Sablefish, *Anoplopoma fimbria*, is a valuable North Pacific Ocean species caught in several commercial fisheries and is often discarded due to size or catch limits. Managers must account for the mortality of discarded fish to assess fish populations and harvest impacts, yet discard mortality rates of specific fisheries are generally unknown. Delayed mortality is one source of undetected mortality in discarded fish. The observance of delayed mortality in discards during laboratory and field studies suggest that capture stressors may induce physiological modifications that result indirectly in future mortality from stress and/or disease. Since the imposition of a stressor disrupts a fish's capability to maintain immunological function, understanding the capacity of the immune system to function after an acute bycatch stressor may be important in assessing the survival of discarded fish and further understanding the mechanisms behind delayed mortality.

The main objective of this study was to describe the immunological health of sablefish exposed to capture stressors. First, experiments were performed to obtain the conditions necessary for [*H]-thymidine incorporation into lymphocytes sensitive to B
and T cell mitogens. Then, in laboratory experiments designed to simulate the capture process, sablefish were subjected to various stressors that may influence survival: towing in a net, hooking, ecologically relevant temperatures of seawater and air, and air exposure time. Following the imposition of stress, the immunological assay was used to assess immune function.

The results demonstrated that regardless of fishing gear type, exposure to elevated seawater temperatures or air times, \([3H]\)-thymidine incorporation into leukocytes from stressed sablefish was significantly diminished in response to the T cell mitogen concanavalin A or the B cell mitogen lipopolysaccharide. Seawater or air temperatures were not found to significantly influence immune responses. The duration and severity of the capture stressors applied in our study were sufficient to induce significantly elevated levels of cortisol and glucose. However, there was no difference in the magnitude of plasma cortisol and glucose responses among stress treatments. These data suggest that immunological suppression occurs in sablefish subjected to capture-related stressors. Functional impairment of the immune system after exposure to capture-related stress is a potential reason for delayed mortality in discarded sablefish. As fishes are returned to the ocean, their ability to perform at the whole organism level may be diminished for an extended period of time and the cumulative strain from maintaining physiological adaptation can reduce their ability to sustain normal immunological functions. Consequently, immunological impairment may predispose a discarded fish to disease and eventual death. However, further studies are needed to determine if delayed
mortality in discarded sablefish can be caused by increased susceptibility to infectious agents resulting from stress-mediated immunosuppression.
CAPTURE-RELATED STRESSORS IMPAIR SABLEFISH, *ANOPLOPOMA FIMBRIA*, IMMUNE SYSTEM FUNCTION

by
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Sarah C. Lupes, Author
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CONTRIBUTION OF AUTHORS

Carl Schreck contributed in developing the experimental design, data analysis and interpretation, and editing of the manuscripts. Michael Davis contributed in developing the experimental design, providing expertise in laboratory capture-related stressors, subjecting sablefish to stressors, and editing of the manuscripts. Bori Olla assisted with the experimental design and provided expertise in working with sablefish. Mary Arkoosh assisted in the development of the immune assay, interpretation and analysis of the immunological data, and editing of the first manuscript.
TABLE OF CONTENTS

INTRODUCTION................................................................................................. .1

Sablefish............................................................................................................... .2

Capture-related Stressors................................................................................... .3

The Stress Response............................................................................................ .4

AN IN VITRO CULTURE SYSTEM FOR SABLEFISH, ANOPLOPOMA FIMBRIA, LYMPHOCYTE MITOGEN-INDUCED PROLIFERATION........................................... .9

Abstract.............................................................................................................. .9

Introduction.......................................................................................................... 9

Material and Results.......................................................................................... 12

Collection and maintenance of sablefish............................................................ 12

Isolation and cultivation of pronephric leukocytes............................................. 12

Osmolality measurements................................................................................... 14

Reagents and mitogens....................................................................................... 14

Kinetics and mitogenic dose assays.................................................................... 15

Leukocyte density............................................................................................... 16

Temperature........................................................................................................ 19

Hypotonic lysis verses density gradient centrifugation........................................ 19

Variability of mitogenic responses...................................................................... 21
## TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discussion</td>
<td>21</td>
</tr>
<tr>
<td>References</td>
<td>27</td>
</tr>
<tr>
<td>CAPTURE-RELATED STRESSORS IMPAIR SABLEFISH, <em>ANOPLOPOMA FIMBRIA</em>, IMMUNE SYSTEM FUNCTION</td>
<td>31</td>
</tr>
<tr>
<td>Abstract</td>
<td>31</td>
</tr>
<tr>
<td>Introduction</td>
<td>32</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>35</td>
</tr>
<tr>
<td>Fish collection and maintenance</td>
<td>35</td>
</tr>
<tr>
<td>Experimental procedure</td>
<td>35</td>
</tr>
<tr>
<td>Data analysis</td>
<td>38</td>
</tr>
<tr>
<td>Results</td>
<td>39</td>
</tr>
<tr>
<td>Discussion</td>
<td>46</td>
</tr>
<tr>
<td>References</td>
<td>56</td>
</tr>
<tr>
<td>CONCLUSION</td>
<td>61</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>62</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Proliferative responses of sablefish pronephric leukocytes to various concentrations of lipopolysaccharide (LPS) or concanavalin A (Con A)</td>
<td>17</td>
</tr>
<tr>
<td>2.</td>
<td>Stimulation indices (SI) for various numbers of sablefish pronephric leukocytes activated with 25 µg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A)</td>
<td>18</td>
</tr>
<tr>
<td>3.</td>
<td>Effects of in vitro culture temperatures on the proliferative responses of sablefish pronephric leukocytes to 25 µg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A)</td>
<td>20</td>
</tr>
<tr>
<td>4.</td>
<td>Proliferation responses of sablefish pronephric leukocytes to 25 µg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A) subjected to density gradient centrifugation or hypotonic lysis</td>
<td>22</td>
</tr>
<tr>
<td>5.</td>
<td>Mean (+SEM) stimulation indices (SI) of sablefish subjected to 2 h of hooking or towing at 8 °C, followed by 15 min air exposure at 10 or 16 °C in comparison to controls (unstressed at 8 °C)</td>
<td>40</td>
</tr>
<tr>
<td>6.</td>
<td>Mean (+SEM) stimulation indices (SI) of sablefish towed for 2 h at 8 °C followed by 15 min exposure to an elevated seawater temperature of 10 or 16 °C in comparison to controls (untowed at 8 °C)</td>
<td>42</td>
</tr>
<tr>
<td>7.</td>
<td>Mean (+SEM) stimulation indices (SI) of sablefish subjected to different air exposure times (15 or 30 min) and air temperatures (10 or 16 °C)</td>
<td>43</td>
</tr>
<tr>
<td>8.</td>
<td>Mean (+SEM) plasma cortisol concentrations (ng/ml) of sablefish subjected to various stressor treatments in comparison to controls (unstressed at 8 °C)</td>
<td>44</td>
</tr>
<tr>
<td>9.</td>
<td>Mean (+SEM) plasma glucose levels (mg/dl) of sablefish subjected to various stressor treatments in comparison to controls (unstressed at 8 °C)</td>
<td>47</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.1</td>
<td>Proliferative responses of leukocytes from pronephros of 25 non-reproductive 2 year-old sablefish to 25 μg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A) on day three of culture</td>
<td>23</td>
</tr>
</tbody>
</table>
CAPTURE-RELATED STRESSORS IMPAIR SABLEFISH, ANOPOLOPOMA FIMBRIA, IMMUNE SYSTEM FUNCTION

INTRODUCTION

The unaccounted mortality associated with the capture and return of non-targeted fish to the ocean, as discarded bycatch, is one of the primary issues currently affecting commercial fisheries management. Although some important steps have been taken to abate bycatch mortality, such as modifications in fishing gear technology (Broadhurst 2000), discard mortality rates of specific fisheries are generally unknown and often speculative (Chopin et al. 1995; Pascoe 1997; NMFS 2003). Delayed mortality is one source of undetected mortality in discarded fishes. The observance of delayed mortality in discards during field and laboratory studies indicates that capture stressors may induce physiological modifications that result indirectly in future mortality from predation, stress, and/or disease (Davis 2002). Since the imposition of a stressor disrupts a fish’s capability to maintain immunological functions (for reviews see: Barton and Iwama 1991; Wendelaar Bonga 1997; Schreck 2000), understanding the capacity of the immune system to function after exposure to a bycatch stressor may be important in assessing the survival of discarded fish and further understanding the mechanisms behind delayed mortality.

