Flatfish stock enhancement: Examining conditioning strategies to promote success

Michelle Lynn Walsh

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Flatfish stock enhancement: Examining conditioning strategies to promote success

Abstract
Conditioning is the process of providing individuals reared for stock enhancement with some degree of "wild" experience prior to release. Flatfish trained for "wild" conditions may more easily and successfully transition to natural environments. This dissertation identifies strategies that optimize feeding-related performance of flatfish in the hatchery and subsequently post release in the wild.

The influence of live feed conditioning on feeding performance of juvenile winter flounder, Pseudopleuronectes americanus, was investigated. In the hatchery, fish reared on live feeds exhibited significantly higher survival ($P < 0.0001$) and growth ($P < 0.01$) than those reared on formulated feed. Once released into cages in the wild, amphipod reared fish had higher mean Stomach Contents Index and RNA/DNA of all feed types, including wild fish. Wild and worm-reared fish exhibited the most similar survival, baseline RNA/DNA values, overall stomach fullness, and diet composition profiles over time.

Pre-release, experimental cage conditioning was conducted for stocking Japanese flounder, Paralichthys olivaceus, in Wakasa Bay, Japan. Recaptured fish were acquired through a cooperative effort between researchers and local fishermen. More conditioned fish were recaptured than non-conditioned fish. Laboratory experiments revealed that conditioned fish had significantly better burying abilities ($p < 0.001$) and enhanced feeding abilities compared to non-conditioned fish.

Video trials were conducted with Japanese flounder to assess the behavior of reared fish directly from hatchery tanks, cage-conditioned, and released-and-recaptured, compared to wild fish. Wild fish buried and attacked most, followed by conditioned, reared-and-recaptured, and non-conditioned fish. Wild and conditioned fish revealed much lower variation in total movement duration, which corresponded with lower levels and variation in prey vertical movement. All fish exhibited a lower number of attacks and off-bottom swimming events, and a lower movement duration when exposed to a moving predator model.

The present research provides information that may promote advances in feeding strategies for flatfish stock enhancement. This work is the first to examine flatfish conditioning strategies using market data and to evaluate the behavior of hatchery-reared flatfish that have been cage-conditioned or released-and-recaptured. In addition, evidence of enhanced performance by cage-conditioned flounder is provided.

Keywords
Agriculture, Fisheries and Aquaculture, Psychology, Behavioral Sciences, Zoology

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FLATFISH STOCK ENHANCEMENT:

EXAMINING CONDITIONING STRATEGIES TO PROMOTE SUCCESS

BY

MICHELLE LYNN WALSH

B.A., Rutgers University, 1998
M.Ed., St. Peter's College, 2000

DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

in

Zoology

September, 2012
This dissertation has been examined and approved.

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8/13/2012
Date
DEDICATION

This dissertation is dedicated to my family. My parents were and are always supportive of me. I know that the only reason I am strong enough to do the things I do in my life is because I know I always have this "safety net" of family to be there for me. I know I will never fall far. And I am not just talking about my parents: aunts, uncles, cousins, brother and sister-in-law, friends - would all break my fall. I know I am incredibly lucky. I know this support has enabled me to take risks in life (like moving halfway across the world AGAIN or starting a Ph.D. program at age 30) that other people do not have the luxury of taking.
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ABSTRACT

FLATFISH STOCK ENHANCEMENT:
EXAMINING CONDITIONING STRATEGIES TO PROMOTE SUCCESS

by

Michelle Lynn Walsh

University of New Hampshire, September, 2012

Conditioning is the process of providing individuals reared for stock enhancement with some degree of "wild" experience prior to release. Flatfish trained for "wild" conditions may more easily and successfully transition to natural environments. This dissertation identifies strategies that optimize feeding-related performance of flatfish in the hatchery and subsequently post release in the wild.

The influence of live feed conditioning on feeding performance of juvenile winter flounder, Pseudopleuronectes americanus, was investigated. In the hatchery, fish reared on live feeds exhibited significantly higher survival (P < 0.0001) and growth (P < 0.01) than those reared on formulated feed. Once released into cages in the wild, amphipod-reared fish had higher mean Stomach Contents Index and RNA/DNA of all feed types, including wild fish. Wild and worm-reared fish exhibited the most similar survival, baseline RNA/DNA values, overall stomach fullness, and diet composition profiles over time.
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The present research provides information that may promote advances in feeding strategies for flatfish stock enhancement. This work is the first to examine flatfish conditioning strategies using market data and to evaluate the behavior of hatchery-reared flatfish that have been cage-conditioned or released-and-recaptured. In addition, evidence of enhanced performance by cage-conditioned flounder is provided.
CHAPTER I

CONDITIONING FLATFISH FOR STOCK ENHANCEMENT:

GLOBAL PROGRESS AND PITFALLS

Introduction

Flatfishes (flounders, halibuts, soles) are among the most desirable and highly priced fishes sold for human consumption (Howell and Yamashita, 2005). Although flatfishes have supported valuable fisheries throughout the world for centuries, catches of many species have declined (Myers and Worm, 2003; Gibson, 2005; Pitcher, 2005; Yamashita and Aritaki, 2010). Many marine fishes release hundreds of thousands of eggs annually, but the small, early life-history stages are vulnerable, there is high natural mortality, and few survive to maturity. Rearing and releasing juvenile flatfish (i.e., stock enhancement) may help rebuild, stabilize, or augment natural populations.

A successful stocking program requires survival of released fish, and to achieve this, released fish must be able to adjust to their new environment, feed successfully, and avoid predation (Howell, 1994). However, hatchery-reared flatfish often exhibit irregular swimming, feeding, and cryptic (burying and color change) behavioral patterns compared with wild conspecifics, and these behavioral "deficits" are assumed to lead to increased predation risk once fish are released (Furuta, 1996; Kellison et al., 2000). Released
flatfish may take days to weeks before they begin feeding normally on wild prey (Furuta et al., 1997; Fairchild, 2010), and this short period of starvation can alter feeding behavior, which in turn may result in an even more pronounced predation risk for reared fish (Miyazaki et al., 2000). Burying ability is essential for flatfish to become both stealthy predators as well as cryptic prey. Thus, these three behaviors (burying, feeding, and avoiding predation) are intricately linked. Conditioning flatfish to natural stimuli before release may offer fish an opportunity to refine these behaviors, which may increase survival in nature and subsequent recruitment to the fishery. Flatfish trained for "wild" conditions may more easily and successfully transition to natural environments upon release (e.g., Kellison et al., 2000; Sparrevohn and Støttrup, 2007).

Examples of conditioning strategies by investigation that have been applied to flatfish in the hatchery include providing sediments in rearing tanks (Tanda, 1990; Miyazaki et al., 1997; Ellis et al., 1997; Fairchild, 2002; Fairchild and Howell, 2004), live feeds (Furuta, 1996), or predator cues (Fairchild, 2002; Hossain et al., 2002). Strategies that can be applied to ease the transition to the wild at, or near, the release site include, conducting "operant conditioning" on fish to respond to light or sound cues (Anraku et al., 1998) for supplemental food provision during the first few days or weeks post release, or short-term release into predator-exclusion cages before full release (Sparrevohn and Støttrup, 2007; Fairchild et al., 2008). Cage conditioning allows hatchery fish to experience natural substrates and sediments, wild (live) food sources, and "safe" predator exposure (fish are able to see predators outside of cages and to detect olfactory predator cues) before actual release. In addition, the short period in the cage enables flatfishes to begin pigment change and recover from transport stress (Fairchild et
al., 2008). Cage conditioning has shown to be effective in increasing post-release survival and recapture of flatfish species such as Atlantic turbot, *Psetta maxima* (Sparrevoehn and Støttrup, 2007).

**Global Progress**

Japan has the highest level per capita of fish consumption in the world, thus it is not surprising that Japanese scientists lead research in marine fish stock enhancement. Japan has been releasing flounder as a fisheries management strategy for over 30 years, and is the most active country with respect to flatfish stocking, both in the range of species reared and the number of fish released. Releases of Japanese flounder, *Paralichthys olivaceus*, marbled flounder, *Pseudopleuronectes yokohomae*, brown sole, *Pseudopleuronectes herzensteini*, barfin flounder, *Verasper moseri*, spotted halibut, *Verasper variegates*, and at least 3 other species of flatfish total over half a billion to date (Svåsand and Moksness, 2004; Howell and Yamashita, 2005; Yamashita and Aritaki, 2010). Japanese flounder, or hirame, is the primary species represented in annual flatfish catch; thus, hirame has been a paramount choice for both aquaculture and stock enhancement for decades and is, in fact, the most important stocked marine finfish in Japan. A total of approximately 25 million Japanese flounder are released annually from federal, prefectural, and private hatcheries throughout the country (Tomiyama et al., 2008).

Japan is the only country that has been successful in exhibiting high recapture rates of stocked flatfish (up to 30%; Fujita, 1996), economic profitability of stocking
efforts (Kitada et al., 1992; Kitada, 1999), stabilization of fisheries catch (Yamashita and Aritaki, 2010), and evidence of biological contribution to wild spawning stocks (Kitada and Kishino, 2006). Indeed, the governing structure of Japan (localized prefectures) has contributed to such successes. Japanese stocking efforts are funded primarily by taxes from citizens and sales income of fishermen (Yamashita and Aritaki, 2010). Although Japanese scientists suggest that feeding behavior of hatchery-reared Japanese flounder can be made more natural by conditioning fish with live or more realistic feeds and by providing fish with sandy substratum before release (Tanaka et al., 1998), few hatcheries engage in conditioning strategies for flatfish stock enhancement. Exceptions include the Obama Laboratory, National Center for Stock Enhancement, Fisheries Research Agency, which has been conducting pre-release, experimental cage conditioning for Japanese flounder since 2008, and the Hyogo Prefecture Hatchery, which provides hatchery-reared juvenile marbled flounder with a diet of frozen mysids before release.

Experimental releases of Atlantic turbot, mostly financed by the European Union, national governments, and the fishing industry, have been conducted in Belgium, Norway, Spain, and the United Kingdom totaling approximately 36,000 fish (Ellis and Nash, 1998; Danancher and Garcia-Vazquez, 2007; Sparrevohn and Støttrup, 2010). Ellis and Nash (1998) showed that the releases significantly increased abundance of the local population. Releases of Black Sea turbot, *Psetta maoticus*, by Russia and Turkey in the 1990s consisted of over 165,000 fish, and evidence suggests that these releases contributed to increased abundance in subsequent years (Maslova, 2002; Sparrevohn and Støttrup, 2010). In Denmark, stocking of Atlantic turbot, European plaice, *Pleuronectes platessa*, and flounder, *Platichthys flesus*, has been conducted since 1988, totaling over 3
million released flatfish to date (Støttrup, 2004; Sparrevohn and Støttrup, 2010). Danish stocking efforts are funded via fees charged for recreational fishing licenses through the National Coastal Fisheries Management Program (Sparrevohn and Støttrup, 2010). Since 2004, Danish scientists have been cage conditioning reared Atlantic turbot before release, and this practice has resulted in a much lower post-release mortality than when fish were not conditioned (Sparrevohn and Støttrup, 2008; Sparrevohn and Støttrup, 2010).

Currently in the United States, flatfish stocking consists of only smaller-scale and mostly experimental efforts. The only official, ongoing program, established by the Texas Parks and Wildlife Department in 2006, exists for southern flounder, *Paralichthys lethostigma*. Details regarding the success of that program, however, are unavailable, although to date approximately 20,000 fish have been released (Sikes, 2011; Tompkins, 2010). An experimental, small-scale release of summer flounder, *Paralichthys dentatus* (N = 1500) was conducted in North Carolina in 1999, and released fish were conditioned to predators by exposure to caged adult blue crabs, *Callinectes sapidus*, prior to release (Kellison et al., 2003). Experimental releases of winter flounder, *Pseudopleuronectes americanus*, have been conducted since 1999 (approximately 27,000 fish released in total to date), and in late summer 2012, a large-scale release (up to 50,000 fish) spearheaded by the University of New Hampshire (UNH) is planned for the salt ponds surrounding Martha's Vineyard, Massachusetts (Zeiber, 2011). UNH protocols have included cage conditioning of flounder before release since 2004, however, evidence arose that the cages themselves attracted 'structure-philic' predators, mostly crabs (Fairchild et al., 2008), so cage design has been modified in recent years to be floating.
Pitfalls

Ensuring that released fish are morphologically, ecologically, genetically, and behaviorally similar to wild conspecifics is necessary for an effective release program (see Le Vay et al., 2007, for review). Nonetheless, conditioning strategies that are easy to implement, economically feasible, and effective are still being developed and tested. In addition, the number of studies that have been able to monitor and track the fate of released conditioned fish is few.

Walsh et al. (in press; Chapter 4) observed that non-conditioned fish, mostly non-feeding individuals, were caught more often than conditioned fish by < 0.5 m/s boat beam trawl when researcher-initiated recapture efforts were applied. Similarly, Sparrevohn and Støttrup (2007) found that the catchability of non-conditioned turbot caught by beam trawl was 10% higher than that of cage-conditioned fish. This may indicate that intensive researcher recollection efforts at, or near, the release site disproportionately sample weak fish that are not feeding or moving. Efforts and money for recapture may be better spent on involving more local fishermen, especially since cooperative efforts generate more interest and publicity in the stocking. Involving more fishermen also may promote the reporting of recaptured hatchery-reared catch by those not directly involved in the project, and thus, amplify the level of monitoring conducted.

Even without implementing a conditioning strategy, one of the greatest difficulties of a stocking exercise is the level of post-release monitoring. In many cases, 1% recapture rates are the norm. Choosing a location that can be monitored adequately may be just as important as choosing a location where stocking is predicted to succeed,
which is essential if the success of a stocking effort will influence future efforts (i.e., funding, resources, support). Stocking agencies have an obligation to conduct post-release monitoring in an attempt to assess stocking effectiveness, especially if the stocking effort is funded with taxation of citizens or fees from fishermen (Yamashita and Aritaki, 2010). In addition, there is a biological and ecological responsibility to evaluate what, if any, effect the stocking has on local fish populations and their habitats.

**Differences in Flatfish Potential for Stocking**

To gauge the success of conditioning for flatfish stock enhancement, two species of two different genera of flatfish were examined: the pseudopleuronectid winter flounder and the paralichthyid Japanese flounder. Each species exhibits characteristic advantages and disadvantages for stocking.

The winter flounder's small mouth size limits cannibalistic tendency, thus, alleviating this as a potential post-release mortality factor. In contrast, cannibalism is an issue for paralichthyids such as Japanese flounder (Kellison et al., 2002) and summer flounder (Bengtson, 1999). The advantage to being a cannibal, however, is that hatchery-reared paralichthyids have a lifetime of experience with live feeds, with their smaller siblings existing as potential prey in rearing tanks. It is likely that even if a reared paralichthyid never has eaten another fish, it probably witnessed this "live feed" predation occurring sometime during its time in the hatchery.

Unlike the largely piscivorous paralichthyids, pseudopleuronectids like winter flounder eat mostly bottom-dwelling invertebrates (e.g., polychaetes and small
crustaceans), and therefore, convert low economic value benthic production into a highly marketable protein source for human consumption (Link et al., 2005) without directly affecting forage-fish populations, which provide a fundamental food web link to higher economic value fish species (and may have economic value in their own right). Indeed, the associated impact on forage-fish populations is one of the central criticisms of aquaculture (Naylor et al., 2000) and, by association, stock enhancement since the rearing of cultured fish often requires a high incidence of wild-fish collection for feed production (either direct or formulated). It follows that fluctuating forage-fish populations impact not only fisheries and aquaculture operations, but also wild ecological processes as well (Alder et al., 2008). Presumably as a result of fluctuating forage-fish availability, diets of many piscivorous flatfishes have shifted over the past few decades, while the diet composition of benthivorous flatfishes, such as winter flounder, indicate little change in bottom-associated prey availability (Link et al., 2002). The omnivorous diet of the winter flounder may also promote the successful use of non-fish based protein sources (e.g., soy-, algae-, or worm-based feeds), which are becoming more commercially available for rearing operations.

Benthic diets also result in different feeding strategies of pseudopleuronectids and paralichthyids. Whereas piscivorous paralichthyids swim off the bottom to feed (Furuta, 1996), pseudopleuronectids rarely leave the benthos for prey acquisition. In studies examining Japanese flounder, Furuta (1996) noted that wild fish quickly returned nearly to their original resting positions after feeding, while hatchery-reared fish spent longer periods off the bottom and resettled a distance away. Although the behavior gap between
hatchery-reared versus wild pseudopleuronectids also may be wide, the manner in which these small-mouth benthivores differ from that of paralichthyids also varies substantially.

Paralichthyids, however, display a trait that gives them a large advantage as a candidate for stock enhancement: fast growth rate. Growth rates of hatchery-reared juvenile Japanese flounder can reach 5-mm total length (TL)/day during peak summer season, wherein that same growth rate at similar temperatures may only be observed in winter flounder over a week's time (pers. obs). Therefore, paralichthyids like Japanese or summer flounder, can reach optimal release size (e.g., 10-cm TL) after a few months in the hatchery, while pseudopleuronectids such as winter or marbled flounder, would take approximately a year to reach a similar size (considering intake ambient water temperatures dictate tank water temperature at many flatfish hatcheries, and winter water temperatures are much lower). This has often led to stocking agencies releasing pseudopleuronectids at much smaller sizes than enacted for paralichthyids (3- to 6-cm TL for marbled flounder and winter flounder; 7- to 12-cm TL for Japanese flounder). Size-at-release is a primary determinant for post-release survival as smaller release size of hatchery-reared fish often is associated with an even higher vulnerability to predation.

**Overall Objective of This Dissertation**

The overall objective of this dissertation is to evaluate conditioning strategies currently executed for flatfish stock enhancement in order to assess whether or not these strategies promote the "success" of released juveniles or the stocking effort. Herein we define "success" of a conditioning strategy in three ways: (1) enhancing performance; (2)
yielding a behavioral repertoire by conditioned fish that more closely matches that of wild fish; and (3) increasing the number of released fish landed at market relative to the amount of non-conditioned fish.

This dissertation reveals a number of unique approaches and insights into flatfish stock enhancement never before reported. Chapter 2 describes the influence of different diets (both live and formulated) on hatchery feeding success, with an explanation of non-traditional live feed culture, including the first detailed description for rearing the common burying amphipod, *Leptocheirus plumulosus*. Chapter 3 evaluates how this hatchery feeding success translated into wild feeding success once individuals were released into the wild. Chapter 4 assesses the success of a large-scale stocking effort where approximately half of the released fish were conditioned in predator-free cages before release. Chapter 5 describes the performance and behavior of fish that underwent cage-conditioning compared to those that were not conditioned.
CHAPTER II

REARING DIETS FOR WINTER FLOUNDER STOCK ENHANCEMENT THAT OPTIMIZE FEEDING-RELATED PERFORMANCE IN THE HATCHERY, WITH NOTES ON THE CULTIVATION OF NON-CONVENTIONAL LIVE FEEDS

Introduction

The commercially and recreationally important winter flounder, *Pseudopleuronectes americanus*, ranges from Labrador, Canada to Georgia, USA, but is most abundant in the Gulf of Maine. Like many groundfish species off the northeastern coast of North America, catches have declined over the past 30 years (NEFSC, 2011). A winter flounder female is capable of releasing hundreds of thousands of eggs annually but because of the vulnerability of the small, early-life stages (fertilized eggs, larvae and newly-settled juveniles), there is high natural mortality, and few fish survive to maturity (Saila et al., 1997). Captively spawning adults and then rearing and releasing juveniles at a size or age beyond this mortality window (a period lasting several months characterized by high predator-induced mortality; Taylor and Collie, 2003) may enhance natural stocks. Winter flounder is currently being evaluated as a stock enhancement candidate by researchers at the University of New Hampshire, USA.
A major challenge of any captive-rearing program, whether for aquaculture or stock enhancement, is to provide an appropriate diet regime during development. Typically, cultured marine fish larvae initially are fed live feeds (e.g., rotifers, *Brachionus* sp., and/or brine shrimp nauplii, *Artemia* sp.), and then are weaned onto formulated diets as they attain a size or developmental state that supports consumption of such artificial feeds. Although formulated feeds can be economical both financially and temporally, live feeds can train hatchery-reared flounder to exhibit more natural behaviors (Tanaka et al., 1998) and can improve the foraging efficiency in fish subsequently exposed to novel prey (Massee et al., 2007). These live feed benefits are especially relevant for fish reared for stock enhancement.

Our objective was to determine how different diets (both live and formulated) influenced feeding success in the hatchery as indicated by survival and growth. In addition, we have included notes on the cultivation of non-conventional live feeds: white worms, *Enchytraeus albidus*, and common burrower amphipods, *Leptocheirus plumulosus*.

**Methods**

**Winter Flounder Rearing and Maintenance**

From April–September 2008, winter flounder eggs, larvae, and young juveniles were reared and maintained at the Coastal Marine Laboratory (CML), Judd Gregg Marine Research Complex, University of New Hampshire (UNH), in New Castle, New
Hampshire (NH), USA, following standard protocols developed over the last several years (Fairchild et al., 2007). After hatch, larvae were grown in 1.8-m diameter round tanks supplied with microalgae (live Nannochloropsis sp.). Tanks received mild aeration and oxygenation, and were maintained under a 12-h light: 12-h dark photoperiod. Larvae were fed a daily ration of enriched rotifers, Brachionus plicatilis, (DHA Selco; INVE, Salt Lake City, UT, USA) three times daily (0800, 1400, and 2000 h) at a density of ~2,000 prey/L. At 20 days after-hatch (DAH), in addition to rotifers, larvae were provided with enriched brine shrimp nauplii, Artemia salina (DC DHA Selco; INVE). After 1.5 wk of co-feeding (30 DAH), rotifers were withdrawn and larvae were fed only brine shrimp nauplii through and beyond settlement (1,500 to 3,000 enriched nauplii/L/tank/d) until initiation of weaning onto the experimental feeds (90 DAH).

