Validation of physiological biopsy methods for sampling live freshwater fishes

by

Laura S.E. Haniford

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science in

Biology

Carleton University Ottawa, Ontario

Abstract

With continuous threats facing our freshwater systems, scientists and fisheries managers require methods to evaluate the health of freshwater fishes. Previously, many health metrics were evaluated using tissue samples lethally taken from fish. However, with technological advances many of the same parameters can be evaluated with only small pieces of tissue taken from a living specimen. Such non-lethal biopsies have been evaluated in laboratory settings to ensure survival after biopsy, however their impact on fine-scale behaviour, fitness, and stress has yet to be evaluated. In this thesis, three separate studies were conducted on male Smallmouth Bass, juvenile Lake Trout, and adult male Walleye to evaluate the consequences of biopsy procedures. Parental care behaviour in Smallmouth Bass (attack scores generated in response to simulated predation, return to nest time) were similar among biopsy methods, however multiple biopsy types (taken from the same fish) was a strong predictor in nest abandonment. Juvenile Lake Trout exploratory behaviour and response to a novel object was not found to be impacted by biopsy treatments, nor was their performance in an exhaustive exercise test. Finally, reflexes and gene expression (Glucocorticoid Receptor 1, Major Histocompatibility Complex Class 2) were not found to differ in adult male Walleye. Collectively, this body of work suggests that biopsy can be conducted on live teleost fish with negligible impacts on welfare or fitness.

General Acknowledgements

I would like to thank my supervisor, Dr. Steven Cooke and committee members Vance Trudeau and Jennifer Provencher, for the guidance provided during my studies. I also wish to thank my friends in the Cooke lab for their support over the last two years and assistance in the field. Further thanks to my sisters Alicia and Paula, to my partner Nigel for editing, and to Oliver for his exemplary supervision. Funding for these projects was provided by an NSERC Discovery Grant to Dr. Cooke as well as the Genome Canada/Ontario Genomics GENFish project and the NSERC FishCast CREATE program through the University of Windsor. Additional funding was provided by the Great Lakes Fishery Commission via the Fisheries Research Program.

Acknowledgements by Chapter

Chapter 2

We are grateful to Cameron, Josh, and Ben Cooke, as well as Connor Reeve for their assistance in the field, and to Connor Reid and Roslyn Dakin for their help with statistical analysis. Additional thanks to Nigel Waite for editing.

Chapter 3

We are grateful to Tim Drew and the staff at the White Lake Fish Culture Station for the support they provided during this project. We would also like to acknowledge Ben Hlina and Chris Elvidge for their guidance during project planning, and to Connor Reid for their assistance in statical analysis. Additional thanks to Nigel Waite for their assistance in the field and for editing.

Chapter 4

Thank you to the Ohio Department of Natural Resources for fish collection, as well as to Matt, Jackson, and Heather for their assistance during the experiment. Further thanks are extended to Mike Dusevic and Christian Bihun for their assistance during the experiment, as well as to

Hossein Haghighi, Declan Burton, Myra Thapar, and Ken Jeffries for their assistance in the laboratory and during analysis.

Table of Contents

Abstract…….2

Acknowledgements……3

Table of Contents……..…….5

Co-authorship Statement……..6

Chapter 1……7

Chapter 2……..12

Chapter 3……..31

Chapter 4……..49

Chapter 5……..66

References…….72

Appendix A……...86

Co-authorship Statement

Due to the collaborative nature of research, the work presented here involved a number of collaborators. Chapter 2 is a manuscript published in the Journal of Applied Animal Behaviour, and all authors contributed to the revision of the manuscript. The complete reference for Chapter 2 is as follows;

Haniford, L.S.E., L. LaRochelle, J.A. Robichaud, D. Burton, and S.J. Cooke. 2023. Evaluating blood, gill, and muscle biopsy methods on the behaviour, reproductive success, and survival of a wild freshwater fish. Applied Animal Behaviour Science, 265, 106004. https://doi.org/10.1016/j.applanim.2023.106004

Chapters 3 and 4 are comprised of two manuscripts that are currently being prepared for submission to peer reviewed journals. Due to the preparation of articles for submission, all manuscripts are written as a group perspective, given that the research involved several collaborators. Those collaborators are listed at the beginning of each chapter.

Chapter 1: General Introduction

Globally, freshwater fisheries contribute significantly to the production of food for human and animal consumption, to recreation, to supporting livelihoods, and to Indigenous cultures (Lynch et al. 2016). Despite the widespread importance of fisheries, freshwater fishes globally are facing numerous challenges. Climate change, habitat loss, and human fishing pressures have depleted populations, causing valued species to become imperiled (Welcomme et al. 2010). Fisheries scientists and managers have implemented various programs to help support native populations (e.g., habitat restoration, fisheries closures, hatchery enhancement), however, without identifying specific threats to fish populations, we fail to offer conservation strategies that are uniquely tailored to the needs of a species or system. Given that health status plays a vital role in the success of fish populations, tools for assessing their health status (e.g., using biomarkers) are imperative to successful management and conservation efforts (Adams et al. 2001).

Traditional methods for assessing fish health have required sacrificing fish and using whole fillets to determine contaminant burden, pathogen load, or health status (reviewed in Adams 2002). However, these techniques are often unfavourable as they require the euthanization of thousands of fish which can deplete populations, which is particularly detrimental in instances where organisms are rare or imperilled (Rolfhus et al. 2008). Furthermore, lethal tissue sampling does not allow researchers to sample the same fish repeatedly over the course of their life eliminating opportunities to understand how the health and condition of organisms change across life stages or relative to different stressors (e.g., before-after studies). Given technological advances it is now possible to use various physiological and genomic assays that require miniscule quantities of tissue. Non-lethal sampling methods have been developed that can

evaluate the health of fishes using only small biopsy samples taken from the gills, muscle, fins, mucus, or blood (Jeffries et al. 2021; Thorstensen et al. 2022). Non-lethal biopsy also benefits researchers as it is suitable to be paired with other technologies, such as electronic tagging and

tracking, which allows researchers to connect fish health to fate (Bøe et al. 2020; Thorstensen et al. 2022). However, concern remains regarding the welfare of the fish during and post-biopsy.

Non-lethal sampling methods have been found to be a highly effective way to examine fish health, as the impact of various stressors can be analyzed using transcriptomics or biochemical assays (such as those assessing enzymes, hormones, osmolytes, or other physiological factors). Collection of biopsies can include drawing blood from the caudal vasculature (Lawrence et al. 2020), taking white muscle tissue using a biopsy punch or needle from the dorsal musculature (Baker et al. 2011), using swabs or forceps to collect mucus and scale samples (Tartor et al. 2020), or using fine scissors to remove the distal ends of 3-6 gill filaments (McCormick 1993; Cornwell et al. 2013). Previous studies (Jeffries et al. 2021; Thorstensen et al. 2022) have outlined the utility of each biopsy type and what metrics can be collected from which tissues. However, given that every technique requires some measure of handling, dermal disturbance (Colotelo and Cooke 2011), and potential to reduce fitness (Portz et al. 2006; Currie et al. 2022), further research is needed to evaluate the impact that biopsy techniques have on welfare (including ecologically relevant endpoints such as behaviour and survival) after release.

Previous work has been done to validate the use of non-lethal biopsy; however, the goals of these studies have largely been focused on determining survival or long-term behaviour after biopsy with a focus on adults. For example, in Cooke et al. (2005), migration success and timing

in Sockeye Salmon was compared between fish that had received gill or blood biopsies and their non-biopsied counterparts. Gill biopsies were further found to have limited impact on growth and salinity tolerance in Atlantic Salmon (McCormick 1993). Similarly, recapture rates in Northern Pike and Lake Whitefish that received muscle biopsies were found to be similar when compared to non-biopsied fish (Baker et al. 2011).

Initial validation work for each biopsy type has been carried out under laboratory conditions (Lockhart et al. 1972; Crawford et al. 1977), however given the highly regulated conditions of a laboratory study (no predators, equal access to food, regulated temperature, etc.) these results may have limited implications to natural settings. Although this previous research has been essential for validation of non-lethal sampling measures, it fails to address the implications on short term behaviour. Moreover, with over 30,000 species of fish (of which over 27,000 are teleost species, Takei et al. 2014), and many contextual variables (e.g., water temperature, body size, disease) that could influence whether biopsies have measurable welfare impacts, there is a need to conduct additional research. When working on fish in the wild, having a detailed understanding on how sampling procedures impact post-release behaviour, condition, and fitness is critical to ensuring fish welfare; failing to do so may make fish vulnerable to predation, disease, or physical injury (Bøe et al. 2020). Should biopsy cause significant impairment such that biopsied fish have altered behaviour, condition, or fitness relative to non-biopsied conspecifics, the benefit of non-lethal sampling is largely lost.

In the following research, we examine how different non-lethal biopsy methods impact the welfare status of three different freshwater teleost fishes. Each study evaluated the effect that blood, gill, muscle, or a combination of two or more biopsy treatments had on the behaviour and fitness of fish. Given results of previous validation studies, we predict that biopsies will be well tolerated in all fish species examined here.

In Chapter 2, we used parental care behaviours of nesting male Smallmouth Bass to study how non-lethal biopsies impacted their ability to care for young. Blood, gill, and muscle biopsies were all evaluated individually and as a combined treatment. Given that bass guard their nests for several weeks during egg development (Philipp et al. 1997; Cooke et al. 2002), we were able to analyze behaviour directly after sampling and we could continue to monitor behaviour and survival for several weeks after. Moreover, given that failure to provide extended care to offspring yields zero reproductive success for that reproductive event, we were also able to assess fitness.

Chapter 3 focused on fine scale behaviour in juvenile Lake Trout. Behavioural assays were used to assess exploratory behaviour and reactivity, which have important implications to how fish can use their environment and avoid predation. This study also looked at organismal performance after biopsy using exhaustive exercise challenges. Moreover, fish were monitored for seven days to determine survival after biopsy, and to assess wound healing at biopsy sites. In this study, both gill and muscle biopsies were assessed independently and in combination.

Finally, in Chapter 4 we used transcriptomics to assess changes in gene regulation to determine the impact of non-lethal biopsy at a higher scale of resolution in adult male Walleye. Blood, gill, and muscle biopsies were all evaluated individually and as a combined treatment, and fish were monitored for ten days after biopsy. Survival was also assessed, and reflexes were tested immediately after sampling. Reflex assessments have previously been found (Davis 2010; Raby et al. 2012) to be a highly effective method at predicting recovery after a stressor such as angling. In addition to biopsy, fish in this study also had V13 tags surgically implanted so we could assess the impact of using non-lethal sampling in combination with electronic tagging.

Collectively, this body of work will inform the further refinement of welfare guidance to ensure that non-lethal biopsy methods are used in manners that minimize welfare impacts on fish. Moreover, validating this tool opens doors for the study of freshwater fishes using novel biomarkers to assess how they respond to various stressors in the lab and in the wild.

Chapter 2: Evaluating Blood, Gill, and Muscle Biopsy Methods on the Behaviour, Reproductive Success, and Survival of a Wild Freshwater Fish

Applied Animal Behaviour Science

Laura S.E. Haniford, Luc LaRochelle, Jessica A. Robichaud, Declan Burton, and Steven J. Cooke

Abstract

Non-lethal biopsy methods (including blood, gill, and muscle biopsies) have been used to study the health and physiological status of wild fishes. Nonetheless, concerns exist regarding the impact of non-lethal sampling on relevant welfare measures such as behaviour and survival. Here, nesting Smallmouth Bass (*Micropterus dolomieu*) were used as a model species to study *in situ* how fish respond to non-lethal sampling. Male Smallmouth Bass provide sole parental care and guard well-defined nests for a period of several weeks, providing a unique opportunity to assess behaviour, reproductive success, and survival in the wild. Fish were captured from their nests by angling and subjected to a biopsy (either blood, gill, or muscle), or a combination of all three biopsy methods prior to release. A control group that was captured but not biopsied as well as a non-angled control were also included. Nests were monitored for a period of four weeks or until the parental males either abandoned offspring, died, or raised a brood to independence. Single biopsies, regardless of the biopsy type, were found to have no impact on parental care and survival, but fish that received the combined treatment took longer to return to their nest and displayed a 6.5 times greater likelihood of nest abandonment. Mortality was only observed in fish that received the combined biopsy treatment. As such, this study reveals that it is possible to maintain the welfare status of Smallmouth Bass in the wild by using individual biopsies, thus emphasizing the importance of making careful decisions about which tissues are needed to achieve desired study objectives. This is one of the few studies to assess the behavioural and fitness consequences of increasingly common non-lethal biopsy methods and provides useful information on the relative consequences of different biopsy methods on wild fish.

2.1 Introduction

Traditional sampling methods for freshwater and marine fish require fish to be lethally sampled to assess fish health and physiological status, contaminant burden, or pathogen load. However,

lethal sampling can be detrimental to fish populations, particularly in instances where the species is rare or imperiled, or species with long life histories and late sexual maturity (Rolfhus et al. 2008). Lethal sampling also prevents researchers from studying behaviour and physiology simultaneously, or linking physiology and health to fate (e.g., survival, spawning; Cooke et al. 2016; Jeffries et al. 2021). As a result of these limitations, a variety of non-lethal sampling methods have been developed that allow for the same research topics mentioned above to be addressed by removing only small pieces of tissue from a living specimen – herein referred to as a non-lethal biopsy (Thorstensen et al. 2022).

Given advances in physiology, omics, technology, and fish health, small quantities of tissue can now be used in laboratory assays or microscopy, opening the door for non-lethal biopsy to be used in fish tissue collection. Previous studies have used blood samples taken from the caudal vasculature (Lawrence et al. 2020), gill clips taken from distal ends of gill filaments (McCormick 1993; Cornwell et al. 2013), or white muscle cores taken using a biopsy punch from the dorsal musculature (Henderson et al. 2016) to assess the physiological and health status of wild fishes. Such techniques are thought to have negligible impact on the post-release survival of the fish (McCormick 1993; Henderson et al. 2016), however, some studies have indicated that non lethally sampled fish had higher mortality rates when compared to their non-biopsied counterparts (e.g., Bass et al. 2020). Little is known about the post-release behaviour of biopsied fish. In one of the few studies to assess behaviour of biopsied fish in the wild, Cooke et al. (2005) noted small differences in the migration speeds of adult sockeye salmon that had received blood and gill biopsies relative to non-biopsied individuals. If non-lethal biopsy methods are resulting in fitness or performance impairments post-release, the benefits of using non-lethal sampling are largely lost given the impact on fish welfare.

Previous validation studies of non-lethal biopsy have occurred on salmonids (Cooke et al. 2005;

Miller et al. 2011) with comparatively little work on warmwater fish species. With a continued emphasis of electronic tagging and tracking studies on wild fish (Hussey et al. 2015), along with the promise of omics, such validation studies will be critical for ensuring that non-lethal biopsy methods do not lead to negative outcomes. To that end, the objective of this study was to evaluate whether non-lethal sampling methods influenced the behaviour and reproductive success of Smallmouth Bass (*Micropterus dolomieu*). Blood, gill, and muscle biopsy were evaluated independently and as a combined treatment to determine their effect on spawning success and nest-guarding behaviour of Smallmouth Bass. We tested the hypothesis that parental care behaviours, survival, and overall nest success will not differ between biopsied fish and their non-sampled counterparts. Male Smallmouth Bass are an effective model species for observing individual-level changes during their nesting season given that they provide sole parental care for a prolonged period. Bass will build a nest and remain guarding the eggs from predation for several weeks, until juveniles develop to the semi-independent stage of free-swimming fry (Philipp et al. 1997; Cooke et al. 2002). During this period, nests (and consequently nesting males) can be individually identified, and routinely monitored for their presence and nest guarding behaviours. Should adult male bass experience significant stress such as extreme temperature fluctuations or predation pressure, there is an increased likelihood of nest abandonment occurring (leading to certain depredation of all zygotes or offspring) (Siepker et al. 2009; Lunn and Steinhart 2010). As such, the behaviour and reproductive success of bass can be assessed after non-lethal sampling in the wild. With interest in ensuring that research methods do not impact the welfare status of wild animals, this study addresses an important knowledge gap on the welfare and behaviour of fish after non-lethal biopsy.

2.2 Methods

All research conducted in this study was carried out following the Carleton University Animal

Care Protocol (REF 110723).

Nest Sites and Selection

Smallmouth Bass were captured from their nests via angling on Big Rideau Lake, Ontario, Canada (44.7706°N, 76.2152°W) between May 16th and May 21st, 2021. Snorkel surveys were conducted to identify nests in similar habitats (gravel shoreline, between one to four metres deep), and nests were marked using a nest tag (a section of PVC pipe with a unique ID code). The number of eggs present in a nest was scored from 1-5; egg scores have been previously described in Siepker et. al. (2006), with a score of 1 representing few eggs and 5 representing many. As brood size has been determined to impact parental care levels (Lunn and Steinhart 2010; Zuckerman et al. 2014), only nests with an egg score of 3 or higher were used. The age of eggs was estimated by the diver (based on transparency and presence of fungus), and only nests with new eggs (less than 2 days old) were selected for use in the study.

