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The Effects of Transport Density on Cortisol Levels in Juvenile Winter Flounder, *Pseudopleuronectes americanus*

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One of the biggest concerns surrounding stock enhancement is promoting a successful juvenile transition from the laboratory to the natural environment. One way to achieve a successful transition is to reduce certain stressors, such as handling and transport densities. For instance, the adverse stimuli produced by initial capture, loading into transport containers, the transport to the release site, unloading, and finally stocking have been shown to induce the hypersecretion of both catecholamines and corticosteroids in adult teleosts (Barton and Iwama 1991; Barnett and Pankhurst 1998). The associated distress of this primary stress response can induce a cascade of secondary affects, including osmoregulatory, metabolic, and immune disturbances, resulting in detrimental effects to fish health, growth, and survival (Carmichael 1984; Barton and Iwama 1991).

Examining the aforementioned disturbances and their related physiological changes has proven useful in modifying aquaculture techniques to reduce stress in reared fish (Pickering 1993). Much of the published literature on teleost stress physiology has focused on adults (e.g., Barton and Iwama 1991; Waring et al. 1992; Wedemeyer 1996), while information describing the specific effects of transport and/or handling stress of juveniles is limited to fewer species (e.g., Barton 2000; Shrimpton et al. 2001; De Carvalho et al. 2002; Barton et al. 2003; Gomes et al. 2003; Urbinati et al. 2004) and is nonexistent for young-of-the-year flounder. The few juvenile species that have been studied suggest that handling and confinement associated with fish transport are likely to impact the performance and scope of juvenile survival (Serafy et al. 1999; Shrimpton et al. 2001; De Carvalho et al. 2002).

The current information presents results from research conducted as part of an ongoing juvenile winter flounder, *Pseudopleuronectes americanus*, stock enhancement project at the University of New Hampshire. The intentions of this study were to determine which transport density produced the least amount of stress when juvenile winter flounder were relocated to the release site.

Materials and Methods

Animal Maintenance

Juvenile winter flounder were reared at the University of New Hampshire's Coastal Marine Laboratory (CML) in Newcastle, New Hampshire, USA, from a wild broodstock that were captured locally in March 2003 by commercial fishing vessels. In August 2003, approximately 10,000 cultured juvenile flounder were individually marked with decimal coded wire tags (0.25 × 1.1 mm; Northwest Marine Technology, Inc., Shaw Island, WA, USA), and approximately 4000 flounder were marked with visible elastomer tags (VIE) (variable tag size; Northwest Marine Technology, Inc.). All tagged fish were approximately the same size (42 mm mean total length ± 0.1 SEM) and age (5 mo). For complete tagging protocol refer to Sulikowski et al. (2005).

Experimental Design

Prior to transport, all tagged fish were acclimated for at least 7 d after tagging. In August

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2003, tagged flounder were stocked at five different densities (100, 200, 300, 400, and 600% of substrate surface area) and transported to the release site (Hampton River, Hampton, NH; GPS location 42°54.532′N by 70°49.491′W) in 1-m^3 insulated containers onboard a UNH research vessel. Stocking densities were calculated using ventral fish surface area to bottom tank area ratio. To measure density, mean fish ventral surface area was estimated by tracing a subsample of 15 live specimens from the entire population onto a 0.5- × 0.5-cm grid paper. Bottom surface area was then determined by counting the number of 0.5 cm^2 squares, or fractions of squares, covered by the fish's outline. The mean fish size for the transport and density experiment was 20.6 cm^2, resulting in 404, 808, 1212, 1616, and 2424 fish to achieve 100, 200, 300, 400, and 600% densities, respectively.

During transport, fresh seawater was continuously pumped through each insulated container. Dissolved oxygen, temperature, and pH levels were monitored to ensure that proper water quality was maintained. Although water temperature gradually decreased during transport from CML temperatures (16.4 ± 1.5 C) to the release site (15.6 ± 2.0 C), this change was minimal and well within the observed tolerance levels for this species (Fairchild 2002). Approximately 50 fish were sampled for measurement of tissue cortisol concentrations at four different times during transport (transport stage) for each stocking density. The transport stages examined were as follows: (1) movement from the CML to the research vessel (approximately 5 min of transport in 5-gal buckets at the appropriate density), (2) midway through the transport process (45 min), (3) upon arrival at the site (90 min), and (4) after 48-h acclimation at the release site. For the fourth and final measurement, juvenile flounder were stocked into predeployed, vinyl-coated wire acclimation chambers (3 × 1.5 × 0.5 m), maintaining the same treatment densities used during transport to the release site. No mortality occurred in any transport density.

