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Selection response of cortisol and lysozyme in rainbow trout and correlation to growth

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Abstract

Progeny groups of rainbow trout (*Oncorhynchus mykiss* Walbaum) selected for high or low post-stress levels of plasma cortisol, or similarly for high or low post-stress lysozyme activity, have been tested for their response to the selection. In four of the four stress exposures, individuals from the line selected for high cortisol responsiveness displayed significantly higher levels of post-stress cortisol than individuals of the low responding line. Phenotypic correlations of cortisol response between samplings, irrespective of line, were highly significant. The realised heritability of cortisol was 0.50, which is very similar to the estimated h^2 based on the parental generation. Only in the last two of the four stress experiments did the high lysozyme selected line exhibit significantly higher lysozyme activity than the low lysozyme line. The timing of vaccination may cause this, since the vaccine is known to affect lysozyme activity. Breeding values of parents were based on vaccinated fish only. The phenotypic correlations between samplings of lysozyme response were weaker than for cortisol, though still significant. The realised heritability of lysozyme was 0.32, which is also in agreement with the previously estimated h^2. The phenotypic correlations between cortisol and lysozyme in individual samplings were in cases of significance negative. There is qualified support for better growth performance in the low cortisol responding line as compared to the high responding line. The data are not conclusive as to establishing whether selection for altered post-stress lysozyme activity affects growth. In conclusion, the present data confirm that the progeny inherit stress-related traits identified in the parents; the response to selection for both cortisol and lysozyme is encouraging. The practical implications or gain of selecting for either trait under aquacultural conditions is still being resolved.

Keywords: Breeding; Cortisol; Lysozyme; Rainbow trout; Selection; Stress

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1. Introduction

Given what is known about the adverse effects of stress, an enhanced tolerance of stressful procedures has been thought to have the potential to improve various performance characteristics of aquacultured fish. Examples of how exposure to stressful stimuli has had a negative impact on fish performance comprise important production traits such as growth (Pickering, 1993; Pankhurst and Van der Kraak, 1997; Gregory and Wood, 1999), reproduction (Pickering et al., 1987; Campbell et al., 1992, 1994; Pankhurst and Van der Kraak, 2000; but see critical remarks by Leatherland, 1999), immunity (Balm, 1997), and flesh quality (Lowe et al., 1993; Sigholt et al., 1997). Under aquacultural conditions, the fish will be exposed to a series of stressors, and it has been suggested that fish with a lower responsiveness to the stressor may be at an advantage compared to fish displaying a more pronounced reactivity to stress (Pottinger and Pickering, 1997). There is, however, as yet no conclusive evidence to suggest that this really is the case. The opposite scenario may also be considered possible, namely that the adaptive potential of a fish with an enhanced ability to respond to stress is at an advantage compared to the performance of a fish with a lowered magnitude of stress response. These alternative assumptions have recently been tested in a multinational selective breeding project, part of whose aim has been to assess the feasibility of generating lines of rainbow trout, *Oncorhynchus mykiss* (Walbaum), which are divergent for stress responsiveness (Fevolden et al., 1999; Pottinger and Carrick, 1999a).

Elevated blood cortisol levels are known to be a direct causal factor in many adverse effects of stress. This, in addition to its role as a robust and reliable index of activation of the neuroendocrine stress response in fish (cf. reviews by Barton 1997; Wendelaar Bonga, 1997), is an important reason for employing the magnitude of post-stress cortisol levels as a selection trait. Differences in cortisol responsiveness to stressors have been demonstrated both among strains (Pottinger and Moran, 1993) and families (Fevolden et al., 1991, 1993, 1994) of salmonid fish, and has thus been adopted as a trait with a genetic component sufficient to be used as a selection criterion (Fevolden et al., 1991; Pottinger et al., 1994).

