J., Fowler, L. & Leonard, J. (1982). *Fish hatchery management*. Washington, D.C.: United States Department of the Interior Fish and Wildlife Service.

Ploeg, A. (2007a). Facts on Mortality in Shipments of Ornamental Fish. In *International Transport of Live Fish in the Ornamental Aquatic Industry* (Ploeg, A., Fossa, S. A., Bassleer, G., Willis, S. & Chuan, L. L., eds.), pp. 115-122. Netherlands: OFI.

Ploeg, A. (2007b). The Volume of the Ornamental Fish trade. In *International Transport of Live Fish in the Ornamental Aquatic Industry* (Ploeg, A., Fossa, S. A., Bassleer, G., Willis, S. & Chuan, L. L., eds.), pp. 48-61. Netherlands: OFI.

Ploeg, A. (2013). The status of the ornamental aquatic industry. *Ornamental Fish International* **72**, 11-13.

Pomerantz, O. & Terkel, J. (2009). Effects of positive reinforcement training techniques on the psychological welfare of zoo-housed chimpanzees (*Pan troglodytes*). *American Journal of Primatology* **71**, 687-695.

Portavella, M., Vargas, J. P., Torres, B. & Salas, C. (2002). The effects of telencephalic pallial lesions on spatial, temporal, and emotional learning in goldfish. *Brain Research Bulletin* **57**, 397-399.

Portz, D. E., Woodley, C. M. & Cech, J. J., Jr. (2006). Stress-associated impacts of short-term holding on fishes. *Reviews in Fish Biology and Fisheries* **16**, 125-170.

Pottinger, T. & Carrick, T. (1999). Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. *General and Comparative Endocrinology* **116**, 122-132.

Pottinger, T. G. (2008). The stress response in fish - mechanisms, effects and measurement. In *Fish Welfare* (Branson, E., ed.). Oxford, UK: Wiley-Blackwell.

Pottinger, T. G. & Carrick, T. R. (2001). Stress responsiveness affects dominant-subordinate relationships in rainbow trout. *Hormones and Behavior* **40**, 419-427.

Pottinger, T. G., Pickering, A. D. & Hurley, M. A. (1992). Consistency in the stress response of individuals of two strains of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **103**, 275-289.

Pounder, K. C., Mitchell, J. L., Thomson, J. S., Pottinger, T. G., Buckley, J. & Sneddon, L. U. (2016). Does environmental enrichment promote recovery from stress in rainbow trout? *Applied Animal Behaviour Science* **176**, 136-142.

Prang, G. (2007). An industry analysis of the freshwater ornamental fishery with particular reference to the supply of Brazilian freshwater ornamentals to the UK market. *Uakari* **3**, 7-51.

R Development Core Team (2015). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Ramsay, J. M., Feist, G. W., Schreck, C. B., Couture, R., O'Neil, J. & Noakes, D. L. G. (2009a). The Effect of Food Deprivation on the Cortisol Response to Crowding in Juvenile Steelhead. *North American Journal of Aquaculture* **71**, 130-133.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. & Schreck, C. B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* **258**, 565-574.

Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L. & Schreck, C. B. (2009b). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture* **297**, 157-162.

Ramsay, J. M., Watral, V., Schreck, C. B. & Kent, M. L. (2009c). Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). *Journal of Fish Diseases* **32**, 931-941.

Ramsay, J. M., Watral, V., Schreck, C. B. & Kent, M. L. (2009d). Pseudoloma neurophilia infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. *Diseases of Aquatic Organisms* **88**, 69-84.

Randall, D. J. & Tsui, T. K. N. (2002). Ammonia toxicity in fish. *Marine Pollution Bulletin* **45**, 17-23.

Reznick, D. & Bryant, M. (2007). Comparative long-term mark-recapture studies of guppies (Poecilia reticulata): differences among high and low predation localities in growth and survival. *Annales Zoologici Fennici* **44**, 152-160.

Robb, D. H. F. (2008). Welfare of Fish at Harvest. In *Fish Welfare* (Branson, E., ed.). Oxford, UK: Blackwell Publishing.

Roberts, H. E. (2010). *Fundamentals of ornamental fish health*. Malaysia: Wiley-Blackwell.

Rose, J. D. (2002). The Neurobehavioral nature of fishes and the question of awareness and pain. *Reviews in Fisheries Science* **10**, 1-38.

