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A Non-Invasive Assay for Monitoring Stress Responses: A Comparison Between Wild and Captive-Reared Rainbowfish (Melanoteania duboulayi)

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KEYWORDS

stress, hatchery, cortisol, predators, reintroduction

ABSTRACT

The stress response of wild and captive reared rainbowfish (Melanoteania duboulayi) following chasing by a simulated predator was examined. Cortisol release rate was monitored using a flow through system by measuring water borne hormone levels. Tests using known cortisol concentrations revealed that the technique yielded 95% of the cortisol present in the water. Cortisol release rates increased several fold in both populations after being chased but peaked at different time periods. Wild fish showed a typical stress response with release rate rising to (2.29±0.22 ng g^{-1} h^{-1}) 2 h after exposure followed by rapid recovery. The captive-reared population by contrast showed an atypical response with cortisol release rate peaking 4 h post exposure but reaching only half the level of the wild fish (1.19±0.11 ng g^{-1} h^{-1}). The implications for the release of hatchery-reared fish for stock enhancement are discussed.

Introduction

It is becoming increasingly evident that the production of fishes in hatcheries for fisheries supplementation or conservation purposes is fraught with difficulties. The list of behavioral and physiological differences between hatchery-reared and wild fish is growing ever longer and has led many to question the validity of stock supplementation from hatchery sources (Brown and Day, 2002; Huntingford, 2004). Some prime examples of these differences include recognition and responses to predators, migration patterns and metabolic rate. In addition to these deficiencies, the reliance of limited parental stock may reduce genetic diversity and may even be a source of “genetic pollution” causing a reduction in fitness over the longer term (Doyle et al., 2001; Utter, 1998).

The natural environment is typically very challenging and wild fish are frequently exposed to a number of potential stressors. One of the most obvious sources of stress is the sudden exposure to predatory attack. There are various stages to the predator–prey interaction (Kelley and Brown, 2010), which are likely to vary between hatchery and wild fishes. Behavioral differences relating to habitat use and risk taking may make encounters more common for naïve hatchery fish. For example, domesticated trout

released into dams took greater risks while foraging and grew faster than wild trout but suffered greater predation when predators were present (Biro et al., 2004). When the fish come into visual contact, hatchery-reared fish may or may not recognize the predator as a threat, depending on their evolutionary history and the extent to which predator recognition is inherited (Houde et al., 2010). In many instances hatchery-reared fishes fail to recognize predators and naïve individuals may even approach out of curiosity (Brown and Warburton, 1999). Lastly, when predator and prey come into contact, hatchery-reared fishes may show inappropriate or poorly developed escape responses such as a lack of schooling behavior (Kydd and Brown, 2009). While much attention has focused on these stages of predator–prey interaction, far less attention has addressed the recovery of prey following a predator attack.

Many of these stages involve both psychological and physiological responses, which commonly involve the release of hormones. Wild animals respond to predatory attacks with the flight or fight response whereby a number of hormones are rapidly released into the bloodstream and target various organs in the body. The overall effect of these hormones is to prepare the animal for action and there are multiple and varied behavioral manifestations of this response not least of which is the adoption of heightened awareness and antipredatory responses such as schooling or hiding. The primary protagonists are adrenaline and epinephrine, which are released into the bloodstream along with a burst of glucose to prepare the fish for an immediate response to threatening stimuli. Such responses occur in the space of seconds in fishes because catecholamines are stored in chromaffin cells which can be released into the bloodstream immediately. As the hormonal cascade proceeds, however, a build-up of other related hormones becomes evident. One of the major components of this latter response is the release of cortisol. Cortisol concentrations in the blood gradually rise following exposure to a stressor, typically peaking an hour after exposure, and then decay over a number of hours before returning to their background state (Barton, 2002; Iwama et al., 2006). While the release of cortisol is slower than adrenaline, its physiological and behavioral effects are far longer lasting (Waring et al., 1996). It's primarily viewed as a homeostatic response by the fish in an attempt to return metabolic activities to normal levels (Reid et al., 1998). Chronic or prolonged cortisol responses have been linked with a series of important behavioral fitness measures including reduced appetitive driven foraging behavior and hierarchy rank establishment (Gregory and Wood, 1999; Pottinger and Pickering., 1992). Both of these factors are largely controlled directly and indirectly by a cortisol induced switch in metabolism (Wendelaar Bonga, 1997). Enhanced metabolic rate is also likely to lead to greater risk taking behavior in order to increase food intake. If the fish are poor foragers, as is often the case with hatchery-reared fish (Brown et al., 2003), this can lead to decreased growth rate and condition factor. So while the immediate flight and fight response to predators is vital, the recovery period is equally important, because sustained levels of stress can be extremely costly in terms of energy expenditure (even over shorter time periods of several hours) and loss of responsiveness to further predatory attacks. Thus it is important that hatchery-reared fish that are destined for release into the wild as part of restocking programs show physiological responses to stressors that are similar to wild individuals if they are to minimize energy expenditure and thereby maximize their chance of survival post-release (Breves and Specker, 2005).