Lysozyme, which is primarily known as a non-specific immune trait with bacteriolytic effects (e.g. Jollés and Jollés, 1984), has also been shown to alter blood concentrations in fish following a stressful stimuli (Moeck and Peters, 1990; Røed et al., 1993a; Demers and Bayne, 1997). According to Moeck and Peters (1990), acute stress causes enhanced lysozyme activity and prolonged stress lowered lysozyme activity. Røed et al. (1993a) observed the opposite trend with initially lowered and prolonged enhanced lysozyme activity following stress. Notwithstanding, it was suggested by Fevolden and Røed (1993) that under certain circumstances, lysozyme activity may be a more stable indicator of stress in fish than is cortisol. This assumption was based on the observation that a line of rainbow trout which was selected for high post-stress cortisol responsiveness, in repeated samplings displayed consistently higher lysozyme activity than the line selected for low cortisol responsiveness, while the difference in cortisol measurements between the two lines was inconsistent (Fevolden and Røed, 1993). Previous studies have demonstrated that there is genetic variation in lysozyme activity of salmonid fish (Røed et al., 1993a,b; Balfry et al., 1997). On this background, the aim of the present

breeding project has been (i) to compare the feasibility of selecting for post-stress cortisol and lysozyme responsiveness, (ii) to evaluate the intercorrelation between the two traits, and (iii) to compare the performance of fish selected for either of the two traits. In a previous paper based on measurements of 50 full-sib families (Fevolden et al., 1999), we presented heritability estimates of the two traits and genetic correlations between them. The present paper describes the responses to selection in progeny groups of parents selected from families (among those 50) with consistent high or low post-stress levels of cortisol or lysozyme. We also evaluate differences in growth performance between our high and low lines for both traits, whereas differences between other performance traits will be presented elsewhere.

2. Materials and methods

2.1. Parent fish

Details about the parental group and their treatment are in Fevolden et al. (1999) but can be summarised as follows. In April 1996, 10 fish were randomly selected from each of 50 full-sib families (=number of dams) of the 1994 year class of rainbow trout from AquaGen . The 50 families comprised 29 paternal half-sib groups (=number of sires). The 500 fish were individually tagged and to ensure identical environmental conditions, they were kept in one common sea cage throughout the experimental phase and up to the time for producing offspring. At approximately 6–8 weeks intervals, the fish were subjected to a confinement stress by netting and transfer to a 2-m^2 tank with shallow (approximately 35 cm) and vigorously running water for 30 min. Two identical stress tanks were used, alternately filled up with fish at 15-min intervals. The stress samplings were repeated three times. Following each stress exposure, the fish was anaesthetised (MS 222) and blood was sampled by severing the caudal peduncle. Blood samples were

Table 1
Dates of handling the experimental rainbow trout
Number and average weight of fish in each sampling are given.

Activity	Sampling date	Number of fish	Weight (g) ± S.D.
Making crosses	March/April 1997		
Hatching	May 1997		
Tagging	November 1997	500	40 ± 12
Transfer to salt water	April/May 1998		
S1 1 stress exposure	May 1998	477	258 ± 73
Vaccination	June 1998		
S2 2 stress exposure	August 1998	551[a]	522 ± 186
S3 3 stress exposure	September 1998	450	820 ± 256
S1 4 stress exposure	February 1999	385	1664 ± 487

[a] Extra fish had been added from reserve stock due to high mortality following S1; topic to be addressed in a separate paper.

left at room temperature for at least 3 h to allow clotting and were then centrifuged (15 min at $2000 \times g$). The serum was stored frozen ($-85°C$) until analysed for plasma concentrations of cortisol and lysozyme.

Based on data from the repeated stress experiments, a family breeding value and an individual breeding value were estimated for post-stress levels of cortisol and lysozyme. This was done by a best linear unbiased prediction (BLUP) procedure, accounting for the