Hatchery Feed Culture

Four experimental hatchery feeds for juveniles were examined. These included a conventional formulated feed and three non-conventional live feeds: (1) a 0.5- to 0.8-mm mix of Skretting Gemma™ commercially-available, formulated pellets; (2) brine shrimp post-nauplii; (3) white worms; and (4) common burrower amphipods (Table 2.1). The selected live feeds were chosen among a number of invertebrate candidates because of availability, rearing and harvesting ease, tendency to thrive with minimal maintenance, and ability to survive in salt/brackish water for prolonged periods (Table 2.2).

Brine shrimp were grown to postnaupliar stages in small, closed raceway systems maintained at 25–30°C following the protocols described by Hoff and Snell (1999). Brine
shrimp were fed a *Spirulina* powdered algae/water solution two times per day for 11–14 d before being provided to juvenile flounder as feed. To harvest, rearing water containing brine shrimp was sieved (105 μm mesh), excess water was wiped from the sieve, and the sieve + brine shrimp was weighed to estimate brine shrimp feedout weight.

A starter culture of white worms was purchased from Aquatic Research Organisms, Inc., Hampton, New Hampshire, USA. Worms were dispersed into clear plastic containers (34.5 x 20.25 x 12.75 cm) filled 5–7 cm high with damp organic potting soil. To feed worms, a trough was formed down the center of each container to the bottom (exposing the clear plastic) and filled to 1 cm with formulated, pellet feed (0.5-mm Skretting Gemma™). Pellet feed was then covered with 4–6 cm of soil, and soil was misted generously with ambient seawater (15–31 ppt) to moisten. Containers were covered with white opaque plastic lids to reduce light exposure, contain moisture, and minimize fouling (e.g., infestation by mites or small flying insects). Food supply was monitored by viewing clear plastic containers from the bottom and added as needed one time per wk. Plastic containers were maintained in low light conditions at room temperature (18–23 C). Containers were left undisturbed except for weekly food and soil moisture maintenance (subsequent misting with tap water to prevent salinity amplification) for 12 wk (i.e., 3 worm reproductive cycles), then split in half to expand culture production as needed. To harvest for flounder feeding, worm containers were placed on heating pads set to the highest heat setting (60 C) for 1–2 h. A small peak of dirt was crafted against the side of the plastic container. Worms migrated to this peak to escape the heat and easily were collected. White worm harvest was weighed directly before feedout.
A starter culture of 1,500 common burrower amphipods was purchased from Aquatic Research Organisms, Inc., Hampton, NH, and distributed to 15 white opaque plastic containers (38 x 30.5 x 15.25 cm) lined with 5 cm of fine sieved mud (< 500 μm grain size). Each container then was filled with ambient seawater (static system) and aerated at room temperature. Three times per week, water was changed and each basin was fed with a solution of wheatgrass powder, ground rabbit food, fish flakes, and powdered zooplankton enrichment. Containers were left undisturbed (except for food provision and water changes) for 12 wk (3 amphipod reproductive cycles), then split in half to expand culture production as needed. To harvest for flounder feeding, amphipods were sieved (1000 μm mesh size) from the sediment and rinsed with seawater, drained into a filter, and then weighed directly.

Samples of white worms (27 May 2008), common burrower amphipods (28 November 2008), and brine shrimp (28 November 2008) were sent to New Jersey Feed Laboratory, Inc. Trenton, New Jersey for proximate composition analysis. Samples were analyzed in triplicate. Proximate composition of Skretting Gemma formulated pellets was provided by the manufacturer and verified by Hossain et al. (2011).

Feeding Trials

At 90 DAH, juvenile winter flounder were distributed into 20-L circular tanks (46-cm diameter x 32-cm deep) constructed with two mesh windows on opposite sides to allow flow-through of ambient seawater pumped directly from the adjacent mouth of the Piscataqua River. Circular tanks were nested into three, large, flow-through rectangular
trays (~6 L ambient seawater/min; two circular tanks per diet per tray; Fig. 2.1a). Each circular tank housed 12 individual fish, resulting in 288 fish with a mean initial standard length (SL) of 30 ± 0.27 mm (± SEM here and throughout). An additional, non-experimental circular tank of pellet-fed fish was maintained under similar conditions to augment numbers available for subsequent caging trials (Chapter 3). All circular tanks were covered with 2.5-cm mesh nets (with a reinforced aperture for feeding) to minimize fish escape (Fig. 2.1b).

Fish were weaned for a total of 14 d before trial initiation. Fish initially were co-fed the experimental feed 1 h prior to brine shrimp nauplii introduction. Over the course of the weaning period, increasing amounts of the experimental feed were provided coinciding with decreasing amounts of brine shrimp nauplii until only the experimental feed was offered. Upon trial initiation, fish in tanks were fed to satiation three times per day (morning, afternoon, and evening). The amount of feed provided to pellet-reared fish was lower than that of all other hatchery feed types (Table 2.1), yet the daily aliquot still surpassed satiation level for pellet-reared fish.

Trials were conducted for 4 wk with a subset of four fish from each circular tank measured weekly for total length (TL; mm), SL (mm), and wet weight (Wt; g); all fish were measured at the culmination of hatchery trials. Each day fish that died were removed. Throughout the study the temperature, dissolved oxygen, and salinity of each tank were monitored. Each tank was siphoned 2 times per week or whenever dissolved oxygen levels dropped below 5 mg L⁻¹. Survival curves were estimated by the Kaplan-Meier method and were compared by applying a log-rank (Mantel-Cox) test. Growth parameters (SL, TL, Wt) were evaluated over time by linear regression with slopes and
intercepts tested for similarity among feed types. In addition, somatic and instantaneous
growth rates were calculated for each growth parameter. Depending on the distribution of
the dataset, overall water quality parameters were compared between trays and feed types
via ANOVA followed by Tukey’s or Kruskal – Wallis (KW) followed by Dunn's Multiple
Comparison Test.

Results

Hatchery Feed Culture

Worm production surpassed 40 g per rearing container; each container was
harvested over a series of 2–4 d. Fish quickly recognized white worms as prey, and began
feeding on them within 1 d. The small-scale common burrower amphipod production
resulted in 48–68 g/wk. We harvested approximately 0.5–1 rearing container per d,
depending on supply and demand. Of all live hatchery feeds provided, common burrower
amphipods required the longest time (~5 d) during weaning to be fully accepted as prey
by fish. Initially, fish appeared startled by the movements of these relatively large, active
prey items. Given their history with the movements of both rotifers and brine shrimp
nauplii within rearing tanks, flounder did not show any pronounced reaction to the
introduction of brine shrimp post-nauplii and began feeding immediately.

White worms contained the highest protein (76%) and lipid (15%) content by
percent non-moisture proximate composition (Table 2.1). The three remaining feed types
(pellets, brine shrimp, common burrower amphipods) were composed of relatively
similar protein content (52-58%). Brine shrimp and common burrower amphipods had the highest percentage of fiber (8-9%) and ash (34-39%), and contained no detectable amount of lipids.

Feeding Trials

**Water Quality.** During trials, tanks ranged from 13.1–20.7 °C ambient seawater temperature, 20–35 ppt salinity, and 2.24–9.3 mg/L DO. Overall temperature and salinity were not significantly different between trays (F = 0.45, P = 0.64 for temperature; F = 2.76, P = 0.06 for salinity) or feed types (F = 0.01, P = 1.0 for temperature; F = 0.22, P = 0.88 for salinity). Dissolved oxygen did not vary between trays (F = 2.27, P = 0.10); however, it differed significantly between feed types (KW = 63.91, P < 0.0001) with pellet-reared tanks having the lowest and most variable DO levels of all feeds (Fig. 2.2).

**Survival.** Survival data of fish reared on the same hatchery feeds among trays were pooled for analyses since there were no significant differences between trays (P > 0.05 within all experimental feed types across trays). When examining hatchery-feeding success in terms of survival, brine shrimp- and amphipod-reared fish showed the highest survival followed by worm-reared fish; mortality of pellet-reared was significantly higher than fish fed other feed types ($\chi^2 = 68.07, P < 0.0001$; Fig. 2.3). Thirty-six percent of all pellet-reared fish died during the course of hatchery trials, the majority during the final 2 wk.
Growth. Growth data of fish reared on the same hatchery feeds among trays were pooled for analyses since there were no significant differences between trays (P > 0.05 within all experimental feed types across trays for all three growth measures). No growth was recorded for pellet-reared fish; pellet-reared fish did not significantly increase in SL, TL or Wt during the 4 wk post-weaning onto pellet feed (P > 0.05; Fig. 2.4). However, the growth trajectories of worm-, amphipod- and brine shrimp-reared fish all significantly increased in all size parameters over time (P < 0.0001). Worm-reared fish had the highest growth rates (somatic and instantaneous) for all growth measures, followed by brine shrimp-, amphipod- and pellet-reared fish (Table 2.3).

Discussion

Hatchery Feeds

During hatchery feeding trials, juvenile winter flounder were provided with live feeds, which included white worms, common burrower amphipods, and brine shrimp. Live feeds may vary not only in size and composition, but also in the response they elicit from predators (James et al., 1993). Live feeds also can train hatchery-reared flounder to exhibit more natural behaviors (Tanaka et al., 1998) and may improve the foraging efficiency in fish subsequently exposed to novel prey (Massee et al., 2007).

White worms occur naturally in the diet of winter flounder (Klein-MacPhee, 1978). Mass white worm cultivation was developed in the former USSR in the 1940s as a result of expanding fish culture programs (Ivleva, 1973). Studies on the biology,
nutrition, and cultivation of white worms are reported in a number of Russian and
Turkish publications, but few are translated for English-speaking audiences (Ivleva,
1973; Vedrasco et. al., 2002; Memiş et. al., 2004). The reviews that do exist in English
describe the production of 100 kg to several tons of white worms cultured per season for
feeding 2.5–3 million juvenile sturgeon (Family Acipenseridae; Ivleva, 1973; Memiş et. al.,
2004).

Common burrower amphipods are easily cultured and readily accepted by
flounder juveniles. There is no published evidence that *L. plumulosus* occurs naturally in
the diet of winter flounder, however, the congeneric and ecologically similar *L. pinguis*
has been documented throughout the winter flounder range (Wells et al., 1973; Klein-
shallower and more estuarine waters than *L. pinguis*, and has a more southern distribution
range. *L. plumulosus* is found south of Cape Cod to Florida, USA, versus Labrador,
Canada to North Carolina, USA for *L. pinguis* (Dickinson et al., 1980). Thus, it is
reasonable to assume that a winter flounder would consume *L. plumulosus* when
encountered in nature. Although not as nutritious as white worms, common burrower
amphipods are a livelier, more challenging feed, and appear to enhance learned predator
behaviors in juvenile flounder. The amphipods, with their quick, 3-dimensional
movements, are much more difficult to capture than the slow, 2-dimensional moving
white worms. Common burrower amphipods actively attempt to escape capture. The
response of fish during initial exposure to the amphipods is not uncommon; Godin (1978)
remarked that some predators withdraw from novel prey for some time before
approaching and attempting to feed.
Brine shrimp, reared to a size appropriate to the foraging capability of the growing predator, are an excellent nursery and weaning diet for many marine fish, and reduce mortalities, cannibalism, and heterogeneous growth of the target cultured species (Sorgeloos et al., 1993). However, brine shrimp do not meet all of the nutritional requirements for marine organisms, especially that of 22:6n-3 docosahexaenoic acid (DHA). Thus, enriching brine shrimp to boost the unsaturated fatty acid content is generally the norm, and many commercial products have been designed for this purpose (e.g., DHA Selco, which we used to enrich pre-experimental brine shrimp nauplii).

Alternatively, *Spirulina* can be provided to brine shrimp, as the nutritional significance of this microalgae lies in its iron and essential unsaturated fatty acid content (Tekelioglu et al., 2005). In addition, *Spirulina*, which we used to feed experimental brine shrimp post-nauplii, is a natural protein source that also contains water soluble pigments such as phycocynanin, allophycocyanin, and beta-carotene, and vitamins B, C, and E. Brine shrimp nauplii are deficient in the amino acids histidine, methionine, phenylalanine, and threonine, and contain only 42% protein by dry weight (Hoff and Snell, 1999). Adult brine shrimp, however, contain the full spectrum of amino acids and are comprised of 60% protein by dry weight. In the present study, analysis of brine shrimp composition revealed 56.6% protein by dry weight, indicating that the nutritional content of brine shrimp we utilized for feeding trials was closer to that of adults than of early nauplii. This is important, as fish derive most of their metabolic energy from oxidizing protein (Brett and Groves, 1979), and winter flounder require a high percentage of protein (45%) in their diet to obtain optimal growth rates (Hebb et al., 1997). Brine shrimp also move in 3-dimensions.
Although brine shrimp nauplii are a conventional feed for pre- and newly settled flounder juveniles, post-nauplii, sub-adults, and adults are rarely provided to larger juveniles since fish of this size are generally capable of consuming and digesting formulated, pellet feeds, which are more economical to prepare and distribute. However, for flatfish reared for stock enhancement that must adapt skills to become efficient predators in the wild, formulated feeds may not promote optimal feeding, growth, or survival skills. First, formulated feeds are generally provided from the surface of tanks, training fish to feed from the upper water column instead of from the bottom substrate (Masuda, 2004). Second, formulated feeds do not move on their own accord. Movement of prey is an important stimulus to attract and cue a visual predator (Holmes and Gibson, 1986; James et al., 1993; Brown et al., 2003; Sarkar et al., 2006) such as a winter flounder. Although tank systems can be modified to prolong suspension of formulated feeds or allow swirling of sunken pellets along the bottoms of tanks, many fish lose interest in settled, motionless (or passively moving) feeds over time (pers. obs). Third, formulated feeds foul rearing waters faster than most live feeds. Unless high water turnover and frequent tank cleaning can be provided, DO levels may fall to a detrimental or lethal level. When examining formulated feeds for juvenile winter flounder, Hebb et al. (2003) noticed that pellets not consumed within 5 min began to break down and leach water-soluble nutrients, which caused lower tank water quality and may have led, in part, to lower fish growth rates. In hatchery feeding trials in the present study, water flow could not be increased high enough to ensure consistent water quality in pellet-reared tanks, as high ambient seawater flow clogged inflow lines and the mesh windows on
tanks that allowed flow-through. Increased siphoning (up to twice each day for some tanks) also did not ensure consistent water quality of pellet-fed tanks.

The brand of formulated feed we chose for this study was inconsequential; we use Skretting Gemma formulated feed to mass-produce the majority of flounder at our facility. Since Lee and Litvak (1996) found no difference with respect to fish growth, feed conversion, or survival between formulated feeds, the choice of pellet feed in this study represents the standard.

Feeding Trials

Survival. Survival rates at the end of 4 wk trials were over 95% for fish reared on white worms, common burrower amphipods, and brine shrimp. This in accordance with Sarkar et al. (2006) who found that survival of young clown knifefish, *Chitala chitala*, fed on live tubifex worms, *Tubifex tubifex*, was 94% at the end of 28 d hatchery trials. We attribute the lower (64%) survival of pellet-reared fish mostly to lower consumption rates and low water quality caused by the decomposition of excess formulated feed in tanks, even with constant water flow and daily siphoning. The amount of feed provided to pellet-reared fish surpassed satiation level, however, we were hesitant to decrease the amount. The percent of pellet feed provided per body weight/d already was much lower than that provided to all other hatchery feeds, and provision of too little feed may not ensure enough encounters to elicit an adequate feeding response in pellet-reared fish (Stoss et al., 2004). Lee and Litvak (1996), who also examined fish in a small-scale, flow-through system, found that average formulated feed consumption of juvenile winter
flounder was about 5% of fish body weight/d and that survival of pellet-reared fish was 68% and 71% at the end of 38-d hatchery trials. They also noted that not all juveniles had completely adapted to the formulated feed 17 d after experiment initiation, which is another reason we may see lower survival for pellet-reared fish. Although their work gave no indication of tank water quality level, they did remove fish from experiments because of fin rot, which is often caused by poor water quality (Mahoney et al, 1973). Survival rates of pellet-reared fish in their study were not much higher from than those observed in the present study.

Due to the extremely low DO levels recorded in the present study, it is difficult to interpret the true influence of a formulated diet on hatchery fish growth and (non-water quality related) survival, so from that perspective, results here should be viewed with caution. Larger scale experimentation with larger tanks and higher flow-through rates may enable a more accurate estimation of the effect of formulated feed on fish growth and survival. However, on this small experimental scale, water quality resulting from formulated feed provision was, for many fish, lethal. Thus, live feeds were much more robust to the constraints of the experimental system.

**Feeding.** Since fish were fed to satiation, the amount of feed presented to hatchery individuals ranged from approximately 8–60 percent body weight/d depending on hatchery feed. Fairchild and Howell (2001) provided 7% body weight/d to juvenile winter flounder fed formulated feed in the hatchery, similar to the 8% we provided to pellet-reared fish. When feeding live and dried diets to 45-mm TL clown knife fish, Sarkar et al., (2006) provided between 5 to 10% body weight/d, which varied with the week of trial. In
the present study, fish consumption rates varied largely because of differences in feed composition (i.e., protein, lipid, fiber, and ash content). White worms were very high in protein and lipid, while brine shrimp and common burrower amphipods had the highest levels of indigestible components (i.e., fiber and ash) with no detectable amounts of lipid. Degani (1991) attributed the higher consumption and growth rates of juvenile three-spot gourami, *Trichogaster trichopterus*, to the palatability and chemical composition of live hatchery feeds compared to formulated diets. When providing live feeds to the common carp, *Cyprinus carpio*, James et al. (1993) found that fish fed *Daphnia* sp. spent more energy on metabolism, showed a poorer rate and efficiency of conversion, and took longer to feed at each feeding event than fish fed more worm-like feeds such as bloodworms, *Chironomus* sp., or mosquito larvae, *Culex* sp.

In the present study, the smallest hatchery feed with the highest provision per fish body weight, brine shrimp post-nauplii, was extremely difficult to weigh without high water weight error. Therefore, the daily brine shrimp rations were weighed more for daily precision rather than for comparative accuracy to other hatchery feeds. In an absolute context, the percent of brine shrimp provided per fish body weight/d should be viewed with caution.

**Growth.** Fish fed live white worms grew the most of all hatchery feeds. James et al. (1993) found that providing fish live bloodworms maximized feeding, absorption, and conversion rates, and suggested that the wriggling motions of these larger, more nutritious prey may have minimized the energy and temporal costs of feeding, and thus maximized fish growth. In a hatchery recirculating system, Sarkar et al. (2006) found that
growth rate was higher for clown knifefish fed on live tubifex worms than on any other non-conventional feed (i.e., live copepods, live bloodworms, fish eggs, floating-type *Spirulina*, dried tubifex worms, freeze-dried *Daphnia* sp., and boiled egg yolk) and remarked that the provision of live tubifex may have stimulated fish feeding behavior and increased acceptance of the prey. During the 4-wk hatchery feeding trials in the present study, pellet-reared fish did not significantly increase in SL, TL or Wt. This may be a result of lower overall growth of pellet-reared fish immediately following weaning from live, moving larval feeds (i.e., rotifers and brine shrimp nauplii), lower consumption rates of formulated feeds, and/or low water quality levels in pellet-reared tanks.

