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Extinction of a conditioned response in rainbow trout selected for high or low responsiveness to stress

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Running head: Conditioned stress response in trout

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Abstract

Two lines of rainbow trout (*Oncorhynchus mykiss*) that exhibit divergent endocrine responsiveness to stressors also display disparate behavioral traits. To investigate whether the high-responding (HR) and low-responding (LR) fish also differ in cognitive function the rate of extinction of a conditioned response was compared between the two lines. Groups of HR and LR fish were exposed to a paired conditioned stimulus (CS; water-off) and unconditioned stimulus (US; confinement stressor). After exposure to 18 CS-US pairings at least 70% of individuals of both lines acquired a conditioned response (CR), manifested as an elevation of blood cortisol levels on presentation of the CS only. Post-conditioning, the fish were tested by presentation of the CS at weekly intervals, for 4 weeks, with no further reinforcement and the extinction of the CR in the two lines was compared. The decline in mean plasma cortisol levels after exposure to the CS over successive tests suggested that the CR was retained for a shorter period among the HR (<14 days) than LR fish (<21 days). The frequency of individuals within each line whose plasma cortisol levels indicated a stress response when exposed to the CS was significantly greater among the LR than HR fish at 14 and 21 days with no HR fish falling into this category at 21 days. At 28 days post-conditioning, there were no HR fish and only three LR fish that were categorized as "stressed". These results suggest that there are differences in cognitive function between the two lines. Possible mechanisms underlying these differences are discussed.

Keywords: conditioning, stress response, cortisol, behavior, rainbow trout, selective breeding, *Oncorhynchus mykiss*

Introduction

The magnitude of the endocrine stress response, defined as post-stress levels of plasma cortisol, is a trait with moderate to high heritability in rainbow trout. This feature has allowed the generation of two lines of rainbow trout (*Oncorhynchus mykiss*) with divergent responsiveness to a standard confinement stressor (Pottinger and Carrick, 1999). In addition to the endocrine divergence for which the lines were selected, they were also found to exhibit disparate behavioral traits. In particular, fish of the low-responding (LR) line displayed a considerably greater degree of aggression than individuals from the high-responding (HR) line in staged fights for dominance (Pottinger and Carrick, 2001a). We have speculated that the endocrine and behavioral characteristics of the HR and LR fish are consistent with the active and passive "coping" strategies described for other species (Koolhaas et al., 1999) and furthermore, that differences in the brain monoaminergic system between the two lines suggest a possible functional link between the endocrine and behavioral traits observed in these lines (Øverli et al., 2001). As part of our continued study into the extent of the behavioral divergence between the lines we have investigated whether the HR and LR lines also exhibit differences in cognitive function.

It has recently been demonstrated that, using the classical Pavlovian conditioning paradigm, a teleost fish (Nile tilapia; *Oreochromis niloticus*) can be conditioned to respond to a visual signal with an activation of the hypothalamic-pituitary-interrenal (HPI) axis (Moreira and Volpato, 2004). In that study, fish were exposed to a localized visual stimulus (conditioned stimulus: CS; light on) paired with a brief confinement stressor (unconditioned stimulus: US). Repeated CS-US pairings resulted in the acquisition of a conditioned response (CR), the elevation of blood cortisol levels, associated with the exposure to the CS alone. Elevation of

corticosteroid levels as a primary CR has previously been reported for rodents (Buske-Kirschbaum et al., 1996; Kreutz et al., 1992) and humans (Sabbioni et al., 1997) but has not been previously demonstrated in fish.

This endpoint offers some advantages in terms of experimental application. Because the endpoint is endocrine and quantifiable in a blood sample, the presence or absence of the CR can be assessed simultaneously in a large group of fish, in contrast to the difficulty of monitoring behavioral endpoints under such conditions. This is helped by the predictable nature of the time-course of the HPI axis response to a stressor. In addition, because of the ease with which minor stressors such as confinement can be applied to fish sharing the same environment, conditioning can take place simultaneously for a group of animals allowing inter-individual differences in the acquisition or loss of the conditioned response to be investigated.

The aims of this study were (i) to confirm that rainbow trout can be conditioned in a similar manner to Nile tilapia to respond to a CS with an endocrine stress response and (ii) to use this approach to compare the extinction of this conditioned response over time in trout of the HR and LR lines as a means of evaluating possible differences in memory retrieval in trout selected for high- or low-responsiveness to stress.