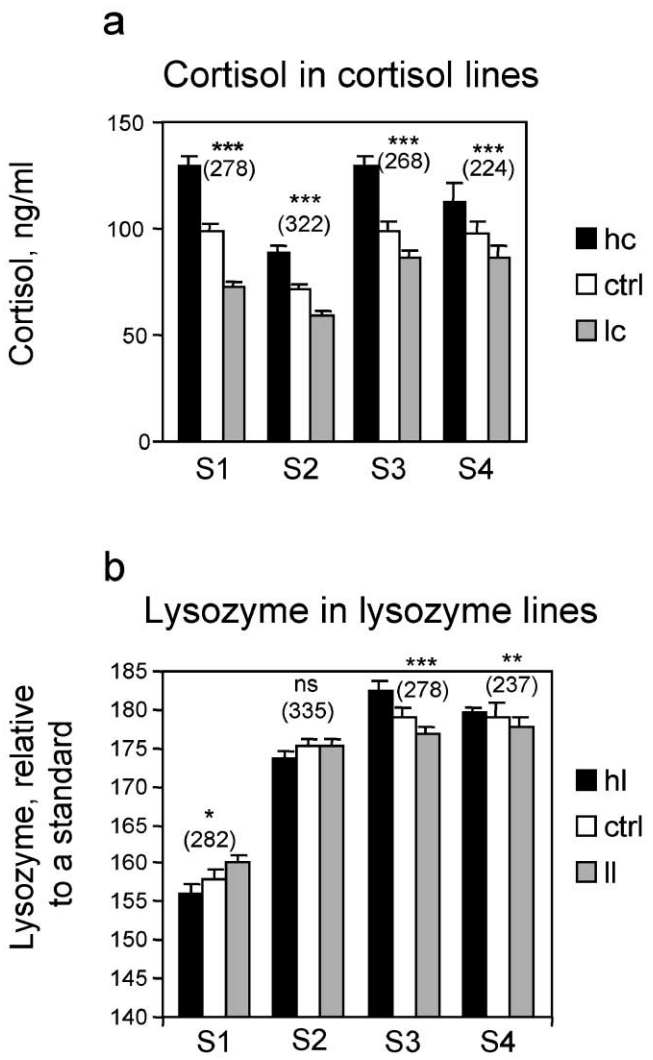

Fig. 1. Mean post-stress levels of plasma cortisol (a) and lysozyme (b) following four stress exposures (S1–S4). Vertical bars: S.E.M. *** $P<0.001$, ** $P<0.01$, * $P<0.05$, ns—not significant in comparison between high (hc, hl) and low (lc, ll) responding lines. S4 are overall means in severely and moderately stressed fish (see text). In brackets are numbers of fish analysed in the three lines together.

additive genetic relationship between sires and dams. The BLUP procedure utilises the available information optimally to provide the most accurate estimates of the breeding candidate's genetic value (for details of BLUP, see e.g. Lynch and Walsh, 1998). Of the initial 500 fish that were randomly selected, 385 survived until the last stress experiment. At the time of making crosses, approximately 300 fish (mean weight >8 kg) were available for the applied family selection scheme.

2.2. Progeny groups

Parental animals were selected from families with the highest and lowest breeding values for each of cortisol and lysozyme post-stress levels, thus, producing four progeny groups or selection lines: high cortisol responders (hc), low cortisol responders (lc), high lysozyme responders (hl), and low lysozyme responders (ll). The number of individuals used to produce the different lines varied between 7 and 17. A control group was made from a random sample of at least 50 families of the actual year class of rainbow trout. These families were not the same as those that were used as parental groups.

The four selected lines plus the control group were kept in separate tanks but under identical conditions until they were individually tagged by PIT-tags in November 1997 (cf. Table 1). They were then transferred to one common tank until they became so large they had to be randomly split between two identical tanks (September 1998). Reserve fish were kept of each line under conditions equal to those of the experimental fish in case of severe mortality following stress exposures or others. The fish were transferred to salt water in April 1998 and thereafter exposed for four stress experiments (Table 1). The stress procedure was as for the parental generation (see above), but since these fish were younger and smaller, they were confined at lower water depths (approximately 15–20 cm). Twenty fish were stressed simultaneously. (It had been shown in the parental generation that the removal of groups of fish from the holding tank for application of the stressor did not affect the fish remaining in the tank significantly since both cortisol and lysozyme in sequential sub-samplings were correlated poorly with the time after the first sampling of the day; Fevolden et al., 1999). In the fourth and last stress exposure (S4, Table 1), half the fish were exposed for the standardised 30-min confinement stress,

Table 2
Pearson correlation coefficients (R) for phenotypic correlations of post-stress levels of cortisol (above diagonal) and lysozyme (below diagonal) in individual fish irrespective of line between the four stress exposures (S1–S4)
ns—not significant. In parenthesis is the number of fish correlated. For sample S4, values are given for severely stressed fish only.

