Variable stress-responsiveness in wild type and domesticated fighting fish

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Received 30 March 2007; received in revised form 30 July 2007; accepted 2 August 2007

Abstract

VERBEEK, P., T. IWAMOTO AND N. MURAKAMI. Variable stress-responsiveness in wild type and domesticated fighting fish. PHYSIOL BEHAV XX(X) 000-000, 2007. We combined behavioral and physiological measures to compare coping style in wild-type *Betta splendens* and a domesticated strain selectively bred for sports fighting. We showed previously that the fighter strain is more aggressive than the wild type during experimental conditions that most closely resemble an actual fight. We predicted that compared to the wild type, the fighter strain would show a more proactive coping style, characterized by lesser cortisol and greater sympathetic responses to non-social challenges. We introduced males to an unfamiliar environment and spatial confinement as challenges that may resemble some of those that *B. splendens* may encounter in its natural habitat. We developed a non-invasive stress assay that enables repeated individual measures of water-borne cortisol. We estimated sympathetic activation through opercular beat rate and recorded the duration of behavioral immobility. We found that exposure to an unfamiliar environment raised cortisol levels in the wild type but not in the fighter strain and that confinement raised cortisol levels in both. In both strains opercular beat rates were significantly reduced during the latter stages of confinement compared to during the early stages. The fighter strain, but not the wild type, adopted a behavioral strategy of immobility from the very beginning of confinement.

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Keywords: Fighting fish; *Betta splendens*; Aggression; Coping styles; Opercular beat rate; Behavioral immobility; Cortisol; Non-invasive stress assay; Context

1. Introduction

*Betta splendens* has been bred selectively for sport fighting for more than 650 years [1]. Recently, we showed that *B. splendens* males bred for fighting are indeed more aggressive than wild type *B. splendens*, but only in experimental contexts that most closely resemble an actual fight [2]. Here we report on our continued work on coping styles in wild type and domesticated fighting fish.

Proactive coping combines high aggressiveness and boldness with a lack of behavioural flexibility. Reactive individuals show the opposite end of the spectrum. Proactive individuals combine low hypothalamus–pituitary–adrenal (HPA) axis activity and reactivity with high sympathetic nervous system and testosterone activity. Again, reactive individuals show opposite patterns [3]. Proactive and reactive coping has been shown in laboratory and natural populations of vertebrates, including fish [4,5], notably rainbow trout (*Oncorhynchus mykiss*) [6,7]. In addition to being associated with high aggressiveness, recent findings also link glucocorticoid deficits with abnormal patterns of aggression. In selected lines of rodents the abnormal aggression involves inappropriate attack targeting [8], while in an unselected population of rainbow trout it consists of sustained aggression after the establishment of dominance [6].

Blood sampling for hormone assay in small fish generally involves sacrificing the animal, which precludes sampling hormone levels over time and under different conditions in the same individuals. Recently, the first studies have been published that developed assays of water-borne hormones in single-housed small fish; notably, cortisol in male convict cichlids (*Archocentrus nigrofasciatus*) [9], and 11-ketotestosterone in male *B. splendens* [10]. These reports follow validation of water-born hormone assays through research that showed that stress levels of water-borne cortisol correspond to plasma levels [11,12]. Here we report
on our newly developed method of detecting cortisol in the holding water of individual *B. splendens* males.

A non-invasive method of estimating sympathetic activation in fish is opercular beat rate, which may be linked to activity as well as metabolic rate [13]. In the present study we measured cortisol and opercular beat rate during environmental challenges that *B. splendens* may encounter in the wild, including exposure to an unfamiliar environment and lowered water level confinement. We predicted that, in addition to the high, context-specific, aggression that we showed in a previous study, males of the *B. splendens* fighter strain would show lesser cortisol and greater sympathetic responses to non-social challenges compared to wild type *B. splendens*.

