Acute Cortisol Stress Response of Juvenile Winter Flounder (*Pseudopleuronectes americanus*, Linnaeus) to Predation

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Abstract. Wild stocks of winter flounder (*Pseudopleuronectes americanus*) in the Northwestern Atlantic have been depleted via fishing pressure to an extent that has warranted increasingly stronger fishing regulations in the last decade. As a means to strengthen the wild populations of winter flounder and to avoid further fishing restrictions the rearing of juvenile fish in hatcheries for release in coastal waters has been viewed as a possibility. Many fishery biologists have concern that hatchery-reared fish are not behaviorally and/or physiologically equipped to survive in the wild due to a lack of predator interaction and conditioning in culture conditions. However, an investigation of the physiological responses linked to predation of hatchery-reared winter flounder has not been described. This study aimed to determine if there is a stress response, as evidenced by an increase in cortisol production, in hatchery-reared juvenile winter flounder to predatory activity. Predation by green crabs (*Carcinus maenas*) and sand shrimp (*Crangon septemspinosa*) have been implicated as a source of juvenile flounder mortality in laboratory and field studies and therefore were chosen as the invertebrate predators in this study. In addition, the summer flounder (*Parylichthys dentatus*) was selected as a predator for this study because of its co-existence with young winter flounder in estuaries at selected times of the year. Juvenile fish (n =12) were subjected to the pressure of predation for 24h in an arena. Results from radioimmunoassay techniques indicate that juvenile winter flounder initiate a cortisol response to sand shrimp and summer flounder predation, while the predation from a green crab does not elicit such a response. The ability of a fish to detect and react to a stress such as predation is vital to the survival of that individual to a reproductive size. This study represents the first step toward understanding the stress physiology of hatchery-reared juvenile winter flounder to predation. Information regarding the physiology of hatchery-reared fish will provide insight into the value of stock enhancement through the release of cultured winter flounder.

1. Introduction

Winter flounder (*Pseudopleuronectes americanus*) stocks in the Northwestern Atlantic have been depleted as a result of several population pressures. In addition to over fishing, the leading cause of the natural stock depletion, an increase in predation as a result of warmer coastal water temperatures has been suggested (Keller and Klein-MacPhee 2000). As a means to strengthen the wild populations of winter flounder, and to avoid further fishing restrictions, the rearing of juvenile fish in hatcheries for release in coastal waters has been viewed as a possibility. Many fishery biologists are skeptical of such an approach because of the uncertainty regarding the fitness of hatchery-reared fish to survive natural environmental pressures such as predation. Juvenile winter flounder are susceptible to the predation of several different types of predators after settlement. An increased understanding of the relationship between juvenile winter flounder of hatchery origin and its predators will aid in appraising the value of stock enhancement by the release of wild fish into coastal waters.

Predation upon newly settled flatfish greatly affects the year-class strength of that season (Van der Veer and Bergman 1987). Crabs have been documented in a variety of regions worldwide, including estuaries in the southern US, as an important predator in flatfish population dynamics (Reichert and Van der Veer 1991). Recently it has been shown in laboratory studies that juvenile winter flounder are vulnerable to green crab (*Carcinus maenas*) predation (Fairchild and Howell 2000). This information is relevant since green crabs and juvenile winter flounder co-exist spatially and temporally in coastal waters.

Decapod crustacean predation has been implicated as a fine control mechanism for yearly class variations in flatfish (Van der Veer and Bergman 1987). In regions such as the Wadden Sea, *Crangon* shrimp species are the most widely documented predator of young-of-the-year flatfish (Fairchild and Howell 2000). Witting and Able (1995) established that the highest mortality rates due to shrimp predation occur around the time of settlement when a flatfish changes from a pelagic to a benthic lifestyle. The sand shrimp (*Crangon septemspinosa*) has been shown to consume juvenile winter flounder in laboratory trials and appears to be an important predator of flatfish in nursery grounds (Van der Veer and Bergman 1987).

Summer flounder (*Paralichthys dentatus*) is a highly piscivorous species that co-occurs temporally and spatially with juvenile winter flounder. Adult summer flounder and juvenile winter flounder are both present in Narragansett Bay from May to October (Stone et al. 1994). An increase in coastal water temperatures, which are presently being experienced, may increase the

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population of summer flounder in coastal waters. The increased abundance of summer flounder may in turn increase pressure upon juvenile winter flounder populations. A better understanding of the relationship between juvenile winter flounder and adult summer flounder may increase awareness about the consequences of coastal ocean warming.

