

THE STRESS RESPONSE OF THE BRASSY CHUB, A LOCAL REEF FISH AND  
CANDIDATE FOR SUSTAINABLE AQUACULTURE

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE  
UNIVERSITY OF HAWAI'I AT MĀNOA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

ANIMAL SCIENCES

MAY 2024

By

Reilly S. Merlo

Thesis Committee:

Andre P. Seale, Chairperson

Bradley K. Fox

Mi-Jeong Lee

Keywords: Brassy chub, Handling stress, Cortisol, Glucose, Antioxidant enzymes

## ACKNOWLEDGEMENTS

I would like to extend my gratitude to all those who have aided and encouraged me throughout this journey of completing my master's thesis. Special thanks to my parents and brother, who have supported me through all my academic endeavors. Also, thank you to my grandparents and wonderful family, who have nurtured and guided me to spread aloha and made me the person I am today. Thank you to Annika for your love and patience; whether you are picking out colors for me or watching me practice my presentation for the 200<sup>th</sup> time, your support has been unwavering.

I would like to express my deepest appreciation to my advisor, Dr. Andre Seale, for providing me the opportunity to pursue a master's degree and join the Lab of Fish Endocrinology and Environmental Physiology. Your knowledge, guidance, and excitement for new data have been contagious. I am also grateful for the help and expertise of my committee members Dr. Andre Seale, Dr. Bradley K. Fox, and Dr. Mi-Jeong Lee. Your experience and constructive feedback have been instrumental in shaping and enriching this thesis.

I would also like to thank all the unsung heroes who support the lab in the Agriculture Science building and keep everything running smoothly including Steve Spielman, the janitorial and custodial staff, and the workers in the front office.

Lastly, I would like to thank the past members of the lab including Dr. Fritzie Celino-Brady, Dr. Tharindu Malintha, and Daniel Woo for greatly expanding my laboratory skillset and outlook on graduate school. To the current graduate students including Tyler Goodearly, Ryan Chang, and Ke Cao, I am truly indebted to you for all your encouragement, support, and camaraderie. To all that have been named, I owe my success in completing this master's thesis and I look forward to continuing work with you on my academic journey.

## ABSTRACT OF CONTENTS

The brassy chub, *Kyphosus vaigiensis*, is a reef herbivore that has been shown to grow rapidly in captivity, though no information is available on how they respond to maintenance-related stress, a major component of captive fish welfare. Generally, in fish, the stress response has been shown to disrupt hydromineral balance and activate endocrine systems including the hypothalamus-pituitary-interrenal (HPI) axis, which are characteristic responses of the alarm phase. During the resistance phase of the stress response, typical changes involve energy metabolism affecting glucose availability and antioxidant enzyme activity. In this study, I characterized the effects of acute handling stress on the physiological responses of brassy chub over a 24-hour (h) time course, by measuring endpoints associated with both the alarm and resistance phases of the stress response. These included plasma and mucus cortisol, plasma glucose and osmolality, and hepatic activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX), and hepatic lipid peroxidation. The results indicated that plasma and mucus cortisol levels were transiently increased in stressed fish by 1 h, suggesting activation of the HPI axis. Moreover, plasma glucose increased by 1, 12, and 24 h while plasma osmolality increased at 1 and 6 h and decreased at 12 and 24 h. SOD activity decreased in the stressed treatment at 6 and 12 h and returned to control levels by 24 h. GPX activity and lipid peroxidation were unaffected by the handling stressor throughout the 24 h period. In addition to validating the use of mucus as a minimally invasive technique to detect HPI axis activation, this study provides fundamental insight on the effects of a maintenance-related stressor on osmoregulatory function, energy mobilization, and the antioxidant defense system. Understanding these responses provides critical insights needed to inform management practices for the welfare and production of the brassy chub.

# TABLE OF CONTENTS

<b>ACKNOWLEDGEMENTS.....</b>	<b>ii</b>
<b>ABSTRACT OF CONTENTS.....</b>	<b>iii</b>
<b>TABLE OF CONTENTS.....</b>	<b>iv</b>
<b>LIST OF FIGURES.....</b>	<b>v</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>vi</b>
<b>CHAPTER I.....</b>	<b>1</b>
<b>CHAPTER II.....</b>	<b>13</b>
Abstract.....	13
1. Introduction.....	14
2. Materials and Methods.....	18
3. Results.....	25
4. Discussion.....	32
<b>CHAPTER III.....</b>	<b>41</b>
<b>References.....</b>	<b>44</b>

## LIST OF FIGURES

1. A) Image of the brassy chub, <i>Kyphosus vaigiensis</i> . B) Schematic of experimental design .....	19
2. A) Plasma and B) mucus cortisol concentrations in control and stressed brassy chub .....	27
3. Plasma osmolality in control and stressed brassy chub .....	28
4. Plasma glucose in control and stressed brassy chub .....	29
5. A) SOD and B) GPX activity in liver tissue of control and stressed brassy chub .....	30
6. Hepatic lipid peroxidation in control and stressed brassy chub .....	31

## **LIST OF ABBREVIATIONS**

### **SYMBOL AND ABBREVIATIONS**

ETC= Electron transport chain

GPX= Glutathione peroxidase

HPI= Hypothalamus-pituitary-interrenal

LPO= Lipid peroxidation

MDA= Malondialdehyde

OWIs= Operational welfare indicators

ROS= Reactive oxygen species

SOD= Superoxide dismutase

## CHAPTER I

### Introduction

As the global population increases, so does the demand for nutritious foods. Finfish provide high quality protein, micronutrients, and essential fatty acids which are not easily found in land-based foods (Kawarazuka and Béné, 2010). As the wild capture of seafood has stagnated since 1990, aquaculture practices have increased their production to meet the rising seafood demand (Costello et al., 2020). Cost and sustainability are key concerns and components of production, and as such, should be considered when choosing species of finfish for aquaculture. In carnivorous species, fishmeal and fish oil from wild capture fisheries have been necessary for production; as these feed ingredients come from fisheries which have stagnated, the industry's reliance on them as an input can limit future growth, in addition to their high carbon footprint and financial costs affecting sustainability (Froelich et al., 2018). Furthermore, open land and freshwater resources suitable for aquaculture have been diminishing. While the culture of herbivorous, marine finfish holds the potential to mitigate these issues, large-scale production of marine herbivores has not begun. Thus, the capacity of candidate species for sustainable aquaculture to handle common rearing practices are understudied as they have yet to be domesticated for optimization to aquaculture conditions (Teletchea and Fontaine, 2014; Teletchea, 2015).

#### *Stress Response in Aquaculture*

Aquaculture is the fastest growing sector of agriculture and is regarded as a necessary sector for a sustainable future. However, this rapidly growing industry is quickly outpacing our knowledge of the health and biology of aquatic species. Hence, immediate efforts are needed to

safeguard the welfare of understudied species, such as the aquaculture candidate species, brassy chub, and other herbivorous marine fishes deemed suitable for aquaculture development (Franks et al., 2021). Fish welfare is crucial for the aquaculture industry, not only for public perception, ethical considerations, and marketing, but also as a key component for production efficiency and quality (Ashley, 2007). There are many potential stressors in aquaculture including handling, confinement, water quality, and pathogen occurrences; these stressors impose an allostatic load on fish, which can be detrimental to long-term health (Broom and Corke, 2002). Creating best practices for fish welfare requires knowledge of stress physiology specific to each species. Therefore, a thorough understanding of the physiological bases of the stress response is necessary to mitigate welfare concerns and maximize the economic and production success of the industry (Martos-Sitcha et al., 2020; Veissier and Boissy, 2007).

The original concept of the biological stress response was first described by Hans Seyle in 1936 as the general adaptation syndrome, "...the symptoms of which are independent of the nature of the damaging agent...and represent a response to damage as such" (Seyle, 1936). Due to the wide range of perceived stressors and responses, an all-encompassing definition of stress is difficult to achieve; however, in a later publication, he specifically defined the stress response as "...the nonspecific response of the body to any demand made upon it" (Seyle, 1973). Stressors can include environmental changes or challenges such as in salinity or temperature, which initiate a stress response by actively demanding physiological action for survival. Stressors can also simply be perceived as a potential harm; consider a scenario where no physical harm is occurring such as a rapid reduction in living space or being startled by tapping on a tank. Recent definitions of stress describe it as a state of threatened homeostasis (Barton and Iwama, 1991). The concept of homeostasis underlies the field of physiology and is considered one of the

characteristics defining life (Billman, 2020; Çengel, 2023). Homeostasis is defined as the coordinated physiological processes that maintain an internal equilibrium or stability. The physiological processes described pertain to interactions between multiple feedback systems that are tightly modulated by higher control centers, allowing for flexibility in responses to adjust to external conditions (Modell et al., 2015). Likewise, the non-specific response to stressors is an adaptive mechanism to minimize potential damage and maintain homeostasis. The stress response is composed of three phases: alarm phase, resistance phase, and exhaustion phase, which are comprised of the primary, secondary, and tertiary responses respectively.

The perception of a stressor in the brain activates the alarm phase, where physiological, adaptive processes are initiated involving multiple endocrine systems. In one such system, the brain uses the sympathetic nervous system to directly signal to chromaffin cells, located near or within the head-kidney in teleost fish. These chromaffin cells release catecholamines including adrenaline and noradrenaline into circulation, causing a spike in concentration typically within seconds of exposure to a stressor (Nilsson et al., 1976; Reid et al., 1998). In addition to increasing glucose mobilization, one of the main functions of catecholamines is to affect cardiovascular and respiratory functions to maintain adequate blood oxygen levels (Mommsen et al., 1988). In fish, this involves functional gill remodeling that increases the surface area exposed to the environment, allowing for a higher rate of oxygen diffusion. However, this also leads to increases in the diffusion of ions and other osmolytes, impacting blood osmolality, a trade-off known as the osmorepiratory compromise (Reid et al., 1998; Wood and Eom, 2021). Crucially, fish exposed to stressors can lose their osmoregulatory capacity, leading to compromised hydromineral balance such as uncontrolled changes in blood solute concentration or osmolality (Breves et al., 2010). The maintenance of a stable hydromineral balance, or osmoregulation, is

critical for cellular functions. Consequently, a rise or fall in osmolality will trigger hypo- or hyperosmoregulatory responses, respectively. These responses, in turn, are largely mediated by neuroendocrine systems, including the hypothalamus-pituitary-interrenal (HPI) axis (Danziger and Zeidel, 2015; Seale and Breves, 2022).

Another endocrine system activated during the alarm phase of the stress response is the HPI axis in fish. The perception of a stressor in the brain triggers the paraventricular nucleus of the hypothalamus to release corticotrophin releasing factor, which travels down the portal system to the pituitary, initiating the production of pro-opiomelanocortin (POMC) from the corticotrophs in the *pars distalis* of the pituitary (Fryer and Lederis, 1986; Scott and Baker 1975). In teleosts, cleavage of the precursor POMC in corticotrophs gives rise to adrenocorticotrophic hormone (ACTH), which is released into circulation (Takahashi and Mizusawa, 2013). ACTH then signals the interrenal cells of the head-kidney to produce and release cortisol into the blood (Mommsen et al., 1999). This process involving the HPI axis is comparatively slower than the release of catecholamines, as the release of cortisol is typically within minutes after initial exposure to a stressor. Because the act of sampling a fish is typically perceived as a stressor, the use of cortisol, rather than catecholamines, for detecting the alarm reaction provides more leeway in sampling time and has become a conventional endpoint of the stress response in the scientific literature (Lemos et al., 2023; Sadoul and Geffroy, 2019; Schreck et al., 2000).

The resistance phase of the stress response is highly adaptive, and during this phase, physiological changes are modulated by circulating levels of cortisol. In fish, cortisol acts as both a glucocorticoid and mineralocorticoid as it affects glucose metabolism, growth, osmoregulation, and innate immune function (Wendelaar Bonga, 1997). Cortisol was originally recognized as a

seawater-adapting hormone, but there is also evidence of cortisol facilitating freshwater adaptation (McCormick, 2001). In seawater, cortisol typically leads to an increase in seawater-adapting ionocytes and increased activity of Na<sup>+</sup>, K<sup>+</sup>-ATPases in the gills (McCormick et al., 2008; Mommsen et al., 1999), while in fresh water, it was recently observed that the expression of glucocorticoid and mineralocorticoid receptors were downregulated, indicating the possible attenuation of cortisol's effects in that environment (Chang et al., 2023). These patterns of environmental regulation of cortisol are generally supportive of restoring or maintaining plasma osmolality to within optimum homeostatic ranges. Another main function of cortisol in response to a stressor is to mobilize energy through gluconeogenesis and glycogenolysis in preparation for the energy demands of stressful situations (Chang et al., 2018; Cheyadmi et al., 2022; Polakof et al., 2012). Increases in circulating glucose, therefore, are typically seen during this phase, as reported in salmonids, *Oncorhynchus mykiss*, *Salvelinus fontinalis*, *Salvelinus namaycush*, *Salmo trutta*, Eurasian Perch, *Perca fluviatilis*, and striped mullet, *Mugil cephalus* (Barton, 2000; Jentoft et al., 2005; Wanshu, 1992), but not in juvenile haddock, *Melanogrammus aeglefinus*, subjected to handling stressors (Afonso et al., 2008). The increases in energy production also affect the rate at which reactive oxygen species (ROS) are generated from the electron transport chain (ETC).

Under stressful conditions, an imbalance between ROS generation and removal occurs due to increased energy demands, cell signaling, and increased non-specific immune functions including respiratory burst (Costas et al., 2011). Increased energy demands during the resistance phase result in an increase in the production of ATP during cellular respiration. A key component of this process is the ETC, including four protein complexes that use redox reactions to create an electrochemical gradient. Under normal conditions, a small portion (0.2-2 %) of

electrons leak out of the ETC and interact with oxygen to form ROS including superoxide ( $O_2^-$ ) or hydrogen peroxide ( $H_2O_2$ ) (Hoseinifar et al., 2021; Zhao et al., 2019). Excess build-up of ROS can lead to oxidative stress including lipid peroxidation (LPO), DNA damage, protein oxidation, and if extensive, cell death (Castro et al., 2018; Chowdhury and Saikia, 2020; Orrenius et al., 2007). Damage from ROS is mitigated by the antioxidant defense system with antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and catalase (CAT), and non-enzymatic antioxidants including glutathione, vitamin C, and vitamin E (Hoseinifar et al., 2021; Mishra et al., 2015). Typically, the first ROS formed from the ETC is superoxide, which is catalyzed by SOD into the less harmful ROS,  $H_2O_2$  and  $O_2$  (Turrens, 2010). GPX and CAT then catalyze the reduction of hydroperoxides, GPX using reduced glutathione, further limiting oxidative damage (Srikanth et al., 2013). Without activity from these enzymes, the hydroperoxides in the presence of iron form highly reactive hydroxyl radicals, which indiscriminately oxidize proteins, lipids, and DNA (Schieber and Chandel, 2014).