In order to assess the effects of capture-related stress on the immune function of sablefish, Anoplopoma fimbria, an important commercial species, we needed to initially establish an assay to characterize or monitor the immunological health of sablefish. In the first chapter of this thesis, we report a number of experiments that evaluated
the conditions necessary to optimize a tritiated-thymidine lymphocyte incorporation assay sensitive to B and T cell mitogens. In the second chapter, our objective was to assess the immunological health of sablefish exposed to capture stressors. Therefore, in laboratory experiments designed to simulate the capture process, we subjected sablefish to various stressors that may influence survival: towing in a net, hooking, ecologically relevant temperatures of seawater and air, and air exposure time. After the application of stressors, the proliferation assay described in the first chapter was used to assess the sablefish immune system function.

Sablefish

Sablefish is an important North Pacific Ocean species that when not targeted in a commercial fishery (McFarlane and Beamish 1983a) is often discarded due to size or catch limits. Sablefish are susceptible to overexploitation due to their long lives with ages over 50 years regularly recorded (Kimura et al. 1983; McFarlane and Beamish 1983b) and their highly migratory nature (Maloney and Heifetz 1997; Rutecki and Varosi 1997) with distribution from northern Mexico to the Gulf of Alaska, westward to the Aleutian Islands, and into the Bering Sea (Wolotira et al. 1993). Sablefish abundance is dependent on strong year classes (McFarlane and Beamish 1992) with juveniles providing the primary source of recruitment into the sablefish fishery (Sigler et al. 2001). During near-shore residency, juvenile survival is associated with sea temperature (Sogard and Olla 1998) and food abundance (McFarland and Beamish 1992). Juvenile sablefish are very susceptible to trawl fisheries during near-shore residency and their incidental
discard can adversely effect sablefish recruitment (Sampson et al. 1997; Sigler et al. 2001). The size-selective price structure of the commercial industry and the minimum size limits also result in the discard of smaller fish potentially leading to greater discard mortality (Schirripa and Methot 2002; Davis and Parker 2004).

Capture-related Stressors

During the capture process, mortality in fish may result from the interactions of various stressors including initial capture, environmental factors, and handling (Davis 2002). Temperature is one of the primary interacting factors that can contribute to increased discard mortality rates. Sablefish are often caught at depths ranging from 155 to 1350 m with long-line or trawl capture methods (Olla et al. 1998; Schrippa and Methot 2002). During the retrieval of fishing gear, sablefish are often exposed to rapid temperature changes ranging from 6 to 17 °C (Olla et al. 1998). Fishing in warmer months will result in sablefish exposed to elevated surface seawater temperatures that may induce greater rates of bycatch mortality. Laboratory and field studies have documented increased stress and mortality related to increased temperature (Barton and Iwama 1991; Olla et al. 1998; Davis et al. 2001; Davis 2002, 2004 in review).

Air exposure is also a critical factor that can increase the level of stress and mortality during the bycatch process (Davis 2002). Air exposure is equivalent to the amount of time a fish remains on deck and the temperature of air. The handling time of a fish is dependent on the size of the catch and the type of gear employed, which can lead to air exposures ranging from several minutes to an hour. The air temperature to which a
fish is exposed on deck will vary in accordance with the time of year fished. Fishing in an el niño year and in warmer seasons will result in exposure to warmer temperatures of air on deck. While sablefish are sensitive to increased temperature, they are considered relatively resilient in air, as mortality in laboratory experiments did not occur until after 30 minutes of air exposure (Davis and Parker 2004).

An important biological factor that modulates stress and mortality is the size of the fish. As commercial value is related to size in the sablefish fishery, there is an incentive to retain larger fish and discard smaller, less profitable fish. In the laboratory, mortality resulting from bycatch stressors differed between size classes of sablefish with smaller fish being more sensitive to the capture process (for review see: Davis and Parker 2004). Smaller sablefish reached higher body core temperatures more quickly than larger fish (Davis 2001). Consequently, smaller sablefish reached 100% mortality at a lower temperature when exposed to gradients of increasing temperatures in laboratory experiments (Davis and Parker 2004).

The Stress Response

The stress response results in physiological and behavioral changes that can be quantitatively measured to reflect the degree of stress a fish experiences (Barton and Iwama 1999). Responses to stress have been classified according to primary, secondary, and tertiary changes that reflect the different levels of biological organization (Mazeaud and Mazeaud 1981; Wedemeyer and McLeay 1981; Wedemeyer et al. 1990). Primary responses are neuroendocrine changes dominated by the hypothalmic-sympathetic-
chromaffin cell axis and the hypothalmic-pituitary interrenal axis (Colombo et al. 1990; Wendelaar Bonga 1997). After a stressful stimulus is perceived by the central nervous system (CNS), catecholamines (CA), epinephrine and norepinephrine, are secreted from chromaffin cells in the pronephros (anterior kidney), stimulating immediate responses in the cardiovascular system, respiratory and osmoregulatory functions, and metabolism (Wedelaar Bonga 1997). Within the HPI axis, the hypothalamus controls the release of the corticotrophin-releasing hormone (CRH) and the thyrotropin-releasing hormone (TRH). These hormones stimulate the pituitary to secrete adrenocorticotropic hormone, alpha melanocyte-stimulating hormone (α-MSH) and β-endorphine, which then controls the production of cortisol in the interrenal cells of the head kidney. Cortisol is important in maintaining homeostasis and mobilizing energy reserves. The elevation of corticosteroids, mainly cortisol, is a widely used indicator of monitoring the severity and duration of the primary stress response. Measurement techniques for cortisol are well established, and are functionally significant in physiological processes affecting fish health (Donaldson 1981; Barton and Iwama 1991).

The secondary stress responses are the blood and tissue modifications that result from the actions of the primary response (specifically CA and cortisol). Secondary changes are apparent in blood and tissue chemistry, hydromineral balance, and hematology, and can include elevated blood sugar and lactate levels (Wedemeyer et al. 1990). Hyperglycemia is initially induced by CA stimulation of glycogenolysis in the liver, and may then be maintained by cortisol action. Measures of glucose are also quick and easy to perform and have been frequently used as indicators of secondary stress responses (Barton et al. 1986).
Tertiary or whole animal (Barton and Iwama 1991) stress responses include changes in metabolic rate, health, behavior, growth, reproduction success, and mortality rates (Pickering 1981; Colombo et al. 1990; Wedemeyer 1990). They have the ability to predict the effects of stress at the population or community levels (Wedemeyer et al. 1984). Though the tertiary changes may be more difficult to assess, they have the highest "ecological relevance" (Adams 1990). Comparisons of measures of stress responses from different biological levels will help to enhance the ecological value of a particular measure and will further illustrate the dynamics of the stress response.

Situations that push physiological systems beyond homeostasis can result in what Sterling and Eyer (1998) have defined as allostasis: the ability of an organism to increase or decrease vital functions to achieve a new form of stasis (Sterling and Eyer 1988; McEwen 1998). Yet, the cumulative strain on the organism from maintaining this allostatic response to stress can result in an allostatic load (McEwen and Stellar 1993), and consequently reduce the organism’s ability to maintain normal physiological functions (Pruett 2003).

The interactions of various capture stressors such as trawling, hooking, temperature and air exposure have been studied in laboratory sablefish with the stress response measured as changes in behavior, physiology, and mortality (Olla et al. 1997, 1998; Davis et al. 2001; Spencer 2000; Davis 2002, Davis and Parker 2004). Gradients of increased bycatch stressors amplified physiological indices of stress such as plasma cortisol, glucose, lactate, potassium and sodium (Olla et al. 1997, 1998; Davis 2002). Though the magnitude of the stress response was related to the intensity of the stressor
there was no correspondence between mortality and physiological measures of stress (Davis et al. 2001).

Since the imposition of a stressor disrupts a fish's capability to maintain immunological function, understanding the capacity of the immune system to function after a bycatch stressor may be important in assessing the survival of discarded fish and further understand the mechanisms behind delayed mortality. It is known that stress can have long-lasting negative effects on the general health of mammals (Dhabhar and McEwen 2001) and teleosts (for reviews see: Anderson 1990; Barton and Iwama 1991, Schreck 1996; Wendelaar Bonga 1997; Weyts et al. 1999). Correlations of immunosuppression and disease outbreaks in fish have been demonstrated in various laboratory settings (Anderson 1990).

