Evaluating Sampling-Induced Stress in Atlantic Cod (Gadus morhua)

Travis S. Ford

University of New Hampshire, Durham

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Evaluating Sampling-Induced Stress in Atlantic Cod (Gadus morhua)

Abstract
Atlantic cod, Gadus morhua, have historically been regarded as one of the most commercially and recreationally important fishes in the Gulf of Maine. All fish involved in fishing and aquaculture are subject to stress, indicated by elevated plasma cortisol concentrations. Given the amount of research conducted on Atlantic cod, stress, and cortisol, little has been done to compare different blood sampling methods and the effects they might have on plasma cortisol concentrations, particularly in adult fish. This study examined three different methods of repeated blood sampling (standard handling caudal vessel puncture, reduced handling caudal vessel puncture, and cannulation) in Atlantic cod and determined if sampling method affected plasma cortisol concentrations. Plasma cortisol concentrations differed significantly between the dates on which the samples were taken, and between the methods of sampling. Vessel puncture blood samples taken using a reduced handling method usually produced lower plasma cortisol concentrations than those obtained via standard methods of handling. Repeated caudal vessel puncture samples, taken 72 hrs apart, did not always produce plasma cortisol concentrations similar to those of the original "pre-stressed" sample. Furthermore, repeated sampling via cannulation was able to generate plasma cortisol concentrations similar to baseline concentrations 48 hours following surgery. These results highlight the importance of reducing sampling induced stress when taking blood samples for plasma cortisol analysis.

Keywords
Agriculture, Fisheries and Aquaculture
Evaluating Sampling-Induced Stress in Atlantic Cod (*Gadus morhua*)

BY

Travis S. Ford

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Thesis Director, W. Huntting Howell, Professor of Zoology, University of New Hampshire

Paul C. Tsang, Professor Animal Sciences and Marine Sciences, University of New Hampshire

James A. Sulikowski, Associate Professor, Department of Marine Sciences, University of New England

Date

12/2/12
Dedication

This thesis is dedicated to anyone who has ever slipped on the ice while carrying a fish.
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ABSTRACT

Atlantic cod, *Gadus morhua*, have historically been regarded as one of the most commercially and recreationally important fishes in the Gulf of Maine. All fish involved in fishing and aquaculture are subject to stress, indicated by elevated plasma cortisol concentrations. Given the amount of research conducted on Atlantic cod, stress, and cortisol, little has been done to compare different blood sampling methods and the effects they might have on plasma cortisol concentrations, particularly in adult fish. This study examined three different methods of repeated blood sampling (standard handling caudal vessel puncture, reduced handling caudal vessel puncture, and cannulation) in Atlantic cod and determined if sampling method affected plasma cortisol concentrations. Plasma cortisol concentrations differed significantly between the dates on which the samples were taken, and between the methods of sampling. Vessel puncture blood samples taken using a reduced handling method usually produced lower plasma cortisol concentrations than those obtained via standard methods of handling. Repeated caudal vessel puncture samples, taken 72 hrs apart, did not always produce plasma cortisol concentrations similar to those of the original "pre-stressed" sample. Furthermore, repeated sampling via cannulation was able to generate plasma cortisol concentrations similar to baseline concentrations 48 hours following surgery. These results highlight the importance of reducing sampling induced stress when taking blood samples for plasma cortisol analysis.
Chapter 1

INTRODUCTION

Cod importance – fisheries and aquaculture

Atlantic cod, Gadus morhua, have historically been regarded as one of the most commercially and recreationally important fishes in the Gulf of Maine (GOM) (Kurlansky 1997). Prior to the twentieth century, GOM cod were fished using sail-powered vessels, but in the early 1900’s steam powered vessels began to rule the fleet, followed by diesel power in the 1920’s and beyond (Kurlansky 1997). In 1892, the first otter trawl was constructed in Scotland, and the technology was brought to New England (Kurlansky 1997). Otter trawls are nets that are deployed from the side or stern of a vessel and dragged across the bottom of the ocean. The net is held open by wooden or steel doors that help herd fish into the net. Otter trawls allowed fishermen to pursue and herd cod over a larger area of ocean floor than the beam trawls previously used in the fishery (Kurlansky 1997). By the 1950’s, due to these and other technological advances, refrigeration, and a demand for fresh fish, commercial catches of cod were increasing around the world (Kurlansky 1997).

Commercial landings of Atlantic cod in the GOM peaked in 1991 at 17,781 mt (Mayo and O’Brien 2006), but this would represent the end of the boom in the cod fishery. By 1999, the cod fishery in the GOM had declined to just 1,636 mt (Mayo and
O'Brien 2006). This decline, caused by overfishing (Myers et al. 1996), led the National Marine Fisheries Service (NMFS) to change the management of the GOM cod to a limited access fishery in 1994. GOM cod was managed by limiting the days-at-sea for each vessel, and introducing a daily possession limit.

On May 1, 2010, NMFS changed the management structure of the GOM cod fishery through the use of "sectors". The New England Fishery Management Council has defined a sector as "a group of persons holding limited access vessel permits under the fishery management plan through which the sector is being formed, who have voluntarily entered into a contract. Sector members agree to certain fishing restrictions for a specified period of time, and are allocated a portion of the Sector’s total allowable catch (TAC)." Amendment 16 to the Fisheries Management Plan created the sectors, and introduced "annual" catch limits (ACL’s) for the all Northeast Multispecies stocks, including GOM cod, thus doing away with the daily possession limit for the majority of the fishery.

Each sector is allowed to manage its collective portion of the ACL for all the permits (fishers) that are members of the sector. For example, if 10 vessels start a sector, and those vessels collectively have been allocated 10% of the commercial ACL for GOM cod, that sector is allowed to land 10% of the commercial ACL without restrictions on possession or effort. If a vessel chooses not to fish in a sector it must fish in the common pool, which manages its portion of the ACL for GOM cod through restrictions on effort (limits on days-at-sea and daily possession). If these ACL’s are exceeded (by a sector or the common pool), some accountability measures (AM’S) are taken, usually in the form of a reduced ACL for the following fishing year or differential days-at-sea counting.
While each of these management strategies manage the fishery in a different way, both result in the discarding of fish. Vessels in sectors and the common pool can only land cod $\geq 22$ inches (56 cm) total length (TL), thus undersized fish are discarded. In addition, vessels in the common pool must discard any fish caught that exceed the daily possession limit. The 3rd Groundfish Assessment Review Meeting (GARM III) from Northeast Fisheries Science Center (NEFSC), Woods Hole, Massachusetts, estimated that 515.7 mt of commercially caught GOM cod were discarded during fishing year 2007 with a discard/kept ratio of 0.129 (NEFSC 2008).

The Atlantic cod stock in the GOM has also been subject to recreational fishing pressure. From 1986-95 an average of 1.6 mt was taken annually, but as much as 2.8 mt have been taken in more recent years (Mayo and O'Brien 2006). Currently, the recreational fishery for cod in the GOM is managed by a ten fish bag limit, a 24-inch (61 cm) TL minimum size, and a seasonal closure from November 1 to April 15. Amendment 16 has created an ACL for recreational GOM cod for fishing year 2010. These size restrictions and bag limits can cause recreationally caught cod to be discarded as well.

In addition to the wild harvest by commercial and recreational fishermen, cod are also being raised in commercial aquaculture operations. The Food and Agriculture Organization of the United Nations (FAO) reports that aquaculture of Atlantic cod first began in 1987 producing 205 mt of cod. In 2007, 12,172 mt of Atlantic cod were raised in the North Atlantic in Norway, Iceland, Canada, the United Kingdom, and the United States.
**Inducers of stress:**

Cod interacting with fishing gear and raised in aquaculture cages all experience stress. For the purpose of this study stress is defined as the response of the cell, or organism, to any demand (stressor) placed on it such that it causes an extension of a physiological state beyond the normal resting state (baseline) (Barton 1997).

A. **Stress Caused by Interaction with Fishing Gear:**

1. **Trawls**

   As fish are herded into an otter trawl the intense swimming causes stress (Herbert and Steffensen 2005). Once the fish has fatigued, and is overcome by the net, it is likely that still other stressors begin to operate. Sablefish caught in a trawl net and dragged across the ocean floor have shown increased stress (Davis et al. 2000), and this is likely true for cod as well. Although low levels of mortality have been seen in undersized Baltic cod that escape from the codend of the net (Suuronen et al. 2005), it is far more dangerous inside the net. As more and more fish are caught, fish inside the net are compacted more tightly, leading to possible crushing and lacerations from the gear (twine, ropes, chain), the spines and teeth of other species, and other debris collected by the net (e.g. rocks and boulders). Due to theses stressors, the duration of the tow can cause stress levels and bycatch mortality to increase (Davis et al. 2001). Further, the rapid ascent from the ocean floor as the net is hauled back can cause rapid decompression and swim bladder rupture (Nichol and Chilton 2006). Once the fish reach the deck they are subject to various air exposure times and extreme temperatures that can increase stress and mortality (Davis et al. 2001). Discarded fish can be lethargic and are subject to predation by sea birds and other predators as they try to descend (Milliken et al. 1999).
Stress caused by interactions with a trawl can also induce sublethal effects. Morgan et al. (1999) found that adult Atlantic cod, experimentally stressed in a manner similar to that of trawl avoidance, performed fewer and altered courtship sequences, and produced more abnormal larvae.

