



The effects of stress on androgen production, spermiation response and sperm quality in high and low cortisol responsive domesticated male striped bass

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Received 6 May 2004; received in revised form 15 December 2004; accepted 17 December 2004

Abstract

Domesticated male striped bass were selected as high cortisol stress responders (HCR) or low cortisol stress responders (LCR) by ranking mean 1-h post-stress plasma cortisol levels following a 2-min standardized net challenge once per month for four consecutive months. During the selection period, HCR and LCR fish maintained a significant divergence in post-stress cortisol levels, but these differences were abolished coincident with the onset of sexual maturation. LCR fish stressed monthly during gonadal maturation and weekly during the spawning season had plasma testosterone (T) and 11-ketotestosterone (11-KT) levels 3–5 times lower than stressed HCR fish. During the simulated spawning season, plasma cortisol and glucose levels were moderately elevated but similar in HCR and LCR fish. There were no differences in sperm quality between HCR and LCR males; however, more HCR fish began spermiating earlier and maintained a spermiation response longer than LCR fish. There were no significant differences in weight, length or coefficient of condition, but HCR fish had a significantly greater specific growth rate on two sample intervals when compared to LCR fish. These results show that male striped bass selected for low cortisol stress responsiveness and exposed to repeated stress have diminished circulating levels of T and 11-KT associated with a shorter duration spermiation response when compared to males selected for high cortisol stress responsiveness. The data suggest that LCR male striped bass subjected to significant stress during maturation and spawning may have impaired reproductive performance.

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Keywords: Stress responsiveness; Striped bass; Cortisol; Reproduction; Androgen

1. Introduction

It is widely accepted that exposure of fish to stressors, such as netting and crowding, evokes a physiological response which includes rapid increases

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in circulating cortisol (Wendelaar Bonga, 1997; Barton, 2002). While the generalized stress response is adaptive in the short term, chronic stress can be maladaptive and may lead to more serious consequences such as impaired growth (McCormick et al., 1998; Procarione et al., 1999), decreased resistance to disease (Maule et al., 1989; Davis et al., 2002) or death (Noga et al., 1994). Exposure to stress can also disrupt reproductive processes by depressing pituitary and plasma levels of gonadotropin (Carragher et al., 1989), decreasing plasma gonadal steroid hormone levels (Pickering et al., 1987; Kubokawa et al., 1999) and by reducing egg size and quality of larvae (Campbell et al., 1992, 1994).

Since stress disrupts growth and reproduction, a considerable effort has been made to evaluate stress responsiveness in economically significant animals including several species of salmonids (Fevolden et al., 1991; Pottinger et al., 1994; Pottinger and Carrick, 1999a). Results of extensive studies with rainbow trout, *Oncorhynchus mykiss*, have demonstrated that the cortisol response to stress is a highly individualized trait that is stable over time (Pottinger et al., 1992). Moreover, crosses of parental fish selected for high and low cortisol responsiveness result in progeny with similar cortisol response traits as their parents (Fevolden et al., 1991; Pottinger and Carrick, 1999b) indicating that cortisol responsiveness is a heritable trait (Pottinger and Carrick, 1999b; Tanck et al., 2001; Fevolden et al., 2002).

Fish maintained in artificial culture environments are often subjected to multiple stressors. Intuitively one might expect that individuals with lower stress responsiveness might be less severely affected than those with a higher degree of stress responsiveness. Consequently, low-stress responding fish could perform better in terms of higher growth rates and better reproductive performance when compared to high-stress responding fish, but there is little evidence supporting this view. A preliminary study in our laboratory found no differences in absolute weight gain between high cortisol stress responsive (HCR) and low cortisol stress responsive (LCR) male striped bass, *Morone saxatilis* (Wang et al., 2004). To our knowledge, only one study has considered the effect of cortisol stress responsiveness on reproductive performance in fish. In that study, there were no detectable differences in a variety of

reproductive indices (e.g. egg weight, sperm count) between HCR and LCR rainbow trout (Pottinger and Carrick, 2000).

Currently, domesticated strains of striped bass are not available for commercial culture. The consequences of selecting striped bass for high and low cortisol stress responsiveness are unknown. The aim of this study was to determine the relationship between cortisol stress responsiveness and reproductive performance in male striped bass. The specific objectives were to: 1) identify a subpopulation of HCR and LCR male striped bass from our domestic broodstock, 2) assess and compare the effect of repeated stress on plasma levels of cortisol, testosterone (T) and 11-ketotestosterone (11-KT) in maturing HCR and LCR fish and 3) compare the spermiation response and sperm quality of selected HCR and LCR striped bass exposed to multiple stressors during a spawning season.