Several techniques have been developed and used for monitoring the immune competence of fish (Anderson 1990). Immunological assays have been standardized for many freshwater (Rosenberg-Wiser and Avtalion 1982; Clem et al. 1984; Kaattari and Yui 1987; DeKoning and Kaatarri 1991) and marine fish (Reitan and Thuvander 1991; Arkoosh et al. 1994; LoPresto et al. 1995) to demonstrate changes due to natural and anthropogenic stressors. Various cellular immune functions in fish have been investigated to determine the status of the immune system (Ellasesser and Clem 1986; Yin et al. 1995; Ortuno et al. 2002). The proliferative responses of leukocytes to a mitogen can be considered a reasonable approximation to show that the immune system of a fish is capable of functioning (Ellasesser and Clem 1986; Luft et al. 1991; Faisal and Hargis 1992). However, to date, there are no established techniques to characterize or monitor the immunological health of sablefish. An assay to measure the responses of fish
leukocytes to *in vitro* stimulation is one method of assessing the function of the sablefish cellular immune system. The ability to assess and measure the immune responses of fish in laboratory-simulated bycatch experiments may lead to further insights regarding the delayed mortality of bycatch (Davis 2002). Further comprehension of the effects of the interactions of capture and environmental stressors on sablefish are critical for providing understanding of what controls delayed discard mortality.
AN IN VITRO CULTURE SYSTEM FOR SABLEFISH, *ANOPLOPOMA FIMBRIA*, LEUKOCYTE MITOGEN-INDUCED PROLIFERATION

Abstract

An *in vitro* cell culture system that supports the proliferative responses of pronephric leukocytes to the B cell mitogen lipopolysaccharide (LPS) and the T cell mitogen concanavalin A (Con A) was established for sablefish, *Anoplopoma fimbria*, a marine teleost. Proliferation was assessed by leukocyte $[^3]$H-thymidine incorporation and expressed as a stimulation indices calculated as the ratios of counts per minute (cpm) of mitogen-stimulated cultures divided by the cpm of unstimulated cultures. Minimal Essential Media made isotonic to sablefish plasma and supplemented with fetal calf serum aided mitogen stimulation. Pronephric leukocytes exhibited similar responses to both mitogens, with maximum stimulation achieved after three days of culture at 17 °C. A higher degree of proliferation occurred in response to LPS when leukocytes were purified from erythrocytes using density gradient centrifugation versus hypotonic lysis. In contrast, the magnitude and kinetic responses of leukocytes to Con A was unaffected by hypotonic lysis, suggesting that sablefish leukocytes that are activated by LPS could be more sensitive to changes in the isotonicity of the surrounding media. These data imply that the *in vitro* mitogenic stimulation of pronephric leukocytes can be utilized for monitoring the immunological health of sablefish in aquaculture and natural populations.

Introduction
Sablefish, *Anoplopoma fimbria*, is an important North Pacific Ocean species in commercial fisheries and aquaculture. Intensive aquaculture practices that can lead to stress-mediated immunosuppression and the need to develop procedures to control disease necessitate the development of methods to measure the immune functions of sablefish. Sablefish are also caught in several commercial fisheries and are often discarded due to size or catch limits. This species is susceptible to overexploitation due to their long lives, with ages over 50 years regularly recorded (Kimura et al. 1983; McFarlane and Beamish 1983) and their highly migratory nature (Maloney and Heifetz 1997; Rutecki and Varosi 1997) with distribution from northern Mexico to the Gulf of Alaska, westward to the Aleutian Islands, and into the Bering Sea (Wolotira et al. 1993). As there is substantial discarding of sablefish in the Northeastern Pacific commercial fishery industry (Sampson et al. 1997), the ability to assess and measure the immune responses in laboratory-simulated bycatch experiments may lead to further insights regarding discard delayed mortality (Davis 2002). However, currently there are no validated techniques to characterize or monitor the immunological health of sablefish.

Sablefish are members of the large order Scorpaeniformes that share the common character of a suborbital stay, formed by the second infraorbital crossing the cheek from the orbit to the preopercle (Bond 1996). Anoplopomatidae have been largely unstudied physiologically, with no information currently on disease resistance capacity. Investigations of the immune functions in species not commonly studied could facilitate further insights into the teleost immune system.

Several techniques have been developed and used for monitoring the immune competence of fish (Weyts et al. 1999). The *in vitro* stimulation of fish leukocytes from

The proliferative responses of leukocytes to a mitogen can be considered a reasonable first approximation to show that the immune system of a fish is capable of functioning (Ellasesser and Clem 1986). However, to date, there are no established techniques to characterize the immunological health of sablefish. The basic aim of this research is to establish an *in vitro* culture system that supports the mitogenic response of sablefish pronephric leukocytes. We report a number of experiments that evaluated the conditions necessary to maximize a tritiated-thymidine lymphocyte incorporation assay
sensitive to B and T cell mitogens. Studies of sablefish cellular immune function can then be used to monitor the health of sablefish in aquaculture and in natural populations.

Methods and Results

Collection and maintenance of sablefish

Juvenile sablefish [20-40 mm total length (TL)] were collected in spring of 2001 offshore of Newport, Oregon and reared at Hatfield Marine Science Center in circular tanks (2.0 m diameter, 0.8 m depth, 31401 volume) supplied with sand-filtered and UV sterilized flow-through seawater (30-32% salinity, 10-13°C, O₂ >90% saturation) at a replacement rate of 101·min⁻¹. Fish were fed to satiation on pelletized salmon food three times a week. During the second year, the fish were maintained at 30-40 fish in large circular tanks (4.5m diameter, 1.0 m depth) supplied with seawater (201·min⁻¹, 30-32% salinity, O₂ >90% saturation, 5.5-7.5°C) and fed a diet of squid, Loligo opalescens, until satiated two times a week.

Isolation and cultivation of pronephric leukocytes

Non-reproductive 2-year-old sablefish were rapidly netted and immediately placed in a lethal dose (400 ppm) of tricaine methanesulfonate (MS-222). The caudal peduncle of each fish was severed and the blood drained in an effort to reduce red blood cells count in the tissues. Individual pronephros were harvested into tissue culture media
(TCM) under aseptic technique. Cells were separated from the surrounding tissue by gently teasing the tissue with the end of a 3 ml syringe plunger through a 40 μm Falcon cell strainer. The single cell suspension was diluted to a total volume of 10 ml with TCM in 50 ml conical tubes (Becton Dickinson) and then washed by centrifugation at 500g for 15 min at 17 °C. Subsequently, the supernatant was decanted, the cellular pellet resuspended with 10 ml of TCM and centrifuged again at 500g for 15 min at 17 °C. Using a hemocytometer, leukocytes from the single cell suspension were counted using the trypan blue exclusion method, and adjusted to $2 \times 10^7$ cells/ml.

Erythrocytes and leukocytes from the kidney were separated according to techniques described by Kaattari and Holland (1990) and adapted by Arkoosh et al. (1994). Briefly, 10 ml of a whole kidney cell suspension ($2 \times 10^7$ cells/ml) were slowly layered over an equivalent volume of Histopaque in 50 ml conical tubes and centrifuged at 500 g for 45 min at 17 °C. The buffy layer of leukocytes at the interface was removed with a pasteur pipette, TCM added for a total volume of 10 ml, and the cells centrifuged at 500g for 15 min at 17 °C. The leukocyte pellet was resuspended in 10 ml of TCM and centrifuged again. After the second wash, the supernatant was aspirated and the pellet resuspended with 0.5 ml TCM. Using the trypan blue exclusion method to determine viability, the isolated populations of leukocytes were counted with a hemocytometer and diluted to $5 \times 10^7$ cells/ml with TCM. For the proliferation assay, cells were added in triplicate to flat bottom 96 well tissue culture plates (Becton Dickinson) at $5 \times 10^7$ cells/ml in 100 μl of TCM, and stimulated with 100 μl aliquots of the appropriate concentration of mitogen. The cultures were maintained at 17 °C in a humidified incubation chamber (C.B.S. Scientific Co.) containing a blood gas mixture (10% O₂, 10%
CO₂, and 80% N₂) and fed on alternative days with 10 µl of a modified nutritional supplement (Kaattari et al. 1986).

**Osmolality measurements**

Osmolalities of freshly separated plasma from 15 individual sablefish and the prepared TCM were measured using a vapor pressure osmometer.

