

1 **Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks**

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Alison M. Bell\*

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Integrative Biology, University of Illinois at Urbana-Champaign

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505 South Goodwin Ave., Urbana, IL 61801, USA

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Phone: (217) 265-5469, Fax: (217) 244-4565, Email: [alisonmb@life.uiuc.edu](mailto:alisonmb@life.uiuc.edu)

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12

Tobias Backström

13

Evolutionary Biology Centre, Dep't of Comparative Physiology, Uppsala University, Sweden

14

15

Felicity A. Huntingford

16

Environmental and Evolutionary Biology, University of Glasgow, UK

17

18

Tom G. Pottinger

19

Centre for Ecology and Hydrology, Lancaster, UK

20

21

Svante Winberg

22

Basic Science and Aquatic Medicine, Norwegian School of Veterinary Sciences, Norway and

23

Evolutionary Biology Centre, Dep't of Comparative Physiology, Uppsala University, Sweden

24 ABSTRACT

25 BELL, A.M., BACKSTRÖM, T.B., HUNTINGFORD, F.A., POTTINGER, T.P., WINBERG, S.  
26 Variable neuroendocrine responses to ecologically-relevant challenges in sticklebacks.  
27 *PHYSIOL BEHAV* 00(0) 000-000, 2006. Here, we compare the behavioral, endocrine and  
28 neuroendocrine responses of individual sticklebacks exposed to either an unfamiliar conspecific  
29 or to a predator. We found that the two stressors elicited a similar hypothalamic-pituitary-  
30 interrenal response as assessed by whole-body concentrations of immunoreactive corticosteroids,  
31 but produced quite different patterns of change in brain monoamine and monoamine metabolite  
32 content as assessed by concentrations of serotonin (5-HT), dopamine (DA), norepinephrine (NE)  
33 and the monoamine metabolites 5-hydroxyindole acetic acid (5-HIAA), homovanillic acid  
34 (HVA) and 3-4-dihydroxyphenylacetic acid (DOPAC). For example, relative to baseline levels,  
35 NE levels were elevated in individuals exposed to a predator but were lower in individuals  
36 confronted by a challenging conspecific. Levels of monoamine neurotransmitters in specific  
37 regions of the brain showed extremely close links with behavioral characteristics. Frequency of  
38 attacking a conspecific and inspecting a predator were both positively correlated with  
39 concentrations of NE. However, whereas serotonin was negatively correlated with frequency of  
40 attacking a conspecific, it was positively associated with predator inspection. The data indicate  
41 that the qualitative and quantitative nature of the neuroendocrine stress response of sticklebacks  
42 varies according to the nature of the stressor, and that interindividual variation in behavioural  
43 responses to challenge are reflected by neuroendocrine differences.

44 **Key words:** stickleback, *Gasterosteus aculeatus*, behavioral syndromes, antipredator  
45 behavior, aggression, glucocorticoid, serotonin, stress, coping styles, individual differences

46 **Running head:** Individual differences in sticklebacks

47 INTRODUCTION

48

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

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

61 Individual differences in behavior are often related to individual differences along both  
62 axes of the stress response [18-22]. With respect to the HPA axis, individual differences in  
63 aggressiveness are negatively correlated with concentrations of plasma glucocorticoids in trout  
64 [23] and chickens [24]. In humans, individual differences in behaviors that are analogous to risk-  
65 taking behaviors and aggression are associated with increased norepinephrine and dopamine  
66 activity [25,26]. Finally, aggression and risk-taking behaviors in several species have been  
67 linked to serotonin turnover. For example, individual differences in aggression are negatively  
68 related to serotonin turnover in monkeys [24,27-29], trout [21] and anolis lizards [30-32].  
69 However, the relationship between 5-HT, stress, the HPI axis and aggression is complex and

70 depends on the duration of the stressor. For example, in salmonids, 5-HT turnover is usually  
71 positively associated with plasma ACTH and cortisol concentrations and negatively associated  
72 with aggression. However, long-term stimulation of the serotonergic system has inhibitory  
73 (negative) effects on the HPI axis [33] and aggression [17].

74 In previous work, we have shown that behavioral reactions to predators and competing  
75 conspecifics covary at the individual level in threespined sticklebacks (*Gasterosteus aculeatus*)  
76 [34-36]. While some individuals are willing to engage in behavior that appears to be dangerous,  
77 such as foraging under predation risk or performing predator inspection, other individuals are  
78 much more cautious around predators. Individuals that take more risks in this context are also  
79 more aggressive toward conspecifics. Covariance among suites of behavioral traits is common  
80 [37,38] and in several species the shy-bold continuum and the proactive-reactive axis have been  
81 associated with individual differences in stress responsiveness [39]. Therefore it is possible that  
82 differences in how individual sticklebacks respond to dangerous situations might be linked with  
83 differences in the stress response.

84 Here, we investigated natural variation in behavioral, glucocorticoid and monoamine  
85 responses of individual sticklebacks to two potentially dangerous situations. We wished to  
86 establish whether wild-caught animals responding to ecologically-relevant challenges show  
87 stress responses that are comparable in nature and extent to those described for laboratory  
88 animals, and whether the stress response might be an underlying root of the covariance of  
89 behavioral responses in sticklebacks. With this in mind, we exposed individuals to either an  
90 unfamiliar conspecific or to a potential predator and recorded their behavior. Although the  
91 danger of predation is greater than the danger posed by a territorial intrusion, we hypothesized  
92 that both situations would induce a stress response because social stress is one of the most

93 effective stressors in inducing a high magnitude response in other animals [40]. We sampled  
94 individuals at 15, 30 or 60 minutes after exposure to determine the time course of the  
95 glucocorticoid and monoaminergic responses to these two threats. This design allowed us not  
96 only to follow the neuroendocrine responses to these stressors through time, but also to  
97 determine whether individual differences in behavioral responses to these challenges could be  
98 related to underlying neuroendocrine physiology.