2. Methods

2.1. Experimental fish

Three weeks prior to the start of the study, 3-year-old domesticated (F_2 , 1999 year class, ranging in size from 2 to 3 kg) male striped bass ($n=67$) were captured, anesthetized in buffered MS-222 (150 mg/L, Fiquel, Argent Laboratories, Redmond WA), implanted with a subcutaneous passive integrated transponder (Avid, Norco, CA) tag, weighed, measured for total length and placed into an 8,600 L tank that was part of a larger recirculating system. High quality filtered water at a salinity of 5 g/L was circulated through the system at a rate of 72 L/min. Dissolved oxygen was monitored continuously and concentrations were maintained at or near saturation. Entrainment of the reproductive cycle was accomplished with computer-controlled software to adjust the lighting to either short (10L:14D) or long (14L:10D) daylengths (King et al., 1995). Dawn and dusk were simulated by ramping the light intensity up or down over a 15-min period. The light intensity at the water surface of the tanks where the fish were maintained or held was 20 lx. Water temperature was maintained using computer controlled chillers and heaters and ranged from 8 to 22 ± 0.5 °C during the

experimental period. Other water quality parameters and their measured ranges were: pH 7.2–7.8, total ammonia <0.5 mg/L, nitrite <0.2 mg/L, nitrate <100 mg/L, salinity 5–6 g/L calcium 150–200 mg/L, and magnesium 300–350 mg/L.

All experimental fish were fed a specially formulated squid-oil based striped bass broodfish diet (Ziegler Brothers, Gardners, PA) ad libitum twice daily. Food was withheld from all fish 2 days prior to each sampling date.

2.2. Identification of high and low cortisol responders

To determine which fish had a consistently high relative cortisol stress response and which fish had a consistently low relative cortisol stress response, all experimental male striped bass ($n=67$) were exposed to a 2-min net challenge once every 4 weeks for four consecutive months. Fish were randomly captured from their population tank in pairs (1 fish/net), held out of water for 2 min and then released into a 1600 L temporary holding tank (6 fish/tank). After 1 h, all of the individuals in each tank were rapidly netted, anesthetized as previously described and bled from their caudal vasculature using heparinized syringes. Body weight and length were measured after bleeding and the fish were placed back into their original population tank for recovery. This process was repeated until all 67 fish were sampled. Blood was maintained on ice in syringes and then transferred to microfuge tubes each containing 250 μg ammonium heparin (Sigma-Aldrich, St Louis, MO). The plasma was collected by centrifugation (4 °C) at $10,000\times g$ and stored at -20 °C for later analysis.

Once the fourth monthly sample had been completed, plasma cortisol levels for each fish were determined by ELISA (see Assays). The means for each fish were ranked and the 10 individuals with the highest mean cortisol values were designated HCR and the fish with the 10 lowest mean cortisol values were designated LCR.

2.3. Comparison of growth parameters

Length and weight data were used to calculate the coefficient of condition, $K=100,000 W/L^3$ where W is body weight and L is total length (Williams, 2000) and the specific growth rate, $\text{SGR}=[(\ln W_2 - \ln W_1)/$

$(t_2 - t_1)]100$, where W_1 and W_2 are the weights at the beginning and end of a sample period and $t_1 - t_2$ is the length of time in days between sample periods for each fish. Data obtained for growth indices were analyzed only for sample dates in August through November. Growth data from December through April were not analyzed due to the confounding effect of sexual maturation on body weight.

2.4. Effect of stress on plasma gonadal steroid hormones during maturation and spawning

Sampling as described above continued at 4-week intervals from November to March during gonadal maturation. Plasma samples taken during this phase of the study were assayed for T, 11-KT and cortisol.