2. Hooks

Atlantic cod that are captured through hooking operations also encounter numerous stressors (Milliken et al. 1999; Milliken et al. 2009). Cod caught on longlines are often lethargic on the surface following discard. Milliken et al. (1999) found that 23% of discarded fish were preyed upon by seabirds and 27% could not descend below the surface, likely due to barotrauma caused by the rapid ascent of the hooked fish to the surface. Further, Milliken et al. (2009) found that depth of capture and sea surface temperature had an effect on survival of Atlantic cod, as did the method of de-hooking the fish.

B. Issue of discarding:

Cod that are either undersized or caught after a daily quota has been met, must be discarded back into the ocean. For example, in the GOM cod fishery, the minimum legal size is currently 22 inches (~56 cm) TL, and the daily quota for common pool vessels at the beginning of fishing year 2010 was 800 lb (~363 kg). If a common pool fisherman catches 1200 lb (546 kg) of cod on his first tow, he is required to discard 400 lb (182 kg). Furthermore, since it is a multispecies fishery, the fisherman may continue fishing for other species for which the daily quota has not been met, and all cod caught in subsequent tows must be discarded.
The fate of discarded fish depends on a number of stress inducing factors, including physical damage (Davis and Ryer 2003), duration of air exposure (Davis et al. 2001), ascent rates (Nichol and Chilton 2006), hook time (Robinson and Carr 1993; Morgan and Carlson 2009), temperature, light level, and sea conditions (Davis and Ryer 2003). It follows that measuring stress can provide insights into ways to modify the fishing activities so as to minimize stress and increase the survival rate of discarded fish. Stress measurements can also be helpful in determining the physiological responses induced by interactions with fishing gear, the amount of time it takes to recover from those interactions, and give insight to the effects that any stressor may have on behavior, growth, reproduction, and the immune system (Morgan et al. 1999; Davis et al. 2001).

The goal of bycatch mortality studies is to determine the likelihood that discarded fishes caught under normal fishing procedures will survive and fully recover from their interactions with fishing gear. Most of these studies include the use of cages that fish are placed in following capture. These cages are deployed back into the ocean and are recovered ~72 hours later. The conditions of the “discarded” fish are recorded (Milliken et al. 1999; Milliken et al. 2009). While survival of Atlantic cod in the Northwest Atlantic longline fishery has been calculated using this method, plasma cortisol concentrations after capture and ~72 hours after discarding have not been reported (Milliken et al. 1999; Milliken et al. 2009). Although these studies documented barotrauma in discarded Atlantic cod, they could not truly assess its effects on mortality because fish were recompressed manually as they descended in the cages. In addition, these studies did not evaluate any physiological blood parameters for stress before or after the 72 hour recovery period. Furthermore, hook gear only accounts for 10% of
GOM cod catch, with the remaining 90% split between otter trawl and gillnet gear (GARM III 2008). To date, no bycatch mortality study has been conducted on Atlantic cod in the Northwest Atlantic otter trawl or gillnet fishery.

C. Stress induced by aquaculture operations

Cultured fish are often subjected to numerous stressors during husbandry that can lead to reduced growth performance and leave them susceptible to disease and other health risks (Robertson et al. 1988; Pickering 1993). Examples of acute stressors associated with aquaculture procedures include transportation (Sulikowski and Howell 2003; Robertson et al. 1988; Barton and Iwama 1991), grading (Barton and Iwama 1991), and netting/handling (Sulikowski and Howell 2003; Barton and Iwama 1991; King and Berlinsky 2006; King et al. 2006). Other chronic stressors such as crowding and poor water quality can also lead to elevated plasma cortisol concentrations (Barton and Iwama 1991).

Cortisol

There are two types of responses to stress in bony fishes; adaptive and maladaptive. Adaptive responses allow the animal to deal with a stressor and maintain homeostasis, while maladaptive responses compromise the animal’s performance (i.e. reproduction, immune-system, growth) (Iwama et al. 2006). Furthermore, there are three levels of stress response in bony fishes, primary, secondary, and tertiary (Iwama et al. 2006). The primary response begins with the fish's perception of an altered state (Iwama et al. 2006). The recognition of this altered state is followed by the release of stress hormones, initially catecholamines (like epinephrine) and shortly followed by cortisol.
Catecholamines are released from chromaffin tissue at the head of the kidney when stimulated by sympathetic fibers, while cortisol is released as part of the hypothalamic-pituitary-interrenal axis (Wendelaar Bonga 1997; Iwama et al. 2006). The secondary level of stress response begins when these stress hormones begin to mobilize energy substrates in order to cope with the stress (Wendelaar Bonga 1997; Iwama et al. 2006). This is often recognized by an increase in plasma glucose, which is used to meet the energy demands of the stressed animal (Wendelaar Bonga 1997; Iwama et al. 2006). Finally, the tertiary response refers to the whole fish or even fish population level. At this level, stress can inhibit growth, reproduction, recruitment, and productivity (Iwama et al. 2006).

Fishes have both behavioral and physiological responses to stressors (Iwama et al. 2006). The behavioral response occurs immediately, and is used to lessen the effects of the stressor, as seen in avoidance (Schreck et al. 1997). However, a fish’s behavior can take minutes to weeks to return to normal following certain stressors, depending on severity (Iwama et al. 2006). Fishes also respond physiologically to stress. The primary physiological response involves the release of the hormone cortisol from interrenal tissue at the head of the kidney, which is part of the hypothalmo-pituitary interrenal (HPI) axis (Figure 1) (Wendelaar Bonga 1997; Iwama et al. 2006). Cortisol is a corticosteroid found in blood plasma that is commonly used as a way to assess stress levels in teleost-fishes (Wendelaar Bonga 1997; Morgan et al. 1998; Davis et al. 2001; Lo et al 2003; Sulikowski and Howell 2003; Herbert and Steffensen 2005; King and Berlinsky 2006; Iwama et al. 2006; Perez-Casanova et al. 2008). Atlantic cod develop this corticosteroid response early in their life history; 8 days post-hatch (King and Berlinsky 2006).
During times of stress, the hormones arginine vasotocin and isotocin (AVT/IT), neuropeptide Y (NPY), corticotrophin-releasing hormone (CRH), melanophore-concentrating hormone (MCH), thyrotropin-releasing hormone (TRH) and dopamine (DA) are all released from the hypothalamus and act on the pituitary gland (Wendelaar Bonga 1997). AVT/IT, NPY, and CRH stimulate the pituitary gland and cause the release of adrenocorticotropic hormone (ACTH). However, NPY and CRH additionally stimulate the release of melanophore-stimulating hormone (α-MSH) and β-endorphin (β-END). TRH and DA also stimulate the release of α-MSH and β-END. MCH, conversely, inhibits the release of ACTH, α-MSH, and β-END (Wendelaar Bonga 1997).

The release of cortisol occurs in response to numerous pituitary hormones acting on the interrenal cells, most notably ACTH, α-MSH, and β-END (Figure 1) (Wendelaar Bonga 1997; Iwama et al. 2006). Upon release, cortisol has an inhibitory effect directly on the interrenal gland to suppress further cortisol production (Bradford et al. 1992; Wendelaar Bonga 1997).
Cortisol has two major actions in fishes. It regulates both hydromineral balance and energy metabolism (Wendelaar Bonga 1997). The hydromineral balance of fishes is regulated by cortisol in several ways. Increased cortisol causes the differentiation of chloride cells that are used in ion transport at the gills, and it amplifies the specific activity of Na+/K+ pumps at the gills, kidneys, and intestines (Jones et al. 1980). In marine fishes, cortisol acts on the gills to stimulate the release of Na⁺ and Cl⁻ (Wendelaar Bonga 1997). Cortisol secretion is thus essential for euryhaline fishes when they move into seawater because it promotes ion exchange at the gills and allows them to osmoregulate in the marine environment (Jones et al. 1980). Conversely, in freshwater
fishes, cortisol promotes the uptake of Na\(^+\) and Cl\(^-\) ions (Laurent and Perry 1989). These changes in the rate of ion exchange caused by cortisol due to stress can upset the delicate balance required to maintain homeostasis, particularly at the gills (Wendelaar Bonga 1997).

