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The Effects of Tagging and Transport on Stress in Juvenile Winter Flounder, *Pseudopleuronectes americanus*: Implications for Successful Stock Enhancement

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The winter flounder *Pseudopleuronectes americanus* is widely distributed in the Northwest Atlantic, ranging from Labrador to Georgia (Collette and Klein-MacPhee 2002), and supports both commercial and recreational fisheries throughout its range. Due to precipitously declining stocks in recent years, *P. americanus* has been classified as over-exploited by the National Marine Fisheries Service (Clark 1998). The causes of this decline in winter flounder abundance are not well understood, but it appears that overfishing is the most likely explanation. While it was hoped that stringent fisheries regulations would allow winter flounder populations to rapidly rebuild to historic levels, it has recently been estimated that more than a decade will be needed for their stocks to fully recover (Clark 1998). The concomitant decline in this natural resource, and documented successes with stock enhancement of other fish species, such as Japanese flounder *Paralichthys olivaceus* (Fukusho 1993), turbot *Scopthalmus maximus* (Iglesías and Rodríguez-Ojea 1994), and white seabass *Atractoscion nobilis* (Drawbridge et al. 1995), led a panel of fisheries and aquaculture experts to conclude that both aquaculture and stock enhancement should be explored as means of increasing the abundance of winter flounder in the US (Waters 1996).

In short, the primary function of stock enhancement for winter flounder, or any other fish, is to raise juveniles from wild broodstock and then release them back to the wild at an optimal size for survival (Cowx 1994; Blankenship and Leber 1995). One of the biggest concerns surrounding stock enhancement is promoting a successful juvenile transition from the laboratory to the wild environment. One way to achieve a successful transition is to reduce or eliminate husbandry related stressors, such as handling and transport durations. For instance, the adverse stimuli produced by initial capture, loading into transport containers, the actual transport, unloading, and finally stocking have been shown to cause hypersecretion of both catecholamines and corticosteroids in adult teleosts (Robertson et al. 1988; Barton and Iwama 1991; Barnett and Pankhurst 1998). This primary stress response, which may occur immediately following extended handling and/or transport, can induce a cascade of secondary effects, including osmoregulatory, metabolic, and immune disturbances resulting in detrimental effects to fish health, growth and survival (Carmichael 1984; Robertson et al. 1988; Barton and Iwama 1991). Examining the aforementioned disturbances and their related physiological changes have proven useful in modifying the aforementioned aquaculture related techniques (Robertson et al. 1988; Pickering 1993). A large portion of the published studies that have used cortisol as a means to study stress have focused on adults (Barton and Peter 1982; Robertson et al. 1988; Barton and Iwama 1991; Waring et al. 1992; Sulikowski and Howell 2003). Only recently has the effect of stress in juveniles been measured by changes in cortisol levels (Shrimpton et al. 2001; Barton et al. 2003; Urbinati et al. 2004). As a final point, the use of this hormone to study this type of stress in juvenile flounder is nonexistent. The 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 manuscript resulted from research conducted as part of an ongoing juvenile winter flounder stock enhancement project at the University of New Hampshire. Two important stressors that have the potential to influence juvenile fish health during stock enhancement are the insertion of identifying tags and transport to the release site. Thus, the intentions of the present study were two-fold: 1) to determine which identifying tag, either decimal coded wire or visible elastomer, produced the least amount of stress when inserted into young of the year flounder; and 2) to determine which transport density, either 100% or 600%, was less stressful to the winter flounder as they were relocated to the stock enhancement site. Together, this information will be used in future enhancement projects to promote a successful juvenile transition from the laboratory to the wild environment.

Materials and Methods

Animal maintenance

Fourteen thousand cultured, juvenile winter flounder were tagged and released into the Hampton River, Hampton, New Hampshire, USA. These fish were reared at the University of New Hampshire's Coastal Marine Laboratory (CML) in Newcastle, New Hampshire, USA from a wild broodstock that was captured locally in March 2003 by commercial fishing vessels. In August 2003, approximately 10,000 juvenile flounder were individually marked with decimal coded wire tags (DCWT) (0.25 x 1.1 mm; Northwest Marine Technology, Inc., Shaw Island, Washington, USA) and approximately 4,000 flounder were marked with visible elastomer tags (VIE) (variable tag size; Northwest Marine Technology, Inc., Shaw Island, Washington, USA). All tagged fish were approximately the same size (42 mm mean total length ± 0.1 SEM) and age (5 mo).