	S1	S2	S3	S4
S1		0.60 (276)***	0.56 (230)***	0.42 (203)***
S2	0.10 (272) ns		0.62 (450)***	0.37 (185)***
S3	0.22 (226)***	0.17 (447)***		0.48 (185)***
S4	0.36 (104)***	0.20 (187)**	0.29 (187)***	

** $P<0.01$.
*** $P<0.001$.

whereas the other half were exposed only for the accompanying handling stress (netting, anaesthesia). The reason for altering the stress protocol was that following S4, slaughter quality should be compared in fish that was severely and moderately stressed (unpublished data).

Shortly following the first stress exposure, all fish were vaccinated with an oil-adjuvant based vaccine against *Vibrio salmonicida*, *V. anguillarum*, and *Aeromonas salmonicida*.

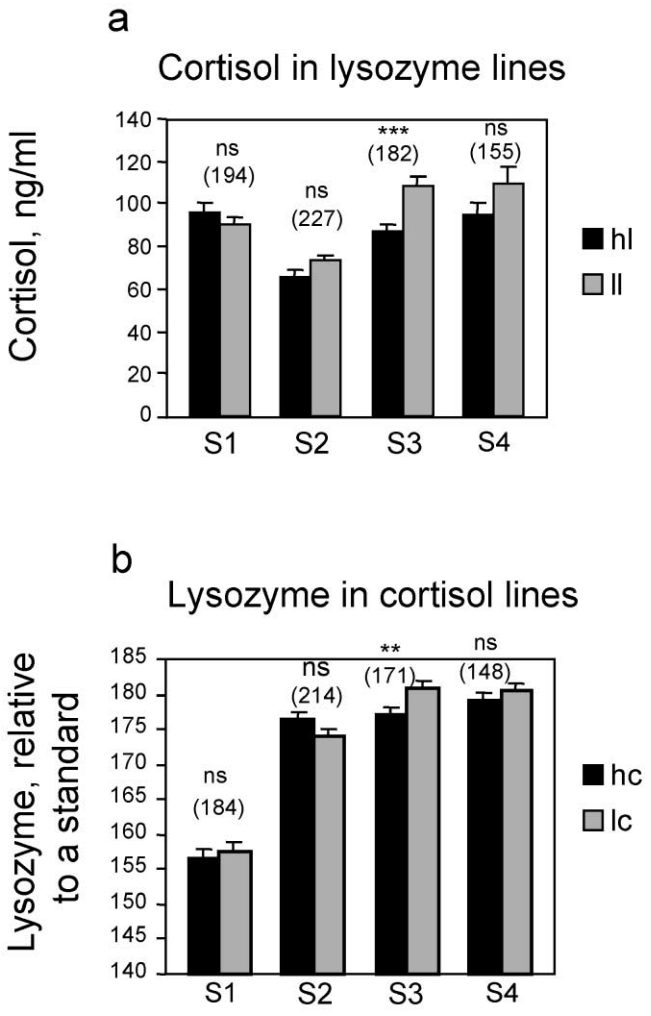

Fig. 2. Mean post-stress levels of plasma cortisol in the lysozyme selection lines (a) and of plasma lysozyme in the cortisol selection lines (b) following four stress exposures (S1–S4). Vertical bars: S.E.M. *** $P<0.001$, ** $P<0.01$, ns—not significant in comparison between high (hc, hl) responding and low (lc, ll) responding lines. S4 are overall means in severely and moderately stressed fish (see text). In brackets are numbers of fish analysed in the high and low responding lines together.

2.3. Chemical analyses

Serum cortisol levels were determined by radioimmunoassay (RIA) as described by Abraham et al. (1977) and modified by Olsen et al. (1992). The antiserum was supplied by Endocrine Sciences (Calbasas, Ca; lot 345-FISH, batch 1255). Samples were counted in Hionic-fluor scintillation fluid (Packard Instrument; Chemical Operations, Groningen, Netherlands), and counted on a Packard Tri-Carb 4000 LC Counter.

Serum lysozyme activity was determined using the *Micrococcus* lysoplate assay of Osserman and Lawlor (1966), incorporating the modifications of Lie et al. (1986) as described by Røed et al. (1993b). The assay is based on measuring the amount of lysed bacteria after adding serum to agarose plates where *Micrococcus lysodeicticus* is incorporated. The lysozyme activity in the sample is directly proportional to the diameter of the zone of lysis.