Parental Male Capture and Biopsy

Bass were angled from their nests using a single hook with a soft plastic lure (ned rig or dropshot). Hooks were removed from the fish within ten seconds of capture and deeply hooked (i.e., in the gills or esophagus) fish were not used for the study. A diver guarded the nest to prevent nest predation by sunfish species during sampling. Once fish were landed, the hook was removed within ten seconds and a timer was started for two minutes to standardize handling time. Fish were placed in a padded trough filled with fresh lake water, measured for total length (in millimetres), and tagged using an anchor tag for individual identification. Fish smaller than 330mm were excluded from this study to prevent size discrepancy between treatments. Angled fish were assigned one of five treatments: an angled control, blood, gill, muscle, or a combined

biopsy treatment of all three biopsy types (hereafter referred to as 'combined treatment'). A second control treatment of fish that were not angled (referred to as 'un-angled control'), were only observed by a snorkeler in the water, and were included to standardize behaviour and nest success without additional stressors. Total length for fish in the un-angled control group were estimated by the diver using their dive slate (21 x 30 centimeters) for scale. After measurement and tagging, all angled fish were held dorsal side down during the two-minute period, except for during the muscle biopsy which was taken using a 4mm biopsy punch (Integra Miltex Disposable Biopsy Punch) from the dorsal musculature below the soft dorsal fin. One mL of blood was taken from the caudal vasculature following established procedures (Lawrence et al. 2020), and three to five millimetres of gill filamentous tissue were taken from the distal edges of the third gill arch using nail scissors.

Nest Return Time

At the end of the two-minute period, fish were released one boat length (5.5 metres) from the nest. A timer was started to record the time it took for the fish to return to the nest, which was observed by the diver. If the fish failed to return after five minutes, testing ceased, and the return time was recorded as greater than five minutes. Previous research has evaluated how different angling stressors (such as durations of air exposure) influence return to nest times where longer times are typically associated with more extreme stressors (Kieffer et al. 1995; Philipp et al. 1997; Hanson et al. 2007). As fish in the un-angled control were not removed from their nest, no return time was collected for fish in this treatment group.

Parental Care

Parental care was assessed 24 hours after sampling. A diver re-assessed egg score and recorded whether the male was present or absent from the nest. For present males, an attack score in response to a predator was assessed using a live Bluegill (*Lepomis macrochrius*) caught earlier

that day and placed in a 4L transparent jar with a perforated lid to allow water flow as per Hanson et al. (2007). The jar was placed directly in the nest (avoiding the eggs) by the diver, who then backed off to allow the bass to settle. After the bass had resumed standard nest guarding behaviour, the diver started a timer for 60 seconds and observed the number of times the bass attacked the jar. If the bass held its mouth open at the jar, the number of seconds were recorded as individual attacks (e.g., three seconds in this position would equal three attacks; Gravel and Cooke, 2009). After 60 seconds, the jar containing the Bluegill was removed from the nest. Nests were then observed via snorkelling every 3-4 days to assess egg development, and to determine whether the male was still present. Nests were checked until the eggs reached the free-swimming fry stage unless nest abandonment (and subsequent total egg predation) occurred. Mortality was assessed opportunistically by searching for fish that had abandoned their nests within 50 m of their nest sites.

Statistical Analysis

All statistical analysis were preformed using R Studio (version 4.1.2, RStudio Team 2021). Total length and egg score were both evaluated using an ANOVA to ensure that average sizes and scores were consistent between treatments. Average attack scores were also compared between treatments using an ANOVA, and a Chi-square test was used to determine whether there was an association between treatment and nest success. Return to nest time and time of abandonment were both assessed by survival analysis using the *Survival* package (Therneau 2021). To generate Kaplan-Meier survival curves for return time, fish that failed to return in the 5-minute window were censored and return times (measured in seconds) were compared between treatments. The number of censored fish were also compared between treatments to determine if one treatment was disproportionately excluded from the analysis. A survival curve was also generated to examine when fish abandoned their nests. As nest checks were conducted every 3-4 days, the passage of time was recorded in terms of number of nest checks and not individual days.

Multivariate survival analysis (Cox proportional hazard regression) was used to determine what factors were significant predictors of return time or abandonment. Consistent with recent perspectives on the rigidity of statistical significance values in ecological and behavioural research we chose to present our findings as strong evidence of significance in p-values fell between 0.001 and 0.01, moderate evidence if p-values were between 0.01 and 0.05, and weak evidence if p-values were between 0.05 and 0.1. P-values that fell above 0.1 were considered to have little or no evidence of significance (Muff et al. 2022).

2.3 Results

A total of 138 Smallmouth Bass were used with n=23 fish per treatment. Total body length of fish and nest egg scores were similar among treatments ($p>0.05$). Mean attack score did not significantly differ among treatments ($F= 1.878$, $p= 0.102$). Fish that were absent after 24 hours when attack scores were generated were excluded from the analysis, but the number of fish excluded was not different among treatments ($\chi^2=0.9127$, $p=0.923$).

There was no evidence that survival curves generated to model return-to-nest time were different from one another ($p = 0.45$). To generate the time-to-event curve, fish that failed to return in the 5-minute window were censored, and the number that failed to return in the five-minute window was compared between treatments. This revealed moderate evidence that the number of fish excluded from analysis differed between biopsy treatments ($\chi^2 =9.020$, $p=0.061$, Figure 2.1). Proportionally less fish from the combined treatment returned within the five-minute window (12 out of 23 total, compared to a minimum of 17 fish in other treatments).

Egg score, attack score, and treatment were analyzed as covariates in a Cox hazard regression to determine if they impacted return time (Table 2.1). There is weak evidence that gill biopsies

influenced return time (p=0.082), with fish from the gill biopsy treatment on average returning faster than fish from the other treatments. There was little evidence that egg score, attack score, or other biopsy treatments had any impact on return time.

Overall nest success (defined by successful development of free-swimming fry) was measured as a binary response and compared among treatments. A Chi-square test was used to evaluate whether there was an association between non-lethal biopsy treatment and nest success. We found a weak relationship between nest success and treatment ($\chi^2 = 8.604$, p= 0.126), however, due to the disparity in nest success between un-angled control fish and fish from the combined treatment (Figure 2.2), we did two additional pairwise comparisons. Specifically, we compared nest success between fish in the un-angled control to fish receiving the combined treatment, and fish in the angled control treatment to fish in the combined treatment. There was strong evidence that the combined treatment negatively affected nest success ($\chi^2 = 5.031$, p=0.025) when compared to the un-angled control, however there was no evidence of nest success being impacted when we controlled for the impact of angling (χ^2=1.493, p=0.222).

A time-to-event curve was also generated to examine when fish abandoned their nests (Figure 3). As nest checks were conducted every 3-4 days, the passage of time was recorded in terms of number of nest checks and not individual days. There was strong evidence to suggest that curves generated differed (p=0.045) shown after the first nest check, indicating that treatment effected abandonment throughout the course of the study. Covariates (including egg score and attack score) were analyzed using a Cox hazard regression, and treatment was found to be a strong predictor of abandonment, with fish from the combined treatment being 6.5 times more likely to abandon nests than fish from the other treatment groups (t= 2.387, p= 0.017, hazard ratio = 6.45, Table 2.2). There was no evidence that other variables (e.g., egg score, attack score) were

directly related to abandonment.

Mortality rate differences were not assessed statistically given that only 3 mortalities were observed during the study. However, all three mortalities observed came from the combined treatment group and were recovered less than a week after sampling took place in proximity to the nest sites. External evaluation of the carcasses revealed bruising surrounding the muscle biopsy site, and discolouration throughout the body. Deceased fish were identified by their external tag.

2.4 Discussion

We were able to assess the consequences of non-lethal biopsy on nesting male Smallmouth Bass over a period of several weeks during parental care. Behaviour immediately after sampling (return to nest time) did not differ between treatments, however more fish from the combined biopsy treatment had to be excluded from the analysis because they did not return to their nest within the five-minute window. Results indicated that sampling using multiple biopsy methods simultaneously leads to impairment in response time after sampling. This could have critical consequences in the wild as responsiveness is a key component in a fish's ability to find shelter, escape predation, or respond to environmental threats (Campbell et al. 2010). The same impairment was not observed in fish from other biopsy treatments; for example, fish that received gill biopsies returned to their nests faster than other biopsy treatments or angled controls.

Previous research has evaluated the consequences of fishing stressors on nesting male bass with prolonged air exposure leading to delays in returning to the nest (Hanson et al. 2007). In our study, fish had minimal exposure to air (i.e., all biopsies were taken with fish held in a water

filled trough). We also had a diver guarding the nest from predators to minimize nest predation, as decreases in brood size during the male's absence have been associated with greater levels of nest depredation and abandonment (Suski et al. 2003). However, it was not possible to guard nests until all fish returned, hence the implementation of a five-minute cut-off window after which we ceased predator defence. This likely contributed to the higher levels of abandonment noted in fish receiving the combined biopsy treatment, given that we found proportionally more fish from this treatment to not return within the five-minute window. Furthermore, it is well established that bass can assess their brood size throughout the nesting period and are more likely to abandon if there has been significant depredation in brood size (Suski et al. 2003). Although significant depreciation in egg score was not noted between initial nest observations and observations conducted 24-hours later, failure to return in the five-minute window left nests of fish from the combined treatment disproportionately vulnerable to predation when compared to fish from other treatments.

Many of the fish excluded from the return time analysis were found to have returned to their nest by the following day when attack scores were measured. Previous studies (e.g., Philipp et al. 1997; Hanson et al. 2007) found that most bass eventually returned to nests after angling events, although return time increased (often due to fish swimming long and indirect routes) when fish were exposed to additional stressors (e.g., air exposure, displacement), indicating distress or disorientation. In this study, no one treatment was disproportionately absent the following day, and attack scores generated in response to a simulated predation event were consistent among treatments, indicating fish were recovered sufficiently to resume routine parental care duties.

Despite the limited impacts on behaviour 24-hours post sampling, as monitoring continued through egg development an increase in nest abandonment was noted relative to controls, particularly in fish from the combined treatment. Previous studies on bass nest abandonment

have concluded that bass trade-off current and future reproductive opportunities (Steinhart and Lunn 2011). In some instances, it is more optimal to abandon a current brood in favour of future reproductive opportunities, especially if the parental fish is in poor condition such that continued care risks their survival (Lukas and Orth 1995). Most of the nests abandoned during this study were done so during the egg-sac or swim-up fry stage which would mean total nest destruction (by predators) and zero reproductive success for that individual for that season. As the combined treatment was found to be a strong predictor of abandonment, this indicates that the stress caused by multiple biopsies may have longer term consequences such immune responses or metabolic costs. Consequently, overall nest success was found to be lowest in fish from the combined treatment, whereas single non-lethal biopsies had no impact on nest success.

Undoubtedly, the stress of collection via angling may also have played a role in parental care behaviours exhibited in fish throughout this study (Kieffer et al. 1995), which is particularly apparent when comparing the nest success in the angled and un-angled controls to single and multiple biopsy treatments. Although we attempted to reduce stressors associated with angling by minimizing air exposure, releasing fish close to their nests, and using heavy gear to decrease fight time (all strategies previously identified as being drivers of nesting bass behavioural impacts; e.g., Philipp et al. 1997), nest success was still higher in the un-angled treatment. Single biopsies had comparable nest success rates to fish from the angled control, and success rates were not found to significantly differ between angled and un-angled control fish. However, when nest success was compared between un-angled fish and fish from the combined biopsy treatment, a substantial reduction in overall nest success can be observed. These results suggest that the impact of angling may be seen when multiple other stressors are present; single biopsies, regardless of type may only cause minor increases in overall stress, but the compounding stress of multiple biopsies in combination with angling presumably exceeded a threshold that contributed to abandonment rates and reductions in overall nest success. Such evidence

emphasizes the need to continuously work to reduce capture stress levels when working with wild fish.

Overall survival was high throughout the study, with only three observed mortalities. All mortalities came from the combined treatment, although other fish may have died but were not recovered. All fish were tagged with external anchor tags, giving opportunity for other mortalities to be reported. No such reports from the public were made. Given that almost all fish returned to the nest for at least some time suggests that the mortality was not the result of the acute stress of biopsy but rather latent consequences that may have been mediated by disease or a general decline in fish health. Given that bruising was noted around the biopsy sites in the three observed mortalities but not on live fish from the same treatments, there is a high likelihood that substantial inflammation possibly caused by a primary or secondary infection could have contributed to the decline of the individual's health. Moreover, additional handling was needed to obtain all three biopsies which can lead to increased dermal disturbance (Colotelo and Cooke 2011) and subsequent infection, particularly as skin and mucus act as an essential part of the teleost immune system (Dash et al. 2018). Although we only took a small amount of blood, minor bleeding can also occur at the site of gill and muscle biopsy which could contribute to anemia (Currie et al. 2022). Ubiquitous water mold (i.e., Saprolegnian fungus) is common in warmwater fish that experience injury and/or chronic stress (Xu and Rogers 1991) and may have contributed to the further decline in fish condition and eventual death of fish exposed to all three biopsies, although fungal growth was not observed at the biopsy sites. Chronic stress caused by biopsy treatments, abiotic factors, or the constant vigilance required for nest defence may have further contributed to a decline in health, particularly as chronic stress has been shown to supress the immune response (Tort 2011), leaving fish vulnerable to infection.

Results from this experiment indicate that a single non-lethal biopsy can be used safely with

negligible impacts on parental care, nesting success, and survival of male Smallmouth Bass. There is however some evidence to suggest that the use of multiple biopsies at once may result in a delayed stress response resulting in nest abandonment. There may be a trade-off between what tissues are needed to answer a given research question and the welfare of fish and as such collected tissues should be chosen carefully and specifically to address desired research outcomes. Given the need to maintain welfare status of individual fish and ensure that research methods do not negatively impact fish populations, this study emphasizes the importance of conducting validation studies of non-lethal biopsy methods using ecologically and biologically relevant endpoints specific to a given species or taxa. With growing interest in linking fish fate to physiological status (e.g., Jeffries et al. 2021), we anticipate a rapid expansion of research involving non-lethal biopsy of fish that are tagged with electronic devices. Research such as we describe here will be essential for developing best practices that maintain fish welfare and ensure that research objectives can be achieved in an effective, ethical, and responsible manner.

Tables

Table 2.1: Cox proportional hazard regression output for return to nest time

Characteristic	Hazard Ratio	CI	P-value
Angled	1.51	0.71, 3.17	0.31
Blood	1.24	0.58, 2.64	0.58
Gill	2.05	0.91, 4.63	0.08
Muscle	1.35	0.62, 2.93	0.45
Combined	1.46	0.79, 3.01	0.41
Egg Score	0.93	0.51, 1.69	0.80
Attack Score	1.00	0.98, 1.01	0.70

[1] CR= Confidence Interval

Table 2.2: Cox proportional hazard regression output for nest abandonment

Characteristic	Hazard Ratio	95% CI[1]	P-value
Angled Control	2.05	0.37, 11.41	0.41
Blood	1.73	0.31, 9.51	0.53
Gill	2.58	0.50, 13.32	0.26
Muscle	2.62	0.50, 13.64	0.25
Combined	6.45	1.40, 29.81	0.02
Egg Score	0.87	0.34, 2.24	0.78
Attack Score	0.99	0.96, 1.02	0.37

[1] CR= Confidence Interval

Figures

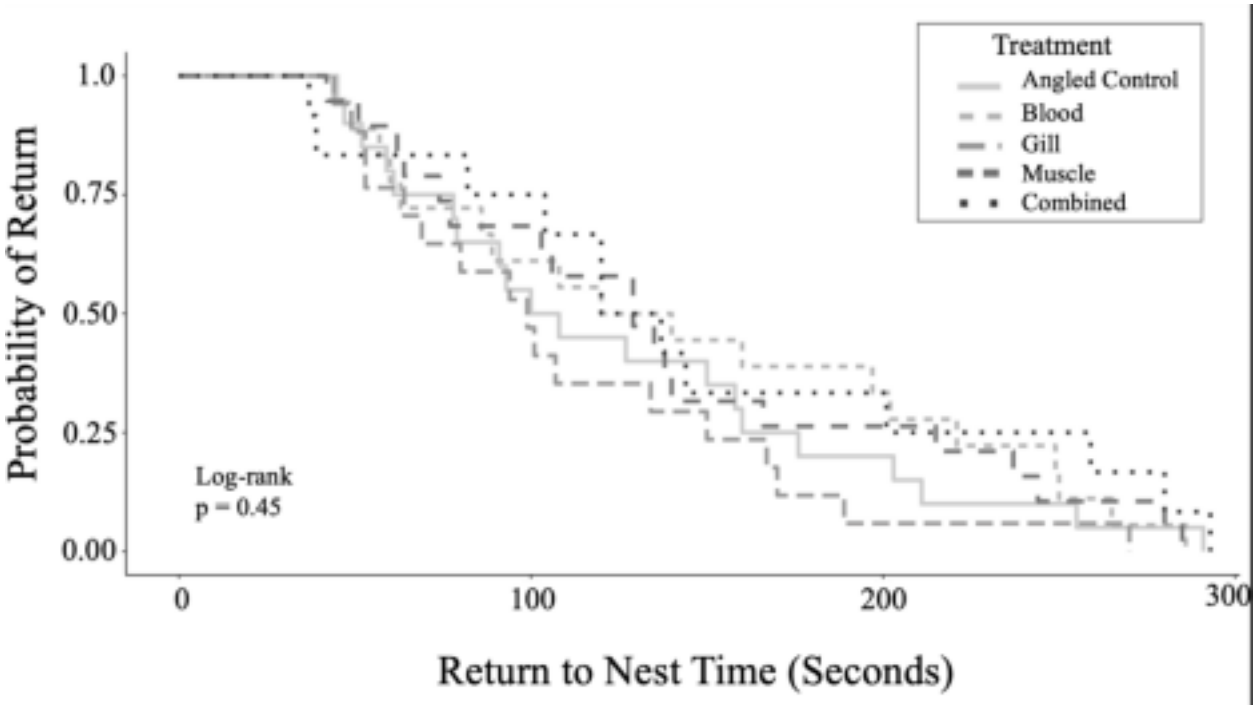

Figure 2.1: Kaplan-Meier time-to-event curve for return to nest time in a 5-minute (300 second) monitoring period. Fish from the Un-angled control group are not included as no return time was measured for fish in this treatment group.

