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The Effects of Passive Integrated Transponder Tagging on Cortisol Release in the Gulf Killifish, Fundulus Grandis

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THE EFFECTS OF PASSIVE INTEGRATED TRANSPONDER TAGGING ON CORTISOL RELEASE IN THE GULF KILLIFISH, *FUNDULUS GRANDIS*

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ABSTRACT

Due to its ease of use, low cost, and essentially limitless number of unique codes, PIT tagging has become the favored technique for tagging individuals in biological studies. However, studies employing PIT tagging generally assume that stress due to the implantation and presence of a PIT tag has no influence on the experimental results. This study investigated the effects of PIT tagging on levels of the stress hormone cortisol in the Gulf Killifish, *Fundulus grandis*, an estuarine fish of the Gulf of Mexico that is prone to daily or seasonal environmental stressors. Cortisol was measured non-invasively by extracting and assaying cortisol excreted by individual fish into their aquarium water. This technique was optimized by acidifying water cortisol samples and processing them by Oasis HLB 1cc solid phase extraction columns. Measurements of cortisol were taken from water samples prior to, immediately after, and over four weeks after PIT tagging. Overall, there was no significant effect of PIT tagging on cortisol release by fish. There was, however, a significant increase in cortisol release by control non-tagged, as well as PIT tagged fish, immediately after handling, suggesting a stress effect of capture, brief emersion from water, or anesthesia. Cortisol release returned to control levels within one week of the procedure, suggesting that fish be allowed to recover one week after handling prior to other experimental manipulations. Future work will measure cortisol release by uniquely PIT tagged fish exposed to natural and anthropogenic stressors.

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INTRODUCTION

Passive integrated transponder (PIT) tagging is a method of identifying and monitoring individuals in biological studies. It entails implanting an individual of interest with an electronic microchip containing an alphanumeric code that is ascertainable by a PIT tag scanner (Gibbons and Andrews, 2004). Although other tagging methods are available, such as the use of leg bands, dart tags, or painted marks, the majority are external in nature and thus susceptible to factors that may affect the legibility of their designated codes (Smith and Nebel, 2013). On the contrary, as an internal method of marking, PIT tagging prevents codes from being lost or becoming indecipherable, making it suitable for application in both short-term (Hooley-Underwood *et al.*, 2017) and long-term (Hua *et al.*, 2015) studies. Additionally, PIT tagging has been shown to yield high retention rates and survivorship (Brewer *et al.*, 2016; Gries and Letcher, 2002; Simard *et al.*, 2017), favoring their application in studies at both the individual (Baras *et al.*, 2000) and population (Sloat *et al.*, 2012) scale.

PIT tagging has been used in studies ranging across all classes of vertebrates, including amphibians (Antwis *et al.*, 2015), birds (Carver *et al.*, 1999), mammals (Walter *et al.*, 2012), reptiles (Buhlmann and Tuberville, 1998), and—of interest to the present study—fish (Musselman, *et al.*, 2017). Among its many areas of application, PIT tagging has often been employed in studies relating to fish physiology and behavior, such as measuring metabolism (Norin and Malte, 2011) and swimming performance (Ficke *et al.*, 2012), respectively. However, such studies assume that the implantation and presence of a PIT tag does not influence measurements of the variable at question. For example, in studies concerning metabolism, it is assumed that PIT tagging does not compromise the oxygen consumption rate (MO_2), or, regarding swimming performance, the critical swimming velocity (U_{crit}). Since the act of tagging

may be considered a physical stressor—a stimulus affecting hormone production, leading to changes in animal performance—the degree to which fish become stressed by tagging must be accounted for in studies using PIT tags (Clark, 2016). This is more critical for studies on smaller fish, for which higher rates of mortality due to PIT tagging have been reported (Dare, 2003).

When exposed to a chemical, physical, or perceived stressor, fish respond by producing and releasing catecholamines and corticosteroids, due to activity of the hypothalamic-pituitary-interrenal (HPI) axis (Figure 1). The hypothalamus of the brain triggers the production of corticotropin-releasing hormone (CRH), followed by that of adrenocorticotropin hormone (ACTH) by the anterior lobe of the pituitary gland. The ACTH then enters the bloodstream, stimulating the production of corticosteroids by interrenal cells in the kidney (Barton, 2002). Once in circulation, the corticosteroid is distributed to target cells that promote adaptive mechanisms to stress, such as the regulation of osmolality, metabolism, and immune responses (Kijewska *et al.*, 2016). One of many corticosteroids produced through the HPI axis, cortisol is considered a principal biomarker of stress in fish (Mommsen *et al.*, 1999) (Figure 2). Though catecholamines (e.g., noradrenaline and adrenaline) may also be indices of stress, cortisol is the most commonly assessed owing to its slow and sustained release by fish, allowing for measurements that are easily and clearly detectable pre-stress and post-stress (Gesto *et al.*, 2015).

Cortisol measurements are frequently obtained by sampling blood plasma (Benfey and Biron, 2000; Clements *et al.,* 2002; Sadler *et al.*, 2000). However, the invasive nature of blood sampling may elicit confounding stress in fish, in addition to that resulting from necessary netting and air exposure (Aerts *et al.*, 2015). For smaller fish, blood sampling is likely to be terminal, allowing for only one measurement of cortisol per individual. Alternatively, Scott *et al.* (2001) developed a non-invasive method of measuring cortisol by sampling water rather than

blood plasma. This method is based on the principle that fish excrete free steroids into water *via* the gills and conjugated (i.e., glucuronidated and sulfated) steroids *via* feces and urine (Felix *et al.*, 2013). In the interest of studying the immediate and extended impact of PIT tagging on cortisol levels in fish, a notable advantage of utilizing this method is that fish remain relatively undisturbed during sampling, allowing for water samples to be collected from the same individual for a desired period of time (Lower *et al.*, 2005).