Prior to tagging, all fish were anesthetized with MS-222 (0.3 g/l). Decimal coded wire tags were injected into the epaxial muscle tissue of their ventral side with the use of an automated tag injector, while VIE marks were injected into the ventral side epaxial muscle tissue with a hand held 1-mL syringe equipped with a 26-gauge needle. To insure homogeneity in handling techniques for both tag types, an approximate subsample of 100 juvenile flounder was hand netted and placed into an aerated 20-L bucket. Approximately 20 fish at a time were hand netted and placed into a 30 x 25 x 7 cm seawater filled holding box where fish were individually marked, and then immediately placed into a new aerated 20-L bucket to recover. This process was repeated until all fish were tagged.

Experimental Design; Tagging Experiment

In order to study changes in cortisol levels, and time periods needed for this hormone to return to baseline values after tagging, the DCWT and VIE experiments were each composed of seven time trials (0, 3, 6, 9, 12, 24, and 48 h after insertion of the tag) with each time trial consisting of approximately 50 juvenile flounder. To minimize any bias between the experiments, the first 50 fish tagged from the initial subsample of 100 (as per above) were used for each time trial. For the initial tagging (time 0), once the 50th fish had been tagged, the group was hand netted out of the aerated 20-L bucket and immediately snap frozen on dry ice (-70 C), and stored at –20 C until further analyses of cortisol levels took place. For the subsequent time trials, 12 (6 for each tag type) circular 1-m diameter fiberglass tanks, supplied with flowing, seawater (16.4 ± 1.5 C) were established. Each of these tanks represented one time trial 3, 6, 9, 12, 24, and 48 h after insertion of the tag. (Note, the clock for each time trial was started once tagging of the first fish in that specific time trial was initiated). After a new set of 50 fish were tagged, this group was placed in their own individual time trial tank where they remained until they were hand netted and snap frozen at the appropriate time. For example, 3 h after tagging, the 50 flounder from that time trial tank (in this case the 3-hr tank) were snap frozen and stored. This scheme continued until the fish designated to be sampled 48 h after having been tagged were snap frozen. In this format each time trial tank was individualized, excluded and unaffected by the netting of flounder at the previous sampling time trial. To avoid any additional stress that might bias our results, all tagged fish were handled in the exact same manner at each time trial. No mortality occurred in either DCWT or VIE tagged fish.

Experimental Design—Transport Density Experiment

In order to determine the optimal transport density based on cortisol levels, tagged flounder were stocked at two different densities (100%, and 600%) and relocated to the release site (Hampton River in -m^3 insulated containers onboard a UNH research vessel. Stocking densities were calculated based on a ventral fish 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 x 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 an *N* of 404 and 2,423 fish to achieve 100% and 600% densities, respectively. Prior to transport, all tagged fish were acclimated for at least 7 d post tagging.

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 later analyses of cortisol at four different time intervals for each density: 1) movement from the CML to the research vessel (5 min); 2) midway through the transport process (45 min); and 3) upon arrival at the site (90 min). For the fourth and final measurement, juvenile flounder were stocked into pre-deployed, vinyl-coated wire acclimation chambers (3 x 1.5 x 0.5 m), at the same density as that of transport to the release site. After 48 h of acclimation to their new environment, the fourth measurements were taken for each density. No mortality occurred in either transport density.

Control fish

Two treatments consisting of 50 untagged juvenile flounders served as controls for the DCWT and VIE tagging experiments. Here the control fish were hand netted from their original 2-m diameter tank, along with those destined for the tagging experiments, and immediately snap frozen on dry ice (-70 C), and stored at –20 C for later use. For the transport and density experiments, two treatments of 50 DWCT and VIE fish were snap frozen on the day of transport to ascertain baseline cortisol levels prior to movement. Again, tagged fish were acclimated for at least 7 d post tagging before these measurements were taken.