2. Materials and methods

2.1. Animals

The fish were bred in our laboratory from wild fish and *B. splendens* fighter stock purchased from a well-established commercial breeder/exporter in Petchaburi Province, Thailand. Rearing and maintenance were as described in [2]. Briefly, fry were moved to grow-out tanks (72 × 45 cm and 23 cm deep) at 30 days after hatching. After 30 days in the grow-out tanks fish from the same spawn were moved to mixed sex community tanks (60 × 36 cm and 29 cm deep). Five months prior to the start of the experiments 10 randomly selected males from each strain were individually housed in oval 1.5-liter plastic bottles (height, including lid: 24.3 cm, cross-diagonal width: 12 cm) separated by white corrugated plastic partitions. These males were subsequently allowed 2 h of exposure to rotating left and right neighbors every day, and a full day of social exposure about every 8 days. Artificial lighting followed a 14-h light/10-h darkness cycle with lights on at 0700. All fish were fed daily with freshly hatched nauplii of *Artemia salina*. Tank water temperature was maintained at 25 °C. The age at the start of testing was 25 months for *B. splendens* and 27 months for *B. splendens* fighter.

The Committee on the Ethics of Animal Experiments at the University of Miyazaki approved the experiments. One *B. splendens* fighter selected for this study died of natural causes prior to the start of the experiments. The remaining fish were kept as part of our longitudinal project on fighting fish.

2.2. Experimental procedures

The stress-responsiveness of the fish was tested by placing them in an unfamiliar environment (relocation test), and by exposing them to lowered water level spatial confinement (confinement test). Water-borne cortisol levels during placement in an unfamiliar environment were compared to cortisol levels measured in the home tank (relocation test). Water-borne cortisol levels and behavior during confinement were compared to a post-confinement period of equal length (confinement test). Based on recent studies that employed newly developed assays to detect water-borne steroids in individually housed small teleosts (*B. splendens*: 10; *A. nigrofasciatus*: 9) we opted for a water volume of 250 ml. for each condition in the two separate tests (see also Section 2.4). The experiments were conducted during afternoon hours to control for diurnal variation of cortisol. The two conditions of the relocation test were separated by eight days. The confinement test was conducted during the course of one day 10 days later. Mutual viewing was disallowed during all experimental conditions. The order in which fish were tested was randomized.

During the first day of testing for the relocation test the fish were quickly netted from their home tanks and transported to plastic tanks (8.5 × 7 cm and 8.5 cm deep). The transparent tanks were covered with an opaque lid and filled with 250 ml of aged, de-chlorinated, municipal tap water, at 25 °C, taken from the laboratory supplies. The tanks were unfamiliar to the fish and placed in an area of the laboratory that was also unfamiliar to them. After 6 h the water was removed from the test tanks one tank at the time for later cortisol measurement. Once all water was removed from a test tank the fish was quickly transferred into a dip net and returned to the home tank. During the second day of testing the fish were transferred to a 1.5 l tank identical to their home tank, filled with 250 ml (rather than 1.5 l) of water (at 25 °C), and placed in the home position. After 6 h the 250-ml of water in each tank was collected and each tank was refilled with 1.5 l of water. The tank water collected from the test- and home tank from which individual water-borne cortisol levels were to be determined (hormone collection water) was stored in 275-ml PET bottles at 4 °C until extraction.

At the start of the confinement test the fish were placed in a clear plastic tank, 25 × 13 cm and 8 cm deep, equipped with a movable clear divider, and filled with 250 ml of our laboratory water (at 25 °C). After 5 min. of acclimating the divider was gently moved to either the right or left side of the tank (randomized). This created a confinement space of 2.5 × 4 × 8 cm. A Canon DM-FV M20 digital video camera was mounted overhead on a tripod (Tecc.Land YT-1100) and zoomed for recordings of the first and last 15 min of confinement.

At the end of the 3-hour confinement period the hormone collection water was stored and the fish were transferred to the experimental tanks used during the first day of testing for the first stage of the relocation test (described above: 8.5 × 7 cm and 8.5 cm deep), filled with 250 ml of water (at 25 °C) for the 3-hour post-confinement period. At the end of 3-hour post-confinement the water was collected and the fish returned to their home tanks. Hormone collection water from the two consecutive experimental periods was stored in 275 ml. PET bottles at 4 °C until extraction.

