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Physiology & Behavior 91 (2007) 15 – 25

PHYSIOLOGY & BEHAVIOR

Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks

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Received 18 October 2006; received in revised form 17 January 2007; accepted 24 January 2007

Abstract

Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks. PHYSIOL BEHAV 00(0) 000-000, 2006. Here, we compare the behavioral, endocrine and neuroendocrine responses of individual sticklebacks exposed to either an unfamiliar conspecific or to a predator. We found that the two stressors elicited a similar hypothalamic–pituitary–interrenal response as assessed by whole-body concentrations of cortisol, but produced quite different patterns of change in brain monoamine and monoamine metabolite content as assessed by concentrations of serotonin (5-HT), dopamine (DA), norepinephrine (NE) and the monoamine metabolites 5-hydroxyindole acetic acid (5-HIAA), homovanillic acid (HVA) and 3-4-dihydroxyphenylacetic acid (DOPAC). For example, relative to baseline levels, NE levels were elevated in individuals exposed to a predator but were lower in individuals confronted by a challenging conspecific. Levels of monoamine neurotransmitters in specific regions of the brain showed extremely close links with behavioral characteristics. Frequency of attacking a conspecific and inspecting a predator were both positively correlated with concentrations of NE. However, whereas serotonin was negatively correlated with frequency of attacking a conspecific, it was positively associated with predator inspection. The data indicate that the qualitative and quantitative nature of the neuroendocrine stress response of sticklebacks varies according to the nature of the stressor, and that interindividual variation in behavioural responses to challenge are reflected by neuroendocrine differences.

Keywords: Stickleback; *Gasterosteus aculeatus*; Behavioral syndromes; Antipredator behavior; Aggression; Glucocorticoid; Serotonin; Stress; Coping styles; Individual differences

1. Introduction

Both attacking a conspecific and confronting a potential predator are dangerous. In addition to energetic costs [1], aggression can result in injury [2] and exposure to predation risk while fighting [3]. Similarly, an encounter with a potential predator can impose energetic costs of escape [4], injury [5] or even death. Not surprisingly, both confrontation by a challenging conspecific [6–11] and exposure to a predator [12–15] elicit a neuroendocrine stress response.

The neuroendocrine stress response involves a coordinated activation of both the hypothalamic–pituitary–adrenal (or interrenal, in the case of fishes, HPI) axis and the brain monoamine neurotransmitter systems [16]. When a stimulus evokes a stress response, both systems are activated by the same central mechanism, resulting in the elevation of plasma corticosteroids and brain monoaminergic activity. In general, exposure to stressors is associated with increased concentrations of plasma glucocorticoids and increased turnover of 5-HT to 5-hydroxyindoleacetic acid (5-HIAA) [17].

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0031-9384/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.physbeh.2007.01.012

Individual differences in behavior are often related to individual differences along both axes of the stress response [18–22]. With respect to the HPA axis, individual differences in aggressiveness are negatively correlated with concentrations of plasma glucocorticoids in trout [23] and chickens [24]. In humans, individual differences in behaviors that are analogous to risk-taking behaviors and aggression are associated with increased norepinephrine and dopamine activity [25,26]. Finally, aggression and risk-taking behaviors in several species have been linked to serotonin turnover. For example, individual differences in aggression are negatively related to serotonin turnover in monkeys [24,27–29], trout [8] and anolis lizards [30–32]. However, the relationship between 5-HT, stress, the HPI axis and aggression is complex and depends on the duration of the stressor. For example, in salmonids, 5-HT turnover is usually positively associated with plasma ACTH [33] and cortisol [8] concentrations and negatively associated with aggression. However, long-term stimulation of the serotonergic system has inhibitory (negative) effects on the HPI axis [34] and aggression [35,36].

In previous work, we have shown that behavioral reactions to predators and competing conspecifics covary at the individual level in threespined sticklebacks (*Gasterosteus aculeatus*) [37–39]. While some individuals are willing to engage in behavior that appears to be dangerous, such as foraging under predation risk or performing predator inspection, other individuals are much more cautious around predators. Individuals that take more risks in this context are also more aggressive toward conspecifics. Covariance among suites of behavioral traits is common [40,41] and in several species the shy-bold continuum and the proactive-reactive axis have been associated with individual differences in stress responsiveness [42]. Therefore it is possible that differences in how individual sticklebacks respond to dangerous situations might be linked with differences in the stress response.

Here, we investigated natural variation in behavioral, glucocorticoid and monoamine responses of individual sticklebacks to two potentially dangerous situations. We wished to establish whether wild-caught animals responding to ecologically-relevant challenges show stress responses that are comparable in nature and extent to those described for laboratory animals, and whether the stress response might be an underlying root of the covariance of behavioral responses in sticklebacks. With this in mind, we exposed individuals to either an unfamiliar conspecific or to a potential predator and recorded their behavior. Although the danger of predation is greater than the danger posed by a territorial intrusion, we hypothesized that both situations would induce a stress response because social stress is one of the most effective stressors in inducing a high magnitude response in other animals [43]. We sampled individuals at 15, 30 or 60 min after exposure to determine the time course of the glucocorticoid and monoaminergic responses to these two threats. This design allowed us not only to follow the neuroendocrine responses to these stressors through time, but also to determine whether individual differences in behavioral responses to these challenges could be related to underlying neuroendocrine physiology.