Control Fish

Two separate treatments consisting of 50 DWCT and 50 VIE fish were acclimated for at least 7 d after tagging to serve as controls. Here, the fish were hand netted, immediately frozen on dry ice (−70 C), and stored at −20 C for later use. Because no statistical differences existed between the tagging types, the control data were pooled.

Cortisol Extraction and Radioimmunoassay

Preparation of juvenile flounder followed techniques used by Sulikowski et al. (2005). Because the flounder were too small to be bled, it was necessary to extract the cortisol from whole specimens. For this procedure, individual juvenile flounder from each transport stage and density were weighed to the nearest gm (Ohaus model TS4K balance) and dissected into smaller segments. To ensure that cortisol levels fell within detectable levels, individual juvenile winter flounder were combined in a 50-mL test tube to yield approximately 5 g of tissue. This 5 g of pooled tissue represented an individual sample. In this scheme, each transport stage of each stocking density treatment consisted of six samples of pooled fish. The control treatments consisted of a total N of 12 (an N of 6 for each tag type).

Individual samples (5 g of pooled tissue) were homogenized in ice-cold phosphate-buffered saline (PBS), centrifuged, and the supernatant removed. Each sample was extracted three times with a threefold volume of ether (anesthesia grade) before the aqueous phase was frozen in an acetone/dry ice bath. Following evaporation of the ether under a stream of nitrogen, the dried extracts were reconstituted in PBS with 0.1% gelatin. Approximately 1000 counts/min of tritiated cortisol was added to plasma samples to account and correct for procedural losses. The overall mean recoveries were 76%. Duplicate samples of cortisol were analyzed by the Atlantic Veterinary College (Prince Edward Island, Canada) using standard radioimmunoassay techniques (e.g., Tsang and Callard 1987; Sulikowski et al. 2004) The intra-assay and interassay coefficients of variance were 4.4 and 5.1%, respectively.

Statistical Analyses

The effects of stocking density (100, 200, 300, 400, and 600%) and transport stage (5 min, 45 min, 90 min, and 48 h) were evaluated on

tissue cortisol concentrations. Data were analyzed with ANOVA and Tukey's test for multiple comparisons of means using SYSTAT version 10 software (SPSS, Inc., Chicago, IL, USA). Results are presented as mean ± 1 SEM (*N*). A probability (*P*) value of <0.05 was considered statistically significant.

Results

Transport to the release site at all stocking densities elicited a stress response in juvenile winter flounder when compared to control fish (Fig. 1); however, distinct differences in the amount and extent of the stress response existed between the densities. The initial transport of juvenile flounder from the CML to the research vessel did not elicit an increase ($P = 0.97$) in cortisol concentration in any of the stocking densities. In contrast, after 45 min of vessel transport, cortisol levels for fish in all stocking densities were statistically higher than for the control. Although cortisol levels of fish stocked at each density were still elevated upon arrival at the release site (90-min vessel transport), all values except those for fish stocked at the 600% density had begun to decrease. Moreover, cortisol levels of fish stocked at the 200 and 300% densities, both returned to near baseline levels. The reduction in cortisol levels continued after 48-h recovery, as levels of this hormone returned to near baseline levels for all transport densities except for those fish stocked at 600%. While cortisol levels in fish stocked at the 600% density showed quantitative declines, these values still remained statistically higher than in the control.

Discussion

The occurrence and consequent physiological responses of juvenile flounders to stress, especially individuals only a few cm in total length, are poorly understood (Sulikowski et al. 2005). This study is one of the first to offer fundamental information that may be useful in modifying practices for successful stock enhancement of juvenile flounder.

The use of cortisol to evaluate stress levels associated with transport and density has been