2.3. Video scoring

Video recordings of minutes 1–5 (interval 1) and 165–169 (interval 2) of the confinement tests were saved in iMovie (Apple Computer, Inc., Cupertino, CA, USA) on a Macintosh G5 computer, and viewed in random order for scoring by PV. Behavioral immobility was defined as a temporary state of complete motor inhibition, and scored with a hand-held stopwatch (Telva Sports). For each minute of interval 1 and 2 the time taken for 20 opercular beats to occur was measured [13]. This measure was then transformed into beats per minute and averaged for both intervals.
2.4. Hormone measurements

Cortisol was extracted from 250-ml water samples using Sep-Pak® C18 solid phase extraction columns (Waters Corp., Milford, MA, USA) fitted to a 12-port vacuum manifold (Waters Corp.). The columns were primed through immersion in methanol for a minimum of 48 h before use. When fitted to the vacuum manifold the columns were washed with 4 ml of methanol followed by 4-ml ddH2O. The 250-ml water samples were pushed through the columns using the vacuum manifold. Hormone was eluted from the columns using 4 ml (60%) methanol. The efficiency of extraction was tested with multiple extractions using the equivalent of 700 pg 3H-labelled cortisol (Daiichiseikagaku Co, Tokyo) in 250-ml water and recovery averaged 99.7%.

Water-borne cortisol levels were measured by radioimmunoassay (RIA). Briefly, following extraction, the 4 ml of solvent was evaporated in a centrifugal evaporator (Tokyo Rikagaku Co, Tokyo) leaving the solid phase hormone. Three hundred µl of 0.05 M phosphate buffer (pH 7.0) including 0.3% gelatin was added to the samples or diluted standard (range from 7.81 to 2000 pg/tube). Then, 15000 cpm/100 µl of 3H-labelled cortisol solution was added to the samples followed by 100 µl of cortisol antibody solution (antibody specificity was 100% for cortisol, 2.4% for deoxycorticosterone, 5.8% for corticosterone, <0.1% for progesterone, 20-hydroxyprogesterone, 17-estradiol, 17-hydroxyprogesterone, androstenedione, testosterone, androsterone, and dehydroepiandrosterone). The samples and standard were then incubated overnight at 4 °C. Bound and free ligands were separated by dextran-coated charcoal. Antibody-bound radioactivity was measured by Packard TriCarb liquid scintillation counter. The intra-and interassay coefficients of variation were 5.5% and 5.2% at 50% binding. Water-borne hormones were not obtained for 2 B. splendens fighter during the location test and 1 B. splendens during post-confinement.

2.5. Statistical analysis

We used GraphPad Prism (GraphPad Software, Inc., San Diego, CA, U.S.A.) for analyses. The data were normally distributed with equal variances. The groups were compared using two factors and repeated measures ANOVA with Bonferroni posttests.

3. Results

3.1. Cortisol measures

Two-way ANOVA was used to analyze the location test, with location (unfamiliar vs. home tank) as the first factor, and strain (wild vs. fighter) as the second factor. There was a significant main effect for location (F1,30 =15.26, P<0.0005) as well as for the location and strain interaction (F1,30 =4.66, P=0.039). Bonferroni posttests showed that cortisol levels were significantly higher in the unfamiliar tank compared to the home tank for the wild fish (P<0.001, N=10, Fig. 1), but not the fighter (N=7, Fig. 1).

Two-way ANOVA was also used to analyze the confinement test, with condition (during vs. after) as the first-and strain (wild vs. fighter) as the second factor. We found a main effect for condition (F1,32 =26.23, P<0.0001). Bonferroni posttests showed that cortisol levels were significantly higher in the unfamiliar tank compared to the home tank for the wild fish (P<0.001, N=9, Fig. 2) and
response in the fighter. This recalls Huntingford’s finding of 3 not trigger a significant hypothalamus during the early stages.