**Reagents and mitogens**

The medium for isolation and maintenance of leukocytes was prepared as described in Milston et al. (2003) and Misumi (2004) but adjusted according to sablefish blood osmolality of approximately 380 mosm. The TCM consisted of a 7% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine (100X), and 9% tumor cocktail in Minimum Essential Media (MEM). The tumor cocktail was prepared by adding 7.5 g dextrose, 75 ml essential amino acids (50X), 100 ml non-essential amino acids (100X), and 100 ml sodium pyruvate (100X), to 630 ml of MEM. The mixture was adjusted to a pH of 7.0 with 10 N NaOH before adding 8.5 g of sodium bicarbonate, 340 mg penicillin, 0.2 mg ml⁻¹ streptomycin sulfate, 0.1 mg ml⁻¹ gentamycin, and 34 µl β2-mercaptoethanol. The cocktail was dispensed through a 0.45 µm filter, aliquotted into 45 ml per conical tube and stored at −20 °C. MEM was purchased from Invitrogen Co., and all other reagents were purchased from Sigma Chemical Co.
The nutritional supplement was prepared by a modification of the method of Kaattari and Holland (1990). Fifteen microliters of TCM was added to 7.5 ml of heat-inactivated fetal bovine serum, 0.347 ml HEPES buffer, 1.0 ml guanosine in TCM (1.0 mg/ml), and a 1 ml mixture containing equal parts adenosine, uridine, and cytidine in TCM (1.0 mg/ml). The solution was adjusted to a pH of 7.4 with 10 N NaOH, filtered through 45 μm filter (Corning), and stored at −20 °C.

Mitogens were purchased from Sigma Chem. Co. Concanavalin A (Con A) a polyvalent carbohydrate-binding protein from *Canalvalia ensiformis* and lipopolysaccharide (LPS) from the bacterium *Echerichia coli* serotype O 55:B5 were obtained as aseptic powders and reconstituted with TCM to a stock solution (10 mg/ml). The stock solution was diluted further to obtain the appropriate concentrations of mitogen per well, and filtered (0.45 μm filter) before use.

*Kinetic and mitogen dose response assays*

To determine the optimal dose of mitogen and to establish the peak day of mitogen-induced proliferation, pronephric leukocytes from three sablefish were incubated in triplicate at 17 °C with concentrations of 5, 25, 150, and 250 μg/well of LPS or 0.5, 5, 25, 50 μg/well of Con A. On days 0, 2, 4, or 6 of culture, 1 μCurie (μCu) of [³H]-thymidine in 10 ul of nutrient supplement was added to each well. Twenty-four hours after the [³H]-thymidine was added, the leukocytes were harvested from their wells with a Wallac Tomtec cell harvester onto a glass fiber filtermat (Wallac). The filtermat was dried and sealed in a plastic filter bag with 4.5 ml of optiphase scintillation fluid
Proliferation was assessed by $[^3 \text{H}]-\text{thymidine}$ incorporation measured as counts per minute (cpm) by a Wallac liquid scintillation counter. Data were derived from triplicate determinations for each fish. The proliferative responses, over background, induced by either LPS or Con A were expressed as stimulation indices (SI), calculated as the ratio of mean counts per minute (cpm) of mitogen-stimulated cultures divided by the mean cpm of non-stimulated cultures. Maximum mitogenic responses for both LPS and Con A were observed on day three of culture (Figure 1). A concentration of 25 μg/ml for each mitogen was determined as the dose required to create a functioning assay.

**Leukocyte density**

To determine the leukocyte density that produced the greatest SI, pronephric leukocytes ranging from concentrations of $5 \times 10^4$ to $5 \times 10^7$ cells/ml were stimulated in triplicate with the optimal concentration of either LPS or Con A and incubated at 17 °C. The cells were subsequently harvested on the culture day that produced the greatest mitogenic response. One μCi of $[^3 \text{H}]-\text{thymidine}$ was added to the leukocytes activated with either LPS or Con A on day 2 of incubation and the cells were harvested and counted on day 3 as described above. A density of $5 \times 10^6$ leukocytes/ml produced the greatest proliferative response to LPS and Con A (Figure 2) and was used in subsequent studies.
Figure 1: Proliferative responses of sablefish pronephric leukocytes to various concentrations of lipopolysaccharide (LPS) or concanavalin A (Con A). The proliferative responses are expressed as stimulation indices calculated as the ratios of \[^{3}\text{H}\]-thymidine uptake of the mitogen stimulated cultures divided by the \[^{3}\text{H}\]-thymidine uptake of the non-stimulated cultures. Data are derived from the means of three sablefish (± SEM) with triplicate determinations for each fish. Control cultures (without mitogen) had a mean count per minute of less than 400.
Figure 2: Stimulation indices (SI) for various numbers of sablefish pronephric leukocytes activated with 25 μg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A). Leukocytes were incubated at 17 °C and harvested on day three of culture. The proliferative responses are expressed as SI calculated as the ratios of [$^3$H]-thymidine uptake of the mitogen stimulated cultures divided by the [$^3$H]-thymidine uptake of the non-stimulated cultures. Data are derived from the means of three sablefish (+SEM) with triplicate determinations for each fish. Control cultures (without mitogen) had a mean count per minute of less than 400.
Temperature

The leukocyte density, mitogen concentration, and duration of culture yielding the maximum mitogenic proliferation were used to determine if a different in vitro temperature would enhance the mitogen-induced proliferative response. The leukocytes were cultured at three temperatures 8 °C, 12 °C, and 17 °C. Cells were pulsed with 1μCu \([^{3}\text{H}]\)-thymidine on day two of culture, harvested twenty-four hours later and counted. Sablefish kidney leukocytes assessed on day 3 of culture maintained a maximum SI for both LPS and Con A at 17 °C (Figure 3). At 8 °C and 12 °C the mitogenic responses to LPS were approximately at a SI of 1.0, while at 12 °C a SI of greater than 2.0 was maintained for Con A (Figure 3).

Hypotonic lysis versus density gradient centrifugation

To determine if a greater SI could be produced with hypotonic lysis instead of density centrifugation, leukocytes were separated from erythrocytes as described by Crippen et al. (2001). Briefly, 2 ml of whole cell suspension was diluted with 9 ml sterile distilled water, the erythrocytes lysed for 20 s, and the cells returned to isotonicity with 1 ml 10X phosphate-buffered saline (PBS). The suspension was centrifuged at 500 g for 7 min at 17 °C, the supernatant aspirated and the pellet resuspended in 2 ml of TCM. Isolated leukocytes at a density of 5 x 10^6 were stimulated with 25 μg/ml of LPS or Con A and kinetic assays were performed in triplicate at 17 °C. On days 0, 2, 4, or 6 of incubation, the cultures were pulsed with 1 μCu \([^{3}\text{H}]\)-thymidine in 10 μl of nutrient
Figure 3: Effects of *in vitro* culture temperatures on the proliferative responses of sablefish pronephric leukocytes to 25 μg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A). Leukocytes were incubated at 8 °C, 12 °C, and 17 °C and harvested on day three of culture. The proliferative responses are expressed as stimulation indices calculated as the ratios of \[^3\text{H}\]-thymidine uptake of the mitogen stimulated cultures divided by the \[^3\text{H}\]-thymidine uptake of the non-stimulated cultures. Data are derived from the means of three sablefish (+ SEM) with triplicate determinations for each fish. Control cultures (without mitogen) had a mean count per minute of less than 400.
supplement, harvested 24 h later, and counted. The greatest SI for LPS was induced on
day 3 of culture with cells separated by density gradient centrifugation (Figure 4). While
the LPS proliferative response was suppressed following hypotonic lysis, the magnitude
and the kinetics of the response to Con A was relatively unaffected by hypotonic lysis
(Figure 4).

Variability of in vitro mitogenic responses

Using the culture conditions previously determined to be optimal, the degree of
variability in the proliferative response to a mitogen was assessed in 25 non-reproductive
2-3 year-old sablefish. Pronephric leukocytes of sablefish were stimulated in triplicate
with either 25 µg/ml of LPS or Con A, pulsed with [³H]-thymidine on day 2 of culture
and harvested 24 hours later. The stimulation indices ranged from 1.7 to 7.8 for LPS and
1.9 to 10.9 for Con A, with a mean SI of 5.0 and 5.8 for LPS and Con A, respectively
(Table 1.1).

Discussion

Leukocytes from the pronephros of the marine teleost Anoplopoma fimbria can be
stimulated to proliferate in vitro with mitogens that are designated as B or T cell
activators in mammals (Ellis 1977). In this study, in vitro cell culture conditions were
defined with the mitogens LPS and Con A that will allow for future evaluation of the
Figure 4: Proliferation responses of sablefish pronephric leukocytes to 25 μg/ml of either lipopolysaccaride (LPS) or concanavalin A (Con A) subjected to density gradient centrifugation or hypotonic lysis. The proliferative responses are expressed as stimulation indices calculated as the ratios of $[^3H]$-thymidine uptake of the mitogen stimulated cultures divided by the $[^3H]$-thymidine uptake of the non-stimulated cultures. Data are derived from the means of three sablefish (± SEM) with triplicate determinations for each fish. Control cultures (without mitogen) had a mean count per minute of less than 400.
Table 1.1: Proliferative responses of leukocytes from pronephros of 25 non-reproductive 2 year old sablefish to 25 μg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A) on day three of culture. The proliferative response for each individual fish is expressed as a stimulation index calculated as the ratio of $[^3]$H-thymidine uptake of the mitogen stimulated cultures divided by the $[^3]$H-thymidine uptake of the non-stimulated cultures. All non-stimulated cultures (without mitogen) had a mean cpm less than 500.