99

## 100 **METHODS**

101

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

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

116 Behavioral observations took place in March and April 2004 in a U-shaped flume with a  
117 live pike (*Esox lucius*) in either arm of the flume. Aquaria that were used for behavioral  
118 observation ('observation tanks', 44 liters, 61x32x22 cm) were placed inside the flume and next  
119 to a window in the flume so that the behavior of the fish could be observed. The window was  
120 covered by a blind with a small opening which allowed the observer to see through the window  
121 with minimal disturbance to the fish. Each observation tank contained a one-liter glass conical  
122 flask, a plastic plant and a length of opaque tube (12 cm diameter, 36 cm tall) that stood  
123 vertically on one side of the tank and allowed fish to be introduced into the tank with a minimum  
124 of disturbance. Exterior lines on the tanks divided them into 16 equally-sized areas.

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

130

131 Procedure:

132 Fish were removed from the stock tank and placed into a settling tank (49 liters,  
133 61x31x26 cm) for two nights in order to acclimate to the flume. After the acclimation period,  
134 sticklebacks were netted from the settling tank and were randomly assigned to one of eight  
135 treatments (see below for a description of the different treatments). The stickleback was  
136 deposited into the tube in an observation tank. After 15 minutes, the tube was lifted, which  
137 allowed the stickleback to swim freely around the tank. After another 15 minutes, the fish was

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

140

141 Treatments:

142       **Unfamiliar conspecific:** We employed a procedure that was designed to simulate a  
143 challenge to the resident fish by an intruding conspecific. Sticklebacks at this size and age (0.373  
144  $\pm$  0.02 g, approximately 7-8 months of age) are not breeding and so do not defend breeding  
145 territories, but they do display aggressive behavior during competition for food and other  
146 resources and can be territorial [41]. Therefore we interpret the behavioural response of  
147 sticklebacks to the unfamiliar conspecific in this experiment as a response to a potential  
148 competitor for food and/or space. It is also worth considering that the sticklebacks' response to a  
149 conspecific might also reflect an affiliative motivation because they were held in isolation.

150       A live conspecific (within 5mm standard length of the resident) was placed into the flask  
151 in the observation tank. Seven different conspecifics were used as intruders throughout the  
152 experiment. A fish was never used as an intruder more than once consecutively. The flask  
153 effectively standardized the behavior of the intruder by minimizing movement. The frequency of  
154 attacking the conspecific (biting) was recorded for 15 minutes after the resident first oriented to  
155 the conspecific because some individuals were facing away from the flask when the intruder was  
156 introduced. Latency to orient to the intruder ranged from 0.4-482.0 seconds (mean=104.6  $\pm$  24.7  
157 s). This procedure is roughly analogous to studies with trout where a resident is challenged by an  
158 intruder [23]. However, an important difference is that in the present case there is no physical  
159 contact between the resident and intruder and the intruder cannot escape. We elected to use this  
160 procedure to minimize stress to the intruder. After the behavioral observation, the flask

161 containing the conspecific was removed from the tank and the resident fish was sacrificed  
162 according to treatment (15 minutes, 30 minutes or 60 minutes after the behavioral observation  
163 was completed).

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

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

182         **Baseline control:** Each day, for ten days, a single stickleback was netted from a stock  
183 tank and sacrificed immediately to contribute to a baseline control value for neuroendocrine and



184 hormonal measurements. These fish were collected at the same time as individuals in the  
185 treatment groups to minimize the amount of disturbance in the stock tank.

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

193

#### 194 Tissue collection

195 Fish were quickly killed by decapitation. The head and body were immediately weighed,  
196 the brain dissected out within three minutes and mounted in Tissue-Tek (Sakura). The brain and  
197 body were immediately frozen on dry ice and stored at  $-80^{\circ}$  C until physiological analyses. A  
198 small amount of tissue from the tail fin was placed in 80% ethanol for DNA extraction for sex  
199 determination. Tissue was collected between 0800 and 1800 hours. As in [42], we found no  
200 evidence for circadian changes in whole-body cortisol ( $r=0.045$ ,  $F_{1,58}=0.118$ ,  $P=0.773$ ).

201

#### 202 Steroid determination

203 Corticosteroids were assessed by measurement of solvent-extractable immunoreactivity in  
204 whole-body homogenates. Corticosteroids were extracted from the tissue by homogenization in  
205 ethyl acetate (5:1 volume:carcass weight). Recovery of steroids from homogenized tissue was  
206 assessed by adding 50 $\mu$ l radio-labelled cortisol tracer to homogenized tissue and equilibrating for

207 one hour before extractions. Immunoreactive steroids were quantified in 20-100  $\mu$ l aliquots of  
208 ethyl acetate extracts of whole-body homogenates using a validated cortisol radioimmunoassay  
209 procedure as described previously [43-46]. We used the rabbit polyclonal antibody to cortisol  
210 produced by the IgG Corporation and supplied by Campro Scientific (code IgG-F-2).  
211 A standard curve of 0-800 pg cortisol per tube was used.

212 We quantified cortisol in whole-body homogenates rather than plasma because successful  
213 extraction of the brain for monoamine analyses required that it be dissected out and frozen as  
214 soon as possible, which precluded rapid blood sampling from the body. The whole-body  
215 homogenate method measures cortisol in multiple body compartments. Therefore in addition to  
216 measuring plasma concentrations of cortisol, this method also detects cortisol derivatives in the  
217 liver and gall bladder that might have cross-reacted with the antibody [47]. This does not detract  
218 from the ability of this method to detect the onset of a stress response, because corticosteroids  
219 are synthesized de novo and not stored prior to release. This method has been employed  
220 previously to monitor the stress response in fish from which, because of their small size, blood  
221 samples could not be obtained, including juvenile trout [48], zebra fish [49] and sticklebacks  
222 [46]. Simultaneous measurement of plasma cortisol and whole-body cortisol in fish exposed to  
223 acute and chronic stressors has confirmed that the method is appropriate for detecting stress-  
224 induced changes in HPI activity [48]. Hereafter we refer to concentrations of corticosteroids we  
225 measured on whole body preps as ng/g of 'whole-body cortisol'.

226

#### 227 Analysis of brain monoamines

228 Brains were sectioned in a frozen state on a cryostat and mounted on glass slides.  
229 Sections of 300  $\mu$ m thickness were cut in the coronal plane. Brain-punch microdissection was

230 performed as described by [30]. The hypothalamus, telencephalon and region posterior to the  
231 hypothalamus ('reticular formation') were identified for punching.