Therefore, the present study aims to investigate the effects of PIT tagging on cortisol release by the Gulf Killifish, *Fundulus grandis*. Native to estuarine habitats prone to variation in salinity, oxygen, temperature, among other abiotic variables, the teleost fish *F. grandis* was used for this study due to its tolerance of environmental stressors, as well as its abundance throughout the Gulf of Mexico coast (Burnett *et al.*, 2007). Cortisol was obtained, extracted, and assayed from water samples of both PIT tagged and non-tagged *F. grandis* one day before, immediately after, and over four weeks following PIT tagging. These measurements were used to assess the effects of PIT tagging on the stress response by these fish.

MATERIALS AND METHODS

HUSBANDRY

Male *F. grandis* (n = 12) were purchased from Joe's Landing Marina in Barataria, LA, transported to the University of New Orleans, and randomly distributed in 40 l aquaria containing aerated, dechlorinated, and filtered tap water. Synthetic sea salt (Instant Ocean Sea Salt, Instant Ocean, U.S.) was added to produce 1/3 strength sea water (1/3 SW). Water oxygen, salinity, and temperature were monitored daily using an oxygen-salinity-temperature probe (YSI Pro2023, Yellow Springs Instruments Co., Inc., U.S.), where dissolved oxygen was maintained

at *c.* 7 mg l^{-1}, salinity at *c.* 12 ppt, and temperature at *c.* 25°C. Fish were maintained at a photoperiod of 14L:10D and treated with parasite medication (General Cure Powder, API, U.S.) as instructed by the manufacturer. Fish were fed dried flake food (TetraMin Tropical Flakes, Tetra, U.S.) amounting to *c.* 1% of their body mass daily and fasted 24 hrs prior to water sampling. All maintenance and experimental protocols complied with national animal welfare regulations and were approved by the University of New Orleans Institutional Animal Care and Use Committee (Protocol 17-004).

After a two-month acclimation period, fish were transferred from the 40 l aquaria to their own 2.5 l chambers. Each chamber was connected to an 80 l sump of 1/3 SW, forming a recirculation system (Figure 3). Water was aerated, filtered, and temperature-regulated such that dissolved oxygen was maintained at 6.96 mg l^{-} (range = 6.52-7.44 mg l^{-}), salinity at 11.3 ppt (range = 9.7-12.4 ppt), and temperature at 24°C (range = 21.6-26.3).

TAGGING PROCEDURE

Fish (10.0 ± 4.7 g; range = 6.7-20.5 g) were maintained in individual chambers for two-weeks prior to PIT tagging. All fish were anesthetized by immersion in tricaine methanesulphonate (MS-222) (0.1 g l^{-1}) until loss of equilibrium (LOE) was reached. LOE, characterized by the inability of fish to right themselves, was reached within 8 to 9 min. Fish were randomized between those that would be PIT tagged and those that would not. Those PIT tagged were injected with an 8.4 mm, 134.2 kHz tag (Biomark MiniHPT8, Biomark, Inc., U.S.) along the ventral side of the intraperitoneal cavity, with the aid of a needle (Biomark N165, Biomark, Inc., U.S.) and implanter (Biomark MK165, Biomark, Inc., U.S.) (Figure 4). Fish that were not PIT tagged were held out of water on moistened paper towels for an equal length of

time (1-2 min). After either procedure, fish were transferred to recovery containers for 2 to 3 min and subsequently returned to their respective chambers.

WATER SAMPLING

Water samples (50 ml) were taken from the 2.5 l chambers *via* a 60 ml syringe and collected in 50 ml plastic tubes. Sampling occurred at six time points before or after PIT tagging: 24 hrs before, immediately after, one week after, two weeks after, three weeks after, and four weeks after. At each time point, water circulation to a specific holding chamber was stopped, and a water sample was taken immediately. A second water sample was taken 2 hrs later, after which water circulation was resumed. All samples were stored in -20 °C until processed.

WATER PROCESSING

An experiment was performed to determine the effects of acidification of water samples and the best solid phase extraction matrix. Four 2.5 l aquaria were filled with 1/3 SW that was taken from the sump of the recirculation system. Two 50 ml water samples were taken, after which cortisol was added to the tanks to achieve the following concentrations: 1.64 pg ml^{-1}; 3.28 pg ml^{-1}; 8.20 pg ml^{-1}; 16.4 pg ml^{-1}. Additional 50 ml water samples were taken after 2 hrs. All samples were frozen at -20°C until processed.

Pairs of water samples were thawed, and one of each pair was acidified to pH 1.6-1.9 by the addition of 1 ml of 2 M hydrochloric acid (HCl) (final concentration: 0.04 M) to release any cortisol bound to proteins in the 1/3 SW or the plastic sampling tube. Cortisol was extracted from water based upon a procedure described by Ellis *et al.* (2004). Water cortisol samples were centrifuged at 3,200 rcf for 5 min to remove any particulates. In the control recovery experiment,

four solid phase extraction matrices were tested: Sep-Pak C18 1cc, Sep-Pak C18 Plus, Oasis HLB 1cc, and Oasis HLB Plus (all from Waters Corp., U.S.). Solid phase extraction matrices were conditioned with 1 ml methanol and 2 ml water, after which 50 ml water samples were applied. Columns were washed with 1 ml deionized water, after which 1 ml ethyl acetate was used to elute cortisol into a clean glass tube. A peristaltic pump (Polystaltic Pump, Haake Buchler Instruments, Inc., U.S.) was utilized to control all flow rates at approximately 1 ml min^{-1}. The control experiment showed higher recoveries from acidified water samples and among the highest recovery using Oasis HLB 1cc columns. Therefore, water samples from fish were processed as described above using this format.

CORTISOL DETERMINATION

Following elution from the solid phase extraction matrix, cortisol samples were kept at -20 °C until assayed by competitive ELISA. Briefly, ethyl acetate was evaporated overnight at 55 °C, the residue was reconstituted in 500 µl of ELISA buffer (Cayman Chemical Co., U.S.), and the solution was mixed by vortexing. All samples were assayed in parallel with cortisol standards as described by the manufacturer. A 96-well microplate spectrophotometer (VersaMax Tunable Microplate Reader, Molecular Devices, LLC, U.S.) was utilized for data acquisition and analysis.