<table>
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Mean (SE) 5.1 (1.9) 5.8 (2.3)
immunological health of sablefish. The sablefish culture system employed the process of adapting mammalian cell culture procedures and medium, which is very similar to the methods used by other teleost systems developed for assessing leukocyte function. Modifications to the TCM included the addition of sodium chloride to be isosmotic with sablefish plasma and the supplementation of 7% heat-inactivated FCS. Although some studies have revealed that the utilization of homologous plasma in leukocyte cultures enhanced the mitogenic response in salmonid pronephros (DeKoning and Kaatari 1992), and the peripheral blood leukocytes of channel catfish (Faulmann et al. 1983), other studies showed that leukocytes from marine species can proliferate in media supplemented with only FCS (Faisal and Hargis 1992; Arkoosh et al. 1994; Galeotti et al. 1999).

Both Con A and LPS at a concentration of 25 μg/ml, induced similar increases in mitotic activities in sablefish pronephric leukocytes when compared to their respective controls. However, in the pronephros of different species the mitogenic stimulation may vary with the type of mitogen presented. Leukocytes from the pronephros showed a differential response to mitogens in chinook salmon (Leith et al. 1984) and Atlantic menhaden, Brevoortia tyrannus (Faisal and Hargis 1992), with cells exposed to LPS responding better than those receiving T cell mitogens, Con A or phytohemagglutinin (PHA). Con A failed to induce any significant mitotic stimulation in pronephric leukocytes in sea bass (Galeotti et al. 1999). Although the magnitudes of the responses of sablefish pronephric leukocytes to Con A and LPS are lower than SI reported for salmonids (Kaattari and Yui 1987; DeKoning and Kaattari 1992), they are similar to those reported for English sole (Arkoosh et al. 1994), plaice, Pleuronectes platessa.
(Grimm 1985), and Atlantic menhaden (Faisal and Hargis 1992). These interspecific variations could be due to differences in mitogen-receptors on leukocytes or differences in leukocyte populations within the pronephros of different species. The responsiveness of the pronephric leukocytes in sablefish may also be reflective of the cultural conditions.

As previously demonstrated in carp (Le Morvan-Rocher et al. 1995) and bluegill, *Lepomis macrochirus* (Cuchens and Clem 1987), the mitogenic response in sablefish was affected by *in vitro* temperature. In channel catfish (reviewed in Bly and Clem 1991), the magnitude of proliferative response of peripheral blood leukocytes to LPS was relatively independent of *in vitro* temperature while the responses to Con A were suppressed at lower temperatures (Clem et al. 1984). In our studies, sablefish proliferative responses to Con A were clearly inhibited at both 8 and 12°C, while pronephric leukocytes were minimally stimulated at 12 °C by LPS. However, whether this temperature sensitivity is due to changes in mitogen kinetics or magnitude cannot be addressed with our data. Bly and Clem (1991) emphasized the importance of *in vivo* temperature in the *in vitro* activation of fish leukocytes, channel catfish cells responded to Con A with maximum stimulation at an *in vitro* temperature 5 °C greater than the *in vivo* holding temperatures. Sablefish acclimated at approximately 8 °C produced the greatest stimulation to both LPS and Con A at an *in vitro* temperature 9 °C greater than their *in vivo* temperature.

Typically, teleost leukocytes from tissues are isolated by a density gradient centrifugation through a medium such as Percoll or Histopaque. Crippen et al. (2001) described a technique that uses hyptonic lysis to separate leukocytes from peripheral blood and pronephros of salmonids. The present study demonstrates that sablefish leukocytes isolated by hypotonic lysis were unable to respond to LPS. Separation
techniques employing hypotonic lysis should be exercised with caution when measuring
the proliferative ability of leukocytes to LPS, especially in marine teleosts. Although
the relative magnitude and kinetic response to Con A was relatively unaffected by
hypotonic lysis, sablefish leukocytes that are activated by LPS could be more sensitive to
changes in the isotonicity of the surrounding media.

The present study demonstrates that pronephric tissue is suitable for use in in vitro
assays to evaluate the proliferative responses of sablefish leukocytes to classic
mammalian B and T cell mitogens. The development of an in vitro culture system for
sablefish leukocytes will allow for future assessment of the function of sablefish cellular
immune system in aquaculture and natural populations.
References


Abstract

Sablefish, *Anoplopoma fimbria*, is a valuable North Pacific Ocean species that, when not targeted in various commercial fisheries, is often a part of discarded bycatch. Predictions of the survival of discarded fish are dependent on understanding how a fish responds to stressful conditions. Our objective was to describe the immunological health of sablefish exposed to capture stressors. In laboratory experiments designed to simulate the capture process, we subjected sablefish to various stressors that may influence survival: towing in a net, hooking, ecologically relevant temperature of seawater and air, and air exposure time. Following the imposition of stress, the *in vitro* mitogen-stimulated proliferation of sablefish leukocytes was used to assess the function of the immune system in an assay we validated for this species. The results demonstrated that the proliferative response of leukocytes from stressed sablefish, regardless of fishing gear type, exposure to elevated seawater temperatures or air times, had significantly diminished responses to the T cell mitogen concanavalin A or the B cell mitogen lipopolysaccharide. A significant difference in the immunological response associated with seawater or air temperature was not detected in sablefish. The duration and severity of the capture stressors applied in our study were harsh enough to induce significantly elevated levels of cortisol and glucose, but there was no difference in the magnitude of levels among stress treatments. These data suggest that immunological suppression occurs in sablefish subjected to capture-related stressors. The proposal that the immune system is functionally impaired after exposure to capture-related stress suggests a
potential reason why delayed mortality is possible in discarded sablefish. Further studies are needed to determine if delayed mortality in sablefish discards can be caused by increased susceptibility to infectious agents resulting from stress-mediated immunosuppression.

Introduction

The unaccounted mortality associated with the capture and release of non-targeted fish to the ocean as discarded bycatch is one of the primary issues currently affecting commercial fisheries management. Managers need to account for the mortality of discarded fish to accurately assess fish populations and harvest impacts, yet discard mortality rates of specific fisheries are generally unknown (Chopin et al. 1995; Pascoe 1997; NMFS 2003). Sablefish, *Anoplopoma fimbria*, is a valuable North Pacific Ocean species caught in several commercial fisheries and is often discarded due to size or catch limits. Sablefish are susceptible to overexploitation due to their long lives with ages over 50 years regularly recorded (Kimura et al. 1983; McFarlane and Beamish 1983) and their high migratory nature (Maloney and Heifetz 1997; Rutecki and Varosi 1997) with distribution from northern Mexico to the Gulf of Alaska, westward to the Aleutian Islands, and into the Bering Sea (Wolotira et al. 1993). Sablefish abundance is dependent on strong year classes (McFarlane and Beamish 1992) with juveniles providing the primary source of recruitment into the sablefish fishery (Sigler et al. 2001). Juvenile sablefish are very susceptible to trawl fisheries during near-shore residency and their incidental discard can adversely effect sablefish recruitment (Sampson et al. 1997; Sigler...
et al. 2001). The size-selective price structure of the commercial industry and the minimum size limits also result in the discard of smaller fish potentially leading to greater discard mortality (Schirripa and Methot 2003; Davis and Parker 2004).

Delayed mortality is one source of undetected mortality in discarded fishes. Studies of fish held in the laboratory or field after capture-related stress, have indicated that discard delayed mortality can vary with species of fish and the presence of physical injury (Davis 2002). For example, in laboratory studies where fish were held for 60 days, delayed mortality has been observed up to 30 days in halibut, *Hippoglossus stenolepis*, 14 days in walleye pollock, *Theragra chalcogramma*, and 35 days in sablefish with physical injury (Davis 2002; 2004 in review). The observance of delayed mortality in discards indicates that capture stressors may induce physiological modifications that result indirectly in future mortality from predation, stress, and/or disease (Davis 2002).

Fish, like other vertebrates, respond to stressful stimuli through a neuroendocrine cascade resulting in physiological and behavioral responses. After the stressor is perceived, a physiological resistance phase follows leading to adaptation or compensation. If the stress is overly severe and enduring, compensation may not be feasible and exhaustion leads to mortality (Selye 1950; Schreck 2000). Situations that push physiological systems beyond homeostasis can result in what Sterling and Eyer (1998) have defined as allostasis: the ability of an organism to increase or decrease vital functions to achieve a new form of stasis (Sterling and Eyer 1988; McEwen 1999). Yet, the cumulative strain on the organism from maintaining this allostatic response to stress can result in an allostatic load (McEwen and Stellar 1993). Over time, the maintenance
of allostasis reduces the organism's ability to maintain normal physiological functions (Pruett 2003).