Methods

Experiment 1: Selection of an appropriate conditioned stimulus

The aim of this experiment was to establish whether the conditioned stimulus (CS) employed previously (light on) to elicit the conditioned response (CR; elevation of plasma cortisol) in tilapia (Moreira and Volpato, 2004) was appropriate for use with groups of rainbow trout held in outdoor tanks. During February 2003, 210 stock

rainbow trout (1+ years old; sexually immature; mixed sex; CEH stock) were transferred from holding tanks to seven experimental tanks. Each tank was 1.8m diameter, glass fiber, containing 1000 l lake water continuously replaced at a constant flow rate of 30l min^{-1} at ambient temperature. The tanks were outdoors and thus exposed to the natural photoperiod. Fish had been held under these conditions from hatch. Prior to and during the study the fish were fed three times per week with commercial feed, at the manufacturer's recommended rate. The fish were allowed to acclimate to these conditions for 10 days. Three cues were evaluated for use as CS: (i) water off: switching off the water supply to tank for a period of 5 minutes; (ii) sound: a metal bar was used to strike the outside tank wall 10 times; (iii) light on: switching on and off for 30 seconds a remotely operated overhead spot light attached to the inside of the tank lid. The unconditioned stimulus (US) comprised a brief period of emersion followed by confinement, imposed by lowering the water level in the experimental tank to expose the fish to air and after 1 minute of emersion raising the water level to a depth of 10 cm. The fish were then kept under these confined conditions for approximately 25 minutes. The total time taken to execute the stressor, including emptying of the tank, was 30 minutes. In every case, the operator took care to remain out of view of the fish. The seven experimental treatments were randomly allocated and comprised (i) a control tank (undisturbed throughout the study); (ii) water off every day (CS only, control); (iii) water off every day plus stressor (paired CS-US); (iv) noise every day (CS only, control); (v) noise every day plus stressor (paired CS-US); (vi) light every day (CS only, control); (vii) light every day plus stressor (paired CS-US). The experimental procedures were carried out for 6 days at the same time each day (starting at 12:00 pm), with a sufficient period allowed between the treatment of successive tanks to accommodate the time required for

sampling on the final day. Significant diel variation in plasma cortisol levels in trout is restricted to the hours of darkness (Pickering and Pottinger, 1983). On day 7 the CS, in tanks previously receiving the paired CS-US, was applied with no accompanying US. The CS only control tanks (water off, noise and light) also received the CS on day 7. One hour after the application of the CS 10 fish were netted from each tank in turn, into a bucket containing anesthetic (2-phenoxyethanol, 1:2000). Complete sedation occurred within 2 minutes and a blood sample was taken from the caudal vessels into a heparinized syringe within 5 minutes of capture. The fish were returned to holding tanks to recover. Blood was kept on ice until it was centrifuged. Plasma was stored at -70^{o}C until required for assay.

Experiment 2: Assessment of the time required to achieve a conditioned response

It was established in Experiment 1 that "water off" was an appropriate CS for this experimental system. The aim of Experiment 2 was to assess how many CS-US pairings were required to elicit an elevation of plasma cortisol levels in response to the CS only (water off) in the majority of individuals sampled. Environmental conditions and husbandry were identical to those of the previous experiment. During April 2003, 840 rainbow trout (mean weight 238 ± 46g; 1+ years old; sexually immature; mixed sex; CEH stock) were transferred from holding tanks to 14 experimental tanks (60 fish tank^{-1}) and allowed to acclimate to conditions for 10 days. The 14 tanks were allocated randomly to seven treatment groups, two tanks per treatment. These comprised 4 control groups and 3 groups conditioned for different periods of time. Within these two categories the following treatment groups were employed: (i) undisturbed controls; (ii) water off (daily interruption of water supply for 5 mins, no associated emersion + confinement stressor; CS only, control); (iii)

emersion + confinement every day including final day (not preceded by water off; US only, control); (iv) emersion + confinement every day except final day (to confirm that the US in the absence of the CS did not elicit a prolonged activation of the HPI axis); (v) conditioning for 8 days (paired CS-US, days 1-8); (vi) conditioning for 12 days (paired CS-US, days 1-12); (vii) conditioning for 18 days (paired CS-US, days 1-18). For groups (v), (vi) and (vii) one day post CS-US pairings (days 9, 13, 19 respectively) only the CS was applied and fish were sampled as previously described for Experiment 1, 10 fish from each treatment tank. In addition, on day 19 only, group (i) was sampled with no disturbance; group (ii) was sampled 30 minutes after the water supply was interrupted; group (iii) was sampled 30 minutes after the 30 minute confinement stressor (US only) and group (iv) was sampled with no disturbance (no confinement). Blood samples were collected as for Experiment 1 and stored frozen at -70^{o}C until required for assay.

Experiment 3: Comparison of the time required to extinction of the conditioned response in HR and LR lines of rainbow trout

The aim of Experiment 3 was to assess whether there was a difference between the HR and LR lines in the time required for extinction of the CR, without further reinforcement. During May 2003, 360 rainbow trout from each of the two selected lines (CEH originated HR and LR lines; Pottinger and Carrick, 1999; F2 generation; 1+ years old; mean weight HR: 241 ± 5 g; LR: 234 ± 5 g) were transferred to 12 experimental tanks (60 fish tank^{-1}), with six tanks each of HR and LR fish. Environmental and husbandry conditions were as described for Experiments 1 and 2. The fish were allowed to acclimate to conditions for 10 days before the start of the experimental procedures. During this period the fish were observed to ensure that

