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Modification of the plasma cortisol response to stress in rainbow trout by selective breeding

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Running head: Selection for stress responsiveness

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ABSTRACT

Male and female rainbow trout were segregated into high- and low-responding individuals (HR, LR) on the basis of their plasma cortisol response to a 3 h period of confinement imposed at five, monthly, intervals. Consistent divergence was obtained in the responsiveness of the two groups, although the difference between LR and HR groups was greater in female fish (56 c.f. 116 ng ml⁻¹) than in males (45 c.f. 69 ng ml⁻¹). Progeny groups (full-sib families) were obtained from the pairing of HR males and females and LR males and females. A third progeny group (US) was obtained by random pairing of parents which were not selected as HR or LR. Post-stress plasma cortisol levels in the progeny were first tested at 6 months after hatch and were significantly correlated with the response of the corresponding parental groups, HR>US>LR (178, 126, 81 ng ml⁻¹ respectively). The difference in responsiveness between LR and HR groups was demonstrated in all four subsequent tests over a 12 month period. There were no significant differences in baseline plasma cortisol levels in LR and HR groups prior to confinement. During a 4h period of confinement, the differences in plasma cortisol levels between LR and HR fish were sustained throughout, indicating that the trait upon which the fish were selected was related to absolute maximum levels of circulating cortisol, not the rate of change of cortisol levels during exposure to a stressor. A moderately high heritability (h^2) for confinement-induced plasma cortisol of 0.41 was obtained by a parent-progeny regression. Manipulation of stress-responsiveness in fish by selective breeding offers scope for optimising performance under intensive rearing conditions but also provides a useful research tool for investigating the operation of the endocrine stress response.

INTRODUCTION

The magnitude of the stress response in vertebrates varies among individuals within a population (Berger *et al.*, 1987; Pottinger *et al.*, 1992; Von Borell and Ladewig, 1992; Cummins and Gevartz, 1993; Marsland *et al.*, 1995). Because growth, reproduction, and disease resistance are affected by chronic or intermittent activation of the hypothalamic-pituitary-adrenal/interrenal axis (Johnson *et al.*, 1992) there have been efforts to modify the responsiveness of this system to stressors in animals of economic significance which are often reared under intensive, unavoidably stressful, conditions (Pottinger and Pickering, 1997). The degree to which the stress susceptibility of an individual might be inherited has been considered in man (Rosch, 1997). Modification of the stress response in domestic fowl by selective breeding has long been considered feasible (Brown, 1959). The stress responsiveness of turkeys (*Meleagris gallopavo*; Brown and Nestor, 1973), chickens (*Gallus domesticus*; Gross and Siegel, 1985) and the Japanese quail (*Coturnix coturnix japonica*; Satterlee and Johnson, 1988) have been modified by selective breeding strategies, with varying degrees of success. A similar approach has been considered with respect to aquacultured fish in which frequent or prolonged exposure to stressors significantly and adversely affects growth (Pickering, 1993; Pankhurst and Van Der Kraak, 1997), reproductive performance (Pickering *et al.*, 1987a; Campbell *et al.*, 1992, 1994), the immune system (Balm, 1997) and flesh quality (Lowe *et al.*, 1993; Sigholt *et al.*, 1997). It is argued that in an environment in which stressful stimuli are frequent or prolonged, fish which possess a low level of responsiveness to stress will be less severely affected than fish displaying a more pronounced reactivity to stress (Pottinger and Pickering, 1997). This assumption is currently being tested in a multi-national selective breeding project, part of whose aim is to assess the feasibility of

modifying endocrine stress-responsiveness in aquacultured rainbow trout, *Oncorhynchus mykiss*.

The level of cortisol in the blood of fish is a robust index of stress (Barton, 1997) and, because cortisol is directly implicated in many deleterious effects of stress, stress-induced cortisol levels provide a well-defined trait of functional significance upon which selection pressure can be directed. Strain differences in cortisol responsiveness to stressors have been demonstrated in fish (Pickering and Pottinger, 1989; Pottinger and Moran, 1993) in that: the relative magnitude of the plasma cortisol response to stress of individual rainbow trout is a stable trait within a proportion of the population (Pottinger *et al.*, 1992); and pooled-gamete crosses of fish selected for low- and high-cortisol response to stress, generate progeny which display similar traits to the parental groups (Pottinger *et al.*, 1994). Quantitative studies have revealed that the heritability of the cortisol response to stress in rainbow trout is moderate to high (Fevolden *et al.*, 1993; 1999). These results suggest that modifying the magnitude of the endocrine stress response in fish, with the attendant benefits this would bring, is technically feasible.

This paper describes the modification of the plasma cortisol response to confinement in rainbow trout by selective breeding. The selection process which was adopted to identify parent fish with divergent cortisol stress responsiveness is described together with an evaluation of the activity of the pituitary-interrenal axis in the progeny of selected individuals.