232 Punches from each of these three regions were collected and homogenized in 50 $\mu$ l ice-  
233 cold 4% perchloric acid containing 40 ng/ml DHBA (dihydroxybenzamine) as internal standard,  
234 using an MSE 100-W ultrasonic disintegrator. Samples were then centrifuged at 13000rpm for  
235 10 minutes at 4°C and the supernatants were analyzed for serotonin (5-HT), dopamine (DA) and  
236 norepinephrine (NE) and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-  
237 dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using high performance  
238 liquid chromatography with electrochemical detection [50] immediately, or stored at -80°C for  
239 no more than two days prior to analysis. Pellets were stored at -80°C for subsequent analysis of  
240 protein content in an Eppendorf Biophotometer by a pre-made program measuring absorbance at  
241 280nm. The monoamines and monoamine metabolites were quantified using standard solutions  
242 and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd.,  
243 the Czech republic). The concentration of monoamines and monoamine metabolites is expressed  
244 as ng per mg protein.

245 We did not detect strong differences between brain regions in concentrations of brain  
246 monoamines: the only effect that we detected was that levels of DA ( $F_{2,81}=3.36$ ,  $P=0.04$ ), 5-  
247 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  
248 reticular formation in the 'predator' treatment (Table 1). Therefore we summed the concentration  
249 of each monoamine across regions and focused our subsequent analysis of treatment differences  
250 on the whole-brain values. However, the failure to detect strong region-specific differences  
251 should not be overinterpreted because we did not have the resolution to detect fine-scale

252 differences. Other studies have found region-specific differences in monoamine turnover during  
253 aggression [32].

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

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

267

#### 268 Determining genetic sex

269 DNA was extracted from each fin clip and genetic sex was determined by genotyping  
270 each individual for a male-specific genetic marker validated for sticklebacks [51].

271

#### 272 Data analysis

273 We compared the behavioral and physiological responses of sticklebacks to an unfamiliar  
274 conspecific and a predator across time using general linear models except when data were non-

275 normal. We tested for the effects of sex, body size, time and treatment on each of the dependent  
276 variables (behavior, whole-body cortisol and brain monoamines in the different regions). We did  
277 not detect sex differences in behavior, whole-body cortisol or brain monoamines and therefore  
278 did not analyze this factor further (all  $P > 0.4$ ). The least-squares difference post-hoc test was used  
279 to test for differences between groups, except when the distribution was non-normal, in which  
280 case we tested for differences between treatments using the nonparametric Mann-Whitney U test.

281 Pearson correlations were used to test for statistically significant relationships between  
282 variables when the data were normally distributed; otherwise, Spearman rank correlation  
283 statistics were computed. Because the same behavioral data was used to test for associations with  
284 brain monoamine concentrations, we used the sequential Bonferroni procedure to correct for  
285 multiple tests. Briefly, for each brain region within a treatment group, we replaced the  
286 correlation statistics with their corresponding P-values and then ranked them from smallest to  
287 largest. Results that were significant ( $P < 0.05$ ) after the sequential Bonferroni procedure are  
288 reported [52]. All tests were two-tailed.

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

291

## 292 **RESULTS**

### 293 Behavioural and physiological responses to an unfamiliar conspecific

294 Presentation of an unfamiliar conspecific elicited a behavioral response; on average,  
295 individuals approached the intruder 8 times and attacked 11 times within the observation period.  
296 However, individuals differed in their behavioral reaction to the simulated intrusion; while one  
297 individual attacked the conspecific over 40 times, other individuals spent most of their time

298 hiding, and scarcely left the refuge. Body size explained some of this individual variation; bigger  
299 fish were more aggressive toward their size-matched opponents (number of attacks:  $r=0.433$ ,  
300  $P=0.024$ ,  $n=27$ ). All of the fish oriented to and approached the conspecific and one-half of the  
301 fish attacked it at least once.

302 Interaction with the unfamiliar conspecific quickly produced a glucocorticoid response  
303 (Figure 1). Whole-body cortisol levels were highest 15 minutes after the simulated intrusion and  
304 then returned to baseline levels by 30 minutes.

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

307 Dopamine turnover to DOPAC was elevated 60 minutes following the aggressive  
308 interaction (Figure 2C and 2D), while levels of norepinephrine were consistently low (Figure  
309 2F).

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

315

### 316 Behavioural and physiological responses to a predator

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

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

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

332

### 333 Comparing responses to the conspecific and predator

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

340 Relative to the conspecific treatment, NE (Figure 2F) and to a lesser extent, DA (Figure  
341 2C) were higher in the predator treatments.

342

343 **DISCUSSION**

344

345 In this experiment, we tested the hypothesis that both the HPI axis and brain  
346 monoaminergic systems are activated in response to fighting with an unfamiliar conspecific and  
347 exposure to a predator. While other studies have found links between these systems in laboratory  
348 animals, the results from this study extends these findings to wild-caught animals that were  
349 confronted by ecologically relevant challenges [28,53]. We found that both stressors elicited a  
350 similar HPI response, but produced very different patterns of change in monoamine content.

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

356

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

359

360 During the present study, both confrontation with an unfamiliar conspecific and exposure  
361 to a predator resulted in activation of the HPI axis and significant alterations in the levels of  
362 brain monoamines in sticklebacks. These results are consistent with other studies which have  
363 shown that both confrontation by a challenging conspecific [10,23] and exposure to a predator  
364 [54] elicit a neuroendocrine stress response in fishes.



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

377 Other studies have found evidence for a more rapid recovery to baseline cortisol levels  
378 following less threatening situations compared to more threatening situations [55]. A longer-  
379 lasting cortisol response to threat of predation as compared to other stressors has been  
380 documented in stonechats [56] and rodents [57,58]. Therefore in this experiment, we hypothesize  
381 that the different time course of the cortisol response to a competitor versus to a predator is  
382 related to the perceived magnitude of the two different challenges. Sticklebacks are social fish,  
383 and frequently interact with other sticklebacks in shoals. Because encounters with conspecifics  
384 are frequent, natural selection might have favored individuals which do not mount a severe stress  
385 response to frequent interactions with conspecifics, and should favor individuals which recover  
386 quickly from fights. In contrast, encounters with predators are less frequent and more threatening

387 than encounters with conspecifics, so selection might have favored individuals with a greater and  
388 longer-lasting stress response.