Approximately 2 weeks after the monthly stress sampling ended, the fish selected as HCR and LCR ($n=10/\text{group}$) were moved from their 8600 L population tank and randomly assigned to one of five 1600 L holding tanks (4 fish/tank). The water temperature during this phase of the study was maintained at 17 ± 0.5 °C. To simulate hatchery operations during spawning, the fish were stress challenged and sampled three times per week for 5 weeks. Each Monday, the fish were anesthetized and blood sampled as previously described. Each Wednesday, the fish were anesthetized, their abdomens were squeezed to check for milt flow, and milt samples (~ 0.5 ml) were collected into 1.5 ml microfuge tubes. The milt was stored on ice and the sperm analyzed after 4 fish had been processed. Each Friday, blood samples were again taken from each fish after anesthesia. On each sample day, individuals were captured from their tanks and held in nets (1 fish/net) out of water for 30 s before being anesthetized.

2.5. Sperm quality assessment

Sperm in each milt sample was analyzed for the percentage of motile sperm and for the duration of the motility. A toothpick was used to remove a small milt sample from each tube. The sample was placed on a Makler counting chamber containing 10 μl of deionized ultrafiltered water for activation. Sperm samples were analyzed at $400\times$ using a phase-contrast microscope (Zeiss model D-7082, Thornwood, NY,

USA) linked to a high contrast video camera (Hitachi model KP-140, Tokyo, Japan). The duration of sperm motility and the percentage of motile sperm were determined for each sample after videotape review. The duration was defined as the difference between when a sample was placed into the counting chamber until forward progression of all sperm cells stopped. The percentage of motile sperm was defined as the total number of cells in a field when movement stopped minus the number of cells that were in that field and not moving at the beginning of activation divided by the total number of cells in the field. Three sperm sub samples from each fish were activated and measured on each sample date.

2.6. Assays

Cortisol and testosterone (T) were measured directly in plasma using ELISA kits (DRG Diagnostics, Mountainside, NJ). Serial dilutions of a striped bass plasma pool and ether extracts from the same pool diluted parallel to the cortisol standard curve. Similarly, T levels determined from serially diluted ether extracts of a male striped bass plasma pool were not different from those obtained from the same serially diluted plasma and both series were parallel to the standard curve. The T antibody crossreacted with 11-ketotestosterone (11-KT) 6.4% at the 50% displacement level while cortisone had a 3% cross reactivity with the cortisol monoclonal antibody. The cross reactivities of all other steroids as tested by the kit's manufacturer were $\leq 1.0\%$. The intra- and interassay C.V.s in the T assay were 8.7% and 4.6% respectively. Intra and interassay variability in the cortisol ELISA was previously published (Wang et al., 2004). Plasma 11-KT was measured directly in a commercially available ELISA kit using acetylcholinesterase as the tracer (Cayman Chemical, Ann Arbor, MI). 11-KT, measured from serially diluted ether extracts of a plasma pool were not different from those obtained from serially diluted plasma and both dilution series were parallel to the standard curve. The intra- and interassay C.V.s were 8.7% and 12.3% respectively. Glucose concentrations were determined using the 96-well plate microassay protocol and hexokinase/glucose-6-phosphate dehydrogenase (Sigma Diagnostics, St. Louis, MO) as the enzyme substrate.

2.7. Statistical analysis

Repeated measures ANOVA analyses were used to assess differences between high and low cortisol responders. If variances were homogeneous, the following covariance structures were tested: unstructured, compound symmetry, first order autoregressive and toeplitz. If variances were heterogeneous, the following variance structures were tested: unstructured, heterogeneous compound symmetry, heterogeneous first order autoregressive and heterogeneous toeplitz. The covariance structure with the best goodness of fit statistics was used for the final mean comparisons and tests of significance. Differences were considered significant at $p \leq 0.05$. Results are presented as means \pm S.E.M. unless otherwise noted. Spermiation response data were analyzed using a mixed model macro specially designed for binomial data. The binomial data was transformed to the logit scale and analyzed using a repeated measures ANOVA. Means and S.E.M.s were then retransformed to the percent scale for data presentation and easy interpretation. Regression equations were used to describe the changes in the data that were occurring over time. All data was analyzed using SAS v8 mixed model analysis (SAS Institute; Cary, NC).

3. Results

3.1. Identification of high and low cortisol responders

Fish identified as HCR had significantly greater mean plasma cortisol levels on all four sample dates when compared to LCR fish (Fig. 1). On three of four sample dates, a small number (2–4 fish per sample) of LCR striped bass did overlap with HCR striped bass. However, none of the higher responding LCR fish had levels above the mean of the HCR group of striped bass.