2. Methods

2.1. Overview

Individuals were presented with one of two potential threats, either an unfamiliar conspecific or a predator, hereafter referred to as ‘conspecific’ and ‘predator’, respectively, and their behavior was recorded. Individuals exposed to the ‘conspecific’ or the ‘predator’ were subdivided into three different treatment groups, sacrificed 15, 30 or 60 min after exposure to the potentially threatening stimulus. Individuals were randomly assigned to a treatment group prior to observing their behavior. The responses to the stressors were compared across time periods and against a ‘baseline control’ group, which consisted of individuals sampled directly from an undisturbed stock tank. Each treatment group comprised ten individuals.

Subadult sticklebacks were collected from the River Endrick in January 2004 and brought to the Glasgow University Field Station, Rowardennan, where all of the behavioral observations were carried out. Groups of fish ($n=10$–40) were maintained in flow-through stock tanks (210 L) at 9±2 °C and on a 14L:10D photoperiod. Fish were fed frozen bloodworms *ad libitum* daily except on the day of observation, when they were unfed.

Behavioral observations took place in March and April 2004 in a U-shaped flume with a live pike (*Esox lucius*) in either arm of the flume. Aquaria that were used for behavioral observation (‘observation tanks’, 44 L, 61×32×22 cm) were placed inside the flume and next to a window in the flume so that the behavior of the fish could be observed. The window was covered by a blind with a small opening which allowed the observer to see through the window with minimal disturbance to the fish. Each observation tank contained a one-liter glass conical flask, a plastic plant and a length of opaque tube (12 cm diameter, 36 cm tall) that stood vertically on one side of the tank and allowed fish to be introduced into the tank with a minimum of disturbance. Exterior lines on the tanks divided them into 16 equally-sized areas.

Each arm of the flume contained one of two live pike (46, 41 cm standard length) and cloth plants which served as hiding places for the pike. The compartments were fitted with a removable opaque cover which created a dark, shaded area for the pike. The pike were caught by hook and line in February 2004 in a small water body near the Glasgow University Field Station (the Duibh Lochan). The two pike were fed dead minnows and dead sticklebacks *ad libitum*.

2.2. Procedure

Fish were removed from the stock tank and placed into a settling tank (49 L, 61×31×26 cm) for two nights in order to acclimate to the flume. After the acclimation period, sticklebacks were netted from the settling tank and were randomly assigned to one of eight treatments (see below for a description of the different treatments). The stickleback was deposited into the tube in an observation tank. After 15 min, the tube was lifted, which allowed the stickleback to swim freely around the tank. After another 15 min, the fish was presented with either an

unfamiliar conspecific or a pike, and the behavioral observation began. Behavioral observations of response to an unfamiliar conspecific and predator were alternated.

2.3. Treatments

2.3.1. Unfamiliar conspecific

We employed a procedure that was designed to simulate a challenge to the resident fish by an intruding conspecific. Sticklebacks at this size and age (0.373±0.02 g, approximately 7–8 months of age) are not breeding and so do not defend breeding territories, but they do display aggressive behavior during competition for food and other resources and can be territorial [44]. Therefore we interpret the behavioural response of sticklebacks to the unfamiliar conspecific in this experiment as a response to a potential competitor for food and/or space. It is also worth considering that the sticklebacks' response to a conspecific might also reflect an affiliative motivation because they were held in isolation.

A live conspecific (within 5 mm standard length of the resident) was placed into the flask in the observation tank. Seven different conspecifics were used as intruders throughout the experiment. A fish was never used as an intruder more than once consecutively. The flask effectively standardized the behavior of the intruder by minimizing movement. The frequency of attacking the conspecific (biting) was recorded for 15 minutes after the resident first oriented to the conspecific because some individuals were facing away from the flask when the intruder was introduced. Latency to orient to the intruder ranged from 0.4–482.0 s (mean=104.6±24.7 s). This procedure is roughly analogous to studies with trout where a resident is challenged by an intruder [22]. However, an important difference is that in the present case there is no physical contact between the resident and intruder and the intruder cannot escape. We elected to use this procedure to minimize stress to the intruder. After the behavioral observation, the flask containing the conspecific was removed from the tank and the resident fish was sacrificed according to treatment (15 min, 30 min or 60 min after the behavioral observation was completed).

2.3.2. Predator

This procedure was designed to simulate a potential predatory threat by a live pike. We lured the pike into a chamber situated next to the observation tank by removing cover over the pike. In general, the pike willingly swam into the chamber, seeking cover. A removable opaque divider was situated between the observation aquarium and the predator chamber. To start the behavioural observation, the divider separating the observation aquarium from the chamber was gently lifted, allowing the stickleback a clear view of the pike on the other side of the glass. The behavior of the individual stickleback was observed for 15 min after the divider was removed and the following behaviors were recorded: predator inspection (swimming next to and orienting to the mouth of the pike) and time orienting (body facing toward the pike). Whether the pike moved or oriented to the stickleback during the observation was also recorded. After the behavioral observation, the opaque divider separating the chamber from the observation aquarium was replaced and the fish was sacrificed according to treatment (15 min, 30 min or 60 min after the behavioral observation completed). In order to eliminate any olfactory cues that might affect subsequent behavioral observations, the water in each of the observation tanks was replaced after each behavioral observation.

The two pike used in this study did not differ in behavior and movement of the pike during the observation period did not have a statistically detectable effect on either the behavior or the physiology of the sticklebacks (all $P>0.05$).

2.3.3. Baseline control

Each day, for ten days, a single stickleback was netted from a stock tank and sacrificed immediately to contribute to a baseline control value for neuroendocrine and hormonal measurements. These fish were collected at the same time as individuals in the treatment groups to minimize the amount of disturbance in the stock tank.

