# A non-invasive assay for monitoring stress responses: A comparison between wild and captive-reared rainbowfish (*Melanoteania duboulayi*)

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## 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 $(2.29 \pm 0.22 \text{ ng g}^{-1} \text{ h}^{-1})$ 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 $(1.19 \pm 0.11 \text{ ng g}^{-1} \text{ h}^{-1})$. The implications for the release of hatchery-reared fish for stock enhancement are discussed.

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## 1. 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 anti-predatory 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

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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.

## 2. Material and methods

### 2.1. 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 ($90 \times 40 \times 40$ 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.

### 2.2. Experimental protocol

One week prior to experimentation, 45 captive-reared rainbowfish, (mean ± S.E., body mass and length $6.78 \pm 0.38$ g and $73.9 \pm 1.33$ mm) and 75 wild rainbowfish (mean ± S.E., body mass and length $3.12 \pm 0.12$ g and $58.0 \pm 0.7$ mm) were re-housed in twelve 25 l volume aquaria ($40 \times 25 \times 25$ cm). The size of the fish differed because we controlled for the age of the fish (16 months). Captive-reared fish are generally in