Previous studies have shown that stress responses can vary dramatically between species, between strains within species and even between individuals (reviewed by Barton, 2002). It is clear that these differences are genetically based and influenced by individual experience (Heath et al., 1993; Overli et al., 2005). Research examining individual differences in behavior, for example, has revealed that coping styles can be linked to underlying hormones (Huntingford et al., 2010; Koolhaas et al., 2007) and that they show a moderate to high degree of heritability (Overli et al., 2005). One of the consistent findings in the literature is that wild and hatchery-reared fish often differ in their response to stressors (e.g. Lepage et al., 2000). In rainbow trout (*Salmo gairdneri*), for example, plasma levels or cortisol, glucose and chloride were all significantly higher in wild trout following confinement to a net and electroshocking than hatchery

fish (Woodward and Strange, 1987). Most of this previous work, however, has examined stress responses following exposure to human related disturbances owing the importance of this information for aquaculture applications. There is currently relatively little information about how different populations respond to more natural events such as chasing by predators (Brown et al., 2005).

Measuring stress hormones in small fishes has traditionally been difficult (Ellis et al., 2004; Scott et al., 2001). This has been a continued source of frustration given that much of the knowledge about fish behavioral ecology has been generated by a few model species which are of a relatively small size (e.g. guppies, *Poecilia reticulata* Peters and sticklebacks, *Gasterosteus aculeatus* L.). For the most part, individuals have to be taken from a large group and sacrificed (e.g. by snap freezing) at different time periods to investigate whole body cortisol levels (Ramsay et al., 2006; Sink et al., 2007). This is problematic because the removal of individuals from a group as part of the sampling regime can induce a stress response in the rest of the group members (Laidley and Leatherland, 1988) by social learning processes (Brown and Laland, 2001). In larger fishes such as salmonids, blood plasma concentration can be measured directly but this is complicated by handling stress and or heavy doses of anesthetic (Oliveira et al., 1999; Pottinger et al., 1992). Sampling plasma in small fishes is technically very difficult and usually terminal. One alternative is to sample cortisol that is released from the gills into the surrounding water (Scott and Ellis, 2007; Scott et al., 2008 for reviews). This procedure has many advantages including the fact that the same fish can be repeatedly sampled over time. Arguably the best approach is to develop a flow through system (sensu Ellis et al., 2004) that enables the sampling of water from the holding aquaria at any point in time without disturbing the fish. Moreover, the use of a flow through system enables the collection of baseline hormone levels and completely eliminates handling related stress (compared with Sebire et al., 2007). In this way the response observed can be entirely attributed to the experimental manipulation. This approach may be particularly useful for analyzing stress responses in small fishes to natural events such as agonistic interactions or predator attacks because the subjects need not be disturbed while the samples and observations are made. It is important to note, however, that the method is straight forward if one is taking a comparative approach within a study, but if comparisons are to be made between species or with other studies, then the water cortisol concentration needs to be calibrated with either whole body or plasma concentrations (Ellis et al., 2004; Zuberi et al., submitted for publication). Despite the obvious benefits of developing a flow-through system for measuring hormones in small fishes, very few studies have ever been conducted using such as system.

Here we developed a flow through system to repeatedly measure the water-borne cortisol concentrations and subsequently release rates in a school of small freshwater fish, *Melanotaenia duboulayi* Castelnau, in response to being chased by a simulated predator. We examined the stress response in a captive-reared population that had been held in captivity for around 15 generations (Kydd and Brown, 2009), a scenario similar to that used in many fish hatcheries, and compared it to a wild caught population. We expected that the captive reared population would show an atypical stress response in comparison to the wild population, which should exhibit relatively rapid responses followed by a quick recovery.