When a stressor persists and becomes chronic, the fish will either physiologically adapt to new homeostatic limits or perish from exhaustion. During the final phase of the stress response, the exhaustion phase, the energetic budget of the fish is limited to maintain solely essential processes. Thus, reductions in appetite, decreased physical activity and growth, lowered immune function, imbalance of ROS production and elimination, and eventually mortality are typically seen during the exhaustion phase (Hoem and Tveten, 2020). Therefore, understanding and detecting the stress response on a species-specific level is critical for minimizing the maladaptive effects of the stress response and successfully producing aquaculture species.

An initial step towards maximizing production is having the ability to detect when aquacultured fish are stressed. To this end, previous studies have commonly used fluctuations in plasma cortisol as an endpoint, as cortisol stored in interrenal cells is directly released into the blood. However, the act of sampling blood from a fish can be perceived as a stressor and negatively impact production performance. Therefore, there is an increasing desire to develop standardized minimally invasive techniques to reduce the intensity of stress from sampling. One such method, developed in trout and European seabass, *Dicentrarchus labrax*, involves the use of skin mucus (Carbajal et al., 2019; De Mercado et al., 2018; Sadoul and Geffroy, 2019). After cortisol is released into the circulatory system, it is metabolized and subsequently excreted into the surrounding water via urine or feces. A small fraction of cortisol also passively diffuses into the skin mucus (Scott and Ellis, 2007). Mucus cortisol detection could provide a minimally invasive procedure for the detection of stress response activation and further act as a potential welfare indicator if validated for individual species.

While detection of the stress response through measurements of plasma and or mucus cortisol are informative, interpreting their significance can be complicated by environmental circumstances, sampling conditions, or species. For example, while activation of the HPI axis from handling stressors with the same magnitude and length led to a ~46 fold increase in plasma cortisol concentration in the lake trout, *Salvelinus namaycush*, only a ~1.3 fold increase was measured in pallid sturgeon, *Scaphirhynchus albus* (Barton 2002). Therefore, a single measurement of cortisol cannot be used to assess whether a fish is responding well to welfare practices, due to the hormone's multitude of biological responses and functions towards adaptive homeostasis. In addition to cortisol, other health parameters or operational welfare indicators (OWIs), such as endpoints in the stress response including the antioxidant defense system,

osmoregulatory capacity, and innate immune function aid in the process of interpreting welfare status (Segner et al., 2012). However, when cortisol is consistently monitored, it is a key indicator for potential issues and can then be followed by diagnostic testing. Inasmuch as understanding physiological processes and the ability to detect the stress response will aid in successful welfare and aquaculture production, more research is needed to strengthen the current knowledge of aquaculture species, particularly newly employed species such as the brassy chub.

### *Brassy Chub as an Aquaculture Candidate*

The brassy chub, *Kyphosus vaigiensis*, is a marine herbivore that is found in tropical and subtropical marine areas around the globe (Figure 1A). As a member of the *Kyphosidae*, a family commonly known for their herbivory, its ecology has been well described, particularly on reefs and inshore habitats (Clements et al., 2017; Okano, 2011; Sakihara et al., 2015; Streit et al., 2015). The brassy chub plays a crucial role in the maintenance of coral reef ecosystems through the consumption of macroalgae; herbivorous reef fish, such as the brassy chub, consume algae that compete with corals for space. If left unchecked, algae can overgrow corals, shading them out and leading to their demise (Michael et al., 2013). The brassy chub in particular is a key player as it consumes both the fronds and thallus of macroalgae, rather than just the fronds (Streit et al., 2015; Wu et al., 2022). Additionally, the brassy chub has the potential to play a major role in slowing the spread of invasive algae, including spiny algae, *Acanthophora spicifera*, mainly by consuming loose algal fragments (Okano, 2011). Stemming from its ecological importance as an herbivorous reef fish and its ease of wild capture, the brassy chub has become a key species in investigating anthropogenic impacts on fish of different trophic levels and herbivorous feeding modes (Cardozo-Ferreira et al., 2021; Serviere-Zaragoza et al., 2021).

Along the same lines, there have been a few studies focused on the brassy chub as a hindgut fermenter. Much of our knowledge on diet and energy metabolism in finfish comes from carnivorous species such as salmonids, and research on this topic in herbivorous marine fish has been widely neglected (Willmott et al., 2005). More recently, understanding fermentation processes in marine fish has become a subject of interest for aquaculture-focused nutritionists and ecologists. The brassy chub and other members of the *Kyphosidae* family house symbiotic gut microbial communities that ferment algal carbohydrates, typically indigestible by non-herbivorous species, into short-chain fatty acids that can be utilized for energy by the host (Mountfort et al., 2002). The brassy chub, in particular, has been shown to have distinct microbial communities in different sections of the gut that aid in nutrient assimilation from seaweeds (Clements et al., 2017; Mountfort et al., 2002; Olivier et al., 2023; Pisaniello et al., 2022, Podell et al., 2023; Sparagon et al., 2022). The ability of the brassy chub to utilize autotrophs as an energy source has been key for maintaining their large, schooling populations in the wild, but also leaves them as an easy target for fishermen.

In Hawai‘i alone, over 8,000 pounds of brassy chub, known locally as nenu (young) or enenu (adults), were landed commercially in 2021 (DLNR, 2021). The brassy chub is a popular game fish and historically it was even reserved for the chiefs. If not prepared correctly, the brassy chub can have a strong odor and flavor due to its herbivory requiring fermentation processes in the intestines as described above, though in certain traditional dishes these flavors were desired (Miyasaki and Fujioka, 2012; Titcomb and Pukui, 2021). Modern preparations of the brassy chub that reduce these odors have been described recently (Morinaka and Ando, 2011; Pardee and Omori, 2023). The brassy chub is commonly caught by throw nets, hook and line, and spearfishing, though more recently it has become a candidate for sustainable aquaculture.

There have been efforts, particularly in Hawai‘i, to develop the brassy chub as an aquacultured food fish (Ocean Era, 2021). Even though Hawai‘i is an island state, it imports the majority of its seafood, leading to a large trade deficit and leaving it vulnerable to natural disasters that may disrupt shipping and the food supply (Geslani et al., 2012; Leung and Loke, 2008). With limited land space and freshwater resources available for commercial land-based aquaculture, there is an increasing need to develop sustainable mariculture to mitigate these issues and feed the increasing population. The brassy chub is a frontrunner as a species for aquaculture development, as it has many beneficial attributes that could aid in its transition. Mainly, as a hindgut fermenter and herbivore, it has the potential to mitigate the industry's reliance on fishmeal, an expensive feed additive with a high carbon footprint. Additionally, its feeding mode as a browser and schooling behavior likely facilitate its acceptance of a multitude of feed options, including floating pellets or attached macroalgae, and high-rearing densities. (Huntingford et al., 2012). Furthermore, it may be beneficial to accrue knowledge of the culture conditions necessary for the brassy chub's production for restocking wild populations, given the ecological functions and rate of capture of the *Kyphosidae* family (Lozano-Muñoz et al., 2022). Despite these qualities that may aid in the brassy chub's adaptation to culture conditions, there is a lack of studies on its physiology with regard to captive rearing. As aquacultured fish are commonly and unavoidably exposed to maintenance-related stressors such as confinement and handling, understanding how the brassy chub responds to these stressors will be paramount for their integration as a sustainable aquaculture species.

## *Goals and Objectives*

The overall goal of this project was to improve our understanding of the brassy chub's suitability to rearing conditions including its capacity to handle acute, maintenance-related stressors. To this end, my objectives were to characterize parameters indicative of the alarm and resistance phases of the stress response in stressed and control fish over 24 hours (h) following a simulated handling stressor. Additionally, I aimed to determine the efficacy of detecting the activation of the stress response using a minimally invasive procedure, the analysis of skin mucus. Specifically, my objectives were to:

**Objective 1:** Characterize the responses of primary stress response endpoints such as plasma cortisol, mucus cortisol, and plasma osmolality.

**Objective 2:** Characterize the responses of secondary stress response endpoints such as plasma glucose and hepatic oxidative endpoints including the activity of SOD and GPX, and LPO.

Overall, I hypothesized that the brassy chub subjected to a handling stressor would elicit primary and secondary stress responses. Inasmuch as the brassy chub is amenable to captive rearing conditions, the simulated handling stressor would affect the primary and secondary response parameters in a manner similar to other well-studied and commonly reared marine species, with responses limited to transient changes followed by restoration to initial levels. Specifically, I hypothesized that the plasma and mucus cortisol would follow similar trends, with transient increases in concentration after the simulated handling stressor, thereby validating their potential utility as markers of the stress response, as reported in other fish species. Additionally, I

hypothesized that the stressor would cause the loss of control of osmoregulatory function, leading to transient increases in plasma osmolality, similar to other fish reared in seawater following a handling stressor. As glucose is an essential energy source for fish and widely used as a general indicator of energy mobilization in response to handling stress, following the simulated stress treatment I predicted a rise in plasma glucose concentration. Lastly, as LPO typically increases from the energy demand of stress, I hypothesized an increase in LPO by 1 h, then decrease as antioxidant activity (SOD and GPX) increases to mitigate damage from increased ROS production. The findings of this work are presented in Chapter II and Chapter III further expands on the interpretation and implications of the results.

## CHAPTER II

### **The effects of handling stress in a reef fish model for aquaculture development, the brassy chub, *Kyphosus vaigiensis*.**

#### **Abstract**

With the expanding global population, interest has increased in the sustainable aquaculture development of locally abundant finfish, such as the brassy chub, *Kyphosus vaigiensis*. Nonetheless, little is known about their resilience in aquaculture settings, where fish are unavoidably exposed to acute, husbandry-related stressors. The response to stressors consists of three phases: alarm, resistance, and exhaustion. The alarm phase includes changes in stress hormone levels, such as catecholamines and corticosteroid hormones. The resistance phase includes changes to metabolic and immune functions. In this study, I characterized the effects of an acute handling stressor on physiological parameters indicative of the alarm and resistance phases of the stress responses of brassy chub over a 24-hour (h) time course. Specifically, I measured plasma and mucus cortisol, plasma glucose, plasma osmolality, liver antioxidant enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX), and liver lipid peroxidation (LPO) at 0, 1, 6, 12, and 24 h after exposure to a handling stressor. The results indicated that both plasma and mucus cortisol levels were transiently increased in stressed fish 1 h after the stressor. Plasma glucose and osmolality were also affected by the handling stressor by 1 h and generally through the 24 h. SOD activity decreased in the stressed treatment at 6 and 12 h and returned to control levels by 24 h. GPX activity and LPO were unaffected by the handling stressor throughout the 24 h period. The physiological response to the handling stressor was similar to other commonly cultured marine fish, suggesting the brassy chub has a comparable capacity to withstand stressors. Overall, this study provides novel information about the stress response of the brassy chub and validates the use of mucus cortisol to detect acute stress.

## 1. Introduction

The culture of marine fish is one of the fastest growing segments of global agriculture, thereby becoming a crucial contributor to sustainable food production systems (Costello et al., 2020). Marine fish, however, can be quite fragile and aquacultured fish are often exposed to acute, maintenance-related stressors (Teletchea, 2015). These stressors, such as handling during transport from one tank to another, can lead to fish mortality and losses in farm profitability (Cheyadmi et al., 2022; Schreck and Tort, 2016). The manner in which fish respond to various stressors reflects their capacity to acclimate to captive conditions and will ultimately determine the intensity of care required for the welfare and successful production of the species (Braithwaite and Ebbesson, 2014).

The challenge of overcoming the impacts of stressors encountered in aquaculture production systems is particularly evident with newly employed species in captive rearing. The process of domestication involves selective breeding for traits that maximize production efficiency including growth rate and feed conversion ratios. Selective breeding can also impact the hardiness of the aquaculture species, and thus domesticated species can be more resilient to aquaculture-related stressors than older filial generations or wild counterparts, as seen in the Eurasian perch, *Perca fluviatilis* (Douxflis et al., 2011; Palińska-Żarska et al., 2021). In Hawai‘i, there is growing interest from both non-profit organizations and commercial aquaculture companies in the sustainable aquaculture development of locally abundant fish, including the brassy chub, *Kyphosus vaigiensis*, commonly known in Hawai‘i as nenu. The brassy chub is an herbivorous reef fish that is distributed circumtropically and commonly targeted by fishermen (Sakihara et al., 2015). In addition to the brassy chub’s market demand, as an aquaculture candidate, the brassy chub benefits from being a schooling herbivore, which could signify its

acceptance of rearing in high densities and low feed costs. Although there is rising interest in the culture of sea chubs, very little is known about their physiology or resilience to stressors in an aquaculture setting.

Biological stress was first described by Hans Selye, and he described an organism's response to stressors as "...the nonspecific response of the body to any demand made upon it" (Selye, 1973). Stress is considered to be a state of threatened homeostasis and the physiological response to a perceived stressor is an adaptive mechanism to minimize potential damage and maintain internal homeostasis. Fish react to stressors through the stress response, which is composed of three phases: alarm phase, resistance phase, and exhaustion phase.

During the alarm phase, fish will perceive a stressor, which initiates a cascade of physiological, adaptive changes including two endocrine pathways. The first is a rapid release of catecholamines that trigger the fight or flight response that may lead to the temporary loss of control of their ability to successfully osmoregulate and keep parameters such as plasma osmolality within a homeostatic range. The second endocrine pathway is initiated through the perception of the stressor in the brain, which activates the hypothalamus-pituitary-interrenal (HPI) axis culminating in the release of corticosteroids, such as cortisol, from the head kidney into the circulatory system (Mommsen et al., 1999). Cortisol mediates energy mobilization through gluconeogenesis and glycogenolysis in preparation for the energy demands of stressful situations (Chang et al., 2018; Cheyadmi et al., 2022). Thus, during the resistance phase, energy is mobilized such as increases in plasma glucose and there are also changes in immune function and antioxidant enzyme activity (Balasch and Tort, 2019; Barton, 2000; Costas et al., 2011). Cortisol is also well known as a seawater-adapting hormone for its role in increasing osmoregulatory capacity (McCormick et al., 2008; Mommsen et al., 1999).

