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Learning strategies during fear conditioning

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ABSTRACT

This paper describes a model of fear learning, in which subjects have an option of behavioral responses to impending social defeat. The model generates two types of learning: social avoidance and classical conditioning, dependent upon (1) escape from or (2) social subordination to an aggressor. We hypothesized that social stress provides the impetus as well as the necessary information to stimulate dichotomous goal-oriented learning. Specialized tanks were constructed to subject rainbow trout to a conditioning paradigm where the conditioned stimulus (CS) is cessation of tank water flow (water off) and the unconditioned stimulus (US) is social aggression from a larger conspecific. Following seven daily CS/US pairings, approximately half of the test fish learned to consistently escape the aggression to a neutral chamber through a small escape hole available only during the interaction. The learning curve for escaping fish was dramatic, with an 1100% improvement in escape time over 7 days. Fish that did not escape exhibited a 400% increase in plasma cortisol and altered brain monoamine response to presentation of the CS alone. Elevated plasma cortisol levels represent classical fear conditioning in non-escaping fish, while a lack of fear conditioning and a decreased latency to escape over the training period in escapers indicates learned escape.

1. Introduction

Fear memories that affect animals living in natural conditions are influenced by contextual information that may be significant for more than just the memory itself. Natural fear learning and conditioning is evoked by stimuli such as territorial competitors and predators that are directly related to how the animal survives. For example, fearful memories of a bigger and stronger territorial competitor include important spatial and social information such as territorial boundaries, food, mates, opponents and rank recognition that add salience to the fear memories of the aggressor. The characteristics of ecologically relevant fear learning and memory formation drove the development of an experimental model that takes specific contextual and social significance into account. Stimuli that evoke social fear are often unpredictable, not habituated, and therefore result in significant stress (Summers et al., 2005). Social stressors have been judged to be the most potent stressors, even for dominant individuals that win aggressive interactions (Koolhaas, de Boer, De Rutter, Meerlo, & Sgoifo, 1997).

Natural and domesticated populations of a wide variety of vertebrates appear to cope with stressful situations, including both social and physical stressors, with a simple dichotomy of heritable strategies; that is, they respond either proactively or reactively (Benus, Bohus, Koolhaas, & van Oortmerssen, 1991; Koolhaas et al., 1999). The proactive phenotype is characterized by behaviorally active coping, such as active avoidance, aggression, or flight responses and low hypothalamus–pituitary–adrenal (HPA) axis responsiveness, but high sympathetic reactivity. The reactive phenotype exhibits passive coping, conservation, immobility, withdrawal, low aggression, elevated HPA responsiveness, and limited sympathetic reactivity. A genetic basis for the expression of behavioral and physiological components of individual coping styles has repeatedly been demonstrated (de Boer, van der Vegt, & Koolhaas, 2003; Driscoll et al., 1998; Ellenbroek & Cools, 2002; Veenema, Meijer, de Kloet, & Koolhaas, 2003).

These divergent characteristics have been artificially selected for in rainbow trout (*Oncorhynchus mykiss*) and exhibit a moderate to high degree of heritability (Pottinger & Carrick, 1999). The two genetically divergent lines differ in their neuroendocrine responsiveness to physical stress (confinement). Trout that respond to confinement stress with highly elevated plasma cortisol levels (high responders, HR) also have a high locomotor response to stress, and do not recover from other stressful events quickly (Øverli, Pottinger, Carrick, Øverli, & Winberg, 2002). In contrast, trout that have a more muted elevation of cortisol response to confinement stress (low responders, LR), have a reduced locomotor response in a territorial intrusion, a more rapid recovery of feeding after transfer to a novel environment, and tend to become socially dominant (Pottinger & Carrick, 2001; Øverli et al., 2002).

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Stress responsiveness has also been used to demonstrate classical conditioning in rainbow trout (including LR and HR fish) to an aversive event (Moreira, Pulman, & Pottinger, 2004). Pairing an auditory conditioned stimulus (turning the water to the tank off = CS) with an aversive unconditioned stimulus (confinement stress = US) over time produces a conditioned response of elevated plasma cortisol concentrations to the CS alone. Strain differences in the ability to form or recall memories of a stressful event after a similar number of training trials are demonstrated by a more rapid rate of extinction of the conditioned response in HR fish (Moreira et al., 2004). This kind of conditioning is suggestive of classical fear conditioning in rodents that pairs electric foot shocks (US) with auditory or visual stimuli (CS) to produce behaviorally conditioned responses such as freezing or potentiated startle (Davis, 1980), but replaces the behavioral effect with a physiological one. This conditioned physiological response suggests that stress coping styles may be an evolutionary adaptation that includes learning (Øverli et al., 2007). In addition, physiological responses to fear conditioning in a natural setting may influence behaviorally relevant outcomes to aversive stimuli and contextual spatial elements of the environment in which they occur.

We propose a model for fear conditioning using rainbow trout that combines the aversive stimulus of a larger territorial competitor as an unconditioned stimulus that promotes fearful behavioral and physiological responses, while providing an opportunity for a smaller test fish to learn to escape from an aggressive interaction that it cannot win. We hypothesized that over a 7 day trial, a majority of the resident trout will learn to escape from an aggressive interaction into the safety of an adjacent chamber. Furthermore, we hypothesized that the escape behavior can be a product of a conditioned response to a neutral stimulus.

Our results suggest two divergent types of learning. In response to the presence of a much larger aggressive conspecific, test fish display two distinctive behavioral responses, escaping or remaining. Learned escape is characterized by a rapid decrease in the latency to escape over seven training periods (see Fig. 1), but notably, an absence of escaping as a conditioned response. Those fish that do not escape, and remain with the aggressive US each of the 7 training days display (see Fig. 1) classical fear conditioning to presentation of the neutral CS alone. The conditioned response is manifest by physiological and neurochemical responses.