2.4. Growth measures

At tagging and at each stress sampling (cf. Table 1), all fish were weighed. Growth performance was considered by means of specific growth rates (SGR) defined as $100[\ln(WT2) - \ln(WT1)]/(T2 - T1)$ where WT2 and WT1 are the current and previous body weight measurements and $T2 - T1$ are days between measurements.

2.5. Statistics

Differences between the high and low responding lines in means of the various traits were tested by *t*-test, whereas phenotypic correlations between variables were expressed with Pearson's correlation coefficients. Realised heritabilities for cortisol and lysozyme were estimated as the ratio of selection response (R) and selection differential (S) (for details see Falconer, 1986).

3. Results

Following all four stress exposures, highly significant differences were seen in mean cortisol levels between the two cortisol lines with the higher values displayed in the line

Table 3
Pearson correlation coefficients (*R*) for phenotypic correlations between post-stress levels of cortisol and lysozyme in individual fish at the four different stress exposures (S1–S4)
ns—not significant. For sample S4, values are given for severely stress fish only.

Correlations	*R*
Cortisol S1–Lysozyme S1	−0.18 (463)***
Cortisol S2–Lysozyme S2	0.03 (547) ns
Cortisol S3–Lysozyme S3	−0.17 (449)***
Cortisol S4–Lysozyme S4	−0.02 (182) ns

*** $P < 0.001$.

selected for high cortisol response (Fig. 1a). The values of the control group are intermediate between the two cortisol-selected lines. Values for sampling 4 are means for "severely" (confinement) and "moderately" (handling without confinement) stressed fish and follow the same pattern as in the preceding samplings with controls intermediate between the high and low responders. When only severely stressed fish at S4 were considered, the mean cortisol level of the high cortisol responders was 160 ± 12.6 (S.E.) ng/ml, which is again significantly higher than in the low responders (117 ± 10 ng/ml).

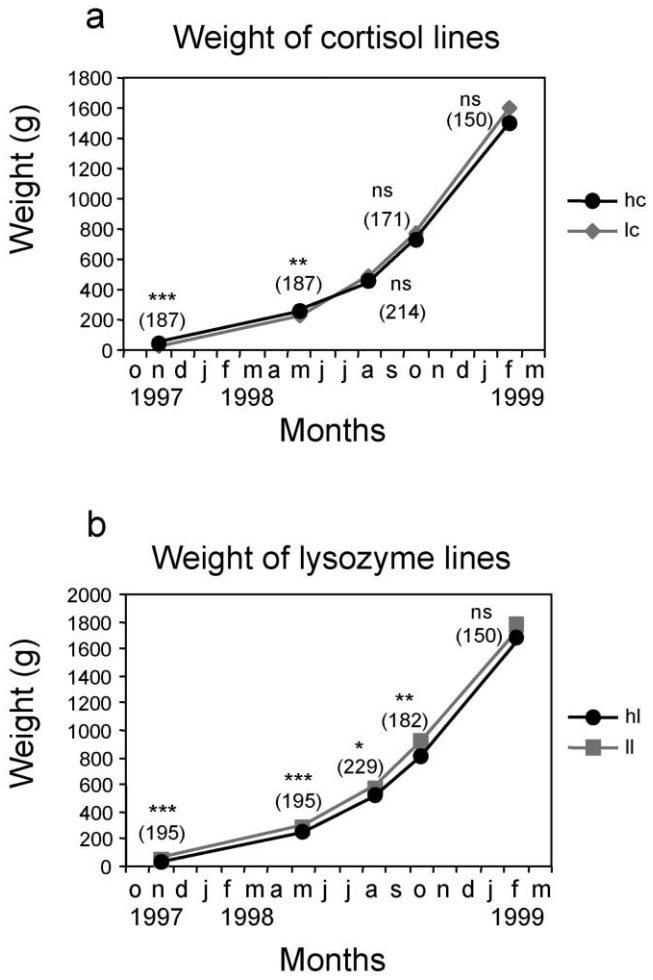

Fig. 3. Mean weight of fish in the two cortisol selection lines (a) and the two lysozyme selection lines (b) at five samplings (at tagging and at four stress exposures). *** $P < 0.001$, ** $P < 0.01$, ns—not significant in comparison between high (hc, hl) and low (lc, ll) responders. In brackets are numbers of fish weighed in the high and low responding lines together.

These values are higher than in the preceding three samplings (cf. Fig. 1a) Phenotypic correlations between samplings for individual fish were highly significant (Table 2).