389         The levels of whole-body cortisol detected in unstressed sticklebacks during the present  
390 study were similar to those previously reported for this species (2 – 8 ng g<sup>-1</sup>; [46]) and levels  
391 detected in the stressed fish in the present study, although slightly higher, were also broadly  
392 consistent with previous observations (50 ng g<sup>-1</sup>;[46]). The difference in magnitude of whole-  
393 body cortisol levels between this and previous studies may be related to the nature of the  
394 stressor.

395         Links between stress-induced blood cortisol levels and behavioral traits have been shown  
396 in fish [10,23], mammals [59] and reptiles [9]. However, while exposure to both stressors elicited  
397 a behavioral and whole-body cortisol response in the treatment groups, we did not detect a  
398 relationship at the individual level between concentrations of whole-body cortisol and behavior.  
399 It is possible that our method might not have had the resolution to detect fine-scale individual  
400 differences.

401         We did not detect any sex differences in whole-body cortisol. The stress response in  
402 vertebrates, including fish [60], is modulated by gonadal steroids with androgens suppressing  
403 and estrogens enhancing corticosteroid responsiveness [61]. However, the fish employed in this  
404 study were not reproductively active and it is therefore unsurprising that no sex-dependent  
405 differences in stress response were observed.

406

407 The monoamine responses to a conspecific and a predator were qualitatively different

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

411           For example, relative to the control group, concentrations of NE were consistently *higher*  
412 in the ‘predator’ treatments, and *lower* in the ‘conspecific’ treatments. Without data on the NE  
413 metabolite, MHPG, we cannot distinguish if reduced concentrations reflect a reduction in NE  
414 release (decrease in NE activity) or an increased turnover to MHPG (increase in NE activity).  
415 However, at an individual level we found that NE was consistently associated with risk-taking  
416 behaviors in both kinds of situations: NE was positively correlated with aggressive behaviors as  
417 well as predator inspection behaviors. These positive correlations suggest that more bold or  
418 aggressive individuals were more ‘aroused’, active or uninhibited, results which are consistent  
419 with other studies showing positive relationships between NE activity and behavioral impulsivity  
420 in monkeys [28] and sensation seeking in humans [62]. The fact that serotonin and NE had  
421 opposite relationships with risk-taking behaviors in this experiment is consistent with the  
422 observation that 5-HT and catecholamines can have antagonistic effects on behavior [17].

423

424 Associations between serotonin, risk-taking behaviors and aggression

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

429           Our results support the view that 5-HT has an inhibitory effect on aggressive behavior  
430 [16,54]. We found a negative relationship at the individual level between concentrations of 5-HT  
431 and aggressive behavior, and that confrontation by an unfamiliar conspecific resulted in lower 5-  
432 HT. Other studies have shown that winners of agonistic interactions have up-regulated brain 5-  
433 HT activity [21,30-32]. One possible explanation for this different pattern is that in our  
434 experiment, there was no physical contact between the resident and the intruder because the  
435 intruders were confined to a flask. As a result, the resident fish were unable to complete their  
436 attacks and therefore might not be analogous to the winners in the forementioned studies. We  
437 remain provisional in our interpretation of these results because 5-HIAA was degraded in many  
438 of the samples in the ‘conspecific’ treatments, preventing us from calculating serotonin turnover  
439 in those treatments. However, it is worth noting that while more aggressive behaviors were  
440 negatively associated with serotonin (Figure 3A), risk-taking behavior under predation risk  
441 showed the opposite pattern – it was *positively* correlated with 5HT (Figure 4B), and negatively  
442 associated with serotonin turnover to 5-HIAA (Figure 4C).

443           Overall, these data provide evidence that the response of fish to stressors is not identical  
444 regardless of the nature of the challenge, but rather that the response varies according to the  
445 magnitude, frequency and predictability of the stressor, as is the case for other vertebrates  
446 [56,63]. Further studies on individual variation in responses to different stressors would benefit  
447 from repeated sampling of the same physiological measures on the same individuals. While it is  
448 currently a challenge to measure brain monoamines noninvasively, noninvasive methods for  
449 measuring glucocorticoids in fish [64] are a promising alternative. In addition, the roles played  
450 by upstream elements of the stress response such as corticotropin releasing hormone (CRH) and  
451 variation in the binding characteristics of corticosteroid receptors and corticotropin binding

452 proteins should also be investigated [65]. Given that other studies have shown that inter-  
453 individual differences in stress responsiveness have a high heritable component [66], further  
454 investigation will provide insight into the mechanisms that have produced adaptive, heritable  
455 behavioral variation in sticklebacks in diverse ecological settings.