The stress response of teleosts as evidenced by corticosteriod production has been documented in a variety of species. The release of cortisol by the interrenal into the blood and tissues represents the link between the initial neuroendocrine perception of stress and the mobilization of energy. Mobilized energy is used for changes in behavior and/or physiology to restore an organism to homeostasis with its environment. Studies have aimed at determining the cortisol response of flatfish to a multitude of stressors, most notably those of interest to the aquaculture industry. Waring et al. (1992) characterized the stress response of the flatfish *Platichthys flesus* to handling and net confinement while Barnett and Pankhurst (1998) examined the stress response of greenback flounder to a variety of aquaculture practices. These two studies showed that crowding, confinement, and handling are stressors capable of producing elevated plasma cortisol levels. To this point studies regarding the physiology of stress in flatfish have not examined predation as a possible stressor.

The purpose of this study was to evaluate the stress response to predation in cultured winter flounder. As a tool to investigate the stress elicited by predation, whole body cortisol levels were used to quantify the physiological response to predation by juvenile winter flounder. In addition to determining if there is a cortisol stress response to predation, our study compared the magnitude of the acute stress response of winter flounder to predators with contrasting feeding habits. Information from this study aims to increase knowledge of the teleost endocrine system and provide new insight into the ecophysiology of a flatfish species.

2. Materials and Methods

2.1 Fish Maintenance

All juvenile winter flounder (55 days after hatch) were acquired from the Lennoco Inc. Hatchery in Chatham, MA and transported to the University of Rhode Island Narragansett Bay Campus in April of 2002. Fish were maintained in flow-through seawater (30 ppt) in a holding tank until time of experiment trials. The temperature of the holding tank ranged from 17° C in the early summer to 22° C at time of last experiment. Juvenile fish were fed HUFA enriched *Artemia nauplii*. Laboratory lighting was maintained at a 12:12 light dark cycle and remained unchanged throughout the duration of the study period.

2.2 Experimental Design

Winter flounder (n=12) were placed into an experimental arena. Each arena consisted of a round tub 45 cm in diameter filled with filtered seawater (30 ppt) at 20-22º C. A 2-3 cm thick layer of fine sediment was placed at the bottom of each arena. Light aeration was maintained throughout each experiment. Fish were housed in the arenas for a 48h acclimation period before addition of a predator.

Green crabs and sand shrimp were collected from Narragansett Bay and maintained in flow-through systems until time of experiment. The carapace width of crabs used in this study ranged from 55–70 mm. The total length of sand shrimp used in this study ranged from 50-60 mm. Prior to addition to predator-prey arenas green crabs and sand shrimp were starved for 48h to encourage predatory activity upon interaction with fish. Summer flounder of 100-110 mm total length were acquired from cultured stocks at the University of Rhode Island's Flounder Facility. Summer flounder predators were housed in a holding tank with juvenile winter flounder as a food source.

Upon completion of the 48h acclimation period predators were added to treatment tanks while control tanks were maintained without the addition of predators. Two sets of predator and control arenas were performed for each experiment. For trials involving the green crabs and summer flounder the predator-prey ratio was 1:12. For trials involving shrimp predation a ratio of 2:12 was established. Predator-prey aquaria were maintained under normal photoperiod patterns that were the same as those maintained in the holding tank system. Predator-prey trials lasted for 24h until sampling was conducted.

2.3 Sampling

Upon completion of the 24h interaction period, predators were removed from trial aquaria and transferred back to a holding tank. Fish were immediately netted and administered a lethal dose of 2-phenoxyethanol (Sigma) in seawater. Fish were then removed from 2-PE, blotted with filter paper, and placed in pre-weighed plastic sample tubes. Individual fish wet weights were determined to the nearest 1.0 mg and samples preserved at –80° C.

2.4 Cortisol Extraction and Radioimmunoassay

Flounder individuals were sectioned and homogenized in 1000 µL of phosphate buffer (PBS) with pH 7.6. Samples were then spiked with a 1000 cpm volume (~10 µL) of radioactive cortisol for extraction efficiency calculation. Cortisol was extracted from fish samples using three 1.5 mL volumes of ether. Sample tubes were allowed to evaporate overnight before RIA analysis was conducted.