2. Materials and methods

All work with fish was conducted at the Gavins Point National Fish Hatchery in Yankton, South Dakota. Prior to experimentation, Rainbow trout *(O. mykiss;* raised from eggs) in size matched groups were housed indoors in six foot diameter circular fiberglass tanks under natural light conditions. Fish were fed daily with Nelson's Silver Cup trout feed at a rate of 1% body weight per day. These experiments were conducted in a manner that minimized suffering of subjects as well as the total number of animals used in accordance with the Declaration of Helsinki and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23), under approved protocol by the University of South Dakota IACUC.

2.1. Tanks

Test aquaria were 75 gallon flow through systems, individually lit, with water inflow spray bars on both sides. Each tank was divided into three separate chambers, with space for a test fish in the middle compartment, a large fish on one side and an empty chamber on the other side (Fig. 1). Chambers were formed by the insertion of opaque barriers which could be easily removed and served to elicit territorial association with a specific space, and to eliminate contact between fish. Two barriers were inserted between the test fish chamber and the empty chamber, such that when one barrier was removed, a small escape hole (2 in. diameter) became available for use during the interaction (the escape hole remained covered at all other times). This escape hole was large enough for the test fish, but not the larger fish to pass through. Importantly, before experimental day one, test fish had no exposure to the escape hole.

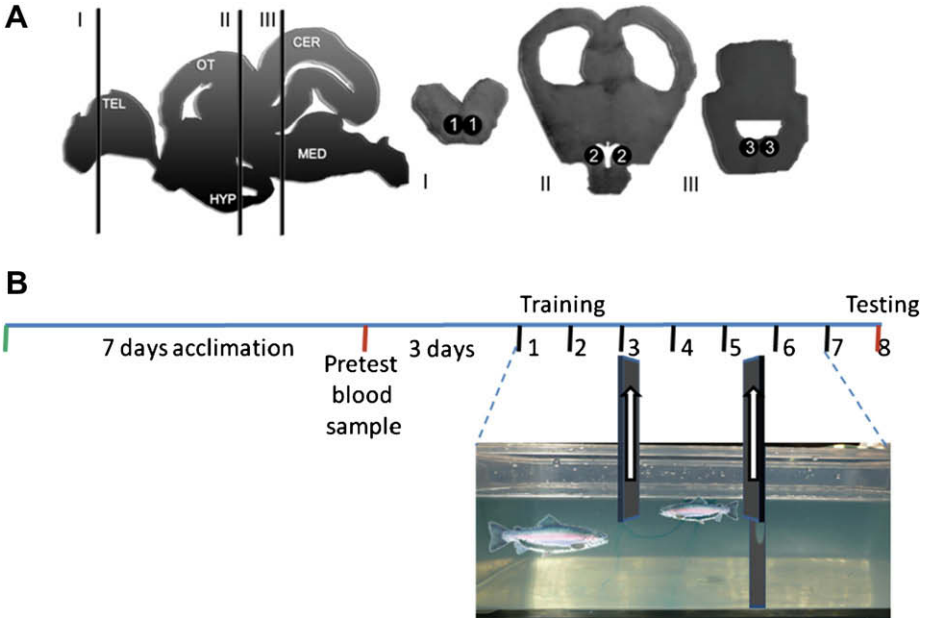

Fig. 1. (A) Anatomical representation for specific regions chosen for microdissection. Sagittal section of a rainbow trout brain; TEL = telencephalon, OT = optic tectum, HYP = hypothalamus, CER = cerebellum, MED = medulla. I Telencephalic coronal section ❶ represents striato–amygdalar complex (subpallium). II Diencephalic coronal section ❷ hypothalamic region sampled. III Brainstem coronal section ❸ raphé. (B) Time line of experimental design. Days 1–7 fish were acclimated to the experimental tanks. A pretest blood sample was taken following acclimation, and fish were allowed to recover for 3 days. Training (CS + US) occurred daily over the next week, with testing on the following day. Fifteen minutes after the initiation of testing, terminal blood and brain samples were collected for analysis.

2.2. Subjects

Smaller (125–150 g) test fish ($N = 24$ total) were netted out of the group tank and placed in an individual compartment in the test aquarium and allowed to acclimate for 10 days. On day 7 of acclimation, a plasma sample was taken from each fish to determine baseline levels of circulating cortisol (Fig. 1). Large (350–450 g) brood-stock ($N = 8$) were used as the aggressive social stimulus (US) in this experiment. These fish were housed separately prior to experimentation, and rotated throughout the experiment to insure a high level of aggression towards the test fish. In preliminary experiments using the new model we observed decreased aggression when interactions were repeated using the same individuals, so for this experiment, each test fish interacted with a new big fish every day. In addition, after 5 days of aggressive social interaction in trials with smaller test fish, each big fish was rested for 1 day by keeping them isolated without social interaction.

2.3. Experimental design

Following the 10 day acclimation period, on experimental day one, water inflow to the tank was turned off (CS), and 15 s later, the barrier between the big fish and small fish was removed, along with the barrier that exposed the escape hole to the empty chamber, making the empty chamber available to the test fish. Fish were allowed to interact for 15 min, and latency to first attack, total number of attacks, submissive behavior and escape time (if applicable) were recorded. If the small fish did not escape within 15 min, the fish were separated, barriers were re-inserted, water inflow was turned back on and the interaction was over. If the small fish did escape however, the water was turned back on, the big fish moved back to his chamber, and the small fish was allowed to remain for 5 min before being ushered back into the home chamber. Fish interacted once daily for 7 days, facing a new large fish each day. On day 8, the water inflow was turned off and the barriers were removed, but there was no large fish present, and fish were observed for 15 min (Fig. 1). Immediately following this observation, fish were rapidly netted, killed, and blood and brains were collected for analysis.

Learned escape, in this paradigm consists of several steps, including finding and using the escape hole for the first time, remembering the location of the hole in subsequent trials and minimizing vulnerability to attack while using the escape hole. Consistently escaping fish develop specific ballistic swimming patterns that presumably serve to limit exposure to the aggressive conspecific during escape. Learned escape did not include randomly leaving the territorial tank for the empty chamber, but was only assigned to fish using ballistic movements directed toward the escape hole in response to the US in at least five of the seven trials. Conversely, non-escapers were fish that did not escape at all.