MATERIALS AND METHODS

Fish

During February 1996, two hundred and fifty two-year old mixed sex rainbow trout (*Oncorhynchus mykiss*; Stirling strain) were divided evenly between ten 1500 liter holding tanks, each supplied with a constant flow of lake water (25 liters min⁻¹). As far as was possible the fish were segregated on the basis of sex, after external examination resulting in six tanks of female fish and four tanks of male fish (approx. 25 fish/tank). Each fish was weighed, measured, and individually tagged with a passive integrated transponder (PIT) tag (Fish Eagle Co.) inserted into the peritoneal cavity of each fish. Subsequently, PIT tags were implanted into the dorsal musculature because of tag loss associated with the ejection of eggs from the body cavity in ripe females.

Stress testing of parent fish

One month after the fish were distributed and tagged, and at monthly intervals following for a total of 5 months (March – July), the fish from each holding tank were transferred, in turn, to four 50 liter confinement tanks, 6-7 fish per confinement tank. Each confinement tank was supplied with a constant flow of lake water (15 liters min⁻¹). After 3 h confinement, the first group of fish was transferred to anaesthetic (2-phenoxyethanol, 1:2000) and a blood sample (~0.5 ml) was removed from the Cuvierian sinus of each fish into a heparinized syringe. After blood sampling, each fish was identified by PIT tag, and weight and length were recorded before the fish was returned to its original holding tank to recover. The blood samples were kept on ice until centrifugation. Plasma was stored frozen (-20°C) until required for assay. Plasma cortisol levels

were determined by a validated radioimmunoassay procedure (Pickering *et al.*, 1987b)

It was intended to complete the selection procedure within 6 months. However, during the period February to May, problems were encountered with a particularly virulent outbreak of fungal disease (*Saprolegnia spp.*). The pathogen did not respond satisfactorily to the normal therapeutic procedure (flush treatment with malachite green) and 80% of the male fish died as a result of the fungal infection or were killed to alleviate suffering. Mortalities among the female fish were minimal (2%). Consequently, an additional group of 100 rainbow trout of the same age (Stannan strain) were obtained to provide the required number of male fish. It was not possible to establish the sex of these fish because no secondary sexual characters are discernible in rainbow trout during the interspawning period, so the fish were distributed randomly between four 1500 liter tanks. These fish were subjected to the same regime of periodic confinement stress and blood sampling as the original group of fish, during the period June - October. All the males used in the breeding study were derived from this group.

Identification and segregation of high- and low-responding parent fish

The mean post-stress plasma cortisol level, across all five episodes of confinement, was calculated for each fish and the fish within each tank were ranked on the basis of this figure. The four most highly ranked fish and the four least highly ranked fish in each tank were designated HR and LR respectively. The fish selected as HR and LR were removed from their Ahome tanks and segregated on the basis of sex into tanks containing only male or female HR or male or female LR. When mature, the HR and LR fish were used to generate 24 HR and 24 LR progeny groups. In order to facilitate the calculation of heritabilities each progeny group comprised of ova from a single female fertilized with milt from a single male. Six groups of male and female fish

were randomly selected from the fish which had not been designated HR or LR and were used to generate six random-bred (unselected, US) progeny groups. Because of restrictions on space and manpower, the original 24 families derived from LR and HR parents were reduced to 15 families of each by discarding 9 randomly selected families from each group. One further batch of LR eggs was lost reducing the total number of LR families to 14.

Measurement of stress-induced plasma cortisol levels in the progeny groups

The fertilized ova were reared in individual family groups within mesh-bottomed trays in incubator troughs and were transferred to stainless steel mesh baskets within 1500 liter holding tanks when swim-up and first feeding occurred. The stress responsiveness of the HR and LR progeny groups was first tested in August 1997. Eight fish (individual weight –10 g) were netted from a holding tank and placed in a 500 ml glass beaker containing water which was aerated. After 3 h the fish were transferred to anaesthetic (2-phenoxyethanol; 1:2000), killed by spinal section, and then frozen individually in labelled polybags. This process was repeated for each of the F1 HR and LR progeny groups. Whole-body total immunoreactive corticosteroid levels were determined for each fish (Pottinger and Mosuwe, 1994). Individual fish were homogenized in distilled water (1:5 weight:volume) using an IKA Ultra-Turrax (T18/10) homogenizer. The homogenate was extracted with ethyl acetate and assayed for immunoreactive corticosteroids as per plasma samples.

On five subsequent occasions (September, October, and November 1997, May and September 1998), sub-samples of six fish from each HR and LR progeny group were subjected to a confinement stressor and plasma cortisol levels were determined. On one occasion (October

1997), the US progeny groups were also tested. The fish were transferred to stainless steel mesh baskets suspended in 50 liter confinement tanks, supplied with a constant flow of lake water, and after a period of 3 h the fish were transferred to a bucket containing anaesthetic (2-phenoxyethanol; 1:2000). Blood samples were obtained from anaesthetized fish either from the caudal vessels after severance of the tail or from the caudal vessels by hypodermic syringe and needle, depending on the size of the fish. All blood samples were heparinized and stored on ice until centrifugation. Plasma was stored frozen until required for assay. The fish were killed by a blow to the head, weighed, measured and sexed. Cortisol levels were determined in each plasma sample.