2.3.4. Settling tank control

At the end of each observation day, 1–2 remaining individuals in the 'settling tank' were quickly netted from the settling tank and sacrificed immediately. This group ($n=10$) was analyzed for corticosteroids to determine whether transfer and housing in the flume produced a stress response. However, it is important to note that this group does not control for the effect of isolation. We did not detect a difference in whole-body between the settling tank control and the baseline control and therefore did not analyze this treatment group further (Fig. 1, $F_{1,18}=0.488$, $P=0.494$).

2.4. Tissue collection

Fish were quickly killed by decapitation. The head and body were immediately weighed, the brain dissected out within three minutes and mounted in Tissue-Tek (Sakura). The

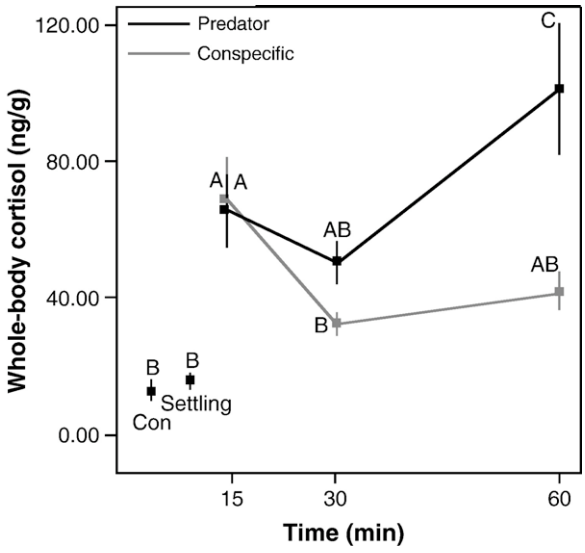

Fig. 1. Whole-body cortisol in the different treatments. Statistically similar means share the same letter.

brain and body were immediately frozen on dry ice and stored at −80 °C until physiological analyses. A small amount of tissue from the tail fin was placed in 80% ethanol for DNA extraction for sex determination. Tissue was collected between 0800 and 1800 h. As in [45], we found no evidence for circadian changes in whole-body cortisol ($r=0.045$, $F_{1,58}=0.118$, $P=0.773$).

2.5. Steroid determination

Corticosteroids were assessed by measurement of solvent-extractable immunoreactivity in whole-body homogenates. Corticosteroids were extracted from the tissue by homogenization in ethyl acetate (5:1 volume:carcass weight). Recovery of steroids from homogenized tissue was assessed by adding 50 µl radio-labelled cortisol tracer to homogenized tissue and equilibrating for one hour before extractions. Immunoreactive steroids were quantified in 20–100 µl aliquots of ethyl acetate extracts of whole-body homogenates using a validated cortisol radioimmunoassay procedure as described previously [46–49]. We used the rabbit polyclonal antibody to cortisol produced by the IgG Corporation and supplied by Campro Scientific (code IgG-F-2). A standard curve of 0–800 pg cortisol per tube was used.

We quantified cortisol in whole-body homogenates rather than plasma because successful extraction of the brain for monoamine analyses required that it be dissected out and frozen as soon as possible, which precluded rapid blood sampling from the body. The whole-body homogenate method measures cortisol in multiple body compartments. Therefore in addition to measuring plasma concentrations of cortisol, this method also detects cortisol derivatives in the liver and gall bladder that might have cross-reacted with the antibody [50], This does not detract from the ability of this method to detect the onset of a stress response, because corticosteroids are synthesized de novo and not stored prior to release. This method has been employed previously to monitor the stress response in fish from which, because of their small size, blood samples could not be obtained, including juvenile trout [51], zebra fish [52] and sticklebacks [49]. Simultaneous measurement of plasma cortisol and whole-body cortisol in fish exposed to acute and chronic stressors has confirmed that the method is appropriate for detecting stress-induced changes in HPI activity [51]. Hereafter we refer to concentrations of corticosteroids we measured on whole body preps as ng/g of 'whole-body cortisol'.

2.6. Analysis of brain monoamines

Brains were sectioned in a frozen state on a cryostat and mounted on glass slides. Sections of 300 µm thickness were cut in the coronal plane. Brain-punch microdissection was performed as described by [30]. The hypothalamus, telencephalon and region posterior to the hypothalamus ('reticular formation') were identified for punching.

Punches from each of these three regions were collected and homogenized in 50 µl ice-cold 4% perchloric acid containing 40 ng/ml DHBA (dihydroxybenzamine) as internal standard, using an MSE 100-W ultrasonic disintegrator. Samples were then centrifuged at 13000 rpm for 10 min at 4 °C and the supernatants were analyzed for serotonin (5-HT), dopamine (DA) and norepinephrine (NE) and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using high performance liquid chromatography with electrochemical detection [53] immediately, or stored at −80 °C for no more than two days prior to analysis. Pellets were stored at −80 °C for subsequent analysis of protein content in an Eppendorf Biophotometer by a pre-made program measuring absorbance at 280 nm. The monoamines and monoamine metabolites were quantified using standard solutions and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd., the Czech republic). The concentration of monoamines and monoamine metabolites is expressed as ng per mg protein.