Dried tubes were reconstituted with 500 µL of standard diluent with a pH of 7.4. Fifty mL of reconstituted sample was then pipetted along with 5 mL of scintillation fluid into vials and placed into a scintillation counter. The recorded counts per minute value was compared to the original count determined for the 1000 cpm volume to determine radioactive cortisol extraction efficiencies for each fish sample. The mean extraction efficiency for all cortisol assays was 44.8% +/- 1.2%.

Fish samples of 200 µL were run in duplicate with 100 µL of labeled cortisol and 100 µL of cortisol antibody at a 1:200 dilution. Tubes containing fish sample, labeled cortisol, and antibody were incubated overnight at 4° C. Following incubation, 400 µL of dextran coated charcoal (DCC) was added to remove unbound cortisol. Tubes were then centrifuged for 15 minutes at 4° C to separate the charcoal from the sample prior to removal of 500 µL of sample for counting in the scintillation counter.

Counts associated with each sample were compared against a standard curve of known cortisol concentrations. The amount of cortisol contained in each tube was then calculated and the whole body cortisol level of the fish determined from the fish's wet weight. Data was then compiled and readied for statistical analysis.

2.5 Analysis

Statistical analysis was conducted using a one-way analysis of variance to determine significance of cortisol responses in regard to treatment. P-values were calculated from log normalized whole body cortisol values to satisfy homogeneity of variance requirements. For all statistical analysis significance was accepted when $P<0.05$.

3. Results

3.1 Experiment #1- Green Crab Predation

Green crab experimental trials resulted in a reduction of the number of individuals in three of the four trial tanks (Table 1). Fish found dead in predator tanks upon inspection appeared not to die of predatory activity from the crab. In trial 1 (Fig 1) whole body cortisol levels in winter flounder exposed to green crab predation (9.86 +/- 2.56 ng/g) was not significantly elevated over the control group (9.60 +/- 2.32 ng/g; P=0.88). In trial 2 (Fig 2), the mean cortisol levels of fish exposed to predation (5.57 +/- 1.64 ng/g) was not significantly greater than the mean cortisol level of control fish (4.94 +/- 0.89 ng/g; P=0.59).

3.2 Experiment #2- Sand Shrimp Predation

Shrimp predation experiments yielded one viable pair of control and predator trials. Sampling of the second trial was unsuccessful due to heavy mortality in the

Winter Flounder Survival

Experiment	Presence of Predator	Trial I	Trial II
Crab	+	92	100
	-	92	83
Shrimp	+	83	17
	-	83	75
S. Flounder	+	83	50
	-	83	83

Table 1. Percent survival of juvenile winter flounder at completion of 24h trial period.

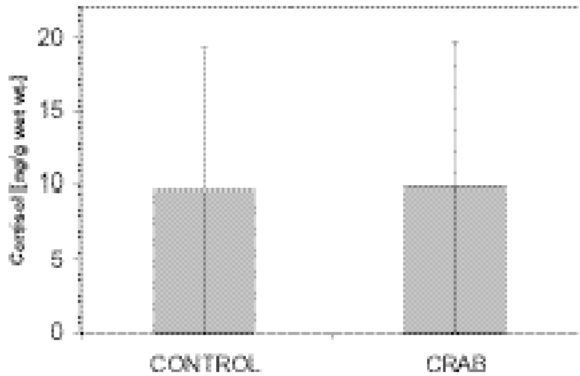

Figure 1. Mean (+/- SEM) whole body cortisol concentration (ng/g of wet wt.) of winter flounder exposed to green crab predation and corresponding control group (Trial 1).

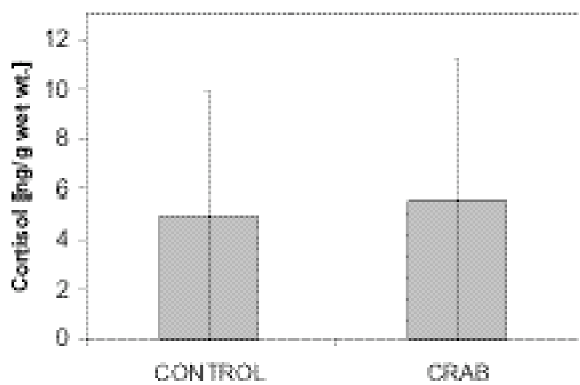

Figure 2. Mean (+/- SEM) whole body cortisol concentration (ng/g of wet wt.) of winter flounder exposed to green crab predation and corresponding control group (Trial 2).