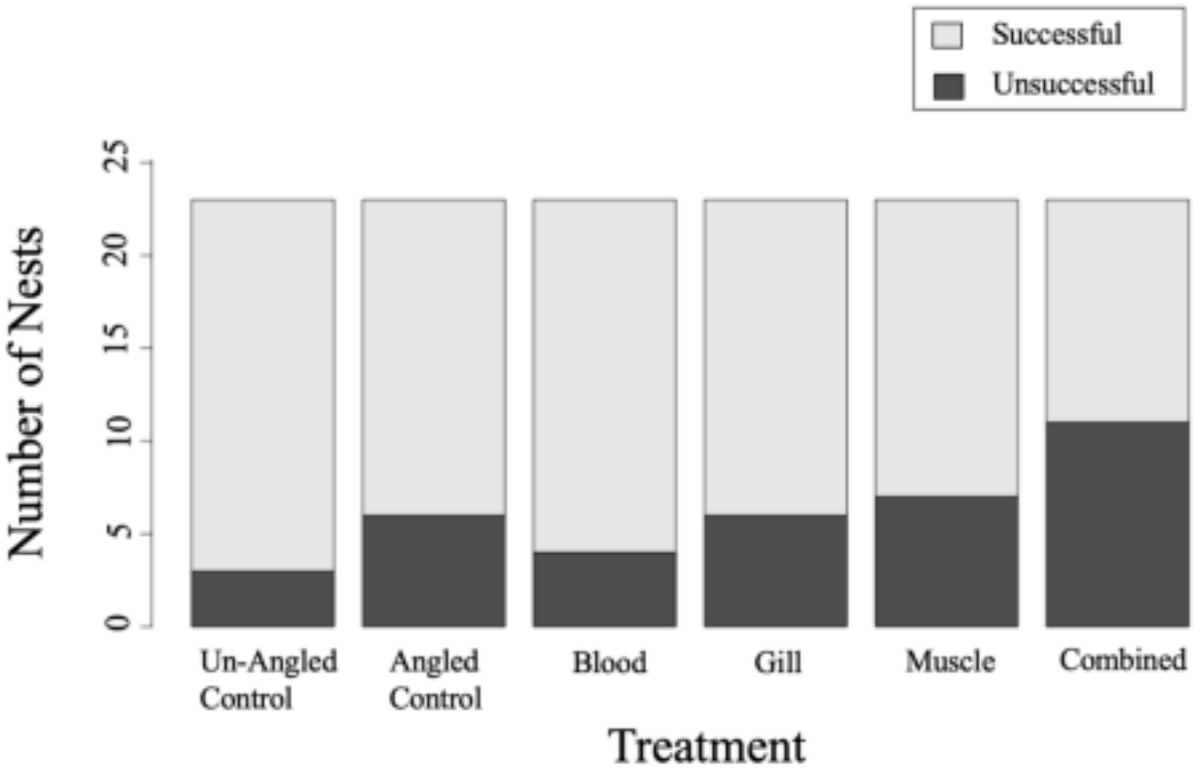

Figure 2.2: Number of successful versus unsuccessful nests for male Smallmouth Bass in each non-lethal biopsy treatment (n=23 nests per treatment). Nest success was determined by eggs hatching and surviving to the free-swimming fry developmental stage.

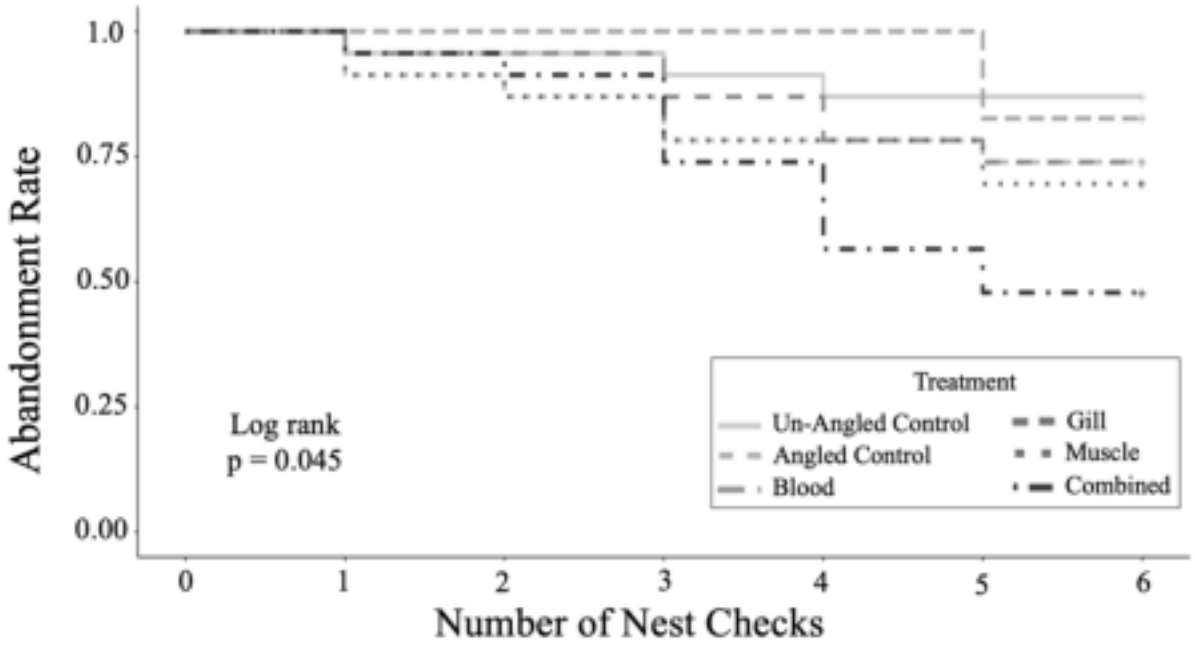

Figure 2.3: Kaplan-Meier time-to-event curve for probability of nest abandonment for each treatment. Nest check 0 was the original sampling event, with nest check 1 occurring 24 hours after sampling. Nest checks 2-6 occurred every 3-4 days, with all nest checks ceasing after nests had been abandoned or eggs had developed into free-swimming fry.

Chapter 3: Effects of non-lethal biopsy on behaviour and fitness in juvenile Lake Trout

Laura S.E. Haniford, Gillian Zorn, Ken Jeffries, Matt Faust, Tom R. Binder, Graham D. Raby, and Steven J. Cooke

Abstract

For researchers to gain a comprehensive understanding of factors impacting fish welfare, behavioural assays can be paired with other technologies, such as non-lethal biopsy. Although previous validation work has been done to determine the impact of biopsies on long-term behaviour and survival, no such studies exist to evaluate fine-scale behaviours (such as reactivity and exploration) post-biopsy. Here, muscle and gill tissue samples were taken (both individually and as a combined treatment) from juvenile Lake Trout (*Salvelinus namaycush*). 24 hours after sampling, we used two behavioural assays (behaviour within a Z maze, and Flight Initiation Distance) as well as an exhaustive exercise test to determine whether biopsies impacted behaviour and fitness when compared to non-biopsied counterparts. There was little evidence that biopsies, regardless of the type, had any influence on exploratory and shelter-seeking behaviour in the maze, on flight initiation distance, or on time to exhaustion (as a proxy for swimming performance). Mortality was very low (2% overall) and limited to fish that received either gill biopsy or the combined biopsy treatment, suggesting it was somewhat less optimal than muscle sampling. This study provides further validation framework for the use of non-lethal biopsy in juvenile salmonids and is one of the few studies to address fine-scale behaviour and swimming performances post-biopsy.

3.1 Introduction

Salmonids are one of the most widely cultured and studied groups of fish. In the wild they contribute to recreational, commercial, and subsistence fisheries, and are cultured to support those activities (Lorenzen et al. 2010); to enhance imperiled populations (Faisal et al. 2019); and for direct sale to consumers (Burridge et al. 2010). Because of the economic, social, cultural, and

ecological value of salmonids, there is great interest in ensuring the health of fish across all life stages.

To assess the health and condition of fish in culture facilities and in the wild, various tissues are collected from salmonids. For example, gill, muscle, and blood samples have been collected to study pathogen load, metabolic status, or contaminant burden (Jeffries et al. 2021; Thorstensen et al. 2022). In some cases, these samples are collected from euthanized animals, but as physiological methods evolve it is possible to conduct assays using relatively small quantities of tissue such that non-lethal sampling is possible. If such sampling can be done without impacting the welfare of fish, it provides a mechanism to reduce mortality (relative to lethal sampling) or even to repeat sample fish over time or to link physiological state to other endpoints such as growth, behaviour, or survival. To do so requires validating that such sampling methods do not impact the fish in a negative manner.

Validation of biopsy studies has occurred for some salmonids, although such efforts have largely focused on adults. For example, in Cooke et al. (2005) blood sampling and gill biopsy were performed on adult Sockeye Salmon implanted with radio transmitters to determine if biopsy impacted migration success. They found little evidence that biopsy affected fish survival. Similar results were found in Jeffries et al. (2014) when gill biopsies were used to assess pathogen load in juvenile Sockeye Salmon. These studies were conducted on wild fish in the field, and although they provide ecological relevance it is challenging to assess fine-scale indicators of potential behavioural changes resulting from biopsy.

Numerous studies have identified that changes in a fish's routine behaviour (such as exploratory behaviour, ventilation, or foraging) may be linked with poor welfare and potential stressors or underlying health concerns (Ashley 2007; Huntingford et. al. 2006; Martins et al. 2012; Yi et al. 2021). As such, there is growing interest in analyzing behaviour of fishes, particularly in hatcheries and other aquaculture facilities, as well as to enhance the ethics of animal research. Furthermore, behavioural indicators provide the advantage of being a quick and non-invasive method to study fish health (Martins et al. 2012).

To maximize researcher's understanding of fish health, behavioural indicators of welfare can be paired with other techniques to provide a robust understanding of overall welfare and fitness (Martins et al. 2012). With the advancement of biochemical and omics technologies we can easily sample fish by taking only small amounts of tissue via non-lethal biopsy (Jeffries et al. 2021). Such sampling methods have been used previously to assess levels of environmental pollutants, to study the prevalence of pathogenic microbes, or to assess physiology in freshwater and marine fishes (Thorstensen et al. 2022).

Previous studies have worked to validate whether non-lethal biopsy causes changes to behaviour and survival of fishes (McCormick 1993; Ackerson et al. 2014; Zhao et al. 2014), but most behavioural outcomes have been of a larger scale, such as migration success (Cooke et al. 2005). As such, there is a need to conduct further validation studies that look at fine scale behavioural changes after biopsy, particularly in the context of hatchery reared fish. In this study, we aim to address this knowledge gap by conducting a series of behavioural and physiological assays to assess whether gill and muscle biopsies impact exploratory and feeding behaviour of hatchery reared juvenile Lake Trout *(Salvelinus namaycush*).

3.2 Methods

All research conducted in this study was carried out following the Carleton University Animal Care Protocol (REF 110723) and was consistent with the fish care guidelines from the Canadian Council for Animal Care.

Fish Collection and Treatments

For this study, 125 juvenile Lake Trout (average total length 171 ± 19mm) were sampled at the White Lake Fish Culture Facility in Sharbot Lake, ON, Canada. Fish were transferred by bucket from rearing pens to flowthrough tanks, with a constant water temperature of 11°C. After transfer, fish received one of five treatments: a gill biopsy, a muscle biopsy, a combined biopsy treatment (both gill and muscle biopsy), a tagged control, and an untagged control. All fish were measured for total length and weight to ensure sizes were consistent between treatments. Fish from the biopsy treatments and the tagged control group were tagged using 8mm Passive Integrated Transponder (PIT) tags (inserted in a small incision made using a scalpel, halfway between the anus and pelvis fins) for individual identification; fish from the untagged control group were identified based on total length and weight measurements.

All fish were held for 2 minutes to ensure handling time was standardized between treatments, with gills kept fully submerged (except for during the gill biopsy during which the head was briefly lifted above the water to take 3-4mm of gill tissue from the distal end of gill filaments on the second gill arch). Muscle biopsies were taken from the dorsal musculature using a 4mm biopsy punch (Integra Miltex Disposable Biopsy Punch).

Behavioural assays

Two behavioural assays were selected for this experiment: behaviour within a Z maze, and Flight Initiation Distance (FID). Previous research has used Z mazes as a method for studying exploratory and shelter-seeking behaviour in a new environment (Chapman et al. 2010; Hlina et al. 2021; Ramsaran et al. 2021), while FID tests have been used to examine how a fish might react to or flee from a new object, simulating predation events or food discovery in the wild (Wilson and Godin 2009; Hlina et al. 2021). As both the Z maze and the FID test examine behaviour directly applicable to hatchery reared fish after release into the wild, these assays were selected as behavioural endpoints for this study.

Fish were first introduced to the Z maze (a rectangular arena constructed from black plexiglass, 100cm x 80cm, filled with fresh water from the flow through tanks to a depth of 20cm), in a gated and covered refuge (20cm x 40cm) at one end of the arena. Three opaque black partitions were used to divide the main area of the arena into a Z pattern (Figure 3.1). Black pieces of plastic were fixed to the bottom of the arena in order to create a gird of 20 equal squares, with 4 squares in each row of the maze. At the beginning of each trial, fish were introduced to the refuge and allowed five minutes to acclimatize to the space before the gate was removed and fish were allowed to explore the maze for a period of 15 minutes. As human interaction may cause a decrease in activity, a GoPro Hero 7 was mounted above the maze, and the fish's behaviour and progression through the maze was observed via video recording. Successful emergence from the refuge, the amount of time each fish spent in the refuge, and swimming activity (quantified as the number of lines fish crossed) were all recorded during the 15-minute exploratory window. The water in the maze was drained and the maze was rinsed thoroughly with fresh water between each fish to reduce the influence of chemokines during the experiment.

Immediately after the Z maze trial, fish were transferred to the FID arena (a cooler, 80cm x 38cm, filled to a depth of 30cm with a measuring tape lining the bottom, and each centimeter marked in different coloured electrical tape, Figure 3.2). Fish were given two minutes to acclimatize to the FID arena, before a novel object (a Carleton University Transit Pass resembling a credit card, 8.5cm x 5.5cm, light blue with holographic details) was lowered into the water column with the reflective side facing towards the fish and moved towards the fish at a steady rate (1cm per second). The approach stopped as soon as the fish moved to avoid the novel object, and the distance between the fish and the novel object was measured.

Exhaustive Exerciser Challenge

Following FID measurements, fish were transferred to a circular arena (diameter of 100cm) with a bucket (diameter 30cm) placed in the centre to create a doughnut shape to assess exercise performance. Such approaches have been widely used as a simple means to assess the swimming performance of fish (Milligan 1996; Kieffer 2000). Although this approach does not generate an actual swimming speed per se, it does provide a robust approach for making inter treatment comparisons (Portz 2007). Electrical tape was used to divide the arena into four equal quadrants to better determine distance travelled. Fish were chased around the arena using a small hand net until they reached the point of exhaustion, classified as the point at which they failed to burst swim away from three consecutive tail-grabs. The amount of time it took to reach the point of exhaustion for each fish and the number of line-crosses were recorded for each fish as per Samson et al. (2014).

After the completion of all assays, fish were returned to the flow-through tank where their survival was monitored for seven days. After this monitoring period, all fish were euthanized using cerebral percussion, and the carcasses were examined for healing at the biopsy sites, and

for signs of infection.

Statistical Analysis

Data generated from this experiment were analyzed using RStudio Version 4.1.2 (R Core Team, 2021). Z-maze behavioural metrics (successful emergence from the refuge, activity in the maze, and time spent in the refuge) were analyzed as dependent variables, with the biopsy treatment treated as the independent variable. All data were assessed for normality using Shapiro-Wilk tests. Successful emergence from the refuge was assessed using a Chi-square test, to determine if there was an association between successful emergence from the refuge and biopsy treatment.

As data for the number of gridlines crossed in the z maze were not normally distributed and transformations were not able to sufficiently correct for normality (due to a large portion of fish not leaving the refuge during the 15-minute exploratory period, thus crossing zero gridlines) a Kruskal-Wallis test was used to compare the number of gridline crosses between biopsy treatments, as was the time spent in the refuge during the 15-minute exploratory period. Values for the time to exhaustion and the number of line crosses were transformed to correct for normality using Log10 and Square Root transformations, respectively. An analysis of variance (ANOVA) was then used to determine if there were differences in average time to exhaustion and number of line crosses between biopsy treatment groups. All associations were considered significant at $p < 0.05$.

3.3. Results

Regardless of biopsy type, emergence success was found to be very low (Figure 3.3). Overall, only 46% of fish emerged during the 15 min trials. Our analysis found no evidence that biopsies, regardless of type, impacted successful emergence from the refuge within the Z maze ($\chi^2 = 4.70$,

DF = 4, p = 0.320). Similarly, biopsy treatment had no effect on the number of gridline crosses made during maze exploration (H= 3.150, DF= 4, p= 0.533). Finally, there was no evidence that the amount of time fish spent in the refuge throughout the experiment was impacted by biopsy type (H= 5.574, DF =4, p= 0.112). There was little evidence that biopsy treatment impacted FID (H= 2.00, DF= 4, p = 0.735, Figure 3.4) as well as in time to exhaustion (F=0.395, DF=4, p= 0.813) and number of lines crossed in the chase tank (F=0.414, DF=4, p=0.798).

Mortality was very low throughout the study with only three mortalities observed (2% mortality rate overall). All three mortalities were observed 24 hours after biopsy, with two fish coming from the gill biopsy treatment group, and one fish coming from the combined biopsy treatment. External examination of the carcasses showed no apparent signs of bruising or infection, but the carcasses were noted to be significantly lighter in colour than other fish sampled on the same day (shown in Figure 3.5). Furthermore, all fish that became observed mortalities were noted to be respiring at the water's surface immediately after sampling upon return to the flow through tank – an observation that was not observed for other fish.