**Conclusions**

Hatchery feeding trials indicated that white worm-reared fish grew the most, pellet-reared fish grew the least, and pellet-reared fish exhibited the lowest survival (although this low performance of pellet-reared fish should be viewed with caution due to low water quality). This study provides information that may promote advances in feeding strategies for flatfish stock enhancement. If we are to promote effective flatfish stocking, weaning, and feeding strategies, we should continue to investigate and identify nutritious, inexpensive, live hatchery-feeds that can be easily mass cultured.
Table 2.1. Feed parameters for experimental hatchery trials. Variation listed as SEM.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Protein</th>
<th>Lipid</th>
<th>Fiber</th>
<th>Ash</th>
<th>% Feed/fish body weight fed per fish/day</th>
<th>Dimensions of movement</th>
</tr>
</thead>
<tbody>
<tr>
<td>White worms</td>
<td>75.90 ±2.84</td>
<td>15.09 ±2.70</td>
<td>2.89 ±0.78</td>
<td>6.12 ±0.54</td>
<td>18.12 ±0.71</td>
<td>2</td>
</tr>
<tr>
<td>Brine shrimp postnauplii</td>
<td>56.60 ±2.76</td>
<td>0 ±3.38</td>
<td>9.09 ±1.04</td>
<td>34.32 ±1.04</td>
<td>60.36 ±2.88</td>
<td>3</td>
</tr>
<tr>
<td>Common burrower amphipods</td>
<td>52.93 ±0.89</td>
<td>0 ±1.09</td>
<td>8.05 ±1.09</td>
<td>39.02 ±0.39</td>
<td>12.60 ±0.78</td>
<td>3</td>
</tr>
<tr>
<td>Formulated pellets*</td>
<td>58.00</td>
<td>14.50</td>
<td>1.10</td>
<td>9.85</td>
<td>8.15 ±0.35</td>
<td>0</td>
</tr>
</tbody>
</table>

*Proximate composition from Hossain et al. (2011).
<table>
<thead>
<tr>
<th>Common name</th>
<th>Culture medium</th>
<th>Culture feed</th>
<th>Survives in salt water?</th>
<th>Required d/wk of labor?</th>
<th>Component of natural winter flounder diet?</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 White worms, <em>Enchytraeus albidus</em></td>
<td>soil, peat, coconut fiber</td>
<td>fish pellet feed, brewery waste, bread, oats, organics, compost, etc.</td>
<td>15 - 45</td>
<td>Yes</td>
<td>1</td>
<td>Yes</td>
<td>when cut apart, each segment continues to move independently; can be fed any organics, including rotten foodstuffs; range of sizes available</td>
</tr>
<tr>
<td>2 Common burrower amphipods, <em>Leptochirerus plumulosus</em></td>
<td>fine mud/silt</td>
<td>mixture of wheatgrass powder, ground rabbit food, fish flakes and powdered zooplankton enrichment</td>
<td>8 - 10</td>
<td>Yes</td>
<td>3</td>
<td>Possible, congeneric <em>L. pinguis</em> documented</td>
<td>hardy; range of sizes available</td>
</tr>
<tr>
<td>3 Brine shrimp post nauplii, <em>Artemia salina</em></td>
<td>salt water</td>
<td>algae</td>
<td>1 - 2</td>
<td>Yes</td>
<td>7</td>
<td>No</td>
<td>readily available</td>
</tr>
<tr>
<td>4 Mysid shrimp, <em>Americamysis bahia</em></td>
<td>brackish water</td>
<td>algae, powdered zooplankton enrichment</td>
<td>2 - 10</td>
<td>Yes</td>
<td>7</td>
<td>No, but <em>Mysidaceae</em> documented</td>
<td>range of sizes available</td>
</tr>
<tr>
<td>Common name</td>
<td>Culture medium</td>
<td>Culture feed</td>
<td>Survives in salt water?</td>
<td>Required d/wk of labor?</td>
<td>Component of natural winter flounder diet?</td>
<td>Pros</td>
<td>Cons</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>--------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>-------------------------------------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>5 Side swimmers/scuds, <em>Hyalella azteca</em></td>
<td>freshwater</td>
<td>fish pellet or flake feed, compost</td>
<td>Yes</td>
<td>1</td>
<td>No</td>
<td>range of sizes available</td>
<td>cannot survive for prolonged periods at salinities above 28 ppt</td>
</tr>
<tr>
<td>1 Micro worms, <em>Panagrellus redivivus</em></td>
<td>oatmeal, cornmeal, bread</td>
<td>oatmeal, cornmeal, bread</td>
<td>Yes</td>
<td>0.75</td>
<td>No, but Nematoda documented</td>
<td>very small; ideal for early weaning</td>
<td>difficult to harvest and weigh out accurately</td>
</tr>
<tr>
<td>1 Vinegar eels, <em>Turbatrix aceti</em></td>
<td>solution of apple cider vinegar and water</td>
<td>apple slice, sugar, apple cider vinegar</td>
<td>Yes</td>
<td>0.5</td>
<td>No, but Nematoda documented</td>
<td>very small; ideal for early weaning</td>
<td>difficult to harvest and weigh out accurately</td>
</tr>
<tr>
<td>5 Tubifex worms, <em>Tubifex tubifex</em></td>
<td>freshwater</td>
<td>fish pellet feed, bread, oats, organics, compost, etc.</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>range of sizes available</td>
<td>risk of parasite infestation, especially if initial culture comes from a wild source</td>
</tr>
<tr>
<td>Common name</td>
<td>Culture medium</td>
<td>Culture feed</td>
<td>Size (mm)</td>
<td>Survives in salt water?</td>
<td>Required d/wk of labor?</td>
<td>Component of natural winter flounder diet?</td>
<td>Pros</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>California black worms, <em>Lumbricus variegatus</em></td>
<td>freshwater and sediments</td>
<td>fish pellets</td>
<td>15 - 50</td>
<td>No</td>
<td>1</td>
<td>No</td>
<td>when cut apart, each segment continues to move; range of sizes available</td>
</tr>
<tr>
<td>Grindal worms, <em>Enchytraeus buchholzi</em></td>
<td>soil, peat, coconut fiber</td>
<td>baby cereal, oatmeal, mashed potato flakes, fish flakes,</td>
<td>4 - 12</td>
<td>No</td>
<td>3</td>
<td>No</td>
<td>small (thin); ideal for early weaning</td>
</tr>
<tr>
<td>Water flea, <em>Daphnia sp.</em></td>
<td>freshwater</td>
<td>algae, yeast, bacteria</td>
<td>1 - 5</td>
<td>No</td>
<td>3</td>
<td>No</td>
<td>can collect seed cultures from local ponds/lakes</td>
</tr>
</tbody>
</table>

1 Ivleva (1973); Klein-MacPhee (1978)  
2 Klein-MacPhee (1978); Aquatic Research Organisms, Inc. (personal communication)  
3 Hoff and Snell (1999)  
4 Stehlik and Meise (2000); Aquatic Research Organisms, Inc. (personal communication)  
5 Aquatic Research Organisms, Inc. (personal communication)  
6 Ivleva (1973); L. Harris (University of New Hampshire, personal communication)
Table 2.3. Mean growth rates (± SEM) of winter flounder over the course of 4-wk hatchery trials.

<table>
<thead>
<tr>
<th>Feed type</th>
<th>Standard length (SL)</th>
<th>Total length (TL)</th>
<th>Weight (Wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Instantaneous growth rate (G_{SL}/d) (mm/d)</td>
<td>Instantaneous growth rate (G_{TL}/d) (mm/d)</td>
<td>Instantaneous growth rate (G_{W}/d) (mm/d)</td>
</tr>
<tr>
<td>Worm-reared</td>
<td>0.0102 ± 0.0007</td>
<td>0.3657 ± 0.0231</td>
<td>0.0112 ± 0.0006</td>
</tr>
<tr>
<td>Brine shrimp-reared</td>
<td>0.0051 ± 0.0005</td>
<td>0.1718 ± 0.0174</td>
<td>0.0059 ± 0.0007</td>
</tr>
<tr>
<td>Amphipod-reared</td>
<td>0.0022 ± 0.0007</td>
<td>0.0696 ± 0.0215</td>
<td>0.0029 ± 0.0008</td>
</tr>
<tr>
<td>Pellet-reared</td>
<td>0.0002 ± 0.0007</td>
<td>0.0083 ± 0.0222</td>
<td>0.0008 ± 0.0008</td>
</tr>
</tbody>
</table>
Figure 2.1. Hatchery experiment set-up: (a) schematic and (b) photograph (Tray 1 in foreground, Tray 2 in background). Gray circles marked "in" denote water flow into system; black circles marked "out" denote water flow out of the system.
Figure 2.2. Mean dissolved oxygen (mg/L) levels per feed type over time for hatchery trials. There were no significant differences in DO between trays so all tanks per feed type were pooled. Letters adjacent to datasets indicate significant differences between hatchery feed types (via overall Kruskal-Wallis test of all time periods combined, followed by Dunn's Multiple Comparison test; P < 0.01 for worm- versus amphipod-reared fish; P < 0.001 for all other significant relationships). Data before Day 0 are of fish during the weaning period.
Figure 2.3. Survival of fish reared on different feed types in the hatchery over time. Letters adjacent to staircase curves indicate significant differences between hatchery feed types ($P < 0.05$ for worm- versus brine shrimp-reared fish; $P < 0.0001$ for all hatchery feeds compared to pellet-reared fish).
Figure 2.4. Mean (± SEM) growth parameters in standard length (SL), total length (TL), and weight (Wt) over 4-wk hatchery feeding trials. Letters adjacent to regression lines indicate significant differences (P < 0.01) in slope between feed types. Data before Day 0 are of fish pre-weaning.
CHAPTER III

REARING DIETS FOR WINTER FLOUNDER STOCK ENHANCEMENT THAT OPTIMIZE FEEDING-RELATED PERFORMANCE IN THE WILD:

SURVIVAL, FEEDING, GROWTH, AND NUCLEIC ACID ANALYSES

Introduction

Weaning onto formulated diets is a stressful time for cultured fish (Sterud et al., 2000), and this may be especially true for flatfish that have just undergone the dramatic morphological and physiological transformations associated with metamorphosis. Weaning occurs an additional time for stocked fish as they transition from formulated hatchery feed back onto live (i.e., wild) diets once released. Reared flatfish may take days (Sparrevohn et al., 2002; Fairchild, 2010) to weeks (Ellis et al., 2002; Furuta et al., 1997) before they begin feeding consistently on wild prey and even then, non-conventional food items may be selected because of size/shape resemblances to formulated hatchery diets (Ellis and Nash, 1998; Ellis et al., 2002). Selection of sub-optimal food items with high inorganic content, such as small stones or bivalves, may lower the physiological fitness of fish and affect survival (Howell, 1973; Ellis and Nash, 1998). This short period of
starvation during the wild transition also can alter feeding behavior (e.g., increasing off-bottom swimming events), which may result in increased predation risk for reared flatfish (Furuta, 1996; Miyazaki et al., 2000). Thus, conforming feeding performance of fish from the hatchery and the wild is paramount for any applied stock-enhancement effort.

Our objective was to quantify feeding-related performance of reared-then-released winter flounder juveniles that were reared on different feeds (both live and formulated) in the hatchery. We aimed to evaluate how feeding history translated to the wild feeding success once individuals were released into nature (caged in-situ) by examining survival, growth, feeding onset and incidence, stomach fullness, diet composition, and nucleic acid-based condition. Nucleic acid-based indices, such as the ratio RNA:DNA, have been used to evaluate growth and nutritional condition of juvenile fishes since protein production varies in accordance with the quantity of RNA produced, while the DNA content of a cell remains relatively constant (Buckley, 1980; Bulow, 1987; Richard et al., 1991; Westerman and Holt, 1994; Buckley et al., 1999). RNA:DNA ratio has been shown to respond to changes in feeding conditions and growth after short periods (1–3 d) in a variety of fish species, including winter flounder (Buckley, 1982; Buckley et al., 1999; Ben Khemis et al., 2000; Mercaldo-Allen et al., 2008).

Methods

Winter Flounder Rearing and Maintenance
From April–September 2008, winter flounder eggs, larvae and young juveniles were reared and maintained at the Coastal Marine Laboratory (CML), Judd Gregg Marine Research Complex, University of New Hampshire (UNH), in New Castle, New Hampshire (NH), USA, as described in Chapter 2. After hatch, larvae initially were fed a daily ration of enriched rotifers _Brachionus plicatilis_ (DHA Selco; INVE, Salt Lake City, UT, USA) and at 20 days after-hatch (DAH), in addition to rotifers, larvae were provided with enriched brine shrimp nauplii, _Artemia salina_ (DC DHA Selco; INVE). After 1.5 wk of co-feeding (30 DAH), rotifers were withdrawn and larvae were fed only brine shrimp nauplii through and beyond settlement until initiation of weaning onto one of four different hatchery feeds (90 DAH): (1) a 0.5-0.8 mm mix of Skretting Gemma™ commercially-available, formulated pellets; (2) brine shrimp, _Artemia salina_, post-nauplii; (3) white worms, _Enchytraeus albidus_; and (4) common burrower amphipods _Leptocheirus plumulosus_.

Wild Caging Trials

Surviving fish from the hatchery feeding trials of Chapter 2 were used in the wild caging trials in an eelgrass-surrounded, mud/silt-bottomed cove adjacent to the CML (43° 04' N; 70° 42' W; Fig. 3.1) from 03 September – 03 October 2008. During the 2-d transitional period between hatchery and cage trials, age-0 wild fish were seined from the cove, and all fish were tagged with color-coded visible implant elastomer tags (Northwest Marine Technology, Shaw Island, WA). Tag color and tag location distinguished wild or cultured individuals and feeding history of the cultured fish. Fish were not fed for 48 h
prior to the initiation of the caging trials. This transitional, 2-d period also enabled fish
time to recover from the handling stress of measurement and tagging. Fish were released
into 0.52 x 0.38 x 0.20 m cages nested into larger, heavier cages to weigh down and
stabilize them in the mud, as well as to provide an additional barrier against predators.
Fifteen fish per cage, three of each feed type plus three wild fish (controls), were
released. Wild fish seined from the cove had already surpassed the size range of
hatchery-reared fish (mean wild fish standard length, SL = 58.29 ± 1.42 mm versus 34.76
± 0.34 mm for hatchery-reared fish). All fish were not released at the same time; rather,
releases were scattered over a 27-h period (3-4 September 2008) so that all retrievals
occurred during daylight hours (Fig. 3.2). Cages were deployed at least 2 m from one
another and were retrieved between 3 h and 30 d post release, resulting in a gradient of 16
distinct cage-hauling events (Table 3.1). Due to mortality during hatchery trials and the
subsequent 2-d transitional period, all hatchery feed types were not represented in all
cages during wild trials. Fish were snap frozen on dry ice upon retrieval. Subsequently,
fish were measured (SL) at the hatchery, and digestive tracts were removed and preserved
in formalin for stomach content analyses. Weight measures were not possible
immediately upon cage retrieval due to the constraints of working in the field, and once
snap frozen, additional water weight accompanying such small specimens made weight
measures highly inaccurate. Therefore, growth data are presented in terms of SL only.

Instantaneous growth rates ($G_{SL}$; Table 3.2) and somatic growth rates (mm/d)
were calculated and analyzed for fish retrieved > 3 d post cage release to ensure that
growth estimates reflected wild feeding activity. The influence of hatchery feed type on
survival upon cage retrieval was assessed via Chi-square association. To simplify
visualization of diet composition and stomach fullness over time, data were compiled into four time groups post cage release: within 1 d (0.125, 0.25, 0.5 and 1 d); from 1 d to 1 wk (1.25, 1.5, 2, 3, and 5 d); approximately 2 wk (9, 12, 16 d); and approximately 1 mo (19, 23, 26, 30 d). The Index of Relative Importance (IRI; Pinkas et al., 1971) was applied to describe prey composition of the stomach contents of the cage-released fish. Stomach Contents Index (SCI) within (over time, by time group) and between (overall) hatchery feed types was compared by Kruskal-Wallis followed by Dunn's Multiple Comparison Test since data did not conform to a Gaussian distribution.

Water quality data were obtained from a DataSonde buoy deployed at the end of the UNH pier (Figs. 3.1 and 3.2) as part of the Great Bay National Estuarine Research Reserve System Wide Monitoring Program and the UNH DataSonde Program. The DataSonde recorded measurements of water temperature, salinity, dissolved oxygen, pH, and turbidity at approximately 30-min intervals; we used temperature and salinity data collected nearest to 12:00 PM for the duration of caging trials.

**Nucleic Acid Analyses**

In preparation for analyses, frozen fish were dissected on a tray set on ice. White muscle tissue samples consisted of the fillet from the dorsal side. Dissecting tools were rinsed with deionized water between dissections to avoid contamination. Each tissue sample was weighed to the nearest 0.001 g and placed in a test tube in an ice slurry bath. The tissue was homogenized in ice-cold distilled water using a Janke and Kunkel Ultra-
Turrax tissue homogenizer. Replicate aliquots immediately were frozen and biochemical analyses of the tissues were completed within 48 h of freezing.

Muscle tissue samples were analyzed using a UV-based method according to Buckley and Bulow (1987) as modified by Kuropat et al. (2002). First, free nucleotides were removed using a series of washes with cold perchloric acid (HClO$_4$). RNA was then hydrolyzed with potassium hydroxide and the hydrolysate was acidified with cold HClO$_4$ to remove the RNA from the DNA and protein. Then DNA was both hydrolyzed and separated from the remaining protein by the addition of hot HClO$_4$. RNA and DNA were estimated from the absorbance of the appropriate hydrolysate at 260 nm using the following extinction coefficient: $A_{260}$ of a 1-mg/ml solution of hydrolyzed RNA or DNA is 0.03. Absorbance was measured using a Ciba-Corning Gilford Response Spectrophotometer. RNA and DNA concentrations were calculated as mg/mg wet tissue weight. As a quality control measure, a large quantity of scup, *Stenotomus chrysops*, muscle tissue was homogenized and frozen in 0.2 g aliquots. One control sample was processed each day along with the tissue samples to verify the accuracy of the run (Buckley et al., 1999).

RNA quantities reflect growth 1–3 d prior to sampling (Kuropat et al., 2002), and fish were not fed for 2 d before cage release; therefore, RNA/DNA ratio and RNA concentrations of fish retrieved ≤ 1 d post cage release were considered baseline values reflective of hatchery feeds. Baseline values of RNA and DNA concentration among hatchery feeds were compared via ANOVA, followed by Tukey's Multiple Comparisons test, while those of RNA/DNA ratio were examined via Kruskal-Wallis, followed by Dunn's Multiple Comparisons test because the variances among hatchery feeds were
unequal. Juvenile winter flounder may take $3-4$ d before they begin feeding on live prey once released (Fairchild, 2010), so only values for fish released $> 3$ d ($5-30$ d post release) were considered to ensure that growth estimates were due to wild-feeding activity. Spearman correlations were conducted to test the strength of the relation between daily instantaneous growth rate, RNA/DNA ratio, RNA and DNA concentrations, temperature, and time post release for fish retrieved $> 3$ d. RNA/DNA ratio, and RNA and DNA concentrations, were plotted by day of cage retrieval, and values within feed types were examined by linear regression to describe general trends over the 30-d period.

**Results**

**Wild Caging Trials**

**Survival.** During the 2-d transitional period between hatchery and wild caging trials, mortalities for hatchery-reared fish equaled 20 for pellet-, 15 for amphipod-, 4 for brine shrimp-, and 3 for worm-reared fish. No wild fish died during the transitional period. For the duration of wild cage release trials, seawater temperatures ranged from 11.4 to 19.0 C (mean $15.2 \pm 0.4$ C) and salinities from 25.7 – 31.4 ppt (mean $29.3 \pm 0.3$ ppt).

Although there were no significant associations between fish raised on different feeds and survival ($\chi^2 = 8.56$, $P = 0.07$), there was a trend of lower cumulative survival.
for pellet-reared fish over time (Fig. 3.3). Wild, worm- and amphipod-reared fish all exhibited over 90% survival during caging.

**Feeding.** The majority of empty guts (stomach + intestines) were observed within the first 6 h after release for all hatchery feed types (data on pellet-reared fish not available; Fig. 3.4). After 1 d post release, wild fish had the highest incidence of empty guts. Overall mean stomach fullness was highest for amphipod-reared fish (1.32) and was lowest for wild fish (0.36; KW = 23.32, P < 0.001, Fig. 3.5a). Over the course of the caging period, worm- and brine shrimp-reared fish showed significant increases in SCI from the first day post release (KW = 12.15, P < 0.01 and KW = 17.83, P < 0.001, respectively; Fig. 3.5b). Although not statistically significant, stomach fullness of amphipod- and pellet-reared fish tended to decrease over time. Wild fish maintained relatively constant SCI during the course of caging. Overall diet composition was similar between fish reared on all hatchery feed types and wild fish. Identifiable prey found in the stomachs of cage released fish included polychaetes, amphipods, copepods, bivalves, cumaceans, nematodes, decapods, isopods, arthropods, gastropods, and tunicates with only the first five prey categories making up the bulk of dietary importance as per IRI (Fig. 3.6). Bivalves were detected in the stomachs of all treatment types; however, wild fish consumed the lowest percentage. The amount of bivalves detected in stomachs became more evident after 2 wk post release. Overall, wild fish ate more amphipods than fish from any other feed type, and the incidence of amphipods in wild fish stomachs increased with time, as the incidence of polychaetes decreased.
Growth. Length-based growth rates for individual fish retrieved > 3 d post cage release ranged from 0.0001 to 0.0242 G_SL/d (instantaneous) and from 0.0087 to 1.01 mm SL/d (somatic). Growth rates of worm- and brine shrimp-reared fish were similar while in the cages (Table 3.3). Overall, wild fish had significantly lower G_SL than all hatchery-reared fish (KW = 49.85, p < 0.001). Among rearing diets of fish retrieved > 3 d post cage release, there were no significant differences in the relation (slope) between size (SL) of fish at the time of retrieval and G_SL (F = 1.31, P = 0.28; Fig. 3.7), although the magnitude of G_SL (y-intercept) was significantly higher overall for the smallest fish, i.e., pellet- and amphipod-reared, followed by brine shrimp- and worm-reared, and lastly wild fish (F = 10.79, P < 0.0001).

Nucleic Acid Analyses

Fish retrieved > 3 d post cage release showed highly significant associations between length-based instantaneous growth rate and both RNA/DNA ratio and RNA concentration (Table 3.4). RNA concentration, DNA concentration, and G_SL were significantly negatively correlated with days post release (time). RNA concentration, DNA concentration, and G_SL were significantly positively correlated with seawater temperature. Water temperature and time post release were not correlated with RNA/DNA ratio (P > 0.05). There were no significant differences in RNA/DNA ratio (KW = 6.39, P = 0.17), RNA concentration (F = 1.37, P = 0.26), or DNA concentration (F = 1.92, P = 0.12) between baseline values for any feed type (Fig. 3.8). Over time, RNA/DNA ratio significantly increased for brine shrimp- (r^2 = 0.15; P < 0.05) and
amphipod-reared fish ($r^2 = 0.30; P < 0.001$), but stayed relatively constant for wild, worm- and pellet-reared fish (Fig. 3.9a). Likewise, RNA concentration significantly increased over time for brine shrimp- ($r^2 = 0.30; P < 0.001$) and amphipod-reared fish ($r^2 = 0.14; P < 0.05$), stayed relatively constant for worm- and pellet-reared fish, but significantly decreased for wild fish ($r^2 = 0.10, P < 0.05$; Fig. 3.9b). DNA concentration remained constant over time for all feed types while in the cages (Fig. 3.9c).

**Discussion**

**Wild Caging Trials**

**Survival.** Mortalities occurring during the 2-d transitional period between hatchery and wild caging trials were attributed to handling stress (measuring and tagging), with smaller fish (i.e., pellet- and amphipod-reared) exhibiting the most pronounced mortality, in combination with lower water quality in rearing tanks towards the end of hatchery trials, especially for pellet-reared fish. Most cage mortality occurred during the last 2 wk of trials. There were 15 fish in each cage, and as fish grew over the course of the trials, it is likely that food availability in the cages became limited over time. Although the percent of empty stomachs did not increase and stomach fullness remained statistically constant or increased with time, fish were growing and thus gaining a higher food demand in a limited feeding environment (and, at least for worm- and brine shrimp-reared fish, were learning to hunt more effectively as evidenced by higher stomach fullness over time) so only the best competitors may have been able to acquire
enough food to survive in the cage. This high density of fish in a small space may mimic the high-density, point source release of a true stocking effort, and thus reflect the intra-specific competition that is likely occurring. Food resources may become available when lesser competitors die, thus resulting in increased stomach fullness for some feed types in conjunction with the lower survival of others over time. Note, however, that all survival trends expressed here represent that of fish in a predator-free environment, and we would expect even higher mortality in the presence of predators. How type of hatchery-feed may influence avoidance behavior and survival in the presence of predators is still unknown.

After 1-d post release, wild fish had the highest incidence of empty guts. Wild fish may have been behaving cautiously, and thus minimizing movements (e.g., feeding activity) while constrained within the cages at high density with hatchery-reared conspecifics. Considering that hatchery-released flounders exhibit a higher incidence of movements and off-bottom swimming behaviors than wild fish (Furuta 1996; Chapter 5), wild fish may have remained especially inactive while in such close proximity to these active, conspicuous fish, which would attract predators to the area. Green crabs, *Carcinus maenas*, which are confirmed predators of juvenile winter flounder, as well as rock crabs, *Cancer irroratus*, and Jonah crabs, *Cancer borealis*, were observed on, under, and around cages during the course of trials and were within visible distance to caged fish.