Rose, J. D., Arlinghaus, R., Cooke, S. J., Diggles, B. K., Sawynok, W., Stevens, E. D. & Wynne, C. D. L. (2014). Can fish really feel pain? *Fish and Fisheries* **15**, 97-133.

Rubec, P. J., Cruz, F., Pratt, V., Oellers, R. & Lallo, F. (2000). Cyanide-free, net-caught fish for the Marine Aquarium Trade. *SPC Live Reef Fish Information Bulletin* **7**, 28-34.

Sadoul, B., Leguen, I., Colson, V., Friggens, N. C. & Prunet, P. (2015). A multivariate analysis using physiology and behavior to characterize robustness in two isogenic lines of rainbow trout exposed to a confinement stress. *Physiology & Behavior* **140**, 139-147.

Sakai, M. (1999). Current research status of fish immunostimulants. *Aquaculture* **172**, 63-92.

Sampaio, F. D. F. & Freire, C. A. (2016). An overview of stress physiology of fish transport: changes in water quality as a function of transport duration. *Fish and Fisheries* **17**, 1055-1072.

Sánchez, J. A., López-Olmeda, J. F., Blanco-Vives, B. & Sánchez-Vázquez, F. J. (2009). Effects of feeding schedule on locomotor activity rhythms and stress response in sea bream. *Physiology & Behavior* **98**, 125-129.

Sandkam, B., Young, C. M. & Breden, F. (2015). Beauty in the eyes of the beholders: colour vision is tuned to mate preference in the Trinidadian guppy (Poecilia reticulata). *Molecular Ecology* **24**, 596-609.

Sarameh, S. P., Falahatkar, B., Takami, G. A. & Efatpanah, I. (2012). Effects of different photoperiods and handling stress on spawning and reproductive performance of pikeperch *Sander lucioperca*. *Animal Reproduction Science* **132**, 213-222.

Saxby, A., Adams, L., Snellgrove, D., Wilson, R. W. & Sloman, K. A. (2010). The effect of group size on the behaviour and welfare of four fish species commonly kept in home aquaria. *Applied Animal Behaviour Science* **125**, 195-205.

Schapiro, S. J., Perlman, J. E. & Boudreau, B. A. (2001). Manipulating the affiliative interactions of group-housed rhesus macaques using positive reinforcement training techniques. *American Journal of Primatology* **55**, 137-149.

Schmidt, C. & Kunzmann, A. (2005). Post-harvest mortality in the marine aquarium trade: A case study of an Indonesian export facility. *SPC Live Reef Fish Information Bulletin* **13**, 3-12.

Schreck, C., Olla, B., Davis, M., Iwama, G., Pickering, A., Sumpter, J. & Schreck, C. (1997). Behavioral responses to stress. In *Fish stress and health in aquaculture*, pp. 145-170. New York: Cambridge University Press.

Schreck, C. B., Contreras-Sanchez, W. & Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* **197**, 3-24.

Schreck, C. B., Jonsson, L., Feist, G. & Reno, P. (1995). Conditioning improves performance of juvenile Chinook salmon, *Oncorhynchus tshawytscha*, to transportation stress. *Aquaculture* **135**, 99-110.

Schreck, C. B., Solazzi, M. F., Johnson, S. L. & Nickelson, T. E. (1989). Transportation stress affects performance of coho salmon, *Oncorhynchus kisutch*. *Aquaculture* **82**, 15-20.

Schreck, C. B. & Tort, L. (2016). The Concept of Stress in Fish. In *Biology of Stress in Fish* (Schreck, C. B., Tort, L., Farrell, A. P. & Brauner, C. J., eds.), pp. 1-34. UK: Academic Press.

Schulte, P. M. (2014). What is environmental stress? Insights from fish living in a variable environment. *The Journal of Experimental Biology* **217**, 23-34.

Schuster, S., Wohl, S., Griebsch, M. & Klostermeier, I. (2006). Animal cognition: How archer fish learn to down rapidly moving targets. *Current Biology* **16**, 378-383.

Scott, A. P., Hirschenhauser, K., Bender, N., Oliveira, R., Earley, R. L., Sebire, M., Ellis, T., Pavlidis, M., Hubbard, P. C., Huertas, M. & Canario, A. (2008). Non-invasive measurement of steroids in fish-holding water: important considerations when applying the procedure to behaviour studies. *Behaviour* **145**, 1307-1328.