Material and methods

Study animals

Rainbowfish were chosen as a model species for several reasons. Firstly they are relatively small and easily maintained in the laboratory setting. Secondly, they have been the subject of behavioral and ecotoxicology studies for decades and long-term captive populations are readily available. Thirdly, some rainbowfish species are endangered and previous attempts to restock fish using traditional captive-breeding programs have failed (Brown and Warburton, 1999).Wild rainbowfish, *M. duboulayi*, were collected using bait traps from the Orara River (30°15′26.91”S, 153°0’42.56”E) and transported to

Macquarie University. Captive reared rainbowfish had been bred and reared in captivity at the EPA for about 15 generations. The parental stock of this captive population was collected from a river in South East Queensland in 1990 (for details see Kydd and Brown, 2009). All fish were initially housed in aquaria (90×40×40 cm) containing river gravel and artificial plants. Light was provided by overhead fluorescent tubing (12:12 light dark) and water temperature was maintained at 22.5 °C. Wild fish were weaned from live food onto commercial flake food (Tetramin) over the first few days and held in captive conditions for a month prior to experimentation. We allowed the wild fish to fully adjust to captive conditions so that their response to the predator model was specific rather than clouded by a generalized response to living in the unfamiliar captive environment.

Experimental protocol

One week prior to experimentation, 45 captive-reared rainbowfish, (mean±S.E., body mass and length 6.78±0.38 g and 73.9±1.33 mm) and 75 wild rainbowfish (mean±S.E., body mass and length 3.12±0.12 g and 58.0±0.7 mm)were re-housed in twelve 25 l volume aquaria (40×25×25 cm). The size of the fish differed because we controlled for the age of the fish (16 months). Captive-reared fish are generally in better condition and grow faster than wild fish as is typical for most hatchery-reared populations. We attempted to control for this by maintaining a stocking density of 9.83±0.15 kg m^{-3} for both populations. Thus there were slight variations in the number of fish in each replicate (7, 8 and 8 for captive and 12, 12 and 13 for wild fish). The twelve aquaria were split into half for the captive and wild populations and these were split in half again for the control and test treatments (n=3 shoals per treatment per species). Small differences in body size do not result in significant changes in cortisol production or metabolism (Bender et al., 2008). The test aquaria were equipped with a small filter and a heater to maintain a constant temperature of 22.5 °C. These aquaria lacked substrate and the sponge was removed from the filter to avoid absorption of free cortisol. In order to maintain a constant rate of flow of 10.01±0.05 ml min^{-1}, each aquarium was connected via Tygon® tubing and a regulator to a reservoir containing aged water equipped with water heater set to 22.5 °C located above the test aquaria. Outflow of water from each aquarium was also controlled by use of Tygon® tubing and a regulator. During the experiment, pH ranged from 6.2 to 6.5, oxygen concentrations was near saturation (~7.5 mg l^{-1}), ammonia was less than 0.25 ppm, and salinity was 0.0 ppm. Fish were fed once daily between 0800 and 0900 h with tetramin flake food.

The experiment involved two treatments; unstressed control schools and schools chased by a simulated predator for 2 min. The predator chasing treatment consisted of a brightly colored, plastic model fish held by its dorsal fin in the researchers fingers and rapidly moved around the aquaria chasing individual fish. The fish could see the hand of the researcher, but they are accustomed to being fed in this manner, so it was unlikely to contribute to the stressor. The 120 mm model depicted a butterfly fish (*Cheatodon spp*) which is a tropical marine species so it was completely novel to both groups of fish. This is important because we did not want to generate stress responses that were dependent on predator recognition, rather we wanted the fish to respond to being chased. This difference is that here we measured differences in physiology whereas if we had chosen a predator known to the wild population our results would be confounded by differences in psychology.

Water sampling and processing

In order to get basal level of cortisol, all of the water in each aquarium was exchanged with fresh dechlorinated water via the flow through system on the day of the experiment. After flushing the aquaria, 500 ml water was collected in a glass bottle from the outflow of the flow through system to avoid disturbing the fish. This was achieved by having a relatively long outflow tube enabling us to collect water without approaching the aquaria too closely. We then recalibrated the flow rate. Further water samples

were collected using the same technique at 0, 0.5, 1, 2, 3 and 4 h after the predator treatment fish were subject to simulated attack. To minimize the possibility of any interference due to background cortisol, water was also sampled from the main aged water aquarium, supplying water to experimental aquaria.