We focus on the concept of cage "survival" and imply that fish not recovered from cages were a result of mortality and not escapement. More accurately these "survival" numbers reflect the number of fish recovered after the field cage experience. The double cage construction we implemented was designed to minimize fish escapement, and the high recovery rates of all fish types support this notion. Sogard
(1992), who examined wild juvenile winter flounder in field cages, makes reference to flaws in initial cage design, which resulted in a paucity of fish recovered during the first year of study; substantial increases in recovery during the second year were attributed to adjustments in cage design. The percent recovery we recorded in the present study (88%) is in accordance with that recorded during the second year of Sogard's (1992) study (mean recovery = 91%) and is in the higher range of those documented in other juvenile winter flounder caging studies using much lower stocking densities (Table 3.5).

**Feeding.** For onset of feeding and feeding incidence analyses, empty guts (stomach + intestines) were considered as opposed to simply empty stomachs, since food presence within any part of the digestive tract would be indicative of recent feeding activity. No interpretation regarding the amount of copepods found in fish stomachs is discussed, as it was impossible to determine whether copepods were actively selected for or whether they originated from the guts of partially digested polychaetes within fish stomachs.

Ellis and Nash (1998) and Ellis et al. (2002) suggested that the occurrence of some sub-optimal "prey" items, such as small stones, in the stomachs of recovered hatchery-reared fish may be due to the resemblance of these items to formulated pellet feeds. In the present study, we did not detect stones in the stomachs of fish, and although there was a high incidence of similar-shaped, high-inorganic-content bivalves in the stomachs of fish, the amount observed in pellet-reared fish did not differ from fish reared on any other hatchery feed (note that other hatchery feed types were never exposed to formulated feeds at any point in their lifetime). The incidence of bivalves in the stomachs...
of all fish types, including wild fish (although wild fish consumed the least amount overall), increased as fish approached 2 wk post release. This may be an indication that more preferred prey items, such as polychaetes, which were more prominent in stomachs within the first week of release for most feed types, were becoming limited inside the cage as time progressed. Fairchild et al. (2005), who examined caged juvenile winter flounder from the same estuary as the present study, found that wild prey availability (i.e., polychaetes, amphipods, nematodes, bivalves, and cumaceans) inside of release cages decreased over a 10-wk period compared to that outside of cages. Polychaetes are a major natural prey component of winter flounder, including those < 1 yr old (Pearcy, 1962; Festa, 1979; Stehlik and Meise, 2000). Amphipod- and pellet-reared fish tended to select polychaetes less than wild, worm- and brine-shrimp reared fish. Wild fish primarily chose polychaetes immediately upon release, but then transitioned to a diet higher in amphipods. Nutritionally, polychaete worms would provide a higher gain with lower foraging cost due to a low fiber and ash content and 2-dimensional movement, while amphipods (3-dimensional movement) would require a higher cost per gain (higher fiber and ash content). Amphipods should still provide more nutrition than the high-inorganic-content (although low-foraging-cost) bivalves. If we were to view wild fish as indicators of optimal foraging, than this switch from polychaetes to amphipods, if and when polychaete abundance declines, would be the norm. Only worm-reared fish mimicked this increase in amphipod consumption with decreasing polychaete intake over time; however, like in all hatchery-reared fish, bivalves comprised a much higher (over 10% in the last 2 wk) dietary importance in worm-reared fish than in wild fish.
Within the first day of release, amphipod- and pellet-reared fish had the highest stomach fullness of all feed types. This higher SCI corresponded with a higher consumption of food items comprised of high inorganic content and slower digestibility, i.e., amphipods and copepods, than that of fish reared on the other hatchery feeds. The subsequent (yet nonsignificant) decrease in stomach fullness over time for these two feed types may have been the result of gorging after 4 wk of feeding on nutritionally suboptimal feeds (these two feed types resulted in the lowest growth in hatchery trials) in conjunction with a period of no food availability (2-d transitional period), followed by a relaxation of such aggressive feeding as food became more consistently available in the wild, at least until prey items potentially became limited in the cages at approximately 2 wk post release. By this time, fish reared on other feed types may have gained enough experience to compete equally with these initially aggressive feeders for limited resources. Alternatively, this may be an indication that in the wild an aggressive feeding strategy may not be sustainable over long periods (i.e., one month), as wild fish exhibited much lower stomach fullness consistently over time. The initial high level of stomach fullness may indicate that amphipod-reared fish may have had adequate training in the hatchery for the life of an active predator in the wild. Although pellet-reared fish also exhibited this high initial stomach fullness, it is important to remember that pellet-reared fish had the lowest survival and growth in the hatchery, and subsequently the lowest survival in wild cages, so the resulting low numbers of these high performing pellet-reared individuals may not justify the cost, waste, and effort involved in rearing them for stock enhancement.
Although in this study we focused on feeding exclusively live or formulated feeds to fish reared for stock enhancement, we are not excluding the possibility that a dried or frozen diet may have merit, or that some combination of feed types (provided either concurrently or transitioned for a short period immediately before release) may optimize hatchery and/or released fish feeding-related performance. Kuhlmann et al. (1981) found that compared to fish fed exclusively formulated feeds, mixed feeding with live mysids increased growth and food conversion in juvenile turbot, Scophthalmus maximus, but growth was even higher for fish fed live mysids only. Marbled flounder, Pseudopleuronectes yokohamae, reared for stock enhancement at Hyogo Prefecture Hatchery in Japan, are sometimes fed a mixture of minced frozen mysids with the addition of formulated feed to boost nutritional content. However, pellet feeding is suspended approximately 2 wk before release to focus fish on the more natural feed (T. Minamiura, Hyogo Prefecture Hatchery, personal communication). Brown et al. (2003) found that the ability of Atlantic salmon, Salmo salar, to switch from one live prey type to another (i.e., bloodworms to brine shrimp) was enhanced when fish were provided with a mixed live/formulated feed in combination with an enriched rearing environment in the hatchery, and suggested that this feeding paradigm had the potential to significantly improve post-release survival of reared-then-released fishes. Ellis et al. (2002) found that hatchery-reared turbot achieved similar feeding rates to wild fish within 9 d of exposure to live feed; thus, we can conclude that even a short transition to live feed in the hatchery before release may enhance wild feeding performance.
Growth. Using cages for studies on fish growth may be suboptimal, as cages may cause growth variability due to fish handling procedures, exclude or concentrate certain types of prey, force fish to remain in unfavorable areas under variable environmental conditions, and may not accurately represent growth of free-swimming, wild fish in these same habitats (Phelan et al., 2000; Kuropat et al., 2002; Fairchild et al., 2005). However, growth of juvenile winter flounder has been estimated previously by caging studies (Table 3.5), which allow a basis for growth comparison, and using cages increases the probability that released fish will be recovered. In addition, the exclusion of predators by cages enables an overview of intraspecific feeding competition without the influence of external predation. The densities at which cages are stocked also can be manipulated to mimic the high densities of a point-source stocking effort.

Somatic growth rates of fish caged > 3 d in the present study correspond to those at the same locale for hatchery-reared juvenile winter flounder reported in earlier years (Fairchild, 1998; Fairchild et al., 2005). These growth rates also overlap the lower range of values for wild juvenile winter flounder reported by previous caging studies conducted at much lower densities in warmer, more southern locations (Table 3.5).

Growth rates of worm-reared fish were slightly lower in the cages compared to growth performance in the hatchery. However, brine shrimp-, amphipod- and pellet-reared fish all exhibited higher growth rates while in the cages. The indefinite supply of nutrient rich worms conspicuously supplied to worm-reared fish in sediment-free tanks most likely exceeded the amount immediately available and visible to fish while in the cages, and since no other prey types were available to worm-reared fish in the hatchery, fish had no choice except to consume worms. In the cages, however, worm-reared fish
likely consumed organisms both easy to catch and within close proximity. Thus, worm-reared fish in the field would prey on less-nutritious food items such as amphipods, copepods, and bivalves, in addition to polychaete worms. Alternatively, brine shrimp- and amphipod-reared fish would have access to nutrient-rich polychaetes once released into cages. Pellet-reared fish growth performance likely increased in the field due to better water quality and more stimulating (moving) prey access while in the cages.

Wild fish grew significantly less than all hatchery-reared fish; however, they also had the most consistent growth rates amongst individuals. Theoretically, wild fish maintained the same diet before and after the initiation of caging (i.e., a wild diet); thus, if we were to assume that the cages had little effect on prey availability, we would expect little difference in wild feeding-related performance (as indicated by instantaneous growth rate) over the course of trials. This consistency of growth rate over a wide range of fish sizes (42- to 75-mm SL) again supports the use of wild fish as reliable control for cage-release experiments.

**Nucleic Acid Analyses**

Since RNA/DNA ratios of hatchery-reared flatfish may decrease in response to short-term starvation periods instilled before release (Gwak et al., 2003), and RNA analysis of white muscle reflects growth 1 to 3 d before sampling (Buckley et al., 1999; Kuropat et al., 2002), we focused on examining fish released > 3 days to evaluate the influence of hatchery feed on subsequent wild feeding performance. The significant correlations between length-based instantaneous growth rate and both RNA/DNA ratio
and RNA concentration are evidence that using these biochemical indicators as a proxy for growth is legitimate. Kuropat et al. (2002) also found a highly significant correlation ($r = 0.83, P < 0.0001$) between RNA concentration and length-based instantaneous growth rate in winter flounder, thus, validating RNA concentration as an indirect measure of growth for this species. RNA/DNA ratio, as an indicator of protein synthesis potential, is a good gauge of nutritional condition (Buckley, 1980; Richard et al., 1991) and can be used to assess diet adequacy (Ben Khemis et al., 2000). The lack of a significant relation between fish size at cage retrieval and instantaneous growth rate within feed types indicates that growth rates did not change as a function of increasing fish size, thus growth indices ($G_{SL}$, RNA/DNA ratio, RNA concentration) can be compared directly between fish of different feed types, regardless of the size differences between them. The lack of correlation between RNA/DNA ratio and both water temperature and time indicates that examining the ratio of these nucleic acid parameters may be more robust to outside variables than examining their concentrations alone, since both RNA and DNA concentration were correlated with time and temperature.

DNA concentrations are virtually constant in somatic tissues (e.g., white muscle) while RNA concentrations are proportional to the amount of protein synthesis (i.e., growth) occurring (Buckley, 1980; Bulow, 1987; Clemmesen, 1994; Buckley et al., 1999). A decrease in RNA concentration while DNA concentration remains constant is an indicator of malnutrition (Richard et al., 1991); in the present study, this trend was detected only in wild fish. Wild fish were larger than all hatchery reared fish, thus their energy demands would be higher than those of the smaller hatchery-reared fish. For wild fish coming from a baseline level of lower density/lower competition (i.e., the wild) and
transferred to higher density/higher competition (i.e., the cages), such a decrease in nutritional condition while maintaining the same diet (i.e., natural prey) in a confined space with limited abundance is not surprising.

The minimum threshold ratio of RNA/DNA necessary to maintain protein synthesis for the normal development and growth of fishes falls between 1–2 (Clemmesen, 1994; Westerman and Holt, 1994), and this critical range also seems to hold true for young flatfish (Richard et al., 1991; Gwak and Tanaka, 2001), including winter flounder (Buckley, 1982; de Montgolfier et al., 2005). The lowest value detected for an individual in the present study was 1.82 (one brine shrimp-reared fish) and only five fish had a value below 2 (one wild, one worm-, and three brine shrimp-reared fish). Thus, most fish were able to maintain themselves above starvation level while in cages.

A number of studies have examined RNA and DNA concentrations of young winter flounder at various developmental and nutritional states (Buckley, 1980, Ben Khemis et al., 2000; de Montgolfier et al., 2005; Mercaldo-Allen, 2008; Fraboulet et al., 2010) and habitats (Kuropat et al., 2002). The mode in the frequency distribution of RNA/DNA values in the present study fell between 3 and 3.5, the same as that recorded by Mercaldo-Allen et al. (2008) when examining wild juvenile winter flounder at similar temperatures. de Montgolfier et al. (2005) and Fraboulet et al. (2010) both recorded lower mean values (< 3) for juvenile winter flounder from colder, Canadian waters.

RNA/DNA ratio and RNA concentration significantly increased for amphipod- and brine-shrimp reared fish once they transitioned to wild prey. However, RNA/DNA ratio and RNA concentration remained constant from hatchery through wild feeding for worm- and pellet-reared fish. RNA/DNA ratio of juvenile Japanese flounder,
Paralichthys olivaceus, fed live mysids for 8 d was 1.7 times higher than those of juveniles fed artificial feeds (Gwak et al., 2003). We saw no evidence of this trend in the present study when examining baseline RNA/DNA values reflective of wild, live, and formulated feeds. Although nucleic acid data for pellet-reared fish were limited due to lower survival rates, the present study indicates that fish reared on formulated feeds are able to maintain a RNA/DNA ratio comparable to fish fed live feeds in the hatchery (and to wild fish) even when growth rates are much lower, and are able to maintain that RNA/DNA ratio after transitioning to natural diets in the wild; however, the impact of a pellet-reared diet on survival must be considered.

Implications for Stock Enhancement

Although wild fish were approximately 2.5-cm larger than hatchery-reared fish at the initiation of caging, their relatively constant survival, stomach fullness, and growth rate indicate they were reliable experimental controls. If we are to consider wild fish as the norm, then the performance of worm-reared fish most consistently matched that of wild performance. Wild and worm-reared fish exhibited the most similar survival, baseline RNA/DNA values, overall stomach fullness, and diet composition profiles over time. This is not surprising, as worms (polychaetes) were the major component of the wild flounder diet overall. However, because of the size difference, caution should be taken in directly comparing performance between wild and hatchery-reared fish in this study.
Cage-released brine shrimp- and amphipod-reared fish had higher mean Stomach Content Index and RNA/DNA ratio among all feed types, possibly indicating these fish were hunting more actively. By being reared on highly motile live feeds that swim in 3-dimensions, these fish may have gained better training for the life of an active predator than those fish reared on other feeds. However, the active foraging exhibited by brine shrimp- and amphipod-reared fish may be sub-optimal to survival with prolonged exposure to predators in the wild. Burke and Masuda (2010) suggested that bold feeding behaviors developed in the hatchery may be a poor strategy for flatfish, which need to be both stealthy predators as well as cryptic prey once released.

Pellet-reared fish had 72% total mortality from the initiation of experiments: 36% from 28-d hatchery feeding trials; 28% from the 2-d transitional period between hatchery and wild trials; and 8% of total mortality from 30-d wild caging trials. Therefore, although pellet-reared fish released in wild cages exhibited similar feeding, growth, and nucleic acid values to those of other hatchery feed types, the impact of lower survival overall cannot be overlooked in the context of a stocking effort. Again, the present study only considers post-release, non-predation induced mortality. We expect that mortality would increase in the presence of predators. In addition, we did not consider any post-release behavioral benefit to rearing fish on live hatchery feeds. However, within the parameters of this study, performance of surviving pellet-reared fish was on par with those fish reared on other hatchery feeds.

**Conclusions**
Cage-release trials indicated that amphipod-reared fish maintained the highest Stomach Contents Index and RNA/DNA ratios, and there were no statistical associations between survival and hatchery feed type. A ranked summary of all hatchery feed types in both hatchery (from Chapter 2) and cage trials reveals that amphipod-reared fish ranked highest in performance overall (Table 3.6). However, worm-reared fish exhibited the highest hatchery performance, and in caging trials worm-reared and wild fish exhibited the most similar survival, baseline RNA/DNA values, overall stomach fullness, and diet composition profiles over time. Therefore, if we designate the performance of wild fish as the ideal, worm-reared fish were the optimal performers. We should also note that overall performance could only be assessed for survivors. Lower performers may have been more likely to experience higher mortality, leaving only higher performers to be ranked. For lower survival feed types (i.e., pellet-reared fish) we may only be ranking the highest performers (those that survived) whereas for hatchery feed types with higher survival (e.g., worm-reared fish) we may be ranking the performance of both low and high performers if both performed above some minimum survival threshold. Thus, examining survival in this rank summary is a prerequisite before considering any other additional performance measure. In addition, how performance would be influenced by the presence of predators is still unknown.
Table 3.1. Cage retrieval schedule for fish reared on different hatchery feeds. Letters denote analyses conducted on fish from specific cages: "a" = survival, "b" = growth, "c" = feeding (onset, stomach fullness, diet composition), "d" = baseline RNA/DNA (ratio, composition), "e" = wild cage feeding RNA/DNA (ratio, composition). Each sampling consists of termination of cage and complete sampling of fish.

<table>
<thead>
<tr>
<th>Hatchery feed</th>
<th>Days post-release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>Worm</td>
<td>abcd</td>
</tr>
<tr>
<td>Brine shrimp</td>
<td>abcd</td>
</tr>
<tr>
<td>Amphipod</td>
<td>abcd</td>
</tr>
<tr>
<td>Pellet</td>
<td>abcd</td>
</tr>
<tr>
<td>Wild</td>
<td>abcd</td>
</tr>
</tbody>
</table>
Table 3.2. Statistics calculated to describe feeding performance.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Formula</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instantaneous growth rate (G)</td>
<td>$G = \ln(Y_z/Y_i)/T$</td>
<td>$Y_i =$ length or weight at initial time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$Y_z =$ length or weight at time $z$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T =$ time period</td>
</tr>
<tr>
<td>Index of relative importance (IRI)</td>
<td>IRI = (N+V)(F)</td>
<td>$N =$ % numerical composition of prey category within the individual</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V =$ % dry weight of prey category within the individual</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$F =$ % frequency of occurrence of prey category within the sample</td>
</tr>
<tr>
<td>Stomach contents index (SCI)</td>
<td>$SCI = (W_{sc} * 100)/(W_f - W_{sc})$</td>
<td>$W_{sc} =$ stomach content dry weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$W_f =$ fish total body dry weight</td>
</tr>
</tbody>
</table>
Table 3.3. Mean growth measures (± SEM) of winter flounder retrieved from cage experiments > 3 d post-release.

<table>
<thead>
<tr>
<th>Feed type</th>
<th>Released size (SL, mm)</th>
<th>Retrieved size (SL, mm)</th>
<th>Instantaneous growth rate (GSL/d)</th>
<th>Somatic growth rate (mm/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worm-reared</td>
<td>38.62 ± 0.73</td>
<td>43.42 ± 0.91</td>
<td>0.0079 ± 0.0007</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>Brine shrimp-reared</td>
<td>35.65 ± 0.84</td>
<td>40.20 ± 1.06</td>
<td>0.0081 ± 0.0008</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Amphipod-reared</td>
<td>33.18 ± 0.71</td>
<td>38.94 ± 1.12</td>
<td>0.0127 ± 0.0011</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>Pellet-reared</td>
<td>31.64 ± 0.81</td>
<td>37.07 ± 1.23</td>
<td>0.0143 ± 0.0023</td>
<td>0.49 ± 0.08</td>
</tr>
<tr>
<td>Wild</td>
<td>60.80 ± 1.76</td>
<td>63.45 ± 1.81</td>
<td>0.0027 ± 0.0005</td>
<td>0.16 ± 0.03</td>
</tr>
</tbody>
</table>
Table 3.4. Correlation coefficients of biochemical parameters of growth, time, and seawater temperature for all fish retrieved from cages > 3 d post-release. *, **, ***, and **** indicate significance at P < 0.05, 0.01, 0.001, and 0.0001, respectively; ns = not significant. Correlations between RNA/DNA ratio and RNA and DNA, respectively, were not calculated due to the confounding nature of these measures.

<table>
<thead>
<tr>
<th></th>
<th>RNA/DNA</th>
<th>RNA</th>
<th>DNA</th>
<th>Instantaneous growth rate</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (C)</td>
<td>-0.09 ns</td>
<td>0.22 *</td>
<td>0.34 **</td>
<td>0.32 **</td>
<td>-1 ****</td>
</tr>
<tr>
<td>RNA/DNA (ratio)</td>
<td></td>
<td></td>
<td></td>
<td>0.36 ***</td>
<td>0.09 ns</td>
</tr>
<tr>
<td>RNA (µg/mg wet tissue Wt)</td>
<td></td>
<td></td>
<td></td>
<td>0.54 ****</td>
<td>0.67 ****</td>
</tr>
<tr>
<td>DNA (µg/mg wet tissue Wt)</td>
<td></td>
<td></td>
<td></td>
<td>0.30 **</td>
<td>-0.34 **</td>
</tr>
<tr>
<td>Instantaneous growth rate (Gₘₗ d⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.32 **</td>
</tr>
<tr>
<td>Time (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.5. Juvenile winter flounder growth rates reported from previous wild caging studies conducted in-situ. All locations are in northeastern USA. SL = standard length; TL = total length; W = wild fish; HR = hatchery-reared fish; N/A = not available.