Predictions of the survival of discarded fish are dependent on understanding how a fish responds to stressful conditions. The capacity of fish to respond to a stressor can be measured by biological, physiological, and behavioral changes that demonstrate the degree of stress expressed (Wedemeyer et al. 1990). Commonly measured stress indicators in sablefish such as plasma cortisol, lactate, and glucose, though useful in quantifying the stress response, have not been correlated directly with capture-related mortality (Davis et al. 2001; Davis 2002).

Since the imposition of a stressor disrupts a fish's capability to maintain immunological function, understanding the capacity of the immune system to function after an acute bycatch stressor may be important in assessing the survival of discarded fish and further understanding the mechanisms behind delayed mortality. It is known that stress can have long-lasting negative effects on the general health of mammals (Dhabhar and McEwen 2001) and teleosts (for reviews see: Anderson 1990; Barton and Iwama 1991; Schreck 1996; Wendelaar Bonga 1997; Weyts et al. 1999). Correlations of immunosuppression and disease outbreaks in fish have been demonstrated in various laboratory settings (Anderson 1990).

The objective of this study was to examine, in the laboratory, the effects of non-lethal capture-related stressors on the immune function of the sablefish. The proliferative response of leukocytes to a mitogen was utilized, in an assay we previously developed and validated for sablefish, as a method of quantifying immune functions. Further comprehension of the effects of the interactions of capture and environmental stressors on
sablefish are critical for providing understanding of what controls delayed discard mortality. Knowledge of the immune response after the imposition of capture-related stressors may lead to a better understanding of why fish die after capture.

Methods and Materials

Fish collection and maintenance

Juvenile sablefish [20-40 mm total length (TL)] were collected with neuston nets in the spring of 2001 about 50 km offshore of Newport, Oregon and reared at the Hatfield Marine Science Center (HMSC) in circular tanks (2.0 m diameter, 0.8 m depth, 31, 401 volume) supplied with sand-filtered and UV sterilized flow through seawater (29-32°/oo salinity, 8-11°C, O₂ >90% saturation) at a replacement rate of 101·min⁻¹. Fish were fed satiation on palletized salmon food three times a week. During the second year, fish were maintained at 30-40 fish per tank in larger circular tanks (4.5m diameter, 1.0 m depth, 159,041 volume) supplied with flow-through seawater (201·min⁻¹, 29-32°/oo salinity, O₂ >90% saturation, 8°C) and biweekly fed a diet of squid, Loligo opalescens, until satiated.

Experimental procedure

In laboratory experiments designed to simulate the capture process, we investigated the impairment of immune system function in 2 year-old non-reproductive sablefish. Each experiment involved three unstressed fish remaining at the maintenance
temperature of 8°C as a negative control and three fish subjected to a stressor. The trials were replicated one week later. All experiments were conducted over a 4 month period at HMSC in Newport, Oregon.

To simulate the stress imposed by trawling in a net and exposure to air that would arise on a commercial vessel, sablefish (mean TL 36.8 cm) were transferred from the maintenance tank by rapid netting to a tank with towing nets as described in Olla et al (1997). Briefly, to simulate the cod-ends of trawls, two 2.5 cm nylon diamond mesh nets (1.2 m length, 0.7 diameter) were attached to rotating arms in a large tank (4.5 m diameter, 1 m depth). For each trial, three fish were sequestered in one net and towed for two hours at 1.1 m/s, a speed at which sablefish could not swim (Olla et al. 1997) and at 8 °C, the temperature at which the fish were reared. Directly following the tow, the fish were transferred in a net to a large basin (90 x 60 x 30 cm) in a temperature-controlled room and exposed to 15 min of air at 10 or 16°C.

The effects of hooking for two hours followed by exposure to 15 min of air at 10 or 16 °C were determined in non-reproductive 2 year-old sablefish (mean TL 36.6). The fish were transferred from the maintenance tank by rapid netting to a circular tank (2.0 m diameter, 0.8 m depth, 8 °C) and hooked through the upper jaw onto commercial long-line gear as described in Davis et al. (2001). Fish were maintained on the lines for two hours, unhooked by hand and treated as described above to air exposures. Sablefish that did not remain hooked on the commercial line for the entire two hours were noted accordingly.

We simulated the capture of sablefish by trawling and exposure to warmer temperatures during gear retrieval by towing non-reproductive 2 year-old sablefish (mean
TL 45.1 cm) for two hours followed by exposure to elevated seawater temperatures. After towing as described above, the fish were transferred by rapid netting to a tank (3.0 m by 1.0 m), containing seawater temperatures of 10°C or 16°C for 15 minutes.

To simulate the stress imposed by air exposure on the deck of a fishing vessel, sablefish (mean TL 36.8 cm) were transferred by dipnet from the maintenance tank into a large basin (90 x 60 x 30 cm) in a temperature-controlled room. The fish were exposed to air at 10 or 16 °C for either 15 or 30 min and then placed back into a recovery tank with 8°C seawater (3.0 m by 1.0 m) for 2 h or 1 h 45 min, respectively. The fish were placed into the recovery tank after air exposure to assure that the interval between the onset of stress and the immune function measurement were equivalent for all laboratory experiments.

After treatments were performed as described above, the fish were immediately placed in a lethal dose (400 ppm) of tricaine methanesulfonate (MS-222). The total length (TL) of each fish was recorded before blood and pronephros samples were obtained from each specimen. Control fish from the maintenance tank (8 °C) were sampled concurrently with the treatment groups. Blood was collected from the caudal vein with 3 mL heparinized vacutainers, the plasma separated by centrifugation and stored at −80 °C until analyzed for cortisol and glucose levels. Plasma cortisol was assayed according to radioimmunoassays techniques by Redding et al. (1984) and validated for sablefish by Olla et al. (1997) and glucose was measured using a NOVA analyzer.

Individual pronephros were harvested into tissue culture media (TCM) under aseptic technique and lymphocytes isolated according to modified methods of Ellsaesser
and Clem (1986), Arkoosh et al. (1994), Milston et al. (2003), and Misumi (2004). Immune function was assessed by quantifying the ability of pronephric leukocytes to proliferate upon *in vitro* stimulation with the B cell mitogen lipopolysaccharide (LPS) or the T cell mitogen concanavalin A (Con A) in an assay we previously validated for sablefish (Lupes chapter 1 2004). Pronephric leukocytes from each sablefish were cultured in triplicate with or without each mitogen. Proliferation was assessed by leukocyte $[^3\text{H}]-\text{thymidine}$ incorporation measured as counts per minute (cpm) by a Wallac liquid scintillation counter.

*Data analysis*

Since each fish was sampled individually, each individual is an independent unit and therefore considered the unit of measure for statistical purposes. Immune assays were performed in triplicate. Therefore, data were derived from triplicate determinations for each fish and individual proliferative responses were derived after the calculated mean of triplicate determinations for the mitogen-stimulated cultures and the non-stimulated cultures. Depending on the assay, in approximately 0.03% of the cases, inclusion of an outlier would significantly affect the calculated stimulation index (SI) according to a Wilcoxon rank-sum test and was disregarded, and the mean was therefore calculated according to duplicated cultures. The proliferative responses were assessed for each individual fish as SI calculated as the ratios of the mean $[^3\text{H}]-\text{thymidine}$ uptake of the mitogen stimulated cultures divided by the mean $[^3\text{H}]-\text{thymidine}$ uptake of the non-stimulated cultures.
For each capture-related laboratory experiment, the medium ± standard error (SE) was calculated per experimental group. The data were analyzed with non-parametric tests as the data did not fit a normal distribution and variances were not equivalent, and calculated with SigmaStat software. A Kruskal-Wallis one-way analysis of variance (ANOVA) of ranks test was utilized to observe any differences due to treatments, followed by a Dunn’s multiple-range test to examine differences between the median values at different levels of capture. Differences were considered statistically significant when P< 0.05.