CALCULATIONS AND STATISTICAL ANALYSES

Total cortisol release (in pg) by fish was the difference between the amount of cortisol in an initial water sample and one taken 2 hrs later. Cortisol release rate (in ng g^{-1} hr^{-1}) was calculated by dividing total cortisol release by the fish biomass and the 2 hr interval. A two-way repeated measures analysis of variance (ANOVA) was performed to assess the effects of PIT

tagging and time of sampling on cortisol release. Post-hoc comparisons on cortisol release of all fish between different time points were done using a Tukey's multiple comparisons test. All statistical analyses were conducted with GraphPad Prism 7 (Graphpad Software, Inc., U.S.), with statistical significance accepted at $P < 0.05$.

RESULTS

The ability of each solid phase extraction matrix to recover cortisol from water was compared by assaying cortisol concentrations of water samples taken from aquaria with known amounts of cortisol added. In addition, the effects of acidifying water samples were evaluated. The amount of cortisol recovered as a function of cortisol added is shown when cortisol was extracted by liquid chromatography using Sep-Pak C18 1cc columns (Figure 5A), Sep-Pak C18 Plus cartridges (Figure 5B), Oasis HLB 1cc columns (Figure 5C), and Oasis HLB Plus cartridges (Figure 5D) with and without prior acidification. Across all formats, acidification of samples increased recoveries 1.4-fold to 2.4-fold higher (compare closed to open symbols on Figure 5), as judged from the slopes of lines fit to recovery data (Table 1). Extraction by Oasis HLB 1cc and Sep-Pak C18 Plus provided the highest cortisol recoveries among the tested matrices, since these matrices generated lines with slopes closest to a slope of 1, which represents 100% cortisol recovery.

Cortisol release by fish was assessed in terms of total cortisol released by fish into aquaria (Table 2). Total cortisol release due to PIT tagging was not statistically significant at any time point (two-way repeated measures ANOVA; $P > 0.05$). However, regardless of whether or not fish were PIT tagged, there was a significant increase in total cortisol release by fish immediately after they were anesthetized and handled (i.e., on Day 0) in comparison to every

other time point (Figure 6A) (Tukey's multiple comparisons test; $P < 0.05$), suggesting that anesthetizing and handling were sufficient to generate a significant stress-response in fish.

Since smaller fish were expected to release smaller amounts of cortisol than larger fish, cortisol release by fish due to PIT tagging was also assessed in terms of the rate of cortisol release (Table 3), in order to objectively compare cortisol release between fish differing in mass. There was no statistically significant difference between cortisol release rates by non-tagged and PIT tagged fish (two-way repeated measures ANOVA; $P > 0.05$). As was observed in total cortisol release analyses, significant variation in cortisol release rates was observed between Day 0 and all other time points regardless of whether or not fish were PIT tagged (Figure 6B) (Tukey's multiple comparisons test; $P < 0.05$). Thus, all post-hoc analyses on cortisol release were completed without regard to whether or not fish were PIT tagged, linking any observances of significant changes in cortisol release to anesthetizing and handling. Cortisol release by fish returned to its basal conditions after a week of being anesthetized and handled and remained low for the duration of the experiment.

DISCUSSION

CORTISOL RECOVERY

Although the sampling of water cortisol has been repeated with up to 95% recovery (Zuberi *et al.*, 2011), most studies that have employed this method have measured free cortisol and not that which may have adhered to steroid-binding elements. Scott and Ellis (2007) question the need to measure bound cortisol on the basis that only free cortisol is excreted through the gills, whereas bound cortisol is presented in a conjugated form in the urine and feces. Indeed, Vermeirssen and Scott (1996) showed that, when the anterior and exterior regions of the

rainbow trout *Oncorhynchus mykiss* are separated by a specialized tank, free steroids are released through the gills in the anterior region, whereas glucuronidated and sulfated steroids are released through bile and urine in the posterior region, respectively. Since the release and conjugation of bound cortisol has been shown to be delayed by rates of defecation and urination (Scott and Sorensen, 1994), this may suggest that sampling only free cortisol is sufficient to accurately determine how much cortisol is in plasma at any desired time point. However, this conclusion does not take into account the possibility that free cortisol, after its released by fish, becomes bound to constituents in the tank water (e.g., exuded proteins, mucous, uneaten food, microbes) or the walls of the sampling vessel.

Therefore, in the present study, neutral and acidified water samples with known concentrations of cortisol were processed through four solid phase extraction matrices to determine if the addition of acid disassociates bound cortisol, as well as to compare how much cortisol may be recovered using each format. As expected, all formats provided positive correlations between amount of cortisol introduced and recovered. However, between formats, water samples that were acidified and processed by Sep-Pak C18 Plus and Oasis HLB 1cc produced the highest recovery of cortisol at 80% and 79%, respectively. Indeed, the Sep-Pak C18 Plus format has also been one of the most commonly utilized cortisol extraction formats in related studies (Diamandis and D'Costa, 1988; Newman *et al.*, 2015; Wong *et al.*, 2008), which may perhaps be on account of such successes in analyte recoveries. However, the Oasis HLB 1cc format was preferred in this experiment because it was less expensive and easier to implement, having been designed to recover analyte in the event of the stationary phase medium experiencing dryness. Regardless of the format, higher cortisol recoveries were yielded for samples that were acidified. Therefore, in the interest of maximizing cortisol recovery, it is

advised that water cortisol samples be acidified whenever this method of cortisol sampling is employed, or at least when similar solid phase extraction matrices are used.

EFFECTS OF TAGGING

Cortisol release due to stressors has been analyzed on a number of different species of fish (Scott and Ellis, 2007), including the European sea bass *Dicentrarchus labrax* (Fanouraki *et al.*, 2008), the rainbow trout *Oncorhynchus mykiss* (Ellis *et al.*, 2005), and the Atlantic salmon *Salmo salar* (Kittilsen *et al.*, 2009). However, few studies have assessed cortisol release by fish due to PIT tagging. Though Jørgensen *et al.* (2017) evaluated the effects of PIT tagging on swimming, hematocrit, and tag retention on the sandeel *Ammodytes tobianus,* cortisol release was not assessed in this study. Likewise, Lower *et al.* (2005) examined the effects of PIT tagging on cortisol release by carp *Cyprinus carpio* and roach *Rutilus rutilus*, but only measured free cortisol and not that which may have become bound to particulates in water or the sampling vessel. Thus, little is known about the effects of PIT tagging on cortisol release by fish—or, specifically, by *F. grandis*.