In response to stress, cortisol mobilizes energy substrates (Iwama et al. 2006) by targeting the liver to increase glucose production through the processes of glycogenolysis and gluconeogenesis. This added glucose is sent to the gills, brain, and muscles to meet the increased demand for energy (Iwama et al. 2006). Increases in plasma glucose concentrations is another indicator used to measure stress levels in fishes (Davis et al. 2001; Sulikowski and Howell 2003; King et al. 2006; Hoffmayer and Parsons 2003; Herbert and Steffensen 2005; Perez-Casanova et al. 2008). This reallocation of energy reserves can contribute to weight loss during times of chronic stress (Wendelaar Bonga 1997).

Perhaps more applicable to fisheries and aquaculture, increased plasma cortisol can cause a reduction in growth rate and suppress both the reproductive and the immune systems (Wendelaar Bonga 1997). Studies of stress related to aquaculture have measured plasma cortisol concentrations to determine if particular husbandry events, such as transportation, netting, grading or vaccination lead to high stress, (Wendelaar Bonga 1997; Sulikowski and Howell 2003; King and Berlinsky 2006; King et al. 2006; Olsen et al. 2008). Sulikowski and Howell (2003) saw plasma cortisol concentrations increase by 241% following the movement of summer flounder (Paralichthys dentatus) into floating net pens. King et al. (2006) observed elevated plasma cortisol concentrations in juvenile Atlantic cod following a netting and air exposure stressor. King and Berlinsky (2006)
found elevated plasma cortisol concentration in juvenile Atlantic cod following a net stressor, and a handling and transport stressor. Finally, Olsen et al. (2008) found elevated plasma cortisol concentrations in juvenile cod after a water level change and chasing stressor. Results from such studies can provide valuable insights on ways to reduce stress, and thus lead to increased production and profits.

Plasma cortisol concentrations in fishes vary depending on species, genetic characteristics, and environment (Barton et al. 2002). Baseline concentrations (pre-stress) of plasma cortisol have been reported from <1 ng/ml in triploid brook trout (Benfey and Biron 2000) to ~ 50 ng/ml in walleyes (Barton et al. 2003). Post-stress concentrations have been reported to be as high as ~300 ng/ml for Arctic char following a handling stressor (Jorgensen et al. 2002) and as low as ~3 ng/ml for Pallid sturgeon following a handling stressor (Barton et al. 2000). Further, the magnitude of change in plasma cortisol concentrations between pre-stress and post-stress also vary by species (Iwama et al. 2006). Following a handling stress, plasma cortisol concentrations are ~90 times higher than baseline (pre-stress: ~1.8 ng/ml; post-stress: ~163 ng/ml) in Arctic Char, while plasma cortisol concentrations only increased about 1.6 times following a handling stressor (pre-stress: ~1.8 ng/ml; post-stress: ~2.9 ng/ml) (Barton et al. 2000) in Pallid sturgeon.

Baseline plasma cortisol concentrations for cultured juvenile cod are have been reported at ~5 ng/ml (King and Berlinsky 2006). These concentrations rose to above 90 ng/ml 30 minutes following a transport and netting stressor. Olsen et al. (2008) also reported pre-stress concentrations for cultured juvenile Atlantic cod at ~5 ng/ml and post-stress concentrations peaking at ~80 ng/ml, following a water level drop and 15 minute
chase with a pole. Because there are ontogenetic changes in stress recovery (Masuda and Ziemann, 2000; Artigas et al. 2005) it is difficult to extrapolate stress levels and recovery times for juvenile Atlantic cod to adults.

Baseline concentrations of plasma cortisol for adult wild Atlantic cod (~46 cm) were reported by Morgan et al. (1998). They found pre-stress plasma cortisol concentrations are <5 ng/ml and saw those levels increase to ~30 ng/ml following a capture and 3 minute confinement stressor. Furthermore, their studies found that resting plasma cortisol concentrations in Atlantic cod were significantly higher in pre-spawning cod compared to post-spawned cod (pre-spawned: ~15 ng/ml; post-spawned: ~5 ng/ml). However, it is important to note that these differences can be explained, not only by individual variation, but by assay specificity, husbandry conditions, and handling procedures as well (Barton and Iwama 1991).

**Sampling Variability**

Because relative plasma cortisol concentrations are routinely used in fisheries studies to measure stress, it is vital to know what the resting (baseline) concentrations are (Morgan et al. 1999; Davis et al. 2001). However, drawing blood samples to measure plasma cortisol has some inherent drawbacks, as netting, handling, and drawing blood from fish will have an effect on plasma cortisol concentrations (Wendelaar Bonga 1997). There are a multitude of techniques for sampling blood from fishes, each with its own advantages and disadvantages, depending on species sampled, fish size, and sampling environment. Two common techniques are caudal vessel puncture (Morgan et al. 1999; Sulikowski and Howell 2003; King et al. 2006) and cannulation (Soivio et al. 1975; Ishimatsu et al. 1988; Lo et al. 2003; Skov and Steffensen 2003).
Cannulation is a technique that has been commonly used in the laboratory (Soivio et al. 1975; Ishimatsu et al. 1988; Lo et al. 2003; Skov and Steffensen 2003). This procedure involves surgically placing a polyethylene tube into a blood vessel that will remain implanted throughout the duration of the sampling period (Soivio et al. 1975; Axelsson and Fritsche 1994). Although this method requires a more invasive surgical procedure to implant the cannula, it should reduce total handling-induced stress during subsequent blood collections (Lo et al. 2003). The fish become acclimated to the presence of the cannula, and the handling time required to obtain a blood sample is drastically reduced, allowing for a sample to be taken from an “unstressed” fish, and the establishment of a more accurate baseline cortisol concentration (Wendelaar Bonga 1997; Lo et al. 2003). Several studies have demonstrated that cannulation is an effective method for collecting repeated blood samples over time (Soivio et al. 1975; Ishimatsu et al. 1988, Lo et al. 2003; Skov and Steffensen 2003), being less stressful than vessel puncture, for example, in the grouper, *Epinephelus malabaricus*, during repeated blood sampling (Lo et al. 2003).

Caudal vessel puncture allows for a quick and easy blood sample to be taken from a fish. This procedure involves restraining the fish, inserting a syringe into the caudal vasculature, and drawing blood. Anesthetization may lessen the cortisol response in fishes compared to those without anesthesia (Wagner et al. 2003), but inevitably, handling, the vessel puncture itself, and anesthetization will stress the fish, potentially causing injury and variability of measured parameters in subsequent samples (Iwama et al. 2006; Wendelaar Bonga 1997). Moreover, the specific anesthesia used can have different effects on stress, recovery, and plasma cortisol concentrations.
MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate) is a popular choice of anesthesia for fishes. However, Mattson and Riple (1989) recommended that the anesthesia Metomidate (1-(1-phenylethyl)-1H-imidazole-5-carboxylic acid methyl ester) be used for anesthetization of Atlantic cod because they found that Atlantic cod are less likely to perish when overdosed with Metomidate rather than with benzocaine and MS-222. Metomidate, a derivative of Etomidate, is a nonbarbituate hypnotic, which induces sleep and not general anesthesia (Amend et al. 1982). In addition to its safety preference, plasma cortisol concentrations in channel catfish (Ictalurus punctatus) following anesthetization with Metomidate are significantly lower than those anesthetized with MS-222. Although the method of cortisol blocking has not been shown for Metomidate, Etomidate blocks activities of the HPI axis by acting on the mitochondrial cytochrome P₄₅₀-dependant enzymes that catalyze the production of cortisol (Vanden Bossche et al. 1984; Wagner et al. 1984). Thus, Metomidate is a preferable choice for use with Atlantic cod when measuring plasma cortisol due to its safety and its suppression of cortisol production during anesthetization (Vanden Bossche et al. 1984; Wagner et al. 1984; Matteson and Riple 1989; King et al. 2005).

**Gaps in knowledge:**

Given the amount of research conducted on Atlantic cod, stress, and cortisol, little has been done to compare different blood sampling methods and the effects they might have on plasma cortisol concentrations, particularly in adult fish. Furthermore, there have been no studies directly comparing the effects of sampling methods and sampling conditions on plasma cortisol for wild and cultured adult Atlantic cod. In addition, only one study has reported baseline plasma cortisol concentrations for adult Atlantic cod
(from Georges Bank; Morgan et al. 1999). Moreover, biological factors and physiochemical factors, such as stage in the reproductive cycle (Pottinger et al. 1996), can cause stress response to vary in fishes (Iwama et al. 2006). Thus, cod in different reproductive stages and/or different stocks may have dissimilar reactions to lab conditions and stressors. Therefore, it is essential that various methods of blood sampling be evaluated and optimized for adult Atlantic cod to increase accuracy of plasma cortisol concentrations and minimize interference in the resultant blood samples.

**Objectives:**

The objectives of this study were to examine three different methods of repeated blood sampling in Atlantic cod and to determine if sampling method affected plasma cortisol concentrations.