We did not detect strong differences between brain regions in concentrations of brain monoamines: the only effect that we detected was that levels of DA ($F_{2,81}=3.36$, $P=0.04$), 5-HIAA ($F_{2,81}=4.57$, $P=0.013$) and 5-HT ($F_{2,81}=5.21$, $P=0.007$) were significantly lower in the reticular formation in the 'predator' treatment (Table 1). Therefore we summed the concentration of each monoamine across regions and focused our subsequent analysis of treatment differences on the whole-brain values. However, the failure to detect strong region-specific differences should not be overinterpreted because we did not have the resolution to detect fine-scale differences. Other studies have found region-specific differences in monoamine turnover during aggression [32].

A decrease in the concentration of a monoamine neurotransmitter could reflect a reduction in the release of the neurotransmitter (decrease in activity) or an increase in turnover to its metabolite (increase in activity). Therefore, it is preferable to use the ratio of the parent neurotransmitter to its metabolite (5-HIAA:5-HT, DOPAC:DA AND HVA:DA) as an index of neurotransmitter activity. However, we were unable to quantify the NE metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) in any of the samples as a consequence of non-identified interfering peaks. In addition, in some samples the monoamines (especially 5-HIAA and 5-HT) became degraded during the sampling procedure, resulting in our failure to detect 5-HIAA. This was particularly a problem for the 'conspecific' treatments (Table 1). Samples with undetectable levels of a monoamine were omitted from that analysis.

Here, we report data on the concentration of both the parent monoamine and metabolite, and we focus our interpretation on differences between treatment groups, rather than on the functional significance of absolute levels.

2.7. Determining genetic sex

DNA was extracted from each fin clip and genetic sex was determined by genotyping each individual for a male-specific genetic marker validated for sticklebacks [54].

Table 1
Concentrations (ng/mg protein) of monoamines in the different brain regions for the different treatments

	NE	DOPAC	5-HIAA	DA	HVA	5-HT
Hypothalamus						
Control	10.48±12.91(10)	4.04±3.76(6)	3.16±.737(4)	2.27±1.57(10)	0.84±0.50(10)	5.83±4.32(10)
Conspecific						
15 min	1.16±0.59(7)	3.60±4.71(3)	und	3.16±1.82(8)	0.78±.55(5)	und
30 min	0.84±0.83(6)	3.03±3.40(3)	und	2.04±1.91(5)	1.00±1.13(6)	2.27±2.54(3)
60 min	1.44±2.47(8)	2.65±3.19(3)	und	4.43±5.01(4)	2.68±3.38(7)	4.26±4.56(8)
Predator						
15 min	25.57±15.63(10)	2.62±.94(6)	3.96±1.77(9)	4.11±4.07(10)	1.13±1.34(10)	4.67±2.48(10)
30 min	21.36±10.53(8)	4.24±.56(5)	2.07±0.74(8)	2.92±3.09(8)	0.21±0.14(7)	4.81±3.45(8)
60 min	27.86±8.21(8)	1.07±1.07(4)	3.33±1.56(8)	3.36±2.73(8)	0.58±0.41(8)	3.75±3.44(8)
Reticular formation						
Control	9.04±11.20(10)	6.10±2.73(6)	2.57±1.41(4)	1.84±1.08(9)	0.41±0.17(10)	3.19±2.45(10)
Conspecific						
15 min	1.32±.84(6)	2.54±2.47(8)	und	3.27±1.58(6)	0.84±.49(5)	20.30±0(1)
30 min	0.83±.56(6)	4.38±3.24(5)	und	1.98±0.80(7)	0.95±0.53(7)	1.80±1.54(3)
60 min	1.08±0.71(8)	4.49±4.18(7)	und	1.57±1.21(4)	0.87±0.69(8)	2.07±2.36(8)
Predator						
15 min	21.51±10.78(10)	und	2.06±.79(8)	2.26±1.04(10)	1.45±1.90(10)	2.11±1.37(10)
30 min	15.53±6.40(8)	und	1.46±.66(8)	2.18±2.14(8)	0.15±0.14(7)	2.10±1.83(8)
60 min	19.88±8.26(9)	und	1.98±.70(8)	1.32±1.23(9)	0.69±.96(8)	2.58±1.45(8)
Telencephalon						
Control	11.44±14.59(10)	3.60±1.80(6)	3.43±1.59(5)	2.63±1.29(10)	0.84±0.49(10)	6.36±4.46(10)
Conspecific						
15 min	1.36±0.68(8)	2.14±2.23(8)	14.39±19.61(2)	2.11±0.72(8)	0.64±.82(3)	und
30 min	7.60±16.94(8)	3.41±2.74(6)	2.12±0(1)	1.31±1.13(6)	0.75±0.53(8)	5.15±2.10(2)
60 min	1.54±2.17(8)	6.48±2.88(6)	1.24±0(1)	1.68±2.37(4)	0.45±0.71(8)	1.92±3.56(10)
Predator						
15 min	24.18±12.70(10)	53.64±0(1)	3.01±2.40(9)	4.71±3.61(10)	2.20±2.59(10)	4.75±3.42(9)
30 min	21.07±14.45(8)	und	2.53±2.79(8)	2.20±1.41(8)	0.42±0.26(8)	3.52±3.93(8)
60 min	29.24±14.30(10)	0.37±0(1)	4.04±2.38(9)	3.87±2.77(10)	0.86±0.71(10)	6.59±4.18(9)

Statistics are presented as mean±SD. Sample sizes are in parentheses. und=undetectable.