A second group of fish ($N = 12$) followed the same experimental design as above, with no access to the escape hole. These fish were included as an aggression control, to compare the effects of daily aggression between escapers and non-escapers. The interactions and time line for these fish were the same as described above. A third and final group of fish ($N = 12$) was included as a CS control group, and was exposed only to the water off CS once a day. The water remained off for 15 min. These fish had no aggressive interactions over the course of the experiment, but followed the same time line as that described above.

2.4. Anesthesia and sampling

Fish were anesthetized in 500 mg/l methane tricane sulfonate (MS222) in a total volume of 10 l. Time to loss of equilibrium was recorded and consistent (~15 s) for all test fish. Blood was taken from the caudal vasculature in a 25 Gauge needle pre-treated with heparin, collected into a micro-centrifuge tube, spun down and plasma was collected then frozen on dry ice. Cortisol levels were analyzed using an enzyme linked immunosorbent assay (Carpenter et al., 2007). Fish were killed by cervical dislocation and brains were collected intact in the cranium and stored at −80 °C until processing.

2.5. Analysis of monoamines

Fish crania were sliced coronally at 300 μm in a temperature controlled (−12 °C) cryostat (Leica-Jung 1800, Wetzlar, Germany). Brain slices were thaw-mounted onto glass microscope slides and individual brain regions to be sampled were microdissected with a 500 μm diameter punch (Fig. 1). The subpallium or "striato–amygdalar" complex was chosen as a major region for analysis, as it contains both limbic and striatal elements (Laberge, Mühlenbrock-Lenter, Grunwald, & Roth, 2006; Northcutt, 2008; Northcutt & Davis, 1983). As our task involves fear conditioning, motivated locomotion and social aggression, this region is likely to be involved. The hypothalamus was chosen as another region for analysis, as previous work in salmonids has shown that serotonergic activity in this region is implicated in the initial stimulation of the hypothalamus–pituitary–interrenal endocrine stress axis secretion (Winberg, Nilsson, Hylland, Soderstöm, & Nilsson, 1997). Monoamine activity in the raphé was also analyzed, as this brain region is the primary source of serotonin in the brain.

Dopamine (DA), and the DA catabolite: 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and its catabolite: 5-hydroxyindoleacetic acid (5-HIAA) were measured using high performance liquid chromatography (HPLC) with electrochemical detection (Emerson, Kappenman, Ronan, Renner, & Summers, 2000; Renner & Luine, 1984, 1986). Following microdissection, samples were expelled into 60 μl of a sodium acetate buffer (pH 5.0) containing 6.82×10^{-5} M DHBA (9.4 pg/μl dihydroxybenzylamine; internal standard). Each sample was subsequently frozen, then thawed and centrifuged at 15,000×*g* for 3 min. The supernatant was removed and 45 μl was injected into a chromatographic system (Waters Associates, Milford, MA) and analyzed electrochemically with an LC-4B potentiostat (Bioanalytical Systems, West Lafayette, IN). The electrode potential was set at +0.6 V with respect to an Ag/AgCl reference electrode. The cerebral tissue remaining from each sample was dissolved in 110 μl 0.4 N sodium hydroxide (NaOH) and protein content was assayed using a spectrophotometer (Bradford, 1976).

2.6. Statistics and calculations

The percentage of escapers versus non-escapers was determined over several trials in two separate experiments, and reflects the average from 2 years. Comparison of latency to escape was made using repeated measures one-way ANOVA followed by the Holm–Sidak multiple comparison test. For cortisol analysis, we utilized two separate two-way repeated measures ANOVAs, one using a sampling time by escape behavior design (Fig. 3A), and the second using a training regimen by sampling time design (Fig. 3B). Monoamine levels were compared utilizing Student's *t*-tests. For all statistical analysis, significance was accepted when α was less than 0.05.

3. Results

3.1. Behavior

In social interactions between paired rainbow trout from adjacent territories, both fish are initially aggressive. During the first 2 days of training, all fish, whether they eventually escaped or

not, swam in close proximity to and visually inspected the escape hole. The mean latency to use the escape hole during the first 2 days of interaction was approximately 10 min, and this time period before escape was filled with both attack and submission behaviors from the test fish, as well as searching for a way out of the tank. Latency to escape decreased dramatically after the first 2 days of training. These aggressive interactions escalate rapidly, from non-contact circling behavior progressing to tail biting and then to lateral and frontal biting attacks. The most aggressive period of the interaction is manifest by biting attacks that are the culmination of high speed ramming, in which the aggressive fish sets up and launches an attack from a distance. These attacks can be severe, causing fin and scale damage. As a new larger fish is used each day for the CS + US training, territorial aggressive interactions occur in every trial during the 7 day training period. Inasmuch as the fish used for the unconditioned stimulus (US) is three times larger than the test fish, the outcome of the aggressive interaction is virtually assured. The interaction remains highly aggressive until social rank is established, at which point the test fish either escapes, or becomes submissive. Non-escaping test fish become behaviorally submissive to the larger fish. Submission includes specific behaviors such as spending time at the top of the water column, staying near edges, and avoiding the center of the tank where the large fish tend to patrol. These behaviors are used to minimize interaction with the dominant larger fish, and are characteristic of subordinate social rank. Escaping test fish also minimize interaction with the larger dominant fish but do not display submissive behavior, instead they escape.

3.2. Learned escape

Over the duration of the entire experiment, 54% of test fish consistently escaped to the empty chamber during aggressive social interaction. Presentation of the CS alone on day 8 however, did not elicit escape behavior in this group. Alternately, 46% of the test fish did not utilize the escape hole, and remained in their home tank for the duration of the experiment.

For those fish that did escape, latency to escape decreased dramatically and significantly (one-way repeated measures ANOVA: $F_{6,45} = 25.49$, $P < 0.001$) over the duration of the experiment (Fig. 2). On experimental day 1 fish escaped in approximately 10 min, and by day 7 fish were escaping on average in less than 60 s.