During confinement a majority of environmental challenges. Apparent water-borne cortisol levels did. Confinement evoked similar physiological responses in the wild males showed strenuous avoidance reactions at first, but compared to the home tank. During confinement a majority of confinement levels fighter males did not show a cortisol response to an unfamiliar environment while wild males did. Confinement evoked similar physiological responses in the home tank levels fighter males did not show a cortisol response to an unfamiliar environment while wild males did. Confinement evoked similar physiological responses in the home tank levels fighter males did not show a cortisol response to an unfamiliar environment while wild males did.

4. Discussion

Our results partially confirm our prediction that fighter males would show a more proactive coping style compared to wild males when faced with non-social challenges. Compared to home tank levels fighter males did not show a cortisol response when exposed to an unfamiliar environment while wild males did. Confinement evoked similar physiological responses in the two strains but differences in behaviour. The wild fish responded predictably and adaptively to environmental challenges. Apparent water-borne cortisol levels for wild males were 4 times higher in the unfamiliar tank compared to the home tank. During confinement a majority of wild males showed strenuous avoidance reactions at first, but reduced energy expenditure during the latter stages by remaining immobile. The wild fish also significantly reduced their opercular beat rate during the latter stages of confinement compared to the early stages.

In contrast to the wild type, relocation to an unfamiliar tank did not trigger a significant hypothalamus–pituitary–interrenal axis response in the fighter. This recalls Huntingford’s finding of 3 decades ago [14] that three-spined sticklebacks (Gasterosteus aculeatus) that were least disturbed by a transfer to a strange environment tended to be most aggressive during the breeding season. However, similar to wild fish, the fighter males did show higher apparent cortisol levels during confinement compared to afterwards. In contrast to the wild fish, we observed little or no struggling by fighter males during the early stages of confinement. Instead, fighter males tended to resort to behavioral immobility from the very beginning of confinement. They also significantly reduced their opercular beat rate during the latter stages of confinement.

Remaining immobile while being faced with inescapable confinement is likely to be an adaptive strategy linked to metabolic rate [15]. Both the wild and fighter males in our study resorted to this strategy during confinement. However, the key difference between the two strains was the fact that fighter males resorted to immobility from the moment they were faced with confinement. In contrast, most of the wild males struggled vigorously during the initial stages of confinement and only later settled down.

A related finding on oxygen deprivation in teleosts underscores the potential benefit of remaining immobile when faced with a stressor that cannot be engaged or escaped from. Rainbow trout from a heterogeneous population exposed to 3 h of severe hypoxia differed significantly in immobility and survival. Individuals that did not panic and remained quiet were significantly more likely to survive than those that showed strenuous avoidance behaviours [16].

Freezing, or tonic immobility, has been linked to high HPA/HPI axis reactivity and a reactive coping style, while flight or fight responses have been associated with a proactive coping style. Context plays a role, however, as the absence of sawdust in the defensive burying test in laboratory rodents elicits freezing behaviour in the proactive animal [3]. In teleosts, immobility during confinement has been linked to proactive coping. For example, in sexually immature rainbow trout individuals that resumed feeding first in a new environment spent more time remaining immobile during confinement [7]. Moreover, rainbow trout selected for low cortisol responses during a confinement test were more likely to become dominant in pair contests [16], and were least disturbed by transfer to a new environment as evidenced by a more rapid resumption of feed intake [17]. Finally, abnormal aggression, consisting of continued attacks by dominant individuals after they established their dominance, was linked to lower cortisol responses to a 30-minute confinement test in an unselected population of rainbow trout [6].

Proactive coping has been associated with high aggressiveness, low HPA/HPI axis activity/reactivity, and high sympathetic nervous system activity. In addition to the high aggressiveness that we showed previously [2], the fighter strain showed low HPI reactivity when faced with an unfamiliar environment. Interestingly, and not predicted by us, the fighter also immediately resorted to immobility when faced with confinement, which, as mentioned earlier, is a pattern of behavior that has been linked to proactive coping in salmonids. Both the fighter and wild strain showed a similar reduction in opercular beat rate during the latter stages of confinement. Moreover, the fighter’s cortisol response to
confinement was statistically indistinguishable from that of the significantly less aggressive and generally more anxious wild type fish. More contextually varied work with our non-invasive individualized assay will be necessary to delineate the glucocorticoid profiles of the fighter and wild *B. splendens*.