The time-course of changes in blood cortisol levels in HR and LR fish during confinement

During May 1998 six fish from each family were netted rapidly from their holding tank into a bucket containing anaesthetic. Blood samples (0.25 ml) were removed from the Cuvierian duct into a heparinized syringe and kept on ice. The fish were individually marked using alcian blue dye administered with a Panjet needleless injector (Wright Dental Group), to allow their identification at subsequent sample points during the time-course study, and transferred to a 50 liter confinement tank supplied with a constant flow of lake water. Additional blood samples were taken from each fish at 1, 2 and 4 h after the initial sample. At the final (4 h) sample the fish were killed by a blow to the head, weighed, measured and sexed. Cortisol levels were determined in each plasma sample.

Calculation of the heritability of the cortisol stress response

The term heritability (h^2), when applied to a metric character, expresses the proportion of the total variance of the trait that is attributable to the average effects of genes (Falconer, 1989) and

is estimated from the degree of resemblance between related individuals. In the present study, the trait was defined as the level of plasma cortisol measured after a 3 h period of confinement. Estimates of cortisol stress responsiveness were available for both parents and offspring allowing the calculation of heritability based on offspring and one parent, or offspring and the mid-parent value. The mean cortisol response to confinement of each parent fish (for the five occasions on which it was tested) were plotted against the mean cortisol response of each corresponding progeny group (for the five occasions on which each was tested). The regression line of parents on progeny provided an estimate of h^2 .

Statistical analysis

Multifactorial analysis of variance (ANOVA, Genstat 5, Lawes Agricultural Trust, Rothamsted Experimental Station) was employed to assess the significance of changes in plasma cortisol levels with time and differences within and between groups. Where appropriate, the data were log-transformed to improve homogeneity of variance.

RESULTS

Identification and segregation of high- and low-responding parent fish

Figure 1 shows the mean plasma cortisol levels following confinement in the fish selected as HR and LR (F0, parents) for each occasion on which the fish were tested. Overall, the difference between plasma cortisol levels in HR and LR females (Fig. 1a) was highly significant and consistent throughout ($P < 0.001$). The overall mean cortisol levels in each group over the entire

testing period were $56.4 \pm 3.4 \text{ ng ml}^{-1}$ (LR, $n = 70$) and $115.7 \pm 5.6 \text{ ng ml}^{-1}$ (HR, $n = 75$). The differences in mean post-confinement plasma cortisol levels between selected male HR and LR fish (Fig. 1b) were not so pronounced as for the females, although overall the difference between the two selected groups was highly significant ($P < 0.001$; LR: $45.2 \pm 3.2 \text{ ng ml}^{-1}$; HR: $69 \pm 5.2 \text{ ng ml}^{-1}$).

Determination of stress-induced plasma cortisol levels in the progeny groups

Stress-induced cortisol levels were determined in the F1 progeny groups (families) on a total of six occasions. On the first occasion, in August 1997, 5 months after hatching, the fish were too small to provide blood samples. Instead, whole-body immunoreactive corticosteroid (IRC) levels were determined following a period of confinement. IRC levels were found to be significantly higher ($P < 0.001$) in the progeny of LR parents ($131 \pm 6.3 \text{ ng ml}^{-1}$ extract) than in those of HR parents ($97.3 \pm 5.1 \text{ ng ml}^{-1}$ extract).

On the subsequent five occasions, when post-confinement blood cortisol levels were determined, the progeny of HR fish consistently displayed plasma cortisol levels which were significantly greater than those in the progeny of LR fish ($P < 0.001$; Fig. 2). There was a significant change in maximum plasma cortisol levels following confinement over time, with levels in HR fish during September 1997 of $93.3 \pm 5 \text{ ng ml}^{-1}$ contrasting with levels in HR fish in May 1998 of $310 \pm 14 \text{ ng ml}^{-1}$ (Fig. 2). Levels in September 1998 were similar to those in September 1997. The significant difference between HR and LR progeny groups was sustained throughout.

Identification of families displaying the most divergent responses

The mean cortisol response to confinement for the five occasions on which blood samples were

collected from the F1 fish are presented for each HR and LR progeny group (family) in Fig. 3. The families have been ranked in order of magnitude of the mean response to confinement and within each selection group the families whose mean response differs significantly from the lowest (for LR) or highest (for HR) responding family are identified. There was a substantial overlap between the families with the lowest mean cortisol response within the HR group and the highest-responding families from the LR group. To maximise the contrast between the HR and LR groups, the families with the most divergent mean responses were used for future work. Consequently the six LR families with lowest mean responses to confinement and the six HR families with the highest responses to confinement were identified.

The plasma cortisol levels in US groups following confinement are presented in Fig. 4 together with plasma cortisol levels in the six most divergent HR and LR families, sampled at the same time. There was a highly significant ($P < 0.001$) difference in plasma cortisol levels between all the groups, with levels in the US fish ($126 \pm 10 \text{ ng ml}^{-1}$) midway between those of the HR ($178 \pm 12 \text{ ng ml}^{-1}$) and LR ($81 \pm 5 \text{ ng ml}^{-1}$) groups.