Cortisol Extraction and Radioimmunoassay

Preparation of juvenile flounder followed a modified technique of Hiroi et al. (1997) and Breves and Specker (2002). Individual juvenile flounder from each tagging time trial and transport interval were weighed to the nearest gram (Ohaus model TS4K balance) and dissected into smaller segments. To ensure cortisol levels fell within detectable levels, three individual juvenile winter flounder were combined/pooled in a 50-mL test tube to yield approximately 5 g of tissue. These 5 g of pooled tissue represented an individual sample. In this scheme, each time trial of the tagging experiments and time interval of the transport/density consisted of six samples of pooled fish. The control treatments consisted of an *N* of 6 for the tag comparison study and a total *N* of 12 for the transport density study (an *N* of 6 for each tag type). Since no statistical differences existed between the control groups of tagged fish, the data were pooled for the transport density study.

The frozen samples were homogenized in a 1:1 volume of ice cold PBS (0.06M $Na_2HPO_4*7H_2O$, 0.04M $NaH_2PO_4*H_2O$, 0.15M NaCl, 0.1% sodium azide) using a Polytron homogenizer and 36-mm generator (Glenn Mills, USA). The homogenate was centrifuged for 5 min at 3,500 rpm, and the supernatant removed and placed into a new 50-mL test tube. Each sample was extracted three times with a threefold volume ether (anesthesia grade) before the aqueous phase was snap frozen in an acetone/dry ice bath. Following evaporation of the ether under a stream of nitrogen, the dried extracts were reconstituted in phosphate-buffered saline with 0.1% gelatin (PBSG). Approximately 1,000 counts/min (cpm) of tritiated cortisol were 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 their standard radioimmunoassay techniques. The intra-assay and inter-assay coefficients of variance were 4.4% and 5.1%, respectively.

Statistical analyses

Results are presented as mean ± 1 SEM (*N*). Statistical analyses were conducted using ANOVA, followed by a Tukey's test using SYSTAT, version 10 software (SPSS, Inc., Chicago, Illinois, USA). A probability (*P*) value of < 0.05 was considered significant.

Results

Tagging Experiments

Pooled results suggest that marking juvenile winter flounder with either DCWT or VIE tags produced perturbations to cortisol levels when compared to pooled control fish (Fig. 1). Moreover, quantitative differences in amount and extent of the stress response existed between the two tag types when compared to the control values. For instance, DCWT samples showed a subtle increase in cortisol levels in the first 6 h post tagging. Statistically significant increases occurred at 9 h post tagging, followed by a peak in cortisol levels at the 12-h sampling period. Although still significantly higher than control levels, cortisol concentrations began to decrease 24-h post tagging and eventually returned to near baseline levels 48 h after the DCWT experiment had begun. In contrast, the VIE samples displayed an increasing trend in cortisol levels that became statistically different than control levels 6 h post tagging. The increasing trend continued through each sampling period thereafter until a peak in cortisol concentration was observed 24 h post tagging. Although cortisol levels had declined at the final sampling period (48 h), the concentration of this hormone was still statistically higher than control levels.

Transport and Density Experiments

Transport to the release site at both 100% and 600% stocking densities elicited a stress response in pooled juvenile winter flounder samples when compared to pooled control fish (Fig. 2). However, similar to the tagging experiments, distinct

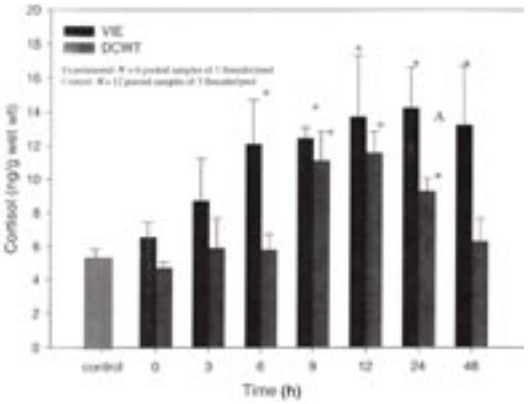

FIGURE 1. *Mean (± SEM) whole body cortisol concentration (ng/g of wet wt) of juvenile winter flounder marked with either visible elastomer tags (VIE) or decimal coded wire tags (DCWT). The corresponding control group represents untagged fish. Asterisk denotes VIE values that are significantly different from the control value (*$P < 0.05$*). Plus sign denotes DCWT values that are significantly different from the control value (*$P < 0.05$*). The letter A denotes statistical difference between VIE and DCWT cortisol levels. Each experimental interval represents an* N *of six pooled samples of three flounder/pool. Each control represents an* N *of 12 pooled samples of three flounder/pool.*