Prior to PIT tagging, there was no difference in the amount of cortisol released between fish that would be PIT tagged and those that would not. Surprisingly, there was a minimal impact of PIT tagging on cortisol release by fish across all time points. This is in contrast to what Lower *et al.* (2005) observed in a study on carp and roach, where there was an immediate increase in cortisol release by both species of fish just 1-2 h following the PIT tagging procedure. This difference in response might be attributed to differing methods of PIT tag implantation. In Lower *et al.* (2005), PIT tags were inserted by means of a *c.* 10 mm surgical incision, whereas in the present study, PIT tags were inserted by injection with a syringe needle. The incision may have

contributed to the increase in cortisol release that was reported by Lower *et al.* (2005). Interestingly, Baras *et al.* (2011) compared these two implantation procedures on the juvenile Nile Tilapia *Oreochromis niloticus*, and discovered that surgically implanted fish had significantly higher survival rates than injected fish after 49 d. Thus, while PIT tagging by injection may minimize stress levels, the trade-off of using this method is that it may also result in lower survival rates in some species of fish.

EFFECTS OF ANESTHESIA AND HANDLING

As surprising was that cortisol release nearly doubled immediately after fish were anesthetized and handled, resulting in dramatic differences in cortisol release between this moment and all other time points. Accordingly, this indicates that while PIT tagging did not significantly affect cortisol release in fish, the very process of handling and anesthetizing did. However, given that anesthetics are administered to reduce the stress that fish may experience during handling and other experimental operations, cortisol levels observed immediately after anesthetizing and handling are likely a fraction of what they would've been had fish not been anesthetized.

Though this hypothesis was not tested in the present study, a study on the three spot gourami *Trichogaster trichopterus* determined that plasma cortisol levels were much lower in fish that were anesthetized and handled in comparison to control groups that were handled without anesthesia (Crosby *et al*., 2006). Another study by Thomas and Robertson (1991) treated the red drum *Sciaenops ocellatus* with two minutes of air exposure and observed pronounced elevations in plasma cortisol in fish that were not administered anesthetics beforehand. These

studies provide support in favor of applying anesthetics to minimize stress experienced by fish during experimental procedures.

Interestingly, both studies tested the effects of using different anesthetics on plasma cortisol and concluded that some anesthetics were more effective in reducing stress than others. Thomas and Robertson (1991) also observed an increase in plasma cortisol in fish anesthetized in 80 mg l^{-1} MS-222 versus 10 mg l^{-1} MS-222, suggesting that there is a dose-related effect of anesthesia on stress. In light of this, the degree to which anesthesia impacts cortisol release while handling fish may depend on a number of factors, such as the type of anesthetic used and the dosage of the anesthetic, in addition to the species, size, and maturity of fish (Popovic *et al*., 2012). These data suggest that repeated measurements of a variable in studies on *F. grandis* should not resume until after one week of anesthetizing and handling, at which cortisol levels are likely to have returned to basal conditions.

CONCLUSION

Cortisol water sampling provided a non-invasive alternative to blood plasma sampling toward assessing stress in *F. grandis* due to PIT tagging that was neither terminal nor poor in recovery. The acidification of water cortisol samples further improved the amount of cortisol recovered, reinforcing the importance of ensuring that both free and bound cortisol be measured in studies investigating cortisol release. Though the injection and presence of a PIT tag did not have an effect on cortisol release in *F. grandis*, cortisol release markedly increased due to anesthetizing and handling. Cortisol release returned to basal conditions a week after fish were anesthetized and handled, suggesting that fish be allowed to recover from anesthetizing and handling for a week prior to commencing experimental procedures.

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TABLE 1: Equations of lines and R^2 values of solid phase extraction formats with and without the addition of HCl.

Matrix Format	Without HCl	With HCl
Sep-Pak C18 1cc	$y = 0.51x - 80.2$ ($R^2 = 0.91$)	$y = 0.70x - 54.7$ ($R^2 = 0.98$)
Sep-Pak C18 Plus	$y = 0.34x - 16.0$ ($R^2 = 0.99$)	$y = 0.80x - 37.0$ ($R^2 = 0.99$)
Oasis HLB 1cc	$y = 0.42x - 18.1$ ($R^2 = 0.95$)	$y = 0.79x - 51.1$ ($R^2 = 0.99$)
Oasis HLB Plus	$y = 0.40x + 23.8$ ($R^2 = 0.99$)	$y = 0.64x + 12.4$ ($R^2 = 0.96$)

TABLE 2: Results of a repeated measures ANOVA of total cortisol release by *F. grandis.*

Sources of Variation	df	F	P
PIT Tagging	1, 10	0.113	0.7432
Time Point	5, 50	7.214	< 0.0001
Interaction	5, 50	1.596	0.1785
Subjects	10, 50`	1.715	0.1033

TABLE 3: Results of a repeated measures ANOVA of cortisol release rate of *F. grandis.*

Sources of Variation	df	F	P
PIT Tagging	1, 10	0.027	0.8719
Time Point	5, 50	9.019	< 0.0001
Interaction	5, 50	1.365	0.2532
Subjects	10, 50`	1.769	0.0912