456

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466

467

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657

658

659 **Figure legends**

660

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

663

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

667

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

671

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

Figure 1

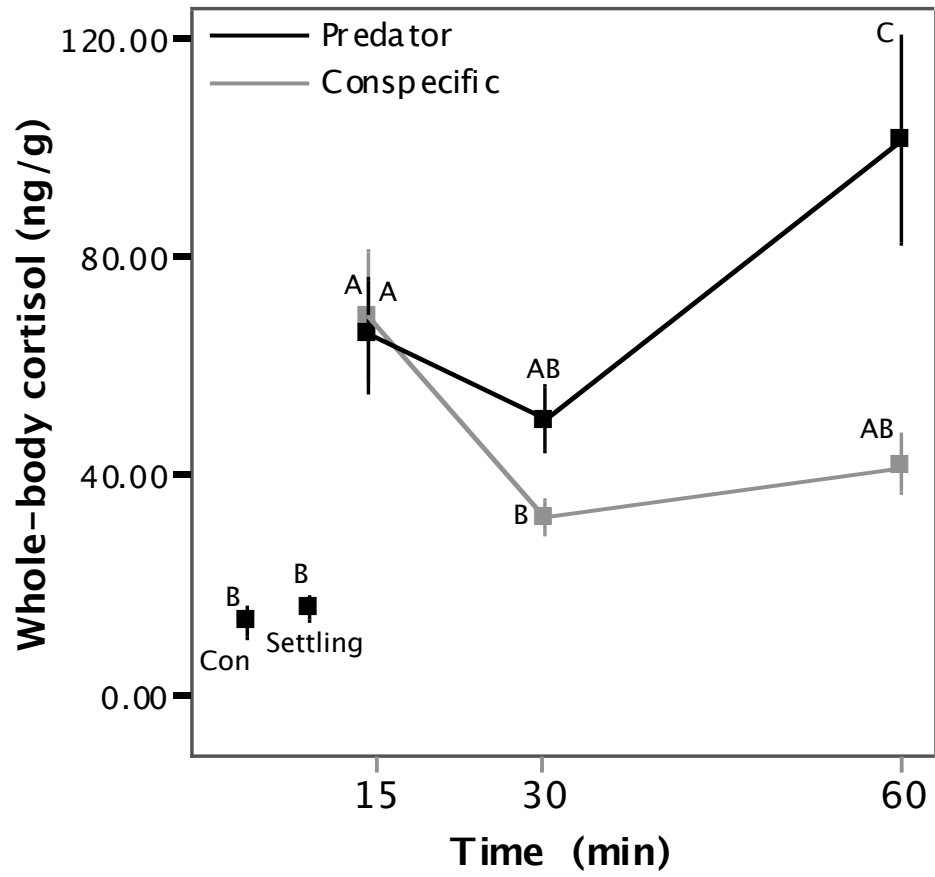
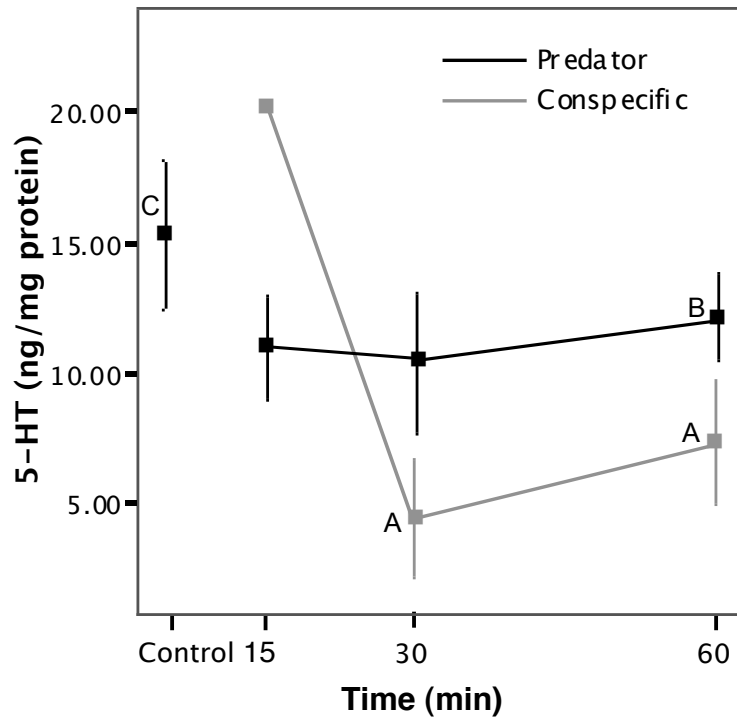