As to the post-stress lysozyme levels, only following the last two stress exposures were the responses according to expectations from the selected parents, that is with significantly higher values in progenies of the high-responding line (Fig. 1b). Moreover,

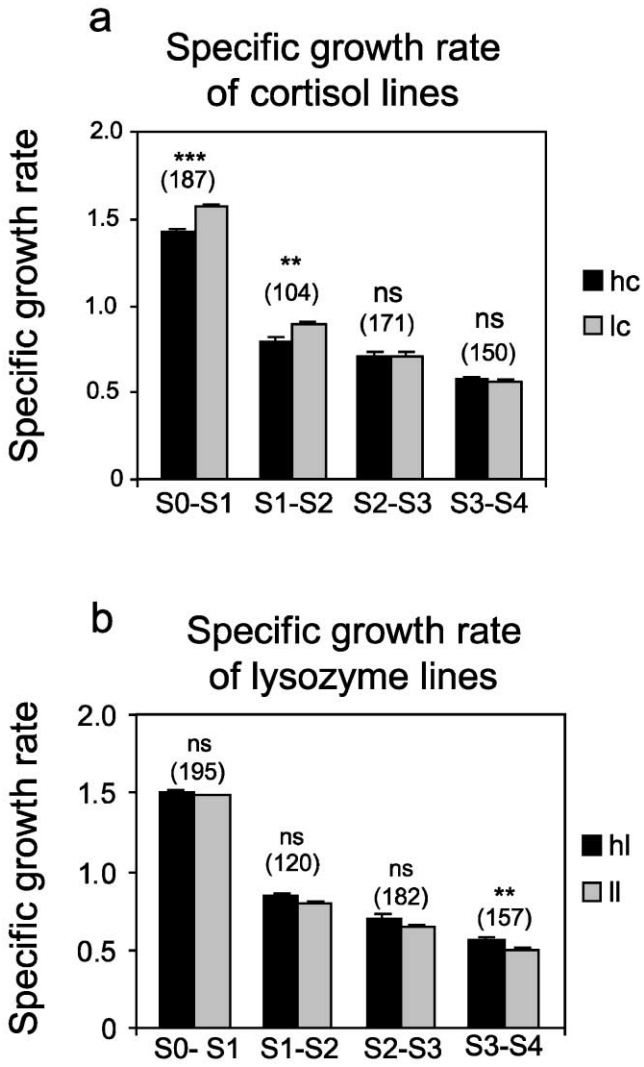

Fig. 4. Specific growth rate for fish of the two cortisol selection lines (a) and for the two lysozyme lines (b). S0–S1: as measured between tagging (S0) and first stress exposure (S1), and so on. *** $P<0.001$, ** $P<0.01$, * $P<0.5$, ns—not significant in comparison between high (hc, hl) and low (lc, ll) cortisol or lysozyme responders. In brackets are numbers of fish measured.

the phenotypic correlations between samplings are weaker for lysozyme than for cortisol, still mainly significant (Table 2).

When comparing cortisol response in the severely and moderately stressed fish of sampling 4 in all lines together, the cortisol levels of the former was significantly higher than that of the second group, 130.3 ± 4.2 (S.E.) ng/ml vs. 71.5 ± 3.2 ng/ml ($P < 0.001$), although the cortisol level of the latter fish clearly demonstrated that it was stressed. The overall lysozyme level of the severely stressed fish, on the contrary, was lower than in the moderately stressed fish, 178.0 ± 0.7 (relative to a standard) vs. 180.7 ± 0.7. The small standard errors make even this difference significant at $P < 0.001$.

Measurements of post-stress cortisol in the high and low lysozyme lines and correspondingly post-stress lysozyme in the high and low cortisol lines, display few significant differences between lines, although there seems to be a trend towards higher cortisol in the low lysozyme line and vice versa (Fig. 2). Comparing cortisol and lysozyme individual for individual, the correlation coefficients are generally low but in cases of significance they are negative (Table 3).

Due to the possible effect that the applied vaccine has on lysozyme activity (see below), the calculation of realised heritabilities were based on the mean of the three post-vaccination samplings (S2–S4; Table 1). The figures were 0.50 for cortisol and 0.32 for lysozyme.