I hypothesized that:

- repeated sampling via cannulation would display the lowest plasma cortisol concentrations due to minimal handling after the initial surgery;

- subsequent samples taken greater than 24 hours post-cannulation surgery would have lower baseline plasma cortisol compared to the vessel puncture studies;

- vessel puncture blood samples taken using a reduced handling method would produce lower plasma cortisol concentrations than those obtained via standard methods of sampling; and

- repeated caudal vessel puncture samples, taken 72 hrs apart, would produce plasma cortisol concentrations similar to those of the original “pre-stressed” sample for all three sampling methods.
Applied Value:

The results of this study will improve the understanding of stress physiology of wild and cultured Atlantic cod held in captivity, and the reaction of these fish to normal research and husbandry procedures such as handling, anesthetization, air exposure, vaccination, and blood draws. This study will also provide insight into the true recovery time (return to pre-stress levels) for fish that have undergone caudal vessel puncture blood draws, cannulation surgeries of the afferent branchial artery, and blood draws from a cannula. Furthermore, the comparison of multiple sampling techniques will help determine the best method for establishing baseline plasma cortisol concentrations, and for taking repeated samples from a single fish.
Experiment 1

Methods

In May 2008, 18 wild cod (average 69.7cm total length +/- 14.4cm) were collected by a commercial fishing vessel off the coast of Seabrook, NH. Fish were collected via a 30-minute tow with an otter trawl. Cod were transported in an oxygenated 1 m³ tank to the University of New Hampshire’s Coastal Marine Laboratory (CML) in New Castle, NH. Following the two hour trip, fish were equally divided into two, 2 m diameter, flow-through seawater tanks, and allowed to acclimate for one week. During the following 7 day sampling regimen, 1-ml blood samples were drawn every 3 days (Days 1, 4, and 7) using two different sampling techniques (see caudal vessel puncture techniques section below for more detail). These procedures were repeated again in December 2008 and April 2009 using cultured cod. Prior to the experiment, these fish were raised in net pens near the Isles of Shoals, NH and were smaller in size (average 49.5 cm total length +/- 6.5 cm). In these second experiments, fish were transported in a 1 m³ tank supplied with flowing seawater, from the aquaculture site to small net pens (~100 m³) beneath the UNH pier in Newcastle, NH. One week prior to the experiment, 20 fish were removed from the net pens and placed into two, 2 m diameter flow-through tanks (10 fish per tank) atop the pier and were allowed to acclimate for one week prior to the experiment.

A. Caudal Vessel Puncture Techniques

1. Standard caudal vessel puncture
The wild fish in Trial 1 were sampled the week of May 30, 2008, while fish in Trials 2 and Trail 3 were sampled the weeks of December 10, 2008 and April 10, 2009, respectively. To initiate each sampling event, individual fish were removed from the tank using a dipnet and placed it in a 100 cm x 30 cm cooler filled with a mixture of seawater and the anesthetic Metomidate (6 mg L\(^{-1}\)). I identified Stage 3 anesthesia when fish began to roll ventral side-up (Schoettger and Julin, 1967). Although the time it took to reach Stage 3 was variable, it took most fish ~5 minutes. Following anesthetization, the total length of each fish was measured, to the nearest 0.5 cm, and a spaghetti tag was inserted into the dorsal musculature just below the first dorsal fin. A 1 ml blood sample was drawn from the caudal vasculature of the animal (ventral approach) via a heparinized tuberculin syringe and 21-gauge needle. The sampled fish was placed in a separate 2 m diameter tank, and its recovery was assisted by running water over its gills, or by back and forth movement, if necessary. This procedure took ~7 minutes per fish, and blood was drawn from 10 fish each day.

2. Caudal vessel puncture with reduced handling

For the second caudal vessel puncture technique, fish were directly anesthetized within their holding tanks. Here, tank depth and volume was reduced from 70 to 20 cm, and from ~4.9 to 1.4 m\(^3\), respectively. Incoming water flow was shut off to the tank, and Metomidate (6 mg L\(^{-1}\)) was added. When all cod exhibited Stage 3 anesthesia (Schoettger and Julin, 1967), a 1.0-ml blood sample was taken as previously described. After blood was drawn from an individual, I placed the sampled fish in a separate 2 m tank and assisted its recovery by running water over its gills, if necessary. This procedure took ~2 minutes per fish, and blood was drawn from 10 fish. After sampling,
all blood samples were immediately placed on ice, and centrifuged as soon as possible (10 - 90 minutes) to isolate plasma. Plasma was removed using a pipette, transferred to 1.5-ml snap-top Eppendorf tubes, and stored at -20°C for future analysis.

**B. Cortisol Extraction**

All plasma samples were extracted twice with 10 volumes of ethyl ether for the cortisol assay. Briefly, ether was added to an aliquot of plasma, vortexed for one minute, flash frozen in an acetone-dry ice bath, decanted, and evaporated using nitrogen. This step was then repeated. The dried samples were reconstituted in one volume of phosphate buffered saline with 0.1% gelatin (PBSG). Buffer blanks, charcoal stripped-plasma, and plasma spiked with steroids were processed in an identical manner.

Approximately 1000 counts min⁻¹ (cpm) of radiolabeled cortisol were added to plasma samples to account for procedural losses. The samples had an average recovery of 78%, ranging from 35%-98% with a SD of 0.09. All samples were corrected for recovery.

**C. Cortisol Radioimmunoassay**

Plasma cortisol concentrations were measured at the University of New Hampshire, Durham, NH using a radioimmunoassay modified and validated for use on Atlantic cod (J. Specker 2007, pers. comm. 7 Jun). Two different aliquots of reconstituted sample extracts in duplicate were incubated with approximately 6,000 – 7,500 cpm of tritiated cortisol (1,2,6,7-³H; Amershan Biosciences, Piscataway NJ) and a cortisol antibody (final dilution: 1:3200; Fitzgerald Industries International, Concord MA) for two hours at room temperature. Following incubation, samples were put on ice for 5
minutes and free steroids were separated from bound using 0.400 ml of an ice-cold suspension of charcoal (1.0%) and Dextran (0.1%) in PBSG. Samples were then vortexed, allowed to incubate for 15 minutes at 4°C, and then centrifuged for 15 minutes at 3,000 rpm (2160 g force) and 4°C. The resulting supernatant was decanted into scintillation vials and 5 ml of Beckman Ready-Safe scintillation cocktail (Beckman Coulter, Somerset, NJ) was added to each vial. Samples were counted in a Beckman LS6000IC (Fullerton, CA) scintillation count. The interassay variation was 13.33% and the intraassay variation was 10.67%.

D. Statistical Analysis

Plasma cortisol concentrations were analyzed using the MIXED procedure of SAS 9.1 (2005) according to the following model:

\[ Y_{ij} = \mu + T_i + c_{ij} + D_k + TD_{ik} + E_{ijk} \]

Where:

\( Y_{ij} \)  = is the dependent variable
\( \mu \)  = overall mean
\( T_i \)  = is the fixed effect of the \( i^{th} \) treatment, \( i = 1,2 \)
\( c_{ij} \)  = is the random effect of the \( j^{th} \) cod within the \( i^{th} \) treatment, \( j=1,\ldots, 58 \)
\( D_k \)  = is the fixed effect of day of experiment, \( k = 1,4,7 \)
\( TD_{ik} \)  = is the fixed effect of the interaction between the \( i^{th} \) treatment source and the \( k^{th} \) day
\( E_{ijk} \)  = is the random residual \( \sim N(0, \sigma^2) \)

In this model, the random effect of cod was used as the error term for the effect of treatment. Residual errors, which are errors within cod across day and represent errors
from repeated measurements in the experimental units (cod) were modeled using a first-order autoregressive covariance structure. Degrees of freedom were calculated using the Kenward-Roger option of MIXED procedure (SAS, 2001). The initial total length of each cod was used as a covariate in this analysis. Least square means were determined for treatment and treatment by week interactions. The PDFF option in SAS® was used to test treatment differences among least squares means. Significant treatment effects were noted at $P \leq 0.05$.

The UNIVARIATE Procedure of SAS 8.2 (1999) was used to determine if any outliers were present. An observation which was greater than 2.5 standard deviations from the mean for plasma cortisol was considered an outlier. The results of the outlier analysis indicated that there was 1 outlier.

Total length was used as a covariate to standardize the data ($P = 0.2108$). The plasma cortisol concentrations seen through the duration of the experiment varied significantly among trials ($P < 0.0001$). Therefore, each of the three trials was analyzed individually. Temperature was not a significant factor amongst the sampling times ($P > 0.05$) and was removed to increase the power of the analysis.

I tested the probability that method of sampling (standard or reduced) did not affect the plasma cortisol concentrations in subsequent samples from the same fish, nor did the sampling itself affect the plasma cortisol concentrations in subsequent samples in wild and cultured fish.