there was no evidence of adverse social interaction or disease. The study comprised three experimental groups: (i) control, undisturbed throughout, one tank each of HR and LR fish; (ii) emersion + confinement every day including final day (not preceded by water off; US only, control), one tank each of HR and LR fish; (iii) conditioned groups, four tanks each of HR and LR fish (paired CS-US for 18 days). All 12 of the experimental tanks were sampled on day 19 (day 0 post conditioning) and 7, 14, 21 and 28 days after the cessation of conditioning (10 fish per tank on each occasion). Post-conditioning, the fish in group (iii) were exposed to the CS only on these weekly test days and were sampled 30 mins afterwards. Sampling was conducted as described for the preceding experiments and blood cortisol levels were determined between each weekly test in order to establish at what point the CR was no longer evident and the study could be terminated. To avoid repeat sampling of the same individuals, fish were marked by dermal staining post sampling with alcian blue dye administered with a Panjet needleless injector (Wright Dental Group). After 28 days the analysis of plasma cortisol levels showed complete extinction of the learned response in both lines of fish and the experiment was terminated.

Cortisol analysis

Cortisol was measured in the plasma samples by a validated radioimmunoassay procedure (Pottinger and Carrick, 2001a).

Statistical analysis

For Experiments 1 and 2, one-way analysis of variance (ANOVA, Genstat 5, Lawes Agricultural Trust) was employed to assess the significance of changes in plasma cortisol levels between treatment groups. For Experiment 3, a two-way

ANOVA was employed with treatment and time as factors. For experiments 2 and 3, the ANOVA was nested, with tank as a blocking term. Significant differences between treatment groups were determined using the estimated standard error of the differences between means provided by the Genstat output. The cortisol data were log-transformed prior to analysis because means and variances were found to be interdependent. In Experiment 3, individual fish were classified as either "unstressed" or "stressed" using their plasma cortisol levels after presentation of the CS in the absence of the US during the post-conditioning period as follows. The individual plasma cortisol values were log-transformed to homogenize the variance and to approximate Normal distributions. For the HR group, the 40 control fish from the retrieval period (28 days) were taken to be a representative sample of "unstressed" fish and the 40 experimentally stressed fish were a representative sample of "stressed" fish. The mean and standard deviation of each sample was calculated and from these the two normal distributions were estimated. Each HR fish in Experiment 3 was classified as "stressed" or "unstressed" according to which normal distribution the cortisol response was most likely to have come from. This was achieved by comparing the two normal distributions and locating the point (threshold) where the two curves intersected. Fish with a cortisol level above the threshold were classified as "stressed" and those below the threshold as "unstressed". The same procedure was followed for the LR group. The relative numbers of HR and LR fish classified by this method as stressed and unstressed were compared at each time-point following the cessation of conditioning using a chi-square test.

These studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986 of the United Kingdom.

Results

Experiment 1: Selection of an appropriate conditioned stimulus

Testing the variance ratio using an $F_{6,72}$ distribution indicated an overall significant effect of treatment (ANOVA, $P<0.001$). Mean plasma cortisol levels in fish exposed to CS only (water off, noise, or light) every day, were statistically indistinguishable from plasma cortisol levels in fish undisturbed throughout the experiment (Fig 1). Plasma cortisol levels in fish exposed to the CS only, water off and noise, were significantly greater than levels in fish repeatedly exposed to the CS only, light ($P<0.001$; Fig. 1). Mean plasma cortisol levels in fish exposed to the CS light in the absence of the US on day 7, were not significantly different from levels in any of the control groups.

Experiment 2: Assessment of the time required to achieve a conditioned response

Testing the variance ratio using an $F_{6,7}$ distribution indicated an overall significant effect of treatment (ANOVA, $P<0.001$). Mean plasma cortisol levels in fish exposed to the CS only (water off) every day, and in fish exposed to the US only (emersion + confinement), on every day except the final day (i.e. control for prolonged activation of the HPI axis arising from prior repeated exposure to US) were statistically indistinguishable from plasma cortisol levels in fish that remained undisturbed throughout the study (Fig. 2). Plasma cortisol levels in fish exposed to the US only, on every day, including the final day, were significantly higher ($P<0.001$) than those in the control fish. After exposure to CS-US pairings for 8 days, the mean plasma cortisol level when exposed to the CS only, on the final day was not significantly higher than levels in the undisturbed, water off and repeat (US) stress

groups. A significant difference in plasma cortisol levels was evident between the control groups and the groups conditioned for 12 days (P<0.001) and 18 days (P<0.01).

Experiment 3: Comparison of the time required to extinction of the conditioned response in HR and LR lines of rainbow trout

Figure 3 depicts the mean plasma cortisol levels in the experimental groups 0, 7, 14, 21 and 28 days after conditioning ceased (following 18 days of CS-US pairings). The ANOVA ($F_{3,366}$) indicated a significant interaction between treatment (control or conditioned), time (0, 7, 14, 21, 28 days) and line (HR, LR) overall (P=0.031).