2.8. Data analysis

We compared the behavioral and physiological responses of sticklebacks to an unfamiliar conspecific and a predator across time using general linear models except when data were non-normal. We tested for the effects of sex, body size, time and treatment on each of the dependent variables (behavior, whole-body cortisol and brain monoamines in the different regions). We did not detect sex differences in behavior, whole-body cortisol or brain monoamines and therefore did not analyze this factor further (all $P>0.4$). The least-squares difference post-hoc test was used to test for differences between groups, except when the distribution was non-normal, in which case we tested for differences between treatments using the nonparametric Mann-Whitney U test.

Pearson correlations were used to test for statistically significant relationships between variables when the data were normally distributed; otherwise, Spearman rank correlation statistics were computed. Because the same behavioral data was used to test for associations with brain monoamine concentrations, we used the sequential Bonferroni procedure to correct for multiple tests. Briefly, for each brain region within a treatment group, we replaced the correlation statistics with their corresponding P-values and then ranked them from smallest to largest. Results that were significant ($P<0.05$) after the sequential Bonferroni procedure are reported [55]. All tests were two-tailed.

All of the procedures were carried out according to institutional guidelines and in accordance with the U.K. Animals (Scientific Procedures) Act of 1986.

3. Results

3.1. Behavioural and physiological responses to an unfamiliar conspecific

Presentation of an unfamiliar conspecific elicited a behavioral response; on average, individuals approached the intruder 8 times and attacked 11 times within the observation period. However, individuals differed in their behavioral reaction to the simulated intrusion; while one individual attacked the conspecific over 40 times, other individuals spent most of their time hiding, and scarcely left the refuge. Body size explained some of this individual variation; bigger fish were more aggressive toward their size-matched opponents (number of attacks: $r=0.433$, $P=0.024$, $n=27$). All of the fish oriented to and approached the conspecific and one-half of the fish attacked it at least once.

Interaction with the unfamiliar conspecific quickly produced a glucocorticoid response (Fig. 1). Whole-body cortisol levels

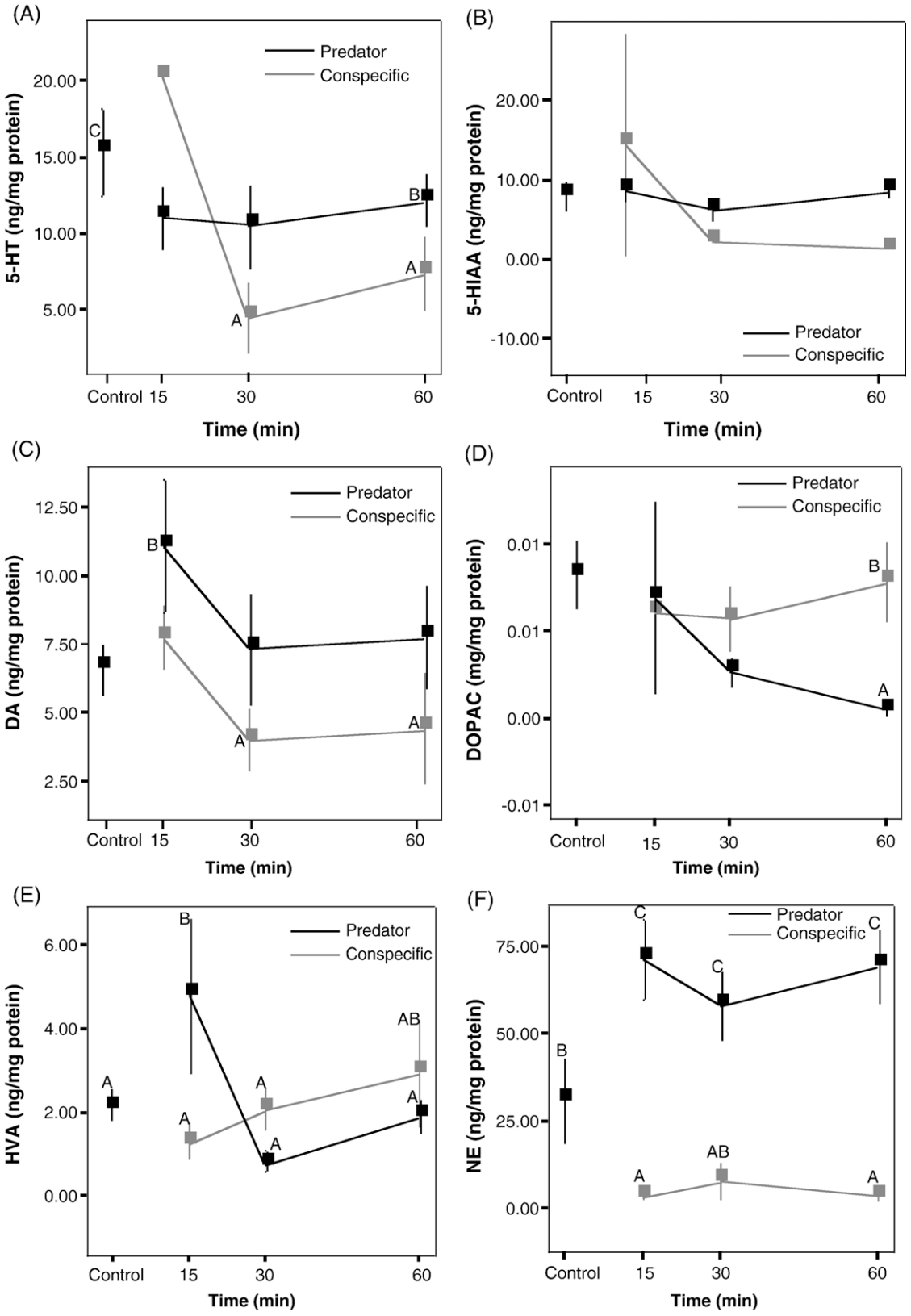

Fig. 2. Whole-brain concentrations of brain monoamines in different treatments. Statistically similar means share the same letter. (A) 5-HT; (B) 5-HIAA; (C) DA; (D) DOPAC; (E) HVA; (F) NE.