- $H_0$: ng/ml plasma cortisol Standard = ng/ml Reduced
- $H_0$: ng/ml plasma cortisol Day 1 = ng/ml Day 4 = ng/ml Day 7
Results

Trial 1 was designed to test the null hypothesis that repeated sampling via caudal vessel puncture would not increase plasma cortisol concentrations in subsequent samples. Wild fish were used, and the research occurred indoors during the week of May 30, 2008. Plasma cortisol concentrations differed significantly between the dates on which the samples were taken, and between the methods of sampling. The mean (+/- SD) plasma cortisol concentration for all three trials was 22.36 (SD = 16.68) ng/ml. The average plasma cortisol concentration for cod 18 (reduced handling method) was 85.22 ng/ml (> 2.5 standard deviations from the mean plasma cortisol concentration), and was therefore removed from the analysis. For the standard method of anesthesia (n=9) and blood sampling, there was a significantly higher mean plasma cortisol concentration on Day 7 (52.1 ng/ml) compared to Days 1 (21.8 ng/ml) and 4 (19.5 ng/ml) (P<0.01; Table 2; Figure 1). However, the reduced handling method of sampling (n=8) returned different results. The mean plasma cortisol concentration on Day 4 (50.1 ng/ml) was significantly higher than on Day 1 (10.4 ng/ml) (P<0.01) and Day 7 (29.8 ng/ml) (P<0.05) (Table 2, Figure 1), but there were no significant differences between Days 1 and 7 (10.4 ng/ml and 29.8 ng/ml, respectively) (P>0.05) (Table 2, Figure 1). There was no significant difference between the two sampling methods on Day 1 (Standard 21.8 ng/ml, Reduced 10.4 ng/ml) (P>0.05) (Table 2, Figure 1). However Day 4 and Day 7 each showed a difference between sampling method. On Day 4 fish undergoing the reduced handling method showed significantly higher plasma cortisol concentrations than the standard method (Reduced 50.1 ng/ml, Standard 19.5 ng/ml) (P<0.01) (Table 2, Figure 1). On Day 7, the plasma cortisol concentrations collected from fish using the standard method
spiked (52.1 ng/ml), while concentrations found in those that had underwent reduced handling were lower (29.8 ng/ml), resulting in significantly higher plasma cortisol concentrations in the standard method (P<0.05) (Table 2, Figure 1).

Trial 2, conducted with cultured fish, was also designed to test the null hypothesis that repeated sampling via caudal vessel puncture would not increase plasma cortisol concentrations in subsequent samples. Results differed from Trial 1. The cultured fish were sampled outdoors during the week of December 10, 2008. In this trial, both methods of sampling resulted in a significant increase in mean plasma cortisol concentrations from Day 1 (Standard 17.9 ng/ml, Reduced 13.2 ng/ml) to Day 4 (Standard 35.3 ng/ml, Reduced 36.9 ng/ml) (P<0.01) (Table 2, Figure 1). On Day 7, plasma cortisol (32.1 ng/ml) using the reduced handling method remained significantly higher than Day 1 (13.2 ng/ml) (P<0.05) but was not different from Day 4 (36.9 ng/ml) (P>0.05) (Table 2, Figure 1). On the other hand, mean plasma cortisol in the fish undergoing the standard method of sampling dropped slightly on Day 7 (32.1 ng/ml), but was not significantly different from either Days 1 (14.2 ng/ml) or 4 (35.3 ng/ml) (P>0.05) (Table 2, Figure 1). There were no significant differences between the two different sampling methods on any day of the trial (P>0.05).

Trial 3 produced much different results than Trials 1 and 2. This trial, like Trial 2, occurred outdoors using cultured fish. Sampling took place during the week of April 10, 2009. For the standard method of sampling, mean plasma cortisol on Day 4 (7.5 ng/ml) was significantly lower than on Day 7 (15.8 ng/ml) (P<0.05), but there was no significant difference between Days 1 (14.2 ng/ml) and 4 or between Days 1 and 7 (P>0.05) (Table 2, Figure 1). There were no significant differences between days
throughout the trial in the reduced handling method methods (P>0.05). However, fish undergoing the reduced handling method had significantly lower values than the standard method on Day 1 and Day 7 (P<0.01) (Table 2, Figure 1).

Blood samples taken on Day 1 of each trial represented an attempt at establishing basal plasma cortisol concentrations. The lowest mean concentrations of plasma cortisol taken on Day 1 were measured in Trial 3 from fish experiencing the reduced handling method (4.6 ng/ml, SE 2.5), while the highest values were measured in Trial 1 using the standard method (21.8 ng/ml, SE 6.2) (Table 1, Figure 3). The standard method of sampling gave consistently higher concentrations in all 3 of the trials (Table 1, Figure 3).

Although there were no statistical differences in plasma cortisol concentrations based on the order that the fish were sampled, the sampling order of fish on Day 1 showed a trend of increasing plasma cortisol concentrations as the sample order progressed in the standard method, while the sampling order in the reduced handling method showed plasma cortisol concentrations that tended to be more sporadic (Figure 2).
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Table 1: Plasma cortisol (mean +/-SE) for the three different trials, and the two handling methods.
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<th>Trial 1</th>
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Table 2: PDIFFs for all interactions
Figure 2: Plasma cortisol concentrations (mean +/- SE) by day and sampling method. Trial 1 represents wild fish sampled in April of 2008, trial 2 represents cultured fish sampled in December 2008, and trial 3 represents cultured fish sampled in June of 2009.

- A and B refer to significant differences (P<0.05) within the same sampling method between days.
- 1 and 2 refer to significant differences (P<0.05) within the same day between the two sampling methods.
Figure 3: Plasma cortisol (mean +/- SE) by order sampled on Day 1 across all three trials.

Figure 4: Plasma cortisol concentrations (mean +/- SE) taken on Day 1. These values represent baseline concentrations because fish were unstressed for one week prior to initiation of the sampling procedure.
Discussion

A. Variation Between Trials

While the procedures for each of the three trials were identical, there were substantial differences in the environment, and in the history and size of fish that could have led to the variability between trials. Barton et al. (2002) found that concentrations of plasma cortisol can vary depending on genetic characteristics and environment. Cod from Trial 1 were caught by trawl from the wild, while cod in Trials 2 and 3 were raised in an open ocean aquaculture net pen. These differences could have contributed to the differences between these trials. For instance, I observed that the wild cod in Trial 1 were much more skittish and active in the tanks than were the cultured fish used in Trials 2 and 3. During sampling, fish in Trial 1 would often react vigorously (high speed escape response) when the capture dip-net was introduced into the tank. This increased activity also happened periodically, for no apparent reason, during the acclimation period and throughout the sampling week. This likely led to the higher plasma cortisol concentrations found in Trial 1. Wild fish are not used to being in a confined space, the light levels present in a laboratory setting, and having interactions with humans. Furthermore, cultured fish tend to have a dulled stress response compared to wild fish of the same species (Woodward and Strange 1987).

Atlantic cod can display significantly higher plasma cortisol concentrations pre-spawning compared to post-spawning (Morgan et al. 1999). Many of the large male cod in Trial 1, caught during the spawning season, were spermiating during the sampling process, while no milt was seen from the cultured fish in Trials 2 and 3. This reproductive condition may have contributed to higher overall plasma cortisol
concentrations seen in Trial 1. In addition, the wild fish were substantially larger than the cultured fish (mean total lengths 69.7cm and 49.5cm, respectively). Although total length was used as a covariate to eliminate this bias, wild fish in Trial 1 were substantially more crowded than the cultured fish in Trials 2 and 3. Crowding such as this can cause elevated plasma cortisol concentrations in fishes (Barton and Iwama 1991).

Furthermore, the environmental conditions were substantially different between trials. Trial 1 was conducted inside the UNH Coastal Marine Laboratory in late-May, but due to time constraints and logistical issues, Trials 2 and 3 were conducted outside, in tanks on the UNH pier, in mid-December and mid-April, respectively. Although a shade cloth blocked direct sunlight, light levels were certainly higher in the tanks on the pier than inside the lab. Although temperature was not a statistically significant variable, the weather conditions undoubtedly had some effect on Trials 2 and 3 that were conducted on the pier. During Trial 2, an ice storm caused a power outage just prior to the 4 days of sampling. A generator maintained the flow-through system, but the harsh conditions may have affected the fish in some unknown manner. These extreme circumstances may have led to the elevated plasma cortisol concentrations that were seen on days 4 and 7 in Trial 2 compared to those seen in Trial 3.