3.3. Cortisol responsiveness

A plasma sample was taken from all fish following tank acclimation for one week. This is termed the "pre-trial" sample and is a baseline cortisol level. There was no difference in the pre-trial cortisol level between fish that would eventually become escapers and those that did not escape (Fig. 3A). Furthermore, following the presentation of CS alone on experimental day 8, non-escapers show significantly elevated levels of cortisol compared to escapers (two-way repeated measures ANOVA, escape effect: $F_{1,10} = 10.76$, $P < 0.008$) as well as to their pre-treatment levels (two-way repeated measures ANOVA, sample time effect: $F_{1,10} = 31.0$, $P < 0.001$). Cortisol levels from post-training samples taken on day 8 were only elevated in animals that did not escape on days 1–7 (two-way repeated measures ANOVA, time by escape interaction effect: $F_{1,10} = 15.43$, $P < 0.003$).

To substantiate the validity of the conditioned response, additional control groups tested daily presentation of the CS or US alone during 7 days of experimentation, followed by presentation of the CS alone on day 8 (CS group) or no stimulus on day 8 (US group). The CS presentation alone was not sufficient to stimulate elevated plasma cortisol (Fig. 3B). Additionally, daily subjugation

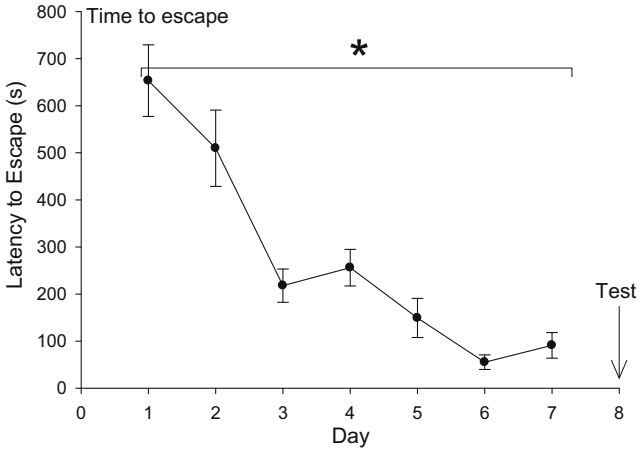

Fig. 2. Mean latency to escape (in seconds, ±SEM) over the 7 days of CS + US training. Fish that learned to escape did so progressively faster over 7 days, each significantly (∗) faster than the latency to first escape. On day 8, only the CS was presented, and no fish escaped.

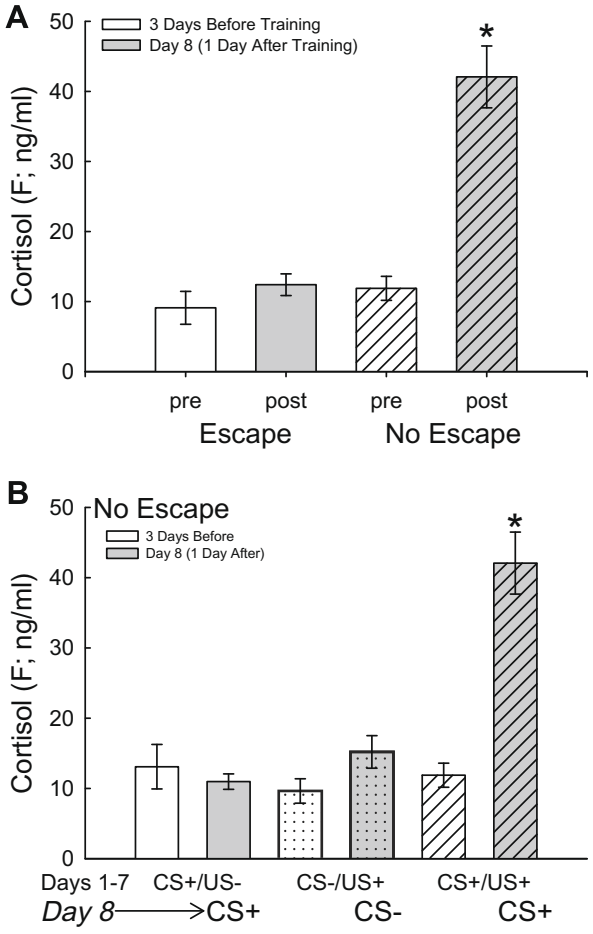

Fig. 3. (A) Pre-trial (clear bars) baseline mean (±SEM) plasma cortisol levels compared to plasma cortisol response to presentation of conditioned stimulus (CS) alone on Day 8 (Gray bars). Presentation of CS alone on day 8 elicited a significant (∗) increase in plasma cortisol compared to pre-trial levels only in non-escapers (hatching), and to post-trial escapers; B) Daily presentation of CS (open bars) or US (dotted bars) alone did not stimulate a conditioned response on Day 8; That is, 7 days of training with CS alone (CS+/US−) or US alone (CS−/US+) was insufficient to produce elevated plasma cortisol levels. A conditioned response to the CS on day 8 is only achieved with daily CS + US pairings (clear hatched bars compared to gray hatched bars).

of the small test fish to social aggression from the larger fish (US) for 7 days was also not sufficient to elevate plasma cortisol on day 8. During the daily bouts of aggressive interaction, it is likely that plasma cortisol increased each day during the social encounter (Øverli, Harris, & Winberg, 1999). However, no changes in plasma cortisol levels are evident on day 8, which is 24 h after the last aggressive encounter. Therefore, neither the CS nor the US alone influenced plasma cortisol levels on day 8 (Fig. 3B).