Evaluation of the biopsy sites after the seven-day monitoring period found bruising at 23% of the muscle biopsy sites in fish from both the muscle and combined biopsy treatments, and abscesses at the muscle biopsy site in 8% of all fish receiving that received muscle biopsies (Figure 3.6). Gill discolouration (light pink in colour, with pink or white tips of the gill filaments) was noted in 16% of all fish receiving a gill biopsy, but not in fish from the control or muscle biopsy groups.

3.4. Discussion

Based on the results of behavioural assays and exhaustive exercise tests, the present study

reveals that there were no detectable impacts of non-lethal biopsy on behaviour, fitness, and survival of juvenile Lake Trout. Of the behaviour assays done in this study, behaviour within the Z maze was severely limited by an overall low emergence rate of fish from the refuge. This limited emergence rate was however found to be consistently low across treatments, and in pilot experiments did not improve with increased time in the Z maze. Nonetheless, even with that limitation in our study, other assessments (e.g., FID test, swimming challenge) failed to detect differences either and mortality was exceedingly low.

Previous work (see Réale et al. 2007; Wilson and Godin 2009) has documented a spectrum of personality in fish, including a personality axis of "boldness" to "shyness"; which could be strongly associated with the low emergence rate observed throughout the study. Within this personality continuum, fish have been observed to engage in more risk-tolerant or bold behaviours such as active exploration, whereas fish with a shyer phenotype are more likely to seek shelter or remain in one place (Wilson et al. 1993). In the wild, boldness may be associated with both positive and negative life histories; in some species, boldness (particularly in males) may increase chances of reproductive success but may also increase risk of predation. Boldness in fish is thought to arise both from genetic inheritance and predation pressure during development. In a hatchery environment where many fish come from the same broodstock and predation pressure is non-existent, boldness may still be selected for during feeding, as more aggressive individuals may be able to increase their food intake during feeding (Sundström et al. 2004; Martins et al. 2012b). Quantifying boldness or shyness in the strain of Lake Trout used here was outside the scope of our study but may be beneficial in future work to further elucidate the combined effect of biopsy treatment and personality.

Similar to emergence from the refuge, swimming activity (in the context of number of gridlines crossed in the maze) and time spent in the refuge were not found to relate to non-lethal biopsy

methods, although results were limited by low emergence rates. Although individual personality may further play a role in behaviours exhibited by fish and measured in this study, other research has found swimming activity to be a proxy determinant of stress levels via blood physiology analysis (Svendsen et al. 2021). Our research found swimming activity throughout the Z maze trials to be consistent between biopsy treatments, and since emergence success, activity, and time spent in the refuge were not found to differ between biopsied fish and our two control groups, observed behaviours likely resulted from personality differences rather than changes in stress levels because of non-lethal biopsy.

Reactivity observed during the FID assays did not reveal differences between non-lethal biopsy types, indicating that non-lethal biopsy does not yield a significant enough amount of stress to impair reactivity or avoidance responses. This has important implications on behaviour in the wild, as reactivity is an essential component of avoiding predation (Dill 1974; Fuiman et al. 2006). Furthermore, exhaustive exercise assays did not find that biopsies impaired the fitness of fish, as both time to exhaustion and total distance swam were consistent across treatments. Given that high intensity exercise is a routine component of fish life histories (e.g., fisheries interactions, migrations, feeding behaviours, and predator avoidance) (Elvidge and Cooke 2020), the findings that non-lethal biopsy has minimal impact on fish performance suggests such methods can be used in the field without impacting essential ecological activities.

Although mortality rates were low overall, previous research (i.e., Van Der Salm et al. 2004; Vitt et al. 2022) has determined that changes in colour (as noted in fish in this experiment prior to mortality) can be highly reflective of the physiological state of the fish (Yi et al. 2021). Pigments (including carotenoids) have been found to be directly affected by oxidative stress, which may be caused by challenges to the immune system (Sefc et al. 2014). Stress-related changes in pigment expression and concentration have been documented across a number of taxa, and as such

colouration is strongly associated with fish condition and welfare (Vitt et al. 2022). In our study, all the mortalities were noted to be much lighter in colour than their counterparts as little as four hours after biopsy. Similar changes in colour were noted in the four fish from the muscle and combined biopsy treatments that were observed to have abscesses at the biopsy site seven days after biopsy. Given the established relationship between colour and condition, these observations suggest a deterioration in health status likely associated with primary or secondary pathogen infection.

Results from this experiment indicate that non-lethal gill and muscle biopsies may be used safely with limited impact on fish exploratory behaviour and reactivity, and do not impair behaviour or swimming performance at 24-hours after sampling in juvenile Lake Trout. Furthermore, multiple biopsy types may be used simultaneously without causing impairment in behaviour and performance. Nonetheless, there was evidence of low levels of mortality with fish that had gill biopsies and some indications that injuries caused by muscle biopsies may not heal well for all fish. As such, care should be taken when conducting muscle biopsy with gill biopsy preferred if it yields the tissues needed for a given purpose. Given the importance of maintaining fish welfare both in hatchery and natural settings, this study provides important validation work on a vital technique for monitoring fish health and condition.

Figures

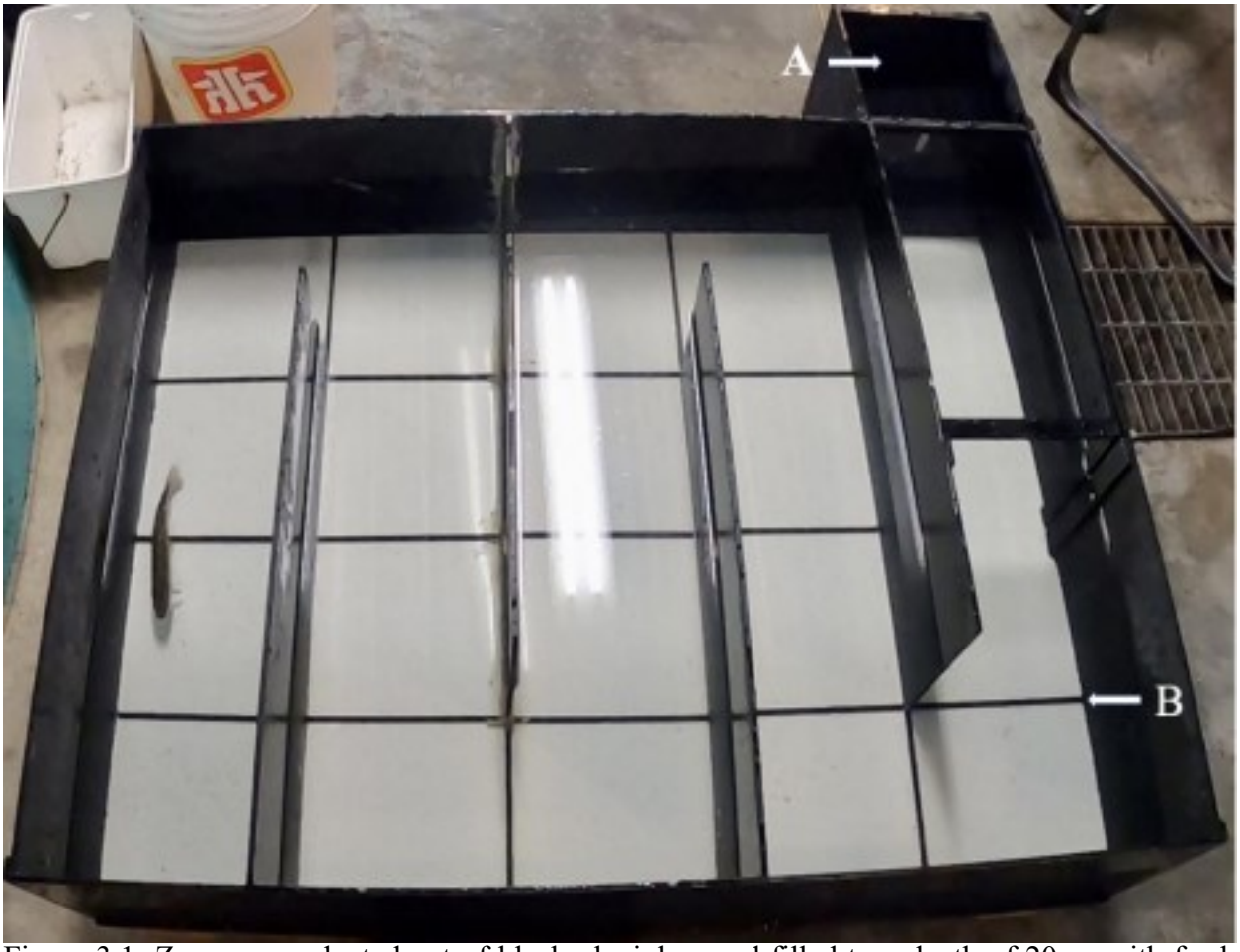

Figure 3.1: Z maze, conducted out of black plexiglass and filled to a depth of 20cm with fresh water. A refuge (A) is located at one end of the maze, which was gated and covered during the 5-minute period fish were given to acclimatize to the maze. A grid of 20 equal squares was created using lines of black plastic (B) laid along the bottom.

Figure3.2: Flight Initiation Distance (FID) arena, constructed using a cooler (80cm by 30cm) filled to a depth of 30cm using fresh water. A measuring tape was fixed to the bottom of the cooler, with different coloured electrical tape marking each centimeter.

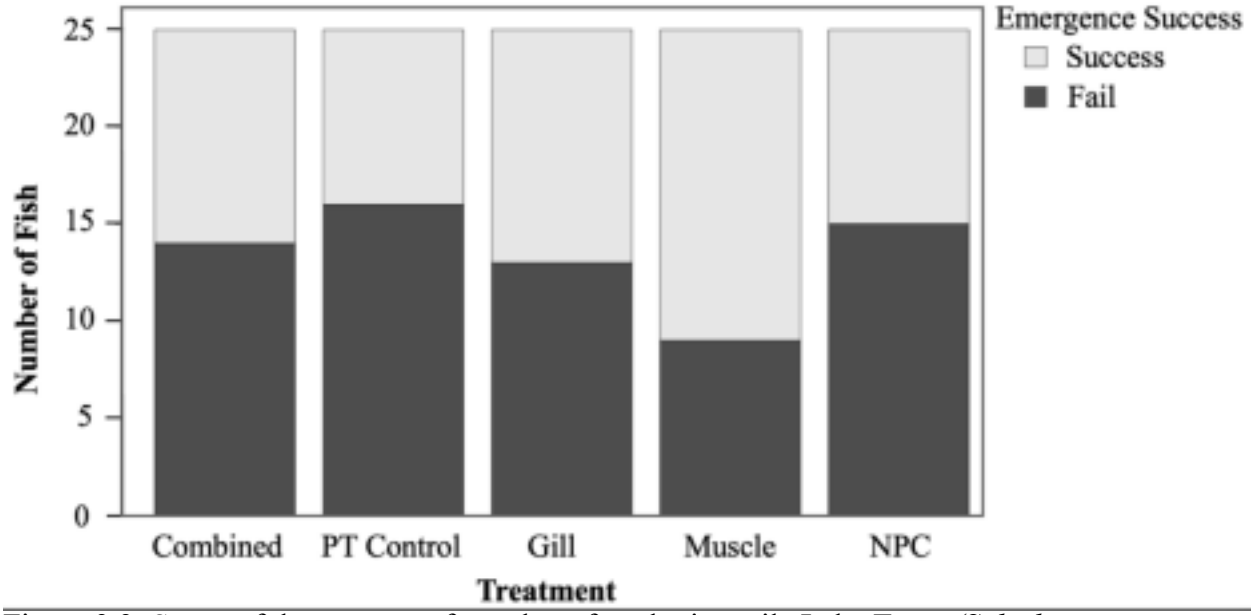

Figure 3.3: Successful emergence from the refuge by juvenile Lake Trout *(Salvelinus namaycush)* for each biopsy treatment type during the 15-minute exploratory window.

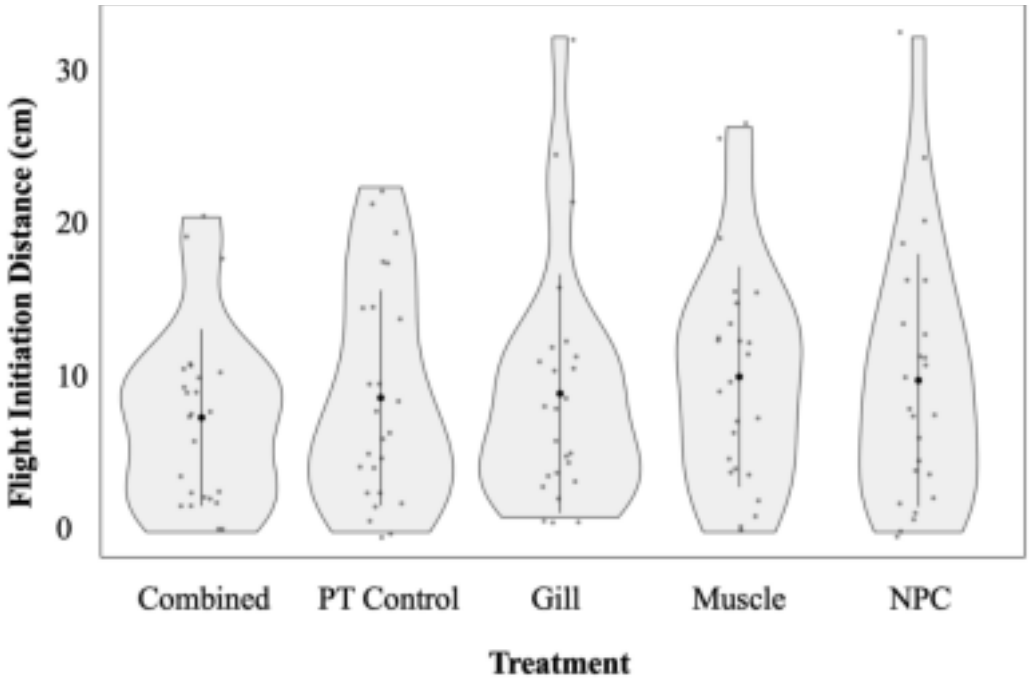

Figure 3.4: Flight Initiation Distance (in cm) measured in juvenile Lake Trout *(Salvelinus namaycush*) from a novel object in different non-lethal biopsy treatment types. Black circles in the centre of each violin indicate the mean, with error bars showing the standard deviation.

Figure 3.5: Juvenile Lake Trout *(Salvelinus namaycush*) pictured 4 hours after non-lethal gill biopsy; Fish A was notably lighter in colour than Fish B (also a gill biopsy), and Fish A was found dead the following day.

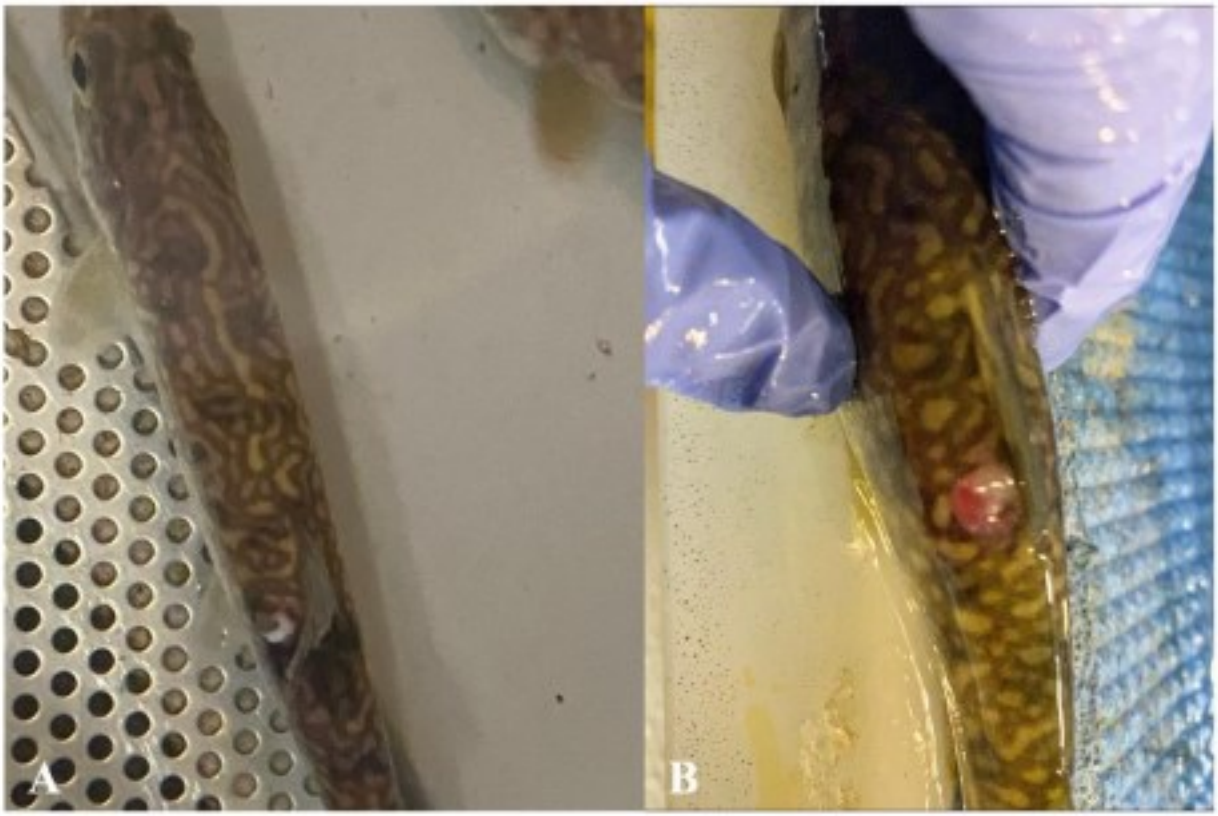

Figure 3.6: Juvenile Lake Trout *(Salvelinus namaycush)* with dorsal muscle biopsies, significant bruising noted at the biopsy site (A), and an abscess noted at the biopsy site (B).