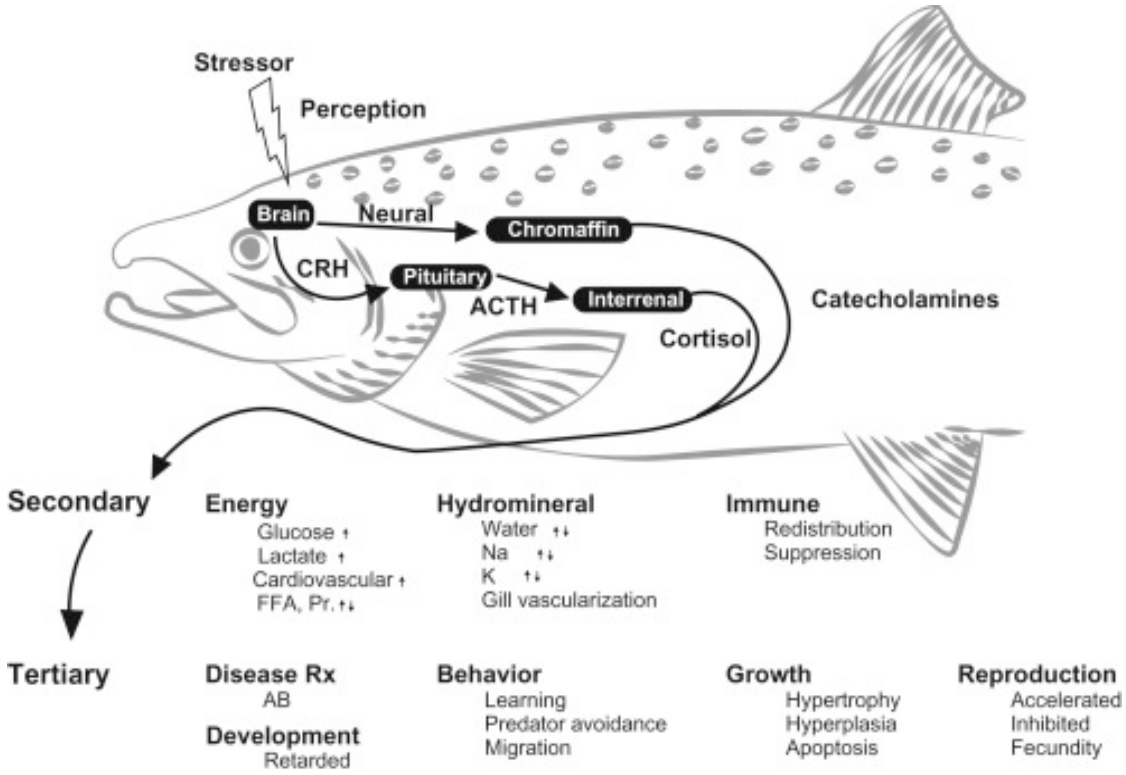

FIGURE 1: The activity of the hypothalamic-pituitary-interrenal axis in evoking primary, secondary, and tertiary adaptive responses to stressors perceived by fish (Schreck and Tort, 2016).

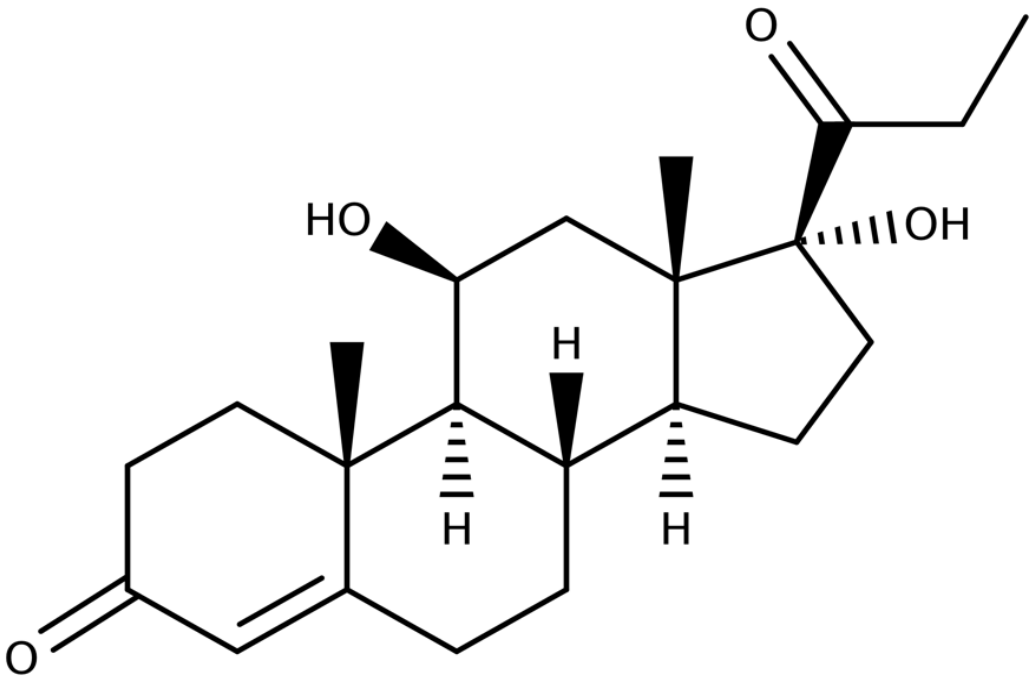

FIGURE 2: The molecular structure of the stress hormone cortisol.