In order to calculate the cortisol release rate we applied an equation based on Ellis et al., 2004:

$$\textbf{Cortisol release rate} = [V(Ct-C_o e^{-kt})kt]/1-e^{-kt})]/w$$

where V is the volume of water, Ct is the concentration at the end of the sampling period t, C_o is the concentration at the beginning of the sampling period, k is the rate of decrease due to dilution over time t and w is the total weight of the fish in the sample. Flow rates were periodically checked throughout the experimental period by recording the time taken to fill a 500 mL beaker from the outflow and the flow rate was adjusted as necessary. The balance between inflow and outflow was achieved by ensuring that the volume of water in the aquaria remained constant.

Immediately after sample collection, the 500 ml water borne hormone samples were filtered (Whatman filters) to remove particulate matter, peristaltically pumped at circa 10 ml min^{-1} through a prefilter (0.45 µm pore size: AcroCap™, GelmanSciences, Ann Arbor, MI, USA) and then either stored at −20 °C or extracted immediately. Freeze storage of water samples does not affect cortisol concentrations (Ellis et al., 2004). Cortisol was extracted from the water samples using an activated LiChrolut® RP-18 solid phase extraction cartridge (500 mg, 3 ml, 40–63 µm, standard PP Merck) fitted to a 24-port vacuum manifold. Columns were primed using two consecutive washes with 2 ml of 100% methanol followed by two consecutive washes with 2 ml de-ionized water (DI). The 500 ml water samples were then pushed through the columns using the vacuum manifold. After pumping, the cartridges were washed with 5 ml DI and free (i.e., unconjugated) cortisol was eluted from the columns into 10 ml borosilicate test tube (12×75 mm) by two consecutive 3 ml washes with ethyl acetate. The 6 ml of eluted solvent was evaporated at 45 °C under nitrogen gas and the residue was re-dissolved in 500 µl of EIA buffer and stored frozen until assayed.

Free cortisol concentrations were measured using Enzyme Immunoassay Kit (Assay Designs Inc., Ann Arbor, Michigan, kit number 900-071). All samples were run in duplicate. The cortisol concentrations obtained from the EIA kit were validated by verifying that slope of the curve obtained by serial dilutions (0, 25, 50, 75%) of sample with EIA buffer matched the standard curve (P=0.97). Waterborne hormone extract was spiked with known high and low concentrations of cortisol standard to ascertain recovery; the slope of the curve plotting observed versus expected cortisol concentrations was 0.98, indicating a significant linear relationship. The precision (intra-assay CV, 7.47%) was calculated by comparing the results from repeated assays (6 times) of two samples differing in cortisol concentration. The reproducibility (inter-assay coefficient of variation, CV, 9.3%) was assessed by repeating three samples in every assay. The lowest detectable limit of the assay, revealed by repeated dilution of water sample, was 56.72 pg ml^{-1}. All the samples were well above the detection limit.

The efficiency of extraction (% recovery) from water sample was assessed by adding radioinert cortisol to water samples collected from main reservoir which supplies dechlorinated water to experimental aquaria to give predicted concentration of 10, 5 and 2.5 $ngml^{-1}$. Water samples (500 ml) were then pumped through extraction cartridges. The cortisol was retrieved from the cartridges using the ethyl acetate elution method and the amountwas quantified by EIA. The efficiency of extraction was found greater than 94%. All values of cortisol from the fish were corrected accordingly.

Cortisol release rate was analyzed using a two-way repeated measures analysis of variance using Statview (SAS inc).