3.2. Comparison of growth parameters

Mean weight, length and coefficient of condition were not different between HCR and LCR fish from August through November. Fish weights and lengths ranged from 2.2 to 3.1 kg and 55 to 60 cm respectively and coefficients of condition were between 1.3 and 1.5

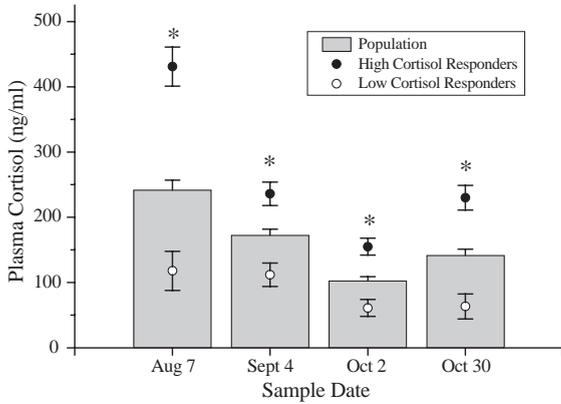


Fig. 1. Mean plasma cortisol levels 1 h after a 2-min net challenge of striped bass males selected as high cortisol responders (HCR; $n=10$) or low cortisol responders (LCR; $n=10$) sampled prior to sexual maturation (Aug–Oct). Bars represent mean cortisol levels for the remaining non-selected fish (population, $n=47$). Asterisks indicate significant differences between HCR and LCR on that sample date. Vertical brackets represent the S.E.M.

(data not shown). HCR fish had a significantly greater SGR for weight when compared to the LCR fish on the first and fourth sample date, and when compared across all five sample intervals (Fig. 2).

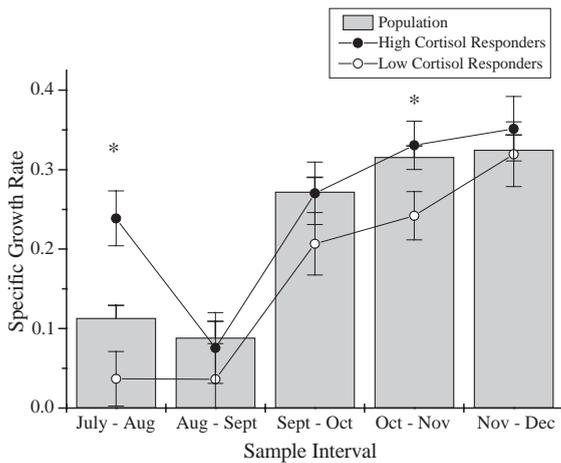


Fig. 2. Mean specific growth rates of male striped bass selected as high cortisol responders (HCR; $n=10$) or low cortisol responders (LCR; $n=10$) and the remaining non-selected fish (population $n=47$) during five sample intervals from July–December. Bars represent mean specific growth rates for the remaining non-selected fish (population, $n=47$). Asterisks denote a significant difference between HCR and LCR groups during that interval. Vertical brackets represent the S.E.M.

3.3. Effect of stress on plasma steroid hormones during maturation and spawning

During gonadal maturation (December–March), mean plasma T and 11-KT levels in HCR individuals were significantly greater than those of LCR fish on all sample dates (Fig. 3). Mean plasma cortisol levels