Scott, A. P. & Sorensen, P. W. (1994). Time-course of release of pheromonally active gonadal steroids and their conjugates by ovulatory goldfish. *General and Comparative Endocrinology* **96**, 309-323.

Sebire, M., Katsiadaki, I. & Scott, A. P. (2007). Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback (*Gasterosteus aculeatus*). *General and Comparative Endocrinology* **152**, 30-38.

Shams, S., Chatterjee, D. & Gerlai, R. (2015). Chronic social isolation affects thigmotaxis and whole-brain serotonin levels in adult zebrafish. *Behavioural Brain Research* **292**, 283-287.

Shepherdson, D. J. (2003). Environmental enrichment: past, present and future. *International Zoo Yearbook* **38**, 118-124.

Sigismondi, L. A. & Weber, L. J. (1988). Changes in Avoidance Response Time of Juvenile Chinook Salmon Exposed to Multiple Acute Handling Stresses. *Transactions of the American Fisheries Society* **117**, 196-201.

Silverstein, J. T., Vallejo, R. L., Palti, Y., Leeds, T. D., Rexroad, C. E., III, Welch, T. J., Wiens, G. D. & Ducrocq, V. (2009). Rainbow trout resistance to bacterial cold-water disease is moderately heritable and is not adversely correlated with growth. *Journal of animal science* **87**, 860-867.

Sloman, K. A., Baldwin, L., McMahon, S. & Snellgrove, D. (2011). The effects of mixed-species assemblage on the behaviour and welfare of fish held in home aquaria. *Applied Animal Behaviour Science* **135**, 160-168.

Sloman, K. A., Motherwell, G., O'Connor, K. I. & Taylor, A. C. (2000). The effect of social stress on the Standard Metabolic Rate (SMR) of brown trout, *Salmo trutta*. *Fish Physiology and Biochemistry* **23**, 49-53.

Smith, M. E., Kane, A. S. & Popper, A. N. (2004). Noise-induced stress response and hearing loss in goldfish (*Carassius auratus*). *Journal of Experimental Biology* **207**, 427-435.

Sneddon, L. U. (2003a). The bold and the shy: individual differences in rainbow trout. *Journal of Fish Biology* **62**, 971-975.

Sneddon, L. U. (2003b). The evidence for pain in fish: the use of morphine as an analgesic. *Applied Animal Behaviour Science* **83**, 153-162.

Sneddon, L. U. (2009). Pain Perception in Fish: Indicators and Endpoints. *Ilar Journal* **50**, 338-342.

Sneddon, L. U. (2015). Pain in aquatic animals. *Journal of Experimental Biology* **218**, 967-976.

Sneddon, L. U., Braithwaite, V. A. & Gentle, M. J. (2003). Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proceedings of the Royal Society B: Biological Sciences* **270**, 1115-1121.

Sneddon, L. U., Lopez-Luna, J., Wolfenden, D. C., Leach, M. C., Valentim, A. M., Steenbergen, P. J., Bardine, N., Currie, A. D., Broom, D. M. & Brown, C. (2018). Fish sentience denial: Muddying the waters. *Animal Sentience: An Interdisciplinary Journal on Animal Feeling* **3**, 1.

Sneddon, L. U., Wolfenden, D. C. C. & Thomson, J. S. (2016). Stress Management and Welfare. In *Biology of Stress in Fish* (Schreck, C. B., Tort, L., Farrell, A. P. & Brauner, C. J., eds.), pp. 463-539. Chennai, India: Academic Press.

Snellgrove, D. L., Alexander, L. G. & Stevenson, A. E. (2007). The effect of Stress Coat® on the excretory loss of cortisol from the common goldfish Carassius auratus following a netting stressor. In *Aquaculture Conference*. San Antonio, Texas, USA.

Sopinka, N. M., Hinch, S. G., Middleton, C. T., Hills, J. A. & Patterson, D. A. (2014). Mother knows best, even when stressed? Effects of maternal exposure to a stressor on offspring performance at different life stages in a wild semelparous fish. *Oecologia* **175**, 493-500.

Sørum, U. & Damsgård, B. (2004). Effects of anaesthetisation and vaccination on feed intake and growth in Atlantic salmon (*Salmo salar* L.). *Aquaculture* **232**, 333-341.