3.4. Monoamine responsiveness

Similar to hormone changes, some central monoamines were elevated by CS stimulation on day 8, following 7 days of CS + US training. While there was a significant ($t_{15} = 2.24$, $P < 0.041$) elevation of the monoamine serotonin (5-HT) in the striato–amygdalar complex (subpallium) of non-escapers compared to escapers, the serotonergic catabolite 5-hydroxyindoleacteic acid (5-HIAA) was also significantly ($t_{15} = 2.2$, $P < 0.046$) greater in subpallium of non-escapers (Fig. 4A and B). Dopamine (DA; $t_{13} = 2.7$, $P < 0.019$) and its catabolite dihydroxyphenylacetic acid (DOPAC; $t_{14} = 2.3$, $P < 0.04$) were both significantly increased in non-escapers compared to escapers in the subpallium (Fig. 4C and D).

In the hypothalamus, there were also significant increases in serotonin (5-HT; $t_{13} = 4.2$, $P < 0.001$) and dopamine (DA; $t_{14} = 2.2$, $P < 0.046$) of non-escapers in response to CS presentation, compared to escapers (Fig. 5A and C). The serotonergic catabolite 5-HIAA ($t_{13} = 1.6$, $P > 0.13$) and the dopaminergic breakdown product DOPAC ($t_{13} = 0.66$, $P > 0.52$) in the hypothalamus were not significantly affected by presentation of the CS at this sampling time.

In the raphé, where serotonergic cell bodies are located, 5-HT was significantly ($t_{15} = 3.73$, $P < 0.002$) increased in non-escapers compared to escapers in response to CS presentation (Fig. 6A). There was no significant ($t_{14} = 0.75$, $P > 0.46$) response to CS of 5-HIAA in the raphé. The raphé levels of DA were also significantly ($t_{12} = 2.8$, $P < 0.016$) elevated in response to the CS in non-escapers compared to escapers (Fig. 6C), however the catabolite DOPAC did not differ significantly ($t_{14} = 0.82$, $P > 0.42$).

4. Discussion

About half of the Rainbow trout paired with a novel, larger, aggressive conspecific daily for a week found and used a previously unknown escape route that provided refuge from social aggression. Escaping fish did so with a progressively reduced latency, suggesting that the response included learning how to accomplish escaping more efficiently with each trial. However, these fish did not escape when presented with the conditioned stimulus alone on day 8 as we had originally hypothesized. This absence of escape behavior may be due to a reluctance to vacate established territorial space (where they are fed) without aggressive provocation. For those animals that did not escape, a different and distinctive type of learning occurred even while under social attack. These fish showed classical Pavlovian conditioning to the neutral stimulus (water off) presented 15 s before introduction of the socially dominant larger fish. The most important outcome of these experiments is the demonstration of two distinctive types of learning being utilized by a single population of fish under circumstances of naturally salient fear conditioning.

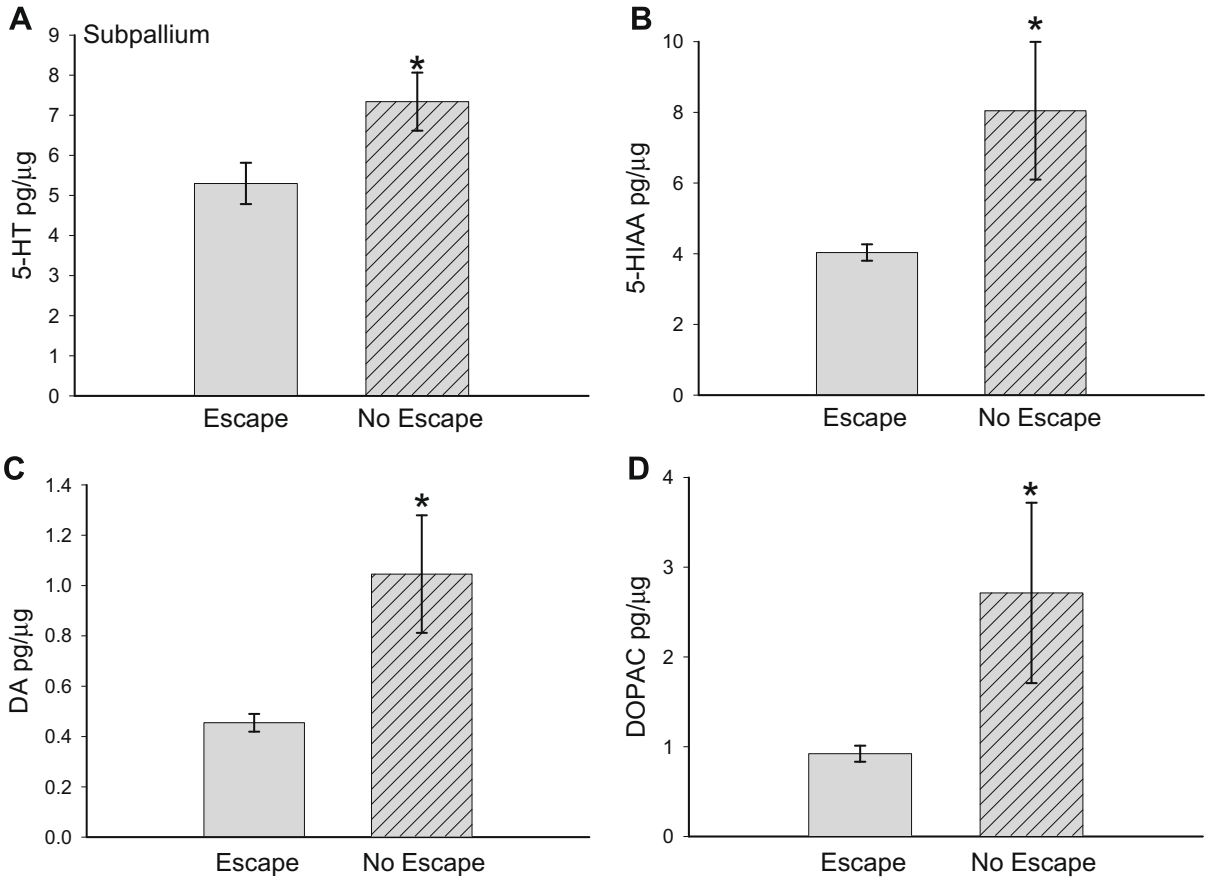

Fig. 4. Mean (±SEM) concentrations of (A) serotonin (5-HT), (B) 5-hydroxyindoleacetic acid (5-HIAA), (C) dopamine, (DA), and (D) dihydroxyphenylacetic acid (DOPAC) are significantly (*) elevated in the striato–amygdalar complex (subpallium) of non-escaping trout (hatched bars) compared to fish that escaped. Subpallial monoamine concentrations were measured following presentation of CS alone on day 8, after 7 days of CS + US pairings.