Figure 3a shows the mean plasma cortisol level in each treatment group when exposed to the CS only, after 18 days of CS-US pairings (day 0). Mean plasma cortisol levels in fish within both conditioned groups (HR, LR) were significantly greater (P<0.001) than levels in the corresponding control groups. There was no significant difference in mean plasma cortisol levels between the HR and LR controls.

Figure 3b shows the mean plasma cortisol level in each treatment group when exposed to the CS only, 7 days after conditioning ended. Mean plasma cortisol levels in fish within both conditioned groups (HR, LR) were significantly greater (P<0.001) than levels in the corresponding control groups. There was no significant difference in mean plasma cortisol levels between the HR and LR controls.

The results after testing at 14 days following the cessation of conditioning are shown in Figure 3c. Mean plasma cortisol levels in conditioned LR fish remained significantly greater than those in the corresponding control group (P<0.001) on exposure to the CS only, whereas plasma cortisol levels in the conditioned HR group

were not significantly different from those in the undisturbed control HR group. There was no significant difference in mean plasma cortisol levels between the HR and LR controls.

At 21 days (Fig. 3d) and 28 days (Fig. 3e) after conditioning ended, mean plasma cortisol levels in both HR and LR conditioned groups, after exposure to the CS, were not significantly different from levels in the undisturbed control fish. There was no significant difference in mean plasma cortisol levels between the HR and LR controls on Day 28 but on Day 21 levels in control LR fish were significantly greater than those in HR fish ($P<0.05$). The control fish, exposed to the US only throughout the conditioning period, responded to emersion + confinement on each test day with a significant elevation of plasma cortisol (data not shown).

The results of Experiment 3 are also presented in Figure 4 as a comparison of the proportion of fish within each line (HR, LR) at each time point following the end of conditioning (0, 7, 14, 21, 28 days) that can be classified as "stressed". There was no significant difference in the proportion of stressed fish among the HR and LR lines immediately following the end of conditioning (day 0) or 7 days after conditioning. However, at 14 and 21 days after the end of conditioning there were significantly more individuals categorized as "stressed" among the LR fish than HR fish, with no HR fish falling into this category at 21 days. At 28 days post-conditioning, there were no HR fish and only three LR fish that were categorized as "stressed".

Discussion

The results of this study clearly demonstrate that rainbow trout can be conditioned to display an elevation of plasma cortisol in response to a conditioned

stimulus (CS), in the same way as has been shown for Nile tilapia (Moreira and Volpato, 2004).

The CS used for single Nile tilapia held in glass aquaria (light on; Moreira and Volpato, 2004) was not appropriate for use in large volume outdoor tanks containing groups of rainbow trout. This was presumably because the fish failed to detect the cue, a failure that was possibly due to the size of the tanks, their outdoor situation, the strength of the signal, or a combination of these factors. Instead, we found that a short (5 mins) interruption of the water supply to the tank was a suitable CS. The cue was not inherently stressful, with no elevation of plasma cortisol evident in fish exposed to the cue on a single occasion or over multiple exposures during this study. Detection of the cue by the fish within the tank may have been related to the cessation of noise/vibration associated with the inflow, the reduction in flow associated with the interruption of the water supply, or alterations in surface patterns/disturbance brought about by the water-off signal. Water-off operated as a classical Pavlovian conditioned stimulus (CS) to the fish within the tank and became associated with the unconditioned stimulus (US), the emersion/confinement stressor, in the majority of individuals following repeated CS-US pairings.

The conditioned response (CR) was acquired by the majority of fish sampled within at least 12 days of exposure to the paired CS-US. However, even by extending the conditioning period to 18 days the range of plasma cortisol levels present among individuals sampled 60 mins after exposure to the CS suggests that not all individual fish within the tank acquired the CR during the conditioning period – a proportion of individuals displayed plasma cortisol levels approaching those of the control, undisturbed, fish. It is possible that the CS was not detected by all individuals within the tank on every CS-US pairing. It may also be the case that the dynamics of the

response exhibited by individuals varied to such an extent that at the selected sampling point plasma cortisol levels remained elevated only in those individuals displaying the most pronounced anticipatory stress responses at 60 mins following the CS. The results of Experiment 3 suggest that at best 70% of individuals exposed to the paired CS-US for 18 days acquired a conditioned response. This uncertainty remains to be resolved if this experimental protocol is to be exploited further but may relate to the normal range of cognitive function evident within any population.

The retention of the CR by HR and LR fish, after an 18-day period of conditioning, was compared in two ways. The decline over successive weekly sampling points in mean plasma cortisol levels for the conditioned HR and LR fish suggests that there was a more rapid loss or extinction of the CR among the HR fish. In this group mean plasma cortisol levels in conditioned fish, on presentation of the CS, were not significantly different to those in control fish from between 7 – 14 days after the end of the conditioning period. In contrast, mean plasma cortisol levels in the LR fish remained significantly greater than those in the control group on presentation of the CS until between 14 and 21 days. A more pronounced difference between the groups is evident if the frequency of individuals whose plasma cortisol levels indicate a stress response, rather than the overall mean plasma cortisol levels of the groups, is compared. In this case the number of individuals responding to the CS with a significant elevation of plasma cortisol level was greater among the LR fish at 14 and 21 days after the end of conditioning. Overall, we interpret these results as indicating that learned responses to external cues are retained for longer by LR fish than HR fish in the absence of any reinforcement.