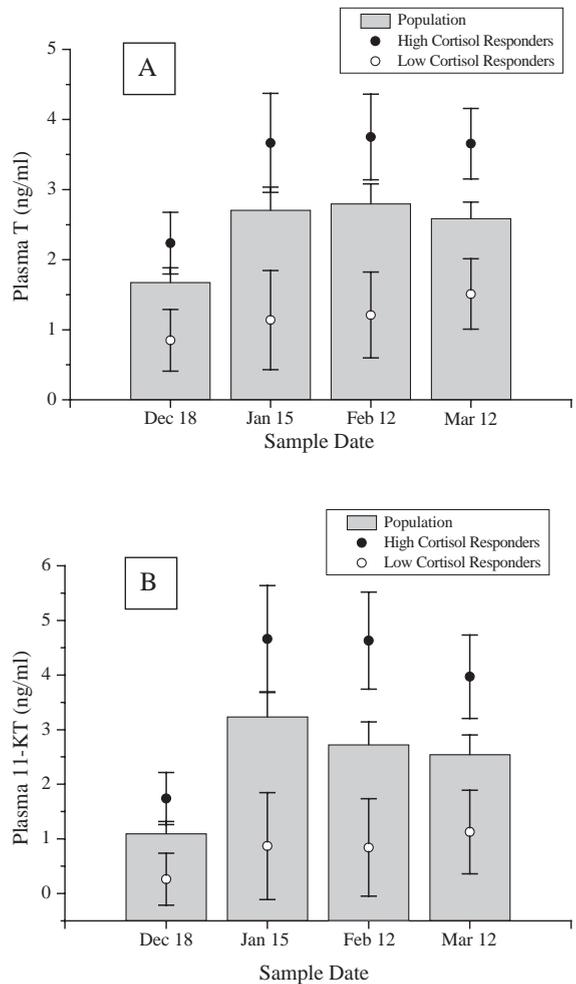


Fig. 3. (A) Mean plasma testosterone (T) and (B) 11-ketotestosterone (11-KT) levels 1 h after a 2-min net challenge of striped bass males selected as high cortisol responders (HCR; $n=10$) or low cortisol responders (LCR; $n=10$) and the remaining non-selected fish (population $n=47$) for samples taken during sexual maturation prior to the spawning season. Mean plasma T and 11-KT levels of HCR fish were significantly greater than those of LCRs on all sample dates and across samples. Vertical brackets represent the S.E.M.

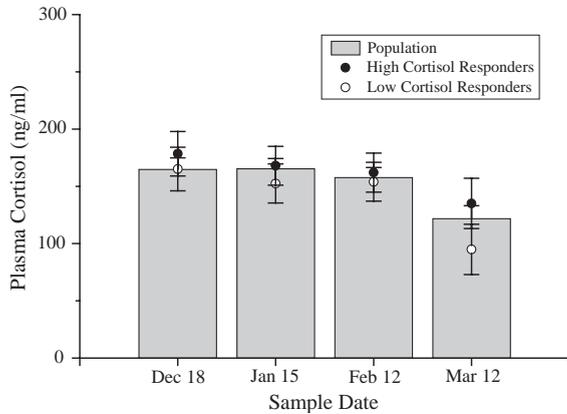


Fig. 4. Mean plasma cortisol levels 1 h after a 2-min net challenge of striped bass males selected as high cortisol responders (HCR; $n=10$) or low cortisol responders (LCR; $n=10$) sampled during sexual maturation (Dec–Mar) prior to the spawning season. Bars represent mean cortisol levels for the remaining non-selected fish (population, $n=47$). Vertical brackets represent the S.E.M.

were not different between HCR and LCR during this period (Fig. 4).

During simulated spawning operations (March–May), there were no differences in mean cortisol levels between HCR and LCR fish (Fig. 5). Mean plasma glucose levels were significantly greater in HCR fish as compared to LCR fish on two sample dates (Fig. 5). Mean plasma T and 11-KT levels were significantly greater in HCR when compared to LCR on all sample dates except May 5 when androgen levels of HCR only tended to be higher (Fig. 6).

3.4. Sperm quality assessment

The mean percentage of motile sperm and the duration of their motility were not different between HCR and LCR individuals on any of the five sample dates (data not shown). However, the majority of HCR fish had a spermiation response which began earlier and lasted longer than LCR fish (Fig. 7).

4. Discussion

The results of this study demonstrate that sexually mature male striped bass, selected as LCR, have significantly lower circulating levels of T and 11-KT when compared to those selected as HCR. This result

was apparent in blood samples taken from fish when spermiation was first detected (December) and continued through the end of the study. The plasma T and 11-KT concentrations in HCR fish during this period ranged from 1.9 to 3.8 ng/ml for T and 1.7 to 4.8 ng/ml for 11-KT and are in general agreement with previously reported androgen levels in unstimulated male striped bass (Mylonas et al., 1997). In contrast, plasma T and 11-KT of LCR fish during the same period were more than 3-fold lower than HCR fish ranging from 0.8 to 1.5 ng/ml for T and 0.3 to 1.2 ng/ml for 11-KT. At times (Dec–Feb), 11-KT levels of