<table>
<thead>
<tr>
<th>Release location</th>
<th>Release size (mm)</th>
<th>Release duration</th>
<th>Water temperature (C)</th>
<th>Recovery (%)</th>
<th>Somatic growth rate (mm/d)</th>
<th>Cage size (m; length x width x height)</th>
<th>Cage density (# of fish /cage)</th>
<th>Fish type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Jersey</td>
<td>22-84 TL</td>
<td>10 d</td>
<td>19-27</td>
<td>23-97</td>
<td>-0.15 to 1.30</td>
<td>1 x 1 x 0.46</td>
<td>3</td>
<td>W</td>
<td>Sogard, 1992</td>
</tr>
<tr>
<td>New Jersey; New York</td>
<td>14-29 SL</td>
<td>11 d</td>
<td>11-26</td>
<td>61-94</td>
<td>-0.06 to 0.53</td>
<td>0.85 x 0.85 x 0.45</td>
<td>3</td>
<td>W</td>
<td>Able et al., 1999</td>
</tr>
<tr>
<td>New Jersey; Connecticut</td>
<td>16-46 SL</td>
<td>9-11 d</td>
<td>16-35</td>
<td>17-91</td>
<td>-0.03 to 0.69</td>
<td>0.72 x 0.72 x 0.45</td>
<td>3</td>
<td>W</td>
<td>Phelan et al., 2000</td>
</tr>
<tr>
<td>New Jersey</td>
<td>18-38 SL</td>
<td>10 d</td>
<td>10-28</td>
<td>56-100</td>
<td>0.23 to 0.56</td>
<td>0.85 x 0.85 x 0.45</td>
<td>3</td>
<td>W</td>
<td>Curran and Able, 2002</td>
</tr>
<tr>
<td>New Jersey</td>
<td>20-24 SL</td>
<td>12 d</td>
<td>13-29</td>
<td>73-89*</td>
<td>0.00 to 0.90</td>
<td>0.75 x 0.75 x 0.40</td>
<td>3</td>
<td>W</td>
<td>Manderson et al., 2002</td>
</tr>
<tr>
<td>Rhode Island</td>
<td>33-37 TL</td>
<td>10-15 d</td>
<td>14-27</td>
<td>N/A</td>
<td>0.29 to 0.44</td>
<td>1 x 1 x 0.70</td>
<td>4</td>
<td>W</td>
<td>Meng et al., 2000</td>
</tr>
<tr>
<td>Rhode Island</td>
<td>30-37 TL</td>
<td>15 d</td>
<td>18-21</td>
<td>94*</td>
<td>0.22 to 0.60</td>
<td>1 x 1 x 0.70</td>
<td>4</td>
<td>W</td>
<td>Meng et al., 2001</td>
</tr>
<tr>
<td>Rhode Island</td>
<td>25-35 TL</td>
<td>14-16 d</td>
<td>14-27</td>
<td>89</td>
<td>0.51 to 0.95</td>
<td>1 x 1 x 0.70</td>
<td>4</td>
<td>W</td>
<td>Meng et al., 2008</td>
</tr>
<tr>
<td></td>
<td>40-58 TL (W)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Hampshire</td>
<td>32-46 TL (HR)</td>
<td>10 wks</td>
<td>7-22</td>
<td>50 (HR)</td>
<td>0.06</td>
<td>1 x 1 x 1</td>
<td>20</td>
<td>W, HR</td>
<td>Fairchild, 1998</td>
</tr>
<tr>
<td>New Hampshire</td>
<td>36 TL</td>
<td>7 wk</td>
<td>11-27</td>
<td>47-56</td>
<td>0.37 to 0.56</td>
<td>1 x 1 x 1</td>
<td>5</td>
<td>HR</td>
<td>Fairchild et al., 2005</td>
</tr>
</tbody>
</table>

*does not include non-recovered fish resulting from non-recovered cages.
Table 3.6. Performance rank summary of fish reared on all hatchery feed types in both hatchery and wild cage trials. The number 4 denotes the highest rank and 1 the lowest rank of all feed types. Fractional ranking was employed in cases where more than one conditioned type shared the same value.

<table>
<thead>
<tr>
<th></th>
<th>Worm-reared</th>
<th>Brine shrimp-reared</th>
<th>Amphipod-reared</th>
<th>Pellet-reared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatchery Survival</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Growth</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Wild cage Survival</td>
<td>3.5</td>
<td>2</td>
<td>3.5</td>
<td>1</td>
</tr>
<tr>
<td>Growth</td>
<td>1.5</td>
<td>1.5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Feeding incidence</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Stomach fullness</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>RNA/DNA ratio</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Totals</td>
<td>Hatchery</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Wild Cage</td>
<td>9</td>
<td>8.5</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>Overall</td>
<td>16</td>
<td>14.5</td>
<td>22.5</td>
</tr>
</tbody>
</table>

*a All survival over 90% was given highest rank.

*b Growth measures for worm- and brine shrimp-reared fish were given equal rank since their values equaled to the 0.001 level.

*c The percent of empty guts (where highest number denotes lowest performance) was converted to its inverse, percent feeding incidence (where highest number denotes highest performance) for ranking purposes.
Figure 3.1. Cove of the Piscataqua River mouth adjacent to the University of New Hampshire Coastal Marine Laboratory (in circle). Star marks the site of cage releases and wild winter flounder seining.
Figure 3.2. Arrangement of cage distribution for fish released in the cove adjacent to the Coastal Marine Laboratory at the University of New Hampshire. Each small rectangle represents one cage. Number inside rectangle denotes day of retrieval after release. Number below rectangle indicates the time of day individual cage was retrieved. Large bold square represents floating dock from which cages were deployed. Figure not to scale.
Figure 3.3. Cumulative survival of caged fish reared on different feeds in the hatchery expressed as percentage over time. There was no association between hatchery feed type and survival upon cage retrieval.
Figure 3.4. Percent empty guts (stomach + intestines) at days post release. Underlines on x-axis values indicate time intervals when no information for pellet-reared fish is available; double underlines on x-axis values indicate time intervals when no information for pellet- or amphipod-reared fish is available. No empty guts were detected after 19 d.
Figure 3.5. Mean Stomach Contents Index (SCI) of fish reared on different hatchery feeds over the course of wild caging trials: (a) overall and (b) over time (± SEM). Letters indicate significant differences between feed types (P < 0.05, 0.01, and 0.001 for brine shrimp-, pellet- and amphipod-reared versus wild fish, respectively). Numbers above error bars indicate the number of fish examined per time period. Duration post-release labels: 1d = up to 1 d, 1w = from 1 d to 1 wk, 2w = approximately 2 wk, and 1m = approximately 1 mo post-cage release. * and ** denote significant differences within feed types from values detected on Day 1 at P < 0.05 and 0.01, respectively.
Figure 3.6. Dietary importance as indicated by Index of Relative Importance (IRI) for caged fish reared on different hatchery feeds overall and over time. Numbers above columns indicate the number of fish examined per time period. Duration post-release labels: 1d = up to 1 d, 1w = from 1 d to 1 wk, 2w = approximately 2 wk, and 1m = approximately 1 mo post-cage release.
Figure 3.7. Instantaneous growth rate (GSL) versus standard length (SL) at time of cage retrieval for fish retrieved > 3 days post release. Each marker represents one individual. Slopes of the regression lines were not significantly different from zero, nor from each other. Letters adjacent to regression lines indicate significant differences (P < 0.01) in intercept between feed types.
Figure 3.8. Baseline nucleic acid measures (≤ 1 d post release) of fish at the start of wild caging trials. There were no significant differences among feed types for any measure. Wt = weight. R/D = RNA/DNA ratio.
Figure 3.9. Nucleic acid measures for post-release fish over time: a) RNA/DNA ratio, b) RNA concentration, and c) DNA concentration. Each marker represents the mean of a feed type per day. Asterisks adjacent to regression lines indicate significant deviations in slope from 0 (* = P < 0.05; *** = P < 0.001). Wt = weight.
CHAPTER IV

POST-RELEASE PERFORMANCE AND ASSESSMENT OF CAGE CONDITIONED

JAPANESE FLOUNDER IN WAKASA BAY, JAPAN

Introduction

Flatfishes (flounders, halibuts, soles) are among the most desirable and highly-priced fishes sold for human consumption (Howell and Yamashita, 2005). Although flatfishes have supported valuable fisheries throughout the world for centuries, catches of many species have declined (Myers and Worm, 2003; Gibson, 2005; Pitcher, 2005; Yamashita and Aritaki, 2010). Many marine fishes release hundreds of thousands of eggs annually, but because of the vulnerability of the small, early life-history stages, there is high natural mortality, and few survive to maturity. Rearing and releasing juvenile flatfish (i.e., stock enhancement) may help augment natural populations.

Hatchery-reared flatfish often exhibit irregular swimming, feeding, and cryptic (burying and color change) behavioral patterns compared with wild conspecifics, and these behavioral "deficits" are assumed to lead to increased predation risk once fish are released into nature (Furuta, 1996; Kellison et al., 2000). Released flatfish may take days to weeks before they begin feeding normally on wild prey (Furuta et al., 1997; Fairchild, 2010), and this short period of starvation can alter feeding behavior, which in turn may
result in an even more pronounced predation risk for reared fish (Miyazaki et al., 2000). Burying ability is essential for flatfish to become both stealthy predators as well as cryptic prey. Thus, these three behaviors (burying, feeding, and avoiding predation) are intricately linked. Conditioning flatfish to natural stimuli before release may offer fish an opportunity to refine these behaviors, which may increase survival, and subsequent recruitment to the fishery. Fish trained for "wild" conditions may transition more easily and successfully upon release (Kellison et al., 2000; Sparrevoorn and Støttrup, 2007).

Predator-free cages may help flatfish adjust to the wild environment, establish burying skills, begin pigment change, recover from transport stress (Fairchild et al., 2008) and experience natural (live) food sources before full release into the wild. Since 2008, Obama Laboratory, Japan Sea National Fisheries Research Institute, Fukui, Japan, has been examining the effects of cage conditioning for Japanese flounder, Paralichthys olivaceus, to establish if it improves flatfish stocking success. Japanese flounder, or hirame, is the primary species represented in annual flatfish catches in Japan; thus, hirame has been a principal species for both aquaculture and stock enhancement for decades (Yamashita and Aritaki, 2010). The objective of this study was to monitor post-release performance of cage conditioned and non-conditioned Japanese flounder by assessing immediate behavioral changes (burying and feeding ability) and longer-term responses (movements and recaptures rates) conducive to successful stock enhancement programs.

**Methods**
Rearing and Marking

From April 2008, 2009, and 2010, Japanese flounder were raised in 20 kL rearing tanks via the "Hottoke shi-iku" method, a low-labor, high-efficiency rearing technique wherein newly hatched larvae (10,000-40,000/m³) are polycultured with L-type rotifers (Takahashi, 1998). The rotifer cultures were maintained via daily addition of the microalgae, *Nannochloropsis oculata*. No water was exchanged and no bottom siphoning occurred in the tanks during the first 2 to 3 wk of rearing. Supplemental rotifers were added when necessary and microalgae was gradually reduced as larvae transitioned from live rotifers to *Artemia* nauplii at approximately 7- to 8-mm total length (TL), approximately 15 d post hatch. When fish reached approximately 10- to 11-mm TL (just before settlement), they were weaned onto a pelleted, formulated diet and then transferred to flow-through tanks. Rearing water temperature started at ~15°C (egg stage) and gradually increased to around 18°C by the time of release when fish reached 10- to 12-cm TL (late June/early July). Hatchery-reared Japanese flounder exhibit a high incidence (> 95%) of black malpigmentation on their blind (abocular) side (Tominaga and Watanabe, 1998) serving as a natural marker of stocked fish. Flounder were identified according to their conditioning treatments using two marking methods: (1) a series of burn marks or brands inflicted on the blind side (Achiha, 2002; Okouchi et al., 2004), and (2) by soaking fish en masse in an alizarin complexone (ALC) dye bath during rearing (Table 4.1).

Study Sites
Two release sites were located in Wakasa Bay in central Honshū along the north coast of Fukui prefecture facing the Sea of Japan: one in Takahama Bay and one in Obama Bay (Fig. 4.1). In 2008 approximately 40,000, and in 2009 approximately 80,000, 9- to 10-cm juveniles were released in Takahama Bay (Table 4.1). In 2010 in the eastern portion of Obama Bay, approximately 13,000, 11- to 12-cm juveniles also were released. All releases occurred along the sandy coastline in waters 1 to 2 m in depth. Both bays deepen into muddy-bottomed basins towards the mouth; Obama Bay depths increase to over 20 m providing appropriate habitat for a shrimp-trawl fishery.

**Conditioning Process**

Seven to 9 d before release, approximately half of the reared fish were moved into 4 x 4 m cages at the Wakasa Bay release site (Fig. 4.1). Cage density was 207 to 324 fish/m² in 2008, 261 to 367 fish/m² in 2009, and 99 to 157 fish/m² in 2010. Fish were fed formulated hatchery feed once per day while in the cages.

**Experimental Trials**

On the day of release, immediately before dismantling conditioning cages and releasing fish along the shallow coast, samples of conditioned fish were collected from within the cages and brought back to the hatchery for experimental trials. In addition, non-conditioned fish directly from rearing tanks were sampled for comparison on the
same day. Initially, all fish were maintained without food or sediments for 24 h to allow acclimation to the experimental container and recovery from transport stress (for cage conditioned fish). Then, fish for burying trials were transferred to identical but sediment-bottomed experimental containers.

To examine the effect of cage conditioning on burying ability, groups of 20 hatchery-reared flounder (both conditioned and non-conditioned exclusively per tank) were placed in black, plastic-sheeting covered, 60 cm x 30 cm x 35 cm, sand-bottomed aquaria (4 replicates per treatment) in 2009. One day before cage conditioning was initiated in Takahama Bay (approximately 7 to 9 d prior), similar baseline trials of burying ability also were conducted with fish directly from rearing tanks. The percentage of fish buried in each aquarium tank was quantified after 5 min. Burying data were analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons post-hoc tests.

To examine the influence of cage conditioning on feeding ability, experimental trials were conducted with hatchery-reared and wild (control) fish using natural, live mysids *Archeomysis sp.* (approximately 1-cm TL) in 2008 and 2009. Wild fish and live mysids were collected at Wada beach in Takahama Bay by beam trawl (net size = 1.5-m width x 40-cm height x 1-mm² mesh). Ten replicates per cultured fish treatment (conditioned and non-conditioned) with 1 flounder per 10 L container were examined. Wild fish replicates varied from five in 2008 to 19 in 2009 due to the incidental numbers of fish collected around the time of trial initiation, and wild fish were substantially smaller (approximately 4- to 5-cm TL) than the 10- to 12-cm TL hatchery-reared fish (Table 4.2). Each flounder was offered 5 mysids and monitored over time to quantify the
number of prey consumed. Monitoring occurred every 30 min until 2 h (in 2008) or 3 h (in 2009) after the introduction of prey items had occurred. Michaelis-Menten nonlinear regression curves were fit to the data profiles of cumulative mysid consumption over time and compared among fish sources (conditioned, non-conditioned, wild). The Michaelis-Menten model is described by the equation

\[ C = \frac{C_{\text{max}} T}{K_m + T} \]

where \( C \) equals the cumulative number of mysids consumed; \( C_{\text{max}} \) equals the maximum number of mysids available for consumption (i.e., 5 as 5 mysids were provided per fish); \( K_m \) is the time at which half of \( C_{\text{max}} \) is consumed; and \( T \) is time after mysid introduction. Calculated values of \( K_m \) were examined to determine differences in mysid consumption profiles. Significance was established via Extra sums-of-squares F-test (Motulsky and Christopoulos, 2004). Differences between the final number of mysids consumed for each fish type at the end of trials was evaluated by Kruskal-Wallis (KW) followed by a Dunn's multiple comparisons test.

Release and Recapture

On the day of release, conditioned fish were corralled out of cages manually while cages were being dismantled. Within 24 h of releasing conditioned fish, additional naive hatchery fish were released directly from tanks nearby (Table 4.1). In 2009 and 2010, researcher-initiated beam trawling (net size = 2-m width x 20-cm height x 8-mm\(^2\) mesh;
< 5-m depth) occurred 1 to 2 times per week for one month post release (beam trawling began 1 and 3 d after full release in 2009 and 2010, respectively; no researcher initiated beam trawling occurred in 2008). Longer-term recaptures were supplied directly by fishermen [set (fyke) net, shrimp trawl, sea cucumber trawl and recreational fishermen] or purchased from the Takahama and Obama fish markets (Table 4.3). Nine set-nets are distributed coastally by fishermen around Takahama Bay (Fig. 4.2a, b). In Obama Bay, out of a fleet of 10 shrimp trawlers, one was hired to collect and preserve all Japanese flounder bycatch for the duration of the fishing season (first week of June through the end of November). In addition, since there are no set-net fishermen in Obama Bay, five stationary fish traps were set up near the release site. Additional Japanese flounder bycatch via sea cucumber trawl was provided in 2011.

Recaptured fish were preserved in 70% ethanol. Otoliths were extracted, and ALC markings were verified by microscope. In 2009 and 2010, presence or absence of prey and/or digested matter in the gut (= stomach + intestines) was noted. Stomach contents were examined and identified. Diet composition was described by the Index of Relative Importance, IRI (Pinkas et al., 1971),

\[ IRI = (N + W) P \]

which combines the frequency of occurrence \(P\), percent numerical composition \(N\), and percent dry weight \(W\), into one number for comparison. Recapture rates of conditioned and non-conditioned fish were analyzed via Chi-square Test.
**Results**

**Experimental Performance Trials**

Differences in burying performance before and after the caging experience were strongly evident \((F = 30.81, p < 0.0001)\), with fish that underwent cage conditioning exhibiting greater burying ability compared to those that did not (Fig. 4.3). The mean percentage of cage-conditioned fish that buried was statistically higher \((\mu = 70.25\% \pm 7.08;\) variation expressed as SEM here and throughout) than that of non-conditioned fish \((\mu = 25.50\% \pm 3.33; q = 10.62, p < 0.001)\), as well as higher than before the caging experience \((\mu = 23.38\% \pm 3.32; q = 8.78, p < 0.001)\). There was no change in burying ability between non-conditioned fish sampled directly from rearing tanks before (baseline) or after the cage conditioning period \((q = 0.48, p > 0.05)\), i.e., between groups of fish with no difference or change in condition.

In mysid feeding trials, significant differences in feeding ability between fish types (conditioned, non-conditioned, wild) were detected (for 2008 and 2009 respectively, overall nonlinear curves: \(F = 42.80\) and 28.75, \(p < 0.0001\); final mysid consumption: \(KW = 10.61\) and 11.84, \(p < 0.01\); Fig. 4.4). Wild fish performed better than hatchery-reared fish in both years \((K_m = 0.29, 0.33, \) respectively), followed by conditioned fish \((K_m = 3.38, 1.56)\), and non-conditioned fish \((K_m = 35.10, 3.48)\). By the end of trial duration in both years, the final number of mysids consumed by conditioned fish was not significantly different from either wild or non-conditioned fish; however,
non-conditioned fish consumed significantly fewer mysids than wild fish (Dunn's difference in rank sum = -12.05 and -14.31 for 2008 and 2009 respectively, p < 0.01).

Recaptured Fish Locations and Movements

In 2008, the first recaptures (one conditioned and one non-conditioned fish) from set net sampling occurred 3 d after release. It took 4 d for a recaptured (conditioned) fish to move 4.5 km to the opposite (western) side of the bay. Recaptures showed that within the first month of release, conditioned fish reached the mouth of the bay before any other release groups. The last confirmed fish location was of a non-conditioned fish at the mouth of the bay on October 13, 2008 (Fig. 4.2a).

In 2009, the first recaptures (8 conditioned and 17 non-conditioned) occurred during initial beam trawl sampling on July 1, the morning after full release. In this year, both conditioned and non-conditioned fish appeared to disperse within a similar time frame. After 6 d, both conditioned and non-conditioned fish were detected 3.5 km across the bay (Fig. 4.2b). Fish captured via researcher initiated beam trawl near (within 0.5 km) the release site, which were mostly non-conditioned fish, showed negligible amounts of food in their stomachs (Fig. 4.5). Fish recaptured with food in their stomachs consumed mainly mysids and small fish (Fig. 4.6), primarily consisting of gobies (Family Gobiidae). The last confirmed location was of a non-conditioned fish still in the lower bay on November 12, 2009.

In 2010, the first recapture (a conditioned fish) was collected the morning after release in a fish trap nearest the release site. Again, fish captured near the release site via
researcher initiated beam trawling were mostly non-feeding individuals (Fig. 4.5) and recaptured feeding fish consumed mostly small fishes (Fig. 4.6), predominantly gobies. Two weeks after release, the first conditioned fish began appearing in shrimp trawls approximately 5.5 km away (Fig. 4.2c). Eighteen days after the first conditioned fish were detected via shrimp trawler, non-conditioned fish began appearing in shrimp trawl nets. The number of recollected conditioned fish (54) quickly surpassed the number of non-conditioned fish (19) recaptured via shrimp trawler (last recapture June 30, 2011). Additional conditioned (8) and non-conditioned (3) fish were captured by sea cucumber trawl in 2011.

Recapture Rates

Overall, the highest recapture rate was recorded in 2010 (0.0096) in Obama Bay, with much lower recapture rates in 2008 (0.0020) and 2009 (0.0024) in Takahama Bay (Table 4.3). Total recapture rates (including researcher initiated beam trawling in addition to all fishermen effort) show that conditioned fish were recaptured more than non-conditioned fish in 2008 (0.0021 for conditioned and 0.0018 for non-conditioned fish) and 2010 (0.0098 for conditioned and 0.0093 for non-conditioned fish), but less than non-conditioned fish in 2009 (0.0023 and 0.0026, respectively). However, since the goal of Japan's flounder stocking efforts is to overcome recruitment limitations by augmenting natural juvenile supply, and thus optimizing fishing harvest (Yamashita and Aritaki, 2010), fishermen recapture rates alone also provide a valuable assessment of stocking success. Using this measure, in both 2008 and 2009, recapture rates of conditioned fish
were more than non-conditioned fish (0.0021 and 0.0018 for conditioned and non-conditioned fish, in 2008 and 0.0018 and 0.0017, in 2009) though the differences were not statistically significant. In 2010, a significantly higher recapture rate was recorded for conditioned fish than for non-conditioned fish (0.0082 and 0.0051, respectively; \( \chi^2 = 3.87, p < 0.05 \)).

Although market recaptures were considered in overall recapture rates and dietary analyses, market landing data were not included in the description of fish movements since specific recapture areas within the bays could not be verified. No market landing data exist as of yet for 2010 released fish since the commercial minimum size limit for Japanese flounder in Fukui prefecture is 25 cm; by the termination of this study, 2010 fish had not recruited to the fishery (Table 4.3).

**Discussion**

**Immediate Benefits of Cage Conditioning**

Fish that underwent one week of cage conditioning exhibited significantly better burying abilities than those that did not. This is in accordance with Fairchild and Howell (2004) who demonstrated via laboratory experiments that conditioning improved the ability of winter flounder, *Pseudopleuronectes americanus*, to bury in sediments. Hatchery-reared winter flounder juveniles that had never before experienced sediments required 2 d to refine burying skills (Fairchild and Howell, 2004), while cultured sole, *Solea solea*, required 12 d of sand exposure to bury as efficiently as wild fish (Ellis et al.,
Miyazaki et al. (1997) suggested that daytime burying ability of reared Japanese flounder exposed to sand might help to elude diurnal visual predators; any deficiency in burying ability could lead to increased predation risk.