During the first months after hatching, there were differences in mean weight between the progeny groups that were due to delay in time of hatching from the first to the last groups (approximately 1 month). Whereas at tagging (S0) and at the first stress exposure (S1), the mean weight of the low cortisol fish was significantly lower than that of the high cortisol line, at the two last samplings, the low cortisol fish had outgrown the high cortisol fish (Fig. 3a). Specific growth rate (SGR) as measured between the five different samplings shows that for the cortisol lines during the first two time intervals, growth was significantly higher in the low cortisol line than in the high cortisol line (Fig. 4a). As to phenotypic correlations between cortisol and weight and between cortisol and SGR, all four significant correlations are negative (Table 4).

Table 4
Pearson correlation coefficients (R) for phenotypic correlations between post-stress levels of cortisol and lysozyme and each of the traits weight and specific growth factor (SGR) in individual fish independent of line at the four different stress exposures (S1–S4)
ns—not significant. For sample S4, values are given for severely stress fish only.

	Cortisol R	Lysozyme R
Weight–S1	−0.11 (472)*	0.09 (466)*
Weight–S2	−0.15(549)***	−0.34 (549)***
Weight–S3	−0.07 (450) ns	−0.19 (449)***
Weight–S4	0.01 (185) ns	0.03 (187) ns
SGR–S1	−0.27 (472)***	−0.01 (466) ns
SGR–S2	−0.13 (279)*	−0.24 (280)***
SGR–S3	−0.05 (450) ns	−0.19 (449)***
SGR–S4	0.05 (185) ns	−0.03 (187) ns

* $P < 0.05$.
*** $P < 0.001$.

Throughout the experimental phase, the high lysozyme fish were smaller than the low lysozyme fish, but the difference was no longer significant at S4 (Fig. 3b). As to SGR only between the two last samplings there was a significant difference between the two lysozyme lines with the higher value displayed in the high lysozyme line (Fig. 4b). The three previous measures also displayed a trend towards higher growth rates in the high lysozyme line. Phenotypic correlations between lysozyme and weight or between lysozyme and specific growth rate, were predominantly negative (Table 4).

4. Discussion

4.1. Response to selection

The results above have shown that selection for cortisol stress response by means of a family selection scheme has been successful. The selection may be considered robust since all stress responses in the parental generation were measured in adult fish (2+ year) kept in seawater net-pens (Fevolden et al., 1999). Yet, in the progeny groups, which were kept in much smaller indoor tanks, the cortisol stress response was in accordance with the parents' performance from young (1 year) to older (2 year) fish. The fact that in all stress exposures of the selected lines there were significantly higher levels of post-stress cortisol in the high cortisol line than in the low cortisol line, correlates well with the relatively high heritability (h^2) of cortisol, which was estimated from repeated stress exposures of the parental generation (0.51) (Fevolden et al., 1999). Moreover, the realised heritability of cortisol (0.50), based on the means of the three post-vaccination samplings, fits in with the former estimated h^2.

For post-stress levels of lysozyme activity, the response of the progeny was seemingly less clear-cut. Only following the two last stress exposures the response was according to the selected parents, that is with the higher lysozyme level in the line selected for high lysozyme activity. Following the first stress exposure, there was in fact significantly higher levels of lysozyme in the low lysozyme line (Fig. 1b). It is likely, however, that this feature has to do with the timing of the vaccination of the fish, which took place between the first and second stress exposure. The vaccine used has oil as adjuvant and such vaccines are known to continuously trigger the immune system, including lysozyme activity. This is also evident from the significant rise in lysozyme activity in all lines between samplings S1 and S2 (Fig. 1b). Since all lysozyme measurements in the parental generation were done after vaccination, the evaluation of the response to selection for lysozyme activity should be considered in vaccinated fish only. Thus, referring to Fig. 1b, one could claim that selection for lysozyme has also been successful with the two last post-vaccinated cases showing significantly higher lysozyme levels in the high lysozyme line. The less divergent levels of lysozyme between the two lysozyme lines even in vaccinated fish, as compared to the divergence of cortisol between cortisol lines, corroborates with the lower h^2 of lysozyme based on data from the parent generation (≈ 0.30; Fevolden et al., 1999). In addition, the realised heritability of lysozyme (0.32), based on the means of the three post-vaccination samplings, is lower than for cortisol but in agreement with the h^2 estimated from the non-selected parental generation. These

figures support the suggestion that cortisol is slightly superior to lysozyme when it comes to selection potential (Fevolden et al., 1999).