The time-course of changes in blood cortisol levels in HR and LR fish during confinement

Data for fish from the six most divergent HR and LR families are presented (see above). There were no significant differences in blood cortisol levels between unstressed HR and LR fish prior to confinement (8.0 c.f. 8.1 ng ml^{-1} , time 0, Fig. 5). Within 1 h of the onset of confinement there was a significant elevation of blood cortisol in both HR and LR. This increase was significantly greater in the HR group than the LR group; the mean increment between the selected HR and LR families was 179 ng ml^{-1} ($P < 0.001$; Fig. 5). Blood cortisol levels remained elevated in both groups, though declining significantly ($P < 0.05$), for the remainder of the confinement period (4

h) and the significant difference between HR and LR fish was sustained throughout.

Calculation of the heritability of the cortisol stress response

The regression of mid-parent cortisol response on progeny cortisol response is presented in Fig. 6. The regression was highly significant ($P = 0.0002$; $y = 0.9034x + 73.401$) and produced an r^2 ($= h^2$) value of 0.4138. Male and female parent-progeny regressions provided estimates of h^2 of 0.27 and 0.41 respectively.

DISCUSSION

The divergence in post-confinement plasma cortisol levels which was achieved in selected parent female fish was substantial. An approximately two-fold difference in stress-induced cortisol levels between high- and low-responding individuals was sustained throughout the selection period (March – July). This is consistent with data from a previous study in which a similar selection procedure was carried out (Pottinger *et al.*, 1992). In male fish the divergence was less pronounced, although significant overall. The males were tested during the later part of the year (June – October), when gonadal steroid levels in maturing male rainbow trout are increasing (Scott *et al.*, 1980). It is known that there is an androgen-dependent attenuation of stress-induced plasma cortisol levels in trout (Pottinger *et al.*, 1995, 1996) and this may have contributed to the lower stress-induced cortisol levels observed in the male fish.

There were no significant systematic differences in the reproductive characteristics of the HR and LR parents; the onset and rate of ovulation was similar in females of both groups, there were no

differences in sperm counts between males from the two groups and the mortality rates of fertilized ova were low for both HR and LR groups (T. G. Pottinger and T. R. Carrick, unpublished). Fifteen families of HR progeny, fourteen families of LR progeny, and six families of unselected (US) progeny were generated. Because of the size of the fish on the first occasion on which the response of the progeny to a confinement stressor was assessed (5 months post-hatch) and the difficulty of obtaining sufficient blood, whole-body corticosteroid (WBC) levels were measured. There was a significant difference in WBC levels between the two progeny groups but not in the expected direction; higher levels of WBC were detected in LR progeny after confinement than in HR progeny. In all subsequent tests, in which plasma cortisol, rather than WBC level, was determined, HR fish displayed a higher response than LR fish. It seems unlikely that the relative responsiveness of the two progeny groups was reversed on this first occasion and more probable that the anomaly is due to the inclusion of corticosteroids from tissue compartments other than the blood. The measurement of WBC levels is adequate for detecting stress-induced activation of the pituitary-interrenal axis (Pottinger and Mosuwe, 1994; Barry *et al.*, 1995) when comparing stressed and unstressed individuals. However, if the post-stress plasma cortisol levels of HR and LR fish result from differences in tissue distribution, metabolism, or clearance rates of cortisol, levels of corticosteroids from whole-body extracts may not necessarily reflect the differences detected in blood cortisol levels.

On every subsequent occasion on which the plasma cortisol levels of the progeny groups were measured following exposure to a confinement stressor, cortisol levels in HR fish were significantly higher than those in LR fish. The consistency of this difference over time was considerably greater than that observed in a previous study which employed pooled gamete crosses (Pottinger *et al.*, 1994) rather than the single male and female crosses used in the present

study. The difference in mean post-confinement plasma cortisol between the HR and LR progeny groups considered as a whole (~25%) was less than that of the corresponding female parents (~50%). However, there was considerable variation in the overall performance of individual families within the HR and LR groups, determined as mean post-confinement plasma cortisol levels. If only the six most divergent HR and LR families are considered, the difference in post-confinement cortisol levels between the HR and LR progeny groups was closer to 50%. The unselected (US), or random-bred fish, whose parents had been randomly selected from the F0 population, displayed a response to confinement which was midway between that of the HR and LR groups. In contrast, in Japanese quail selected for divergence in post-immobilization serum corticosterone levels, deviation from the random-bred controls line was more rapid in the high-response line than the low-response line, which required several generations of selection to achieve a significant change (Satterlee and Johnson, 1988). In turkeys, significant differences in the plasma corticosterone response to cold stress between the selected lines were not observed until the second selected F2 generation (Brown and Nestor, 1973).

The change in plasma cortisol levels in HR and LR fish during a 4 h period of confinement indicates that the difference in post-confinement plasma cortisol levels between HR and LR fish at 3 h arises from a difference in the maximum plateau of circulating cortisol during confinement, and is not due to differences in the dynamics of the response. Whether the different plateau values are due to differences in pituitary ACTH secretion, in the sensitivity of the interrenal to ACTH, in the steroid synthetic capacity of the interrenal, or in clearance rates, is the subject of continued investigation. In Japanese quail selected for a high serum corticosterone response, the differences between selected and random-bred birds may reside at the level of the adrenal gland. Both adrenal mass and adrenocortical cell function contribute to the difference in responsiveness

with high-responding birds possessing adrenals which are heavier and show a greater response to ACTH and 8Br-cAMP at the cellular level *in vitro* (Carsia *et al.*, 1988). Similarly, differences in the stress-induced plasma corticosterone level in various genetically defined strains of rat are suggested to arise from differences in adrenal sensitivity to ACTH, rather than differences in pituitary ACTH secretion (Gomez *et al.*, 1996; Sarrieau *et al.*, 1998). However, if differences in interrenal sensitivity underlie the divergent stress responsiveness of the HR and LR lines, this is not reflected in plasma cortisol levels in unstressed fish. There was no significant difference between the cortisol levels in the blood of unstressed HR and LR fish during the time-course study and no differences in baseline levels of cortisol have been observed in comparisons of the HR and LR fish carried out since the studies reported here (T. G. Pottinger & T. R. Carrick, unpublished).