Chapter 4: Evaluation of biopsy methods on walleye implanted with electronic tags

Laura S.E. Haniford, Matt Faust, Ken M. Jeffries, Declan Burton, Myra Thapar, Christian Bihun and Steven J. Cooke

Abstract

Biopsy techniques that involve collection of small quantities of tissue have been used to evaluate the health of native freshwater fishes. Although previous validation has been carried out in laboratory settings on how fish respond to biopsy, researchers have yet to evaluate the impact biopsy has on health and fitness after sampling in the field. Furthermore, there is little research regarding the suitability of combining biopsy with other methods, such as electronic tracking and tagging studies, with Cooke et al. 2005 being one of the few documented studies. Here, we analyze how adult male Walleye respond to gill, muscle, and blood biopsies when also implanted with electronic V13 tags. Reflex action mortality predictors (RAMP) and qPCR were used to evaluate reflex impairment and expression of Major Histocompatibility Complex Class II and Glucocorticoid Receptor 1. Fish were monitored for a total of 10 days to assess how biopsy impacted survival, and only one mortality occurred in this monitoring period. There was little evidence that biopsies, regardless of type, caused impairment or changes in regulation after sampling, and biopsy had no impact on survival.

4.1 Introduction

Global freshwater fisheries play a critical role in recreation, nutrition, economics, and culture (Welcomme et al. 2010). Despite the social and intrinsic value of freshwater systems and fisheries, anthropogenic effects have cause widespread changes to freshwater systems, the impacts of which are still being studied (Naiman and Turner 2000). To successfully mitigate challenges caused by anthropogenic activities, scientists must have a detailed understanding of how freshwater systems are being impacted, and what effect these changes have on freshwater fish. As such, developing tools to identify how changes are impacting freshwater systems is critical to successful and targeted conservation efforts. Several recent technological developments (reviewed in Cooke et al. 2022) are reshaping how wild fish can be studied to

understand the ways in which various stressors influence fish behaviour, physiology, and fitness (e.g., survival). Most notable are electronic tagging and tracking tools and the suite of omics technologies.

Electronic tagging and tracking tools are used to monitor fish movement and activity (Thorstad et al. 2013; Cooke et al, 2013), and are essential for identifying critical habitat as they allow researchers to identify spawning grounds, migration corridors, and nursery habitat (Brownscombe et al. 2022; Crossin et al. 2017). Omics tools allow researchers to study expression of genes and proteins related to stress and health status (Connon et al. 2018). Although both technologies are critical for successful management, additional strength can arise from combining electronic tagging and tracking with biopsy, particularly as tag implantation gives researchers an opportunity to collect tissue samples.

Some studies have already combined electronic tagging with omics, such as in Miller et al. 2011, where genomic signatures were used to predict spawning failure in Sockeye Salmon, or in Kristensen et al. 2021 in which multiple ecotypes of Atlantic Cod (*Gadus morhua*) were identified using telemetry and genotyping. Tissue biopsies used for omics work previously required lethal sampling, which limited researchers to smaller sample sizes and unprotected species, however, given the increased sensitivity of sequencing tools, most assays now only require very small amounts of tissue which can be taken from a living specimen. As such, small quantities of blood, muscle, gill, or other tissue can be collected to answer the same research questions as traditional lethal methods. Non-lethal samples can further be used to assess energetics, contaminant burden, or pathogen load, and can be highly impactful when combined with electronic tracking studies.

Although previous validation work has been done in a laboratory setting to evaluate survival after biopsy (McCornick 1993; Ackerson et al. 2014; Zhao et al. 2014), there are no current studies that address the impact biopsy has on health and stress. This knowledge gap is a major barrier to the widespread use of biopsy with other technologies; given that biopsy is an additional procedure beyond tag implantation, researchers have justifiable concerns regarding the welfare of fish after biopsy. Furthermore, behaviour and survival are major endpoints in tagging and tracking studies. Should biopsy be found to alter these endpoints, the benefit of combining non lethal sampling with other techniques is largely lost.

In this study we aim to evaluate if biopsy influences the survival and physiological status of fish implanted with electronic tags. Gill, muscle, and blood biopsy were all evaluated individually and as a combined treatment with fish held in net pens and assessed for survival and physiological status after 10 days. Previous studies (e.g., Raby et al. 2012) have found that reflex action mortality predictors are a highly effective method of measuring stress and vitality in fish and have been successfully used to predict post-release mortality (Davis 2010). Collected tissue samples can be further analyzed to evaluate expression of genes related to health and immune function. Walleye are a highly motile species, and their movement is currently being studied throughout the Great Lakes (Hayden et al. 2014; Brooks et al. 2019; Matley et al. 2020; Euclide et al. 2021; Elliott et al. 2023). Given the wealth of telemetry work that is already ongoing with this species, we chose the male spawning population in Sandusky, Ohio, for initial validation studies.

4.2 Methods

All research conducted in this study was carried out following the Carleton University Animal Care Protocol (REF 110723).

Fish collection and initial holding

Adult male Walleye (n= 135, average total length 425mm ± 38mm) were collected from the Sandusky River (Ohio, USA) on March 22nd and April 4th, 2022, via electrofishing, and held overnight in a hatchery truck tank (left running, to maintain temperature and dissolved oxygen levels). Before starting the experiment, fish were transferred to cattle troughs fed with fresh lake water, where they were held until surgery.

Electronic tag implantation and biopsy

Tagging methods used in this study followed previously established protocols (Elliott et al. 2023). The sex of all fish was confirmed by applying light pressure to the abdomen to stimulate the release of gametes. Each fish was also measured for total length (in mm). Fish were placed supine in a cradle created using soft mesh, with a hose inserted through the fish's mouth to keep the gills oxygenated. Fish were immobilized using a TENS unit throughout the surgery and biopsy process. A small incision (approximately 2cm in length) was cut using a scalpel on the ventral side of the fish midway between the anus and pelvic fin, and a dummy V13 tag (35 x 10mm, Vemco, Halifax NS) was inserted into the abdominal cavity through the incision. The incision was then closed using 2-3 interrupted stiches using 4-0 coated vicryl dissolvable sutures (Ethicon).

Fish were then assigned to one of five treatments; a tagged control, blood, gill, or muscle biopsy, or a combined biopsy treatment in which all three biopsy types were done simultaneously. Methods for taking biopsy samples were done as outlined in Chapter 1. Gill biopsies from both the gill and combined treatment were transferred to RNA Later (Invitrogen, ThermoFisher Scientific) for later analysis. Finally, an 8mm PIT tag was inserted using a needle into the soft tissue under the jaw. Surgery and biopsy were timed using a stopwatch so handling time could be

including in the analysis. An additional group of non-tagged control fish had gill biopsies collected immediately after capture in order to establish a baseline of expression in fish without biopsies or implanted tags.

Reflex impairment assessment

Previous work (Davis 2010) has validated the use of reflex impairment in predicting fish stress and mortality. Given that this method has been shown to be quick and effective, reflex impairment assessments were used to evaluate impairment after biopsy and surgery. Fish were transferred to a cooler filled with fresh lake water where reflexes (bursting and equilibrium) were assessed. Reflexes were tested at five-minute intervals, and once fish had passed both bursting and equilibrium assessments they were moved to an open net pen (3x4m) suspended in Lake Erie. All fish were transferred to the net pen within ten minutes of sampling.

Survival Assessment

The net pen was checked daily for mortalities, and fish were monitored for a total of ten days. After this monitoring period, gill biopsies were taken from all fish prior to euthanization using cerebral percussion. Healing at the muscle and surgery site was noted for all fish.

RNA extraction, cDNA Synthesis and Chip Analysis

RNA extraction was done using RNeasy Plus Mini Extraction Kits (Qiagen 74034). Extraction was done following the Qiagen protocol with several minor modifications (outlined in Appendix A). After extraction, RNA concentration and purity was determined using Nanodrop, and gel electrophoresis (done on a 1% agarose gel) was used to verify the quality of RNA. Samples were then made to a standard concentration of 1500ng/uL for cDNA library synthesis (done using High-Capacity cDNA RT Kit, ThermoFisher Scientific).

An STP (Stress Response Transcription Profile) chip developed by Gen-FISH was used to determine expression of 25 different genes related to health and immune function (listed in Table 1) using qPCR on a TAQMAN Open Array Block. For the purpose of this thesis, only results for Glucocorticoid Receptor 1 (GR1) and the Major Histocompatibility Complex Class 2 (MHC-II) have been analyzed. The 60S Ribosomal Protein L7, 40S Ribosomal Protein S9, and Ribosomal Protein L13A were all used as endogenous controls. All samples were run in duplicate, and data was filtered to select only amplified samples with a CRT standard deviation value below 3. The average CT values were then calculated for samples where both duplicates were included after filtration. After filtration, all samples were normalized using the three endogenous reference genes following the normalization process described in Vandesompele et al. 2002.

Data analysis

All statistical analyses were preformed using R Studio (version 4.1.2, R Core Team 2021). Successfully passing the reflex assessment was measured as a binary response (pass or fail), and the time at which fish passed was treated as categorical. A Chi-square test was used to determine whether there was an association between biopsy treatment and the time it took to pass reflex assessments.

An analysis of variance (ANOVA) was used to evaluate whether average delta CT values for MHC-II and Glucocorticoid Receptor 1 differed between biopsy treatments for all samples taken at the end of the 10-day monitoring period. Paired T-tests were used to determine whether expression of MHC-II and Glucocorticoid Receptor 1 changed in the same fish between initial sampling and at 10 days post-sampling.

4.3 Results

Regardless of biopsy treatment, there was no association between biopsy treatment and the time at which fish passed the equilibrium ($\chi^2 = 8.05$, df = 8, p = 0.43) and bursting assessments ($\chi^2 = 6.61$, df = 8, p = 0.58). Most fish passed both assessments immediately after sampling, shown in Figures 4.1 and 4.2, and no fish took longer than 10 minutes to pass both assessments.

There was no association in the average CT values for MHCII (F= 0.15, df= 2, P =0.10) and Glucocorticoid Receptor 1 (F=0.19, DF= 2, P=0.10) when gill and combined biopsy treatments were compared with untagged control fish between samples taken during the initial tagging. Furthermore, there was little evidence that treatment impacted CT values for MHCII (F = 0.02, df = 5, p = 0.98, Figure 4.3) and GR1 (F= 0.12, df = 5, p = 0.97, Figure 4.4) in samples taken from fish at the end of the 10-day monitoring period. When CT values were compared in the same fish initially and after 10 days or monitoring, there was no evidence that biopsy altered expression of MHCII and Glucocorticoid Receptor 1 (Table 4.2)

At the end of the 10-day monitoring period, one fish was euthanized because of advanced concurrent infections of Lymphocystis and Saprolegnia. That fish was in the muscle biopsy treatment. No other mortalities were observed throughout the study and fish were generally vigorous at the conclusion of the study.

4.4 Discussion

Based on the results of the reflex impairment assessment for bursting and equilibrium, the present study indicates that biopsy methods had no impact on reflexes immediately after sampling. Furthermore, there were no detectable impacts of biopsy on Major Histocompatibility Complex Class II and Glucocorticoid Receptor 1 expression immediately after sampling or at 10

days post-sampling. In addition to this, expression of both MHCII and Glucocorticoid Receptor 1 was found to be similar when compared in the same fish during initial sampling and after 10 days. Here I discuss key findings.

Previous studies have used reflex assessment scores to quantify stress in fish; given that a reflex is an involuntary response to a stimulus, multiple reflexes can be assessed to produce a real-time reflex index which has been shown to be strongly correlated with stress (Raby et al. 2012). In this study, nearly all fish passed both the bursting and equilibrium assessments immediately after surgery, indicating that both V13 tag implantation and biopsy caused minimal disturbance to overall homeostasis. This is further confirmed when comparing the change in gene expression between biopsy treatments taken immediately after surgery. For this study we used electrosedation. The use of electrosedation during surgery in fish has become increasingly popular due to the shorter duration and recovery time (Reid et al. 2019). The impact of electrosedation on blood cortisol levels has been found to be dependent on species (Abrams et al 2018); here, we did not detect an effect of electrosedation on stress response when examining gene expression.

Both Glucocorticoid Receptor 1 and Major Histocompatibility Complex Class II play crucial roles in the stress response of fish. During a stressor, cortisol is secreted into the bloodstream from adrenal glands (Aedo et al. 2023). Once secreted, cortisol binds to Glucocorticoid Receptor 1 and initiates a molecular cascade which allows the fish to respond to the stressor, such as by increasing metabolism and energy production which may be crucial for burst swimming required for predator avoidance or during angling interactions (Lawrence et al. 2019; Kennedy and Janz 2023). Although beneficial for short term stress response, long-term upregulation may sequester energy from other essential functions (e.g., growth, reproduction, osmoregulation) (Kennedy and Janz 2023). The degree to which cortisol levels (and subsequent upregulation of Glucocorticoid

Receptor 1) can vary widely between fish species (Vallejos-Vidal et al. 2022), and quantification of stress based on expression requires specific previous knowledge on species and population (Aedo et al. 2023). Given that no baseline is currently known for the Sandusky River spawning population, the important distinction in our study is that Glucocorticoid Receptor 1 expression was found to be consistent both across time periods (pre and post sampling) and across treatment groups (gill biopsy and combined treatment). Furthermore, Glucocorticoid Receptor 1 expression for all biopsy types was not found to be detectably different in control fish which had no tag implanted. As such, results from our study suggest that non-lethal biopsies, regardless of the type, do not impact regulation of Glucocorticoid Receptor 1, nor do they indicate an increase in stress caused by biopsy.

Similar to GR1 expression, no alteration in MHCII expression was observed in this study. Given that surgery, handling, and biopsy all have the potential to introduce pathogens through dermal disturbance and associated injury, changes in immune system regulation would be expected to occur. MHCII plays a vital role in the adaptive immune system of fishes as it is responsible for presenting foreign peptides to T cells, resulting in the release of cytokines which in turn signal and recruit other immune cells (Dijkstra et al. 2013; Li et al. 2017). Here, expression was found to be consistent between time periods, indicating that no one treatment made fish particularly vulnerable to pathogenic infections. Interestingly, some fish were observed to have Lymphocystis prior to sampling, and the outliers shown in Figure 4.3 correspond to those fish. Most wild fish harbour pathogens although the extent to which they become pathogenic may be influenced by the condition of the fish.

One limitation of this study was that no positive control was included to determine what MHCII and GR1 expression profiles would resemble in Walleye from the Sandusky River spawning population experiencing acute or chronic stress. Here, biopsy treatments were only compared to wild fish that had not been electronically tagged or biopsied. The use of a positive control would

clarify what level of gene expression could be expected given the scale of the stressor but was outside the scope of the current study.

In conclusion, the findings of this study indicate that non-lethal biopsies, regardless of the type, do not cause reflex impairment, nor do they alter expression of Glucocorticoid Receptor 1 and Major Histocompatibility Complex Class II. Although careful experimental design should always be undertaken in order to maximize animal welfare, our findings suggest that gill, muscle, or blood biopsies are suitable for combination with electronic tagging and tracking studies. This creates opportunities for tagging fish and obtaining a biopsy prior to release and then subsequently assessing relationships between fish behaviour and fate with various biomarkers assessed using the non-lethal biopsies. Although there has been much validation work with salmonids (e.g., Cooke et al. 2005), this is among the first few studies to assess these methods in warm-water systems where opportunistic infections like saprolegnia are common.