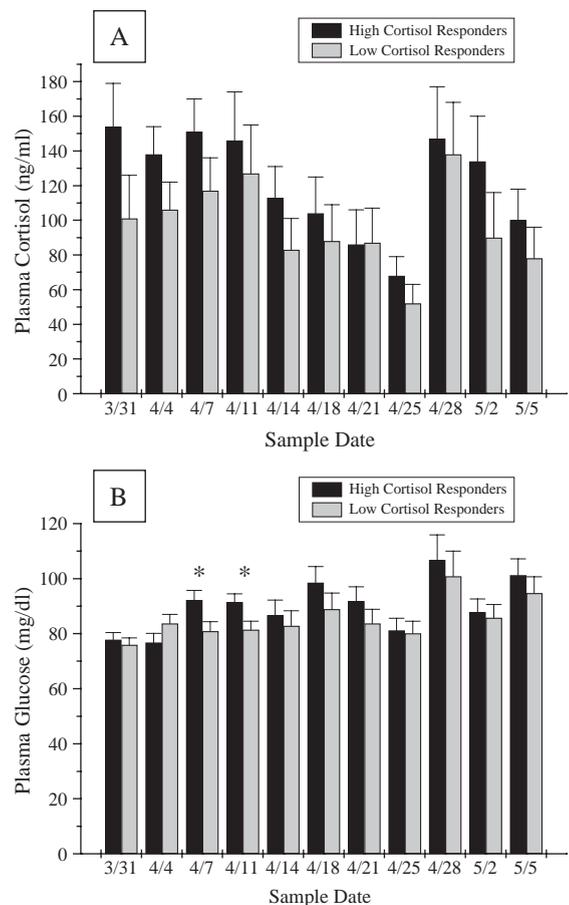


Fig. 5. (A) Mean plasma cortisol and (B) glucose levels in samples taken immediately following a 30-s net challenge during the spawning season in striped bass males selected as high cortisol responders (HCR; $n=10$) or low cortisol responders (LCR; $n=10$). Asterisks denote significant differences between HCR and LCR on that sample date. Vertical brackets represent the S.E.M.

LCR fish were more than 5 times lower than those of HCR fish. Furthermore, LCR fish started spermiating later and stopped spermiating sooner than HCR fish. On most of the sample dates where milt was collected, there were a greater number of HCR fish spermiating than LCR fish. These results show that low cortisol stress responsiveness is linked to decreased plasma levels of androgens in these fish and suggests that the low steroid levels may have affected reproductive performance.

The data on the effects of stress or cortisol administration on plasma levels of gonadal steroids in fish are somewhat equivocal. Stress has been shown to significantly decrease plasma levels of T

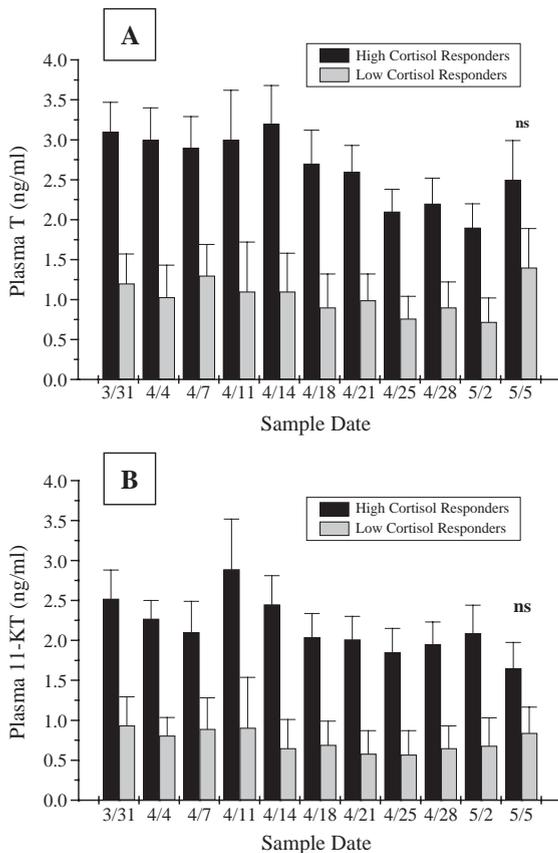


Fig. 6. (A) Mean plasma testosterone (T) and (B) 11-ketotestosterone (11-KT) levels in samples taken immediately following a 30-s net challenge during the spawning season in striped bass males selected as high cortisol responders (HCR; n=10) or low cortisol responders (LCR; n=10). Plasma T and 11-KT were significantly greater in HCRs when compared to LCRs on all sample dates except May 5 (ns=not significant). Vertical brackets represent the S.E.M.