Conditioned fish feeding performance exceeded that of non-conditioned fish in both 2008 and 2009. In both years, mysid consumption by non-conditioned fish was significantly lower than that of wild fish, yet there was no statistical difference between conditioned and wild fish. Wild juvenile flounder mainly consume mysids from approximately 1.5-cm TL (settlement) to over 15-cm TL if small fish prey (such as gobiids) are unavailable (Yamashita and Yamada, 1999). The overall shapes of the feeding performance curves were similar for 2008 and 2009, with wild fish performing highest, non-conditioned fish performing lowest, and conditioned fish falling in between the two. This reveals a repeatable precision of conditioned and non-conditioned fish feeding performance. Non-conditioned, hatchery-reared flatfish may take days (Fairchild, 2010) to weeks (Furuta et al., 1997) before feeding normally on wild prey, and this short period of starvation can alter feeding behavior (e.g., starving Japanese flounder exhibit prolonged off-bottom swimming behavior) that may, in turn, result in even greater predation risk for reared fish (Miyazaki et al., 2000).

Taking into consideration the 2 to 12 d needed to refine burying ability (Ellis et al., 1997; Fairchild and Howell, 2004) coupled with the days to weeks before feeding normally on wild prey (Furuta et al., 1997; Fairchild, 2010), it becomes evident that the first days post release may present a drastically heightened predation risk for non-conditioned, hatchery-reared fish. Those fish that live beyond these first days likely have
learned these survival skills; however, conditioning has the ability to decrease this period of susceptibility and may result in more released fish surviving to recruitment.

Longer-term Benefits of Cage Conditioning

Overall, conditioned fish exhibited higher overall performance than non-conditioned fish in terms of movement and fishermen's recapture rates. Sparrevoehn and Støttrup (2007) investigated the effects of transferring turbot, *Psetta maxima*, to enclosures at the release site 6 d prior to the release and found that such a conditioning period had a positive effect on flatfish survival; mortality of cage-conditioned fish was half that of non-conditioned fish. Kellison et al. (2003) found that hatchery-reared summer flounder, *Paralichthys dentatus*, released in cages showed no difference in habitat-specific growth rates than those of wild fish, whereas stocked (non-conditioned) Japanese flounder landed in the fishery tended to be smaller than wild conspecifics (Tomiyama et al., 2008). Released hatchery-reared flatfish have been shown to have lower residence time than their wild counterparts (Kellison et al., 2003), but site fidelity can be increased by transferring fish to *in-situ* cages before release (Fairchild et al., 2009). Conditioned fish in this study moved more actively towards the mouths of the bays than non-conditioned fish. This movement would be expected of healthier, fitter fish since (1) high coastal water temperatures (up to 30° C) in the shallows of the release area during the summer release time would prompt Japanese flounder, whose optimal temperature for growth is 20-25° C (Iwata et al., 1994), to move into cooler, deeper waters, and (2) potentially higher concentrations of prey exist in deeper waters of the bay.
(e.g., shrimp populations in the middle of Obama Bay, which form the basis of the shrimp trawling industry centered at the bay mouth).

Given the state of (non)feeding of many fish recaptured near the release site, this research indicates that slow boat (< 0.5 m/s) beam trawling efforts may target weak, nonfeeding, nonmoving fish. The amount of small fish prey at the study sites should have been adequate to give the >10-cm TL released flounder juveniles ample opportunity to feed. Mysids are a predominant prey item of post-settled juvenile Japanese flounder and piscivorous feeding becomes evident at approximately 5-cm TL (Yamada et al., 1998; Tanaka et al., 1999). Non-conditioned fish, mostly nonfeeding individuals, were caught more often by beam trawl than conditioned fish for all years when researcher initiated recapture efforts were applied. Similarly, Sparrevoorn and Støttrup (2007) found that the catchability of non-conditioned turbot caught by beam trawl was 10% higher than that of cage conditioned fish. Efforts and money for recapture may be better spent on involving more local fishermen, especially considering shrimp trawlers tow faster (approximately 1.8 m/s), use a larger net (16-m length x 6-m width x 4-m height, 18-mm² cod-end mesh), and are capable of surveying deeper (> 20 m) waters.

Overall recapture rate in 2010 was approximately 1% (0.0096 in 1 yr), which is within the range of other stocked flatfish recapture rates such as for turbot (0.16 to 11% in 7.5 yr; Støttrup et al., 2002), summer flounder (2% in 3 mo; Kellison et al., 2003), winter flounder (0% in 1 wk; Fairchild, 2002), and similar to other Japanese flounder stocking efforts in this area of Japan (0.35 to 4.47% in 1 mo; Tanaka et al., 2006). Recapture rates of Japanese flounder in northeastern Japan tend to be higher (Yamashita et al., 2006) due mostly to differences in mysid abundance (Yamashita et al., 2006).
Mysid productivity in Japanese flounder nursery grounds is significantly higher in northern Japan than in southern Japan, resulting in a higher surplus for stocked juveniles (Tanaka et al., 2006). Recaptures in 2008 (over 3 yr) and 2009 (over 2 yr) in Takahama Bay were lower than expected due in part to the difficulty in monitoring the wide, deeper portions at the mouth of the bay. Thus, Obama Bay, with its tapered mouth and deep-water shrimp trawling industry, provided the better environment for monitoring in this study. We also expect recaptures rate of the 2010 released Japanese flounder to increase as fish continue to be recaptured in subsequent years.

In 2009, 80,000 fish were released - the highest number of all three years - yet this year had the lowest recapture rate by fishermen. Successful stocking endeavors require post-release densities below the carrying capacity of the release environment (Munro and Bell, 1997). The large number of fish released into Takahama Bay at one location may have exceeded the immediate carrying capacity of the environment, and thus, poor overall recapture may have resulted. Both Tanaka et al. (2005) and Sparrevoorn and Støttrup (2007) found that during years of higher release numbers, the fraction of recaptured flatfish containing fish-prey in their stomachs was lower than in years when fewer numbers were released. The sudden increase in the number of flatfish predators at the release site may cause short-term, density-dependent ecological changes in the dynamics of the prey species (e.g., in prey numbers as well as the behavior of prey; Sparrevoorn and Støttrup, 2007), and these changes may affect the overall success of the stocking effort as well as impact wild conspecifics (Tanaka et al., 2005; Yamashita et al., 2006) and/or other species (Bell, 2004).
Pre-release conditioning cages may provide flatfish time for wild behavioral adjustment, which may increase burying and feeding ability, and decrease predator mortality (Sparrevohn and Støttrup, 2007). Nevertheless, conditioning cages in themselves may attract predators simply by providing structure to a barren bottom (Fairchild et al., 2008). Swimming crabs, *Portunus gladiator*, confirmed predators of juvenile Japanese flounder (Saitoh et al., 2003), were observed crawling on the outside of conditioning cages in Obama Bay and around the release site in Takahama Bay; however, the crab density was much less than the green crab, *Carcinus maenas*, density in Fairchild's study (pers. obs).

**Conclusions**

Conditioned fish exhibited better overall performance than non-conditioned fish. Immediate benefits of cage conditioning included a higher percentage of cage-conditioned fish burying immediately upon release compared to non-conditioned fish, and conditioned fish identified and consumed natural prey more than non-conditioned fish. Long-term benefits of cage conditioning were evident; non-conditioned fish lingered near the release site while conditioned fish dispersed soon after the conditioning cage was dismantled. Significantly more conditioned fish were recaptured via fishermen's efforts than non-conditioned fish in Obama Bay in 2010, but no detectable differences in recapture rate were detected between conditioned and non-conditioned fish in Takahama Bay either in 2008 or 2009, despite the increased number of fish released in 2009. For monitoring purposes, the narrow-mouthed and readily fished deeper waters of Obama
Bay provided a much better environment to assess the impact of a stocking effort, which is essential if the success of a stocking effort will influence future efforts. Therefore, choosing a location that can be monitored adequately may be just as important as choosing a location where stocking is predicted to succeed.

This study is the first to examine flatfish conditioning strategies using commercially landed data and shows that cage conditioning can favorably alter the attributes and recapture rates of released fish. The large number of juveniles released during routine Japanese management efforts can provide researchers with an unparalleled opportunity to examine and evaluate the scope and scale of a flatfish stocking effort not yet realized in other parts of the world. International fisheries managers and scientists, therefore, can regard Japanese flounder stocking efforts in Japan as case studies from which to model and base their own developing flatfish stocking protocols.
Table 4.1. Demographics of conditioned (C) and non-conditioned (NC) fish released in all years. ALC = alizarin complexone dye.

<table>
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<th>2009 Takahama Bay</th>
<th>2010 Obama Bay</th>
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<tr>
<td></td>
<td>35N 29' 38&quot;, 135E 32' 44&quot;</td>
<td>35N 29' 38&quot;, 135E 32' 44&quot;</td>
<td>35N 31' 59&quot;, 135E 45' 17&quot;</td>
</tr>
<tr>
<td>Release location coordinates</td>
<td>C</td>
<td>NC</td>
<td>C</td>
</tr>
<tr>
<td>Condition status</td>
<td>Date of fish to acclimation cages</td>
<td>24-Jun</td>
<td>22-Jun</td>
</tr>
<tr>
<td>Release location</td>
<td>Date of release</td>
<td>3-Jul</td>
<td>4-Jul</td>
</tr>
<tr>
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<td>Number of fish released</td>
<td>23400</td>
<td>21300</td>
</tr>
<tr>
<td>Obama Bay</td>
<td>Mean TL of fish released in cm (size range)</td>
<td>9.79 (7.05 - 12.69)</td>
<td>10.02 (7.23 - 12.29)</td>
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<td></td>
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</table>
Table 4.2. Demographics of conditioned (C), non-conditioned (NC), and wild fish used in experimental feeding trials.

<table>
<thead>
<tr>
<th>Year</th>
<th>Status</th>
<th>2008</th>
<th>2009</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>NC</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>TL (cm) ± SD</td>
<td>10.66</td>
<td>11.22</td>
<td>4.89</td>
</tr>
<tr>
<td></td>
<td>± 0.59</td>
<td>± 1.02</td>
<td>± 1.58</td>
</tr>
</tbody>
</table>
Table 4.3. Demographics of conditioned (C) and non-conditioned (NC) fish recaptured in all years. Total recapture includes researcher-initiated beam trawling (RBT) in addition to fishermen recapture methods. Fishermen recapture includes market landing data (Market), setnets (Setnet), shrimp trawler (Shrimp), sea cucumber trawler (Seacuc), passive fish traps (Trap), and recreational fishermen (Recreat) catch. Last recaptures updated on June 30, 2011. * denotes a significant difference in recapture rate between conditioned and non-conditioned fish at P < 0.05.

<table>
<thead>
<tr>
<th>Release year</th>
<th>Bay</th>
<th>Status</th>
<th>Last recapture</th>
<th>Total recapture</th>
<th>Fishermen recapture</th>
<th>Recaptured number by fishing method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Date</td>
<td>TL (cm)</td>
<td>Number</td>
<td>Percent</td>
</tr>
<tr>
<td>2008</td>
<td>Takahama</td>
<td>C</td>
<td>4-Aug-10</td>
<td>46</td>
<td>49</td>
<td>55.68</td>
</tr>
<tr>
<td></td>
<td>NC</td>
<td>7-Apr-11</td>
<td></td>
<td></td>
<td>39</td>
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<tr>
<td></td>
<td>Total</td>
<td></td>
<td>88</td>
<td></td>
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</tr>
<tr>
<td>2009</td>
<td>Takahama</td>
<td>C</td>
<td>18-May-11</td>
<td>45</td>
<td>93</td>
<td>49.21</td>
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<tr>
<td></td>
<td>NC</td>
<td>3-Jun-11</td>
<td></td>
<td></td>
<td>96</td>
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</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>189</td>
<td></td>
<td></td>
<td>0.0024</td>
</tr>
<tr>
<td>2010</td>
<td>Obama</td>
<td>C</td>
<td>30-Jun-11</td>
<td>25</td>
<td>80</td>
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<td>42</td>
<td>34.43</td>
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<td>Total</td>
<td></td>
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<td></td>
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<td>0.0096</td>
</tr>
</tbody>
</table>
Figure 4.1. Study sites in Wakasa Bay, central Honshū, Japan. The black square marks the release and cage-conditioning site in Takahama Bay (2008, 2009) and the white square marks the release and cage-conditioning site in Obama Bay (2010). White circle denotes the Obama Laboratory, Japan Sea National Fisheries Research Institute.
Figure 4.2. Recapture locations of hatchery-released Japanese flounder in (a) Takahama Bay in 2008 and (b) 2009 and (c) Obama Bay in 2010. White squares mark the release and cage-conditioning locations. Single-ringed circles denote fish recaptured by set-nets or traps, double-ringed circles by beam trawl, triple-ringed circle by sea cucumber trawler and mesh-ringed circle by shrimp trawler. Size of the circle reflects number of fish recaptured at each location. Degree of shading of the circle reflects the ratio of conditioned (white) to non-conditioned (gray) fish recaptured at each location with numbers on the tops and bottoms of circles indicating actual numbers of conditioned and non-conditioned fish recaptured, respectively. Solid black circles show set net locations where no released flounder were recaptured.
Figure 4.3. Burying abilities of fish before (left of dashed line) and after (right of dashed line) the cage-conditioning period. Black bar denotes fish directly from rearing tanks before the cage-conditioning period. White bar denotes conditioned fish after the cage-conditioning period. Gray bar denotes non-conditioned fish, directly from rearing tanks after the cage-conditioning period. Error bars display standard error. *** denotes significance at p < 0.001.
Figure 4.4. Feeding abilities of conditioned (white), non-conditioned (gray) and wild (black) fish immediately after the conditioning cage period in 2008 and 2009. The number of mysids consumed was monitored at select intervals after mysid introduction. Michaelis-Menten nonlinear regressions are fitted to mean number of mysids consumed. Error bars display standard error.
Figure 4.5. Number of fish with food in guts (stomach + intestines) recollected within 2 mo of release in 2009 and 2010. Numbers on the tops of bars reflect the number of guts examined per fish conditioning type. Numbers in parentheses reflect fish recollected via researcher-initiated beam trawl near the release site, which predominated recollection through the first week post release.
Figure 4.6. Dietary composition as indicated by Index of Relative Importance (IRI) of collected conditioned (C), non-conditioned (NC), and wild fish within the first 5 mo of release in 2009 and 2010. Mean size in cm (TL ± SD) of examined fish is indicated after the respective year. Numbers on the tops of bars reflect the number of fish examined per conditioning type. Only prey items that comprised 1% or more of total dietary importance were included. No wild fish were examined in 2010.
CHAPTER V

THE INFLUENCE OF CAGE CONDITIONING ON THE PERFORMANCE AND

BEHAVIOR OF JAPANESE FLOUNDER REARED FOR STOCK ENHANCEMENT:

BURYING, FEEDING, AND THREAT RESPONSE

Introduction

Stock enhancement, the spawning and rearing of organisms in captivity and releasing large numbers of young back into nature, is one of the few proactive strategies available to fisheries managers to restore, stabilize, or augment fish populations and thus fisheries catch. However, many stocked fish exhibit pronounced mortality immediately after release, attributed largely to behavioral deficiencies instilled by the unnatural hatchery environment (Furuta, 1996; Flagg et al., 2000; Hossain et al., 2002; Le Vay et al., 2007). For example, the higher incidence of off-bottom swimming behavior observed in hatchery-reared flatfish has been implicated as a leading cause of increased predation (Furuta, 1996; Kellison et al., 2000). In addition, released flatfish may take days to weeks before they begin feeding normally on wild prey (Furuta et al., 1997; Fairchild, 2010), and this short period of starvation can alter feeding behavior, which in turn may result in an even higher predation risk (Miyazaki et al., 2000). These behaviors (feeding and avoiding predation) thus are intricately linked.
These hatchery-induced behavioral deficiencies may be mitigated by providing some level of training or conditioning to reared flatfish, either in the hatchery or immediately before release in the wild. Examples of conditioning strategies that may be applied to flatfish in the hatchery include providing rearing tanks with sediments (Tanda, 1990; Miyazaki et al., 1997; Ellis et al., 1997; Fairchild, 2002; Fairchild and Howell, 2004), feeding fish live feeds, (Furuta, 1996; Walsh et al., in press; Chapter 4), or introducing predator cues (Fairchild, 2002; Hossain et al., 2002). Strategies that can be applied to ease the wild transition at, or near, the release site include conducting “operant conditioning” on fish to respond to light or sound cues for supplemental feed provision during the first few days/weeks post release (Anraku et al., 1998), or short-term release into predator-excluding cages before full release (Sparrevohn and Støttrup, 2007; Fairchild et al., 2008; Walsh et al. in press; Chapter 4). Cage conditioning allows hatchery fish to experience substrates and sediments, wild (live) food sources, and “safe” predator exposure (fish are able to see predators outside of cages and to detect olfactory predator cues) before actual release. In addition, the short period in the cage enables flatfishes to begin pigment change and recover from transport stress (Fairchild et al., 2008). Cage conditioning has shown to be effective in increasing post-release survival and recapture of flatfish species such as turbot, *Psetta maxima* (Sparrevohn and Støttrup 2007), and Japanese flounder, *Paralichthys olivaceus* (Walsh et al. in press; Chapter 4).

Since 2008, Obama Laboratory, National Center for Stock Enhancement, Fisheries Research Agency (NCSE-FRA), in Fukui, Japan, has been examining the effects of cage conditioning for Japanese flounder stock enhancement to establish if the strategy improves fitness of released individuals (i.e., to perform more like wild fish) or
stocking success (i.e., the number of fish landed at market). From these releases, Walsh et al. (in press; Chapter 4) assessed that recapture rates of conditioned fish caught by local fishermen were significantly greater than those of non-conditioned fish (i.e., fish released directly from hatchery tanks to the wild). In addition, laboratory experiments revealed that conditioned fish exhibited enhanced burying and feeding performance compared to non-conditioned fish. However, the degree to which behaviors refined during the cage experience contributed to enhancements in these performance measures remained unknown.

Video analyses allow the assessment of not only the end result of a performance measure (e.g., a buried fish; a full stomach), but also the means by which the end result was achieved (e.g., the sequence of behavioral events that lead to a buried fish or a full stomach). For example, in Walsh et al. (in press; Chapter 4), burying ability was assessed by releasing recently conditioned and non-conditioned fish into tanks and returning after 5 min to quantify the number of fish buried. Likewise, feeding ability was assessed by providing tanks of conditioned and non-conditioned fish with prey and returning every 30 min to quantify how many prey remained. The form of this experimental design did not allow observing of the behavioral mechanisms behind the differences in performance.

Our objective was to assess not only whether cage conditioning enhances the performance of released juvenile flounder, but also to elucidate the behavioral mechanisms behind the performance. We approached this question by examining burying, feeding, and threat response behaviors, which we assessed by video-based experimental trials conducted in the laboratory immediately following the cage conditioning experience. We compared the performance and behavior of four fish types:
(1) "conditioned" fish which spent 7 d in a predator-free conditioning cage; (2) "non-conditioned" fish directly from tanks; (3) "wild" fish; and (4) non-conditioned fish that were released and recaptured (NCRR) after 6 d at large in the wild.

Methods

Cage Conditioning Protocols and Fish Condition Types

We based our experimental trials on the protocols instilled at Obama Laboratory, NCSE-FRA. Each of Obama Station's 4 x 4 m cages holds between 2500-5000 fish depending on the year of release. We constructed conditioning cages on a smaller size scale (1 x 1 m; 1/16th) size of Obama Station's cages but maintained the Obama-cage density (Fig. 5.1). Like Obama Station's cages, the cage in the present study consisted of a metal piped frame supporting a soft mesh enclosure on all sides. Cage density matched those of Obama Station's 2010 Japanese flounder release in Obama Bay, Fukui, Japan; thus, approximately 150 fish from the hatchery were released into the predator-free enclosure.

Cages were erected in the shallow coast in 1-2 m of water in a cove adjacent to Kyoto University's Maizuru Fisheries Research Station (MFRS), Maizuru, Kyoto, Japan. To encourage burying and to mimic Obama Station release sites, approximately 5 cm of sand was distributed over the bottom of cages before fish introduction. Once introduced to cages on June 24, 2010, fish were fed once per day with the hatchery-provided feed
(formulated, commercially available pellets), as per Obama Station protocol. Fish were conditioned in cages for 1 wk before trial initiation.

Wild fish and (inadvertently) NCRR fish were seined from Kanzaki Beach, Maizuru, Japan, 1 to 2 d before trial initiation. NCRR fish were identified by dark pigment spots located on the abocular side. These permanent markings (hypermelanosis) occur in over 95% of hatchery-reared fish but in less than 5% of wild fish, thus providing a "natural" marker (Tominaga and Watanabe 1998). This evidence combined with a larger size compared to the local wild population, supported the assessment that these wild caught fish were hatchery reared. Investigation revealed that these wild-caught yet hatchery-reared fish were raised at Miyazu Laboratory, NCSE-FRA, and released by Kyoto Prefecture on June 23, 2010 (total length, TL [mean, range] of released fish = 10.3, 8.7 to 11.9 cm). Captured wild fish were smaller on average than all hatchery reared fish examined in trials (wild fish = 6.3, 5.0 to 8.0 cm TL; NCRR fish = 10.4, 8.8 to 12.5 cm TL; non-conditioned fish = 10.6, 9.4 to 12.3 cm TL; conditioned fish = 10.6, 9.9 to 11.8 cm TL). Once collected, wild and NCRR fish were maintained together in a separate cage (identical to the conditioning cage) 1 to 2 m away from the conditioning cage in the shallow coast until trial initiation.