At the exhaustion phase, the fish focuses its limited energy on maintaining homeostasis in the most essential processes for life, leading to decreased physical activity and growth, immunosuppression, and imbalance of production and elimination of reactive oxygen species (ROS) (Hoem and Tveten, 2020).

Previous studies have widely used cortisol as an endpoint for detecting the alarm stage of the stress response in fish (Breves et al., 2010; Sadoul and Geffroy, 2019). After the perception of a stressor, the release of cortisol into circulation occurs within minutes (min), thus, the cortisol concentration in plasma can be used as a proxy to indicate the perceived stressor in fish (Kalamarz-Kubiak, 2018). To minimize the negative impacts of detecting stress from the commonly used invasive procedures, such as drawing blood for plasma cortisol detection, there is an increasing desire from aquaculture and fisheries management for non-invasive or minimally invasive procedures in the determination of stress levels. After cortisol is released into the circulatory system, a small fraction can passively diffuse into the skin mucus (Scott and Ellis, 2007). In salmonids, *Oncorhynchus mykiss*, and European seabass, *Dicentrarchus labrax*, there were positive correlations between concentrations of cortisol in the mucus and plasma (Carbajal et al., 2019; Sadoul and Geffroy, 2019). With adequate validation, therefore, mucus cortisol detection could provide a minimally invasive procedure for the determination of stress levels in other fish species.

Under stressful conditions, an imbalance of ROS generation and removal occurs, mainly due to increased energy demands, cell signaling, and non-specific immune function. The excessive build-up of ROS, or oxidative stress, can damage the structure of proteins and nucleic acids. It also damages lipids through lipid peroxidation (LPO) (Castro et al., 2018; Chowdhury and Saikia, 2020). This damage is mitigated by the activity of antioxidant enzymes such as

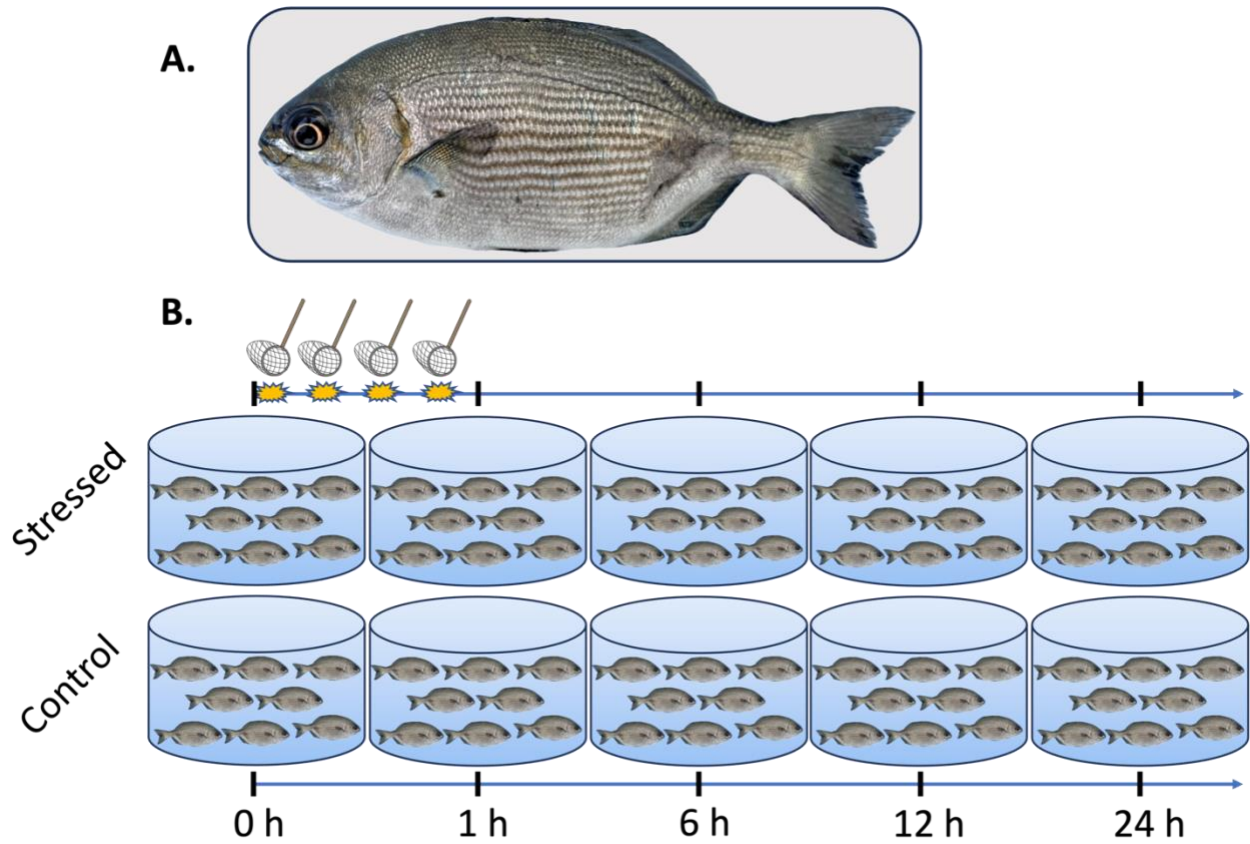
superoxide dismutase (SOD) and glutathione peroxidase (GPX). SOD catalyzes the conversion of  $O_2^-$  into less harmful ROS,  $H_2O_2$  and  $O_2$  (Turrens, 2010). GPX catalyzes the reduction of hydroperoxides using reduced glutathione, further limiting oxidative damage (Srikanth et al., 2013). Previous studies using European seabass (Castro et al., 2015) and gilthead sea bream, *Sparus aurata* (Sanz et al., 2012) have shown measurements of LPO and antioxidant enzyme activity to be good biomarkers for detecting stress, as these markers tend to fluctuate in response to stressors.

To better understand the suitability and resiliency of the brassy chub as an aquaculture candidate, focusing on osmotic balance, energy metabolism, and innate immune function, I investigated factors indicative of the alarm and resistance phases of the stress response following an acute handling stressor in brassy chub over a 24 h time course. Specifically, I measured plasma osmolality, plasma glucose, plasma and mucus cortisol, and hepatic antioxidant enzyme activity of GPX and SOD, and LPO in the liver. I hypothesized that the fish subjected to a simulated handling stressor would elicit transient responses indicative of the alarm and resistance phases of the stress response, through temporarily increased levels of cortisol in the plasma and mucus, plasma osmolality and glucose, hepatic antioxidant activity, and lipid peroxidation. By comparing stressed and unstressed brassy chubs throughout a 24 h time course, I obtained fundamental, baseline information on the stress response, and its detection, from a sustainable aquaculture candidate.

## **2. Materials and Methods**

### *2.1 Animals and rearing conditions*

Eighty juvenile brassy chubs from the same cohort weighing  $24.7 \pm 1.0$  g (average  $\pm$  standard error of the mean) were acquired from a local supplier (Ocean Era, Kailua-Kona, HI) and acclimated into 10 flow-through tanks (stocked at 8 fish per tank) with Kāneʻohe Bay seawater (32-36 ‰, 6.8-8.4 mg/L DO) at Waikalua Loko Iʻa (Kāneʻohe, Oʻahu, HI) for 8 days prior to the experimental trial. Fish were fed Trout Chow pellets (Skretting, Tooele, UT) twice a day to satiation. Water temperatures were maintained between 24 and 29 °C. All housing and experimental procedures were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee of the University of Hawaiʻi.



**Figure 1.** A) Image of the brassy chub, *Kyphosus vaigiensis*. B) Schematic of the experimental design, where brassy chub were subjected to simulated handling by confinement stress and netting for 1 h. Fish were sampled prior to the stressor at time 0 and then following the handling stress at 1, 6, 12 and 24 h (n=8).

## *2.2 Experimental procedure and sampling*

On the day of the experimental trial, feed was withheld from all tanks. Each of the 10 tanks were randomly assigned a treatment group (stressed vs unstressed controls) and sampling time point (0, 1, 6, 12, and 24 h; Figure 1B). For the stressed treatment, the water level was dropped such that the dorsal fins of the fish were exposed (10 cm), and a net was used to agitate the tanks for the first 5 min of every 15 min for 1 h. The stressed fish, therefore, were subjected to both netting and confinement stress, which hereafter will be generally referred to as handling stress. At each sampling time, 8 fish from one stressed and one control tank were sampled; fish were sampled 4 at a time to reduce the effects of time and handling stress due to removal from their tanks. At time 0 (7 a.m.), fish from two tanks were anesthetized using 2-phenoxyethanol (0.3 mL/L, Sigma Aldrich, St. Louis, MO). Mucus was collected from the skin using a glass slide and placed into a 50 mL falcon tube. Blood was extracted from the caudal vasculature using heparinized syringes (200 U/mL, Sigma Aldrich, St. Louis, MO), and then weight and length measurements were taken. To minimize the effects of handling, mucus and blood samples were taken within 5 min of being anesthetized. The fish were euthanized by rapid decapitation and removal of the brain tissue. Fish were kept on ice for the sampling of liver tissues, which were snap-frozen in liquid nitrogen and then stored at -80 °C for analysis. Sampling procedures were repeated for the stressed and control tanks at subsequent time points.

## *2.3 Plasma and mucus parameters*

To extract the plasma, the blood was centrifuged at 10,000 g at 4 °C for 10 min. The plasma supernatant was then transferred to a new 1.5 mL Eppendorf vial. Plasma osmolality was measured using a vapor pressure osmometer (Wescor 5100C, Logan, UT). Similarly, the falcon

tubes with mucus were centrifuged at 10,000 g at 4 °C for 10 min to condense the mucus on the bottom of the tube. The mucus was then transferred to a new 1.5 mL Eppendorf vial.

Plasma and mucus cortisol were measured using ELISA protocols modified from Carey and McCormick, 1998 as recently described and validated for fish plasma in Chang et al., 2023. Microtiter plates were prepped by adding 150 µL/well of rabbit anti-cortisol (Fitzgerald Ind. Int'l, MA) diluted 1:30,000 in coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 100 mL ddH<sub>2</sub>O), sealed, and incubated for 4 h at 37 °C. The solution in the plates was then discarded and the plates were washed 5 times with wash buffer (0.15 M NaCl, 0.05 % Tween 20, ddH<sub>2</sub>O to 1 L). 250 µL of EIA buffer (19.5 mL of 0.2 M NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O, 30.5 mL of 0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, 0.1 % BSA, 50 mL of ddH<sub>2</sub>O) was then added to each well and incubated for 30 min. After which, the buffer was discarded and replaced with 150 µL per well of fresh EIA buffer. 100 µL of Cortisol-HRP conjugate (Fitzgerald Ind. Int'l, MA; diluted 1:6,000 in EIA buffer) and 2.5 µL of sample (plasma or mucus) or cortisol standard (Sigma) dissolved in Ringer's solution (140 mM NaCl, 10 mM NaHCO<sub>3</sub>, 2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 4 mM KCl, ddH<sub>2</sub>O added to 1 L and pH adjusted to 7.8) were added. Plates were then sealed and incubated overnight at 25 °C. The next day, the solution in the plates was discarded and rinsed 5 times with wash solution. 200 µL/well of TMB Peroxidase Substrate (Sigma, T0440) was added, then the plates were incubated at room temperature with gentle shaking (60-70 rpm) for 10 min. After incubation, 50 µL of 0.5 M HCl was added to each well and absorbance was read at 450 nm using a multimode plate reader (Synergy LX, BioTek). Standards were run in triplicate and samples were run in duplicate.

Plasma glucose was determined using a Glucose Assay Kit (Sigma, GAGO-20) following the standard protocol. 10 µL/well of sample plasma or glucose standard was added to 96 well

plates followed by 200  $\mu\text{L}$ / well of assay reagent (0.8 ml o-Dianisidine Reagent (Sigma, D 2679) in 39.2 ml Glucose Oxidase/Peroxidase Reagent (Sigma, G 3660)). The plates were then sealed and incubated for 30 min at 37 °C. After incubation, 100  $\mu\text{L}$ /well of 12 N  $\text{H}_2\text{SO}_4$  was added to stop the reaction. Absorbance was read at 540 nm using the multimode plate reader.

#### *2.4 Liver lipid peroxidation and antioxidant enzyme activity*

LPO was assayed by measuring malondialdehyde (MDA), which is commonly used as a marker for LPO (Castro et al. 2015; Fernández et al. 1997). LPO in the liver was determined using a Lipid Peroxidation Assay Kit (Cayman Chemical, 700870) following standard protocol. 25 mg of frozen liver tissue in a 2 mL tube was finely ground with a pestle, and then 250  $\mu\text{L}$  of RIPA buffer (Cayman Chemical, 10010263) was added. Samples were vortexed, then centrifuged at 1,600 g for 15 min at 4 °C. 100  $\mu\text{L}$  of supernatant was then transferred to new, 2 mL tubes. 100  $\mu\text{L}$  of TCA Assay Reagent (Cayman Chemical, 700016) and 800  $\mu\text{L}$  of Color Reagent (848 mg of thiobarbituric acid, 80 mL acetic acid, 80 mL NaOH) were then added to each tube. The tubes were then vortexed and added to a heat block at 95 °C to boil for 1 h. After boiling, the tubes were placed in ice baths for 10 min to stop the reaction, then centrifuged for 10 min at 1,600 g at 4 °C. 200  $\mu\text{L}$  from each vial was then plated on a 96 well plate and absorbance was read at 540 nm. The results are presented as nmol MDA per mg protein.