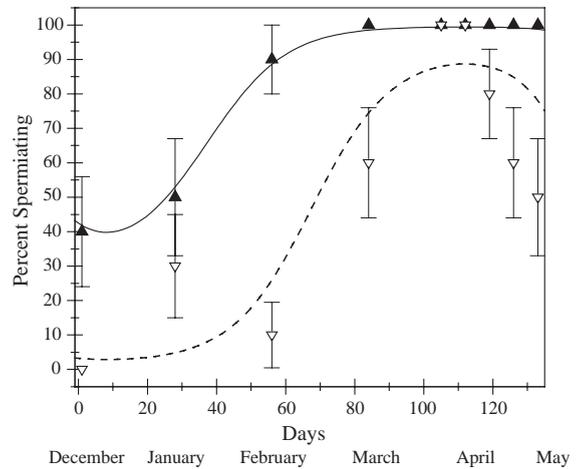


Fig. 7. Mean percentage of striped bass males selected for high cortisol responsiveness (HCR, solid triangles) and low cortisol responsiveness (LCR, white triangles) that had a spermiation response on the sample dates shown. Shown with regression lines fitting the data, HCR (logit) $y = -0.303 - 0.0275\text{day} + 0.00180\text{day}^2 - 0.0000183\text{day}^3$, LCR (logit) $y = -3.40 - 0.0275\text{day} + 0.00180\text{day}^2 - 0.0000183\text{day}^3$. Samples were taken monthly prior to the spawning season and weekly during the spawning season. The difference between the number of HCR and LCR spermiation across all sample dates was very highly significant ($p < 0.0001$). Vertical brackets represent the S.E.M.

(Carragher et al., 1989) and 11-KT in confined male rainbow and brown trout, *Salmo trutta* (Pickering et al., 1987; Campbell et al., 1994). Similar findings have been reported for male black bream, *Acanthopagrus butcheri* (Haddy and Pankhurst, 1999). In contrast, cortisol-implanted sexually maturing male brown and rainbow trout had no differences in plasma 11-KT when compared to sham injected fish (Carragher et al., 1989). In females, stress may lead to no significant change in T and E₂ (Campbell et al., 1994) or to decreased levels of T and E₂ (Campbell et al., 1994; Haddy and Pankhurst, 1999; Cleary et al., 2000; Pottinger and Carrick, 2000).

This effect of stress on gonadal steroid levels has been attributed to a direct inhibition by cortisol on ovarian steroidogenesis of rainbow trout ovarian follicles (Carragher and Sumpter, 1990). However, Pankhurst et al. (1995) reported that neither stimulated (gonadotropin) nor unstimulated ovarian follicles of snapper (*Pagrus auratus*), goldfish (*Carrasius auratus*) or the common carp (*Cyprinus carpio*) cultured in vitro had a diminished capacity to produce T and E₂ even in the presence of high (1000 ng/ml) levels of

cortisol. A recent study demonstrated that dexamethasone can inhibit secretion of 11-KT from testicular tissue of pubertal and adolescent carp cultured in vitro (Consten et al., 2002). In the present study, mean T and 11-KT levels in LCR fish were very low (≤ 1 ng/ml) during most of sexual maturation, approaching levels previously observed during early recrudescence in captive striped bass males (Woods and Sullivan, 1993). In contrast, circulating levels of T and 11-KT in HCR striped bass were significantly higher and within the same range of previously reported values (Woods and Sullivan, 1993; Mylonas et al., 1997). Stress has been shown to cause decreases in gonadotropin (GtH) levels in white sucker, *Catostomus commersoni* (Stacey et al., 1984), which could lead to lower circulating levels of T and 11-KT. Selecting for low cortisol responsiveness may have selected for other characteristics such as a decreased sensitivity of the testes to GtH or differences in clearance of steroids from the blood. Clearly, more study is required to describe the nature of these differences in striped bass.

The significantly lower androgen levels taken together with the shorter spermiation period of LCR fish suggests that these factors may have led to diminished reproductive performance. Spermiating fish (HCR) were first identified in December, approximately 1 month prior to any detectable spermiation response in LCR fish. All HCR fish were spermiating in March and continued through the end of April. In contrast, the majority of the LCR fish did not have a spermiation response until March 12 and only 50% were spermiating at the end of the study. These differences could not be attributed to differences in size or age of individuals. This result suggests that the seasonal sperm production was far lower in the LCR group when compared to the HCR group. However, further study to compare maximum milt volumes as well as sperm counts must be conducted to verify this observation.