Results

No cortisol was detected in water supplying to experimental aquaria. Cortisol release rate was significantly higher in wild fish than captive-reared fish and significantly higher in predator exposed fish than control fish (Table 1). The significant interaction between Treatment and Population indicated that the manner in which the two populations responded to the treatments varied. If one examines this result more closely it is apparent that cortisol release rate was higher in the predator exposed wild fish than predator exposed captive-reared fish and no difference was apparent in the control treatment. The repeated measure was also significant indicating that cortisol release rates changed over time. The significant three-way interaction between time, population and treatment is indicative of the different patterns displayed by fish from both populations in both treatments over time. To examine this interaction more closely we split the data by treatment. During the control treatment there was no differences between the two populations ($F_{1,4}$=1.012, P=0.371), no change in excretion rate over time ($F_{6,24}$=1.931, p=0.117) and no interaction between these two variables ($F_{6,24}$=0.823, P=0.563). In contrast during the predator treatment there was a significant difference between the two populations ($F_{1,4}$=136.142, P<0.001), a change in release rate over time ($F_{6,24}$=29.426, P<0.001) and an interaction between these two variables ($F_{6,24}$=33.649, P<0.001). Cortisol release rate in wild fish was significantly elevated at 0.5 h and peaked at 2 h post stress (2.29±0.22 ng g^{-1} h^{-1}) and then started declining whereas cortisol release rate in captive reared fish gradually built up and peaked at 4 h post stress (1.19±0.11 ng g^{-1} h^{-1}) during the experimental phase (Fig. 1).

Recovery following extraction of known concentrations of cortisol at 2.5 ng l^{-1}, 5 ng l^{-1} and 10 ng l^{-1} yielded 2.3, 4.7 and 9.7 ng l^{-1} (92%, 94% and 97%) respectively.

Table 1. Results of the two-way repeated measures ANOVA examining free cortisol release rate (ng g^{-1} h^{-1}) in wild and captive reared rainbowfish based on water samples extracted from aquaria during the control and predator exposed treatments.

Source	DF	F value	P value
Population	1,8	125.254	<0.001
Treatment	1,8	617.088	<0.001
Population×Treatment	1,8	108.917	<0.001
Time	6,48	30.146	<0.001
Population×Time	6,48	32.813	<0.001
Treatment×Time	6,48	28.165	<0.001
Population×Treatment×Time	6,48	33.839	<0.001

Discussion

Our results demonstrate that the captive reared population showed a substantially different stress response after being chased compared to the wild population. In general, wild rainbowfish showed a typical stress response characterized by an increase in cortisol release rate to the two hour mark post-stressor followed by rapid recovery (Barton, 2002; Iwama et al., 2006) whereas the captive-reared fish showed an atypical, attenuated response. We can be certain that the differences in response are not due to variation in predator recognition (e.g. Brown and Warburton, 1999) because we used model predators that were unfamiliar to both populations and manually chased the fish in each population in a similar way. Thus the variation in response is entirely physiological not psychological. These results add to the

growing collection of negative effects of hatchery rearing on fish behavior and physiology and show that the flow through method of analyzing hormones is highly advantageous in this context.

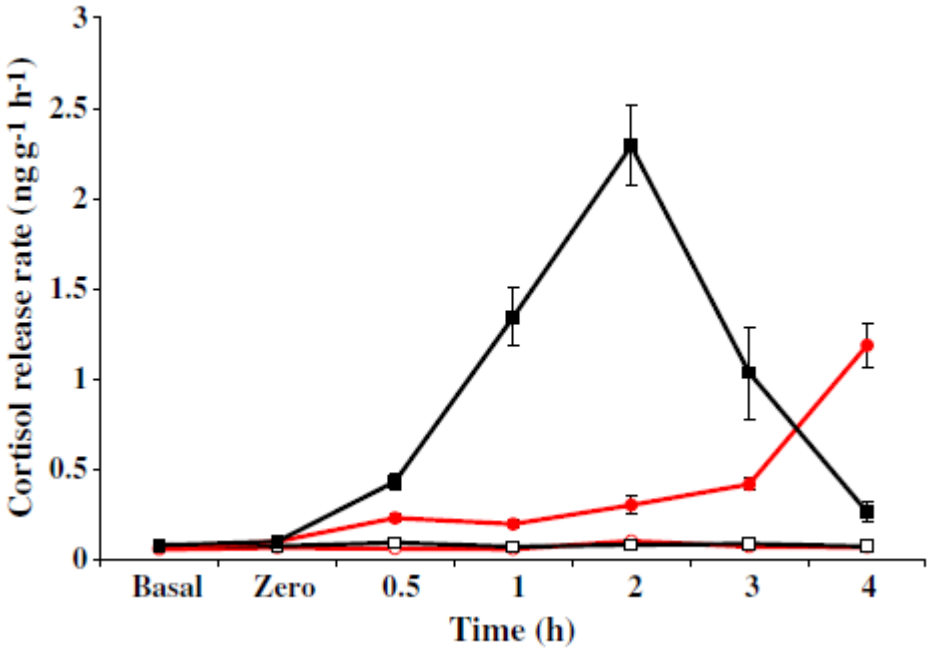

Fig. 1. Mean (±S.E.) Cortisol release rate in water from wild (black squares) and captive reared rainbowfish (red circles) in the control treatment (open shapes) and exposed to a predator (filled shapes). N=3 groups of fish in each case.