**Experimental Set-up**

At the end of the conditioning period, fish from the conditioning cage, fish directly from tanks, and the wild fish and NCRR fish from the holding cage were distributed to 16, static-system, glass aquarium tanks at MFRS (Fig. 5.2). Tanks were
randomly assigned three fish of one condition type (i.e., exclusively conditioned, non-conditioned, wild, or NCRR fish) per tank (N = 4 tanks per condition type) and were maintained in a temperature controlled room at 19°C for 3 d under a 14 h light: 10 h dark regime, which roughly corresponded to natural diurnal light fluctuation. The experimental room had one window, which allowed some provision of natural light intensity. Tanks were sand-bottomed and covered on three sides by light blue plastic sheeting, which prevented fish from viewing adjacent tanks and provided contrast and reduced light refraction (glare) for video. An aerated model predator (14 cm length, 9 cm width, 11 cm height; Penn Plax Aerating Eel #RR874 - Aquarium Air Decoration) was set in the corner of each tank hidden by a blind (Fig. 5.3). The aeration system was engineered so that air in all tanks could be turned on and off simultaneously from one valve. Default aeration was set to constant “on” during the 3-d experimental period to oxygenate the static water system and to acclimate fish to the sound of aeration, which accompanied the model predator’s mouth-opening motion. Cameras were initially positioned above tanks for burying analysis but were moved laterally on Days 2 and 3 of trials to record off-bottom feeding movements and threat responses better.

Behavior Analyses

We examined a series of Japanese flounder behaviors via video over the 3-d period (Table 5.1). For the first 24 h, we assessed burying ability as the degree of concealment (0, 25, 50, 75, 100% concealed) of individuals by viewing still video frames approximately every 30 min for the first 8 h (Day 1) of trials, and resuming burying
analysis an additional 5.5 h the following morning (Day 2). Then (24 h after trial initiation), each tank was provided with ten, 2-cm Japanese flounder as prey. We examined one “period” (45 min, 24 s) of video to assess feeding behavior once all prey were distributed to tanks ("feeding" treatment). Scored behaviors included those based on number of events, total durations, and swimming distances. The following day (Day 3), we analyzed one baseline “period” of behavior without introduced prey and without the model predator in view ("baseline" treatment), followed by one “period” with the model predator revealed (blind removed; "threat response" treatment). When the predator was in view, a schedule of air "on" and "off" was followed (Table 5.2) so that fish would not get habituated to the movement of the predator and to simulate the predator becoming satiated and less active over time. Then ten, 2-cm Japanese flounder again were introduced to tanks as prey, and one “period” of feeding in the presence of the model predator was assessed ("threat response with feeding" treatment). The duration of the “period" was the maximum amount of time that maintained congruency between all treatments (i.e., baseline, feeding, threat response, threat response with feeding). For behavior analyses when the model predator was in view, equal amounts of time with the model predator moving (air "on") and not moving ("air off") were analyzed (22 min, 42 s each).

Swimming course of off-bottom events was defined as per Furuta (1996) and Miyazaki et al. (2000). Course A was defined as a fish swimming off-bottom and then returning close (≤ 1 TL or less) to initial position, Course B as a fish returning in the same direction but > 1 TL from initial position, and Course C as fish settling in a different position and direction after the off-bottom event. The vertical movements of the
2-cm, juvenile prey flounder also were assessed by tallying the number of times prey crossed the mid-water column threshold (located 9 cm from the surface/sand bottom) within tanks.

At the end of trials, all experimental predator fish were euthanized with an overdose of MS222 and preserved in ethanol. Fish were weighed and measured, stomachs were dissected, and the number of prey consumed per fish was recorded.

**Data Analyses**

The overall experimental design consisted of four fish condition types (conditioned, non-conditioned, wild, NCRR fish) and four behavioral treatments (baseline, feeding, threat response, threat response with feeding). To assess burying ability, the mean degree of concealment for each tank was calculated and plotted over time. Regressions were fit for each fish condition type for two time frames: (1) the initial 8 h after tank introduction on Day 1, and (2) the 5.5 h on the day after tank introduction on Day 2. In addition, overall burying ability was assessed for Day 1 and Day 2 by 1-way ANOVA.

Feeding behaviors (i.e., number of attacks and off-bottom swimming events; attack distance; off-bottom height and return-to-bottom distance; movement and off-bottom duration) with or without the presence of the model predator were assessed by 2-way ANOVA (repeated measures by treatment when matching was effective and by traditional ANOVA when not), followed by Bonferroni post-tests. Total movement duration (sum of movements of all fish per tank over all treatments) and vertical prey
movement (number of times prey crossed the mid-water column threshold) were compared via Kruskal-Wallis test. The total number of off-bottom events for each swimming course (A, B, or C) was tallied for each treatment, and fish condition type and percentages of the total were calculated.

Stomach Contents Index (SCI) was calculated for each individual where SCI equals (weight of stomach contents x 100)/(body weight - weight of stomach contents).

**Results**

**General Behavioral Observations**

Active fish of all conditioned types displayed a variety of distinct behaviors including a “head-up” posture, “yawning” or throat clearing, and fin waving. When no prey were provided and no model predator was in view, conditioned fish spent the most time in motion, often exhibiting a “head-up” posture. Many times, fish maintained long durations in motion while in a very slow creep.

The 2-cm prey fish mainly behaved in one of two ways: either they remained relatively motionless on or buried in the sand on the bottoms of tanks until detected by the 10-cm predator fish, or they remained in the water column close to the surface of the tank securing themselves against the sides of the tank or swimming constantly. Remaining near the surface did not ensure safety as many predator fish swam to the surface to attack and feed on off-bottom prey. Most prey maintained their position at the
bottom or at the surface, but occasionally prey would move between the two locales, especially when startled by the movement of the predator fish.

**Burying, Feeding, And Threat Response**

Burying during Day 1 was significantly highest for wild, conditioned, and NCRR fish, with non-conditioned fish burying less but not significantly different from NCRR fish ($F = 8.15$, $P < 0.001$; Fig. 5.4a). Examining burying over time, NCRR fish were the only condition type to have a slope ($m$) significantly less than zero ($m = -0.06 \pm 0.01$ SEM; $F = 21.84$, $P < 0.0001$; Fig. 5.4c), although overall, differences between the slopes of all condition types were not quite significant ($F = 2.34$, $P = 0.07$). On Day 2, burying was significantly highest for wild fish, followed by conditioned fish, and then NCRR fish and non-conditioned fish, which were not significantly different from each other ($F = 95.27$, $P < 0.0001$; Fig. 5.4b). Wild fish were the only condition type to increase burying significantly over time ($m = 0.04 \pm 0.01$ SEM; $F = 8.51$, $P < 0.01$), although overall, differences between the slopes of all condition types were not significant ($F = 0.20$, $P = 0.90$).

Wild fish exhibited the highest number of attacks and successful attacks of all fish condition types (Fig. 5.5). NCRR and conditioned fish exhibited similar numbers of attacks, with NCRR fish attacking slightly more successfully. Non-conditioned fish performed the fewest number of attacks, although when the model predator was hidden, most of these attacks were successful. In the presence of the model predator, overall number of attacks and successful attacks was lower for all condition types than when the
predator was hidden. Since repeated measures matching was not effective (P > 0.05), traditional 2-way ANOVAs indicated both a significant treatment and condition effect in attack number between fish condition types (F = 6.32, P < 0.05), and a significant treatment effect in number of successful attacks (F = 5.76, P < 0.05). Post-tests did not elucidate where these differences were most pronounced.

Fish treatments had significant differences in off-bottom durations between treatments (repeated measures ANOVA; F = 3.49; P < 0.05), although post-tests did not elucidate where these differences were most pronounced. No statistical differences were detected between fish condition types (F = 1.33, P = 0.32) but trends were evident. Wild fish spent the less amount of time off-bottom than all other fish types (Fig. 5.6). When no prey were provided and no model predator was in view (i.e., baseline treatment), conditioned fish spent the most time off-bottom, followed closely by NCRR fish and then non-conditioned fish. After prey were provided to tanks, off-bottom duration of non-conditioned fish peaked and that of conditioned and non-conditioned fish decreased from baseline levels. All hatchery-reared fish (conditioned, non-conditioned, and NCRR) responded to the model predator similarly with respect to a lower time spent moving with and without the presence of prey, while wild fish maintained their minimal time off-bottom, especially in the absence of prey.

The overall swimming course profile among fish condition types was similar with > 50% of off-bottom events conducted via “A” course, approximately 10% “B” course, and approximately 30% “C” course (Table 5.3). Conditioned fish engaged in the highest number of off-bottom events, but the majority of these events occurred when no predator was in view and no prey were available (i.e., baseline). All fish decreased in number of
off-bottom events when the model predator was in view, although wild fish maintained the same (low) number of off-bottom events with or without the model predator when feeding was an option. Conditioned fish exhibited the least amount of off-bottom events when the model predator was in view and prey were provided. Repeated measures 2-way ANOVA did not indicate any significant differences between fish condition type ($F = 1.16, P = 0.37$) or treatment type ($F = 2.00, P = 0.13$) with respect to the number of off-bottom events.

In threat response trials, the duration fish spent moving while the model predator was moving (air "on") was significantly different between fish condition types ($F = 3.84, P < 0.05$; Fig. 5.7a), with post-tests indicating the most pronounced differences between wild and non-conditioned fish ($t = 3.00, P < 0.05$). Wild, NCRR, and conditioned fish moved less when the model predator was moving, while non-conditioned fish maintained the same level of activity regardless of the activity of the predator (although this trend was not significant). After prey were provided to threat response treatments, the number of off-bottom swimming events and attacks were significantly lower overall when the model predator was moving versus when it was not moving ($F = 5.51, P < 0.05$ for off-bottom events, $F = 7.80, P < 0.05$ for attacks, Figs. 5.7b, c).

Prey (2-cm fish) behavior differed within treatments; more prey vertical movements were observed in tanks of NCRR and non-conditioned fish (Fig. 5.8a). Although the trend was not significant among fish conditioned types, the prey movement variation detected within NCRR and non-conditioned fish treatments was higher than those within wild and conditioned fish treatments. Activity levels of prey fish mirrored
that of predator fish activity; NCRR and non-conditioned fish treatments exhibited the highest total movement durations (Fig. 5.8b).

Wild fish contained significantly more prey in their stomachs than any other fish condition type at the end of Day 3, after the threat response with feeding trials (Fig. 5.9). All hatchery-reared fish performed similarly, although variation was lowest for conditioned fish.

**Discussion**

**Burying**

Overall, wild fish buried most, followed by conditioned, NCRR, and non-conditioned fish. On Day 1, all fish were acclimating to the new tank environment, so gauging absolute performance may have been premature, however, differences in burying ability were already evident; wild fish buried significantly more than NCRR and non-conditioned fish. Conditioned fish performance fell in between: not significantly different from wild or NCRR fish, but higher than non-conditioned fish. This is in accordance with Walsh et al. (in press; Chapter 4) who showed that burying performance of 10-cm, cage-conditioned Japanese flounder was significantly higher than that of non-conditioned fish. Day 2 elucidated a clearer assessment of burying ability among fish condition types. Fairchild and Howell (2004) also demonstrated that conditioning improved the ability of winter flounder, *Pseudopleuronectes americanus*, to bury in sediments.
By examining burying performance regularly over a time continuum, we were able to assess not only whether fish were burying, but also the pattern of burying over time. Fish tended to bury less (and thus, remain exposed more) as the day progressed (mid-day to nighttime), although this relationship was only significant for NCRR fish. This pattern may be attributed to fish becoming more acclimated to the tank over time. However, the following day the opposite trend was observed: fish buried least in the hours immediately after lights "on" and progressively buried more as the day progressed (morning to mid-day), although this trend was only significant for wild fish. Non-conditioned fish tended to follow these patterns weaker than other fish condition types. This may be evidence of nocturnal or diel activity where fish remain buried more during the day, and less at night, as observed by Miyazaki et al. (1997). They suggested that daytime burying ability of Japanese flounder might help to elude diurnal visual predators and that any deficiency in burying ability could lead to increased predation risk.

Feeding

The period of high activity observed during baseline treatments was attributed to searching behavior, where fish maintained a “head-up” posture, also observed in active, congeneric summer flounder, *Paralichthys dentatus* (Olla et al., 1972), as well as winter flounder (Olla et al., 1969). Olla et al. (1972) associated “yawning” behavior in summer flounder with changes in fish activity. Crossing the mid-tank threshold by 2-cm prey flounder was interpreted as a higher level of prey activity. In addition, it suggested an
attempt to change the predator avoidance strategy from being cryptic on the bottom nearer to predators versus being evident at the surface farther from predators.

Of all fish condition types, non-conditioned fish spent the longest amount of time in motion. This high level of predator activity corresponded with higher levels of vertical prey movement suggesting that activity levels of the predator influence behavior of the prey. Juvenile European flounder, *Platichthys flesus*, have been shown to induce anti-predator behaviors in amphipod prey, resulting in decreased prey availability and lower gut fullness and growth rates for the flatfish (Grønkjær, 2011). Examining total movement duration (sum of movements of all fish per tank over all treatments) allowed the best evaluation of the overall activity of a fish condition type because this measure provided an estimate that could not be elucidated by simply considering the mean. A mean per tank, even when surrounded by an estimate of variation, will not reflect the true total activity level of that tank, considering that fish within each tank were not moving independently. The movements of an individual fish often instigated movements of the other fish in the tank, as well as the 2-cm prey (when present). This often resulted in an amplification of activity, whether fish were searching for food or actively feeding (with or without the model predator). This level of amplification would be lost if we simply considered the mean. Examining three fish per tank rather than one, however, better represents the true scenario of a stocking event. When released for stocking, fish are not independent, so evaluating activity independently is not a realistic estimation. It is important to consider the variation exhibited between tanks of a fish condition type, since it only may take a few fish within a population to instigate activity of conspecifics and surrounding prey. Enhanced activity at the release site also may attract predators to the
area (Sparrevohn and Støttrup, 2007). Wild and conditioned fish revealed much lower variation in total movement duration (with much lower maximums and upper quartile limits), which corresponded with lower levels and variation in prey vertical movement for those fish condition types.

Using live fish as prey was an important component of this study for adequately assessing the behavioral influence newly released flounder would have on the behavior of prey. The predominant prey items of Japanese flounder > 5-cm TL are small fishes, if sufficient numbers are available (Yamashita and Yamada, 1999). Juvenile 2-cm prey flounder were readily accessible from Obama Laboratory, who continually rear an abundance of juvenile flounder throughout the spring and summer months. Therefore, acquiring a large quantity of 2-cm fish of the same size and developmental state was easy and reliable. Alternatively, wild gobies could have been sampled, but assuring collection of enough for all experimental tanks while maintaining a congruent size for experimental standardization would not have been feasible.

Japanese flounder are highly cannibalistic. Cannibalism generally is observed when the TL size differential in lengths between fish exceeds 2 in rearing tanks or 3 in the wild (Tanaka et al., 1989; Yamashita and Yamada, 1999; Kellison et al., 2002). Thus, using Japanese flounder as both predator and prey for these experimental trials portrayed a realistic scenario. Size differentials of this magnitude often were witnessed during routine sampling for juvenile Japanese flounder in the Yura River Estuary, Japan (pers. obs).

All hatchery-reared fish spent more time off-bottom than wild fish, and this trend was most pronounced during baseline treatments when fish were not in the presence of
prey (thus, it appeared they were searching for prey) and did not perceive the threat of the model predator. Furuta (1998) also observed a higher off-bottom duration for hatchery-reared Japanese flounder when comparing swim-up time for feeding to wild fish. Once prey were provided, conditioned and NCRR fish drastically decreased off-bottom duration (and the number of off-bottom events exhibited by conditioned fish approximately halved), perhaps in an effort to become more cryptic and stealthy predators. In contrast, non-conditioned fish increased off-bottom duration once prey were provided.

Differences in off-bottom swimming course of 6-cm TL Japanese flounder have been observed. Furuta (1998) found that wild flounder were more likely to exhibit rapid feeding behavior and return to the bottom close to their initial position (deemed "Course A"), while hatchery-reared flounder tended to settle back on the bottom a distance away from their initial position (deemed "Course B", if fish returned facing initial direction, or "Course C", if fish faced a different direction). Unlike Furuta's (1998) study, no such pattern was observed in 10-cm Japanese flounder in the present study. All four condition types exhibited a similar breakdown of overall swimming course, in which Course A was performed over 50% of the time. Wild fish in Furuta's (1998) study exhibited Course A only 38% of the time, while in the present study 6-cm TL wild fish exhibited Course A 63% of the time. Differences may be explained in part by experimental design: Furuta (1998) examined one fish per tank, while the present study examined three. Since observational learning has been documented for Japanese flounder (Arai et al., 2007), it is possible that fish could have learned more optimal swimming courses by observing each other over the 3-d trial period. Hatchery-reared fish of this larger (10-cm TL) size may
have already experienced and observed ample piscivory through in-tank cannibalism to refine optimal swimming course for feeding, as returning to the bottom far from initial location not only puts fish at an increased predation risk, but also startles and stirs potential prey in the new location, and thus eliminates the element of predatory surprise.

Wild fish attacked and consumed prey most successfully both in the presence and absence of the model predator. Conditioned and NCRR fish, which both spent approximately one week in the presence of wild prey, had similar levels of attack success. Non-conditioned fish attacked least and least successfully. This is supported by Walsh et al. (in press; Chapter 4) who showed that feeding performance of 10-cm Japanese flounder on mysid prey was significantly highest for wild fish and lowest for non-conditioned fish, with conditioned fish performance falling in between and not significantly different from either.

The levels of prey consumption indicated by the number of successful attacks in the Day 3 threat response with feeding trials correspond to the final stomach content analyses at the end of trials. Stomach contents revealed slightly higher numbers of prey consumed, since a time period existed after prey provision but before videoed behavior was assessed (because all prey could not be distributed to all tanks simultaneously) in addition to the time period at the end of trials before all prey could be removed from tanks (because all experimental fish could not be removed from all tanks simultaneously). For both measures of prey consumption, wild fish successfully consumed more prey than all hatchery-reared fish.

**Threat Response**
Conditioned fish performed the fewest off-bottom events when prey were provided in the presence of the model predator. During conditioning, many gobies (Family Gobiidae), a common, small-fish prey of Japanese flounder, took refuge within the cage, even though it was filled with a high density of potential predators. Juvenile jack mackerel, *Trachurus symmetricus*, also took refuge within the water column of the cage. Thus, conditioned fish may have learned that in the presence of a lot of other fish activity (which would result after adding ten, 2-cm Japanese flounder to an experimental tank that already contained three, 10-cm flounder and a moving model predator), minimal movement may be the optimal strategy to ensure the stealthy capture of prey.

In the presence of the model predator, all hatchery-reared fish reduced the time they spent off-bottom to similar levels, with slightly more time spent off-bottom before prey were introduced. Fish of all condition types exhibited a lower number of attacks and off-bottom swimming events, and a lower movement duration when the model predator was in motion versus when it was still. Regardless of whether experimental fish perceived the model as a true predator, the reduced movement duration, off-bottom duration, and number of off-bottom events and attacks exhibited by fish while in view of the model predator suggests that the model predator was being perceived as a threat.

An advantage of using model predators in lieu of live predators for experimental trials is that it is then possible to replicate predator behavior among experimental units. In addition, live predators do not always actively participate once trials begin (Fairchild, 1998). A disadvantage of using model predators is that prey may not respond as they would to a real predator and/or only a small range of anti-predator behaviors may be
performed (Magurran and Girling, 1986). The model predator in the present study provided only visual and not olfactory cues, but represented a realistic size/shape/figure of a potential threat that fish would be expected to encounter in the wild, such as the longfin snake-eel, *Pisodonophis cancrivorus*. This is important since some fish, such as minnows, *Phoxinus phoxinus*, are able to discriminate between realistic and unrealistic predator models (Magurran & Girling, 1986).

Predator avoidance of hatchery-reared juveniles improves through the learning process (Hossain et al., 2002). Cage conditioning can provide hatchery-reared fish with “safe” predator exposure, wherein fish are able to see predators outside of cages and to detect olfactory predator cues before being released. Masuda et al. (2005) found that taurine enrichment of hatchery feeds also is efficient for improving the anti-predator performance of released fish, as fish fed enriched feeds swam farther from the release site and had a higher incidence of recapture than fish fed standard feeds.

**Implications for Stock Enhancement**

Recapture of sufficient abundances of hatchery-reared fishes to assess post-release performance is rare, especially recapture of a quantity suitable to standardize an experimental regime and of a release duration sufficient enough to make an assessment of the transition to a wild lifestyle. The present study is unique in that it assesses behavior and performance of similar-sized fish directly from tanks (i.e., non-conditioned), cage conditioned, and reared-than-released (i.e., NCRR) to a local wild population. In addition, the duration that conditioned fish were caged and hatchery-reared fish were
released before recapture was analogous (7 d versus 6 d, respectively), allowing a comparison of fish undergoing a simulated release (i.e., cage conditioned) and those actually undergoing one (i.e., NCRR) over a similar time period.

Overall, there was a trend for NCRR fish to perform worse than conditioned fish. This phenomena is supported by observations during routine release monitoring where non-conditioned fish recaptured at the release sight were captured more, lingered longer, dispersed slower, and ate less than their conditioned counterparts (Walsh et al., 2012; Chapter 4). This leads us to consider the possibility that recapture efforts near release sites may target weaker, nonfeeding, unmoving, individuals with possibly lower stocking success potential.

Walsh et al. (in press; Chapter 4) assessed recapture rates of cage conditioned Japanese flounder and found that numbers caught by local fishermen were significantly greater than those of non-conditioned fish (i.e., fish released directly from hatchery tanks to the wild). In addition, laboratory experiments conducted on fish removed from cages immediately prior to release revealed that conditioned fish exhibited enhanced burying and feeding performance compared to non-conditioned fish, which is in accordance with the present study.

**Conclusions**

Wild fish buried most, followed by conditioned, NCRR, and non-conditioned fish. Fish had a tendency to remain most exposed at the beginning and end of the day (i.e., around hours of darkness), and bury more mid-day (i.e., around hours of maximum light),
but this trend was weakest for non-conditioned fish. Wild fish attacked and consumed prey most successfully, followed by conditioned and NCRR fish, with non-conditioned fish attacking least and least successfully. Of all fish condition types, non-conditioned fish spent the most amount of time moving. Wild and conditioned fish revealed much lower variation in total movement duration, which corresponded with lower levels and variation in prey vertical movement. All hatchery-reared fish spent more time off-bottom than wild fish, and this trend was most pronounced during baseline treatments when fish were not in the presence of prey. In the presence of the model predator, all hatchery-reared fish reduced the time they spent off-bottom to similar levels. Fish of all condition types exhibited a lower number of attacks and off-bottom swimming events, and a lower movement duration when the model predator was in motion compared to when it was still. This study is the first to evaluate the behavior of hatchery-reared flatfish that have been cage-conditioned or released-and-recaptured. In addition, we provide evidence that cage conditioning of flounder can enhance the performance of released fish.
Table 5.1. Schedule of video trials and fish behaviors examined. One time period of video equalled 45 min, 24 s.