In previous papers (e.g. Fevolden and Røed, 1993; Fevolden et al., 1994), we suggested that short-time stress may enhance the levels of plasma lysozyme in fish; a suggestion which has been lent support by other studies (Moeck and Peters, 1990; Demers and Bayne, 1997). If there is a direct mechanistic link between cortisol elevation and lysozyme elevation, this would evoke a positive phenotypic correlation between post-stress cortisol and lysozyme levels. However, both cortisol and lysozyme could be elevated during stress by mechanisms that are independent and the results of the present study show that this correlation is in fact weak, and in the cases of significance it is negative (Table 3). Genetic correlations between cortisol and lysozyme of the parental group were also negative, but large standard errors precluded the significance (Fevolden et al., 1999). The observation from the fourth stress experiment in the present study also give qualified support for an antagonistic effect of cortisol and lysozyme responsiveness; in severely stressed fish, there was more cortisol but less lysozyme than in moderately stressed fish. There is reason to moderate the earlier suggestion of lysozyme being a more "reliable" stress indicator than cortisol (Fevolden and Røed, 1993). As stated above that assumption was based on the observation that a line of rainbow trout that was intentionally selected for high post-stress cortisol responsiveness, in repeated samplings displayed higher lysozyme activity than the line selected for low cortisol responsiveness. The post-stress cortisol response in the two lines was inconsistent. A contributing factor to this feature could have been that the selection for cortisol response in those lines had not been successful and that an unintended selection for high post-stress lysozyme levels may have occurred. One obvious reason why the selection for enhanced cortisol response in the present study was more successful is that breeding values were based on three repeated samplings of the parental generation, compared to only one (F-0) or two (F-1) in the previous study (Fevolden et al., 1991; Fevolden and Røed, 1993).

4.2. Correlations to growth

The initial difference in mean weight between the progeny lines can in part be ascribed to time delays between hatching of the different groups. The selection of parents to produce the cortisol and lysozyme lines was done without taking into account the weight of the fish. There was, however, no significant difference in weight among the parental groups used to produce the different lines. A better measure for growth than weight alone is specific growth rate. Based on group means of SGR and phenotypic correlations between post-stress cortisol values and SGR, qualified support for better growth in low cortisol responders than in high cortisol responders is provided. The data for lysozyme are more inconclusive. SGR means tend to be higher in the high lysozyme line than in the low lysozyme line, which would be expected considering the negative correlation between lysozyme and cortisol. This trend is disrupted, however, by the two highly significant negative phenotypic correlations between lysozyme and SGR overall (Table 4). When the phenotypic correlation is being made only within the two lines selected for lysozyme responsiveness, the negative correlation between growth and lysozyme is weakened (only one significant case of four possible at the $P < 0.05$ level).

Notwithstanding, although there is the possibility that a behavioural trait linked to stress responsiveness may underlie the differences observed between lines in SGR, we are yet in a position to claim only qualified support for cortisol stress responsiveness being a more influential determinant of growth than lysozyme responsiveness.

5. Conclusion

In conclusion, the present data together with those of Fevolden et al. (1999), and of Pottinger and Carrick (1999a,b, 2000), have manifested that selection for cortisol stress responsiveness is indeed feasible. Pottinger and Carrick (1999b) estimated a post-stress cortisol heritability in rainbow trout based on parent–offspring regressions of 0.41, which is only slightly lower than our estimate based on a large family material (Fevolden et al., 1999). Moreover, it has been shown herein that selecting for lysozyme activity is also feasible, although somewhat less prosperous than selecting for cortisol. The two traits may be less interrelated than formerly suggested, and it is likely that the type and duration of the stressor is determinative of whether the correlation is negative or positive. Yet, the additive value of genes controlling cortisol and lysozyme activity are such that both traits could be considered as breeding objectives in salmonids. However, before recommending that either trait be implemented in selection regimes, better knowledge of possible correlations to other production traits is essential. A series of performance characteristics other than growth have been measured in the same progeny lines, e.g. various immune parameters, disease resistance, and selected measures of slaughter quality. Those data are being processed and will be published in due time.

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