Tables

Table 4.1: Genes selected for STP Chip Developed by GEN-FISH

GENE FUNCTION	GENE SYMBOL	GENE NAME
STRESS RESPONSE	*nr3c1* (or *gr1*)	Glucocorticoid receptor 1
STRESS RESPONSE	*nr3c2* (or *mr*)	Mineralocorticoid receptor
STRESS RESPONSE	*hsd11b1*	11β-hydroxysteroid dehydrogenase 1
STRESS RESPONSE	*serpinh1a / serpinh1b*	Serpin Family H1 (hsp47)
STRESS RESPONSE	*hsp70a* / *hsp70b*	Heat shock protein 70a / 70b
STRESS RESPONSE	*hspa4a* / *hspa4b*	Heat shock 70 kDa protein 4
STRESS RESPONSE	*hspa8*	Heat shock cognate 71 kDa protein (hsc70)
STRESS RESPONSE	*hsp90ba* / *hsp90bb*	Heat shock protein 90 (constitutive)
STRESS RESPONSE	*cirbpa* / *cirbpb*	Cold inducible RNA binding protein
STRESS RESPONSE	*mmp2*	matrix metalloproteinase 2 (constitutive)
ENDOCRINE DISRUPTION	*esr1*	Estrogen receptor 1
ENDOCRINE DISRUPTION	*esr2a*	Estrogen receptor 2a
ENDOCRINE DISRUPTION	*esr2b*	Estrogen receptor 2b
ENDOCRINE DISRUPTION	*ar*	Androgen receptor
ENDOCRINE DISRUPTION	*cyp19a1a*	cytochrome P450, family 19, subfamily A, polypeptide 1a (ovarian aromatase)
GROWTH AND METABOLISM	*ghra/ghrb*	Growth hormone receptor
GROWTH AND METABOLISM	*igf1*	Insulin-like growth factor 1
GROWTH AND METABOLISM	*igf2a/igf2b*	Insulin-like growth factor 2
GROWTH AND METABOLISM	*cpt1b*	Carnitine palmytoltransferase 1B
GROWTH AND METABOLISM	*ctsd*	Cathepsin D
IMMUNE FUNCTION	CaM/CALM	Calmodulin
IMMUNE FUNCTION	MHC-I	Major histocompatibility complex class 1
IMMUNE FUNCTION	MHC-II	Major histocompatibility complex class 2
IMMUNE FUNCTION	STAT1	Signal transducer and activator of transcription 1
IMMUNE FUNCTION	TAPBP	Tapasin
ENDOGENOUS CONTROL	*rpl7*	60S ribosomal protein L7
ENDOGENOUS CONTROL	*rps9*	40S ribosomal protein S9
ENDOGENOUS CONTROL	*rpl13a*	ribosomal protein L13a

Table 4.2: Comparison of delta CT values for MHCII and GR1 in adult male Walleye *(Sander vitreus)* from the gill and combined biopsy treatments before and after the 10-day monitoring period

	MHCII	GR1
Gill	t = -0.35, df = 16, p-value = 0.73	t = -0.11, df = 21, p-value = 0.92
Combined	t = 0.04, df = 20, p-value = 0.98	t = -0.34, df = 23, p-value = 0.74

Figures

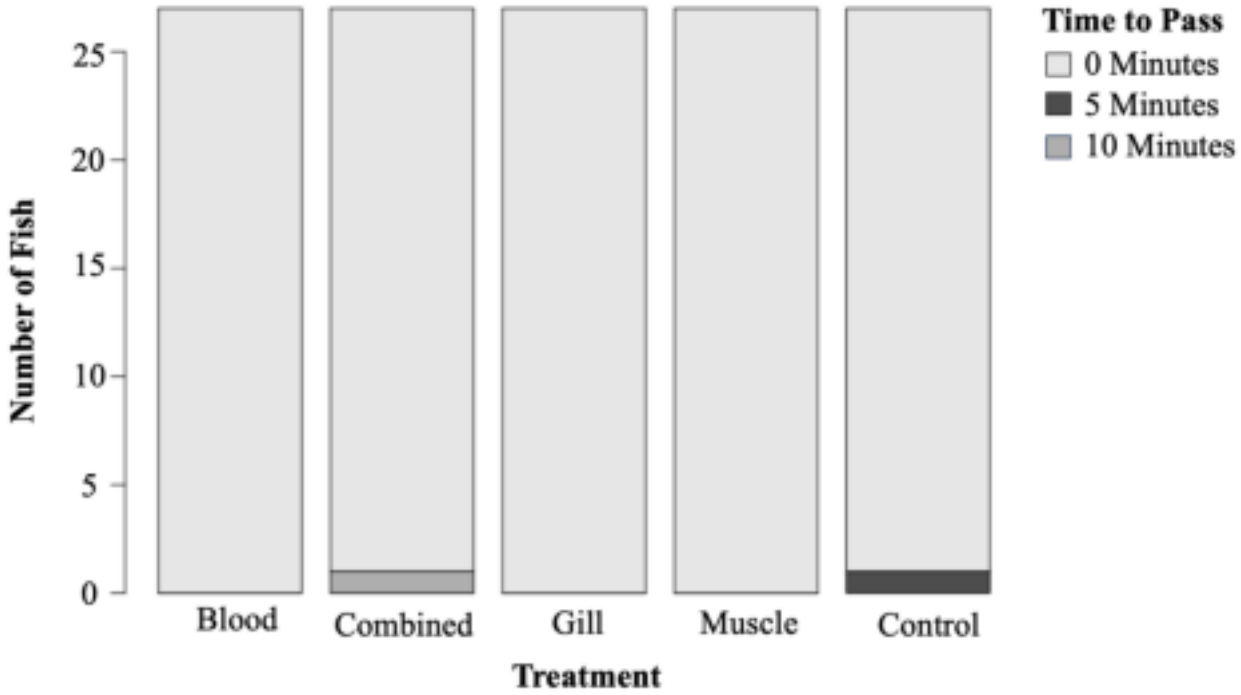

Figure 4.1: Biopsy treatments done on adult male Walleye *(Sander vitreus*), and the time required to pass the reflex assessment (bursting)

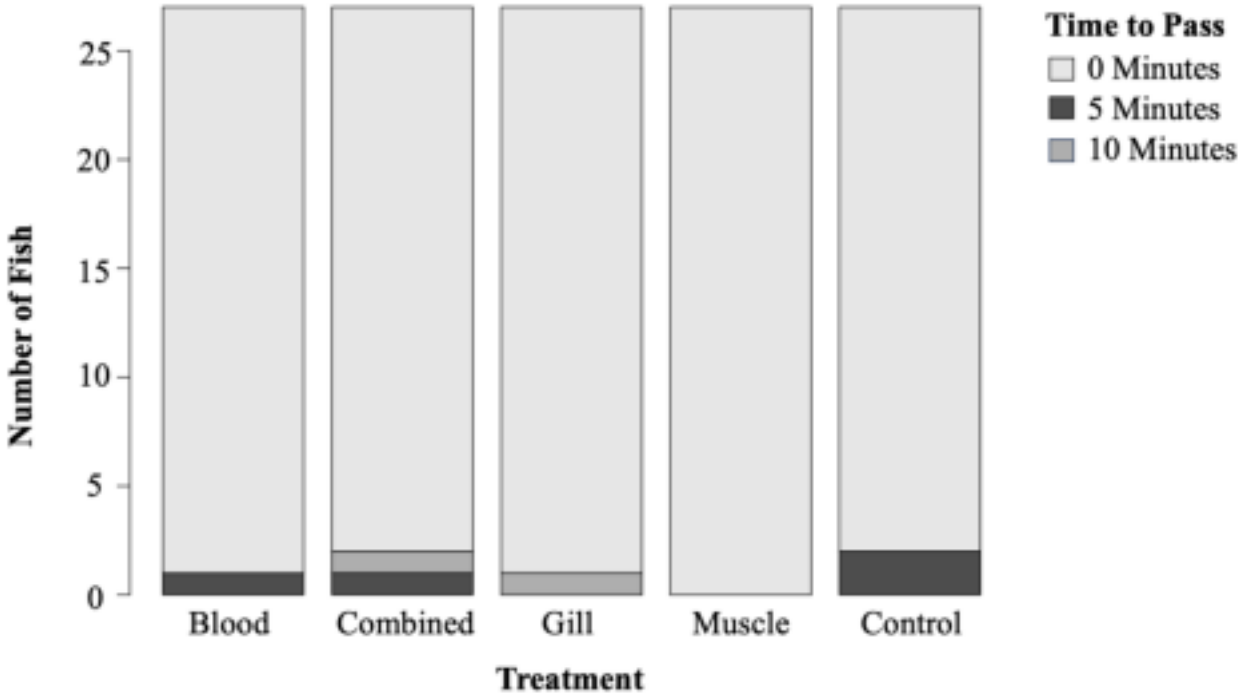

Figure 4.2: Biopsy treatments done on adult male Walleye (*Sander vitreus*) and the time required for fish to pass the reflex assessment (equilibrium)

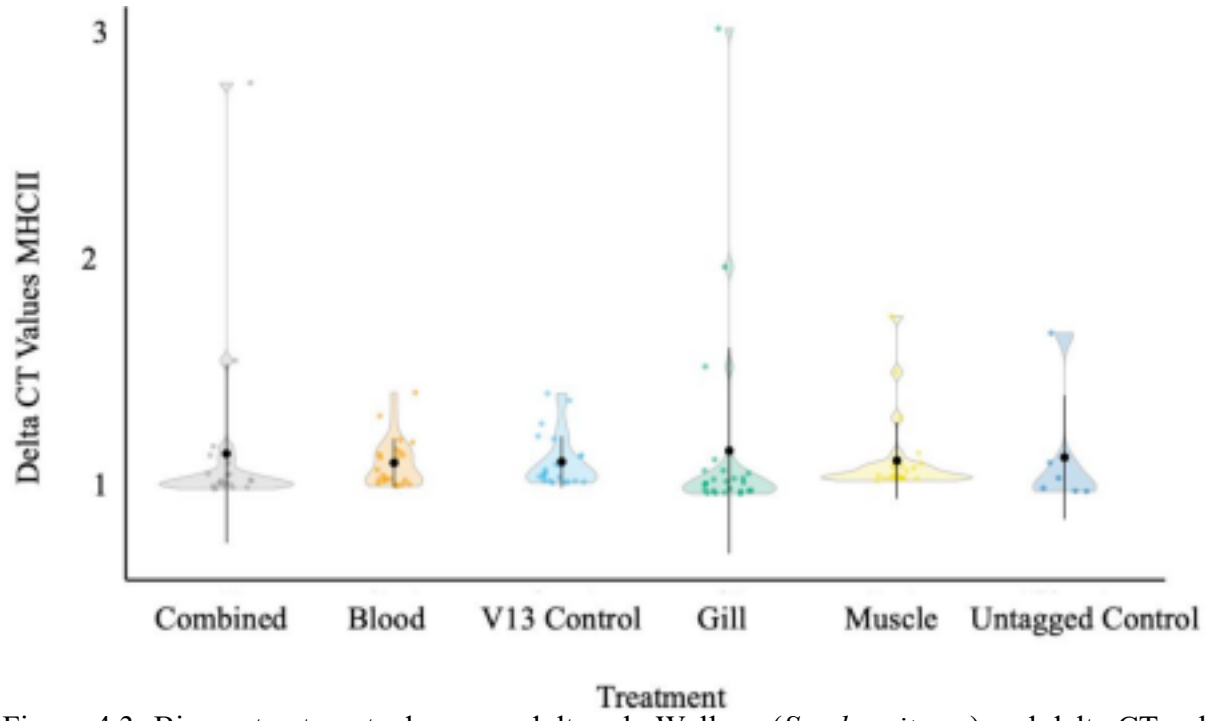

Figure 4.3: Biopsy treatments done on adult male Walleye (*Sander vitreus*) and delta CT values measuring gene expression of MHCII in gill tissue samples collected 10 days after initial biopsy and tagging. Black circles represent the mean, with standard error bars showing standard deviation,

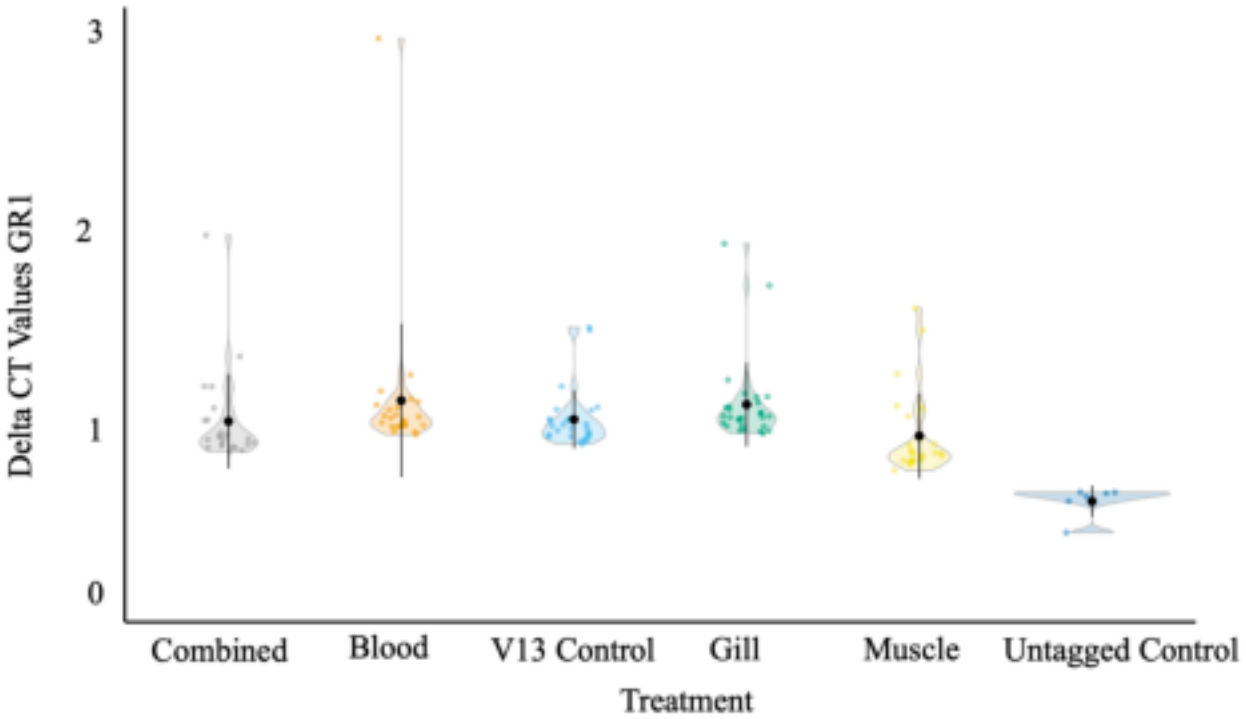

Figure 4.4: Biopsy treatments done on adult male Walleye (*Sander vitreus*) and delta CT values measuring gene expression of GR1 in gill tissue samples collected 10 days after initial biopsy and tagging. Black circles represent the mean, with standard error bars showing standard deviation,

Chapter 5: Conclusions and future research directions

The research outlined in this thesis offers an in-depth analysis of how biopsy techniques influence the behaviour, fitness, and health of three freshwater fishes. In Chapter 2, male Smallmouth Bass were assessed for their parental care abilities for a period of four weeks after sampling. Results from that study revealed that behaviour was largely unaffected, however the biopsy treatment comprised of multiple biopsy types was found to be a strong predictor of nest abandonment. Similar results were observed in Chapter 3, where muscle and gill biopsies were not found to influence exploratory and reactivity behaviour in juvenile Lake Trout. Furthermore, that chapter revealed that biopsies, regardless of type, did not hinder swimming performance 24 hours after biopsy. Finally, in Chapter 4, reflexes and expression of Glucocorticoid Receptor 1 and Major Histocompatibility Complex Class 2 genes were found to be similar across all biopsy treatments, regardless of when samples were collected. Across the board, mortality was negligible emphasizing that this approach is indeed a non-lethal sampling method.

The consequences of physiological biopsy were largely negligible in the three studies that were part of this thesis, with the combined biopsy treatment (blood, gill, and muscle) in the Smallmouth Bass chapter being the only instance of biopsies being observed to impair behaviour. Here, the combined biopsy treatment was found to increase nest abandonment rates in male bass. Given that nest abandonment has been shown to occur in fish when stressors outweigh the benefit of continued parental care (Siepker et al. 2009; Lunn and Steinhart, 2010) results indicate that latent stress caused by biopsy may be sufficient to exceed this stress threshold. Furthermore, nest abandonment did not occur immediately after biopsy, and mortalities in this study were not detected until a week after sampling.

Previous definitions of stress have been repeatedly refined in order to include both ecological and physiological definitions (Steckler et al. 2005). The term “allostasis” was introduced to describe the physiological response to daily and seasonal variations, such as changes in temperature, reproductive state, or environmental conditions (McEwen and Wingfield, 2003). The concept of allostasis was further refined in Romero et al. 2009, via the introduction of the Reactive Scope Model, a model which seeks to combine stress, allostasis, and homeostasis into one model. Here, determinants of the physiological stress response are broken into four ranges, which encompass predictable (Predictive Homeostasis) and unpredictable (Reactive Homeostasis) environmental changes, and the pathological effect of short-term and long-term stressors that exceed an organism’s ability to maintain homeostasis (Homeostatic Failure and Homeostatic Overload, respectively) (Romero et al. 2009). The Reactive Scope Model can provide additional context for the response of fishes to biopsy in our three studies; nesting failure in Smallmouth Bass from the combined biopsy treatment could fit well within the range of Homeostatic Overload, as here the combined effect of angling, nest defence, and multiple biopsies was found to exceed the threshold for stress identified in previous studies during the nesting season. In contrast, given that no impact of biopsy was observed in Walleye and Lake Trout, fish likely were in the Reactive Homeostasis state, where fish were able to respond to biopsy without suffering long term changes in behaviour or survival.

To try and capture physiological changes during the 7-day period noted to be significant in Smallmouth Bass, gill samples were taken from all male Walleye in Chapter 4, both initially during the experiment, and again 10 days later. Previous work (e.g., Bortoletti et. al. 2021) that has evaluated the stress response in fish using expression of Glucocorticoid Receptor 1 has indicated that short-term stressors (e.g., air exposure, handling) result in increased expression or GR1 in a time and tissue dependent manner. Although this may be specific to individual species, generally GR1 expression is found to decrease within 24 hours of exposure to the

stressor (Vallejos-Vidal et al. 2022). In the case of chronic stress, however, GR1 expression remains elevated for longer durations (days to weeks; Tort 2011). Based on the nest abandonment rates of Smallmouth Bass, I would have expected to observe increased GR1 expression in Walleye from the combined treatment. Moreover, I predicted that GR1 expression would have remained elevated in Walleye from the combined treatment in samples collected 10 days post-biopsy. Given that expression of GR1 remained consistent throughout the Walleye study, results of Chapter 4 indicate that either chronic stress was not experienced, gill tissue was not able to sufficiently capture GR1 expression, or 10 days was too far outside the range to adequately capture elevated GR1 expression and fish had fully recovered.

Throughout these three studies, mortality rates were consistently very low, with between 0.5-2% of all fish sampled in each study resulting in mortalities. This is consistent with findings in Ackerson et al. (2014), in which a survival rate of 97% was determined in Smallmouth Bass that received muscle biopsies. Other studies (e.g., Lockhart et al. 1972; Osmundson et al. 2000; Baker et al. 2004) have noted repeated capture of biopsied individuals, further indicating that biopsies may be well tolerated, even in natural settings where predation, feeding requirements, and competition are ecological realities. As such, results found suggest that the majority of biopsies do not cause mortality, and cases where they cause mortality are uncommon.