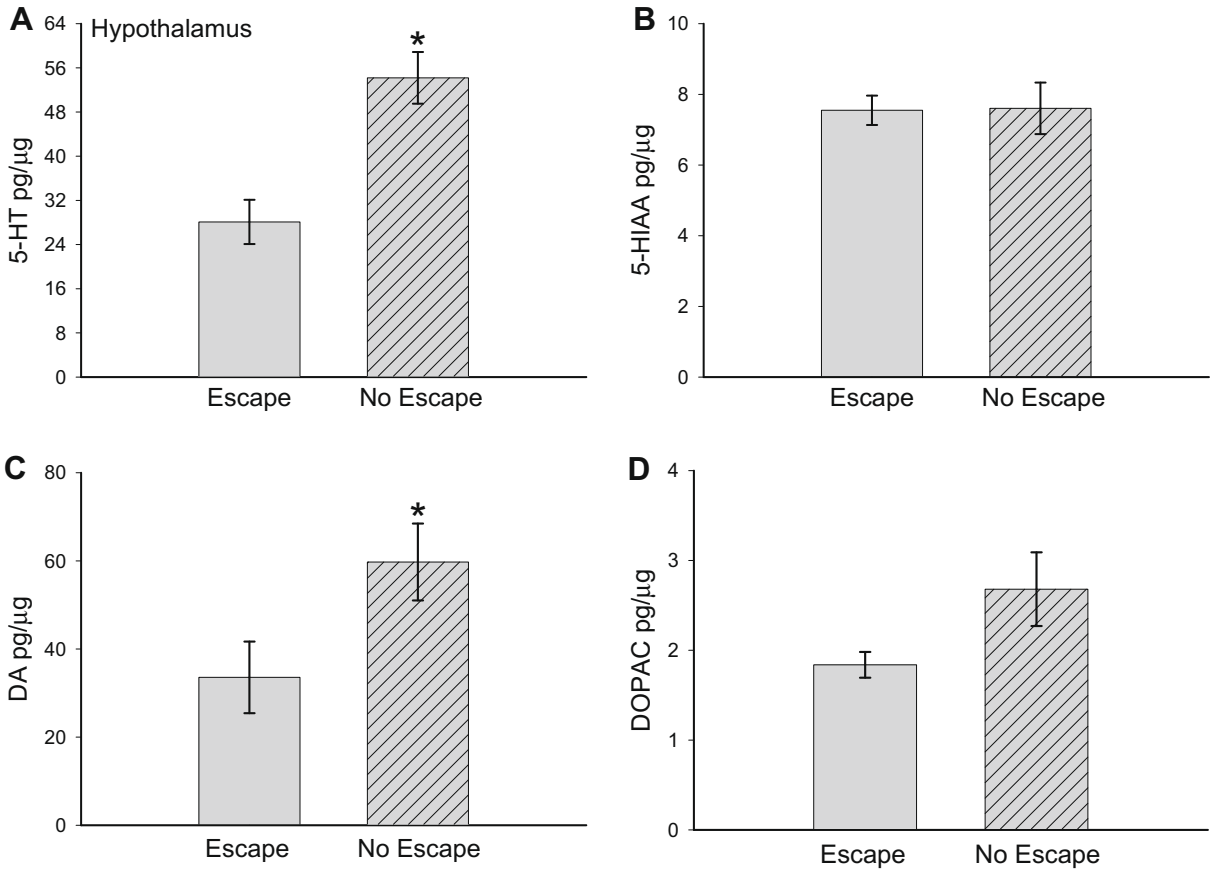

Fig. 5. Mean (±SEM) concentrations of (A) serotonin (5-HT) and (C) dopamine (DA) are significantly (*) elevated in the hypothalamus of non-escaping trout (hatched bars) compared to fish that learned to escape. Hypothalamic monoamine concentrations were measured following presentation of CS alone on day 8, after 7 days of CS + US pairings. There was no significant effect of CS + US pairing on concentrations of (B) 5-hydroxyindoleacetic acid (5-HIAA) or (D) dihydroxyphenylacetic acid (DOPAC) in this region.

The learning curve that describes the decreased latency to escape suggests that the subpopulation of fish that make use of the escape hole learn how to do so more efficiently with each trial. Among the tasks that appear to accrue during "learned escape" are (1) finding the escape hole, (2) using the escape hole for the first time, (3) remembering the location of the escape route, (4) minimizing vulnerability from attack while using the escape hole, (5) developing ballistic escape movements to safely accomplish escape, and (6) assessing the safety of the novel environment on the first escape, plus remembering this security in future attempts. It is important to remember that a novel environment in the wild is not always a safe environment, and that conditioning to remain in a territory replete with resources may confer adaptive advantage, even if submission is required.

One value of our model is to demonstrate in a fear conditioning paradigm that more than one adaptive response is possible to an unconditioned fearful stimulus. Current animal models that examine spatial and fear learning treat each learning style as a separate event. For example fear conditioning models such as auditory/olfactory-foot shock pairings result in a single behavioral response: freezing. Acoustic startle fear conditioning models focus primarily on potentiated startle response. Even the context rich conditioned defeat model in hamsters focuses primarily on complete submission of the test animal (Potegal, Huhman, Moore, & Meyerhoff, 1993). In a spatial learning model like the Morris Water Maze (Morris, 1984) that is at least somewhat stressful, while the animal may traverse the enclosure in any fashion it wishes, there is still primarily one measurable effect, latency to find the area where the platform is located. In the goldfish shuttle-box model, an aversive shock produces an escape response, but staying to be shocked has no adaptive value for the animal (Gallon, 1972; Zhuikov, Couvillon, & Bitterman, 1994), whereas remaining in the presence of a dominant conspecific presents the opportunity for use and/or territorial acquisition in the future. In addition, it is important to note that while we often refer to our model as "learned escape", it is not similar to "escape from fear" models (Cain & LeDoux, 2007; Miller, 1941).