Spence, R., Ashton, R. & Smith, C. (2007). Oviposition decisions are mediated by spawning site quality in wild and domesticated zebrafish, *Danio rerio*. *Behaviour* **144**, 953-966.

Spence, R. & Smith, C. (2007). The Role of Early Learning in Determining Shoaling Preferences Based on Visual Cues in the Zebrafish, *Danio rerio*. *Ethology* **113**, 62-67.

Stefansson, M. O., FitzGerald, R. D. & Cross, T. F. (2002). Growth, feed utilization and growth heterogeneity in juvenile turbot *Scophthalmus maximus* (Rafinesque) under different photoperiod regimes. *Aquaculture Research* **33**, 177-187.

Strange, R. (2009). Nutrition in Fish. In *Fundamentals of Ornamental Fish Health* (Roberts, H. E., ed.), pp. 89-101. Malaysia: Wiley-Blackwell.

Sullivan, M., Lawrence, C. & Blache, D. (2016). Why did the fish cross the tank? Objectively measuring the value of enrichment for captive fish. *Applied Animal Behaviour Science* **174**, 181-188.

Sundbaum, K. & Näslund, I. (1998). Effects of woody debris on the growth and behaviour of brown trout in experimental stream channels. *Canadian Journal of Zoology* **76**, 56-61.

Sunyer, J. O., Gómez, E., Tort, L., Navarro, V. & Quesada, J. (1995). Physiological responses and depression of humoral components of the immune system in gilthead sea bream (*Sparus aurata*) following daily acute stress. *Canadian Journal of Fisheries and Aquatic Sciences* **52**, 2339-2346.

Swaney, W., Kendal, J., Capon, H., Brown, C. & Laland, K. N. (2001). Familiarity facilitates social learning of foraging behaviour in the guppy. *Animal Behaviour* **62**, 591-598.

Swanson, C., Mager, R. C., Doroshov, S. I. & Cech Jr, J. J. (1996). Use of Salts, Anesthetics, and Polymers to Minimize Handling and Transport Mortality in Delta Smelt. *Transactions of the American Fisheries Society* **125**, 326-329.

Templeton, C. N. & Shriner, W. M. (2004). Multiple selection pressures influence Trinidadian guppy (*Poecilia reticulata*) antipredator behavior. *Behavioral Ecology* **15**, 673-678.

The European Parliament and the Council of the European Union (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Thomas, R. (2015). *Data Analysis with R Statistical Software: A Guidebook for Scientists*. Newport Printing, UK: Eco-explore.

Thompson, R. R. J., Paul, E. S., Radford, A. N., Purser, J. & Mendl, M. (2016). Routine handling methods affect behaviour of three-spined sticklebacks in a novel test of anxiety. *Behavioural Brain Research* **306**, 26-35.

Tlusty, M., Dowd, S., Cooper, R., Chao, N. L. & Whittaker, B. (2005). Shipping Cardinal Tetras from the Amazon - understanding stressors to decrease shipping mortality. *Ornamental Fish International* **48**, 21-23.

Turner, J. W., Nemeth, R. & Rogers, C. (2003). Measurement of fecal glucocorticoids in parrotfishes to assess stress. *General and Comparative Endocrinology* **133**, 341-352.

Turschwell, M. P. & White, C. R. (2016). The effects of laboratory housing and spatial enrichment on brain size and metabolic rate in the eastern mosquitofish, *Gambusia holbrooki*. *Biology Open*.

UNEP-WCMC (2007). International Trade in Aquatic Ornamental Species. Cambridge, UK.

Van de Weerd, H. A., Van Loo, P. L. P., Van Zutphen, L. F. M., Koolhaas, J. M. & Baumans, V. (1998). Strength of preference for nesting material as environmental enrichment for laboratory mice. *Applied Animal Behaviour Science* **55**, 369-382.

Vanderzwalmen, M., Eaton, L., Mullen, C., Henriquez, F., Carey, P., Snellgrove, D. & Sloman, K. A. (2018). The use of feed and water additives for live fish transport. *Reviews in Aquaculture* **0**, 1-16.

Varsamos, S., Flik, G., Pepin, J. F., Bonga, S. E. W. & Breuil, G. (2006). Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, *Dicentrarchus labrax*. *Fish & Shellfish Immunology* **20**, 83-96.