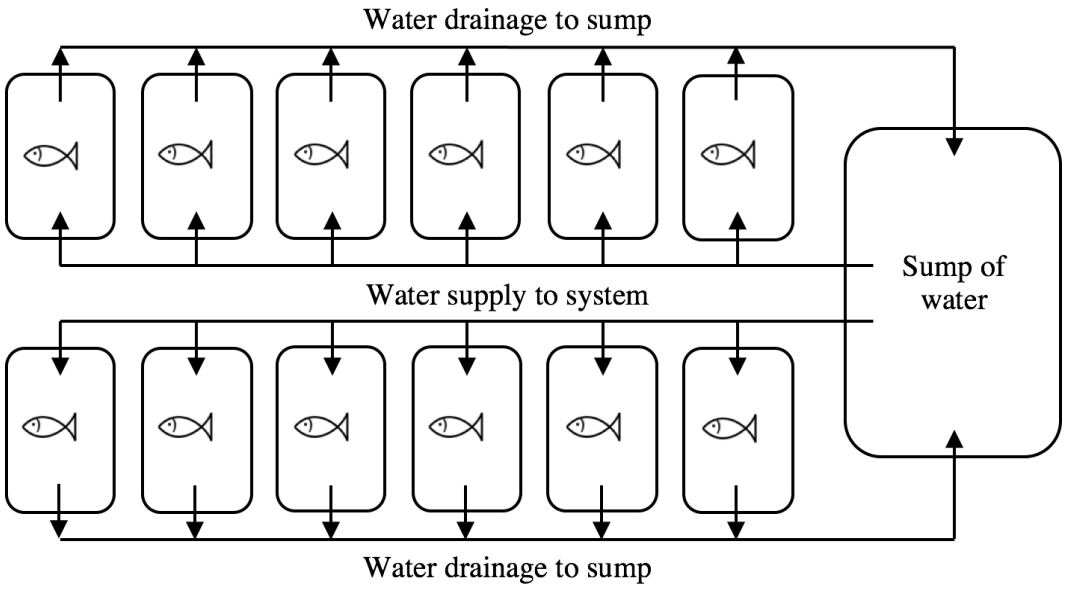

FIGURE 3: The recirculation system in which water was exchanged between the 2.5-l chambers where fish were held and an 80-liter sump of water, where water was aerated, filtered, and temperature controlled (24°C).

FIGURE 4: The length of an 8.4 mm PIT tag relative to that of adult *F. grandis*.

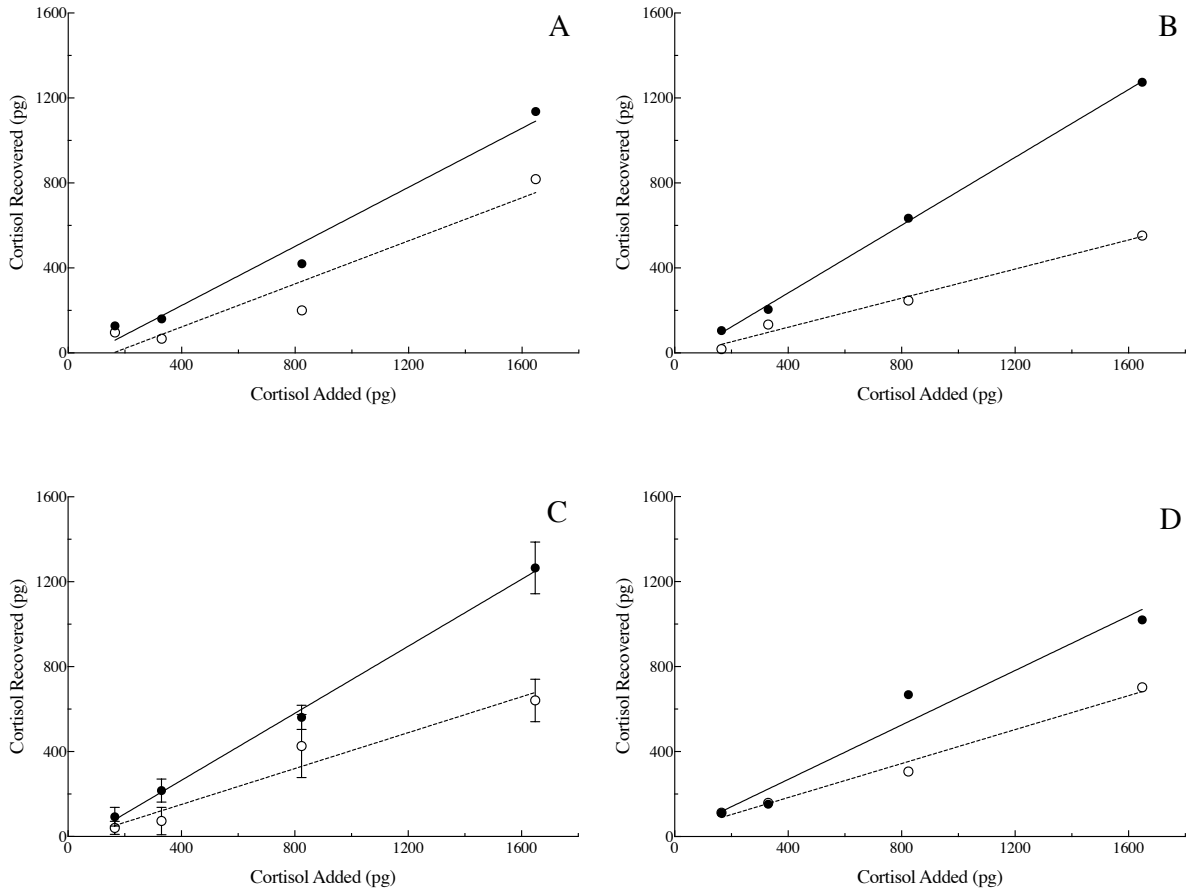

FIGURE 5: The recovery of known amounts of cortisol using Sep-Pak C18 1cc (A), Sep-Pak C18 Plus (B), Oasis HLB 1cc (C), and Oasis HLB Plus (D), with (—●—) and without (- - ○ - -) the addition of HCl. Points on A, B, and D represent the mean of replicate readings of a single recovery experiment. Points on C represent the mean of readings from two replicate experiments. Error bars on C represent a range of values. Cortisol recoveries using the matrices in A, B, C, and D were 1.37-fold, 2.35-fold, 1.89-fold, and 1.60-fold higher, respectively, as a result of adding HCl to water.