Wild caught rainbowfish displayed a typical stress response characterized by a marked increase in cortisol release rate 30 min after being chased by a simulated predator for 2 min. Cortisol release rate rapidly increased until 2 h after predator exposure. They then showed an equally rapid recovery, but had not fully recovered by 4 h post exposure (Fig. 1). Captive reared fish, in contrast, showed a small response by 30 min post exposure with only a gradual increase until the three hour mark. At 4 h post predator exposure, the cortisol release rate increased significantly and may have continued to increase after the cessations of our sampling period. Although cortisol release rate may have continued to increase in the hatchery-reared fish, most fish species are well into the recovery phase by this point in time (Barton, 2002; Iwama et al., 2006) which suggests that release rates may well have begun to decline after this point. It is evident that future experiments with captive-reared rainbowfish will have to extend beyond the four hour mark to fully capture the cortisol response.

While the pattern of cortisol release rate between captive and wild fish differed over the 4 h following predator exposure, there was also a quantitative difference. The peak cortisol release rate in the captive reared fish (1.19 ng g^{-1} h^{-1}) was only half that displayed in the wild fish (2.29 ng g^{-1} h^{-1}). Control fish which were not exposed to predators, showed no change in their cortisol concentrations relative to background levels irrespective of their origin. Mounting a delayed cortisol response to a stressor is not likely to be adaptive in this species in any sense. A lack of immediate response to the predator is likely to end in death. Equally a prolonged and late response is a waste of energy. The latter is particularly confounded in animals that may already be struggling to find food. We are aware of only a single study

that has shown such a long delay between the onset of a stressor and the cortisol response peak. That example involved the sea raven *Hemitripterus americanus* which is a relatively inactive fish with a low metabolic rate and the authors suggest that this may be an adaptive response to help conserve energy (Vijayan and Moon, 1994).

The results obtained for the wild fish were similar to those obtained in other species where the stress response typically occurs between 0.5 and 4 h after exposure to a stressor (Barton, 2002; Scott et al., 2008). For example, plasma cortisol levels in rainbow trout, *Oncorhynchus mykiss* Walbaum, under a mild confinement stress peaked just 30 min after the onset of the stressor (Pottinger and Moran, 1993) whereas exposure to a single bout of handling stress produced water cortisol concentrations that peaked at 2 h post handling followed by recovery (Ellis et al., 2004). Similarly, cortisol levels in carp, *Cyprinus carpio* L., captured and held in angler's keep nets returned to basal levels after 4 h (Pottinger, 1998). It is apparent that the cortisol response varies depending on the severity of the stressor, varies between species and between individuals, but in most instances recovery is achieved within 4 h, further highlighting the odd response displayed by the captive stock.

One important question remains; are population and species differences the result of experience during ontogeny, maternal effects or do they reflect underlying genetic variation that has built up over several generations as the result of natural or artificial selection? Observations suggest that large changes in behavior can occur in a single generation under hatchery conditions (Álvarez and Nicieza, 2003; Salonen and Peuhkuri, 2006). Estimates of heritability (h^2) of stress responses can vary dramatically depending on the population and species under consideration. Heritability of plasma cortisol increase in responses to repeated stressors has been estimated to be 0.56 in rainbow trout (Fevolden et al., 1999). Fevolden et al., 1999, in contrast, estimated heritability of cortisol stress response in hatchery-reared Atlantic salmon and rainbow trout using a standard confinement technique and generated h^2 values of 0.05 and 0.27 respectively. Despite this variation, however, there is a general expectation that selection for low stress lines can be achieved in aquaculture lines (Pottinger and Pickering, 1997). It is likely that captive-reared lines have been selected for low stress responses to suit the hatchery environment either by design or accident.