Finally, as sampling progressed and procedures began to run smoother, finding the caudal vein became easier and researchers perfected their techniques. This may have led to less external and internal damage to the caudal peduncle and the caudal vessels (Iwama et al. 2006, Wendelaar Bonga 1997). Additionally, the ease of sampling led to less handling of individual fish that would have decreased plasma cortisol concentrations (Wendelaar Bonga 1997; Iwama et al. 2006).
B. Variation Between Sampling Days

1. Standard Method

The standard method of sampling used in this study most closely resembled the vessel puncture procedure conducted by Lo et al. (2003). They found that plasma cortisol concentrations increased steadily following repeated vessel punctures of *Epinephelus malabrus*, and noted consistently elevated plasma cortisol concentrations up to 13 days following repeated vessel punctures occurring every other day. The current study found different reactions to repeated vessel punctures when compared to Lo et al. (2003) due potentially to a variety of variables (sampling intervals, sampling procedures, species, fish size, genetic characteristics, environmental factors). Only Trial 2 documented consistently elevated plasma cortisol concentrations in subsequent samples (Day 4 and Day 7). This was potentially due to the ice storm that occurred prior to sampling on Day 4, in addition to the repeated anaesthetization and possible internal bleeding that can occur (Wendelaar Bonga 1997; Lo et al. 2003; Iwama et al. 2006).

Trial 1 provided significantly higher plasma cortisol concentrations on Day 7 compared to Day 1. These elevated concentrations can be attributed to the repeated anaesthetization and possible internal bleeding (Wendelaar Bonga 1997; Lo et al. 2003; Iwama et al. 2006). However, no significant increase was seen between Day 1 and Day 4.

Overall, Trial 3 had lower plasma cortisol concentrations throughout the week long experiment, but there was still a significant increase in plasma cortisol between Days 4 and 7, which was consistent with the results of Trial 1. During all trials it was often difficult to get sample quickly because fish often thrashed during the vessel
puncture despite being at Stage 3 anesthesia. Small (2003) saw a similar reaction in channel catfish (*Ictalurus punctatus*) that reached Stage 3 anesthesia induced by Metomidate at 6-ppm. Catfish had small reflex movements when their container was struck. This reaction can be attributed to Metomidate being a nonbarbituate hypnotic, and therefore induces sleep and not general anesthesia (Amend et al. 1982).

2. Reduced Handling Method

Trial 1 saw a sharp increase in plasma cortisol concentrations from Days 1 to 4 when using the reduced handling method. This could be a result of an unintentional over anesthetization that occurred as researchers became familiar with Metomidate. This is consistent with the findings of Kakizawa et al. (1995), in which plasma cortisol increased following a lowering of water level and chasing of rainbow trout. However, plasma cortisol returned to Day 1 levels by Day 7.

Plasma cortisol concentrations in Trial 2 were significantly higher on Day 4 than on Day 1, but by Day 7 they had dropped slightly to concentrations that were not significantly different from either Day 1 or Day 4 (Figure 1). These elevated concentrations were likely due to the complications caused by the ice storm that occurred prior to sampling on Day 4, in addition to the repeated anaesthetization and water level change (Kakizawa et al 1995; Wendelaar Bonga 1997; Iwama et al. 2006).

Trial 3 showed no significant differences between all three sampling days in the reduced handling method. The reduced stress response of the cultured fish under ideal conditions is a probable cause for low plasma cortisol concentrations, as well as the increased ease of sampling (Woodward and Strange 1987; Barton et al. 2002).

C. Variation Between Sampling Methods
There were no significant differences between sampling methods on Day 1 in Trial 1, however, on Day 4 plasma cortisol concentrations were significantly higher for the reduced handling method (Standard Method = 19.93 ng/ml, Reduced Handling Method = 50.02 ng/ml, $P < 0.01$). A possible explanation for the elevated concentration is that the wild cod became more stressed by the drop in water level in the tank than they were by being pursued by a net, particularly when compared to cultured fish (Woodward and Strange 1987, Kakizawa et al. 1995, Olsen et al. 2008). Another potential cause of this is an over-anesthetization that occurred on Day 1 during the reduced sampling. On Day 1, fish sampled via the reduced handling method were accidentally over-anesthetized while trying to determine the proper dosage. Although Metomidate has been shown to block plasma cortisol (Thomas and Robertson 1991, Olsen et al. 1995), Ledingham and Watt (1983) saw elevated mortality following surgery in human patients that were anesthetized using Metomidate. Thomas and Robertson (1991) suggested these deaths may have been due to Metomidate blocking cortisol production. They propose that a corticosteroid stress response is necessary to recover from a severe stressor. Cortisol serves an important function in the mobilization of energy substrates during times of stress (Wendelaar Bonga 1997), and this may be a necessity for the animal to recover. It is possible that the initial plasma cortisol block caused by the Metomidate overdose was responsible for the elevated concentrations in the reduced handling method two days later. Conversely, on Day 7 the standard method of sampling resulted in significantly higher plasma cortisol concentrations than did the reduced handling method (52.54 ng/ml, 28.64 ng/ml, respectively; $P = 0.03$).
Trial 2 produced no significant differences between sampling methods on any sampling day. This may have been a result of the ice storm that occurred prior to Days 4 to 7.

In Trial 3, the plasma cortisol concentrations for the reduced handling method were significantly lower than the standard method on Days 1 and 7 (Figure 1). These lower concentrations were likely due to the differences between the two sampling procedures, and are consistent with the hypothesis that vessel puncture blood samples taken using a reduced handling method will produce lower plasma cortisol concentrations than those taken via the standard method of sampling (Barton and Iwama 1991; Wendelaar Bonga 1997; Sulikowski and Howell 2003; King and Berlinsky 2006; King et al. 2006 Olsen et al. 2008), i.e. the pursuit with a net and longer sampling time per tank (1.45 hr) for the standard method, compared to a change in water level and shorter sampling time (0.28 hr) for reduced handling method. The longer sampling times allowed enough time for the HPI axis to respond with a corticosteroid response caused by the occurring sampling event (net pursuit) (Figure 2) (See Sampling Order section below).

D. Baseline Levels

I hypothesized that the lowest concentrations would be found only on Day 1 for both methods of sampling, and I would define those values as-baseline concentrations. However, the lowest plasma cortisol concentrations were seen in Trial 3 on Day 4 in the reduced handling method (1.14 ng/m). This value was not significantly different than the Days 1 and 7 of the same trial and method (4.63 ng/ml and 4.35 ng/ml, respectively, P > 0.05). These values were similar to pre-stress basal concentrations seen by King et al.
(2006) for juvenile cod at a similar temperature (4°C). Trial 3 was the only trial that detected significantly lower baseline plasma cortisol concentrations on Day 1 for the reduced handling method compared to the standard method (4.63 ng/ml and 14.20 ng/ml, respectively, P = 0.01). I expect that this difference was due to the longer sampling time for the standard method on Day 1 for Trial 3 (1.38 hr standard method compared to 0.33 hr reduced handling method).

E. Sampling Order

The results of the study suggest that the order in which the fish were sampled may have influenced plasma cortisol concentrations (Figure 2). A general increasing trend can be seen for the standard method in all trials, while concentrations for the reduced handling method have a more sporadic distribution, likely due to individual variation (Figure 2). Fish that had blood drawn later experienced a longer period of net pursuit than those fish that were sampled earlier in that sampling event, causing plasma cortisol concentrations to increase. This is consistent with the reaction of other fishes to a chasing/net stressor along with the timing of the corticosteroid response seen in juvenile Atlantic cod (Barton and Iwama 1991; Wendelaar Bonga 1997; King and Berlinsky 2006, King et al. 2006).

F. Summary

- Drawing blood from adult fish induces some stress, which is reflected in elevated plasma cortisol concentrations. Once quantified by comparing the results of different sampling methods, these biases can be minimized by adjusting experimental design. Wild adult Atlantic cod appear to react differently to experimental conditions than do cultured cod. It is clear that differences in the origin of the fish and sampling method
contributed to the variability of plasma cortisol samples. Thus, minimizing the time a fish is stressed during a sampling event is crucial to obtaining accurate plasma cortisol concentrations, and identifying any potential stressors, and quantifying their intensity, is essential for interpreting results of plasma cortisol analysis in Atlantic cod.
### Experiment 2

#### Methods

**A. Cannulation**

From October of 2008 to March of 2009, cultured cod taken from the UNH Open Ocean Aquaculture net pen just off the Isle of Shoals in NH (average 49.5 cm total length) were moved to net pens under the pier at the University of New Hampshire’s (UNH) Coastal Marine Laboratory (CML) in New Castle, NH. Twenty-six fish were randomly selected to undergo a cannulation procedure. To begin this procedure, an individual fish was immersed in a 100 cm x 30 cm cooler filled with a seawater/Metomidate mixture (6 mg L\(^{-1}\)) until Stage 3 anesthesia was reached. The fish was then transferred to a surgery platform that allowed a continuous flow of sea water/Metomidate mixture over the gills. Once cannulated (procedure described below), the fish was isolated in a separate 2 m diameter flow-through tank. Blood was sampled once per day via the cannula for 7 days. At the completion of each trial, fish were euthanized and sexed.