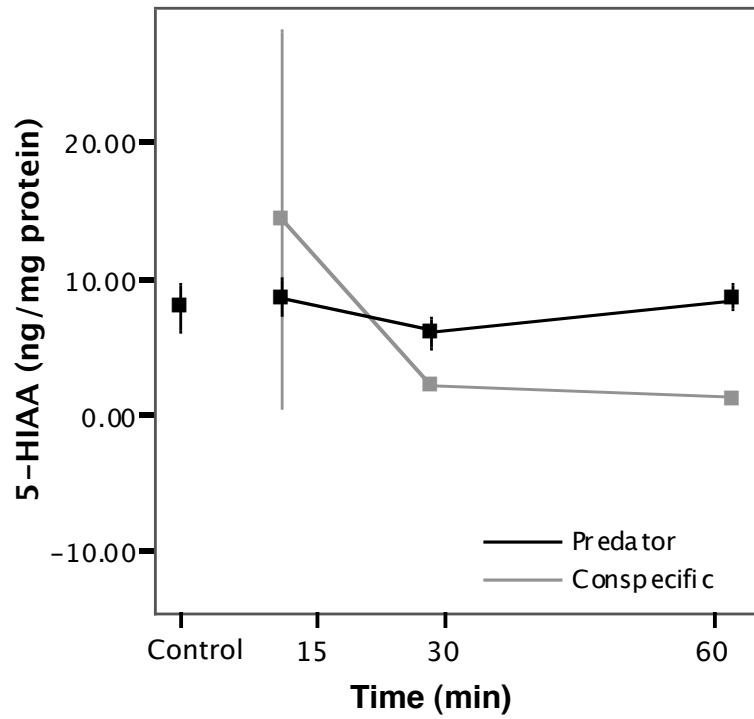


Figure 2

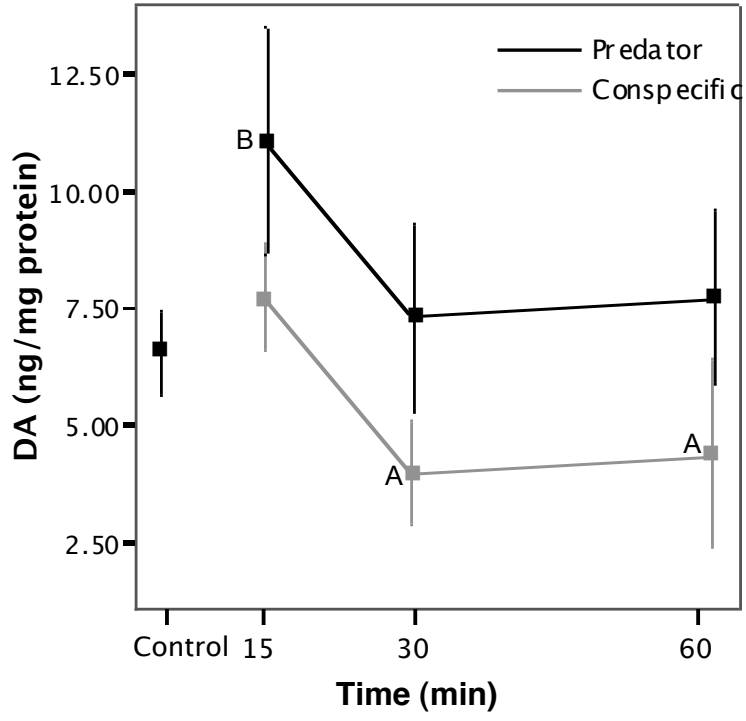
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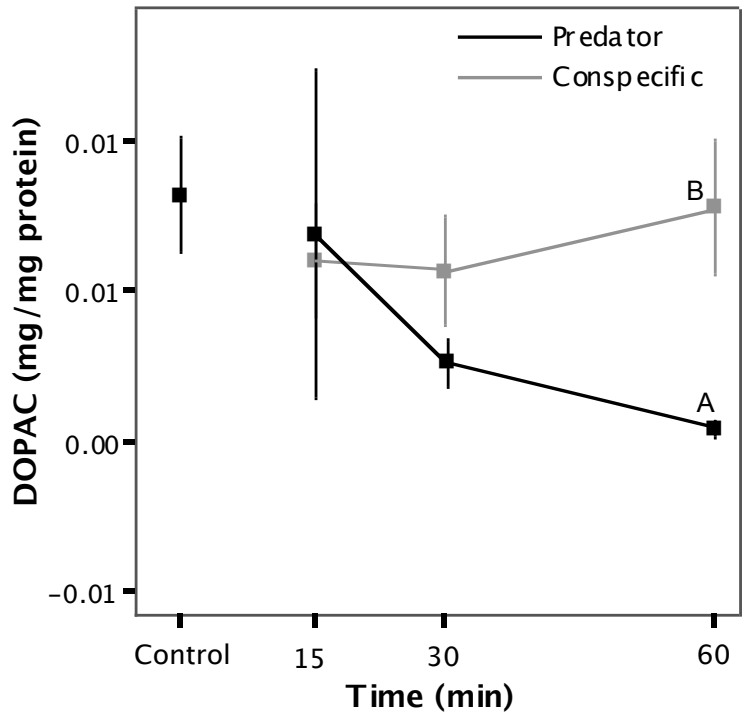
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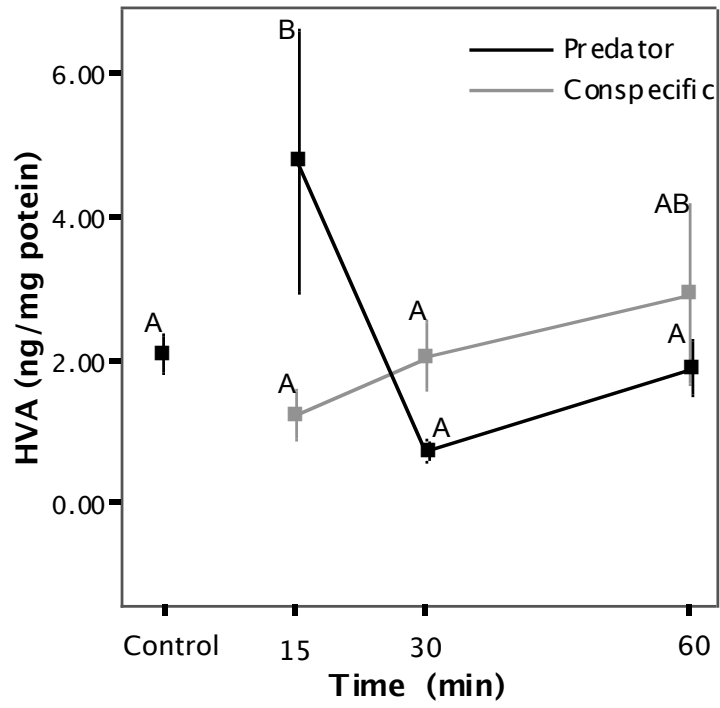
(C)



(D)



(E)



(F)