Results

We studied the functional capability of pronephric leukocytes from sablefish subjected to various capture-related stressors to respond in vitro to the B cell mitogen LPS or the T cell mitogen Con A. Cells from control fish (unstressed at 8 °C) were capable of responding to LPS and Con A (SI of 5.0 and 6.0 respectively), while the proliferative responses were significantly depressed (P< 0.001) in fish that were towed for 2 h at 8 °C followed by 15 min of air at 10 or 16 °C (Figure 5). A significant difference in the immunological response associated with air temperature was not detected in sablefish exposed to 15 min of air at 10 or 16 °C (P>0.5). In comparison to control fish, sablefish that were hooked for 2 hours and then transferred to air at 10 or 16 °C for 15 min also presented significantly diminished (P<0.001) levels of leukocyte proliferation upon in vitro stimulation with LPS and Con A (Figure 5). No significant
Figure 5: Mean (+SEM) stimulation indices (SI) of sablefish subjected to 2 h of hooking or towing at 8 °C, followed by 15 min air exposure at 10 or 16 °C comparison to controls (unstressed at 8 °C). The SI of stressed fish was significantly lower than in controls denoted (*) (P < 0.001).
difference was discerned between the 10 or 16 °C air temperature following the hook stress for either one of the mitogens (P>0.05).

SI of sablefish towed for 2 hours at 8 °C followed by a 15 min exposure to an elevated seawater temperature of 10 or 16 °C were significantly (P< 0.001) diminished in comparison to controls for both LPS and Con A (Figure 6). A significant difference in the immunological response associated with seawater temperature was not detected in sablefish exposed to 15 min at 10 or 16 °C. Sablefish that were directly exposed to air temperatures of 10 or 16 °C for 15 or 30 min and then placed in a recovery tank with 8 °C seawater also presented a loss of mitogen responsiveness. The SI for LPS and Con A were significantly reduced (P<0.05) in comparison to the control fish (unstressed at 8 °C) (Figure 7). However, the proliferative responses did not vary significantly (P>0.05) due to air temperature or minutes of air exposure.

Physiological variables measured directly after each treatment indicated that the sablefish were undergoing significant stress responses. Cortisol plasma levels increased significantly (P<0.001) from a median of 17.95 ng/ml in control fish (unstressed at 8 °C) up to a median of 170.8 ng/ml in fish towed and held in air at 10 °C and to a median of 192.4 ng/ml in fish towed and held in air at 16 °C (Figure 8). Fish towed and exposed to elevated air temperatures of 10 or 16 °C had median cortisol levels of 220.9 ng/ml and 176.4 ng/ml, respectively. In comparison to the controls, cortisol plasma levels were also significantly elevated (P<0.001) in sablefish exposed to various air temperatures and times and in fish towed and exposed to elevated seawater temperatures (Figure 8). However, there were also no significant differences in the magnitude of cortisol levels between the different treatments (Figure 8). Regardless of capture stressor, the cortisol
Figure 6: Mean (+SEM) stimulation indices (SI) of sablefish towed for 2 h at 8 °C followed by 15 min. exposure to an elevated seawater temperature of 10 or 16 °C in comparison to controls (untowed at 8 °C). The SI of stressed fish was significantly lower than in controls denoted (*) (P < 0.001).
Figure 7: Mean (+SEM) stimulation indices (SI) of sablefish subjected to different air exposure times (15 or 30 min) and air temperatures (10 or 16°C). The SI of stressed fish was significantly lower than in controls denoted (*) (P < 0.001).
Figure 8: Mean (+SEM) plasma cortisol concentrations (ng/ml) of sablefish subjected to various stressor treatments in comparison to controls (unstressed at 8 °C): towed or hooked for 2 h at 8 °C, followed by 15 minute air exposure at 10 or 16 °C (a); towed for 2 h at 8 °C followed by 15 min exposure to an elevated seawater temperature of 10 or 16 °C (b); different air exposure times (15 or 30 min) and air temperatures (10 or 16 °C) (c).
Figure 8
levels did not vary significantly (P>0.05) due to air temperature or minutes of air exposure. Fish towed and exposed to elevated air temperatures of 10 or 16 °C had median blood glucose levels of 165.0 mg/ml and 158.5 mg/ml, respectively (Figure 9). Fish captured by hooking and exposure to elevated air temperatures of 10 or 16 °C had median blood glucose levels of 114.0 mg/ml and 121.5 mg/ml, respectively (Figure 9). Blood glucose levels significantly increased from a median of 50.0 mg/ml to elevated glucose levels immediately after the induced stress, and were not significantly different between capture by tow or hook (P > 0.05) (Figure 9). In comparison to the controls, glucose levels were also significantly elevated (P<0.001) in sablefish exposed to various air temperatures and times and in fish towed and exposed to elevated seawater temperatures (Figure 9). However, there were also no significant differences in the magnitude of glucose levels between the different treatments. Regardless of capture stressor, the glucose levels did not vary significantly (P>0.05) due to air temperature or minutes of air exposure.

Discussion

Regardless of treatment, the duration and combination of capture and environmental stressors in our study were severe enough to result in a significant reduction in the ability of pronephric leukocytes from stressed sablefish to respond in culture to B and T cell mitogens. Immunosuppression as a result of stress is frequently associated with decreased disease resistance to opportunistic pathogens (Wendelaar
Figure 9: Mean (+SEM) glucose levels (mg/dl) of sablefish subjected to various stressor treatments in comparison to controls (unstressed at 8 °C): towed or hooked for 2 h at 8 °C, followed by 15 min air exposure at 10 or 16 °C (a); towed for 2 h at 8 °C followed by 15 min exposure to an elevated seawater temperature of 10 or 16 °C in (b); different air exposure times (15 or 30 min) and air temperatures (10 or 16 °C) (c).
Figure 9
Bonga 1997) and our study demonstrates the cellular basis at which captured and released sablefish could be more susceptible to infectious diseases. The fact that the immune system is functionally impaired after exposure to capture-related stress sheds some light on a potential reason why delayed mortality is possible in discarded sablefish. As a fish is discarded back into the ocean, their ability to perform at the whole organism level may be diminished for an extended period of time. Changes in feeding, social interactions, schooling, swimming ability, orientation, predator evasion, and mortality corresponded with increased capture stress intensity in previous laboratory studies in sablefish, Pacific halibut, walleye pollock, and lingcod, Ophiodon elongatus (for review see: Davis 2002). “Injury, infection and delayed mortality have been observed in Atlantic herring that escaped from a trawl, walleye Pollock that were towed in a net, and in Pacific halibut that were towed in a net or hooked” (Davis 2004 in review). Davis (2004 in review) suggested that in his laboratory study with sablefish towed in a net, delayed mortality resulted from external skin and fin infections at the site of injury.

Over time the cumulative strain from maintaining physiological adaptation or allostasis reduces an animal’s ability to sustain normal physiological functions, including immunological functions (McEwen and Stellar 1993; Pruett 2003); this would also be the case with fish. Therefore, the ability of the immune system to protect the fish from injury is impaired and can predispose the fish to disease and eventual death. Continuous harsh environmental conditions can add to the cumulative damage, which accelerates the pathophysiological processes and results in mortality sooner (McEwen 1999).

Based on the available evidence there is little doubt that the imposition of a stressor will result in altered immune responsiveness (Kusnecov and Rabin 1994;
Modulation of the immune system due to stressors has been demonstrated in both mammals and fish. In studies designed to evaluate immune function after the imposition of stress, deficiencies in both cell-mediated and humoral-mediated responses have been observed. The decreased lymphocyte responsiveness to mitogen stimulation, antigen stimulation, and a reduction in lymphocyte cytotoxicity are a few of the deficiencies that have been demonstrated in fish (Wendelaar Bonga 1997). The in vitro stimulation of fish lymphocytes from the peripheral blood, thymus, pronephros, and spleen is one of the most commonly used methods to assess the function of the cellular immune system (Luft et al. 1991; Faisal and Hargis 1992). The mitogen-induced proliferation of lymphocytes from the pronephros was used in this study as it can be considered a reasonable first approximation to show that the immune system of a fish is capable of functioning (Ellasesser and Clem 1986).

Depending on the type of stress, exposure to a stressor can have a profound effect on the immune system resulting in either enhancement or suppression of immune function (Barton and Iwama 1991; Dhabhar and McEwen 1999; Weyts et al. 1999; Pruett 2003). The enhancement of the immune system in fish has been observed following an acute stress (Demers and Bayne 1997; Ruis and Bayne 1997) and immunosuppression has been observed as a consequence of a chronic stress (Yin et al. 1995; Bly et al. 1997). Although this effect of stress on the fish immune system is generally accepted, variability in the response of the immune system after stress has been reported in both mammals and fish. The variability in immune responses after the imposition of stress can be attributed to the use of different species, different cell populations and different immunological assays (Weyts et al. 1998). Considerations of stressor influences on immune function
also need to account for the duration, intensity, and frequency of the stressor, as this will influence the magnitude and the duration of the response to the stressor (Pruett 2003).