**B. Cannulation Surgery**

1. **Afferent Branchial Artery**

   All surgeries were performed at the UNH CML by me and UNH veterinarian Dr. Dean Elder. The cannulation procedure was based on that described by Skov and Steffensen (2003). Each fish was anesthetized in Metomidate and placed on a surgical board with constant mixture of water and Metomidate flowing over the gills. Prior to the
surgery, a 10 cm piece of 4/0 silk suture material was tied and glued to a 40 cm piece of PE-50 tubing about 3 cm from the insertion end. The insertion end of the tubing was cut at a 60° angle to allow for a smooth entrance into the blood vessel. The end of the PE-50 line was fitted with an access port that allowed it to float freely on the water surface for sampling at a distance from the fish. Both the cannula and access port were flushed with heparin-saline solution prior to the surgery. To begin cannulation, the gill filaments were separated about 2 cm dorsal to base of the 2nd gill arch to reach the 2nd afferent branchial artery that lies on the posterior portion of the gill arch beneath the filaments. A pair of thin forceps was used to puncture the artery and blood began to flow freely. Keeping the filaments separated, we inserted the cannula into the punctured artery and advanced the cannula ~2 cm. Once the cannula was inserted properly we wrapped the suture that had been previously fastened to the cannula around the gill arch and secured it by tying a surgeons knot. Making sure that blood was still flowing freely through the cannula, we fastened the tubing to the lateral portion of the fish just caudal to the operculum using a suture needle and nylon suturing material. The tubing was fastened using a Chinese finger cuff knot to prevent the cannula from sliding. Finally, the tubing was secured to the fish at a point just cranial to the dorsal fin.

When the cannula was in place, a 1 ml blood sample was taken (time: 0 hr), and the fish was placed in a recovery tank filled with fresh seawater and allowed to recover. The entire procedure took approximately 20 minutes. The fish was allowed to recover for one hour in the recovery tank before being placed back in the flow-through tank where it remained for the remainder of the experiment.

2. Sampling Times
In the fall of 2008, cannulated fish (n=13) were sampled daily. Due to complications with blood coagulation in the cannula and concern/interest in the initial cortisol recovery period, sampling times were changed for fall 2008 and spring 2009. Plasma cortisol samples were obtained from the seven successfully cannulated fish at 0h (blood drawn when cannula was inserted), and then again 24, 48, and 72h post-cannulation. In spring 2009, we took blood samples on a finer scale from 13 fish on Day 1 at times 0.0, 0.5, 1.0, 3.0, 6.0, and 24 h, and then twice more at 48 h and 72 h post-cannulation. After sampling, all blood samples were immediately placed on ice, and centrifuged as soon as possible to isolate plasma. Plasma was removed using a pipette, transferred to 1.5-ml snap-top Eppendorf tubes, and stored at -20°C for future analysis.

Of the 26 cannulation surgeries, 7 were considered “successful”, yielding blood samples for \( \geq 72 \) hours. Of the other 19, 3 fish died due to complications during surgery, and consistent samples could not obtained from the remaining 16 fish due to cannula malfunctions, i.e. lack of blood flow or dislodged cannula.

**C. Cortisol Extraction**

All plasma samples were double extracted with 10 volumes of ethyl ether for cortisol assay. Briefly, ether was added to an aliquot of plasma, vortexed for one minute, flash frozen in an acetone-dry ice bath, decanted, and evaporated using nitrogen. Samples were reconstituted in one volume of phosphate buffered saline with 0.1% gelatin (PBSG). Buffer blanks, charcoal stripped-plasma, and plasma spiked with steroids were processed in an identical manner.

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Approximately 1000 counts min\(^{-1}\) (cpm) of radiolabeled cortisol were added to plasma samples to account for procedural losses. The samples had an average recovery of 78%, ranging from 35%-98% with a SD of 0.09. All samples were corrected for recovery.

**C. Cortisol Radioimmunoassay**

Plasma cortisol concentrations were measured at the University of New Hampshire, Durham, NH, using a radioimmunoassay modified and validated for use on Atlantic cod (J. Specker 2007, pers. comm. 7 Jun). Two different aliquots of reconstituted sample extracts were incubated in duplicate with approximately 6,000 – 7,500 cpm of tritiated cortisol (1,2,6,7-\(^{3}\)H; Amershan Biosciences, Piscataway NJ) and a cortisol antibody (final dilution: 1,3200; Fitzgerald Industries International in Concord MA) for two hours. Following incubation, samples were put on ice for 5 minutes and free steroids were separated from bound using 0.400 ml of an ice cold suspension of charcoal (1.0%) and Dextran (0.1%) in PBSG. Samples were then vortexed, allowed to incubate for 15 minutes at 4°C, and then centrifuged for 15 minutes at 3,000 rpm (2160 g force) and 4°C. The resulting supernatant was decanted into scintillation vials and 5ml of Beckman Ready-Safe scintillation cocktail (Beckman Coulter, Somerset NJ) was added to each vial. Samples were counted in a Beckman LS6000IC (Fullerton, CA) scintillation counter. The inter-assay variation was 13.33% and the intra-assay variation was 10.67%.

**E. Statistical Analysis**
Data were analyzed using the MIXED procedure of SAS 9.1 (2005) according to the following model:

\[ Y_{ij} = \mu + H_i + c_{ij} + E_{ijk} \]

Where:

\( Y_{ij} \) = is the dependent variable  
\( \mu \) = overall mean  
\( H_i \) = is the fixed effect of the \( i^{th} \) hour, \( i = 1, ... , 7 \).  
\( c_{ij} \) = is the random effect of the \( j^{th} \) cod within the \( i^{th} \) treatment, \( j=1, ... , 4 \) or \( 7 \)  
\( E_{ijk} \) = is the random residual \(~N(0, \sigma^2_e)\)  

In this model, the random effect of cod was used as the error term for the effect of treatment. Residual errors, which are errors within cod across hour, and represent errors from repeated measurements in the experimental units (cod), were modeled using a first-order autoregressive covariance structure. Degrees of freedom were calculated using the Kenward-Roger option of MIXED procedure (SAS, 2001). Least square means were determined for treatment and treatment by week interactions. The PDIFF option in SAS® was used to test treatment differences among least squares means. Significant treatment effects were noted at \( P \leq 0.05 \). Total length was used as a covariate to normalize the data (\( P=0.2397 \)). A test for outliers was performed and none were identified. Data were analyzed by two separate methods to avoid missing data points. Temperature was not a significant factor amongst the sampling times (\( P>0.05 \)) and was removed to increase the power of the analysis.
**Results**

The plasma cortisol concentrations did not vary significantly over time in either of the two sampling schedules in the fall or the spring. (P=0.2447 for sampling every 24 hrs; P= 0.1337 for fine scale sampling).

Although no significant differences were found between time intervals, some trends were evident in the plasma cortisol concentrations for both sampling schedules. Daily samples averaged 10.0 ng/ml for 0 h, immediately following the surgery. Concentrations peaked at 24 h at 25.9 ng/ml (Figure 4, Table 3). Finally, plasma cortisol began to return to 0 h concentrations, averaging 12.9 ng/ml and 11.5 ng/ml for 48 h and 72 h, respectively (Figure 4, Table 3).

Fish that were sampled on a finer scale showed similar trends during the first 72 hours. The mean at 0 h was 13.4 ng/ml, but concentrations quickly dropped to 2.4 ng/ml at 0.5 h and 3.1 ng/ml at 1 h (Figure 5, Table 4). Plasma cortisol then began to gradually rise to 7.0 ng/ml at 3 h and 13.8 ng/ml at 6 h (Figure 5, Table 4). Finally, concentrations peaked at 30.1 ng/ml at 24 h (Figure 5, Table 4). After the 24 h sample, plasma cortisol concentrations began to fall, dropping to 8.4 ng/ml at 48h and 10.0 ng/ml at 72 h (Figure 5, Table 4). Subsequent daily samples remained in this range.
Figure 5. Plasma cortisol concentrations (ng/ml) (mean +/- SE) over time for 7 successfully cannulated cod.

<table>
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<tr>
<th>Time (hour)</th>
<th>Mean (ng/ml)</th>
<th>SE</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>24</td>
<td>25.9</td>
<td>5.8</td>
</tr>
<tr>
<td>48</td>
<td>12.9</td>
<td>7.3</td>
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<tr>
<td>72</td>
<td>11.5</td>
<td>6.4</td>
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</tbody>
</table>

Table 3. Plasma cortisol concentrations (ng/ml) (mean and +/- SE) over time for 7 successfully cannulated cod
Figure 6. Fine scale plasma cortisol (mean +/-SE) for 4 successfully cannulated cod.