The moderate to high heritability estimate obtained for the cortisol response to confinement ($h^2 = 0.41$) in this study is consistent with an estimate of 0.56 obtained by Fevolden *et al.* (1999) for the heritability of the cortisol response to confinement in three-year old seawater-reared rainbow trout. Similar, or lower estimates have been reported for other species such as Japanese quail ($h^2 = 0.22$, mid-parent/progeny regression; Satterlee and Johnson, 1988) and turkeys ($h^2 = 0.2 - 0.4$, realized; Brown and Nestor, 1973). It is interesting to speculate why the cortisol stress response in rainbow trout appears to have a high heritability. Low heritabilities are generally associated with phenotypes which are critically important for survival (Tave, 1986) and in which evolutionary pressure has already exploited a large proportion of the available genotypic variation. The high heritabilities reported for the magnitude of the cortisol response to stress in rainbow trout suggests that the magnitude of the response may not be a critical element of its value to the fish in its natural environment. However, Falconer (1989) also points out that the

value of the heritability for a trait may differ between populations and, in particular, small populations which are isolated long enough for fixation to occur may display lower heritabilities than larger populations. This may account for the lower estimates of h^2 reported for turkeys and quail. Further work will establish whether there are costs/benefits associated with the magnitude of the cortisol response to stress in fish within an intensive rearing environment. The availability of trout with divergent cortisol responses to imposed stressors offers a valuable tool for the investigation of the function of the hypothalamic-pituitary-interrenal axis in fish.

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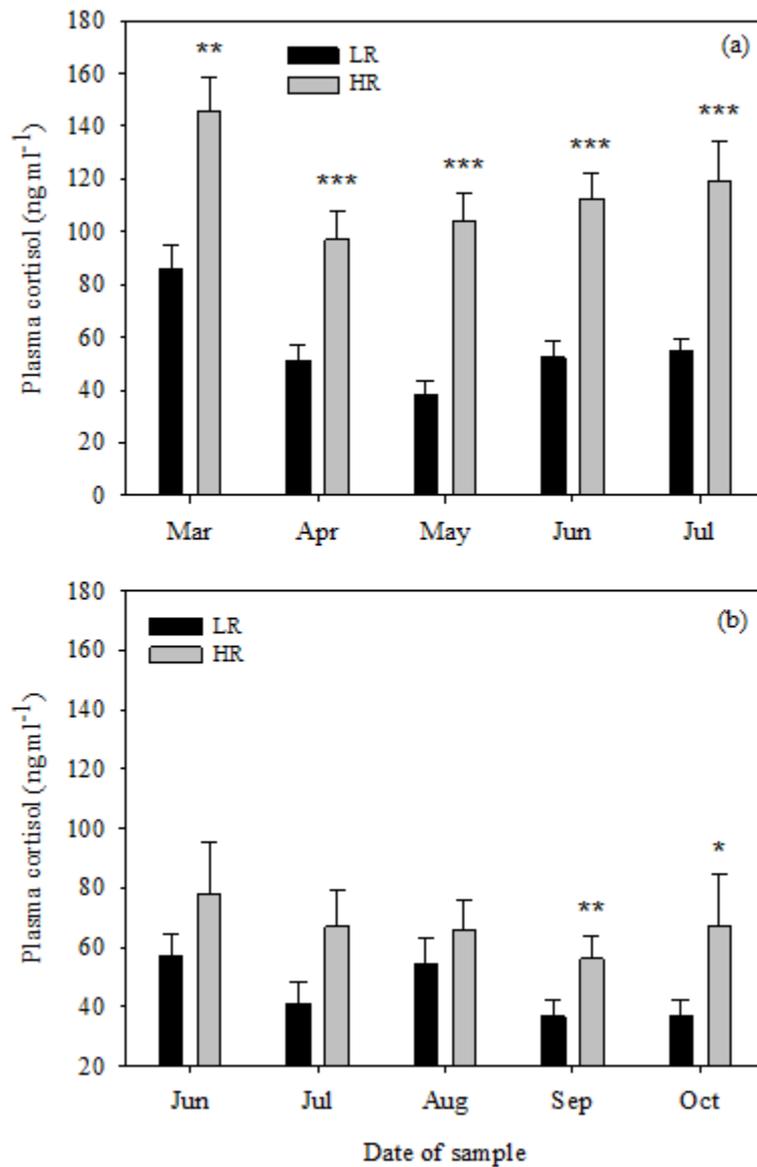