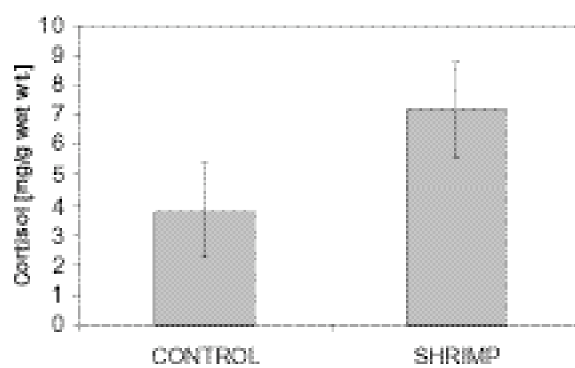

Figure 3. Mean (+/- SEM) whole body cortisol concentration (ng/g of wet wt.) of winter flounder exposed to sand shrimp predation and corresponding control group.

predator tank (Table 1). It did not appear that the mortality was a result of shrimp predation but rather of abiotic factors. In addition to a poor number of sampled fish, RIA analysis yielded cortisol levels that were extremely variable. Data from the second trial was discarded due to an extremely small number of samples and a low level of confidence in the values. In the first trial (Fig. 3) the whole body cortisol levels of fish exposed to shrimp predation (7.19 +/- 1.60 ng/g) was significantly greater than that of the control fish (3.82 +/- 1.54 ng/g; P=0.04).

3.3 Experiment #3- Summer Flounder Predation

The cortisol level of fish from trial one (Fig. 4) exposed to summer flounder predation (13.20 +/- 6.60 ng/g) was greater than control fish (5.56 +/- 0.74 ng/g). Despite the difference in cortisol levels between the control and treatment fish the values were not significantly different (P=0.62). Similar to trial one, cortisol levels of fish exposed to a summer flounder predator in trial two (Fig. 5) had a greater mean cortisol level than control fish. However, in trial two the difference in means was statistically significant (P=0.03). Fish subjected to summer flounder predation experienced a mean cortisol level of 5.92 +/- 1.23 ng/g while control fish experienced a mean cortisol level of 2.96 +/- 0.77 ng/g.

4. Discussion

The most important finding of this study is that predation from sand shrimp and summer flounder is capable of eliciting a cortisol stress response in hatchery-reared juvenile winter flounder. In the only viable experiment with shrimp predation statistical analysis supported the difference in cortisol levels observed between control and treatment fish. Summer flounder predation produced a significant elevation of cortisol levels in one of two trials. However, in the trial in which cortisol was not found to be significantly elevated by predation there is a considerable difference in the mean values between groups. In this case, a large standard error may be strongly influencing statistical analysis.

Predation from green crabs did not result in the elevation of whole body cortisol levels in both trials. It is possible that the predatory behavior of green crabs may not require flatfish to mobilize energy for sustained behavior to avoid predation. Another potential reason for the lack of a cortisol response is that the abundance of green crabs in local estuaries historically has not been great enough to warrant the development of a stress response. Green crabs are a non-indigenous species that were introduced on the eastern seaboard in the early nineteenth century (Levinton 2001). Despite the presence of blue crabs in coastal waters, it is possible that an interaction between green crabs and winter flounder for less than two hundred years is not a sufficient amount of time to initiate a physiological link between the two species.

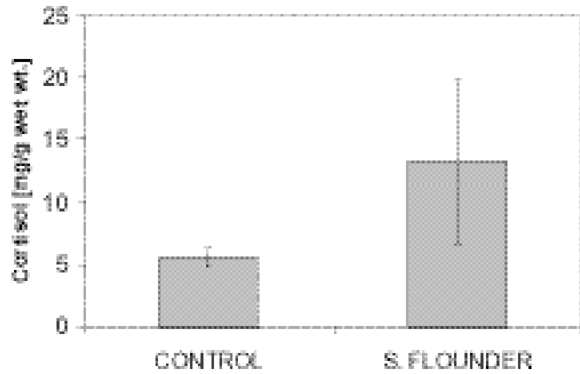

Figure 4. Mean (+/- SEM) whole body cortisol concentration (ng/g of wet wt.) of winter flounder exposed to summer flounder predation and corresponding control group (Trial 1).