Overall, these models have provided a wealth of information about acquisition, consolidation, retention, expression, and extinction of specific learned responses, and often their molecular mechanisms (Davis, 2006; Monfils, Cowansage, & LeDoux, 2007; Schaaf et al., 1999; Sung et al., 2008; Walker & Davis, 2008; Xu, 1997), which in our model has yet to be tested. In real world fearful learning and conditioning situations for humans or animals, there are most often more than one choice of behavioral response. It is possible that with a choice of responses, the molecular mechanisms for acquisition through extinction will be different, but perhaps related. Our model is designed to consider the possibility of real world type behavioral choices in response to conditioning in conjunction with aversive stimuli. Previous studies show divergent responses to common stimuli in other animal models. For example, proactive rats intentionally bury noxious stimuli (such as a shock probe), actively removing negative elements from their environment, whereas reactive rats passively avoid these stimuli (de Boer & Koolhaas, 2003). In a population of rainbow trout reacting to hypoxic conditions, some animals simply move to the bottom of the tank and wait, while other animals exhibit wildly fluctuating patterns of locomotion, moving rapidly around the tank as though

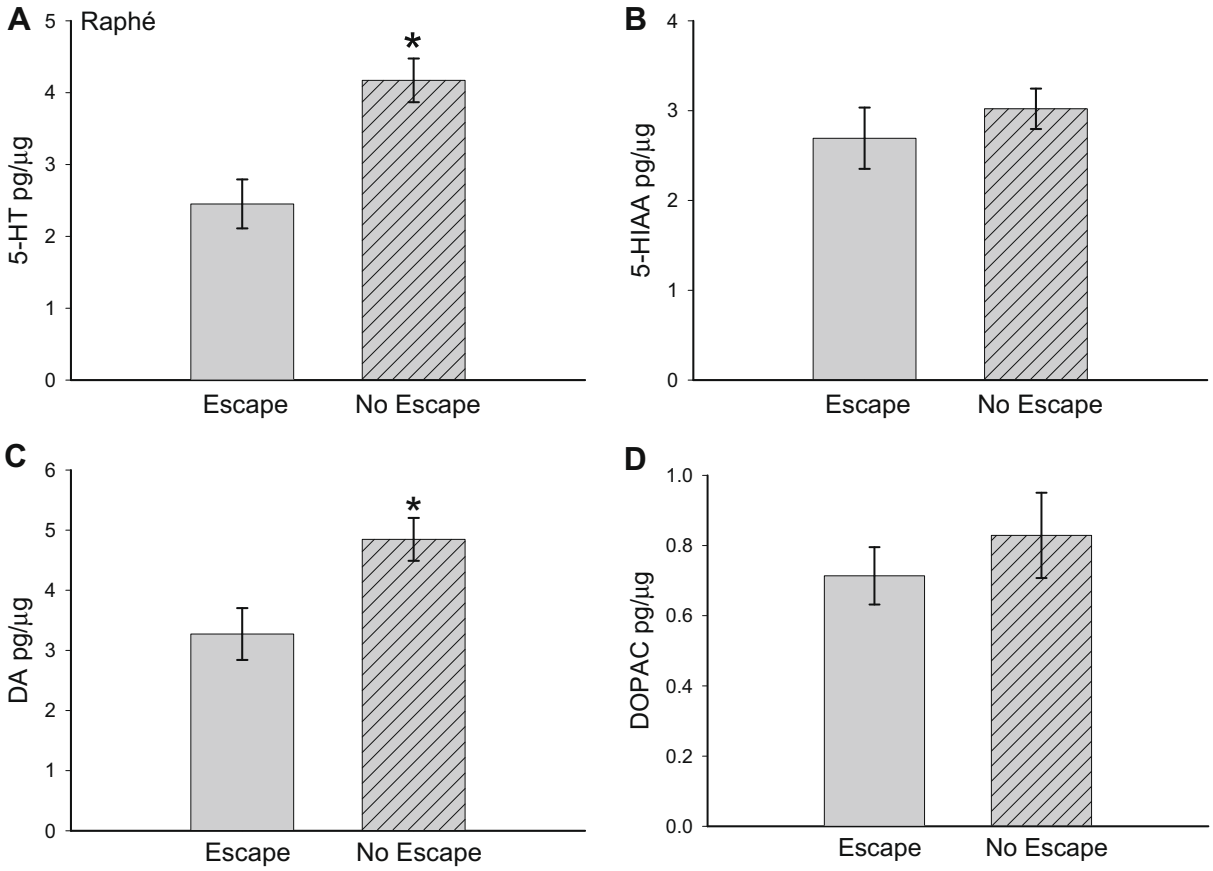

Fig. 6. Mean (±SEM) concentrations of (A) serotonin (5-HT) and (C) dopamine (DA) are significantly (∗) elevated in the raphé of non-escaping trout (hatched bars) compared to fish that escaped. Monoamine concentrations in the raphé were measured following presentation of CS alone on day 8, after 7 days of CS + US pairings. There was no significant effect of CS+US pairing on concentrations of (B) 5-hydroxyindoleacetic acid (5-HIAA) or (D) dihydroxyphenylacetic acid (DOPAC) in this region.

they are trying to escape (Schjolden, Pulman, Metcalfe, & Winberg, 2006). Like conditioned defeat, we use social aggression as the aversive stimulus. Social defeat is a context rich stimulus because it includes the elements of social rank dynamics, which in natural settings allows for more than one appropriate behavioral response. Among the appropriate responses is a spatially defined behavior in which the smaller animal recognizes impending social aggression and chooses to escape, thereby eliminating the stressor. A separate, but also evolutionarily advantageous behavioral response is submission in the face of impending social defeat, which is comparable to the conditioned defeat model used in hamsters (Huhman et al., 2003). It is important when studying conditioned responses to fearful aversive stimuli that choice be an element of the potential behavioral reactions if we are to understand fear conditioning in natural animal and human situations.