Figure 1. Plasma cortisol levels following confinement in (a) female and (b) male fish (F0, parents) selected as high-responding (HR, light bars) and low-responding (LR, dark bars) on each of five occasions on which the fish were tested. Each bar represents the mean \pm SEM. $n = 14$ (LR) or 15 (HR). Significant differences between HR and LR fish at each time are denoted by *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

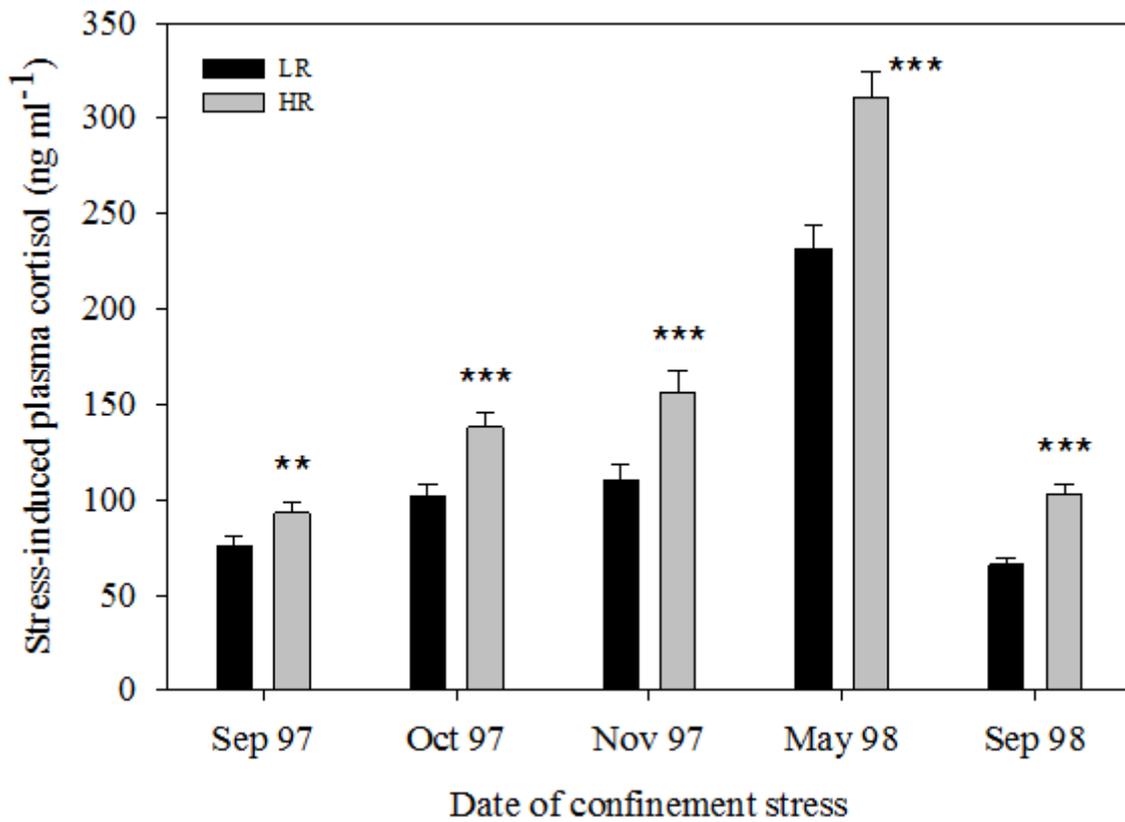


Figure 2. Plasma cortisol levels in the progeny of high-responding (HR, light bars) and low-responding (LR, dark bars) parents following confinement on each of five occasions. Six fish from each progeny group (family) were tested at each time. Each bar represents the mean \pm SEM. $n = 84$ (LR) or 90 (HR). Significant differences between HR and LR fish at each time are denoted by ** $P < 0.01$, *** $P < 0.001$.