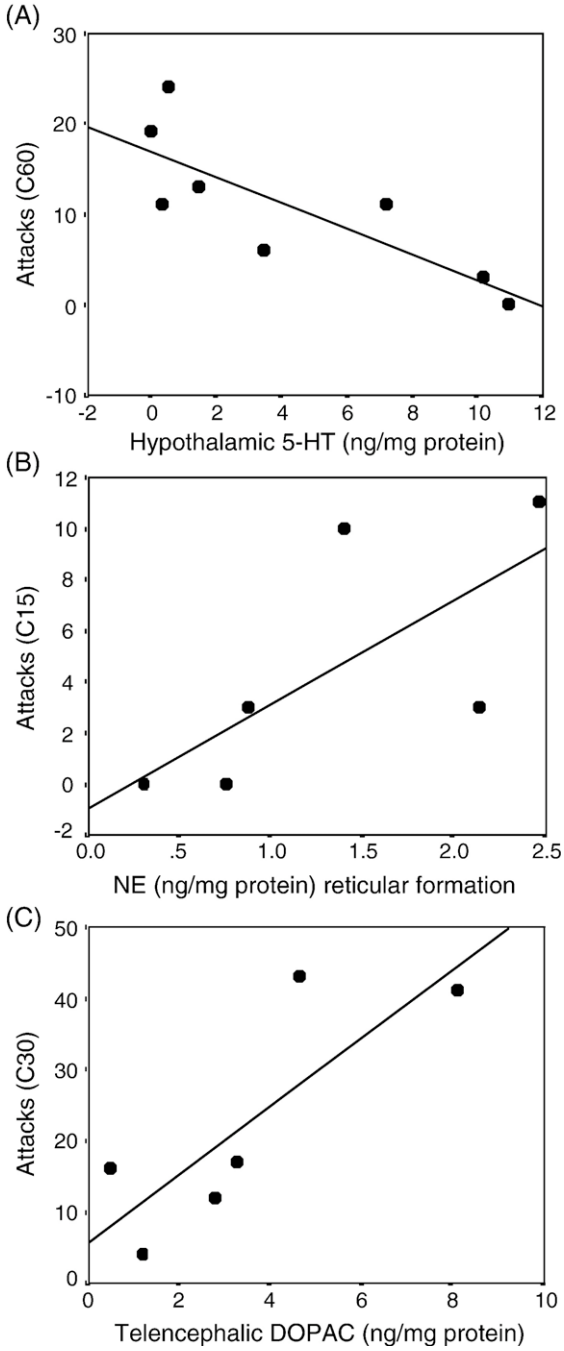

Fig. 3. Correlations between monoamine concentrations and aggressive behavior (attacks). (A) Hypothalamic 5-HT 60 min after a fight; (B) NE in reticular formation 15 min after a fight; (C) Telencephalic DOPAC 30 min after a fight.

were highest 15 min after the simulated intrusion and then returned to baseline levels by 30 min.

The serotonergic system was quickly suppressed in response to the presence of the unfamiliar conspecific, as indicated by reduced whole-brain levels of 5-HT (Fig. 2A, Table 1).

Dopamine turnover to DOPAC was elevated 60 min following the aggressive interaction (Fig. 2C and D), while levels of norepinephrine were consistently low (Fig. 2F).

Individual differences in concentrations of brain monoamines were related to differences among individuals in aggressiveness. Individuals with lower hypothalamic 5HT were more aggressive ($r=-0.806$, $P=0.016$, $n=8$, Fig. 3A), while norepinephrine ($r=0.883$, $P=0.020$, $n=6$, Fig. 3B) and DOPAC ($r=0.815$, $P=0.048$, $n=6$, Fig. 3C) were positively associated with aggressiveness.