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Mean (ng/ml)</th>
<th>SE</th>
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<tbody>
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Table 4. Fine scale plasma cortisol concentrations (mean +/-SE) for 4 successfully cannulated cod.
Discussion

A. Surgery

There are multiple techniques for cannulation of fishes (Soivio et al. 1975; Axelsson and Fritsche 1994; Lo et al. 2003). Cannulation of an afferent branchial artery was chosen for adult Atlantic cod because no incision was needed, there were multiple points where the cannula could be secured to the fish (Soivio et al. 1975, Axelsson and Fritsche 1994), and it has been used in adult Atlantic cod previously (Skov and Steffensen 2003). The surgical procedure generally lasted approximately 20 minutes. Surgeries were completed in 10 minutes under ideal circumstances, but took up to 30 minutes if there were complications, i.e., difficulty inserting cannula, difficulties keeping blood flowing through the cannula, or excessive movement by anesthetized fish. The three mortalities that occurred were likely due to extended exposure to Metomidate during more complicated surgeries. Ledingham and Watt (1983) reported increased post-surgery mortality in fish anesthetized with Metomidate, and Thomas and Robertson (1991) proposed that these mortalities occurred because of the lack of a corticosteroid stress response. They argued that the corticosteroid response was necessary to recover from a severe stressor, and thus Metomidate, a cortisol blocker, may have prevented the animal's healthy recovery. However, Mattson and Riple (1989) saw no mortalities in cod due strictly to Metomidate at various concentrations.

It was often difficult to insert the cannula because it had to be inserted blindly (Axelsson and Fritsche 1994). There were occasional issues with keeping blood flow through the cannula and maintaining blood flow. Normally, if the cannula was reinserted, flushed with additional heparin-saline solution, or inserted into an afferent
branchial artery on another gill arch, adequate blood flow could be maintained. In addition, cod would thrash occasionally despite reaching Stage 3 anesthesia, which made surgery difficult. Small (2003) described a similar reaction in channel catfish (*Ictalurus punctatus*) that reached Stage 3 anesthesia induced by Metomidate at 6-ppm. Catfish had small reflex movements when their container was struck. This reaction can be attributed to Metomidate being a nonbarbituate hypnotic, and therefore induces sleep and not general anesthesia (Amend et al. 1982). In addition, Mattson and Riple (1989) found that cod maintained opercular respiration for twice as long when anesthetized with Metomidate compared to MS-222. This can be troublesome, particularly when performing surgery on the afferent branchial artery, although this occurred only sporadically in our experiments. For these reasons, Metomidate is not an ideal anesthesia to use when sedating fish for surgery of the afferent branchial artery.

B. **Blood Drawing**

There were numerous problems associated with drawing blood from the cannula on the majority of the cod. Blood could only be drawn for \( \geq 72 \) hours on 7 of the 23 fish that completed the surgery. At the beginning of the experiment, six of the cannulas became dislodged which led to a new cannula design. Following the implementation of the new design, blood flow generally ceased due to clots in the cannula, despite the daily flushing with heparin-saline solution. Following the failure to draw blood, additional heparin-saline was used to dislodge the clot, but this met with mixed results.

The sampling did not seem to stress cod, which is consistent with other experiments (Soivio et al. 1975; Axelsson and Fritsche 1994; Lo et al. 2003). However, if the cod noticed the researcher, the fish would often swim calmly to the other side of the
tank. The cannula seemed to visibly irritate two of the fish. These fish would rub their operculum on the side of the tank, eventually damaging or dislodging the cannula and the operculum rubbed raw. These fish were not used in the analysis because blood could not be drawn repeatedly.

C. Plasma Cortisol Concentrations

Although no significant differences were observed in the data, some trends emerged. Average initial plasma cortisol concentrations, taken directly after surgery, were slightly elevated (13.4 ng/ml). This was likely due to the netting and air exposure that occurred during the capture procedure (Barton and Iwama 1991; Morgan et al. 1999; Davis et al. 2001; Sulikowski and Howell 2003; King and Berlinsky 2006; King et al. 2006). At 0.5 h and 1.0 h following surgery, average plasma cortisol concentrations decreased (2.5 ng/ml and 3.1 ng/ml, respectively). This can be explained by the properties of Metomidate, which is chemically related to the known cortisol blocker Etomidate (Vanden Bossche et al. 1984; Wagner et al. 1984). Once fish were anesthetized, it is likely that the Metomidate blocked the activities of the HPI axis, and prevented the release of cortisol during surgery. However, following the surgery, as fish recovered and became aware of the cannula, the average plasma cortisol concentrations began to rise. Following surgery, average plasma cortisol concentrations increased to 7.0 ng/ml after 3 hours and 43.8 ng/ml after 6 hours. Average plasma cortisol concentrations peaked 24 hours after the surgery at 30.1 ng/ml. Lo et al. (2003) found slightly elevated concentrations 24 hours after sampling, but instead peaked 3 and 5 days following sampling. In the present experiment, concentrations decreased on days 2 and 3 (8.4 ng/ml and 10.0 ng/ml, respectively). These differences could be due to variations among
species, experimental design, or their cannulation of the dorsal aorta as opposed to the afferent branchial artery (Wendelaar Bonga 1997; Barton et al. 2002). Plasma cortisol concentrations likely decreased 48 hours and 72 hours following the surgery because fish were acclimated to the cannula and recovered from the procedure.

There is some concern with cannulating the afferent branchial artery to sample blood for plasma cortisol analysis. In marine fishes, cortisol acts on the gills to stimulate the release of $\text{Na}^+$ and $\text{Cl}^-$ (Wendelaar Bonga 1997). The elevations in plasma cortisol, caused by stress, can interfere with the fish’s ability to maintain homeostasis, particularly at the gills (Wendelaar Bonga 1997). Thus, the gills serve an important function in dealing with stress, and the damage inflicted by cannulation on the gills and the resulting stress may exacerbate stress reaction in the fish. Because the gills play such a pivotal role in osmoregulation and stress management, it may be preferable to cannulate other sites on the fish that are less crucial to the stress response, although in the present study, the damage to the gills did not appear to cause a problem with the stress response or recovery.

Cannulation of the afferent branchial artery appears to be a viable form of repeated blood sampling from Atlantic cod, particularly over short periods of time (0-72 hrs). Some fish samples were taken for up to 8 days, but this was rare. Later surgeries were faster and problem free compared to earlier ones, as we became more familiar with procedures, and the cannula design was also perfected. Future cannulations would likely be more successful, and sampling would last longer, as techniques are refined. While cannulation of the afferent branchial artery is a viable method of blood sampling from Atlantic cod, it appears that the effects of the cannulation, both the acute stress of the
surgery and the chronic stress of having the cannula in place, have some effect on plasma cortisol concentrations for at least 48 hours. Therefore, one must wait at least 48 hours following surgery to allow circulating cortisol concentrations to return to pre-surgery conditions.
Conclusion

Atlantic cod, like other bony fishes, respond to stressors with elevated plasma cortisol concentrations. Through the three methods of repeated sampling (standard handling caudal vessel puncture, reduced handling caudal vessel puncture, and cannulation) it was found that:

- vessel puncture blood samples taken using a reduced handling method usually produced lower plasma cortisol concentrations than those obtained via standard methods of handling;
- repeated caudal vessel puncture samples, taken 72 hrs apart, do not necessarily produce plasma cortisol concentrations similar to those of the original “pre-stressed” sample;
- wild and cultured Atlantic cod respond differently to similar stressors
- cannulation is a viable method of repeated sampling for Atlantic cod, but with some difficulties; and
- repeated sampling via cannulation can produce plasma cortisol concentrations similar to baseline concentrations 48 hours following surgery.

Future work done to complement this research would include:

- a study to obtain baseline plasma cortisol concentrations from Atlantic cod in the wild, immediately following a rod and reel capture;
• a study comparing concentrations of plasma cortisol to other blood parameters, including glucose and estradiol or testosterone, during times of stress and recovery;
• a large scale discard mortality projects on the trawl, gillnet, and recreational fisheries for GOM cod that takes into account the barotraumas that occurs during capture, and allows fish to descend on their own; and
• additional cannulation projects that look to obtain plasma cortisol profiles for stressors of various types and intensity
Literature Cited


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Mattson, N.S. and T.H. Riple (1989). Metomidate, a better anesthetic for cod (Gadus morhua) in comparison with benzocaine, MS-222, chlorobutanol, and phenoxyethanol. Aquaculture 83: 89-94


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Specker, J. (June 7, 2007), Department of Biological Sciences, University of Rhode Island, Personal Communication


Project: A comparison of two blood sampling techniques in Atlantic cod

Approval Date: 28-Mar-2008

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. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2403.

For the IACUC,

[Signature]

Jessica Bulle, Ph.D.
Chair

cc: File