The disparity observed in the time required for extinction of the CR between the lines may have arisen because of differences in cognitive function between the

lines of fish either: during learning and memory consolidation; during the delay phase (between consolidation and retrieval); or at the time of retrieval. There are data indicating that rodent lines divergent for behavioral and endocrine traits that are analogous to those observed in the HR and LR trout lines also exhibit differences in learning (see for example Cabib et al., 1996; Uvnas-Moberg et al., 1999). Examination of the possible mechanistic bases for the apparent difference in memory retention of the HR and LR fish is complicated by the fact that it is unclear from this preliminary study how rapidly the CR was initially acquired by individuals within each line. One obvious possibility, given the divergent endocrine response to stress between these lines, is that the cognitive processes involved in acquiring and sustaining the CR were differentially modulated by elements of the HPI axis.

In mammals corticosteroids are believed to both enhance and impair learning and memory (Roozendaal, 2002; Wolf, 2003), the effect exhibited being dependent upon the circulating levels of corticosteroids and the timing of the elevation. For example, early developmental exposure to elevated corticosteroids may impair subsequent cognitive performance (Kitaysky et al., 2003). The possibility of unavoidable exposure to stress during early development of the HR and LR lines cannot be excluded. Chronically elevated corticosteroid levels have adverse effects on learning and memory (Starkman et al., 2001) and memory retrieval is severely impaired by exposure to short-term elevation of corticosteroid levels when administered prior to testing (de Quervain et al., 1998). In the present study the fish were not subject to any stressors before, during or after conditioning, other than the acute stressor (emersion + confinement) used for conditioning itself. Nor were they exposed to any stressful stimuli during the 18-day retrieval trial. Elevation of plasma cortisol levels, where it occurred during this period, arose as a consequence of the

acquired CR. Therefore, if cortisol plays any role in the observed results it is more likely that this occurs during the acquisition/consolidation of the CR, not retrieval.

In addition to reports of adverse effects of corticosteroids on cognitive processes, the apparently positive role played by corticosteroids under certain circumstances is also well-documented. Roozendaal (2002) reviewed the evidence for positive effects of corticosteroids on memory formation and concluded that the elevation of blood corticosteroid levels immediately post-training may enhance memory consolidation, a conclusion supported by others (Beylin and Shors, 2003; Shors, 2001). In the present study plasma cortisol levels were higher in the HR line than in the LR line during exposure to the paired CS-US. Subsequent testing did not suggest that acquisition of the CR and/or memory consolidation was in any way enhanced in the HR line, given that the CR was lost more rapidly by this line than the fish of the LR line.

The influence of corticosteroids on memory function is not restricted to positive or negative effects of supra-baseline levels. Manipulation of plasma cortisol levels at or below baseline levels in human subjects has demonstrated that memory recall could be adversely affected by reducing circulating levels of cortisol (Lupien et al., 2002). In this study, and in others (Pottinger and Carrick, 1999), we did not observe any systematic differences in baseline plasma cortisol levels between the HR and LR lines.

Overall, there seems little evidence at this stage to suggest that the divergence in post-stress plasma cortisol levels by which the HR and LR lines are characterized is causally linked to differences in the rate of extinction of the CR between the two lines. It is nonetheless possible that other elements of the HPI axis could contribute to the observed differences. For example, CRH may be directly involved in the

acquisition of a conditioned response (Croiset et al., 2000; Eckart et al., 1999; Wu et al., 1997). However, we have no evidence that there are differences in hypothalamic CRH secretion in the HR and LR lines – plasma ACTH levels are indistinguishable in the two lines during stress (Pottinger and Carrick, 2001b). In the CNS, selection for divergent stress responsiveness in the HR and LR lines of rainbow trout has resulted in regional divergence in brain monoaminergic activity. In HR fish exposed to a stressor there was an increase of serotonin and dopamine in the brain stem, and norepinephrine in the optic tectum and telencephalon. These changes were not observed in LR fish exposed to the same stressor (Øverli et al, 2001). The significance of these differences to cognitive processes are difficult to speculate upon - while it is accepted that the serotonergic system plays a role in cognition (Cassel and Jeltsch, 1995) the precise nature of that role remains controversial (Sarihi et al., 2000). There is also strong evidence in higher vertebrates that the noradrenergic system is required for the enabling of corticosteroid-mediated effects on cognition. These effects of the noradrenergic system are focused upon the basolateral amygdala, a key structure that regulates wider effects within the brain on memory consolidation and retrieval (Roozendaal, 2003). In the absence of appropriate data from the HR and LR lines the relevance of these findings to the present study is difficult to assess.