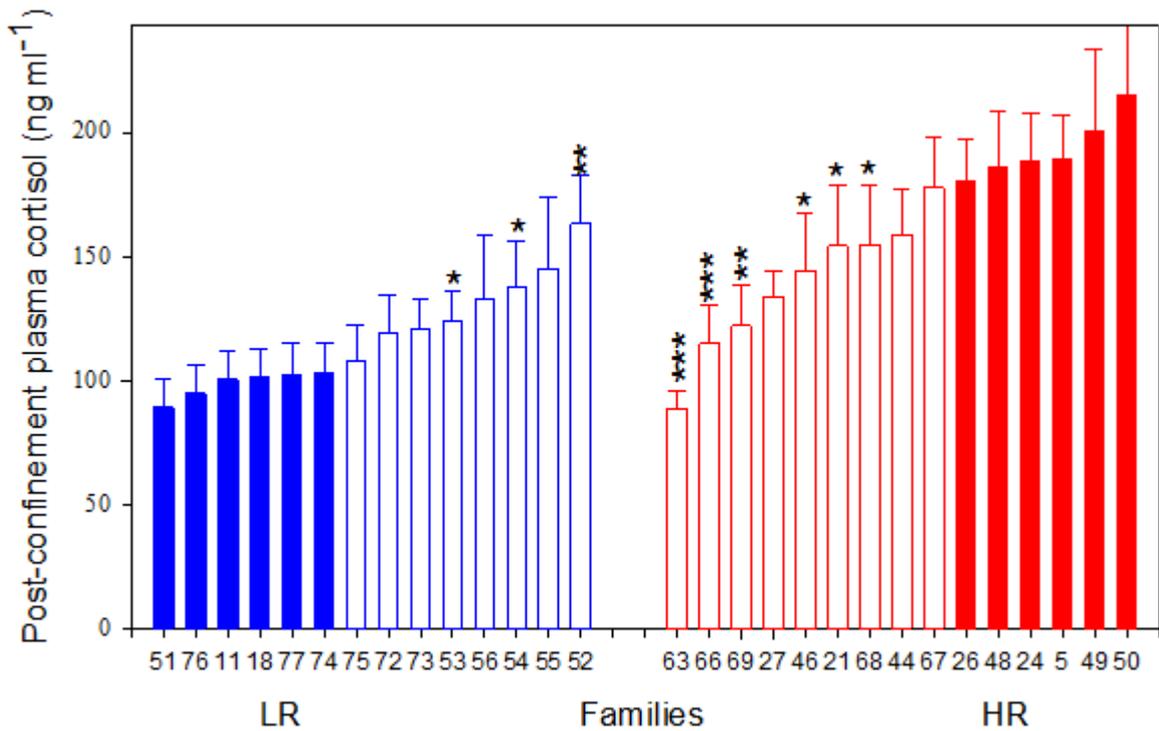


Figure 3. Plasma cortisol levels following confinement in each of 14 progeny groups derived from low-responding (LR) parents and 15 progeny groups derived from high-responding (HR) parents, ranked in order of the magnitude of the mean cortisol response. Each bar is the mean \pm SEM of the plasma cortisol level following five separate episodes of confinement ($n = 30$). The families identified as most divergent are shaded (LR, dark; HR, light). Families within either the LR or HR group, and which have mean cortisol response levels which are significantly different from the lowest LR group, or highest HR group, are denoted by asterisks. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. The numbers along the x axis denote the identifying number of each family.

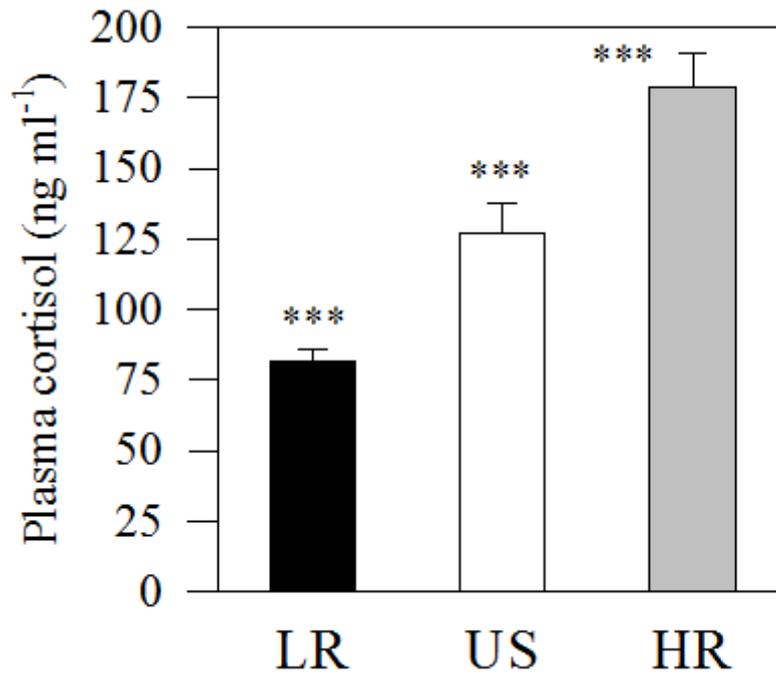


Figure 4. Plasma cortisol levels following a 3 h period of confinement in the progeny of low-responding (LR), unselected (US), and high-responding (HR) parents. Each bar is the mean \pm SEM of 36 fish, comprising six fish from each of six families. The HR and LR families were the most divergent in responsiveness, based on five episodes of confinement over a 12 month period. *** denotes significant differences from the other groups ($P < 0.001$).