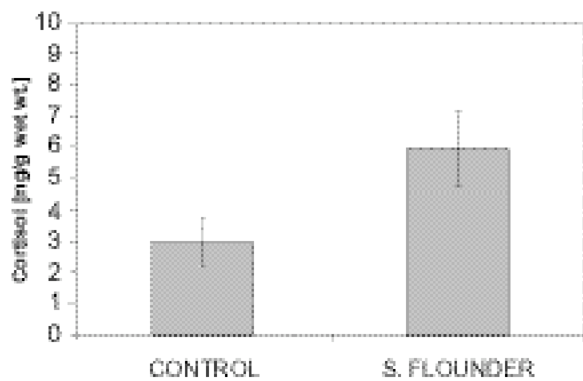

Figure 5. Mean (+/- SEM) whole body cortisol concentration (ng/g of wet wt.) of winter flounder exposed to summer flounder predation and corresponding control group (Trial 2).

The mean cortisol level of fish in control arenas varied from trial to trial in each experiment. Abiotic factors such as temperature, dissolved oxygen, and lighting were tightly controlled, and are unlikely to be the cause of the observed variation. Differences in control values may be due to natural variation of cortisol levels of fish in captivity. Further studies regarding cortisol levels of juvenile winter flounder in captivity would prove valuable in clarifying the reasons for the variation in whole body cortisol levels we observed.

Despite the variation in control values it is possible to compare the magnitude of the cortisol response to different predators in statistically significant trials. In the shrimp experiment (Fig. 2) the mean cortisol levels of fish exposed to predation were 1.9 times greater than the mean levels of control fish. In the second trial of the summer flounder experiment (Fig. 5) the mean cortisol levels of fish exposed to predation were 2.0 times greater than the mean cortisol levels of control fish. It is possible that the stress response to predation in juvenile winter flounder may be of the same magnitude irrespective of the predator involved.

A survival rate greater than 80% was observed in the majority of arenas at the conclusion of the 24h time frames. Inspection of the deceased fish suggested that they did not die of wounds inflicted by the added predator. Deceased fish did not resemble illustrations presented by Seikai et al. (1993) of flatfish preyed upon by a Crangonid shrimp. Further evidence for mortality not associated with predation is that fish were found dead in control arenas as well. We are unsure as to the cause of mortality in the trial arenas. It is possible that a certain percentage of fish in each trial were not capable of acclimating sufficiently to the change in aquaria.

The use of cortisol as a tool to investigate predatory stress between species may prove to be valuable. Further work needs to be done to determine if there is a relationship between a cortisol response elicited by a predator and the probability of a successful predatory interaction between the two species. Olla et al. (1992) determined that an elevation in cortisol levels in juvenile coho salmon reduces the ability of salmon to avoid predation soon after stress initiated by handling. A study of this nature upon juvenile winter flounder involving predator stress elicited cortisol levels would provide insight into the pathway between the neuroendocrine perception of stress and behavior in flatfish species.

Another area for future research would involve the comparison of the physiology associated with predation between cultured juvenile winter flounder and wild juvenile winter flounder. In a behavioral study, Kellison et al. (2000) observed that fish from a hatchery environment spent significantly more time swimming in the water column and took longer to become cryptic on the benthos than wild conspecifics. The authors concluded that hatchery-reared fish are not as well suited for survival in the wild due to a lack of predator interaction in rearing conditions. A comparison of the physiology involved in predator detection may elucidate the reasons for the differences in behavior of cultured flatfish in comparison to wild flatfish.

Future research focused upon understanding the ecophysiology of juvenile winter flounder will better equip fishery biologists to evaluate restocking techniques. Yamashita et al. (2001) discussed how differences in ecological responses via physiological mechanisms effects the growth and survival of individuals and populations. They made the case that there is a vast array of biotic and abiotic factors, including predation, that affect the life cycles and survival of flatfish. Further work surrounding the physiological stress response of juvenile winter flounder to predation will educate biologists about the ecophysiology of flatfish and other teleost fish.

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