Volpato, G. L. (2009). Challenges in Assessing Fish Welfare. *Ilar Journal* **50**, 329-337.

Volpato, G. L., Goncalves-de-Freitas, E. & Fernandes-de-Castilho, M. (2007). Insights into the concept of fish welfare. *Diseases of Aquatic Organisms* **75**, 165-171.

Wabnitz, C. & Nahacky, T. (2014). Rapid aquarium fish stock assessment and evaluation of industry best practices in Kosrae, Federated States of Micronesia. Australia: Secretariat of the Pacific Community.

Wabnitz, C., Taylor, M., Green, E. & Razak, R. (2003). From Ocean to Aquarium. Cambridge, UK: UNEP-WCMC.

Wagner, G. N., Singer, T. D. & McKinley, R. S. (2003). The ability of clove oil and MS-222 to minimize handling stress in rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquaculture Research* **34**, 1139-1146.

Walster, C. (2008). The Welfare of Ornamental Fish. In *Fish Welfare* (Branson, E., ed.), pp. 271-290. Oxford, UK: Blackwell Publishing.

Ward, A. J. & Hart, P. J. (2003). The effects of kin and familiarity on interactions between fish. *Fish and Fisheries* **4**, 348-358.

Waring, C. P., Poxton, M. G. & Stagg, R. M. (1997). The physiological response of the turbot to multiple net confinements. *Aquaculture International* **5**, 1-12.

Watson, I. & Roberts, D. (2015). The benefits of wild-caught ornamental aquatic organisms. Durrell Institute of Conservation and Ecology: University of Kent.

Wendelaar-Bonga, S. E. (1997). The stress response in fish. *Physiological Reviews* **77**, 591-625.

Wilkes, L. (2011). Potential environmental enrichment for zebrafish used in regulatory toxicology. In *Biological Sciences*: University of Exeter.

Wilkes, L., Owen, S. F., Readman, G. D., Sloman, K. A. & Wilson, R. W. (2012). Does structural enrichment for toxicology studies improve zebrafish welfare? *Applied Animal Behaviour Science* **139**, 143-150.

Williams, T., Readman, G. & Owen, S. (2009). Key issues concerning environmental enrichment for laboratory-held fish species. *Laboratory Animals* **43**, 107-120.

Winberg, S. & Olsén, K. H. k. (1992). The influence of rearing conditions on the sibling odour preference of juvenile arctic charr, *Salvelinus alpinus* L. *Animal Behaviour* **44**, 157-164.

Woodward, J. J. & Smith, L. S. (1985). Exercise training and the stress response in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **26**, 435-447.

Wright, D. & Krause, J. (2006). Repeated measures of shoaling tendency in zebrafish (*Danio rerio*) and other small teleost fishes. *Nature Protocols* **1**, 1828.

Young, P. S. & Cech Jr, J. J. (1993). Effects of Exercise Conditioning on Stress Responses and Recovery in Cultured and Wild Young-of-the-Year Striped Bass, *Morone saxatilis*. *Canadian Journal of Fisheries and Aquatic Sciences* **50**, 2094-2099.

Young, P. S. & Cech Jr, J. J. (1994). Effects of Different Exercise Conditioning Velocities on the Energy Reserves and Swimming Stress Responses in Young-of-the-Year Striped Bass (*Morone saxatilis*). *Canadian Journal of Fisheries and Aquatic Sciences* **51**, 1528-1534.

Young, R. J. (2013). *Environmental enrichment for captive animals*. Cornwall, UK: Wiley-Blackwell.

Zahl, I. H., Kiessling, A., Samuelsen, O. B. & Olsen, R. E. (2010). Anesthesia induces stress in Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*). *Fish Physiology and Biochemistry* **36**, 719-730.

Zandonà, E., Dalton, C. M., El-Sabaawi, R. W., Howard, J. L., Marshall, M. C., Kilham, S. S., Reznick, D. N., Travis, J., Kohler, T. J. & Flecker, A. S. (2017). Population variation in the trophic niche of the Trinidadian guppy from different predation regimes. *Scientific Reports* **7**, 5770.

Zuberi, A., Ali, S. & Brown, C. (2011). A non-invasive assay for monitoring stress responses: A comparison between wild and captive-reared rainbowfish (*Melanoteania duboulayi*). *Aquaculture* **321**, 267-272.

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