SOD activity was detected using a SOD Colorimetric Activity Kit (ThermoFisher, EIASODC) following the standard protocol. 50 mg of frozen liver tissue was finely ground using a pestle and 0.5 mL of phosphate-buffered saline (PBS, 0.12 M NaCl, 0.016 M  $\text{Na}_2\text{HPO}_4$ , 0.004 M  $\text{NaH}_2\text{PO}_4$ , pH adjusted to 7.25) was then added to each sample. The tubes were vortexed and centrifuged at 10,000 g for 15 min at 4 °C. 10  $\mu\text{L}$  of supernatant was then transferred to a new

tube and diluted 1:8 in assay buffer. Standards were prepared from lyophilized bovine SOD reconstituted in assay buffer to an activity level of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0 U/mL, vortexed, and incubated for 5 min at room temperature before the assay. When running the assay, 10  $\mu$ L of sample or standard was added in duplicate to a 96 well plate. 50  $\mu$ L of substrate and 25  $\mu$ L of xanthine oxidase were then added to each well. The plate was incubated at room temperature, then the absorbance was read at 450 nm. The results are presented as U/mg protein. One unit is the amount of enzyme necessary to produce 50 % inhibition of 1.5 mM nitroblue tetrazolium reduction at pH 7.8 and 25 °C.

GPX activity in liver homogenates was determined through a Glutathione Peroxidase Assay kit (Cayman Chemical, 703102). GPX was measured through monitoring the decrease in absorbance at 340 nm, representing the oxidation of NADPH to NADP<sup>+</sup>. 5 mg of frozen liver was cut, rinsed with PBS, and then finely ground with a pestle. 250  $\mu$ L of GPX sample buffer (Cayman Chemical, 703112) was then added. The samples were vortexed and then centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was then extracted and transferred to a new tube. When running the assay, 50  $\mu$ L (70  $\mu$ L for blanks) of assay buffer was added to each well. 50  $\mu$ L of Co-substrate mixture (Cayman Chemical, 703111), 50  $\mu$ L of NADPH (Cayman Chemical, 703119), and 20  $\mu$ L of GPX positive control (Cayman Chemical, 703114) or liver homogenate sample was then added. 20  $\mu$ L of Cumene hydroperoxide (Cayman Chemical, 703118) was quickly added, the plate was shaken on a plate shaker for 10 s at 100 rpm, then the plates were read at 340 nm once a minute for 6 min. Results are presented as the amount of enzyme required to oxidize 1  $\mu$ mol of NADPH per min per mg protein.

Total protein was measured using a Pierce BCA Protein Assay Kit (23225, ThermoFisher). Standards were prepared using BSA (Thermoscientific, 23209) diluted in RIPA

buffer, PBS, or sample buffer (for LPO, SOD, and GPX respectively) to concentrations 250, 125, 50, 25, 5, and 0 µg/mL. 25 µL of standard or sample (liver tissue after homogenization in solution per standard protocol of LPO, SOD, and GPX) were added to a 96 well microplate. Then, 200 µL/ well of working reagent (50mL Reagent A (Pierce, 23228) and 1 mL Reagent B (Pierce, 23224)) was added and the plate was thoroughly mixed on a plate shaker at 100 rpm for 30 s. The plate was then covered and incubated at 37 °C for 30 min. After incubation, the plate was cooled to room temperature and the absorbance was read at 562 nm.

### *2.5 Statistical Analysis*

The data collected was analyzed using two-way ANOVA with time (0, 1, 6, 12, and 24 h) and treatment (control and stressed) as the main effects. Significant effects ( $p < 0.05$ ) were followed by protected Fisher's LSD test. When necessary, data was log-transformed or square root transformed to fit the normality and equal variance assumptions of ANOVA. All statistical analyses were performed using Prism 10 (GraphPad).

### **3. Results**

#### *3.1 Cortisol*

For plasma cortisol, there were significant interaction and time effects. The time 0 cortisol concentrations were elevated and there were no significant differences between control and stressed groups. 1 h after the initiation of the trial, the stressed group had significantly higher plasma cortisol concentrations (~2x) compared with the control. The plasma cortisol for the stressed fish decreased to levels similar to those of controls at the 6 and 12 h time points. The control fish maintained plasma cortisol levels mainly at ~75 ng/mL after time 0, when it was elevated to ~300 ng/mL (Fig. 2A).

Similarly, there were significant interaction and treatment effects for the mucus cortisol. There were no significant differences between the control and stressed fish at time 0, but by 1 h after the initiation of the trial, the stressed group had elevated cortisol levels (~3x) compared with the control group. There were no significant differences between the control and stressed groups at times 6, 12, and 24 h following the stressor. The control fish maintained mucus cortisol levels around 2-5 ng/mL throughout the 24 h period, with time 0 significantly lower than 12 h (Fig. 2B).

#### *3.2. Plasma Osmolality*

There were significant interaction and time effects for the plasma osmolality. At time 0, there were no significant differences between the control and stressed fish. By 1 h after initiating the trial, stressed fish had significantly higher plasma osmolality (~15 mOsm/kg) compared with the controls and maintained this trend through 6 h. At 12 and 24 h, the stressed groups had significantly lower osmolality (~10 mOsm/kg) compared with the controls. The control fish

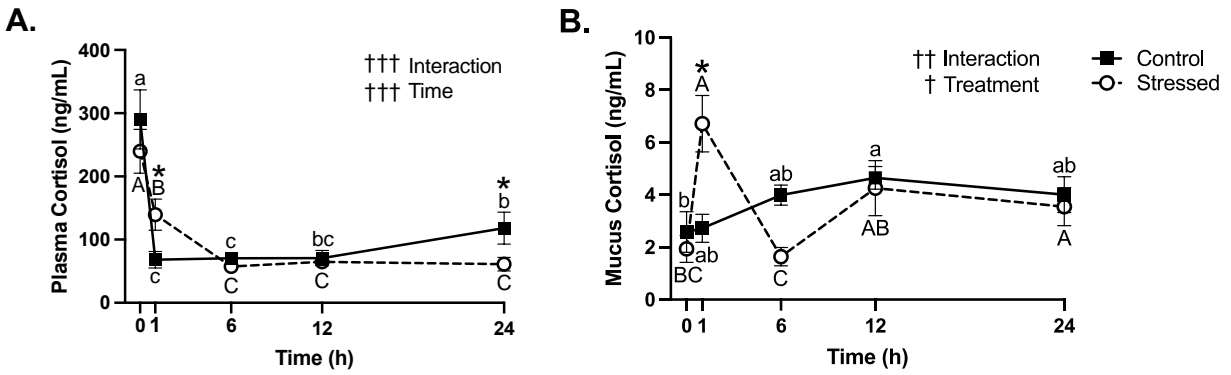
maintained a range of ~345-375 mOsm/kg throughout the 24 h period, with the 12 h time point significantly higher than 0, 1, 6, and 24 h time points (Fig. 3).

### *3.3 Plasma glucose*

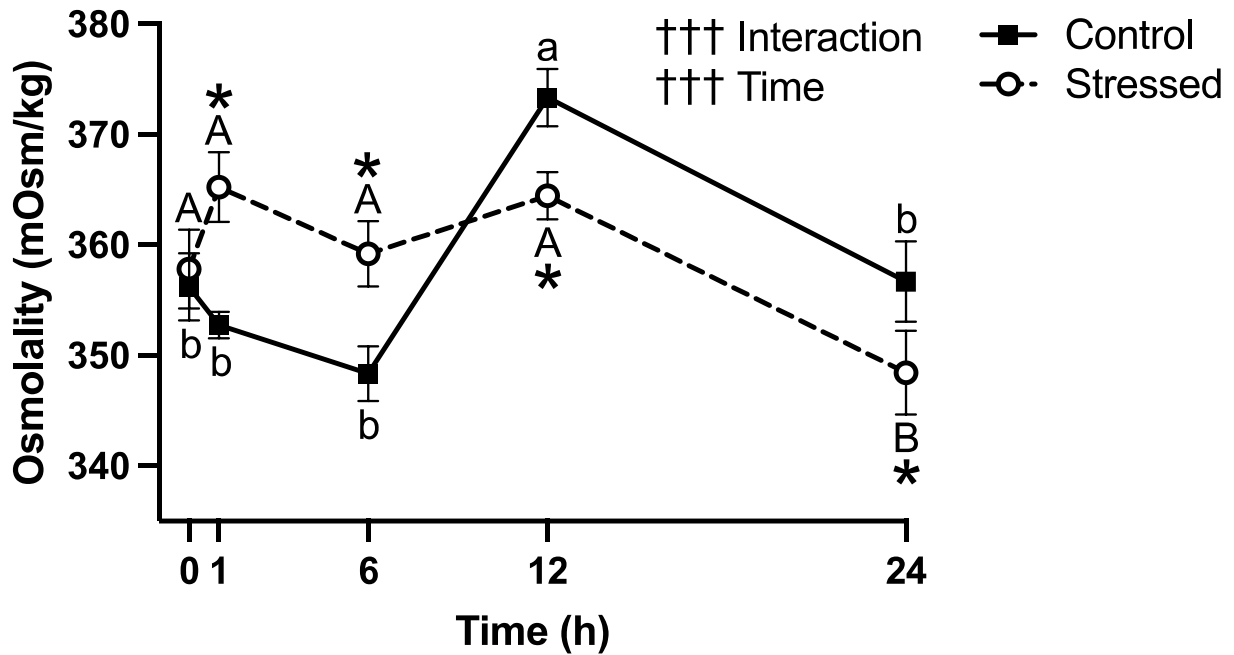
There were significant effects of treatment and time for plasma glucose. At time 0, there were no significant differences between the control and stressed fish. At times 1, 12, and 24 h after the initiation of the trial, the stressed fish had significantly higher plasma glucose (~30 to 50 mg/dL) compared with controls. The control fish maintained plasma glucose levels between 60-80 mg/dL with the 1 h time point significantly lower than time 0. The 12 h time point had the highest concentration (Fig. 4).

### *3.4. Antioxidant Activity*

There were significant effects of treatment and time for liver SOD activity. At times 0 and 1 h after the initiation of the stressor, there were no significant differences between the control and stressed groups. However, at times 6 and 12 h after the initiation of the stressor, the SOD activity was significantly lower in the stressed group, which returned to control levels by 24 h (Fig. 5A). Within the control group, the SOD activity was highest at the 12 h time point. Liver GPX activity was only significantly affected by time, with times 6 and 12 h having significantly higher activity than 0, 1, and 24 h (Fig. 5B). There were no significant main effects for liver lipid peroxidation (Fig. 6).

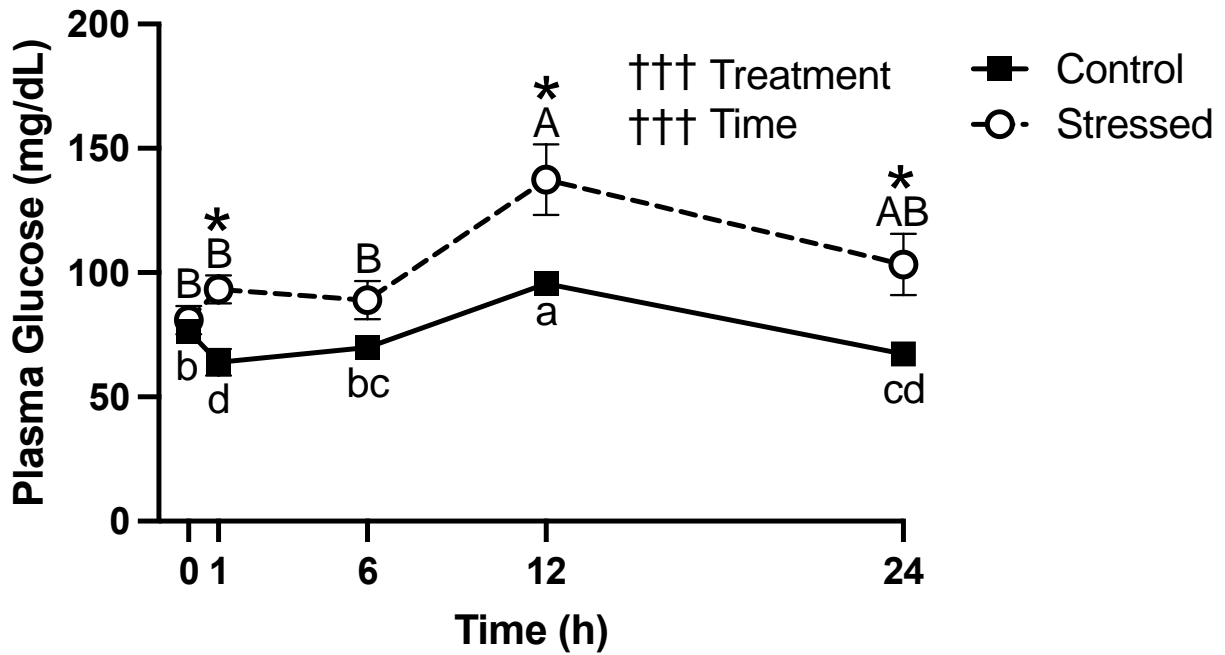


**Figure 2.** A) Plasma and B) mucus cortisol concentrations in control and stressed brassy chub over a period of 24 h. Significant effects are denoted with † representing  $p < 0.05$ , †† representing  $p < 0.01$ , and ††† representing  $p < 0.001$ . Significant effects were followed by protected Fisher's LSD test. Time points that do not share a lowercase letter represent significant differences in the control group while time points that do not share an uppercase letter represent significant differences in the stressed group. \* represents a significant difference between the control and stressed groups within the time point ( $p < 0.05$ ).