In our study immunosuppression was observed in sablefish that were towed or hooked and then exposed to air or increased temperatures. An undetectable significant difference in immune responses between the two capture methods suggests that the two gear types are equally damaging to the ability of leukocytes to proliferate. In these experiments sablefish were exposed to two sequential stressors for a cumulative duration of 2.25 or 2.5 hours. The combinations of sequential stressors used were nonlethal, resulting in no immediate mortality or delayed mortality in sablefish during previous capture-related laboratory experiments (Davis 2004 per comm.). Nonlethal conditions were employed to avoid dealing with samples from morbid individuals and to ensure consistency in sample sizes.

We assayed sablefish leukocytes directly after the imposition of bycatch stressors and demonstrated that the immune system of sablefish is initially compromised directly following capture-related stressors. However, there can also be a large range of immune responses from suppression to enhancement within a session of stress exposure and/or the time elapsing post-stress as the immune system recovers or fails to adapt to the stressor (Maule et al. 1989; McEwen 1999; Ortuno 2001). The interval between the stress and the immune function measurement are important; the timing of the immunological assay has a large effect on the evaluation of the immunological endpoint (Cupps and Fauci 1982). For example, in mice exposed to an auditory stressor there is a significant depression in mitogenic responsiveness for the first two weeks, followed by a significant increase in immune function response for the next two weeks (Borysenko and Borysenko 1982).
Maule et al. (1988) indicated that the fish immune response to a stressor operates similar to mammalian systems in which there is a rapid immune depression, followed by a transient immunostimulation, and ending in a more chronic suppression of the immune function.

There is little information on the immunological response of fish to exposure to two or more sequential stressors (Schreck 2000). Schreck (2000) presented a conceptual physiological stress response model to sequential stressors suggesting that if the stressors occur temporally close together, then there can be cumulative effects. Schreck’s (2000) model could explain why the combination of bycatch stressors, though possibly considered acute in nature, resulted in a suppressive immune response rather than an immune function enhancement. The cumulative effect of stress is apparent in other laboratory studies in sablefish, Pacific halibut, walleye pollock, and lingcod, where stress increased with stressor intensity as measured by changes in feeding, orientation, predator evasion, and mortality (Olla et al. 1997, 1998; Davis et al. 2001; Davis and Olla 2001, 2002; Davis 2004 in review). In these studies the initial capture (towing or hooking) caused stress, which was then magnified by the addition of environmental stressors such as air or increased seawater temperature, resulting in impaired behavior and increased mortality. It was therefore surprising in our study that at treatment severities that were just below lethal levels, increased environmental air or seawater temperatures did not cause further immunosuppression. Previous sablefish studies demonstrated that exposure to above-ambient seawater temperature after simulated capture at 8 °C accentuated lactate and potassium levels in sablefish (Olla et al. 1998, Davis et al. 2001). Increased levels of physiological stress and mortality have been observed to increase under field conditions
of increased temperature (Barton and Iwama 1991). Various studies also show that an abrupt change in temperature can induce immune suppression in both specific and nonspecific immunity (Ellis 1981, Wendelaar Bonga 1997). Because we did not quantify immune function after each successive stressor, we do not know if the initial capture stress depressed the immune system so severely, not allowing for further immunosuppression induced with each sequential stressor. However, we cannot disregard the possibility that the sequence of stressors even at nonlethal severities were debilitating to the immune system due to the cumulative effect of stress. Given the different natures of the stress stimuli in our study and the complexity of the immune response, it is difficult for us to classify our sequence of stressors as a combination of acute stressors or a cumulative chronic stressor simply based on the immune response observed.

Sablefish directly exposed to air exposure for 15 or 30 minutes also did not reflect the general acute immunological response to a stressor, resulting instead in decreased lymphocyte mitogen-induced proliferation. However, these results are consistent with other studies that have demonstrated that non-lethal aerial emersions of salmonids even as brief as 30 seconds (Maule et al. 1988) caused a significant depression in immune function. Many nonlethal acute stressors including confinement, crowding, acute handling, transport, and increased water temperature can also induce immunosuppression (Wendelaar Bonga 1997). Sublethal levels of stress produced by hypoxia have also demonstrated impairment in immune function (Mellergaar and Nielsen 1995; Ortuno et al. 2002). In previous experiments, sablefish mortality was only observed after 30 minutes in air and increased dramatically between 30 and 50 min (Davis and Parker
Davis and Parker (2004) also demonstrated that there was behavioral impairment after just 10 minutes in air possibly reflecting physiological injury.

The lack of air temperature effects on the immune function was consistent with lack of air temperature effects on mortality (Davis and Parker 2004). Davis and Parker (2004) suggested that the warming of sablefish is not very efficient in air and therefore body core temperature remains low. The warming of sablefish is more efficient in water with smaller fish reaching higher body core temperature more quickly than larger fish (Davis et al. 2001). One possible explanation for the lack of air temperature effect on immune function is that the direct exposure to the air for 15 or 30 minutes so severely depressed the immune system such that further immunosuppression was not possible in sablefish subjected to increases in air temperature.

Our study found suppressed immune function in sablefish subjected to capture-related stressors, but we do not know the mechanisms underlying the immune suppression. How the function of lymphocytes, macrophages and other cells of the immune system are modified by the duration and frequency of the stressor are not well understood (Krusnecov and Rabin 1994). Prolonged stress exposes lymphocytes to continuously elevated levels of hormones, costicosteriods, catecholamines (CA) and other neuropeptides from lymphoid organs. The stress-related immune dysfunction appears to be driven by these neuroendocrine responses with glucocorticoids as the major mediators of immunosuppression (Pickering 1981; Adams 1990; Wendemeyer et al. 1990; Wendelaar Bonga 1997; McEwen and Seeman 1998; Pruett 2003).

The duration and severity of the capture stressors applied in our study were harsh enough to induce significantly elevated levels of cortisol and glucose. However, there
was no difference in the magnitude of plasma cortisol and glucose among stress treatments. Davis et al. (2001) observed greater plasma cortisol, lactate, and potassium levels in towed sablefish followed by elevated temperature and air than in hooked sablefish followed by elevated temperature and air. However, in that study the stress-induced capture was inflicted upon sablefish for a total of 4 hours, two times the duration of our study. The greater duration of capture stress in their study possibly contributed to further increases in cortisol and glucose levels observed after the towing stress.

Our study demonstrates that the sablefish immune system is functionally impaired after exposure to capture-related stressors. As a fish is discarded back into the ocean, their ability to perform at the whole organism level may be diminished for an extended period of time. Stress relocates metabolic energy from performance activities such as growth, reproduction and immune responses towards activities required to restore homeostasis such as cardiac output, oxygen uptake, and hydromineral balance (Barton and Iwama 1991). Thus, the energetic costs associated with adapting and recovering from the stressor are significant and reduce the performance capacity of the fish (Scheck 1981). Over time the cumulative strain on a discarded fish from maintaining physiological adaptation reduces its ability to sustain normal immunological functions. Therefore, the ability of the immune system to protect the fish from damage is impaired and can predispose the fish to disease and eventual death. However, further studies are needed to determine if delayed mortality in sablefish discards can be caused by increased susceptibility to infectious agents resulting from stress-mediated immunosuppression.
References


CONCLUSION

The present study demonstrated that pronephric leukocytes are suitable for use in
in vitro assays to evaluate the proliferative responses of sablefish cells to classic
mammalian B and T cell mitogens. The development of an in vitro culture system for
sablefish leukocytes will allow for future assessment of the function of the sablefish
cellular immune system in aquaculture and natural populations. This immunological
assay should be usable at sea for evaluation of fish health and assessment of the effects of
fishing tactics and gear. Use of this assay to assess and measure the sablefish immune
response to laboratory-simulated bycatch conditions leads to further insights regarding
delayed discard mortality. The functional impairment of the immune system after
exposure to capture-related stress proposes a potential reason why delayed mortality is
possible in discarded sablefish. As fish are returned to the ocean, their ability to perform
at the whole organism level may be diminished for an extended period. Over time the
cumulative strain on a fish from maintaining physiological adaptation reduces its ability
to sustain normal immunological function. Therefore, the ability of the immune system
to protect the fish is impaired and can possibly predispose a discarded fish to disease and
eventual death. Consequently, following the imposition of nonlethal capture stressors,
morbidity is not sudden and mortality is not observed until weeks later, as reported in
previous field and laboratory experiments (Davis 2002; 2004 in review). However,
further studies are needed to determine if delayed mortality in sablefish discards can be
caused by increased susceptibility to infectious agents resulting from stress-mediated
immunosuppression.
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