In conclusion, this study provides preliminary evidence that there are differences in cognitive function between two lines of rainbow trout selectively bred for divergent plasma cortisol responses to stressors. This is consistent with earlier findings that the two lines exhibit different behavioral traits and provides evidence for a link between endocrine function, behavioral traits, and cognitive performance in fish. Further studies are required to establish the functional significance of these findings and whether the apparent differences in cognitive function observed in this

study are causally linked to the neuroendocrine divergence evident in the selected lines.

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Figure Legends

Figure 1. Plasma cortisol levels in undisturbed rainbow trout (control) and in rainbow trout exposed to a conditioned stimulus only (water, noise, light; CS only) or, after a 6 day period of exposure to CS-US pairings exposed to the CS only, (paired CS-US). Each bar denotes the mean ± SEM, n = 10. Significant differences between CS only groups and the corresponding conditioned group are denoted by *** $P < 0.001$; NSD: no significant difference.

Figure 2. Control groups: plasma cortisol levels in undisturbed rainbow trout (undisturbed); trout exposed to the CS only on every day (water off); trout exposed to the US only on every day except day 19 (-US final day); trout exposed to the US on every day including day 19 (+US final day). Conditioned groups: trout exposed to the paired CS-US on every day for periods of 8, 12 and 18 days (8d, 12d, 18d) except the day of sampling (one day after the end of conditioning) when they received only the CS. Each bar denotes the mean ± SEM, n = 10. Significant differences between treatment groups and the control group are denoted by *** $P < 0.001$, * $P < 0.05$.

Figure 3. Plasma cortisol levels in undisturbed rainbow trout of the HR and LR lines (control) and in HR and LR fish exposed to the CS only (conditioned) at intervals of (a) 0 days; (b) 7 days; (c) 14 days; (d) 21 days and (e) 28 days post-conditioning. Each bar is the mean ± SEM, n = 10 (control); n = 40 (conditioned). Significant differences between HR and LR conditioned groups and their corresponding controls are denoted by asterisks immediately above the bars. The results of comparisons between HR and LR fish within control treatment groups are indicated by asterisks

adjacent to horizontal lines above the bars: *** $P<0.001$, * $P<0.05$, NSD = no significant difference.

Figure 4. The proportion of HR and LR fish that can be categorized as stressed at each time point post-conditioning on the basis of their individual plasma cortisol levels. Significant differences between lines in the proportion of fish that could be categorized as stressed are denoted by: *** $P<0.001$, NSD = no significant difference.