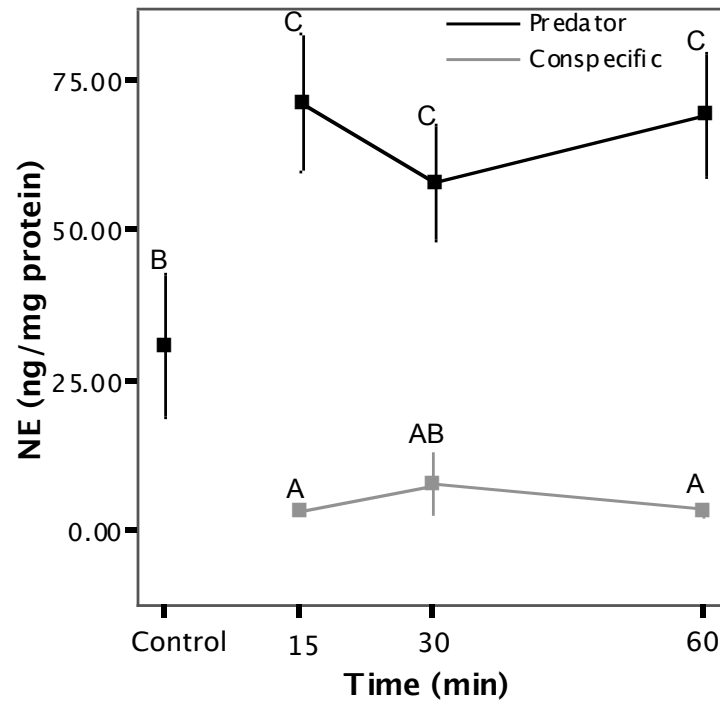
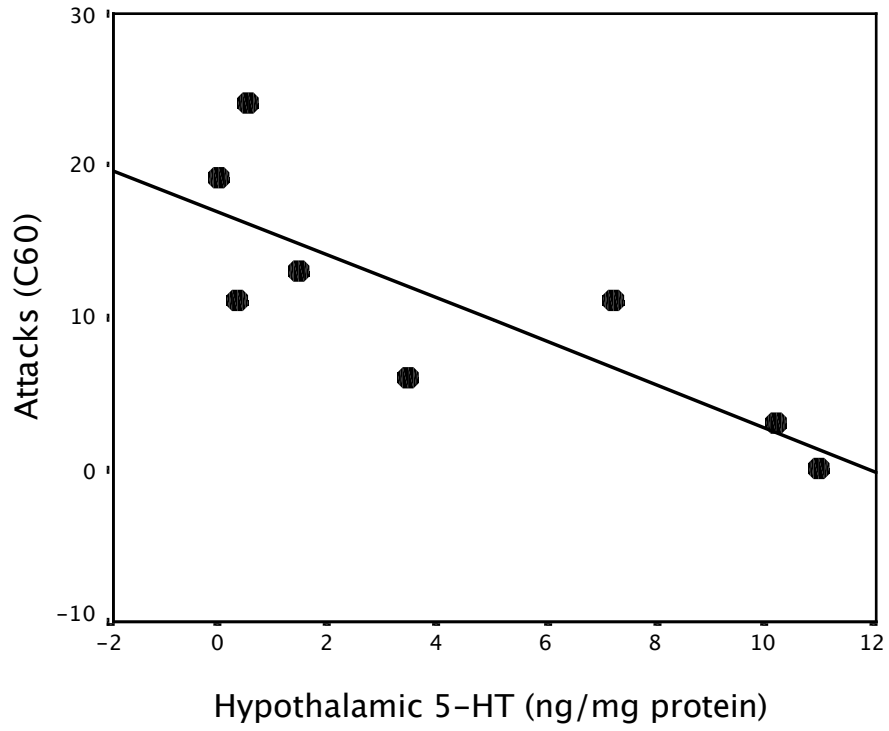
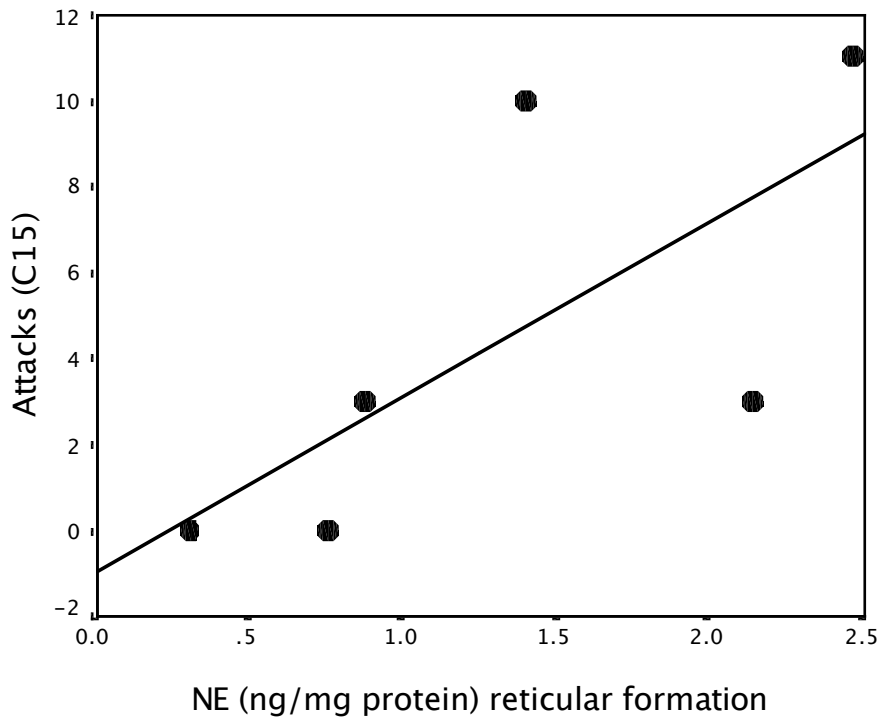


Figure 3

(A)



(B)



(C)