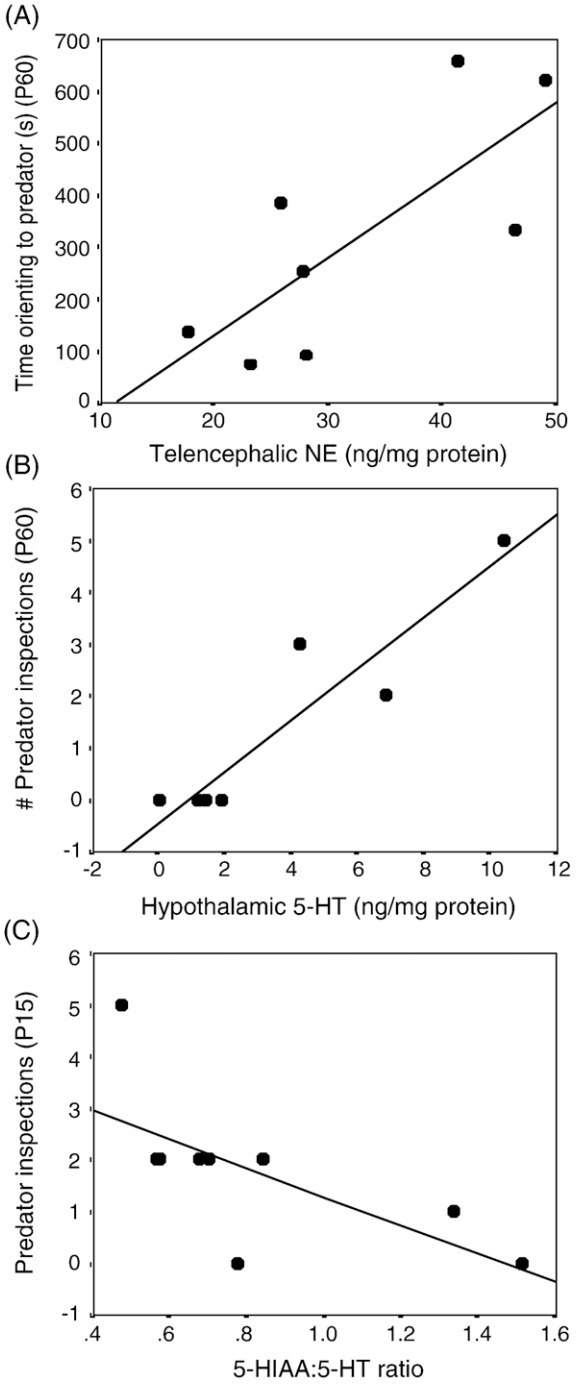

Fig. 4. Correlations between monoamine concentrations and behavior under predation risk. (A) Telencephalic NE 60 min after exposure and time orienting to the predator; (B) Hypothalamic 5-HT 60 min after exposure and predator inspections; (C) Whole-brain 5-HIAA:5-HT ratio 15 min after exposure and predator inspections.

3.2. Behavioural and physiological responses to a predator

When presented with the pike, most individuals inspected the predator at least once and oriented to it more than nine times. As in the 'conspecific' treatment, individuals differed in their behavior: some individuals inspected the pike as many as seven times during the 15-min observation period, while others spent the entire observation period hiding in the refuge.

Exposure to the predator elicited a significant glucocorticoid response within 15 min which reached a maximum 60 min after exposure to the predator (Fig. 1). Concentrations of DOPAC fell at 60 min (Fig. 2D) while concentrations of HVA increased at 15 min (Fig. 2E), indicating that predator-induced stress stimulated the rapid turnover of DA to HVA.

Activity under predation risk and predator inspection behavior (both of which potentially involve a risk of predation) were positively associated with neurotransmitter concentrations. For example, individuals with greater levels of NE engaged in riskier behavior ($r=0.766$, $P=0.027$, $n=8$, Fig. 4A). Serotonin turnover was also associated with predator inspection behavior: the number of predator inspections was significantly positively correlated with hypothalamic serotonin ($r=0.928$, $P=0.003$, $n=7$, Fig. 4B) and negatively correlated with whole-brain serotonergic activity ($r=-0.669$, $P=0.049$, $n=9$, Fig. 4C).

3.3. Comparing responses to the conspecific and predator

Both confrontation by a conspecific and exposure to a predator elicited a cortisol response, but the time course of the cortisol response differed between treatments (Fig. 1), as evidenced by the significant interaction between time and treatment ($F_{2,58}=5.5$, $P=0.006$). Moreover, the magnitude (average across the three time periods) of the cortisol response was greater to the predator compared to a conspecific (Conspecific: 47 ± -4.97 ng/g, Predator: 72 ± 8.24 ng/g, $P=0.002$).

Relative to the conspecific treatment, NE (Fig. 2F) and to a lesser extent, DA (Fig. 2C) were higher in the predator treatments.

4. Discussion

In this experiment, we tested the hypothesis that both the HPI axis and brain monoaminergic systems are activated in response to fighting with an unfamiliar conspecific and exposure to a predator. While other studies have found links between these systems in laboratory animals, the results from this study extend these findings to wild-caught animals that were confronted by ecologically relevant challenges [28,56]. We found that both stressors elicited a similar HPI response, but produced very different patterns of change in monoamine content.

Our design permitted us to determine the time course of the neuroendocrine response to these stressors and to ascertain whether individual differences in behavioral responses to the stressors were related to underlying physiology. We showed that not only do these challenges elicit a neuroendocrine response, but that different behavioral responses of individuals were related to their particular neuroendocrine profiles.

4.1. The cortisol response to a conspecific and predator were broadly similar, but exposure to a predator was more stressful

During the present study, both confrontation with an unfamiliar conspecific and exposure to a predator resulted in activation of the HPI axis and significant alterations in the levels of brain monoamines in sticklebacks. These results are consistent with other studies which have shown that both confrontation by a challenging conspecific [10,23] and exposure to a predator [57] elicit a neuroendocrine stress response in fishes.

In the present study both exposure to a conspecific or to a predator resulted in highly significant increases in whole-body cortisol concentrations within 15 min relative to controls. In the conspecific-exposed group, whole-body cortisol levels were statistically indistinguishable from control fish after 30 min and remained so at 60 min. In contrast, whole-body cortisol concentrations in the predator-exposed group remained highly elevated after 60 min, significantly exceeding levels attained after 15 min. We interpret these data to indicate that the magnitude of the initial response to both stressors was similar, resulting in similar whole-body cortisol concentrations at 15 min, but that the HPI axis in the predator-exposed fish remained active for longer, resulting in a greater accumulation of whole-body cortisol with time. The overall significant difference in total cortisol between the two treatment groups detected across all time points indicates a quantitative difference in the response of the fish to the two stressors.