In our laboratory fighter males and females are easily manageable as they show little or no fear for handling or exposure to novelty. For all intent and purposes they are domesticated. In contrast, handling and changes to their captive environment easily frighten the wild fish, including the fish bred in our laboratory from wild-caught stock. It seems as if the fighter strain does not experience non-social stressors as fear inducing to the same extent as the wild fish do. Taken together, we suggest that the differences we observed between the two strains can be explained as relating to differences in coping style, with the fighter generally exhibiting a proactive coping style comparable to what has been observed in other teleosts [5].

One important difference between the two strains that we have not yet quantified and reported in a formal way is the style of aggression. In our laboratory, the wild fish show the highly ritualized territorial aggression that has been extensively described elsewhere [18], with extended periods of mutual flaring, and chases and bites commonly directed at less vulnerable body-parts, including fins and caudal muscular regions. In contrast, the quick to trigger aggression of the fighter is characterized by a relentless focus on the most vulnerable body parts of an opponent, especially the head area, including eyes and gills. Thai breeders claim to have selectively bred for this pattern of aggression for many years, and the informal observations in our laboratory bear witness to their efforts. We plan to formally document the abnormal aggression of the fighter soon.

Our newly developed measurement of cortisol in water allows us to further investigate important HPI axis differences between the two strains. However, at the moment this new method is time-consuming [12], and we will be working toward more expedient procedures. Less time-consuming procedures will allow us to test larger samples of wild and fighter males and expose them to a variety of repeated ecologically valid challenges [19]. Even fish selectively bred for high or low cortisol responses have been shown to exhibit different responses on subsequent stress sessions [20], illustrating the variability of the cortisol response and the need for repeated testing.

As sampling water-borne cortisol levels for individual fish is a new method potential problems in interpreting steroid concentrations remain to be overcome [12]. For example, in contrast to plasma steroid concentrations that provide a snapshot of a single point in time, water samples represent levels over a longer period [ibid.,] which limit their application. Moreover, there is evidence that in addition to releasing steroids into the water fish also take up steroids from water. It has therefore been suggested to report water-borne steroid levels as ‘apparent’ or ‘uncorrected’ [12], which suggested convention we have followed in this paper.

We have shown that experimental contexts matter when testing aggression in fighting fish. The fighter and wild males showed similar levels of aggression in response to their mirror image and a video recording of an aggressive conspecific male, but quite different levels of aggression when faced with an interactive conspecific male across a transparent divider [2]. A significant body of research on high and low responding rainbow trout lines as well as new research on sticklebacks shows that a careful consideration of the particular social and non-social experimental context is equally essential for valid measurements of endocrine responses to aggression and stress [5,21]. In sum, success in the work on coping styles and the related work on animal models of abnormal aggression depends on an integrated toolkit of carefully considered behavioral and physiological assays; one cannot properly function and offer insight without the other [8,19].

Finally, a growing body of research suggest a complex interplay between glucocorticoids and serotonin in the neural regulation of aggression [22]. Promising new research suggests a complex role for serotonin in the expression of aggression in *Betta splendens*. Acute treatment with the 5-HT1A receptor agonist 8-OHDPAT decreased aggressive behavior in males of a domesticated strain, while chronic treatment with the selective serotonin reuptake blocker fluoxetine caused no changes in aggression in these males [23]. Recent research in rats has shown that glucocorticoid-deficient rats that show abnormal aggression by preferentially targeting vulnerable body-parts of the opponent dramatically increased biting attacks following the administration of the 5-HT agonist buspirone. In contrast, buspirone dose-dependently decreased aggression in normal rats [24]. It will be important to compare and contrast manipulations of serotonergic activity in the fighter and wild type *B. splendens*.

Acknowledgements

We thank the editor and 3 anonymous reviewers for their constructive and insightful comments. We thank Mamiko Sakai for assistance with animal care. The research was supported, in part, by research funds from Miyazaki International College and the University of Miyazaki.

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