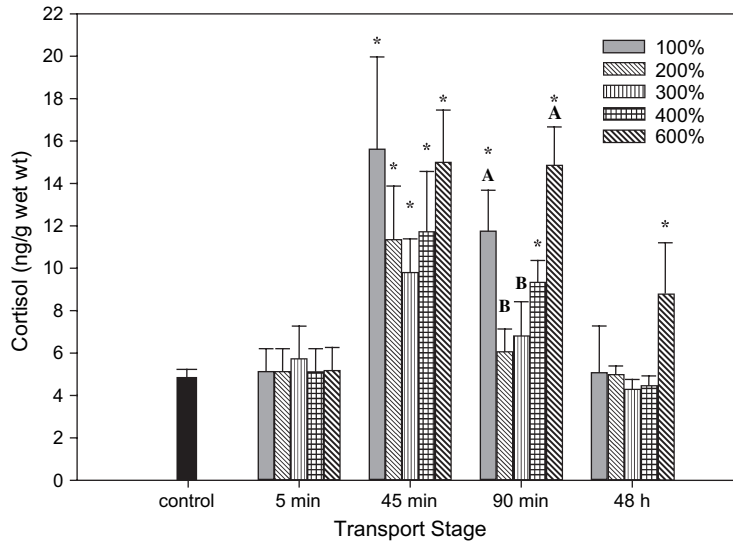

FIGURE 1. *Mean (±SEM) whole-body cortisol concentration (ng/g of wet weight) of tagged juvenile winter flounder transported at 100, 200, 300, 400, and 600% densities for each transport stage. The corresponding control group represents tagged fish that were not transported. Asterisks denote transport density values that are significantly different from the control value (*$P < 0.05$*). Transport densities designated with different letters are significantly different from each other (*$P < 0.05$*). Each experimental stage represents six pooled samples (5 g of tissue per pool). Each control represents 12 pooled samples (5 g of tissue per pool).*

used in other juvenile species. For example, Gomes et al. (2003) found that increased mortality and higher concentrations of plasma cortisol were associated with higher transport densities in juvenile tambaqui, *Colossoma macropomum,* while Congleton et al. (2000) found higher cortisol levels in juvenile chinook salmon *Oncorhynchus tshawytscha* when transport densities were at their maximums. Moreover, the study of four juvenile salmonid fish indicated that all species were stressed by transport and that species-specific responses existed (Barton et al. 2003). In this study, transport density also produced elevated cortisol levels in juvenile winter flounder when compared to control (nontransported) specimens. Although statistical comparison between the transport densities only revealed significant differences between fish stocked at 200 and 300% transport densities to those stocked at 100 and 600% after 90-min vessel transport, we believe that the quantitative differences in cortisol levels between the densities are biologically significant (Yoccoz 1991; Sulikowski and Howell 2003). For example, although cortisol levels for fish in all stocking densities were statistically higher than the control when sampled after 45 min of transport, fish stocked at 100 and 600% density showed increases in cortisol levels that were up to 5 ng/g wet weight higher than the other measured transport densities. Moreover, these quantitative differences continued in measurements taken at the release site (90 min after transport). At this stage of transport, cortisol levels for fish stocked at 100 and 600% densities were between 6 and 9 ng/g wet weight higher than those observed at the other three densities. Increased transport density has been shown to adversely effect the physiology of many species (i.e., Montero et al. 1999), so the significantly elevated cortisol levels in fish stocked at the 600% transport density is not surprising. However, the disproportionate changes in cortisol levels of fish stocked at the 100% density, the lowest transport density, during movement to the release site were unexpected. The 100% transport density was lower than the culture tank density (approximately 200%) in which the juvenile flounder were reared at the CML. It is possible that the reduction in density acted as a stressor and elicited and perhaps augmented the transport stress response. However, future studies are needed to test this possibility.

We believe that the protracted stress response exhibited at the 48-h sampling stage by fish stocked at the 600% density may decrease the fitness of these individuals upon release in the wild. Fish with elevated levels of corticosteroids after transport probably face additional stress, both biotic and abiotic, when released into the stock enhancement site (Wanat 2002). For instance, Breves and Specker (2002) have shown that predation from sand shrimp, *Crangon septemspinosa*, and summer flounder, *Paralichthys dentatus,* elicited a cortisol stress response in juvenile winter flounder. Both species are important predators of juvenile winter flounder (Witting 1995) and are prevalent within our enhancement study sites (Fairchild and Howell 2000; Jones 2000; Fairchild 2002). Accumulation of stressors has been shown to intensify physiological responses in other species (Carmichael 1984; Maule et al. 1988; Iversen et al. 1998). For example, chinook salmon subjected to multiple acute stressors showed an increased predator avoidance time (Sigismondi and Weber 1988) and were found to have lower resistance to pathogens and poorer seawater adaptability (Schreck et al. 1995). Based on this information, it is possible that exposure to predators, or other stressors, could produce an additive stress response in juvenile winter flounder stocked at the 600% transport density that could compromise their survival after release. Future studies are needed to test this hypothesis.

Conclusion

The results of this study suggest that transport of juvenile winter flounder produced a stress response at all stocking densities. However, only fish stocked at the 600% density failed to recover to baseline levels within 48 h. To ensure the successful transport of this species from the hatchery to the wild, we recommend transporting the fish at a 400% stocking density to the release site, whereupon they should be placed into acclimation cages for a minimum of 48 h prior to their release into the wild.

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