Furthermore, evaluation of the euthanized fish revealed no evidence of disease, and in the Walleye study although expression of MHCII and GR1 were elevated in fish with observed lymphocystis infection, expression did not significantly differ from other fish with no evidence of infection. Moreover, there was little evidence that expression of both MHCII and GR1 increased in diseased fish after initial biopsy, which may further indicate that biopsy did not cause additional stress. MHCII expression was not able to be quantified in the one mortality from the Walleye study due to insufficient amplification during PCR, however MHCII expression

captured in the initial biopsy was higher than in other samples (although not statistically different), indicating likely prior infection.

Of the species studied here and in previous work, many of these species are the target of electronic tagging and tracking studies (GLATOS 2023). When combining biopsy with fish that have been tagged, researchers are able to identify the connection between the internal state of a fish and the fish's behaviour and fate (Cooke et al. 2005; Cooke et al. 2008; Boe et al. 2020). Such studies have already been critical in identifying mechanisms behind successful or impaired migration in Pacific (Miller et al. 2011) and Atlantic Salmon (Benthal et al. 2022), and the validation work done here further emphasizes the plausibility of combining sampling methods to elucidate mechanisms behind behaviour and fate in a wide range of freshwater species spanning families and body sizes.

Although this work provides important validation framework for future studies, concern remains regarding biopsy on more delicate species. Observations from other researchers have noted that not all biopsy types are suitable for all species. Freshwater Drum (*Aplodinotus grunnies*), for example, were observed to be in extremely poor condition to an extent which required euthanization after a muscle sample was taken using a muscle punch (Dr. Caleb Hasler, personal communication). In our research, muscle biopsy was only found to cause mortality for one individual across all three studies, and the Walleye in which the mortality occurred was observed to be impaired via lymphocystis infection prior to sampling. As such, continuing to ensure that biopsy is well tolerated in individual species is critical to maintaining welfare status during experimentation, and research should be expanded to include additional species.

Furthermore, the results of Chapter 4 failed to capture any measurable response to non-lethal

sampling when using transcriptomics to measure MHCII and GR1 expression. Future analysis will include expression of genes in Table 4.1, which may be able to capture stress or immune response more accurately to sampling. Additionally, taking samples from different tissues and expanding upon timepoints at which tissues were collected may provide greater context to the stress and immune response. In Vallejos-Vidal et al. 2022, cortisol levels were noted to spike in blood and skin samples of Sea Bream (*Sparus aurata*) between 1- and 6-hours post air exposure. In our work, samples were only taken either immediately after tag implantation, or 10 days after, and so time periods selected would have missed any significant changes in gene regulation in the hours which followed biopsy. Evaluating the stress response to biopsy in shorter time intervals could provide crucial insight to the impacts which biopsy have at a cellular level.

An additional future direction for evaluation of biopsy would be an extension of the monitoring period in order to assess biopsy through all stages of healing. Although previous research (Zhao et al. 2014; McCormick 1993) noted that cuts from gill biopsies were found to be fully healed within 7 days of biopsy, the time required to regenerate tissue lost may exceed 40 days (as identified in the model species Danio rerio) (Ramel et al. 2021). Furthermore, muscle biopsies have been noted to fully healed in Smallmouth Bass within two months of biopsy (Ackerson et al. 2014). Due to the longer timeframe noted in previous research, continuing to evaluate how biopsy impacts behaviour, survival, and transcriptomic expression throughout the healing process is essential to help ensure the safety of biopsy.

In the broader context of relevance to the field, this work illustrates that biopsy has negligible consequences on the welfare and fitness in Smallmouth Bass, Walleye, and juvenile Lake Trout. This further adds to previous validation work, in which Atlantic Salmon (Boe et al. 2020), Sockeye Salmon (Cooke et al, 2005), Northern Pike (Baker et al. 2004), and Lake Whitefish (Baker et al. 2004) were found to be largely unimpaired by biopsy. Given that putative non-lethal

sampling methods are increasing in popularity, this research emphasizes that it is possible to collect tissue samples from live fish while still maintaining adequate welfare.

Thesis References

Abrams, A. E. I., A. M. Rous, J. L. Brooks, and M. J. Lawrence. 2018. Comparing immobilization, recovery, and stress indicators associat- ed with electric fish handling gloves and a portable electrosedation system. *Transactions of the American Fisheries Society* 147:390–399.

Ackerson, J.R., M.J. Mckee, C.J. Schmitt, W.G. Brumbaugh. 2014. Implementation of a non-lethal biopsy punch monitoring program for mercury in Smallmouth Bass, *Micropterus dolomieu* Lacepede, from the Eleven Point River, Missouri. *Bull Environ Contam Toxicol* **92**, 125–131 (2014). https://doi.org/10.1007/s00128-013-1145-x

Adams, S. M. (2002). *Biological indicators of aquatic ecosystem stress*. *American Fisheries Society.*

Aedo, J. E., R. Zuloaga, D. Aravena-Canales, A. Molina, and J. A. Valdés. 2023. Role of glucocorticoid and mineralocorticoid receptors in rainbow trout (Oncorhynchus mykiss) skeletal muscle: A transcriptomic perspective of cortisol action. *Frontiers in Physiology,* 13, 1048008. https://doi.org/10.3389/fphys.2022.1048008

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

Baker, R. F., P.J. Blanchfield, M.J. Paterson, R.J. Flett, and L. Wesson. 2004. Evaluation of nonlethal methods for the analysis of mercury in fish tissue. *Transactions of the American Fisheries Society (1900)*, *133*(3), 568–576. https://doi.org/10.1577/T03-012.1

Bass, A. L., C. F. Stevenson, A. D. Porter, E. L. Rechisky, N. B. Furey, S. J. Healy, A. M. Kanigan, A. G. Lotto, D. W. Welch, and S. G. Hinch. 2020. In situ experimental evaluation of tag burden and gill biopsy reveals survival impacts on migrating juvenile sockeye salmon. *Canadian Journal of Fisheries and Aquatic Sciences,* 77(12), 1865-1879. https://doi.org/10.1139/cjfas-2020-0134

Bernthal, F.R., J.D. Armstrong, J.D., K.H. Nislow and N.B. Metcalfe. 2022. Nutrient limitation in Atlantic salmon rivers and streams: Causes, consequences, and management strategies. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 32(6), 1073-1091. https://doi.org/10.1002/aqc.3811

Bøe, K., M.J. Robertson, I.A. Flemming, and M. Power. 2022. Evaluating the effect of dorsal muscle biopsies on adult Atlantic salmon growth and marine return rates, *Conservation Physiology*, 8(1), coz099, https://doi.org/10.1093/conphys/coz099

Bortoletti M., L. Maccatrozzo, G. Radaelli, S. Caberlotto, and D. Bertotto. 2021. Muscle cortisol levels, expression of glucocorticoid receptor and oxidative stress markers in the teleost fish *Argyrosomus regius* exposed to transport stress. *Animals (Basel),* 11(4), 1160- . doi: 10.3390/ani11041160.

Brooks, J. L., J. D. Midwood, L. F. G. Gutowsky, C. M. Boston, S. E. Doka, J. A. Hoyle, and S. J. Cooke. 2019. Spatial ecology of reintroduced walleye (Sander vitreus) in Hamilton Harbour of Lake Ontario. *Journal of Great Lakes Research* 45(1).

Brownscombe, J.W., L. P. Griffin, J. L. Brooks, A. J. Danylchuk, S. J. Cooke, and J. D. Midwood. 2022. Applications of telemetry to fish habitat science and management. *Canadian Journal of Fisheries and Aquatic Sciences*. **79**(8): 1347-1359. https://doi.org/10.1139/cjfas-2021-0101

Burridge, L., J. S. Weis, F. Cabello, J. Pizarro, and K. Bostick. 2010. Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture*, 306(1), 7–23. https://doi.org/10.1016/j.aquaculture.2010.05.020

Campbell, M. D., R. Patino, J. Tolan, R. Strauss, and S. L. Diamond. 2010. Sublethal effects of catch- and-release fishing: Measuring capture stress, fish impairment, and predation risk using a condition index. *ICES Journal of Marine Science*, 67(3), 513-521. https://doi.org/10.1093/icesjms/fsp255

Chapman, B. B., Morrell, L. J., & Krause, J. (2010). Unpredictability in food supply during early life influences boldness in fish. *Behavioral Ecology*, 21(3), 501-506.

Colotelo, A. H., and S. J. Cooke. 2011. Evaluation of common angling-induced sources of epithelial damage for popular freshwater sport fish using fluorescein. *Fisheries Research*, 109(2), 217-224, https://doi.org/10.1016/j.fishres.2010.12.005

Connon, E.R., K.M. Jeffries, L.M. Komoroke, A.E. Todgham and N.A. Fangue. (2018). The utilitiy of transcriptomics in fish conservation, *Journal of Experimental Biology,* 221, 148833. doi:10.1242/jeb.148833

Cooke, S. J., G. T. Crossin, D. A. Patterson, K. K. English, S. G. Hinch, J. L. Young, R. F. Alexander, M. C. Healey, G. Van Der Kraak, and A. P. Farrell. 2005. Coupling non-invasive physiological assessments with telemetry to understand inter-individual variation in behaviour and survivorship of sockeye salmon: development and validation of a technique. *Journal of Fish Biology,* 67(5), 1342-358. https://doi.org/10.1111/j.1095-8649.2005.00830.x

Cooke, S. J., S.G. Hinch, A.P. Farrell, D.A. Patterson, K. Miller-Saunders, D.W. Welch, and G. Van der Kraak. 2008. Developing a mechanistic understanding of fish migrations by linking telemetry with physiology, behavior, genomics and experimental biology: an interdisciplinary case study on adult Fraser River sockeye salmon. *Fisheries,* 33(7), 321-339.

Cooke, S.J., J.D. Midwood, J.D. Thiem, P. Klimley, M.C. Lucas, E.B. Thorstad, J. Eiler, C. Holbrook, and B.C Ebner. 2013. Tracking animals in freshwater with electronic tags: past, present and future. *Anim Biotelemetry* 1, 5. https://doi.org/10.1186/2050-3385-1-5

Cooke, S. J., E. G. Martins, D. P. Struthers, L. F. G. Gutowsky, M. Power, S. E. Doka, J. M. Dettmers, D. A. Crook, M. C. Lucas, C. M. Holbrook, and C. C. Krueger. 2016. A moving target—incorporating knowledge of the spatial ecology of fish into the

assessment and management of freshwater fish populations. *Environmental Monitoring and Assessment,* 188(4), 239. https://doi.org/10.1007/s10661-016-5228-0

Cooke, S. J., J. F. Schreer, D. H. Wahl, and D. P. Philipp. 2002. Physiological impacts of catch-and-release angling practices on Largemouth Bass and Smallmouth Bass. *American Fisheries Society Symposium*, 31, 489- 512.

Cooke, S.J., M.F. Docker, N.E. Mandrak, N. Young, D.D. Heath, K.M. Jeffries, A. Howarth, J.W. Brownscombe, J. Livernois, C.A.D. Semeniuk, P.A. Venturelli, A.J. Danylchuk, R.J. Lennox, I. Jaric, A.T. Fisk, C.S. Vandergoot, J.R. Britton, A.M. Muir. 2022. Technoscience and the modernization of freshwater fisheries assessment and management. *Environmental Technology and Innovation,* 28, 102865. https://doi.org/10.1016/j.eti.2022.102865

Cornwell, E. R., C. A. Bellmund, G. H. Groocock, P. T. Wong, K. L. Hambury, R. G. Getchell, and P. R. Bowser. 2013. Fin and gill biopsies are effective nonlethal samples for detection of viral hemorrhagic septicemia virus genotype IVb. *Journal of Veterinary Diagnostic Investigation,* 25(2), 203-209. https://doi.org/10.1177/1040638713476865

Crawford, B.A., S.A. Leider, J.M. Tipping. 1977. Technique for rapidly taking samples of skeletal muscle from live adult steelhead trout. *Progress Fish-Culturist*, 39, 125-125.

Crossin, G.T., M.R. Heupel, C.M. Holbrook, N.E. Hussey, S.K. Lowerre-Barbieri, V.M. Nguyen, G.D. Raby, and S.J. Cooke. 2017. Acoustic telemetry and fisheries management. *Ecol Appl*, 27: 1031-1049. https://doi.org/10.1002/eap.1533

Currie, A. R., D. Cockerill, M. Diez-Padrisa, H. Haining, F. L. Henriquez, and B. Quinn. 2022. Anemia in salmon aquaculture: Scotland as a case study. *Aquaculture*, 546, 737313. https://doi.org/10.1016/j.aquaculture.2021.737313

Dash, S., Das, S. K., Samal, J., and Thatoi, H. N. (2018). Epidermal mucus, a major determinant in fish health: A review. *Iranian Journal of Veterinary Research,* 19(2), 72-81.

https://doi.org/10.22099/ijvr.2018.4849

Davis, M. W. 2010. Fish stress and mortality can be predicted using reflex impairment. *Fish and Fisheries*, 11, 1-11.

Dijkstra, J. M., U. Grimholt, J. Leong, B. F. Koop, and K. Hashimoto. 2013. Comprehensive analysis of MHC class II genes in teleost fish genomes reveals dispensability of the peptide-loading DM system in a large part of vertebrates. *BMC Evolutionary Biology* 13(1):1–14.

Dill, L. M. 1974. The escape response of the zebra danio (Brachydanio rerio) I. The stimulus for escape. *Animal Behaviour*, 22(3), 711–722. https://doi.org/10.1016/S0003-3472(74)80022-9

Elliott, C. W., M. S. Ridgway, P. J. Blanchfield, and B. L. Tufts. 2023. Novel insights gained from tagging walleye (Sander vitreus) with pop-off data storage tags and acoustic transmitters in Lake Ontario. *Journal of Great Lakes Research*, 49(2):515–530.

Elvidge, C. K., and S. J. Cooke. 2020. Predation risk mediates cognitive constraints following physical exertion in schoolmaster snapper. *Physiology and Behavior,* 214, 112767. https://doi.org/10.1016/j.physbeh.2019.112767.

Euclide, P. T., J. Robinson, M. Faust, S. A. Ludsin, T. M. MacDougall, E. A. Marschall, K.-Y. Chen, C. Wilson, M. Bootsma, W. Stott, K. T. Scribner, and W. A. Larson. 2021. Using genomic data to guide walleye management in the Great Lakes. Yellow Perch, Walleye, and Sauger: Aspects of ecology, management, and culture. *Fish and Fisheries,* 41, 115–139.

Faisal, M., T. P. Loch, M. Shavalier, M. G. VanDeuren, I. Standish, A. Winters, G. Glenney, J. Aho, M. Wolgamood, J. VanAmberg, E. Eisch, and G. E. Whelan. 2019. Resurgence of salmonid Herpesvirus-3 infection (Epizootic Epitheliotropic Disease) in hatchery-propagated Lake Trout in Michigan. *Journal of Aquatic Animal Health*, 31(1),

31-45.

Fuiman, L. A., K. A. Rose, J. H. Cowan, and E. P. Smith. 2006. Survival skills required for predator evasion by fish larvae and their relation to laboratory measures of performance. *Animal Behaviour, 71*(6), 1389–1399. https://doi.org/10.1016/j.anbehav.2005.11.013

Glatos. GLATOS. (2023.). https://glatos.glos.us/

Gravel, M. A., and S. J. Cooke. 2009. Influence of inter-lake variation in natural nest predation pressure on the parental care behaviour of Smallmouth Bass (Micropterus dolomieu). *Ethology,* 115(6), 608-616. https://doi.org/10.1111/j.1439-0310.2009.01641.

Gunderson, L., B. A. Cosens, B. C. Chaffin, C. A. T. Arnold, A. K. Fremier, A. S. Garmestani, R. K. Craig, H. Gosnell, H. E. Birge, C. R. Allen, M. H. Benson, R. R. Morrison, M. C. Stone, J. A. Hamm, K. Nemec, E. Schlager, and D. Llewellyn. 2017. Regime shifts and panarchies in regional scale social-ecological water systems. *Ecology and society : a journal of integrative science for resilience and sustainability* 22(1):1.

Hanson, K. C., S. J. Cooke, C. D. Suski, and D. P. Philipp. 2007. Effects of different angling practices on post-release behaviour of nest-guarding male black bass, Micropterus spp. *Fisheries Management and Ecology,* 14(2), 141-148. https://doi.org/10.1111/j.1365-2400.2007.00534.

Hayden, T. A., C. M. Holbrook, D. G. Fielder, C. S. Vandergoot, R. A. Bergstedt, J. M. Dettmers, C. C. Krueger, and S. J. Cooke. 2014. Acoustic telemetry reveals large-scale migration patterns of Walleye in Lake Huron. *PLOS ONE,* 9(12). 114833.

Henderson, C. J., T. F. Stevens, and S. Y. Lee. 2016. Assessing the suitability of a non-lethal biopsy punch for sampling fish muscle tissue. *Fish Physiology and Biochemistry,* 42(6), 1521 1526. https://doi.org/10.1007/s10695-016-0237-z

Hlina, B. L., D. M. Glassman, A. D. Chhor, B. S. Etherington, C. K. Elvidge, B. K. Diggles, and S. J. Cooke. 2021. Hook retention but not hooking injury is associated with behavioral

differences in Bluegill. *Fisheries Research*, *242*, 106034–. https://doi.org/10.1016/j.fishres.2021.106034

Huntingford, F.A., Adams, C., Braithwaite, V.A., Kadri, S., Pottinger, T.G., Sandøe, P. and Turnbull, J.F. (2006), Current issues in fish welfare. *Journal of Fish Biology*, 68: 332-372. https://doi.org/10.1111/j.0022-1112.2006.001046.