Other studies have found evidence for a more rapid recovery to baseline cortisol levels following less threatening situations compared to more threatening situations [58]. A longer-lasting cortisol response to threat of predation as compared to other stressors has been documented in stonechats [59] and rodents [60,61]. Therefore in this experiment, we hypothesize that the different time course of the cortisol response to a competitor versus to a predator is related to the perceived magnitude of the two different challenges. Sticklebacks are social fish, and frequently interact with other sticklebacks in shoals. Because encounters with conspecifics are frequent, natural selection might have favored individuals which do not mount a severe stress response to frequent interactions with conspecifics, and should favor individuals which recover quickly from fights. In contrast, encounters with predators are less frequent and more threatening than encounters with conspecifics, so selection might have favored individuals with a greater and longer-lasting stress response.

The levels of whole-body cortisol detected in unstressed sticklebacks during the present study were similar to those previously reported for this species (2–8 ng g^{-1}; [49]) and levels detected in the stressed fish in the present study, although slightly higher, were also broadly consistent with previous observations (50 ng g^{-1};[49]). The difference in magnitude of whole-body cortisol levels between this and previous studies may be related to the nature of the stressor.

Links between stress-induced blood cortisol levels and behavioral traits have been shown in fish [10,23], mammals [62] and reptiles [9]. However, while exposure to both stressors elicited a behavioral and whole-body cortisol response in the treatment groups, we did not detect a relationship at the individual level between concentrations of whole-body cortisol and behavior. It is possible that our method might not have had the resolution to detect fine-scale individual differences.

We did not detect any sex differences in whole-body cortisol. The stress response in vertebrates, including fish [63], is modulated by gonadal steroids with androgens suppressing and estrogens enhancing corticosteroid responsiveness [64]. However, the fish employed in this study were not reproductively active and it is therefore unsurprising that no sex-dependent differences in stress response were observed.

4.2. The monoamine responses to a conspecific and a predator were qualitatively different

Whereas the cortisol response was broadly similar across stressors, the monoamines showed a differential response across the two stressors, some being suppressed in response to a conspecific but elevated in response to the predator.

For example, relative to the control group, concentrations of NE were consistently *higher* in the 'predator' treatments, and *lower* in the 'conspecific' treatments. Without data on the NE metabolite, MHPG, we cannot distinguish if reduced concentrations reflect a reduction in NE release (decrease in NE activity) or an increased turnover to MHPG (increase in NE activity). However, at an individual level we found that NE was consistently associated with risk-taking behaviors in both kinds of situations: NE was positively correlated with aggressive behaviors as well as predator inspection behaviors. These positive correlations suggest that more bold or aggressive individuals were more 'aroused', active or uninhibited, results which are consistent with other studies showing positive relationships between NE activity and behavioral impulsivity in monkeys [28] and sensation seeking in humans [65]. The fact that serotonin and NE had opposite relationships with risk-taking behaviors in this experiment is consistent with the observation that 5-HT and catecholamines can have antagonistic effects on behavior [33,36].

4.3. Associations between serotonin, risk-taking behaviors and aggression

In agreement with other studies which have shown that risk-taking behaviors are negatively associated with brain serotonergic activity [24,27–29], we found that risk-taking behaviors performed while under predation risk (e.g. inspection) were negatively correlated with serotonin turnover to 5-HIAA (Fig. 4C).

Our results support the view that 5-HT has an inhibitory effect on aggressive behavior [36]. We found a negative relationship at the individual level between concentrations of 5-HT and aggressive behavior, and that confrontation by an unfamiliar conspecific resulted in lower 5-HT. Other studies have shown that winners of agonistic interactions have up-regulated brain 5-HT activity [30–32,53]. One possible explanation for this different pattern is that in our experiment, there was no physical contact between the resident and the intruder because the intruders were confined to a flask. As a result, the resident fish were unable to complete their attacks and therefore might not be analogous to the winners in the forementioned studies. We remain provisional in our interpretation of these results because 5-HIAA was degraded in many of the samples in the 'conspecific' treatments, preventing us from calculating serotonin turnover in those treatments. However, it is worth noting that while more aggressive behaviors were negatively associated with serotonin (Fig. 3A), risk-taking behavior under predation risk showed the opposite pattern — it was *positively* correlated with 5HT (Fig. 4B), and negatively associated with serotonin turnover to 5-HIAA (Fig. 4C).

Overall, these data provide evidence that the response of fish to stressors is not identical regardless of the nature of the challenge, but rather that the response varies according to the magnitude, frequency and predictability of the stressor, as is the case for other vertebrates [59,66]. Further studies on individual variation in responses to different stressors would benefit from repeated sampling of the same physiological measures on the same individuals. While it is currently a challenge to measure brain monoamines noninvasively, noninvasive methods for measuring glucocorticoids in fish [67] are a promising alternative. In addition, the roles played by upstream elements of the stress response such as corticotropin releasing hormone (CRH) and variation in the binding characteristics of corticosteroid receptors and corticotropin binding proteins should also be investigated [68]. Given that other studies have shown that inter-individual differences in stress responsiveness have a high heritable component [69], further investigation will provide insight into the mechanisms that have produced adaptive, heritable behavioral variation in sticklebacks in diverse ecological settings.

Acknowledgements

We thank Stuart Wilson, David Alvarez, Susie Coyle for help with the fish, Andy Young for catching the pike, Per-Ove Thörnqvist and Joachim Schjolden for technical assistance with HPLC and brain punches, Kim Pulman for technical help with the cortisol RIA. Funding was provided by an NSF International Postdoctoral Research Fellowship to AMB. AMB was supported by a fellowship from the American Association of University Women during preparation of the manuscript. Work in the Winberg lab was supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

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