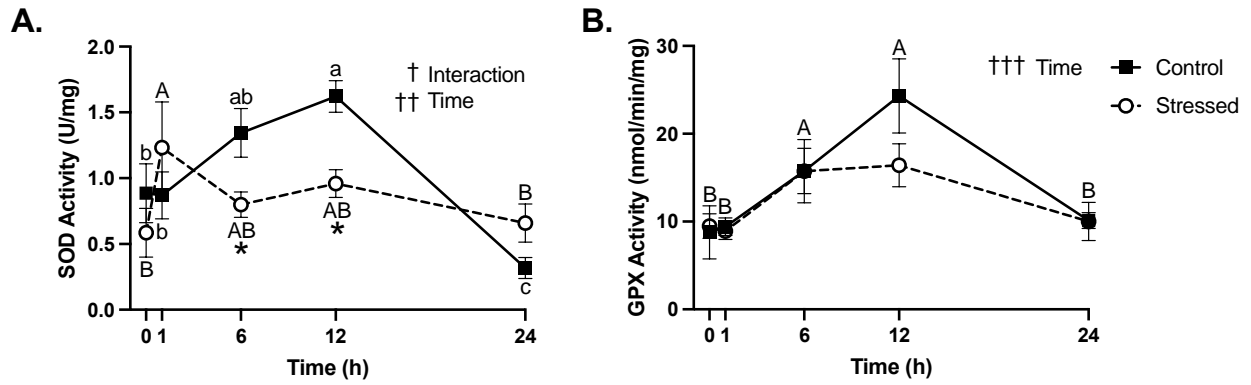


**Figure 3.** Plasma osmolality in control and stressed brassy chub over a period of 24 h.

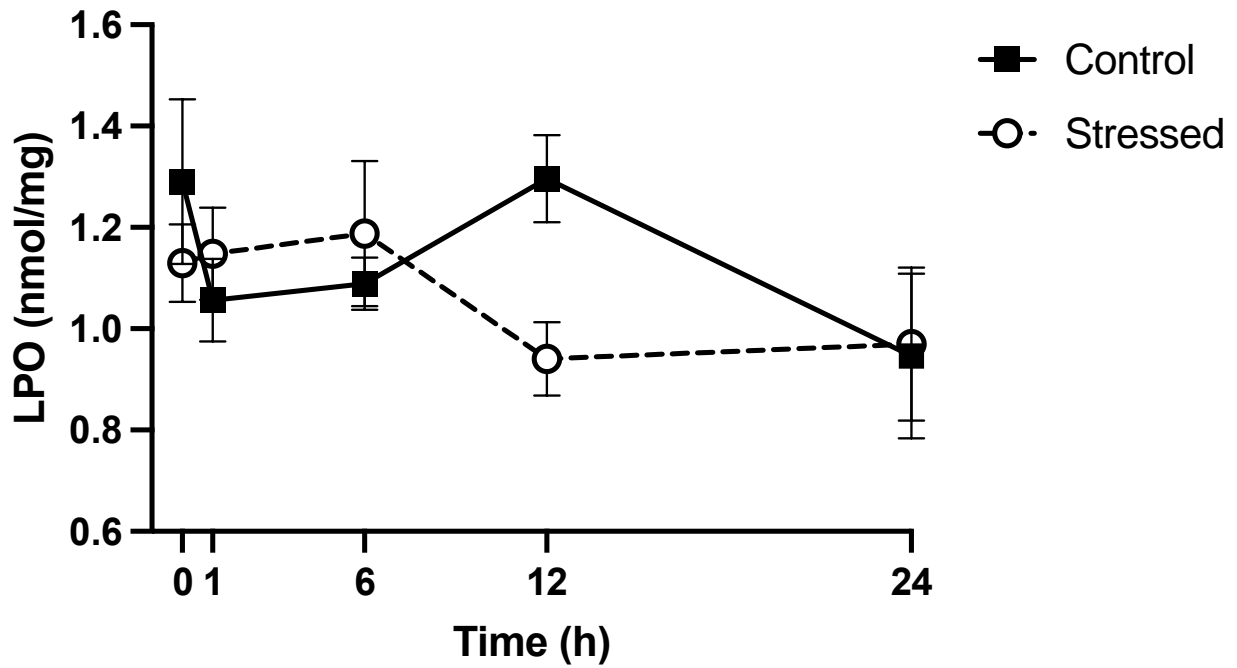
Significant effects are denoted with ††† representing  $p < 0.001$ . Significant effects were followed by protected Fisher's LSD test. Time points that do not share a lowercase letter represent significant differences in the control group while time points that do not share an uppercase letter represent significant differences in the stressed group. \* represents a significant difference between the control and stressed groups within the time point ( $p < 0.05$ ).



**Figure 4.** Plasma glucose in control and stressed brassy chub over a period of 24 h. Significant effects are denoted with ††† representing  $p < 0.001$ . Significant effects were followed by protected Fisher's LSD test. Time points that do not share a lowercase letter represent significant differences in the control group while time points that do not share an uppercase letter represent significant differences in the stressed group. \* represents a significant difference between the control and stressed groups within the time point ( $p < 0.05$ ).



**Figure 5.** A) SOD and B) GPX activity in liver tissue of control and stressed brassy chub over a period of 24 h. Significant effects are denoted with † representing  $p < 0.05$ , †† representing  $p < 0.01$ , and ††† representing  $p < 0.001$ . Significant effects were followed by protected Fisher’s LSD test. Time points that do not share a lowercase letter represent significant differences in the control group while time points that do not share an uppercase letter represent significant differences in the stressed group. \* represents a significant difference between the control and stressed groups within the time point ( $p < 0.05$ ).



**Figure 6.** Hepatic lipid peroxidation in control and stressed brassy chub over a period of 24 h.

Lipid peroxidation is represented by malondialdehyde. No significant effects were found.

#### 4. Discussion

The stress responses of most highly aquacultured species have been well studied (Liu et al., 2022; Schreck and Tort, 2016). The characteristics of individual parameters of the stress response can be highly variable between species and dependent on the intensity and longevity of the stressor (Barton, 2000; Eissa and Wang, 2014). Thus, this study characterized endpoints indicative of the alarm and resistance stages of the stress response often reported in commonly aquacultured species. For the first time, these endpoints were measured in the brassy chub, a candidate for sustainable aquaculture production. The endpoints measured in the current study included plasma and mucus cortisol, plasma osmolality and glucose, liver SOD and GPX activities, and LPO. Overall, the majority of the responses of the brassy chub were transient, similar to general trends found in other commonly cultured marine species following an acute stressor.

To detect the alarm phase of the stress response, plasma cortisol was measured. Consistent with the transient activation of the stress response, stressed brassy chub had significantly higher plasma cortisol compared to unstressed controls by 1 h, but this difference was no longer observed at 6 h. Baseline plasma cortisol in unstressed fish was ~75 ng/mL. Historically, it was thought that basal (pre-simulated stress) cortisol levels in fish were <40 ng/mL, however, basal cortisol levels can fluctuate widely between species, with welfare conditions, and time of day (Barton and Iwama, 1991). For example, in European seabass the basal plasma cortisol has been reported between 13 and 335 ng/mL (Barton, 2002). Additionally, cortisol is influenced by circadian rhythm with temporary increases in cortisol having been reported in trout and goldfish, *Carassius auratus*, with the onset of morning light (Rance et al., 1982; Spieler and Noeske, 1984). Hence, the elevations in the plasma cortisol of the brassy chub

at times 0 and 24 h in the control fish may be attributed to circadian rhythm. Additionally, elevated cortisol at time 0 in both control and stressed fish could be impacted by the length of sampling or initial stress of sampling. The elevation of cortisol concentration in stressed fish compared with control fish at 1 h following the onset of the experiment suggests that the brassy chub perceived the simulated handling as a stressor, thereby triggering the activation of the HPI axis and subsequent release of cortisol into the blood. A rise in plasma cortisol following handling stress was also observed in other commonly cultured species including Mozambique tilapia, *Oreochromis mossambicus*, haddock, *Melanogrammus aeglefinus*, wolffish, *Anarhichas minor*, and striped bass, *Morone saxatilis* (Breves et al., 2010; Hosoya et al., 2007; Kenter et al., 2021; Le François et al., 2013). For acute handling stressors, in addition to strain and species-specific responses, environmental parameters, severity and longevity of the stressor, age, diet, and history of previous stressors all impact the rate and amount of cortisol released (Barton et al., 1998; Barton, 2000; Foo and Lam, 1993; Kenter et al., 2021). Following an acute stressor, the brassy chub elevated plasma cortisol levels to ranges similar to those reported in other species (40-200ng/ml; Barton and Iwama, 1991; Barton, 2002). The same factors that influence the amount of cortisol released can also affect the rate of cortisol clearance (Davis and Small, 2006; Scott and Ellis, 2007). In most fish species, maximum increases in cortisol following exposure to a stressor occur within 0.5-1 h (Barton and Iwama, 1991). Because the stressor lasted for 1 h in the current study, the 1 h time point may not have captured the peak in cortisol. However, the increased cortisol levels in stressed fish subsided by 6 h compared with those of controls, indicating our ability to detect cortisol's transient response and subsequent clearance in the brassy chub. The timeline of plasma cortisol returning to pre-stress levels following the stressor was similar to that reported in other aquaculture species (2-8 h) including Atlantic salmon, *Salmo*

*salar*, catfish, *Ictalurus punctatus*, and seabream, *Sparus aurata* (Arends et al., 1999; Davis et al., 1984; Madaro et al., 2023).

As a means to detect the alarm stage of the stress response through minimally-invasive sampling, the mucus cortisol was also measured throughout this trial. Similar to the pattern observed in plasma cortisol, mucus cortisol transiently increased by 1 h and returned to pre-stress levels by 6 h. However, there were some crucial differences between the two methods of cortisol detection. Mainly, the concentration of mucus cortisol was much lower than that of plasma, as previously observed in other species comparing both approaches (Carbajal et al., 2019; Fernández-Alacida et al., 2019; Sadoul and Geffroy, 2019). Remarkably, the increase in cortisol concentration of stressed relative to control fish at 1 h was similar in magnitude regardless of whether plasma or mucus was used (2-3x control levels). Because cortisol in the mucus requires passive diffusion from the blood plasma, a time delay is expected in the peak of cortisol, with the mucus following the plasma; in Atlantic salmon, this delay is ~30 min (Madaro et al., 2022). Inasmuch as this study did not have the resolution in sampling times to determine the time delay, future studies would be needed to further characterize the peak and clearance of cortisol in plasma and mucus following a stressor in brassy chub. Nonetheless, in the current study, it was still possible to capture the elevation of both plasma and mucus cortisol at the 1 h time point. Finally, the amount of attainable mucus from this size class of fish is quite small (~2-10  $\mu$ L). This led to the loss of some replicate fish, due to the minimum quantity of mucus needed for running the assay. This could likely be avoided by sampling older, larger fish that may produce more mucus or adjusting sampling protocols for stripping more mucus, though the latter may have adverse effects on the fish health and may be considered more invasive. Overall, the use of mucus cortisol can be a useful tool as a minimally invasive technique for the detection of the

alarm stage of the stress response in brassy chub, with the caveats of the time delay, reduced sensitivity compared with plasma, and the necessity of larger fish.

The ability of a fish to maintain a stable internal environment, including plasma osmolality, is a fundamental characteristic needed for survival. In the current study, the plasma osmolality of the brassy chub in the control group ranged from ~345 to 375 mOsm/kg throughout the 24 h period, with the 12 h time point significantly higher than all other times. As the fluctuations in basal osmolality do not correlate with the salinity and temperature fluctuations in the tanks, two environmental factors that are known to affect osmolality (Malintha et al., 2023; Seale et al., 2019), the increase seen at the 12 h time point may be attributed to other external factors. Goldfish, carp, sailfin molly, *Poecilia latipinna*, and snook, *Centropomus undecimalis*, for example, have been shown to have fluctuations in plasma osmolality that have been attributed to circadian rhythms, though the acrophase varies greatly between species and the time of year (Kühn et al., 1986; Peterson and Gilmore, 1988; Spieler and Noeske, 1984).

Fish in seawater face a continuous osmotic imbalance with the surrounding water, which has ~3-fold higher salt concentration than fish blood. As described in other fish species, stressors can impair the fish's ability to successfully osmoregulate (Arends et al., 1999; Breves et al., 2010; Carneiro et al., 2007; Costas et al., 2011). Thus, I expected that the stressor would impair the capacity of fish to extrude ions, leading to transient increases in plasma osmolality. In the current study, the acute handling stressor led to an increase in plasma osmolality by 1 and 6 h, then a reduction in plasma osmolality at 12 and 24 h compared with control fish. The transient rise in plasma osmolality can be a consequence of stress-induced increases in respiration rates and consequent increase in branchial permeability to ions, as postulated by the osmorepiratory compromise (Breves et al., 2010; Reid et al., 1998; Wood and Eom, 2021). Additionally, the

increases in plasma osmolality following initial responses to stress could also be attributed to increases in free amino acids, glucose, and fatty acid metabolites in preparation for the demands of further stress events, as seen in Senegalese sole, *Solea senegalensis*, and gilthead seabream (Arends et al., 1999; Costas et al., 2011). Notably, the increase in osmolality in the stressed fish was still below the highest osmolality measured in the unstressed group, suggesting the increase was still within a tolerable range for the brassy chub. Further, this stress-induced rise in plasma osmolality in the brassy chub was lower in magnitude than seen in Mozambique tilapia following a similar handling stressor, even though the intensity of the stressors was higher for the brassy chub (stressed 5 min every 15 min for brassy chub vs 5 min every 30 min for Mozambique tilapia; Breves et al., 2010). This may suggest that brassy chub has a higher capacity to handle stressors in seawater than other species, though physiological responses to stressors and ultimately the resiliency of fish to handling stressors can be complicated by a multitude of other factors. Finally, as cortisol typically promotes seawater acclimation, its increase in circulation in stressed fish could have contributed towards the reduction in plasma osmolality compared with the unstressed fish seen at 12 and 24 h following the stressor.

The process of osmoregulation is energetically costly, with some estimates indicating it requires up to 50 % of the total metabolic energy allocation of fish (Bœuf and Payan, 2001). This energy can come in a variety of forms including lipids, proteins, and carbohydrates (Tseng and Hwang, 2008). I focused on glucose in the current study as it is known to be rapidly mobilized in fish after acute stressors (Polakof et al., 2012). Additionally, its interactions with the stress hormone, cortisol, have been well described as cortisol has been shown to increase glucose mobilization through glycogenolysis and gluconeogenesis and causes functional remodeling of the gill to improve ion extrusion capacity (Faught and Vijayan, 2016; Laiz-Carrión et al., 2002;

Mommsen et al., 1999). In the current study, the basal levels of glucose in the brassy chub (60-80 mg/dL) was within range of other actinopterygii (Polakof et al., 2012). I hypothesized that the plasma glucose in stressed fish would increase, then return to control levels by 24 h, as seen in some salmonids and gilthead seabream (Arends et al., 1999; Barton, 2000). In the current study, however, plasma glucose was increased in the stressed fish as early as 1 h and had a generally sustained increase throughout the 24 h period. The increase in glucose after the stressor was approximately 30 to 50 mg/dL. When compared with the Mozambique tilapia subjected to a handling stressor, Breves et al., 2010 reported stressed fish having increases in glucose approximately 70 to 100 mg/dL (Breves et al., 2010). Salmonids also have been shown to have higher basal glucose levels and increases after handling stressors compared to the brassy chub (Barton, 2000). The low levels of glucose and its response to stressors in the brassy chub may be due to its diet and gastrointestinal processes and subsequent changes in glycogen storage and gluconeogenesis rates. Additionally, glucose homeostasis in fish can be impacted by a variety of environmental and preconditioning factors including temperature, pH, and salinity, further complicating the physiological stress responses and interspecies comparisons (Polakof et al., 2012). In the current study, lipids were not measured, but this may be an area that warrants future studies since hindgut fermenters, such as the brassy chub, may use lipids as a preferential metabolic substrate for energy (Willmott et al., 2005).