Studies conducted on poeciliids have shown that stress responses are not only heritable but can also vary by differential exposure to stressors during ontogeny (Brown et al., 2005; Kelley and Brown, 2010). Repeated exposure to stress during ontogeny can both sensitize and desensitize fish to stress depending on the context. The latter is the most relevant to the present study because desensitization to mild stresses can result in attenuated neuroendocrine and metabolic responses (Reid et al., 1998). In a hatchery there is little point in repeatedly responding to every little disturbance, so desensitization or habituation to stressors makes energetic sense in this context. Indeed, the cortisol release rate in our captive population was only half that of the wild population during the study interval which is entirely consistent with this hypothesis. Moreover, a reasonably rapid cortisol response followed by a rapid recovery period after exposure to a stressor such as predators makes physiological sense given that the fish need to respond appropriately to stressors, but the expense of maintaining a prolonged response in the wild needs to be minimized given the energetic costs. Based on these studies, it is apparent that the captive-reared rainbowfish showed an atypical response to the simulated predation event that may be symptomatic of both incidental artificial selection and desensitization during ontogeny. Of course these two mechanisms are not mutually exclusive and it is likely that the results herein are the product of both mechanisms.

There are a number of issues regarding the validity of the flow through approach for studying stress responses in small fishes. Firstly, water cortisol concentration is positively correlated with plasma concentration in rainbow trout (Ellis et al., 2004) and whole body cortisol concentration in rainbowfish

(Zuberi et al., submitted for publication), but the methodology would have to be validated for every species prior to adopting this approach. Secondly, while cortisol is metabolized to sulfated and glucuronidated conjugates, only the free steroid fraction that enters solution can be measured using the extraction kits we employed. Free steroid measurements are the most widely adopted assay for stress responses and are a sufficient proxy for the latter stages of the stress response in fishes (Sorensen et al., 2005) but further information could be gained by examining conjugates. Thirdly, the rate at which cortisol passes from the gills into the water is potentially influenced by a number of factors such as gill surface area, ventilation rate, the presence and afinities of plasma or target binding proteins, and the concentration gradient (Scott et al., 2008). The former is particularly relevant here, because the ratio of gill surface area to body mass decreases as body size increases (Pauly, 1997), thus smaller fishes captured from the wild could potentially release more cortisol into the water even if they had a similar plasma cortisol concentration to the larger captive-reared fish. Note, however that there was no difference in the control treatments, nor can differences in body size explain the variation in the temporal pattern of cortisol release. Indeed, Bender et al. (2008) showed very similar amounts of steroid release over the same time period for cichlid fish (*Neolamprologus pulcher*) varying in size between 3 and 12 g. Thus the variation in fish size are unlikely to explain the observed differences between wild and captive-reared rainbowfish but certainly warrants further investigation. Lastly, and most importantly, the fact that the fish can remain undisturbed while cortisol measurements are taken is the great benefit of this approach. Theoretically one could make simaltaneous behavioral observations and tie these in with hormone release rate with little difficulty. Thus, despite some limitations, this method provides an excellent, non-invasive method of repeatedly measuring hormonal responses in small fishes over long time frames in response to a variety of stimuli.

It is becoming apparent that hatchery-reared fish differ from their wild counterparts in a number of important ways (Brown and Day, 2002; Huntingford, 2004). Further studies are required to determine the relative contributions of genes and experience in the development of stress responses in hatchery-reared fishes and how these responses differ from their wild counterparts. From a restocking perspective, the focus needs to shift towards responses to natural stressors in particular. With a greater appreciation of these mechanisms it may be possible to manage hatchery stocks destined for release in such a way as to minimize the detrimental effects of the hatchery environment and enhance post-release survival. One method might be to reduce desensitization to stressors by lowering disturbance frequency during development. One could take the opposite approach to minimize stress responses in aquaculture where the fish are destined for the table. Moreover, some of the many behavioral differences between wild and captive stocks likely have underlying hormonal origins and the method employed here enables us to investigate such differences in closer detail.

Conclusion

Our results demonstrate the effect of rearing environment on the physiology of fish. Fifteenth generation captive reared rainbowfish showed a substantially different stress response after being chased than their wild counterparts. It is likely that this variation is the outcome of long-term artificial selection in the captive population in combination with desensitization as a result of repeated exposure to potential stressors in the captive stock. We can be sure that this variation is solely due to differences in physiology and is not the result of differential predator recognition because we employed a novel model predator. The method developed here for studying the stress response in small fishes will be helpful in the future to provide a greater insight into the hormonal basis of behavior differences generated in response to variation in rearing environment. Moreover, far more subtle experiments could be conducted examining the psychological stress profile of fishes exposed to a variety of different predators or conspecifics.

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