<table>
<thead>
<tr>
<th>Day of Trial</th>
<th>Examined Video/Tank</th>
<th>Treatment</th>
<th>Behavior Quantified/Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1 image every 30 min (first 8 hr after tank introduction)</td>
<td>burying (initial)</td>
<td>degree of concealment (0, 25, 50, 75, or 100%)</td>
</tr>
<tr>
<td>Day 2</td>
<td>1 image every 30 min (18-23.5 hr after tank introduction)</td>
<td>burying</td>
<td>same as &quot;burying (initial)&quot; above</td>
</tr>
<tr>
<td></td>
<td>1 time period of video after prey introduction</td>
<td>feeding</td>
<td># attacks (success/failure);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>attack distance (cm);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>movement duration (s);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>off-bottom swimming duration (s);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td># off-bottom swimming events;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>off-bottom swimming course (A, B, or C);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>off-bottom swimming height (cm);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>return distance after off-bottom event (cm)</td>
</tr>
<tr>
<td>Day 3</td>
<td>1 time period of video with no model predator and no prey</td>
<td>baseline</td>
<td>movement duration (s);</td>
</tr>
<tr>
<td></td>
<td>introduced</td>
<td></td>
<td>off-bottom swimming duration (s);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td># off-bottom swimming events;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>off-bottom swimming course (A, B, or C);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>off-bottom swimming height (cm);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>return distance after off-bottom event (cm)</td>
</tr>
<tr>
<td></td>
<td>1 time period of video with predator exposure</td>
<td>threat response</td>
<td>movement duration (model predator moving/still; s);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td># off-bottom swimming events (model predator moving/still);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>same as &quot;baseline&quot; above</td>
</tr>
<tr>
<td></td>
<td>1 time period of video with predator exposure after prey</td>
<td>threat response +</td>
<td># attacks (model predator moving/still);</td>
</tr>
<tr>
<td></td>
<td>introduction</td>
<td>feeding</td>
<td>same as &quot;feeding&quot; above;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>same as &quot;threat response&quot; above</td>
</tr>
</tbody>
</table>
Table 5.2. Model predator movement (air turned on/off) schedule.

<table>
<thead>
<tr>
<th>ACTUAL TIME (min)</th>
<th>TOTAL TIME (min)</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>remove predator partition (action aerator &quot;predator&quot; now visible to fish)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>no motion</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>motion</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>no motion</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>motion</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>no motion</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>motion</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>no motion</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>motion</td>
</tr>
<tr>
<td>5</td>
<td>43</td>
<td>no motion</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>motion</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>no motion</td>
</tr>
<tr>
<td>60-120</td>
<td></td>
<td>REPEAT FOR HOUR 2 DURING PREY INTRODUCTION</td>
</tr>
</tbody>
</table>
Table 5.3. Number of off-bottom swimming events and swimming course (A, B, or C) for fish in all treatments. NCRR = non-conditioned, released, and recaptured fish.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Swimming Course</th>
<th>Baseline Events</th>
<th>Baseline Events %</th>
<th>Feeding Events</th>
<th>Feeding Events %</th>
<th>Threat Response + Feeding Events</th>
<th>Threat Response + Feeding Events %</th>
<th>Condition Type Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild</td>
<td>A</td>
<td>4</td>
<td>80</td>
<td>5</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td>20</td>
<td>5</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>NCRR</td>
<td>A</td>
<td>26</td>
<td>52</td>
<td>24</td>
<td>51</td>
<td>16</td>
<td>73</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>20</td>
<td>40</td>
<td>17</td>
<td>36</td>
<td>6</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>47</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>141</td>
</tr>
<tr>
<td>Conditioned</td>
<td>A</td>
<td>60</td>
<td>54</td>
<td>44</td>
<td>73</td>
<td>24</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>16</td>
<td>14</td>
<td>3</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>36</td>
<td>32</td>
<td>13</td>
<td>22</td>
<td>15</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>112</td>
<td>60</td>
<td>49</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>240</td>
</tr>
<tr>
<td>Non-conditioned</td>
<td>A</td>
<td>33</td>
<td>66</td>
<td>50</td>
<td>68</td>
<td>3</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8</td>
<td>16</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9</td>
<td>18</td>
<td>19</td>
<td>26</td>
<td>10</td>
<td>77</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>50</td>
<td>74</td>
<td>13</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>154</td>
</tr>
<tr>
<td>Treatment Total</td>
<td></td>
<td>217</td>
<td>191</td>
<td>85</td>
<td>68</td>
<td>561</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1. Cage protocols at Maizuru Fisheries Research Station (MFRS) were smaller-scale representations based on Obama Station protocols. Like those of Obama Station, cages in the present study (a) consisted of a metal piped frame supporting a soft mesh enclosure on all sides. Cage density (b) matched that of Obama Station's 2010 Japanese flounder release in Obama Bay, Fukui, Japan.
Figure 5.2. Experimental design of video trials.
Figure 5.3. Individual experimental tank configuration. The model predator (a) was positioned in the back corner of tanks and concealed with a polyvinyl chloride (PVC) blind (b) until Day 3 of trials. Tanks were sand-bottomed and covered on three sides with light blue plastic sheeting. Cameras were positioned above tanks to document fish burying ability, but were moved laterally to record feeding and threat response behaviors.
Figure 5.4. Burying ability for fish of different condition types. For overall burying ability on (a) Day 1 and (b) Day 2, letters above bars indicate significant differences between condition types. (c) For the degree of burying over time, each marker represents a condition type mean at a different point in time. Error bars and regression lines have been removed to simplify the visual. ** and **** represent significant differences from zero at P < 0.01 and 0.0001, respectively, in the slope of burying over time. NCRR = non-conditioned, released, and recaptured fish.
Figure 5.5. Total number of attacks and successful attacks in the presence and absence of the model predator. * indicates a significant difference in attack number of all condition types overall while in the presence and absence of the model predator at P < 0.05. There was a significant difference in attack number between fish condition types (P < 0.05), although post-tests did not elucidate where those differences could be attributed. There were no significant differences in successful number of attacks between fish condition types. Error bars represent SEM. NCRR = non-conditioned, released, and recaptured fish.
Figure 5.6. Total off-bottom duration for all treatments and fish condition types. * indicates a significant difference in off-bottom duration of all condition types overall between treatments at P < 0.05. There were no significant differences between fish condition types. Error bars represent SEM. NCRR = non-conditioned, released, and recaptured fish.
Figure 5.7. The influence of model predator movement on fish activity: (a) duration of fish movement, and the (b) number of off-bottom events and (c) number of attacks after prey introduction. Means represent that of all individuals moving, swimming off-bottom, or attacking during the respective treatment. Error bars represent SEM. NCRR = non-conditioned, released, and recaptured fish.
Figure 5.8. Movement of predators and prey: (a) number of times prey fish vertically crossed the mid-water column threshold within tanks, and (b) total movement duration of predator fish for all treatments combined. Error bars represent SEM. NCRR = non-conditioned, released, and recaptured fish.
Figure 5.9. Stomach Contents Index (SCI) of fish at the end of Day 3, after the threat response + feeding trials. *** indicates a highly significant difference between fish condition types. Error bars represent SEM. NCRR = non-conditioned, released, and recaptured fish.
SYNTHESIS AND FUTURE DIRECTIONS

All reared fish are not equal. The end products of fish reared for aquaculture and those reared for stock enhancement vary greatly. For aquaculture, the ideal end product is a large-sized individual that was grown most economically (in both a financial and temporal sense) with preferably a pleasant flavor. For stock enhancement, the ideal end product is an individual that can survive in the wild until recruitment (e.g., to the fishery; to the spawning stock). Conditioning fish for stock enhancement can increase survival and recapture rates of released fish.

In the last 20 yr, we have seen great advancements in formulated feed production, including the design of feeds that take longer to break down in water or contain more non-fishmeal based ingredients, as well as feeds accepted by younger and smaller stages of fish. These are great feats for aquaculture. However, how do these advances in formulated feeds affect fish reared for stock enhancement - other than increasing the temporal gap between the last time reared fish saw live prey in the hatchery (probably in the larval or early juvenile stages) and encountering them as released fish in the wild? The degree to which fish reared for stock enhancement need live prey training before release will depend on the type of fish reared and its particular ecological niche and associated behaviors. As discussed in Chapter 1, cannibalistic fish generally have the opportunity to experience "live feeds" before release regardless of exclusively being fed formulated feeds in the hatchery.
In the hatchery, worm-reared fish grew the most with over 90% survival. If we are to define "success" of a conditioning strategy as enhancing performance, worm-reared fish were most successful (Chapter 2). In caging trials, worm-reared and wild fish exhibited the most similar survival, baseline RNA/DNA values, overall stomach fullness, and diet composition profiles over time (Chapter 3). This is not surprising if we consider that (polychaete) worms are a predominant prey item of wild juvenile winter flounder. Thus, a diet of worms would provide reared fish with ample training for life in the wild. If we are to define "success" of a conditioning strategy as yielding a behavioral repertoire by conditioned fish that more closely matches that of wild fish, worm-reared fish were most successful (Chapter 3).

In light of the small numbers of pellet-reared fish examined in Chapter 3 (due to low survival from experiments of Chapters 2 and 3), a logical next step of this research would be to assess the extent to which juvenile winter flounder reared on formulated pellet feeds in the hatchery successfully transition to wild diets once released. To this end, we have already conducted some experimental caging to determine the onset of feeding, stomach fullness and diet composition of released pellet-reared fish. Preliminary results indicate that after 18 hr most fish had food in their stomachs, stomach fullness increased with time, diet was composed mainly of polychaetes and crustaceans (copepods and amphipods), and inorganics, although common in the first few hours post release, were minimal later.

Cage-conditioned fish exhibited better overall performance than non-conditioned fish (Chapters 4 and 5). Immediate benefits of cage conditioning included a higher percentage of cage-conditioned fish burying immediately upon release compared to non-
conditioned fish, and conditioned fish identified and consumed natural prey more than non-conditioned fish. If we are to define "success" of a conditioning strategy as yielding a behavioral repertoire by conditioned fish that more closely matches that of wild fish, cage-conditioned fish were most successful (Chapters 4 and 5). Long-term benefits of cage conditioning were evident; non-conditioned fish lingered near the release site while conditioned fish dispersed soon after the conditioning cage was dismantled. Significantly more conditioned fish were recaptured via fishermen's efforts. If we are to define "success" of a conditioning strategy as increasing the number of released fish landed at market, cage-conditioned fish were most successful (Chapter 4).

Japan has been releasing flounder as a fisheries management strategy for over 30 years; therefore, there is great potential for international scientists and managers to regard these large-scale Japanese efforts as case studies from which to model and base their own developing flatfish stocking protocols. Comparative work on congeneric species for stocking may allow for more direct application of Japanese strategies to other regions of the world. We have already conducted videoed behavior trials similar to those of Chapter 5 for 4- and 7-cm Japanese flounder, 4-cm marbled flounder, 4- and 7-cm winter flounder, and are being planned for 4- and 7-cm Southern flounder. Analyses are ongoing. In this way, the influence of cage-conditioning on behavior and performance can be compared between species regionally as well as ontogenically. These comparisons then can be used to identify which species, and when during its life history, the strategy is best applied.
APPENDIX A

POTENTIAL OF WHITE WORMS ENCHYTRAeus ALBIDUS AS A COMPONENT FOR AQUACULTURE AND STOCK ENHANCEMENT FEEDS

Introduction

Marine invertebrates can provide a valuable substitute-supplementary nutritional source that may decrease the demand for fishmeal ingredients in the production of aquaculture feeds. Large-scale production of live aquatic worms, as well as other invertebrates, also may be useful for marine/estuarine stock enhancement and sea ranching efforts, as well as for aquarium, terrarium, laboratory, and personal maintenance of fishes, amphibians, reptiles, birds, and larger invertebrates. Ideally, the mass cultured invertebrate would be fast-growing, precocious, fecund, easy to rear, and able to thrive at high densities (Ivelva 1973). White worms (Enchytraeus albidus) are 2- to 4-cm long, globally distributed, intertidal oligochaetes that feed on decaying organic matter. They are found on a wide variety of moist terrestrial soils, as well as in fresh and brackish waters, in the marine littoral zone, and on aquatic plants washed ashore. They even have been found in large densities within the gravel filters of irrigated fields as well as in urban water pipes (Ivelva 1973). Their nonfastidious culture may enable the production of a natural, sustainable feed for marine aquaculture.
Experimental work on the mass culture of invertebrates for production purposes began in Russia at the end of the nineteenth century (Ivleva, 1973). White (or pot) worm cultivation was developed in the 1940s, as a result of expanding fish culture programs in the USSR. Studies on the biology, nutrition, and cultivation of white worms are reported in a number of Russian publications, but few have been translated for English-speaking audiences (Vedrasco et al., 2002). The reviews that do exist describe rooms of wall-to-wall racks stacked with wooden boxes (50 x 40 x 12 cm) of worms (100 to 300 g/box). Many fish breeding plants maintained over 1,000 wooden boxes and produced from 500 kg to several tons of white worms per season for feeding 2.5 to 3 million juvenile sturgeon (Ivelva 1973; Memiş et al., 2004). Peak production biomasses have been recorded at 2–3 kg/m² with mean production at 1.2 kg/m² (Ivleva, 1973). Over the course of its lifetime (L₅₀ = 200 d), an individual white worm can produce approximately 1,000 highly viable eggs, of which 93-95% will successfully develop. The species has a high survival rate at all growth and developmental stages. Although some aquarium hobbyist and research organism suppliers produce white worms on a small-scale for use as feed by at-home aquarists as well as for biological and toxicological studies, currently no targeted, large-scale production appears to be ongoing. The reasons for this are unclear, but the cease of production appears to correspond with the breakup of the USSR and perhaps the increased availability of formulated feeds to that area of the world.

White worms are composters and feed on decaying plant- and animal-based
organic matter under natural conditions. Thus, these worms can be cultured on a wide variety of feeds otherwise considered "wastes", including the byproducts of breweries, bakeries, and other food industries, as well as the proteolyzed yeasts prepared in paper and pulp plants (Ivelva 1973). This dietary flexibility provides the culturist with the potential to develop interesting local partnerships, collaboration, and publicity. The use of "wastes" as worm fodder keeps materials that would normally end up in a landfill within the sustainable production system. In addition, these "wastes" provide the culturist with an inexpensive and easily obtained food supply for worm production.

Instructions for the small-scale production of white worms to feed ornamental fish, such as discus, bettas, angelfish, as well as killifish, can be found widely on the internet. However, mass culture is successful only in exceptional cases, with the difficulty lying in the transition from a satisfactory, modest production to an efficient, large-scale operation expected to produce hundreds and thousands of kilograms (Ivleva, 1973). For small-scale production at the University of New Hampshire, worms were reared in clear plastic shoeboxes (34.5 x 20.25 x 12.75 cm) filled 5–7 cm high with damp organic potting soil. Maintenance (feeding and moistening of the soils) of all cultures (up to N = 25) took less than one hour, once per week. Feed (e.g., stale formulated fish pellets, baby cereal flakes, hot dog rolls, a mixture of moistened dry dog food and oil) was distributed against the bottom of containers and covered with soil to minimize infestation by mites or small flying insects. Feed levels could be monitored simply by viewing the underside of the clear container and replenishing feed when necessary. Worms were easily harvested by placing the rearing container on a heat source, in this case, a heating pad (Fig. A.1). A small mound of rearing soil was constructed adjacent to
the side of the container, and over the course of 1–2 hours, the thermophobic white worms migrated to the surface (and even up the sides of the plastic container) where they were easily, and cleanly, harvested.

**As a Feed Component**

White worms consist of high protein (75%) and lipid (15%) content with relatively low levels of mineral compounds (6%; Chapter 2). Fatty acid analyses revealed white worms as a substantial n-3 LC-PUFA source, though they may be limiting in terms of DHA (Fig. A.2). The worms provide a balanced supply of amino acids including tyrosine, tryptophan, arginine, histidine, cystine, and methionine, as well as calcium, phosphorus, iron, carotene, and vitamins A and B2 (Malikova, 1956, in Ivelva 1973). They are readily accepted by juvenile sturgeon (Ivelva 1973) as well as winter flounder (Klein-MacPhee 1978; Chapter 2), indicating that as a feed, white worms provide adequate palatability as well as amino acid balance, energy, and digestibility - all requirements of an appropriate protein source for aquaculture feeds.

The use of marine worms as a protein source in formulated fish feeds is not a new concept. Dragonfeeds, a derivative of UK-based Blue Marine Feeds Limited (Kent, UK), combines the cultured polychaete, *Nereis virens*, with vegetable proteins to produce a fishmeal-less feed with the full amino acid profile of fishmeal. With a 70% protein and 2% lipid composition, the Dragonfeeds product is marketed for both finfish and shrimp aquaculture. Alternatively, Aquathrive, manufactured by Reed Mariculture/Reef Nutrition (Campbell, CA, USA), combines fishmeal and oil with Terebellid polychaetes
to produce a 46% protein, 11% lipid feed that is marketed mainly to aquarium hobbyists. White worms provide distinct advantages to these polychaete-based feeds: (1) the small size of white worms enables the option for feeding directly (live) to small and/or juvenile fishes, and (2) white worms can be reared in damp soils/substrates eliminating the need for a water-based culture system. Terrestrial, air-breathing organisms can congregate in much higher densities than those of aquatic animals (Ivelva 1973). Preliminary work has been conducted to minimize even the amount of soil necessary for production by growing white worms on agar plates (Springett 1964).

The benefits of using white worms as a live feed include their tolerance to a wide range of temperatures and salinities (Ivelva 1973). Although optimal temperature for growth and reproduction peaks at 15–21°C, white worms will survive in freezing temperatures as well as persist for over 30 min at 33°C if necessary for introduction as live feed. Salinity tolerance spans the spectrum of most natural waters, and the worms will continue moving (i.e., eliciting a behavioral feeding response from predators) in fresh, brackish, estuarine, as well as full salinity seawater. The nature of white worms as oligochaetes allows for the severing of individuals into smaller pieces if needed, and these pieces themselves will continue to move when fully submerged. Providing white worms as a live feed can improve rearing water quality, since any excess feed remains alive (and thus does not breakdown) in tanks, remaining available (and moving) until the target aquacultured species becomes hungry again.

The potential economic benefits of white worm production for commercial aquaculture might include incorporation into formulated diets or development of alternative organic diets for carnivorous marine fishes. The mass culture of invertebrate
live feeds as an advanced diet for estuarine/marine stock enhancement for species such as salmonids, flatfish, or shad may increase stocking effectiveness, survival, and recruitment of released fish, translating to higher landings for fishermen and an economic boost for fishing communities. The development of systems to grow white worms for freshwater, brackish, or marine baitfish and/or fee fish operations is worth investigation as sourcing nutritionally balanced diets for small-scale operations is a serious hurdle for producers. Identification of diets that are reared and harvested easily, thrive with minimal maintenance, and survive in salt/brackish water for prolonged periods also may decrease overall feed costs by reducing feed waste and water quality maintenance.

To summarize, white worms are an interesting candidate for aquaculture feeds because of their (1) rearing and harvesting ease (i.e., they thrive on neglect), (2) nonselective, composting feeding nature (i.e., it is cheap to feed them), (3) ability to survive in a wide range of temperatures (0–33°C) and salinities (0–35 ppt; even when they are cut into pieces), and (4) high nutritional content (75% protein, 15% lipid). White worms present the potential for mass scale production involving interesting, local collaboration with an inexpensive materials cost. In addition, production of white worms as an aquaculture feed or feed ingredient may enable a reduced reliance on fishmeal and the opportunity to culture marine species on a natural, sustainable feed.
Figure A.1. Small-scale white worm harvest procedures at the University of New Hampshire. Thermophobic worms progressively move to the surface and even up the sides of the container (a-d) after being placed on a heat source, in this case, a heating pad (e). Worms are then easily and cleanly collected from the surface (f).
Figure A.2. Percent fatty acid methyl ester (FAME) composition of white worms. Analyses conducted for M. Walsh by J. Trushenski of Southern Illinois University at Carbondale.
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23-Jun-2008

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Zoology, Spaulding Life Sciences Ctr
Durham, NH 03824

IACUC #: 080407
Project: Spawning movement and habitat use of winter flounder in the southern Gulf of Maine
Category: C
Approval Date: 16-May-2008

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol
submitted for this study under Category C on Page 5 of the Application for Review of Vertebrate
Animal Use in Research or Instruction - the research potentially involves minor short-term pain,
discomfort or distress which will be treated with appropriate anesthetics/analgesics or other
assessments.

Approval is granted for a period of three years from the approval date above. Continued
approval throughout the three year period is contingent upon completion of annual reports on
the use of animals. At the end of the three year approval period you may submit a new
application and request for extension to continue this project. Requests for extension must be
filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the
   UNH Occupational Health Program for persons handling animals. Participation is mandatory
   for all principal investigators and their affiliated personnel, employees of the University and
   students alike. A Medical History Questionnaire accompanies this approval; please copy and
distribute to all listed project staff who have not completed this form already. Completed
questionnaires should be sent to Dr. Glad Porschke, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at
862-2003.

For the IACUC,

Jessica Bolker, Ph.D.
Chair

cc: File
University of New Hampshire

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11-Dec-2009

Fairchild, Elizabeth A
Zoology, Spaulding Life Sciences Ctr
Durham, 03824

IACUC #: 091101
Project: Evaluating the Benefits of Cage-Conditioning for Flatfish Stock Enhancement
Category: D
Approval Date: 20-Nov-2009

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category D on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - Animal use activities that involve accompanying pain or distress to the animals for which appropriate anesthetic, analgesic, tranquilizing drugs or other methods for relieving pain or distress are used.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Notes:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladis Porsche, UNH Health Services.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.,
Chair

cc: File
Welsh, Michelle
LIST OF REFERENCES


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