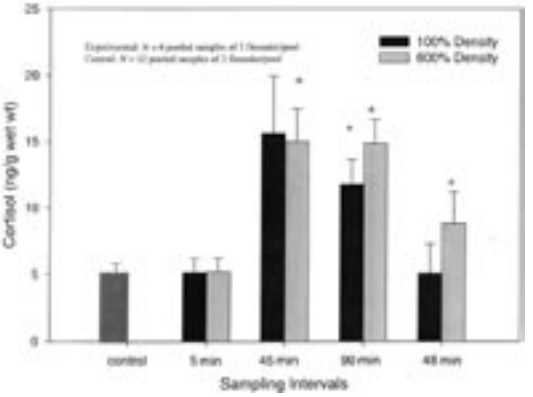

FIGURE 2. *Mean (± SEM) whole body cortisol concentration (ng/g of wet wt) of tagged juvenile winter flounder transported at either 100% or 600% density. The corresponding control group represents tagged fish that were not transported. Asterisk denotes 100% density values that are significantly different from the control value (*$P < 0.05$*). Plus sign denotes 600% density values that are significantly different from the control value (*$P < 0.05$*). Each experimental interval represents an* N *of six pooled samples of three flounder/pool. Each control represents an* N *of 12 pooled samples of three flounder/pool.*

differences in 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 appear to produce a stress response at either stocking density. In contrast, at the midpoint (45 min) of transfer, cortisol levels for fish in both stocking densities were statistically higher than the control values. Cortisol levels of fish stocked at 600% density remained quantitatively unchanged at the third transport measurement (90 min). Although cortisol levels of fish stocked at 100% density were still statistically higher than control levels, the concentration of this hormone was quantitatively lower than values of fish stocked at 600% density, and had begun to decrease at the 90-min interval. At the fourth and final measurement (48 h), cortisol levels of fish stocked at 100% density returned to near baseline levels while those stocked at 600% density were still statistically higher than the control.

Discussion

The occurrence of stress related reactions following handling and transport of adult teleosts, especially the salmonids, is well established in the literature (Bourne 1986; Roberston et al. 1988; Barnett and Pankhurst 1998; Sulikowski and Howell 2003) as are those involving different stocking densities (Martinez-Tapia and Fernandez-Pato 1991; Bjornsson 1994; Procarione et al. 1999). However, the consequent physiological responses of juvenile flounder subjected to the same types of stressors are poorly understood, and those that pertain to juvenile winter flounder, especially individuals only a few cm in total length, are nonexistent. Since the protocols for rearing winter flounder in a laboratory setting have been developed (Fairchild 1998), the next essential step in stock enhancement of this species is establishing a procedure for the successful transition to the wild. As a result, the present study offers fundamental information for future studies towards modifying aquacultural related practices for successful stock enhancement of juvenile flounder.

Tagging experiments

Using either DCWT or VIE tags to mark juvenile winter flounder produced significant perturbations to cortisol levels in pooled fish when compared to the controls. The maximum values, and those for the controls, represented in the present study are similar (13.2 ng/g and 5.6 ng/g wet wt., respectively) to cortisol levels in juvenile winter flounder subjected to a predation stressor (Breves and Specker 2002). Moreover, the tagging experiments on juveniles of other species have produced some degree of stress response. For instance, increased cortisol levels were exhibited by juvenile chinook salmon *Oncorhynchus tshawytscha* (Sharpe et al. 1998) marked with DCWT, while juvenile herring *Clupea h. harengus* marked with plastic anchor tags displayed a significant increase in mortality rates when compared to nontagged fish (Stobo et al. 1992).

Although statistical comparison between the DCWT and VIE only revealed significant differences within one time interval (24 h), consistent quantitative differences in amount and scope of the stress response existed between the two tag types. Since all fish were treated in an equivalent manner after tagging, the observable differences in the amount and scope of stress responses are most likely attributed to differences in the methods used to insert each tag. Interestingly, the DCWT samples displayed a lag period before cortisol levels quantitatively and significantly rose above control levels. While we are unsure why this lag period took place, it is possible that the delayed response is part of a natural reaction to the amount and type of stress the DCWT presented. However, although all fish were treated in an equivalent manner after tagging, it is possible that the DCWT fish were subjected to some other stress that we were unaware of at those time intervals.