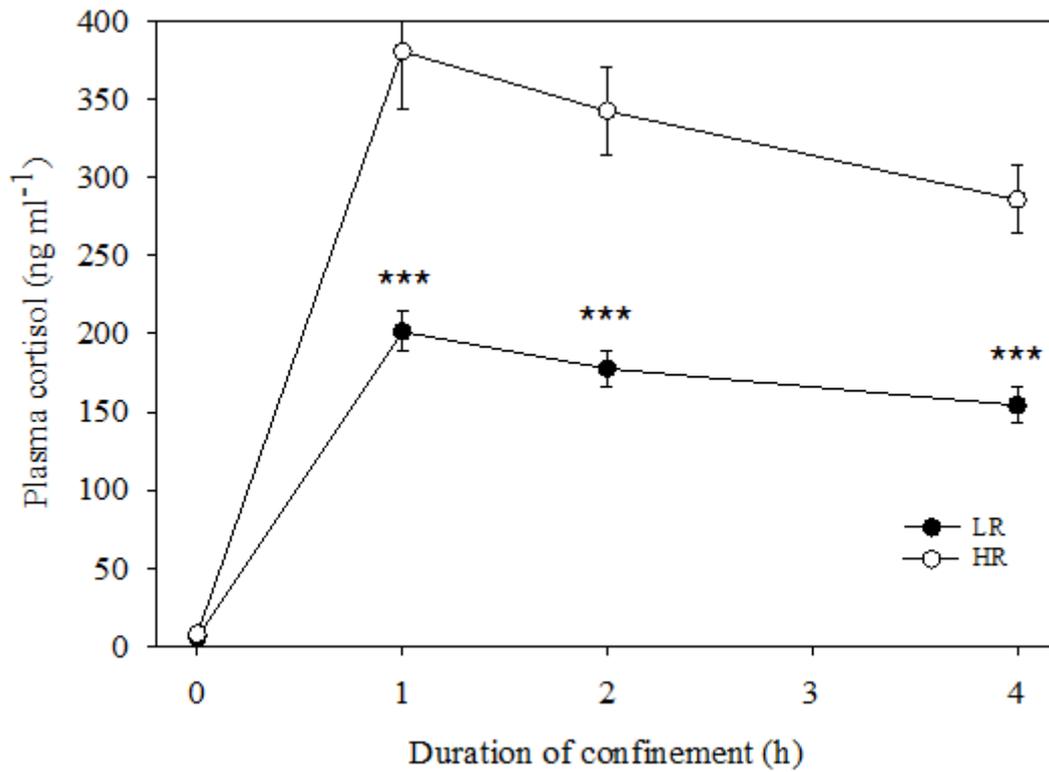


Figure 5. Plasma cortisol levels in the progeny of high-responding (HR, ○) and low-responding (LR, ●) parents during a 4 h period of confinement. Each point is the mean \pm SEM, $n = 36$ (6 fish from each of the six most divergent families). Significant differences between HR and LR fish are denoted by *** $P < 0.001$.

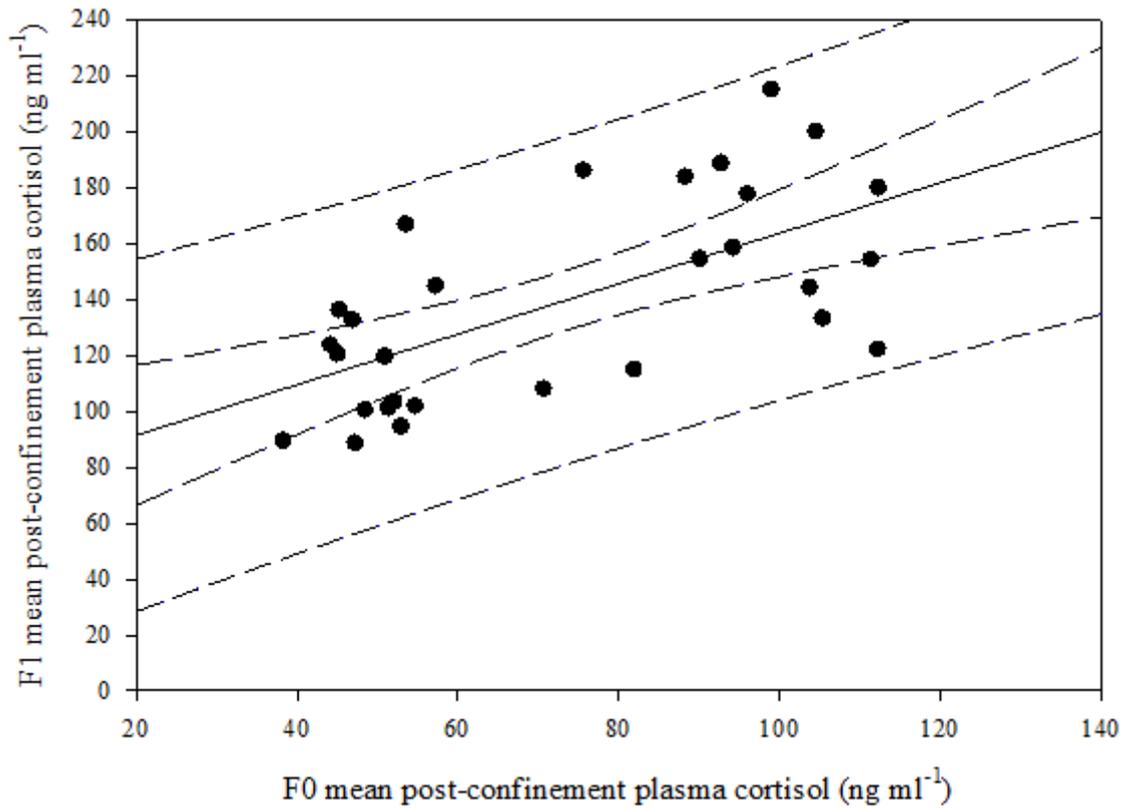


Figure 6. A regression of the mean plasma cortisol response to confinement in each progeny group (family) against the mean [(male + female)/2] response of the corresponding parent fish. $Y = 0.9034x + 73.4$, $r^2 = 0.4138$. The 95% confidence intervals and prediction limits are indicated by dotted lines