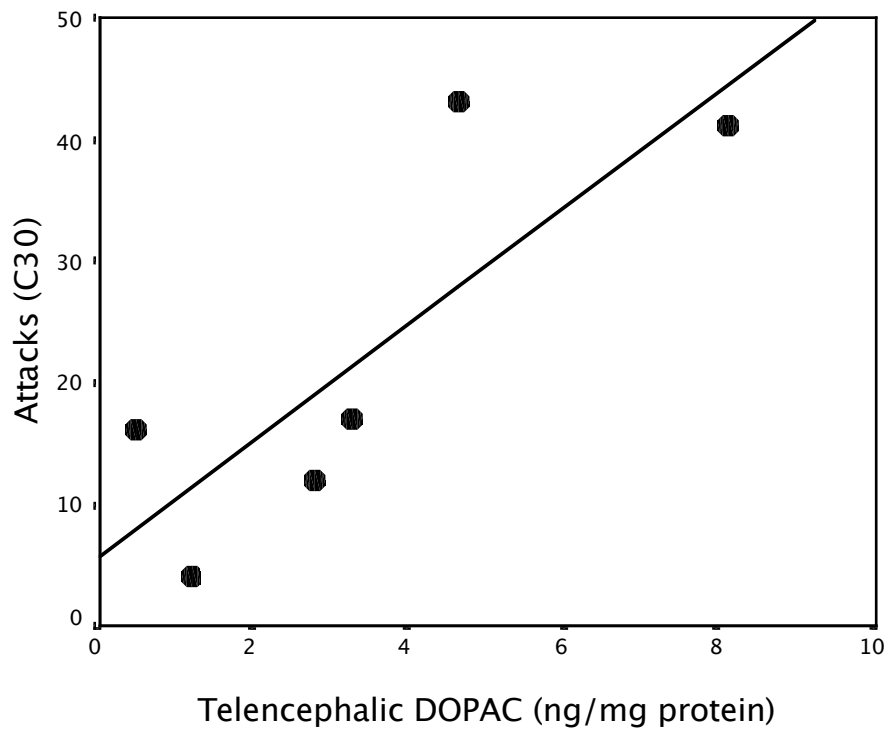
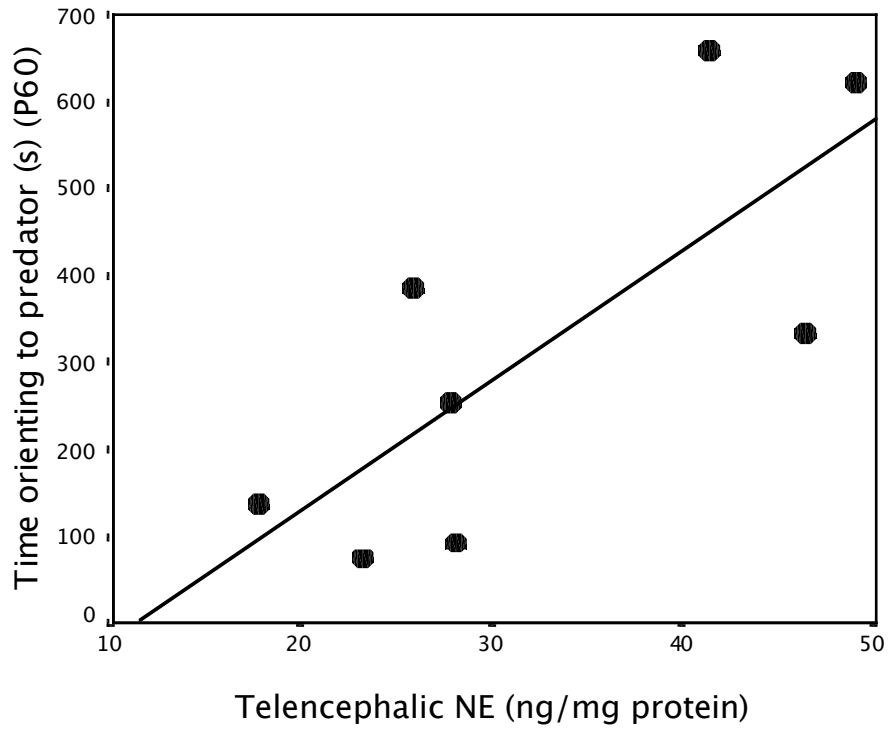
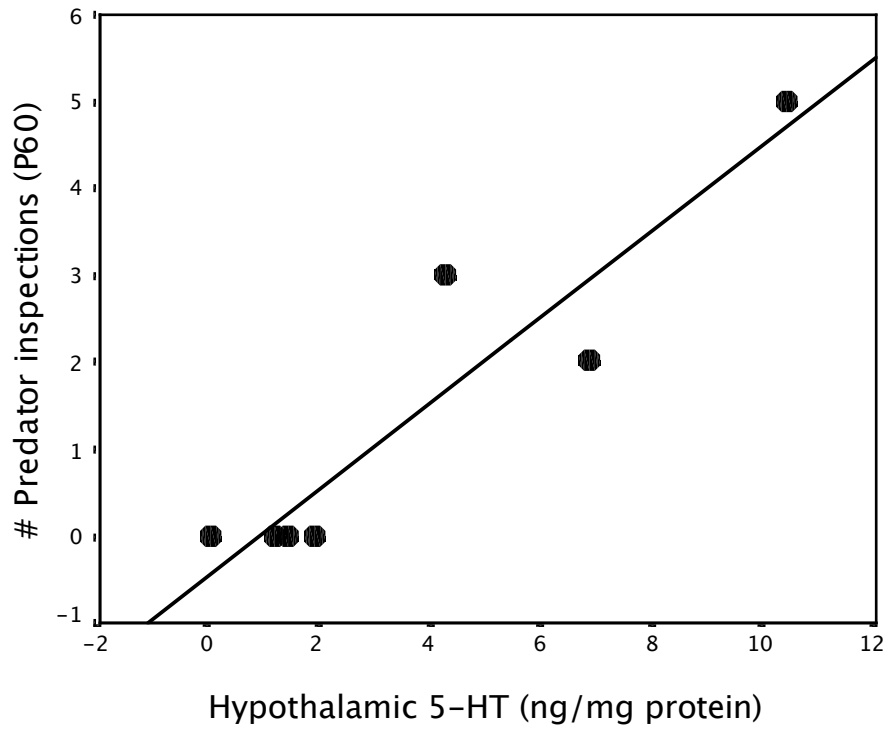


Figure 4

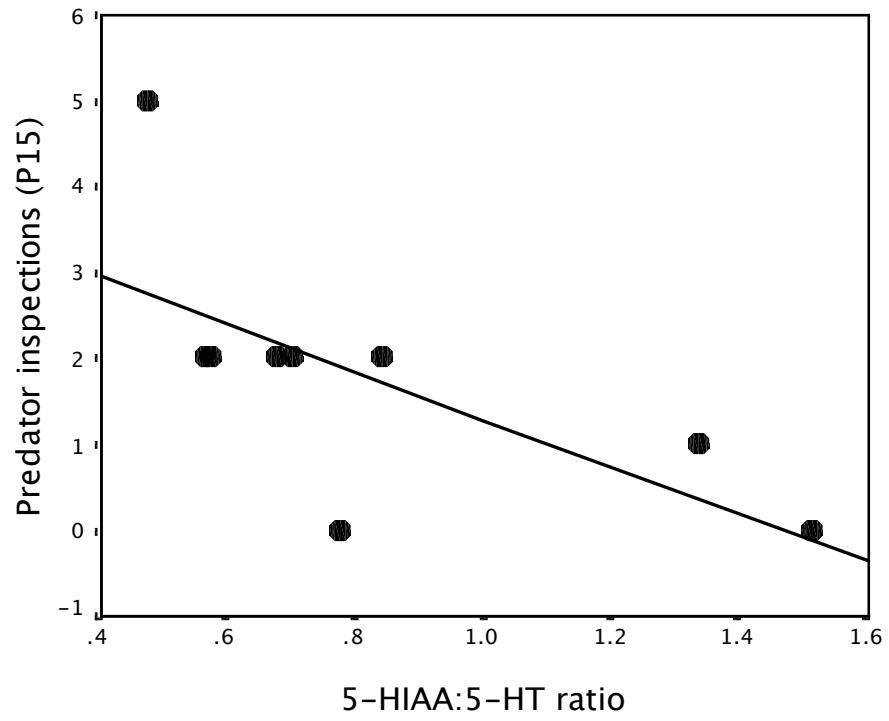
(A)



(B)



(C)



**Table 1.** Concentrations (ng/mg protein) of monoamines in the different brain regions for the different treatments. Statistics are presented as mean  $\pm$  sd. Sample sizes are in parentheses.

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