Many authors have discussed the relative merits of judging biological significance based on statistical tests, and suggested that it is not always associated with statistical significance (Yoccoz 1991; Natanson et al. 1999). This may be the case in our study of fry winter flounder as there appears to be a different physiological reaction for each tag type. This is not surprising since the tagging methodology between the two tag types was notably different. For example, the amount of aerial exposure varied from a few seconds for DCWT to over 15 sec for the VIE tag insertion. In addition, the amount of time the needle remained within the flesh was also prolonged in

VIE tagged fish. Waring et al. (1996) found that augmented aerial exposure increased plasma cortisol levels in turbot *Scophthalmus maximus*. Likewise, Sulikowski and Howell (2003) suggested that increased aerial exposure of summer flounder *Paralichthys dentatus* resulted in initially high values of cortisol. Moreover, the quantitative differences in the cortisol levels between the tag types were not limited to the initial handling/aerial exposure, but continued at the 48-h sampling time interval. Previous studies suggest that the prolonged stress response exhibited by the VIE tagged flounder could induce a cascade of secondary effects resulting in disturbances to fish health, growth and survival especially if other stressors are added (Carmichael 1984; Robertson et al. 1988; Barton and Iwama 1991). In addition, mortality due to accumulation effects of different stressors has been documented in other species such as red drum *Sciaenops ocellatus* L. (Serafy et al. 1999) and Atlantic salmon *Salmo salar* L. (Iversen et al. 1998). Based on these studies, it is reasonable to suggest that the quantitative differences in cortisol levels represent a biologically significant event, which would place the VIE tagged fish at a higher risk of mortality if immediately subjected to another type of stressor. Thus, we suggest that marking juvenile winter flounder with DCWTs offers a less stressful alternative to marking the fish with VIE tags.

Transport and stocking density

Transport and stocking densities of juvenile winter flounder also resulted in elevated cortisol levels when compared to control fish. The effects of transport density on juvenile health appear to be species specific. For example, Gomes et al. (2003) found 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. Handling and confinement of juvenile American shad *Alosa sapidissima* also produced concomitant increases in plasma cortisol (Shrimpton et al. 2001). In contrast, cortisol concentrations were inversely related to stocking density in juvenile matrinxa *Brycon cephalus* (Urbinati et al. 2004).

As with the tagging portion of our study, we believe that the quantitative differences in cortisol levels between the two densities, especially at the 48-h sampling interval, represent a biologically significant event (Yoccoz 1991; Sulikowski and Howell 2003). We suspect that the prolonged stress response exhibited at the 48 hr sampling interval by fish stocked at 600% density for transport would be less fit to survive in the wild, especially if these fish had been recently tagged (within 24 h). 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, predation from sand shrimp *Crangon septemspinosa* and summer flounder *Paralichthys dentatus* elicited a cortisol stress response in juvenile winter flounder (Breves and Specker 2002). Both species are important predators of juvenile winter flounder (Witting 1995), and sand shrimp are prevalent within our stock enhancement site (Fairchild and Howell 2000; Jones 2000; Fairchild 2002). Although not measured in the present study, fish transported at high densities may be exposed to mechanical abrasion due to contact among them (Ross and Ross 1999). This can cause loss of fish scales and mucus, leading to infection and disease (Wedemeyer 1996). Accumulation of stressors has been shown to intensify physiological responses (Carmichael et al. 1983; Maule et al. 1988; Iversen et al. 1998). For example, chinook salmon subjected to multiple acute stressors displayed an increased predator avoidance time (Sigismondi and Weber 1988). It is conceivable that exposure to predators or injury during transport, along with the already augmented cortisol levels of the 600% density 48-h time interval, could produce an additive stress response that compromised the survival of those fish after release. However, future studies are needed to test this assumption.

Conclusion

The results of the present study suggest that VIE tags provoked a quantitatively stronger stress response when compared to DCWT, and that juvenile winter flounder tagged with VIE tags need more time to recover. Although transport to the release site produced a stress response at both

100% and 600% densities, fish stocked at 100% density began to recover more rapidly, returning to baseline levels within 48 h. To ensure the successful transition of this species from the laboratory to the wild, we recommend waiting a minimum of 48 h after insertion of DCWTs before transporting the fish at a 100% stocking density.

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