Regardless of the metabolic substrate, aerobic metabolism uses the ETC to obtain ATP as the functional energy source during cellular respiration. As stressors trigger metabolic changes to meet potential energy demands, the increase in aerobic metabolism leads to alterations in the ETC and production of excess ROS, which have a variety of damaging reactions including with proteins, lipids, and DNA (Nimse and Pal, 2015). The liver is especially susceptible to ROS

damage as it is a crucial site for lipid metabolism (Sanz et al., 2012). One of the first enzymatic defenses against ROS damage is SOD, which works by converting superoxides into hydrogen peroxide. Catalase and GPX then reduce the hydrogen peroxide to water. In the current study, I hypothesized that the antioxidant activity of liver SOD would increase in response to the stressor, due to the increased energy demands of stress leading to increased ROS production. Following the stressor, however, there was actually a reduction in SOD activity by 6 and 12 h, which would suggest a decrease in the capacity to mitigate damage from ROS. Similar studies with acute stressors have reported decreases (European seabass and lumpfish, *Cyclopterus lumpus*) or increases (trout, *Oncorhynchus mykiss*, and black jaw tilapia, *Sarotherodon melanotheron*) in hepatic SOD following acute stressors (Adeogun et al., 2020; Castro et al., 2018; de Santa Lopez et al., 2023; Özdemir and Bayir, 2023). The SOD response to an acute stressor, therefore, is likely species-specific. In addition, Özdemir and Bayir, 2023 and de Santa Lopez et al., 2023 also found that there were differences in hepatic SOD expression between males and females following stress, which the current study in brassy chub did not account for as the fish were too young to visibly detect phenotypic sex differences. I also hypothesized that the antioxidant activity of GPX would increase in response to the stressor, however, the stressor did not affect the GPX activity, similar to stressed European seabass when given feed containing fish oil (Castro et al., 2018). There were overall increases in GPX activity at the 6 and 12 h time points, likely due to temporal variations in photoperiod leading to increased swimming activity and subsequent increase in energy metabolism as reported in the blunt snout bream, *Megalobrama amblycephala* (Tian et al., 2019). Lastly, unlike other acute handling stress studies, the applied simulated handling stressor had no effect on hepatic LPO in the brassy chub. This could be due to a variety of reasons including ROS production not exceeding the capacity of

the antioxidant defense system including peroxy scavengers, as seen in the sheepshead minnow, *Cyprinidon variegatus*, after a hypoxia stressor (Jimenez et al., 2018). Overall, these results indicate that the simulated handling stressor negatively impacted hepatic SOD activity, but did not lead to excess oxidative damage of lipids, suggesting that brassy chub are resilient to oxidative stress after an acute handling stressor.

#### *4.4 Conclusions*

In this study, I characterized physiological endpoints of the alarm and resistance phases of the stress response in the brassy chub. Notably, measurable elevations in plasma and mucus cortisol, following handling stress, support their effectiveness as indicators for detecting acute stress. The activation of the alarm phase followed similar trends to other commonly aquacultured species in seawater, with transient increases in stress hormones and an elevation in plasma osmolality. These were followed by secondary responses during the resistance phase including metabolic changes such as a sustained rise in plasma glucose, indicating the mobilization of energy. Additionally, there was a minor reduction in the antioxidant enzyme activity of SOD, though this did not lead to detectable lipid oxidative damage in the liver. Together, these results support the capacity of brassy chub to endure temporary (1 h) handling stress. However, because the physiology of the brassy chub is poorly understood, more research is needed to shed light on the adaptive stress responses to environmental and anthropogenic stressors, including health-related parameters, other endpoints of the HPI axis, and effectors of osmoregulation and growth. Additionally, it is crucial to understand how the brassy chub responds, not only to a single handling stressor, but also to sequential stressors and other factors including water quality, light,

diet, and feeding regimens over longer periods. Overall, this knowledge stands to inform and benefit both environmental management and aquaculture practices.

## **CHAPTER III**

### **Final Remarks**

In this master's thesis, I designed and conducted a study to characterize the effects of an acute handling stressor on parameters indicative of the alarm and resistance phases of the stress response in the brassy chub. To the best of my knowledge, this is the first study using F1, bred in captivity with parents from the wild, brassy chub and the first study focused on its stress response. As the fastest growing sector of agriculture, aquaculture is at the forefront of maximizing sustainable food production for our growing population. As a rapidly growing marine herbivore, the brassy chub could play a role as a critical component for the expansion of aquaculture production by limiting our reliance on freshwater resources and wild capture fisheries for fish feed inputs. Given the potential benefits of increasing sustainable aquaculture through the development of the brassy chub, the overall goal of this project was to improve our understanding of the brassy chub's suitability to culture conditions and the stressful handling practices that are often associated with the captive rearing of fish, thereby providing a fundamental basis for welfare inputs. Additionally, I aimed to validate the use of mucus samples as a potential minimally invasive technique to measure stress in the brassy chub. In this study, I measured endpoints that are foundational in the study of stress in fishes, with specific regard to understanding the resiliency of fish to environmental stimuli such as handling stress.

I hypothesized that the simulated handling stressor would affect the alarm and resistance phase response parameters in similar manners to other commonly aquacultured, marine species. I also predicted that the responses of plasma and mucus during the alarm phase would follow similar overall trends in response to the simulated stressor, validating the mucus as a minimally invasive technique for detecting stress.

After exposure to the handling stressor, the measurable fluctuations in both plasma and mucus cortisol confirmed my hypothesis and indicated the utility of these methods for detecting acute stress. Additionally, confirming that mucus samples can be used as a minimally invasive technique for the brassy chub benefits the fish by reducing sampling stress, in addition to reducing potential economic losses from reduced growth or mortalities. The plasma osmolality in the stressed fish increased by 1 and 6 h, indicating the stressor negatively impacted the fish's ability to properly maintain salt and water balance. As the energetic demands of osmoregulation can be costly, the loss of osmotic balance from the simulated handling stressor may lead to a reduction in energy put towards growth, a crucial endpoint in aquaculture production. For the secondary response endpoints, plasma glucose increased by 1h, likely as a response to the increase in energy demand during the fight or flight response of the alarm phase and from the increase in cortisol triggering glycogenolysis from the liver and gluconeogenesis for glucose mobilization at subsequent time points. On the other hand, contrary to the expected stress-induced rise in LPO and activity of antioxidant enzymes, the activity of SOD decreased at 6 and 12 h while GPX and LPO remained unaffected by the stressor in brassy chub. The reduction in SOD suggested a reduced capacity to mitigate damage from ROS, however, there was no excess LPO damage which indicated the ROS production after the stressor did not exceed the capacity of the antioxidant defense system. Further exploration of the brassy chub's ROS production and removal after the stressor is needed to fully understand the extent to which the brassy chub may be resilient to handling stress; as a hindgut fermenter, the brassy chub may have preadaptations to limit LPO in the liver, but may be more susceptible to other oxidative damage endpoints. Increased intensity or longevity of stressors may be needed for detectable changes in LPO in the brassy chub as seen in other aquaculture species. Overall, the results of this study support the

resiliency of brassy chub to an acute handling stressor. Nonetheless, there is still much more that needs to be investigated in future studies. For example, the ability to detect stress is helpful, but needs to be combined with other endpoints such as health, growth, or immune function parameters for a full interpretation of health status and development of OWIs. On that point, a physically healthy fish does not signify optimal welfare conditions, as they could be adapted to the suboptimal environments; suboptimal environmental conditions along with anthropogenic stressors may impact the fish's allostatic load, cumulative impacts of stress events or chronic stress, and the changes in magnitude of the responses to stress as such. Thus, more studies are necessary to determine optimal welfare practices. Additionally, a stressor or even a stimulus that is perceived as a stressor, may not lead to detectable damage to the brassy chub as seen in the current study. However, the physiological changes that take place can be energetically taxing, leading to a reduction in the capacity to endure future stressors. As the brassy chub is newly employed as an aquaculture candidate, domestication and selective breeding may aid in this aspect. Overall, the findings in this thesis provide novel insights into the capacity of the brassy chub to endure a handling stressor. This knowledge can provide the framework for a plethora of future studies in informing and enhancing fish welfare and aquaculture production performance.

## References

- Adeogun, A. O., Ibor, O. R., Omiwole, R., Chukwuka, A. V., Adewale, A. H., Kumuyi, O., & Arukwe, A. (2020). Sex-differences in physiological and oxidative stress responses and heavy metals burden in the black jaw tilapia, *Sarotherodon melanotheron* from a tropical freshwater dam (Nigeria). *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 229, 108676. <https://doi.org/10.1016/j.cbpc.2019.108676>
- Afonso, L. O. B., Hosoya, S., Osborne, J., Gamperl, A. K., & Johnson, S. (2008). Lack of glucose and hsp70 responses in haddock *Melanogrammus aeglefinus* (L.) subjected to handling and heat shock. *Journal of Fish Biology*, 72(1), 157–167. <https://doi.org/10.1111/j.1095-8649.2007.01697.x>
- Arends, R. J., Mancera, J. M., Muñoz, J. L., Wendelaar Bonga, S. E., & Flik, G. (1999). The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *The Journal of Endocrinology*, 163(1), 149–157. <https://doi.org/10.1677/joe.0.1630149>
- Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science*, 104(3–4), 199–235. <https://doi.org/10.1016/j.applanim.2006.09.001>
- Balasz, J. C., & Tort, L. (2019). Netting the Stress Responses in Fish. *Frontiers in Endocrinology*, 10, 62. <https://doi.org/10.3389/fendo.2019.00062>
- Barton, B. A., Schreck, C. B., & Fowler, L. G. (1988). Fasting and Diet Content Affect Stress-Induced Changes in Plasma Glucose and Cortisol in Juvenile Chinook Salmon. *The Progressive Fish-Culturist*, 50(1), 16–22. [https://doi.org/10.1577/1548-8640\(1988\)050<0016:FADCAS>2.3.CO;2](https://doi.org/10.1577/1548-8640(1988)050<0016:FADCAS>2.3.CO;2)
- Barton, B. A. (2000). Salmonid Fishes Differ in Their Cortisol and Glucose Responses to Handling and Transport Stress. *North American Journal of Aquaculture*, 62, 12–18.
- Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*, 42(3), 517–525. <https://doi.org/10.1093/icb/42.3.517>
- Barton, B. A., & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, 1, 3–26. [https://doi.org/10.1016/0959-8030\(91\)90019-G](https://doi.org/10.1016/0959-8030(91)90019-G)
- Billman, G. E. (2020). Homeostasis: The Underappreciated and Far Too Often Ignored Central Organizing Principle of Physiology. *Frontiers in Physiology*, 11, 200. <https://doi.org/10.3389/fphys.2020.00200>
- Boeuf, G., & Payan, P. (2001). How should salinity influence fish growth? *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 130(4), 411–423. [https://doi.org/10.1016/s1532-0456\(01\)00268-x](https://doi.org/10.1016/s1532-0456(01)00268-x)
- Braithwaite, V. A., & Ebbesson, L. O. E. (2014). Pain and stress responses in farmed fish: *Revue Scientifique et Technique de l'OIE*, 33(1), 245–253. <https://doi.org/10.20506/rst.33.1.2285>
- Breves, J. P., Hirano, T., & Grau, E. G. (2010). Ionoregulatory and endocrine responses to disturbed salt and water balance in Mozambique tilapia exposed to confinement and handling stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 155(3), 294–300. <https://doi.org/10.1016/j.cbpa.2009.10.033>
- Broom, D. M., & Corke, M. J. (2002). Effects of Disease on Farm Animal Welfare. *Acta Veterinaria Brno*, 71(1), 133–136. <https://doi.org/10.2754/avb200271010133>

- Carbajal, A., Soler, P., Tallo-Parra, O., Isasa, M., Echevarria, C., Lopez-Bejar, M., & Vinyoles, D. (2019). Towards Non-Invasive Methods in Measuring Fish Welfare: The Measurement of Cortisol Concentrations in Fish Skin Mucus as a Biomarker of Habitat Quality. *Animals*, 9(11), 939. <https://doi.org/10.3390/ani9110939>
- Cardozo-Ferreira, G. C., Calazans, T. L., Benevides, L. J., Luiz, O. J., Ferreira, C. E. L., & Joyeux, J.-C. (2021). Ecological Traits Influencing Anthropogenic Debris Ingestion by Herbivorous Reef Fishes. *Frontiers in Marine Science*, 8, 717435. <https://doi.org/10.3389/fmars.2021.717435>
- Carey, J. B., & McCormick, S. D. (1998). Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. *Aquaculture*, 168(1–4), 237–253. [https://doi.org/10.1016/S0044-8486\(98\)00352-4](https://doi.org/10.1016/S0044-8486(98)00352-4)
- Carneiro, P. C. F., Urbinati, E. C., & Bendhack, F. (2007). Osmoregulation and Fish Transportation. In B. Baldisserotto, J. M. Mancera, & B. G. Kapoor (Eds.), *Fish Osmoregulation* (1st ed., pp. 235–248). CRC Press. <https://doi.org/10.1201/9780429063909-8>
- Castro, C., Pérez-Jiménez, A., Coutinho, F., Corraze, G., Panserat, S., Peres, H., Teles, A. O., & Enes, P. (2018). Nutritional history does not modulate hepatic oxidative status of European sea bass (*Dicentrarchus labrax*) submitted to handling stress. *Fish Physiology and Biochemistry*, 44(3), 911–918. <https://doi.org/10.1007/s10695-018-0480-6>
- Castro, C., Pérez-Jiménez, A., Coutinho, F., Díaz-Rosales, P., Serra, C. A. dos R., Panserat, S., Corraze, G., Peres, H., & Oliva-Teles, A. (2015). Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. *British Journal of Nutrition*, 114(10), 1584–1593. <https://doi.org/10.1017/S0007114515003360>
- Çengel, Y. A. (2023). Eighteen distinctive characteristics of life. *Heliyon*, 9(3), e13603. <https://doi.org/10.1016/j.heliyon.2023.e13603>
- Chang, C.-H., Huang, J.-J., Yeh, C.-Y., Tang, C.-H., Hwang, L.-Y., & Lee, T.-H. (2018). Salinity Effects on Strategies of Glycogen Utilization in Livers of Euryhaline Milkfish (*Chanos chanos*) under Hypothermal Stress. *Frontiers in Physiology*, 9, 81. <https://doi.org/10.3389/fphys.2018.00081>
- Chang, R. J. A., Celino-Brady, F. T., & Seale, A. P. (2023). Changes in cortisol and corticosteroid receptors during dynamic salinity challenges in Mozambique tilapia. *General and Comparative Endocrinology*, 342, 114340. <https://doi.org/10.1016/j.ygcen.2023.114340>
- Cheyadmi, S., Chadli, H., Nhhala, H., El Yamlaoui, B., El Maadoudi, M., Kounoun, A., Cacciola, F., Ez-Zaaim, A., & Chair, H. (2022). Primary and Secondary Physiological Stress Responses of European Sea Bass (*Dicentrarchus labrax*) Due to Rearing Practices under Aquaculture Farming Conditions in M'diq Bay, Moroccan Mediterranean: The Case of Sampling Operation for Size and Weight Measurement. *Life*, 13(1), 110. <https://doi.org/10.3390/life13010110>
- Chowdhury, S., & Saikia, S. K. (2020). Oxidative Stress in Fish: A Review. *Journal of Scientific Research*, 12(1), 145–160. <https://doi.org/10.3329/jsr.v12i1.41716>
- Clements, K. D., German, D. P., Piché, J., Tribollet, A., & Choat, J. H. (2017). Integrating ecological roles and trophic diversification on coral reefs: Multiple lines of evidence identify parrotfishes as microphages. *Biological Journal of the Linnean Society*. <https://doi.org/10.1111/bij.12914>