Previous studies in rainbow trout have shown the capacity for these animals to be classically conditioned through a physical stressor to produce a measurable increase in stress hormone secretion (Moreira et al., 2004). These studies demonstrate that trout have the capacity to respond to auditory and physical conditioned stimuli, including cessation of tank water inflow. It is unclear as to whether the presentation of the CS 15 s before the US represents trace or delay conditioning. While the condition of cessation of water flow is extant at the time of the presentation of the US, which would represent delay conditioning, the sensory signal is only presented at the onset of water off, as silence itself presents no sensory input to be registered at the time of US presentation, which would suggest trace conditioning. Different responses in magnitude of cortisol secretion in response to a confinement stress can be seen in a single population of rainbow trout (Pottinger & Carrick, 1999). Within that population, individuals with a low cortisol response to stress have higher locomotor activity and become socially dominant, compared with trout that respond with a higher cortisol response (Pottinger & Carrick, 2001; Schjolden et al., 2006).

Individual differences in Pavlovian conditioning in rodents predict stress induced corticosterone release and altered mesolimbic levels of brain monoamines (Tomie, Aguado, Pohorecky, & Benjamin, 2000). In our study, both elevated plasma glucocorticoid (Moreira et al., 2004) and central monoamine concentrations gave evidence of a potent conditioned response following a week of CS + US training, and presentation of the CS on day 8 to non-escaping fish (Figs. 3–6). Glucocorticoids have been demonstrated to modulate aversive learning and memory (Bartolomucci, de Biurrun, Czeh, Van Kampen, & Fuchs, 2002; Donley, Schulkin, & Rosen, 2005; Luine, Spencer, & McEwen, 1993). One mechanism for this effect may be the influence of glucocorticoids remodeling anatomical fine structure and function of hippocampal circuits (Fuchs et al., 2001; McEwen, 2000; Weiss, Krupka, Bahner, Both, & Draguhn, 2008). Glucocorticoids also inhibit hippocampal brain derived neurotrophic factor (BDNF) activity in mammals (Kumamaru et al., 2008). Similarly, early evidence suggests elevated BDNF gene expression in the hippocampus (dorsolateral pallium) of escaping trout, but not in non-escaping trout that exhibit a conditioned cortisol response (Carpenter, Sabirzhanov, Arendt, Smith, & Summers, 2008).

Trait anxiety is a critical defining behavior in characterizing stress coping styles of various vertebrate species (Koolhaas, de Boer, Buwalda, & van Reenen, 2007; Øverli et al., 2007). Proactive

individuals show blunted plasma glucocorticoid response to various stressors, similar to the cortisol response of escaping trout in our model (Koolhaas et al., 1999). Conversely, reactive individuals typically show a heightened glucocorticoid and central monoaminergic response to various stressors, similar to the cortisol and monoamine response of non-escaping trout in our model (Korzan & Summers, 2007; Øverli, Pottinger, Carrick, Øverli, & Winberg, 2001). Increased serotonergic and dopaminergic activity suggests that the conditioned response is evident in central neurotransmitter systems. Regionally specific changes in monoaminergic activity may underlie specific elements necessary to stimulate motivation, stress responsiveness and neuroendocrine sensitivity necessary for escape behavior and submissiveness. In socially subordinate fish, long term elevation of serotonergic activity is seen in both telencephalon (which includes striato–amygdalar complex) and brain stem (including raphé) (Winberg & Lepage, 1998; Winberg & Nilsson, 1993; Winberg, Nilsson, & Olsen, 1992). Dopaminergic activity in the striato–amygdalar complex may influence motivation and submissive behavior associated with the stress of submission, as CRF treated losers of aggressive interaction show elevated dopaminergic activity in this region, while increased serotonergic activity in this region is associated with fear and stress (Carpenter et al., 2009). Elevated serotonin in the hypothalamus stimulates the endocrine stress cascade in salmonids (Winberg et al., 1997), which may be the direct stimulus for the cortisol conditioned response in non-escaping trout. Dopamine in the mammalian hypothalamus stimulates another potent pathway involved in the stress response, arginine vasopressin (Onaka, 2000). Increased 5-HT in raphé suggests enhanced serotonergic synthetic activity, which may be regulated by DA (Korzan, Summers, Ronan, Renner, & Summers, 2001), which also increases with fear conditioning. Non-escaping fish are behaviorally submissive to the larger fish, employing a reactive or passive coping style, with specific behaviors that include spending time at the top of the water column, staying near the edges, and avoiding the center of the tank where the large fish tend to patrol. The behavioral characteristics of escaping and non-escaping fish appear to mirror those of proactive and reactive fish, respectively. Therefore, it suggests that the two distinctive forms of learning evoked by the escape paradigm may be evolutionarily conserved adaptive strategies, used by different elements of natural populations to cope with stressful conditions that occur in the wild.

In conclusion, our model demonstrates that the natural response to aggressive social interaction includes at least two distinct responses: escape or submission. What is more, these behavioral strategies are facilitated by two distinct kinds of learning, each expressed during specific responses to the possibility of escape. Learned escape from the larger fish produces a time dependant but not a conditioned response. Fish that are submissive to the larger individual display a classically conditioned fear response of elevated plasma stress hormones and central monoamines. The two responses, escape/no-escape, appear to be traits similar to those used to characterize distinctive stress coping strategies, evident in trout but also in a variety of other vertebrates. Therefore, the distinctive learning modes evident for escape and submission may be evolutionarily conserved elements of adaptive stress coping strategies used by all vertebrates.

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