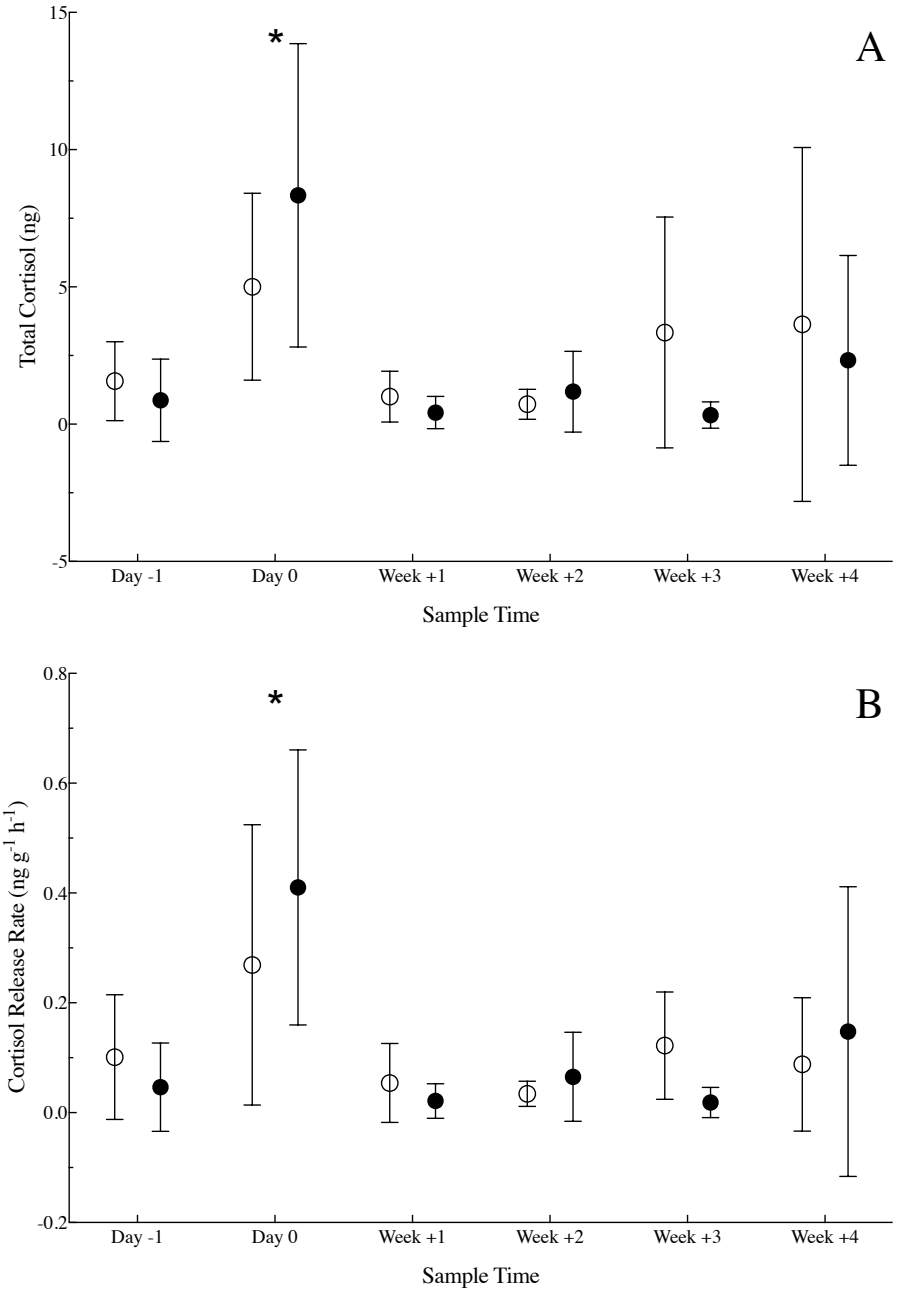

FIGURE 6: The effects of PIT tagging on mean ± 95% c.i. total cortisol released (A) and cortisol release rates (B) by non-tagged (n = 6) (—○—) and PIT tagged (n = 6) (—●—) *F. grandis*. Asterisks represents significant differences between the cortisol release by all fish on Day 0 and at every other time point (Tukey's multiple comparisons test; $P < 0.05$).

APPENDIX 1: Cortisol recovery data.

Assay Date	Time (hr)	+/- HCl	Format	Average Result (pg)	Actual (pg) (2 hr - 0 hr)	Expected (pg)	Percent Recovery
1/12/17	0	-	Oasis 1cc	22.05			
1/12/17	0	-	Oasis Plus	22.66			
1/12/17	0	-	SP 1cc	0.99			
1/12/17	0	-	SP Plus	49.32			
1/12/17	0	+	Oasis 1cc	73.23			
1/12/17	0	+	Oasis Plus	41.08			
1/12/17	0	+	SP 1cc	1.64			
1/12/17	0	+	SP Plus	0.00			
1/12/17	2	-	Oasis 1cc	95.35	71.59	164.80	43.44
1/12/17	2	-	Oasis 1cc	161.33	137.57	329.60	41.74
1/12/17	2	-	Oasis 1cc	598.87	575.11	824.00	69.79
1/12/17	2	-	Oasis 1cc	765.06	741.30	1648.00	44.98
1/12/17	2	-	Oasis Plus	136.38	112.62	164.80	68.33
1/12/17	2	-	Oasis Plus	182.57	158.81	329.60	48.18
1/12/17	2	-	Oasis Plus	330.39	306.63	824.00	37.21
1/12/17	2	-	Oasis Plus	726.69	702.93	1648.00	42.65
1/12/17	2	-	SP 1cc	120.38	96.62	164.80	58.63
1/12/17	2	-	SP 1cc	91.06	67.30	329.60	20.42
1/12/17	2	-	SP 1cc	224.19	200.43	824.00	24.32
1/12/17	2	-	SP 1cc	841.59	817.83	1648.00	49.63