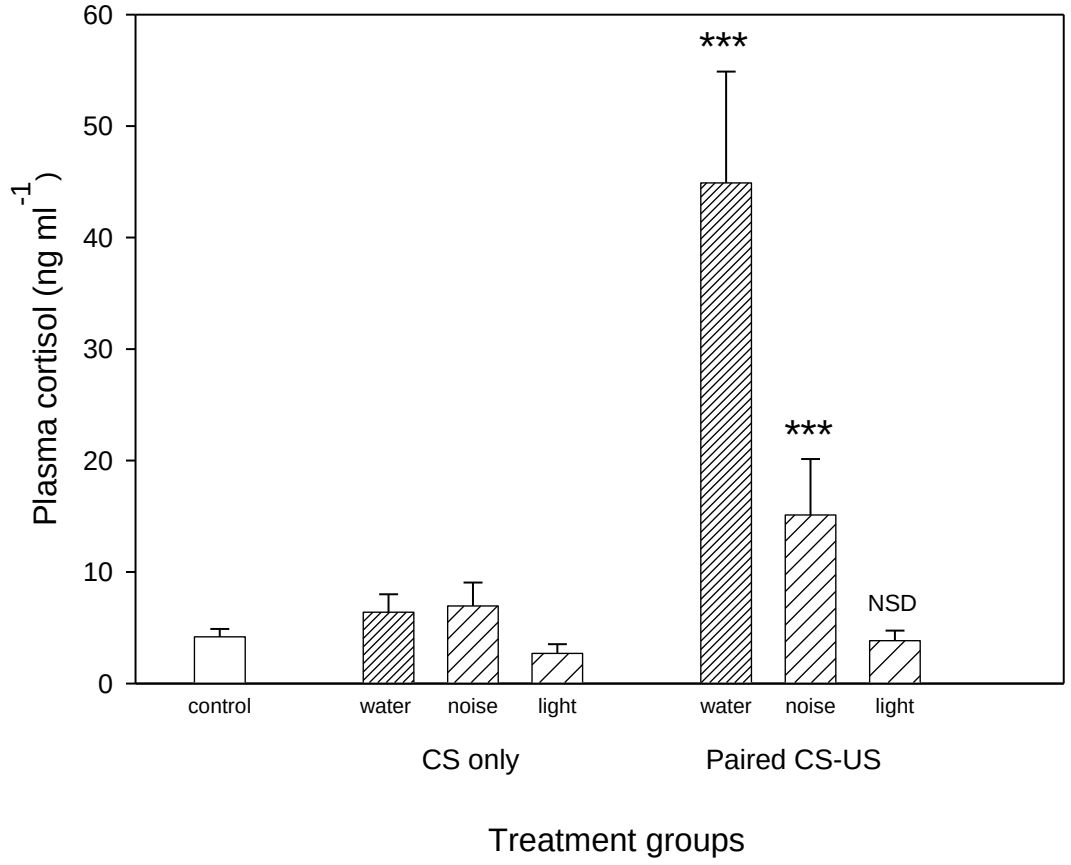

Figure 1. Moreira, Pulman, Pottinger

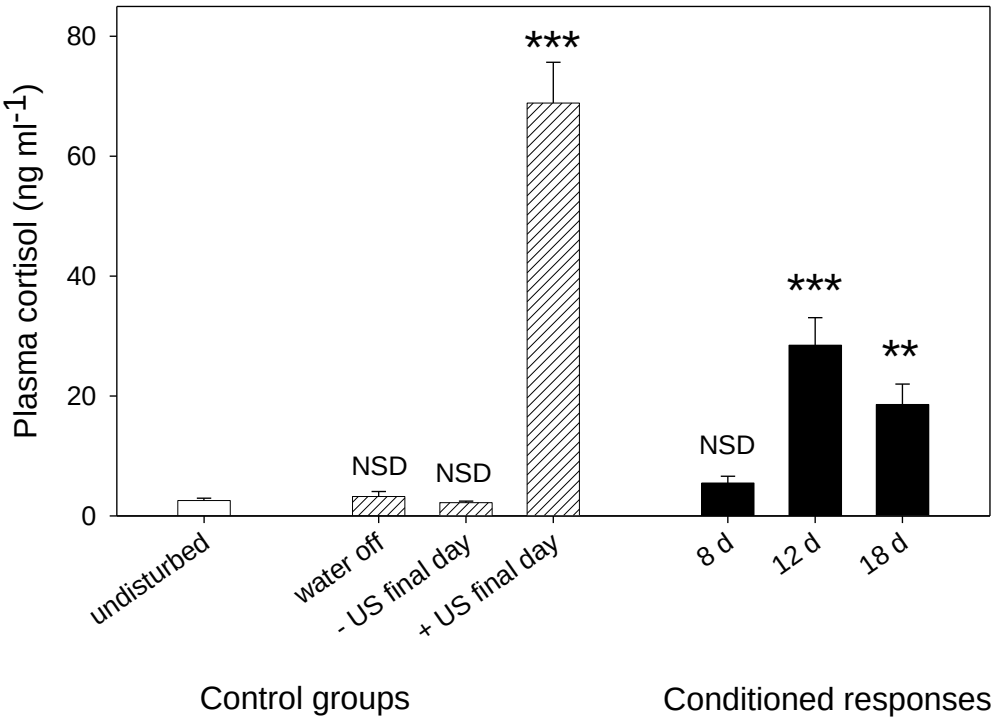

Figure 2. Moreira, Pulman, Pottinger.