- Costas, B., Conceição, L. E. C., Aragão, C., Martos, J. A., Ruiz-Jarabo, I., Mancera, J. M., & Afonso, A. (2011). Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: Effects on non-specific immune parameters, plasma free amino acids and energy metabolism. *Aquaculture*, 316(1–4), 68–76. <https://doi.org/10.1016/j.aquaculture.2011.03.011>
- Costello, C., Cao, L., Gelcich, S., Cisneros-Mata, M. Á., Free, C. M., Froehlich, H. E., Golden, C. D., Ishimura, G., Maier, J., Macadam-Somer, I., Mangin, T., Melnychuk, M. C., Miyahara, M., de Moor, C. L., Naylor, R., Nøstbakken, L., Ojea, E., O'Reilly, E., Parma, A. M., ... Lubchenco, J. (2020). The future of food from the sea. *Nature*, 588(7836), 95–100. <https://doi.org/10.1038/s41586-020-2616-y>
- da Santa Lopes, T., Costas, B., Ramos-Pinto, L., Reynolds, P., Imsland, A. K. D., & Fernandes, J. M. O. (2023). Exploring the Effects of Acute Stress Exposure on Lumpfish Plasma and Liver Biomarkers. *Animals*, 13(23), 3623. <https://doi.org/10.3390/ani13233623>
- Danziger, J., & Zeidel, M. L. (2015). Osmotic homeostasis. *Clinical Journal of the American Society of Nephrology*, 10(5), 852–862. <https://doi.org/10.2215/CJN.10741013>
- Davis, K. B., & Small, B. C. (2006). Rates of cortisol increase and decrease in channel catfish and sunshine bass exposed to an acute confinement stressor. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 143(1), 134–139. <https://doi.org/10.1016/j.cbpc.2006.01.003>
- Davis, K. B., Suttle, M. A., & Parker, N. C. (1984). Biotic and Abiotic Influences on Corticosteroid Hormone Rhythms in Channel Catfish. *Transactions of the American Fisheries Society*, 113(4), 414–421.
- De Mercado, E., Larrán, A. M., Pinedo, J., & Tomás-Almenar, C. (2018). Skin mucous: A new approach to assess stress in rainbow trout. *Aquaculture*, 484, 90–97. <https://doi.org/10.1016/j.aquaculture.2017.10.031>
- DLNR. (2021). Commercial Marine Landings Summary Trend Report Calendar Year 2021. *Department of Land and Natural Resources State of Hawaii*, 1-17.
- Douxflis, J., Mandiki, S. N. M., Marotte, G., Wang, N., Silvestre, F., Milla, S., Henrotte, E., Vandecan, M., Rougeot, C., Mélard, C., & Kestemont, P. (2011). Does domestication process affect stress response in juvenile Eurasian perch *Perca fluviatilis*? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 159(1), 92–99. <https://doi.org/10.1016/j.cbpa.2011.01.021>
- Eissa, N., & Wang, H. (2014). Transcriptional stress responses to environmental and husbandry stressors in aquaculture species. *Reviews in Aquaculture*, 8(1), 61–88. <https://doi.org/10.1111/raq.12081>
- Faught, E., & Vijayan, M. M. (2016). Mechanisms of cortisol action in fish hepatocytes. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 199, 136–145. <https://doi.org/10.1016/j.cbpb.2016.06.012>
- Fernández, J., Pérez-Álvarez, J. A., & Fernández-López, J. A. (1997). Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry*, 59(3), 345–353. [https://doi.org/10.1016/S0308-8146\(96\)00114-8](https://doi.org/10.1016/S0308-8146(96)00114-8)
- Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M., & Ibarz, A. (2019). Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus. *Aquaculture*, 499, 185–194. <https://doi.org/10.1016/j.aquaculture.2018.09.039>

- Foo, J. T. W., & Lam, T. J. (1993). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. *Aquaculture*, 115(1–2), 145–158. [https://doi.org/10.1016/0044-8486\(93\)90365-6](https://doi.org/10.1016/0044-8486(93)90365-6)
- Franks, B., Ewell, C., & Jacquet, J. (2021). Animal welfare risks of global aquaculture. *Science Advances*, 7(14), eabg0677. <https://doi.org/10.1126/sciadv.abg0677>
- Froehlich, H. E., Jacobsen, N. S., Essington, T. E., Clavelle, T., & Halpern, B. S. (2018). Avoiding the ecological limits of forage fish for fed aquaculture. *Nature Sustainability*, 1(6), 298–303. <https://doi.org/10.1038/s41893-018-0077-1>
- Fryer, J. N., & Lederis, K. (1986). Control of Corticotropin Secretion in Teleost Fishes. *American Zoologist*, 26(4), 1017–1026. <https://doi.org/10.1093/icb/26.4.1017>
- Geslani, C., Loke, M., Takenaka, B., & Leung, P. (2012). Hawai‘i’s Seafood Consumption and its Supply Sources. Pelagic Fisheries and Research Program.
- Hoem, K. S., & Tveten, A. (2019). Current approaches in decoding the molecular mechanisms of long-term stress in adult farmed Atlantic salmon (*Salmo salar*). *Reviews in Aquaculture*, raq.12405. <https://doi.org/10.1111/raq.12405>
- Hoseinifar, S. H., Yousefi, S., Van Doan, H., Ashouri, G., Gioacchini, G., Maradonna, F., & Carnevali, O. (2021). Oxidative Stress and Antioxidant Defense in Fish: The Implications of Probiotic, Prebiotic, and Synbiotics. *Reviews in Fisheries Science & Aquaculture*, 29(2), 198–217. <https://doi.org/10.1080/23308249.2020.1795616>
- Hosoya, S., Johnson, S. C., Iwama, G. K., Gamperl, A. K., & Afonso, L. O. B. (2007). Changes in free and total plasma cortisol levels in juvenile haddock (*Melanogrammus aeglefinus*) exposed to long-term handling stress. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, 146(1), 78–86. <https://doi.org/10.1016/j.cbpa.2006.09.003>
- Huntingford, F., Kadri, S., & Jobling, M. (2012). Introduction: Aquaculture and Behaviour. In F. Huntingford, M. Jobling, & S. Kadri (Eds.), *Aquaculture and Behavior* (1st ed., pp. 1–35). Wiley. <https://doi.org/10.1002/9781444354614.ch1>
- Jentoft, S., Aastveit, A. H., Torjesen, P. A., & Andersen, Ø. (2005). Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (*Perca fluviatilis*) and domesticated rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 141(3), 353–358. <https://doi.org/10.1016/j.cbpb.2005.06.006>
- Jimenez, A. G., Braun, E., & Tobin, K. (2019). How does chronic temperature exposure affect hypoxia tolerance in sheepshead minnows’ (*Cyprinodon variegatus variegatus*) ability to tolerate oxidative stress? *Fish Physiology and Biochemistry*, 45(2), 499–510. <https://doi.org/10.1007/s10695-018-0583-0>
- Kalamarz-Kubiak, H. (2018). Cortisol in Correlation to Other Indicators of Fish Welfare. In A. G. Al-kaf (Ed.), *Corticosteroids*. InTech. <https://doi.org/10.5772/intechopen.72392>
- Kawarazuka, N., & Béné, C. (2010). Linking small-scale fisheries and aquaculture to household nutritional security: An overview. *Food Security*, 2(4), 343–357. <https://doi.org/10.1007/s12571-010-0079-y>
- Kenter, L. W., Breton, T. S., & Berlinsky, D. L. (2021). Comparing stress responses of F1 and domesticated striped bass (*Morone saxatilis*) following a repeated acute stressor. *Aquaculture Research*, 52(10), 4786–4798. <https://doi.org/10.1111/are.15312>

- Kühn, E. R., Corneillie, S., & Ollevier, F. (1986). Circadian variations in plasma osmolality, electrolytes, glucose, and cortisol in carp (*Cyprinus carpio*). *General and Comparative Endocrinology*, 61(3), 459–468. [https://doi.org/10.1016/0016-6480\(86\)90234-0](https://doi.org/10.1016/0016-6480(86)90234-0)
- Laiz-Carrión, R., Sangiao-Alvarellos, S., Guzmán, J. M., Martín Del Río, M. P., Míguez, J. M., Soengas, J. L., & Mancera, J. M. (2002). Energy Metabolism in Fish Tissues Related to Osmoregulation and Cortisol Action. *Fish Physiology and Biochemistry*, 27(3/4), 179–188. <https://doi.org/10.1023/B:FISH.0000032725.96481.b8>
- Le François, N. R., Tremblay-Bourgeois, S., Dupont Cyr, B.-A., Savoie, A., Roy, R. L., Imsland, A. K., & Benfey, T. J. (2013). Cortisol and Behavioral Response to Handling (Acute) and Confinement (Chronic) Stressors in Juvenile Spotted Wolffish, *Anarhichas minor*. *Journal of Applied Aquaculture*, 25(3), 248–264. <https://doi.org/10.1080/10454438.2013.815142>
- Lemos, L. S., Angarica, L. M., Hauser-Davis, R. A., & Quinete, N. (2023). Cortisol as a Stress Indicator in Fish: Sampling Methods, Analytical Techniques, and Organic Pollutant Exposure Assessments. *International Journal of Environmental Research and Public Health*, 20(13), 6237. <https://doi.org/10.3390/ijerph20136237>
- Leung, P., & Loke, M. (2008). Increasing Hawai‘i’s Food Self-Sufficiency. College of Tropical Agriculture and Human Resources, University of Hawai‘i and Mānoa, EI-16
- Liu, Z., Zhou, T., & Gao, D. (2022). Genetic and epigenetic regulation of growth, reproduction, disease resistance and stress responses in aquaculture. *Frontiers in Genetics*, 13, 994471. <https://doi.org/10.3389/fgene.2022.994471>
- Lozano-Muñoz, I., Castellaro, G., Bueno, G., & Wacyk, J. (2022). Herbivorous fish (*Medialuna ancietae*) as a sustainable alternative for nutrition security in Northern Chile. *Scientific Reports*, 12(1), 1619. <https://doi.org/10.1038/s41598-021-04628-3>
- Madaro, A., Nilsson, J., Whatmore, P., Roh, H., Grove, S., Stien, L. H., & Olsen, R. E. (2023). Acute stress response on Atlantic salmon: A time-course study of the effects on plasma metabolites, mucus cortisol levels, and head kidney transcriptome profile. *Fish Physiology and Biochemistry*, 49(1), 97–116. <https://doi.org/10.1007/s10695-022-011634>
- Malintha, G. H. T., Woo, D. W., Celino-Brady, F. T., & Seale, A. P. (2023). Temperature modulates the osmosensitivity of tilapia prolactin cells. *Scientific Reports*, 13(1), 20217. <https://doi.org/10.1038/s41598-023-47044-5>
- Martos-Sitcha, J. A., Mancera, J. M., Prunet, P., & Magnoni, L. J. (2020). Editorial: Welfare and Stressors in Fish: Challenges Facing Aquaculture. *Frontiers in Physiology*, 11, 162. <https://doi.org/10.3389/fphys.2020.00162>
- McCormick, S. D. (2001). Endocrine Control of Osmoregulation in Teleost Fish. *American Zoologist*, 41(4), 781–794. <https://doi.org/10.1093/icb/41.4.781>
- McCormick, S. D., Regish, A., O’Dea, M. F., & Shrimpton, J. M. (2008). Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and isoform mRNA levels in Atlantic salmon. *General and Comparative Endocrinology*, 157(1), 35–40. <https://doi.org/10.1016/j.ygcen.2008.03.024>
- Michael, P., Hyndes, G., Vanderklift, M., & Vergés, A. (2013). Identity and behaviour of herbivorous fish influence large-scale spatial patterns of macroalgal herbivory in a coral reef. *Marine Ecology Progress Series*, 482, 227–240. <https://doi.org/10.3354/meps10262>

- Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H., & Prajapati, J. (2015). Probiotics as potential antioxidants: A systematic review. *Journal of Agricultural and Food Chemistry*, 63(14), 3615–3626. <https://doi.org/10.1021/jf506326t>
- Miyasaki, T., & Fujioka, Y. (2012). Flavor Compounds of Several Herbivorous Fishes (2). *Japanese Society for Aquaculture Science*. <https://doi.org/10.11233/aquaculturesci.60.189>
- Modell, H., Cliff, W., Michael, J., McFarland, J., Wenderoth, M. P., & Wright, A. (2015). A physiologist's view of homeostasis. *Advances in Physiology Education*, 39(4), 259–266. <https://doi.org/10.1152/advan.00107.2015>
- Mommsen, T. P., Vijayan, M. M., & Moon, T. W. (1999). Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, 9, 211-268
- Mommsen, T. P., Walsh, P. J., Perry, S. F., & Moon, T. W. (1988). Interactive effects of catecholamines and hypercapnia on glucose production in isolated trout hepatocytes. *General and Comparative Endocrinology*, 70(1), 63–73. [https://doi.org/10.1016/0016-6480\(88\)90094-9](https://doi.org/10.1016/0016-6480(88)90094-9)
- Morinaka F., & Ando W. (2011). Study of Making Food on *Kyphosus vaigiensis*. *The Japanese Society of Fisheries Engineering*. [https://doi.org/10.18903/fisheng.48.1\\_73](https://doi.org/10.18903/fisheng.48.1_73)
- Mountfort, D. O., Campbell, J., & Clements, K. D. (2002). Hindgut Fermentation in Three Species of Marine Herbivorous Fish. *Applied and Environmental Microbiology*, 68(3), 1374–1380. <https://doi.org/10.1128/AEM.68.3.1374-1380.2002>
- Nilsson, S., Abrahamsson, T., & Grove, D. J. (1976). Sympathetic nervous control of adrenaline release from the head kidney of the cod, *Gadus morhua*. *Comparative Biochemistry and Physiology. C: Comparative Pharmacology*, 55(2), 123–127. [https://doi.org/10.1016/0306-4492\(76\)90034-4](https://doi.org/10.1016/0306-4492(76)90034-4)
- Nimse, S. B., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Advances*, 5(35), 27986–28006. <https://doi.org/10.1039/C4RA13315C>
- Ocean Era. (2021). Preliminary Environmental Review for the Ocean Era Offshore Aquaculture Farm off 'Ewa Beach, O'ahu, Hawai'i. 1–62
- Okano, R. L. (2011). Potential Influences of Submarine Groundwater Discharge, Nutrients, and Herbivory on Hawaiian Reef Algae. University of Hawaii.
- Oliver, A., Podell, S., Kelly, L. W., Sparagon, W. J., Plominsky, A. M., Nelson, R. S., Laurens, L. M. L., Augyte, S., Sims, N. A., Nelson, C. E., & Allen, E. E. (2023). Enrichable consortia of microbial symbionts degrade macroalgal polysaccharides in *Kyphosus* fish [Preprint]. *Microbiology*. <https://doi.org/10.1101/2023.11.28.568905>
- Orrenius, S., Gogvadze, V., & Zhivotovsky, B. (2007). Mitochondrial oxidative stress: Implications for cell death. *Annual Review of Pharmacology and Toxicology*, 47, 143–183. <https://doi.org/10.1146/annurev.pharmtox.47.1.20505.105122>
- Özdemir, E., & Bayır, M. (2023). Molecular cloning and characterization of Cu-Zn superoxide dismutase (sod1) gene in brown trout and its expression in response to acute aquaculture stressors. *Animal Biotechnology*, 34(6), 1968–1978. <https://doi.org/10.1080/10495398.2022.2061505>
- Palińska-Żarska, K., Król, J., Woźny, M., Kamaszewski, M., Szudrowicz, H., Wiechetek, W., Brzuzan, P., Fopp-Bayat, D., & Żarski, D. (2021). Domestication affected stress and immune response markers in *Perca fluviatilis* in the early larval stage. *Fish & Shellfish Immunology*, 114, 184–198. <https://doi.org/10.1016/j.fsi.2021.04.028>

- Pardee, C., & Omori, C. (2023). *Hawai'i's Reef Fish Food, Science, Tradition*. Legacy Isle Publishing.
- Peterson, M. S., & Gilmore, R. G. (1988). Hematocrit, osmolality, and ion concentration in fishes: Consideration of circadian patterns in the experimental design. *Journal of Experimental Marine Biology and Ecology*, 121(1), 73–78. [https://doi.org/10.1016/0022-0981\(88\)90024-X](https://doi.org/10.1016/0022-0981(88)90024-X)
- Pisaniello, A., Bojarski, L. D., Handley, K. M., White, W. L., Angert, E. R., & Clements, K. D. (2022). Sources of variation in community composition of the hindgut microbiota in two tropical *Kyphosus* species. *Coral Reefs*, 41(5), 1523–1535. <https://doi.org/10.1007/s00338-022-02299-8>
- Podell, S., Oliver, A., Kelly, L. W., Sparagon, W. J., Plominsky, A. M., Nelson, R. S., Laurens, L. M. L., Augyte, S., Sims, N. A., Nelson, C. E., & Allen, E. E. (2023). Herbivorous Fish Microbiome Adaptations to Sulfated Dietary Polysaccharides. *Applied and Environmental Microbiology*, 89(5), e0215422. <https://doi.org/10.1128/aem.02154-22>
- Polakof, S., Panserat, S., Soengas, J. L., & Moon, T. W. (2012). Glucose metabolism in fish: A review. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, 182(8), 1015–1045. <https://doi.org/10.1007/s00360-012-0658-7>
- Rance, T. A., Baker, B. I., & Webley, G. (1982). Variations in plasma cortisol concentrations over a 24-hour period in the rainbow trout *Salmo gairdneri*. *General and Comparative Endocrinology*, 48(2), 269–274. [https://doi.org/10.1016/0016-6480\(82\)90026-0](https://doi.org/10.1016/0016-6480(82)90026-0)
- Reid, S. G., Bernier, N. J., & Perry, S. F. (1998). The adrenergic stress response in fish: Control of catecholamine storage and release. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 120(1), 1–27. [https://doi.org/10.1016/S0742-8413\(98\)00037-1](https://doi.org/10.1016/S0742-8413(98)00037-1)
- Sadoul, B., & Geffroy, B. (2019). Measuring cortisol, the major stress hormone in fishes. *Journal of Fish Biology*, 94(4), 540–555. <https://doi.org/10.1111/jfb.13904>
- Sakihara, T. S., Nishiura, L. K., Shimoda, T. E., Shindo, T. T., & Nishimoto, R. T. (2015). Brassy chubs *Kyphosus vaigiensis* display unexpected trans-island movement along inshore habitats. *Environmental Biology of Fishes*, 98(1), 155–163. <https://doi.org/10.1007/s10641-014-0245-8>
- Sanz, A., Furné, M., Trenzado, C. E., De Haro, C., & Sánchez-Muros, M. J. (2012). Study of the Oxidative State, as a Marker of Welfare, on Gilthead Sea Bream, *Sparus aurata*, Subjected to Handling Stress. *Journal of the World Aquaculture Society*, 43(5), 707–715. <https://doi.org/10.1111/j.1749-7345.2012.00602.x>
- Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Current Biology: CB*, 24(10), R453–462. <https://doi.org/10.1016/j.cub.2014.03.034>
- Schreck, C. B. (2000). Accumulation and long-term effects of stress in fish. In G. P. Moberg & J. A. Mench (Eds.), *The biology of animal stress: Basic principles and implications for animal welfare*. (1st ed., pp. 147–158). CABI Publishing. <https://doi.org/10.1079/9780851993591.0147>
- Schreck, C. B., & Tort, L. (2016). The Concept of Stress in Fish. In *Fish Physiology* (Vol. 35, pp. 1–34). <https://doi.org/10.1016/B978-0-12-802728-8.00001-1>
- Scott, A. P., & Baker, B. I. (1975). ACTH production by the pars intermedia of the rainbow trout pituitary. *General and Comparative Endocrinology*, 27(2), 193–202. [https://doi.org/10.1016/0016-6480\(75\)90233-6](https://doi.org/10.1016/0016-6480(75)90233-6)

- Scott, A. P., & Ellis, T. (2007). Measurement of fish steroids in water—A review. *General and Comparative Endocrinology*, 153(1–3), 392–400. <https://doi.org/10.1016/j.ygcen.2006.11.006>
- Seale, A. P., & Breves, J. P. (2022). Endocrine and osmoregulatory responses to tidally-changing salinities in fishes. *General and Comparative Endocrinology*, 326, 114071. <https://doi.org/10.1016/j.ygcen.2022.114071>
- Seale, A. P., Pavlosky, K. K., Celino-Brady, F. T., Yamaguchi, Y., Breves, J. P., & Lerner, D. T. (2019). Systemic versus tissue-level prolactin signaling in a teleost during a tidal cycle. *Journal of Comparative Physiology B*, 189(5), 581–594. <https://doi.org/10.1007/s00360-019-01233-9>
- Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K. S., Mathieu, C., Ruane, N., Jutfelt, F., Toften, H., & Vaughan, L. (2012). Health of farmed fish: Its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry*, 38(1), 85–105. <https://doi.org/10.1007/s10695-011-9517-9>
- Selye, H. (1936). A Syndrome produced by Diverse Nocuous Agents. *Nature*, 138(3479), 32–32. <https://doi.org/10.1038/138032a0>
- Selye, H. (1973). The evolution of the stress concept. *American Scientist*, 61(6), 692–699.
- Serviere-Zaragoza, E., Lluch-Cota, S. E., Mazariegos-Villarreal, A., Balart, E. F., Valencia-Valdez, H., & Méndez-Rodríguez, L. C. (2021). Cadmium, Lead, Copper, Zinc, and Iron Concentration Patterns in Three Marine Fish Species from Two Different Mining Sites inside the Gulf of California, Mexico. *International Journal of Environmental Research and Public Health*, 18(2), 844. <https://doi.org/10.3390/ijerph18020844>
- Sparagon, W. J., Gentry, E. C., Minich, J. J., Vollbrecht, L., Laurens, L. M. L., Allen, E. E., Sims, N. A., Dorrestein, P. C., Kelly, L. W., & Nelson, C. E. (2022). Fine scale transitions of the microbiota and metabolome along the gastrointestinal tract of herbivorous fishes. *Animal Microbiome*, 4(1), 33. <https://doi.org/10.1186/s4252302200182-z>
- Spieler, R. E., & Noeske, T. A. (1984). Effects of Photoperiod and Feeding Schedule on Diel Variations of Locomotor Activity, Cortisol, and Thyroxine in Goldfish. *Transactions of the American Fisheries Society*, 113(4), 528–539. [https://doi.org/10.1577/1548-8659\(1984\)113<528:EOPAFS>2.0.CO;2](https://doi.org/10.1577/1548-8659(1984)113<528:EOPAFS>2.0.CO;2)
- Srikanth, K., Pereira, E., Duarte, A. C., & Ahmad, I. (2013). Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish—A review. *Environmental Science and Pollution Research International*, 20(4), 2133–2149. <https://doi.org/10.1007/s11356-012-1459-y>
- Streit, R. P., Hoey, A. S., & Bellwood, D. R. (2015). Feeding characteristics reveal functional distinctions among browsing herbivorous fishes on coral reefs. *Coral Reefs*, 34(4), 1037–1047. <https://doi.org/10.1007/s00338-015-1322-y>
- Takahashi, A., & Mizusawa, K. (2013). Posttranslational modifications of proopiomelanocortin in vertebrates and their biological significance. *Frontiers in Endocrinology*, 4, 143. <https://doi.org/10.3389/fendo.2013.00143>
- Teletchea, F. (2015). Domestication of Marine Fish Species: Update and Perspectives. *Journal of Marine Science and Engineering*, 3(4), 1227–1243. <https://doi.org/10.3390/jmse3041227>
- Teletchea, F., & Fontaine, P. (2014). Levels of domestication in fish: Implications for the sustainable future of aquaculture. *Fish and Fisheries*, 15(2), 181–195. <https://doi.org/10.1111/faf.12006>

- Tian, H., Zhang, D., Li, X., Jiang, G., & Liu, W. (2019). Photoperiod affects blunt snout bream (*Megalobrama amblycephala*) growth, diel rhythm of cortisol, activities of antioxidant enzymes and mRNA expression of GH/IGF-I. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 233, 4–10. <https://doi.org/10.1016/j.cbpb.2019.03.007>
- Titcomb, M., & Pukui, M. K. (2021). Native Use of Fish in Hawaii. University of Hawaii Press.
- Tseng, Y.-C., & Hwang, P.-P. (2008). Some insights into energy metabolism for osmoregulation in fish. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 148(4), 419–429. <https://doi.org/10.1016/j.cbpc.2008.04.009>
- Turrens, J. F., Mott, A., Pannell, L. F., & Ruiz, J. C. (2010). Modification of specific amino acid side chains during hydroxyl radical-dependent bovine serum albumin (BSA) oxidation. *The FASEB Journal*, 24(S1). [https://doi.org/10.1096/fasebj.24.1\\_supplement.1001.2](https://doi.org/10.1096/fasebj.24.1_supplement.1001.2)
- Veissier, I., & Boissy, A. (2007). Stress and welfare: Two complementary concepts that are intrinsically related to the animal's point of view. *Physiology & Behavior*, 92(3), 429–433. <https://doi.org/10.1016/j.physbeh.2006.11.008>
- Wanshu, H. (1992). Plasma cortisol and glucose concentrations in the striped mullet (*Mugil cephalus L.*) subjected to intense handling stress. *Chinese Journal of Oceanology and Limnology*, 10(1), 40–43. <https://doi.org/10.1007/BF02844298>
- Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiological Reviews*, 77(3), 591–625. <https://doi.org/10.1152/physrev.1997.77.3.591>
- Willmott, M. E., Clements, K. D., & Wells, R. M. G. (2005). The influence of diet and gastrointestinal fermentation on key enzymes of substrate utilization in marine teleost fishes. *Journal of Experimental Marine Biology and Ecology*, 317(1), 97–108. <https://doi.org/10.1016/j.jembe.2004.11.008>
- Wood, C. M., & Eom, J. (2021). The osmorepiratory compromise in the fish gill. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 254, 110895. <https://doi.org/10.1016/j.cbpa.2021.110895>
- Wu, Y., Wang, X., Zhang, X., Shi, Y., & Li, W. (2022). Locomotor posture and swimming-intensity quantification in starvation-stress behavior detection of individual fish. *Computers and Electronics in Agriculture*, 202, 107399. <https://doi.org/10.1016/j.compag.2022.107399>
- Zhao, R.-Z., Jiang, S., Zhang, L., & Yu, Z.-B. (2019). Mitochondrial electron transport chain, ROS generation and uncoupling (Review). *International Journal of Molecular Medicine*, 44(1), 3–15. <https://doi.org/10.3892/ijmm.2019.4188>