1/12/17	2	-	SP Plus	41.95	18.19	164.80	11.04
1/12/17	2	-	SP Plus	157.73	133.97	329.60	40.65
1/12/17	2	-	SP Plus	270.52	246.76	824.00	29.95
1/12/17	2	-	SP Plus	576.44	552.68	1648.00	33.54
1/12/17	2	+	Oasis 1cc	166.11	137.12	164.80	83.21
1/12/17	2	+	Oasis 1cc	299.66	270.67	329.60	82.12
1/12/17	2	+	Oasis 1cc	533.65	504.66	824.00	61.24
1/12/17	2	+	Oasis 1cc	1416.41	1387.42	1648.00	84.19
1/12/17	2	+	Oasis Plus	139.31	110.32	164.80	66.94
1/12/17	2	+	Oasis Plus	181.72	152.73	329.60	46.34
1/12/17	2	+	Oasis Plus	697.00	668.01	824.00	81.07
1/12/17	2	+	Oasis Plus	1049.06	1020.07	1648.00	61.90
1/12/17	2	+	SP 1cc	156.21	127.22	164.80	77.20
1/12/17	2	+	SP 1cc	189.50	160.51	329.60	48.70
1/12/17	2	+	SP 1cc	448.57	419.58	824.00	50.92
1/12/17	2	+	SP 1cc	1165.75	1136.76	1648.00	68.98
1/12/17	2	+	SP Plus	134.44	105.45	164.80	63.99
1/12/17	2	+	SP Plus	234.16	205.17	329.60	62.25
1/12/17	2	+	SP Plus	663.22	634.23	824.00	76.97
1/12/17	2	+	SP Plus	1303.19	1274.20	1648.00	77.32
3/15/18	0	-	Oasis 1cc	52.25			
3/15/18	0	-	Oasis 1cc	45.71			
3/15/18	0	+	Oasis 1cc	32.67			

3/15/18	0	+	Oasis 1cc	40.67			
3/15/18	2	-	Oasis 1cc	59.07	10.09	164.80	6.12
3/15/18	2	-	Oasis 1cc	57.65	8.67	329.60	2.63
3/15/18	2	-	Oasis 1cc	326.87	277.89	824.00	33.72
3/15/18	2	-	Oasis 1cc	589.96	540.98	1648.00	32.83
3/15/18	2	+	Oasis 1cc	86.07	49.40	164.80	29.97
3/15/18	2	+	Oasis 1cc	199.24	162.57	329.60	49.32
3/15/18	2	+	Oasis 1cc	654.46	617.79	824.00	74.97
3/15/18	2	+	Oasis 1cc	1180.43	1143.76	1648.00	69.40

APPENDIX 2: Cortisol from PIT tagging data.

Fish Number	Tagged?	Sample Time	Fish Mass (g)	Total Cortisol Release (ng)	Cortisol Release Rate (ng g^{-1} h^{-1})
1	No	Day -1	7.38	0.79	0.05
1	No	Day 0	7.38	2.52	0.17
1	No	Week +1	7.23	0.61	0.04
1	No	Week +2	7.56	0.28	0.02
1	No	Week +3	7.13	1.74	0.12
1	No	Week +4	7.01	0.05	0.00
2	No	Day -1	12.33	1.23	0.05
2	No	Day 0	12.33	6.89	0.28
2	No	Week +1	12.73	0.99	0.04
2	No	Week +2	13.03	0.40	0.02
2	No	Week +3	13.11	1.25	0.05
2	No	Week +4	13.95	1.42	0.05
3	Yes	Day -1	9.29	0.13	0.01
3	Yes	Day 0	9.29	11.06	0.60
3	Yes	Week +1	9.96	0.41	0.02
3	Yes	Week +2	9.96	2.38	0.12
3	Yes	Week +3	9.71	1.08	0.06
3	Yes	Week +4	10.21	2.04	0.10
4	Yes	Day -1	9.78	1.47	0.08

4	Yes	Day 0	9.78	7.04	0.36
4	Yes	Week +1	9.41	1.50	0.08
4	Yes	Week +2	8.83	3.45	0.20
4	Yes	Week +3	7.78	0.03	0.00
4	Yes	Week +4	7.34	9.59	0.65
5	Yes	Day -1	6.77	0.07	0.01
5	Yes	Day 0	6.77	4.56	0.34
5	Yes	Week +1	6.81	0.26	0.02
5	Yes	Week +2	6.96	0.75	0.05
5	Yes	Week +3	7.31	0.72	0.05
5	Yes	Week +4	7.10	1.34	0.09
6	Yes	Day -1	11.41	0.00	0.00
6	Yes	Day 0	11.41	17.80	0.78
6	Yes	Week +1	11.78	0.00	0.00
6	Yes	Week +2	12.81	0.19	0.01
6	Yes	Week +3	13.06	0.04	0.00
6	Yes	Week +4	13.54	0.89	0.03
7	No	Day -1	6.83	3.98	0.29
7	No	Day 0	6.83	10.19	0.75
7	No	Week +1	6.81	2.60	0.19
7	No	Week +2	7.19	0.68	0.05
7	No	Week +3	7.26	3.33	0.23
7	No	Week +4	7.53	0.16	0.01

8	No	Day -1	12.51	1.09	0.04
8	No	Day 0	12.51	1.88	0.08
8	No	Week +1	12.9	0.62	0.02
8	No	Week +2	13.51	1.72	0.06
8	No	Week +3	13.67	2.24	0.08
8	No	Week +4	14.73	3.93	0.13
9	Yes	Day -1	9.28	3.55	0.19
9	Yes	Day 0	9.28	5.09	0.27
9	Yes	Week +1	9.54	0.00	0.00
9	Yes	Week +2	9.30	0.26	0.01
9	Yes	Week +3	9.13	0.00	0.00
9	Yes	Week +4	9.28	0.10	0.01
10	No	Day -1	20.52	0.09	0.00
10	No	Day 0	20.52	5.90	0.14
10	No	Week +1	20.74	1.22	0.03
10	No	Week +2	21.46	0.52	0.01
10	No	Week +3	23.73	11.24	0.24
10	No	Week +4	26.03	15.82	0.30
11	No	Day -1	6.69	2.24	0.17
11	No	Day 0	6.69	2.66	0.20
11	No	Week +1	6.89	0.00	0.00
11	No	Week +2	7.52	0.75	0.05
11	No	Week +3	7.72	0.23	0.01

11	No	Week +4	8.24	0.42	0.03
12	Yes	Day -1	19.51	0.00	0.00
12	Yes	Day 0	19.51	4.45	0.11
12	Yes	Week +1	19.68	0.37	0.01
12	Yes	Week +2	19.65	0.07	0.00
12	Yes	Week +3	19.90	0.11	0.00
12	Yes	Week +4	20.97	0.00	0.00