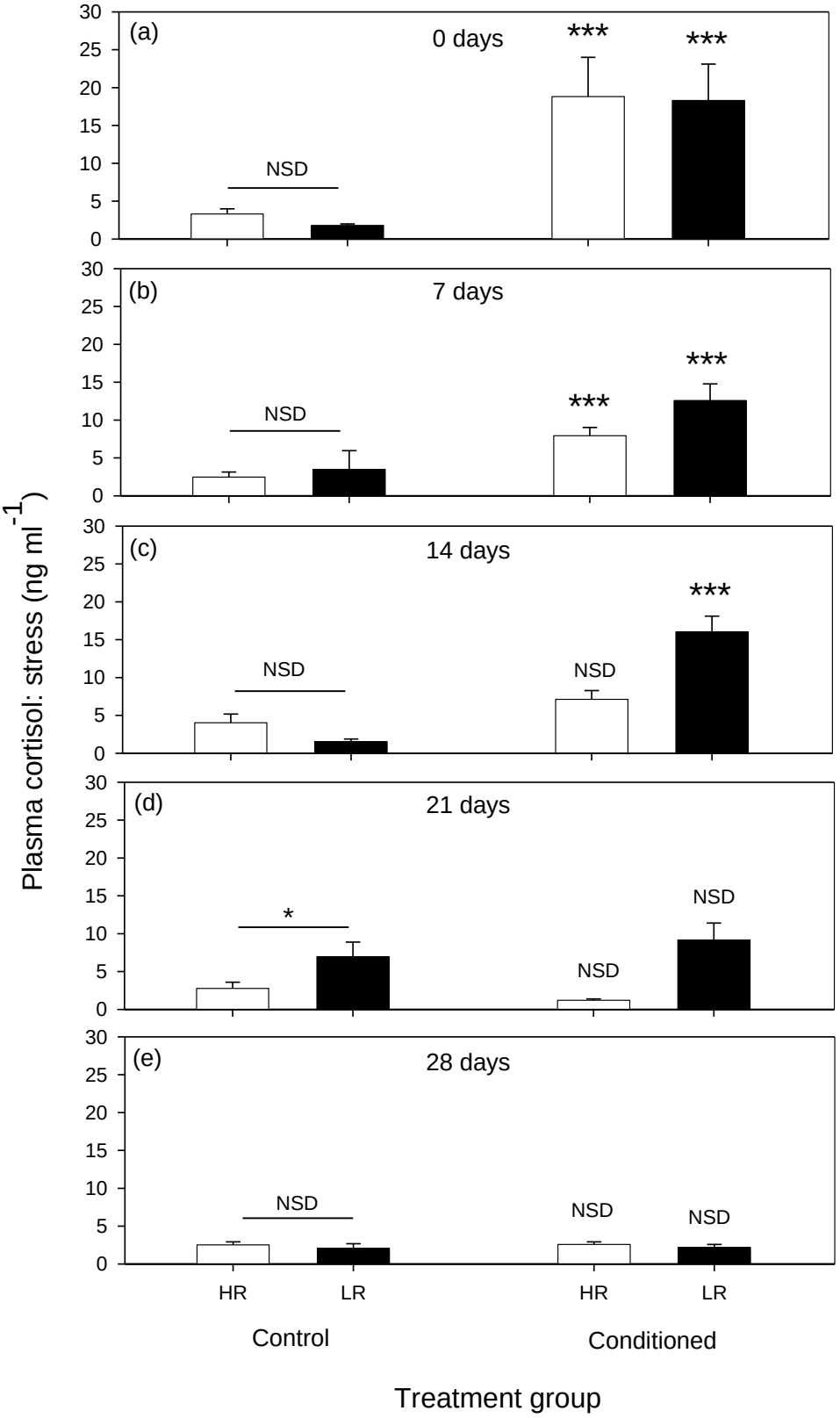

Figure 3. Moreira, Pulman, Pottinger

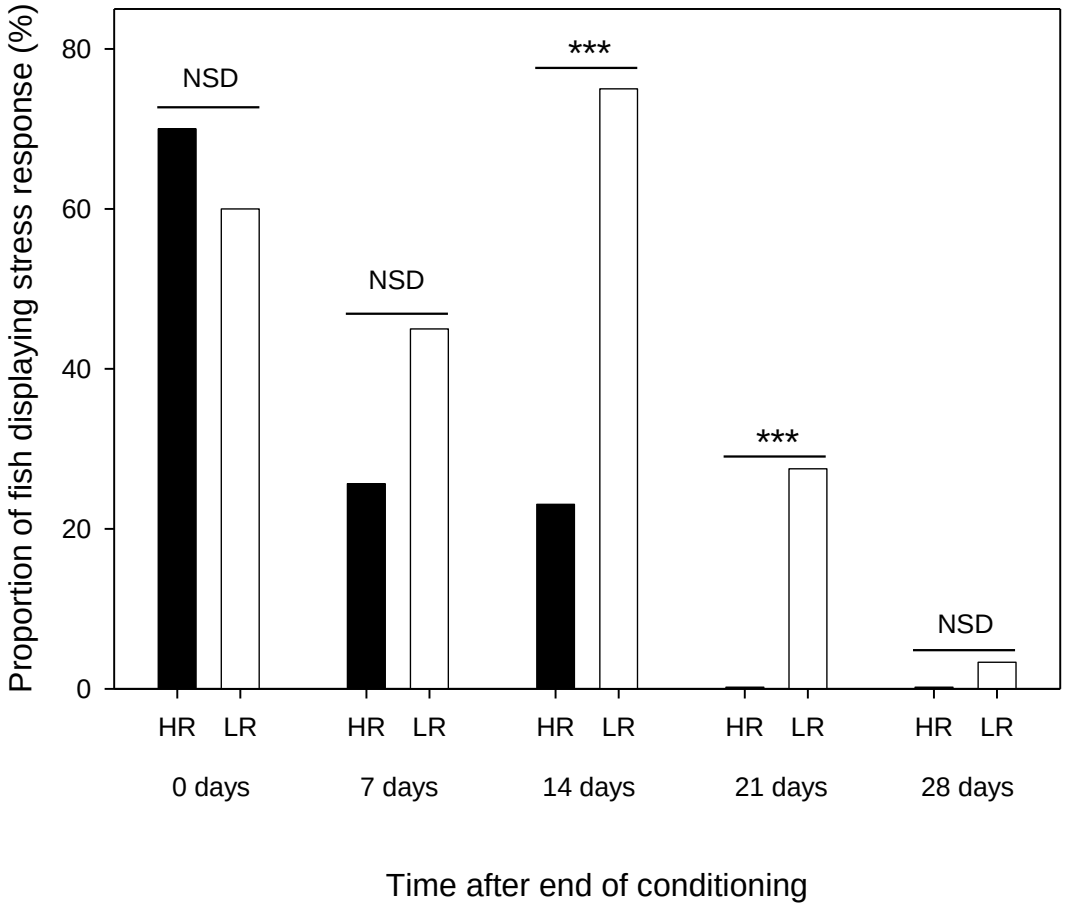

Figure 4. Moreira, Pulman, Pottinger.