Hussey, N. E., S. T. Kessel, K. Aarestrup, S. J. Cooke, P. D. Cowley, A. T. Fisk, R. G. Harcourt, K. N. Holland, S. J. Iverson, J. F. Kocik, J. E. M. Flemming, and F. G. Whoriskey. 2015. Aquatic animal telemetry: A panoramic window into the underwater world. *Science,* 348(6240), 1221-1221. https://doi.org/10.1126/science.1255642

Jeffries, K. M., Hinch, S. G., Gale, M. K., Clark, T. D., Lotto, A. G., Casselman, M. T., Li, S., Rechisky, E. L., Porter, A. D., Welch, D. W., & Miller, K. M. (2014). Immune response genes and pathogen presence predict migration survival in wild salmon smolts. *Molecular Ecology*, *23*(23), 5803–5815. https://doi.org/10.1111/mec.12980

Jeffries, K. M., A. Teffer, S. Michaleski, N. J. Bernier, D. D. Heath, and K. M. Miller. 2021. The use of non-lethal sampling for transcriptomics to assess the physiological status of wild fishes. *Comparative Biochemistry and Physiology B*, 256, 110629. https://doi.org/10.1016/j.cbpb.2021.110629

Kennedy, E. K. C., and D. M. Janz. 2023. Can scale cortisol concentration be quantified non-lethally in wild fish species? *Conservation Physiology* 11(1), coac081. https://doi.org/10.1093/conphys/coac081

Kieffer, J. D., M. R. Kubacki, F. J. S. Phelan, D. P. Philipp, and B. L. Tufts. 1995. Effects of catch-and-release angling on nesting male Smallmouth Bass. *Transactions of the American Fisheries Society,* 124(1), 70-76. https://doi.org/10.1577/1548-8659(1995)124%3C0070:EOCARA%3E2.3.CO;2

Kieffer JD. Limits to exhaustive exercise in fish. 2000. *Comp Biochem Physiol A Mol Integr*

Physiol, 126(2):161-79. doi: 10.1016/s1095-6433(00)00202-6.

Kristensen, M. L., E. M. Olsen, E. Moland, H. Knutsen, P. Grønkjær, A. Koed, K. Källo, and K. Aarestrup. 2021. Disparate movement behavior and feeding ecology in sympatric ecotypes of Atlantic cod. *Ecology and Evolution*, 11(16):11477–11490.

Lawrence, M. J., G. D. Raby, A. K. Teffer, K. M. Jeffries, A. J. Danylchuk, E. J. Eliason, C. T. Hasler, T. D. Clark, and S. J. Cooke. 2020. Best practices for non-lethal blood sampling of fish via the caudal vasculature. *Journal of Fish Biology,* 97(1), 4-15. https://doi.org/10.1111/jfb.14339

Li, X., H. Du, L. Liu, X. You, M. Wu, and Z. Liao. 2017. MHC class II alpha, beta and MHC class II-associated invariant chains from Chinese sturgeon (Acipenser sinensis) and their response to immune stimulation. *Fish and Shellfish Immunology*, 70, 1-12.

Lockhart, W. L., Uthe, J. F., Kenney, A. R., and P.M Mehrle. 1972. Methylmercury in Northern Pike (Esox lucius): Distribution, elimination, and some biochemical characteristics of contaminated fish. *Canadian Journal of Fisheries and Aquatic Sciences*, *29*(11), 1519–1523. https://doi.org/10.1139/f72-240

Lorenzen, K., K. M. Leber, and H. L. Blankenship. 2010. Responsible approach to marine stock enhancement: An update. *Reviews in Fisheries Science,* 18(2).

Lukas, Joseph A., and Donald J. Orth. 1995. Factors affecting nesting success of Smallmouth Bass in a regulated Virginia stream. *Transactions of the American Fisheries Society,* 124(5), 726-735. https://doi.org/10.1577/1548-8659(1995)124<0726:FANSOS>2.3.CO;2

Lunn, B. D., and G. B. Steinhart. 2010. Effect of brood reduction on nest abandonment of Smallmouth Bass. *Transactions of the American Fisheries Society,* 139(2), 586 592. https://doi.org/10.1577/T09-060.1

Lynch, A. J., Cooke, S. J., Deines, A. M., Bower, S. D., Bunnell, D. B., Cowx, I. G., et al. (2016). The social, economic, and environmental importance of inland fish and

fisheries. *Environmental Reviews*, *24*(2), 115-121.

Martins, C. I. M., L. Galhardo, C. Noble, B. Damsgård, M. T. Spedicato, W. Zupa, M. Beauchaud, E. Kulczykowska, J. C. Massabuau, T. Carter, S. R. Planellas, and T. Kristiansen. 2012. Behavioural indicators of welfare in farmed fish. *Fish physiology and Biochemistry,* 38(1):17–41.

Matley, J. K., M. D. Faust, G. D. Raby, Y. Zhao, J. Robinson, T. MacDougall, T. A. Hayden, A. T. Fisk, C. S. Vandergoot, and C. C. Krueger. 2020. Seasonal habitat-use differences among Lake Erie's walleye stocks. *Journal of Great Lakes Research,* 46(3).

McCormick, S. D. 1993. Methods for nonlethal gill biopsy and measurement of Na +, K + -ATPase activity. *Canadian Journal of Fisheries and Aquatic Sciences,* 50(3), 656–658. https://doi.org/10.1139/f93-075

McEwen, B.S., and J.C. Wingfield. 2003. The concept of allostasis in biology and biomedicine. *Hormones and Behaviour,* 43, 3-15.

Miller, K. M., S. Li, K. H. Kaukinen, N. Ginther, E. Hammil, J. M. R. Curtis, D. A. Patterson, T. Sierocinski, L. Donnison, P. Pavlidis, S. G. Hinch, K. A. Hruska, S. J. Cooke, K. K. English, and A. P. Farrell. 2011. Genomic signatures predict migration and spawning failure in wild Canadian Salmon. *Science,* 331(6014), 214–217. https://doi.org/10.1126/science.1196901

Milligan C. L. (1996). Metabolic recovery from exhaustive exercise in rainbow trout. *Comparative Biochemistry and Physiology -- Part A: Physiology*, *113*(1), 51–60. https://doi.org/10.1016/0300-9629(95)02060-8

Muff, S., E. B. Nilsen, R. B. O'Hara, and C. R. Nater. 2022. Rewriting results sections in the language of evidence. *Trends in Ecology and Evolution,* 37(3), 203-210. https://doi.org/10.1016/j.tree.2021.10.009

Naiman, R. J., and M. G. Turner. 2000. A Future Perspective on North America's Freshwater

Ecosystems. *Ecological Applications,* 10(4):958–970.

Osmundson, B.C., T.E. May, and D.B. Osmundson. 2000. Selenium concentrations in the Colorado pikeminnow (Ptychocheilus lucius) : Relationship with flows in the Upper Colorado River. *Archives of Environmental Contamination and Toxicology*, *38*(4), 479–485. https://doi.org/10.1007/s002449910063

Philipp, D. P., C. A. Toline, M. F. Kubacki, D. B. F. Philipp, and F. J. S. Phelan. 1997. The impact of catch-and-release angling on the reproductive success of Smallmouth Bass and Largemouth Bass. *North American Journal of Fisheries Management,* 17(2), 557–567. https://doi.org/10.1577/1548-8675(1997)017<0557:TIOCAR>2.3.CO;2

Portz, D. E., Woodley, C. M., & Cech, J. J. (2006). Stress-associated impacts of short-term holding on fishes. *Reviews in Fish Biology and Fisheries*, *16*, 125-170.

Raby, G. D., M. R. Donaldson, S. G. Hinch, D. A. Patterson, A. G. Lotto, D. Robichaud, K. K. English, W. G. Willmore, A. P. Farrell, M. W. Davis, and S. J. Cooke. 2012. Validation of reflex indicators for measuring vitality and predicting the delayed mortality of wild coho salmon bycatch released from fishing gears. *Journal of Applied Ecology,* 49:90–98.

Ramel, M.C., F. Progatzky, A. Rydlova, M. Wane, J. Schymeinsky, C. Williams, B. Jung, J.

Lamb, M.J. Thomas, L. Bugeon, and M.J. Dallman. 2021. Dynamics of repair and regeneration of adult zebrafish respiratory gill tissue after cryoinjury. *BioRxiv.* https://doi.org/10.1101/2021.05.27.445469

Réale, D., S. M. Reader, D. Sol, P. T. McDougall, and N. J. Dingemanse. 2007. Integrating animal temperament within ecology and evolution. *Biological reviews of the Cambridge Philosophical Society,* 82(2), 291–318. https://doi.org/10.1111/j.1469-185X.2007.00010.

Reid, C.H., C.S. Vandergoot, J.D. Midwood, E.D. Stevens, J. Bowker, S.J. Cooke. 2019. On the elctromobilization of fishes for research and practice; Opportunities, challenges, and

research needs. *Fisheries,* 44(12), 576-585.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rolfhus, K. R., M. B. Sandheinrich, J. G. Wiener, S. W. Bailey, K. A. Thoreson, and C. R. Hammerschmidt. 2008. Analysis of fin clips as a nonlethal method for monitoring mercury in fish. *Environmental Science and Technology,* 42(3), 871–877. https://doi.org/10.1021/es071427+

Samson, E., Brownscombe, J., & Cooke, S. (2014). Behavioural and reflex responses of mottled mojarra Eucinostomus lefroyi (Gerreidae) to cold shock exposure. *Aquatic Biology*, *23*(1), 101–108. https://doi.org/10.3354/ab00609

Sefc, K. M., A. C. Brown, and E. D. Clotfelter. 2014. Carotenoid-based coloration in cichlid fishes. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *173*(100), 42–51. https://doi.org/10.1016/j.cbpa.2014.03.006

Siepker, M. J., D. H. Wahl, D. P. Philipp, and K. G. Ostrand. 2006. Evidence of reduced reproductive success of nesting largemouth bass sampled with standard electrofishing procedures. *North American Journal of Fisheries Management,* 26(3), 631-635.

Siepker, M. J., S. J. Cooke, D. H. Wahl, and D. P. Philipp. 2009. Individual reproductive success of Largemouth Bass and Smallmouth Bass subjected to different components of competitive angling events. *Transactions of the American Fisheries Society,* 138(4), 818–825. https://doi.org/10.1577/T07-269.1

Steckler, T., *N.H. Kalin, J.M.H.M. Reul.* Handbook of Stress and the Brain, Elsevier, Amsterdam, the Netherlands (2005), 3-23

Steinhart, G. B., and B. D. Lunn. 2011. When and why do Smallmouth Bass abandon their broods? The effects of brood and parental characteristics. *Fisheries Management and Ecology*, 18(1), 1–11. https://doi.org/10.1111/j.1365-2400.2010.00758.x

Sundström, L. F., E. Petersson, J. Höjesjö, J. I. Johnsson, and T. Järvi. 2004. Hatchery selection promotes boldness in newly hatched brown trout (Salmo trutta): Implications for dominance. *Behavioral Ecology*, 15(2), 192–198. https://doi.org/10.1093/beheco/arg089

Suski, C. D., J. H. Svec, J. B. Ludden, F. J. S. Phelan, and D. P. Philipp. 2003. The effect of catch-and-release angling on the parental care behavior of male Smallmouth Bass. *Transactions of the American Fisheries Society,* 132(2), 210-218. https://doi.org/10.1577/1548-8659(2003)132<0210:TEOCAR>2.0.CO;2

Svendsen, E., M. Føre, F. Økland, A. Gräns, R. D. Hedger, J. A. Alfredsen, I. Uglem, C. M. Rosten, K. Frank, U. Erikson, and B. Finstad. 2021. Heart rate and swimming activity as stress indicator for Atlantic salmon (Salmo salar). *Aquaculture*, 531, 735804-. https://doi.org/10.1016/j.aquaculture.2020.735804

Takei, Y., J. Hiroi, H. Takahashi, and T. Sakamoto. 2014. Diverse mechanisms for body fluid regulation in teleost fishes. *American Physiological Society,* 307(7), R778-R792. https://doi.org/10.1152/ajpregu.00104.2014

Tartor H, L., A. Monjane, and S. Grove. 2020. Quantification of defensive proteins in skin mucus of *Atlantic salmon* using minimally invasive sampling and high-sensitivity ELISA. *Animals (Basel).* 0(8):1374. doi: 10.3390/ani10081374.

Therneau, T. M. (2020). *A Package for Survival Analysis in R*. https://CRAN.R-project.org/package=survival

Thorstad, E., A. Rikardsen, A. Ahmet and F. Økland. 2013. The Use of Electronic Tags in Fish Research–An Overview of Fish Telemetry Methods. *Turkish Journal of Fisheries and Aquatic Sciences.* 13. 881-896. 10.4194/1303-2712-v13_5_13.

Thorstensen, M. J., C. A. Vandervelde, W. S. Bugg, S. Michaleski, L. Vo, T. E. Mackey, M. J. Lawrence, and K. M. Jeffries. 2022. Non-lethal sampling supports integrative movement research in freshwater fish. *Frontiers in Genetics,* 13, 795355.

https://doi.org/10.3389/fgene.2022.795355

Tort, L. 2011. Stress and immune modulation in fish. *Developmental and Comparative Immunology,* 35(12), 1366-1375. https://doi.org/10.1016/j.dci.2011.07.002

Vallejos-Vidal, E., B. Sanz-Milián, M. Teles, S. Reyes-Cerpa, J. M. Mancera, L. Tort, and F. E. Reyes-López. 2022. The gene expression profile of the glucocorticoid receptor 1 (gr1) but not gr2 is modulated in mucosal tissues of gilthead sea bream (*Sparus aurata*) exposed to acute air-exposure stress. *Frontiers in Marine Science* 9, 977719-https://doi.org/10.3389/fmars.2022.977719

Van Der Salm, A. L., M. Martínez, G. Flik, and S. E. Wendelaar Bonga. 2004. Effects of husbandry conditions on the skin colour and stress response of red porgy, Pagrus pagrus. *Aquaculture,* 241(1), 371–386. https://doi.org/10.1016/j.aquaculture.2004.08.038

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* 3, https://doi.org/10.1186/gb-2002-3-7-research0034

Vitt, S., C. E. Bakowski, and T. Thünken. 2022. Sex-specific effects of inbreeding on body colouration and physiological colour change in the cichlid fish Pelvicachromis taeniatus. *BMC Ecology and Evolution*, 22(1), 124–. https://doi.org/10.1186/s12862-022-02074-x

Watson, J., K. Hyder, R. Boyd, R. Thorpe, M. S. Weltersbach, K. Ferter, S. J. Cooke, S. Roy, and R. M. Sibly. 2020. Assessing the sublethal impacts of anthropogenic stressors on fish: An energy-budget approach. *Fish and Fisheries* 21(5):1034–1045.

Welcomme, R. L., I. G. Cowx, D. Coates, C. Béné, S. Funge-Smith, A. Halls, and K. Lorenzen. 2010. Inland capture fisheries. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 365(1554):2881–2896.

Wilson, A. D. M., and J. G. J. Godin. 2009. Boldness and behavioral syndromes in the bluegill

sunfish, Lepomis macrochirus. *Behavioral Ecology*, 20(2), 231–237. https://doi.org/10.1093/beheco/arp018

Wilson, D. S., K. Coleman, A. B. Clark, and L. Biederman. 1993. Shy-bold continuum in pumpkinseed sunfish (Lepomis gibbosus): an ecological study of a psychological trait. *Journal of Comparative Psychology,* 107(3), 250–260. https://doi.org/10.1037/0735-7036.107.3.250

Yi, M., H. Lu, Y. Du, G. Sun, C. Shi, X. Li, H. Tian, and Y. Liu. 2021. The color change and stress response of Atlantic salmon (Salmo salar L.) infected with Aeromonas salmonicida. *Aquaculture Reports*, 20, 100664–. https://doi.org/10.1016/j.aqrep.2021.100664

Xu, D., and W. A. Rogers. 1991. Electron microscopy of infection by Saprolegnia spp. in channel catfish. *Journal of Aquatic Animal Health,* 3(1), 63-69, https://doi.org/10.1577/1548-8667(1991)003<0063:EMOIBS>2.3.CO;2

Zhao, F., P. Zhuang, T. Zhang, L. Zhang, J. Liu, and J. Hou. 2014. Non-lethal gill biopsy of juvenile hybrid sturgeon (Acipenser ruthenus ♀ × A. schrenckii ♂): Validity and impact on growth and osmoregulation. *Journal of Applied Ichthyology,* 30(6), 1243–1245. https://doi.org/10.1111/jai.12554

Zuckerman, Z. C., D. P. Philipp, and C. D. Suski. 2014. The influence of brood loss on nest abandonment decisions in Largemouth Bass Micropterus salmoides. *Journal of Fish Biology,* 84(6), 1863– 1875. https://doi.org/10.1111/jfb.12404

Appendix A

Qiagen RNA Extraction Mini-Kit Extraction Protocol (Modified from manufacturer's guidelines)

1. **Cells**: Harvest 3-4 gill filaments and blot any access RNAlater on a Kimwipe.

   Add 600uL of Buffer RLT and homogenize for 10 minutes. Add 1 volume (500ul) of 70% ethanol to the lysate, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 3.
2. Transfer up to 700 µl of the sample, including any precipitate, to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 1 minute at 13000x g. Discard the flow-through.
3. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 1 minute at 1300 x g. Discard the flow-through.
4. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 1 minute at ≥1300x g. Discard the flow-through.
5. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 min at ≥1300 x g. Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 2 min to dry the membrane.
6. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 50 µl RNase-free water (warmed to 60 °C) directly to the spin column membrane. Close the lid, and centrifuge for 2 min at ≥1300 x g to elute the RNA.