Sperm quality indices such as the percentage of motile sperm and the duration of motility are not very good predictors of fertilization potential in striped bass (He and Woods, in press; He et al., 2004). In the present study, there were no differences between the sperm quality indices in LCR and HCR fish. Previous studies in salmonids have shown that stress can significantly accelerate or delay the time

of ovulation, decrease egg size and lower progeny survival (Campbell et al., 1992, 1994; Contreras-Sanchez et al., 1998) or decrease sperm counts (Campbell et al., 1992; Pottinger and Carrick, 2000). When the reproductive performance of rainbow trout, selected as high or low responders, was evaluated, there were no differences in fertilization rate, percent hatch or survival of embryos (Pottinger and Carrick, 2000). It was concluded that there was no advantage or disadvantage in terms of reproductive performance when considering fish on the basis of cortisol responsiveness. In the present study, the shorter and less productive spermiation period suggests that the overall output of sperm for the season was lower in the LCR group as compared to the HCR group.

Sexual maturity and the androgens, T and 11-KT in particular, have an attenuating effect on the cortisol response to stress in male salmonids (Pottinger et al., 1995; Pottinger et al., 1996; Young et al., 1996). The results of the present study suggest that this too may be the case with male striped bass. During the selection period (Aug–Oct), post-stress plasma cortisol levels of HCR fish were significantly greater than the corresponding values for LCR fish on each of the 4 sample dates. In December, androgen levels began to rise signaling the onset of maturation for striped bass at the mid-Atlantic latitude (Woods and Sullivan, 1993). During the same period the differences in post-stress cortisol levels between HCR and LCR fish were eliminated. Beginning in March, the fish were subjected to continual and repeated stress 3 times per week for 5 consecutive weeks and mean plasma cortisol levels were only moderately elevated. It may be that our sampling protocol failed to detect divergent cortisol levels because the fish were bled a single time 48–72 h following the stressors (Monday and Friday). Results from our previous study suggest that this would be sufficient time for recovery of cortisol levels from a similar acute stressor (Wang et al., 2004). However, the chronic nature of the stress in the spawning trial and the presumption that sexual maturation attenuated the cortisol response as reported for salmonids (Pottinger et al., 1995; Pottinger et al., 1996) may also help to explain the modest elevations in cortisol and glucose that we observed. The exact mechanism for this effect of androgens on the cortisol stress response is

unknown. There is some data indicating that treatment with androgens can lead to reduced production of ACTH and cortisol in confined rainbow trout (Pottinger et al., 1996). Clearly, further study is required to determine the basis for this effect in striped bass.

Available evidence concerning the effects of cortisol responsiveness on growth and condition factor is equivocal. In some studies, low cortisol responders grew at faster rates than high cortisol responders (Fevolden et al., 2002) while in other studies, high cortisol responders had better growth rates or simply weighed more than the low cortisol responders (Pottinger and Carrick, 1999a). In the present study, there were no differences for absolute growth in weight or length, nor coefficient of condition between HCR and LCR fish. However, HCR fish did have a higher SGR for weight compared to LCR fish. This is in general agreement to a previous study in our lab (Wang et al., 2004) which also found no differences in absolute growth in length and weight. Selecting for cortisol responsiveness may have an effect on growth rates, but our data do not indicate a strong link between cortisol responsiveness and growth in male striped bass broodstock.

In conclusion, male striped bass selected as low cortisol responders had low post-stress cortisol levels and dramatically lower levels of T and 11-KT when compared to high cortisol responders. Concurrently, the low cortisol responders had a significantly shorter and less productive spermiation period when compared to high cortisol responders suggesting impaired reproductive performance.

Acknowledgements

We are grateful for the statistical guidance we received from Dr. Larry Douglass of the University of Maryland, Department of Animal and Avian Sciences. We would like to acknowledge Mr. Dan Theisen, Chongmin Wang and Dr. Shuyang He for assistance with the experimental stress challenges and blood collection, as well as care and feeding of the animals. This study was funded by the Maryland Agricultural Experiment Station Competitive Grants, Number 5-25866 and 5-25971 to L.C. Woods III. This study was approved by the University of

Maryland's Institutional Animal Care and Use Committee, Research Protocol Number R-01-26.

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