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Consequences of Environmental Manipulation on Behavioural and Neuromorphological Plasticity as It Relates to the Reintroduction of Atlantic Salmon (Salmo salar) to Lake Ontario

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Consequences of environmental manipulation on behavioural and neuromorphological plasticity as it relates to the reintroduction of Atlantic Salmon (*Salmo salar*) to Lake Ontario

By

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

The key ideas, primary contributions, experimental designs, data analysis, interpretation, and writing for each chapter were performed by the author under the supervision of Trevor Pitcher.

Chapter 1 incorporates unpublished material developed and drafted by the author, Ali Mokdad, under the supervision of Trevor Pitcher.

Chapter 2 of this thesis includes the outcome of a publication which has the following co-authors: The primary author, Ali Mokdad, honours thesis students Mohamed Elsheikh and Olivia Sulja, and Trevor Pitcher. Ali Mokdad developed the experimental design, collected, and analyzed the data and drafted the manuscript. Mohamed Elsheikh helped collect the behavioural data. Olivia Sulja helped collect neuromorphology data. Trevor Pitcher provided input for the experimental design, data analysis, and manuscript.

Chapter 3 of this thesis includes the outcome of a publication which has the following co-authors: The primary author, Ali Mokdad, Shawn Garner, Bryan Neff, and Trevor Pitcher. Ali Mokdad developed the experimental design, collected, and analyzed the data and drafted the manuscript. Shawn Garner helped rear and maintain the Atlantic Salmon prior to stream release; as well as provided

input for the experimental design, data analysis, and manuscript. Bryan Neff provided the facilities for rearing the fish prior to stream release; as well as provided input for the experimental design and manuscript. Trevor Pitcher provided input for the experimental design, data analysis, and manuscript.

Chapter 4 incorporates unpublished material developed and drafted by the author, Ali Mokdad, under the supervision of Trevor Pitcher. Ali Mokdad developed the experimental design, collected, and analyzed the data, and drafted the manuscript. Trevor Pitcher provided input for the experimental design, data analysis, and manuscript.

Chapter 5 of this thesis includes the outcome of a submitted publication which has the following co-authors: The primary author, Ali Mokdad, Jeremy Kraus (United States Geological Survey), Marc Chalupnicki (United States Geological Survey), James McKenna Jr. (United States Geological Survey), and Trevor Pitcher. Jeremy Kraus helped to rear and maintain the salmon, collected morphological data, performed the odorant exposures, and collected behavioural data; as well as provided input for the experimental design and manuscript. Marc Chalupnicki helped to rear the salmon, collected morphological data, and helped to collect behavioural data; as well as provided input for the manuscript. James McKenna Jr. provided the Atlantic Salmon and facilities where the experiment was conducted; as well as provided input for the experimental design and manuscript. Trevor Pitcher provided input for the experimental design, data analysis, and manuscript.

Chapter 6 incorporates unpublished material developed and drafted by the author, Ali Mokdad, under the supervision of Trevor Pitcher.

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This thesis includes three original papers that have been previously published/submitted to journals for publication, as follows:

Thesis Chapter	Publication title/full citation	Publication status

Chapter 2	Mokdad, A. I., M. Elsheikh, O. M. Sulja, and T. E. Pitcher. 2022. Neuromorphological and behavioural effects of early developmental exposure to alarm cue on captive-reared Atlantic Salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences:cjfas-2022-0100.	Published
Chapter 3	Mokdad, A. I., S. R. Garner, B. D. Neff, and T. E. Pitcher. 2022. Upstream and Downstream Dispersal Behavior of Hard- and Soft-Released Juvenile Atlantic Salmon. North American Journal of Fisheries Management 42(2):438–446.	Published
Chapter 5	Mokdad, A. I., J. M. Kraus, M.A. Chalupnicki, J.E. McKenna Jr., and T,E, Pitcher. Testing for behavioural evidence of olfactory imprinting at parr and smolt stage of Atlantic Salmon. North American Journal of Fisheries Management	Submitted

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ABSTRACT

The reintroduction of imperilled species has become an important tool in conservation biology and relies on the captive-rearing of remaining individuals, or a subset thereof, as a lifeline to prevent extinction. The success of reintroduction efforts has generally been low, mainly due to poor post-release performance of captive-reared animals. Captive-breeding programs tend to produce behaviourally and neurologically compromised animals that deviate from wild phenotypes and are less fit in natural settings. While genetic adaptation can account for some of the behavioural deficiencies expressed by captive-reared animals, phenotypic plasticity has been shown to play a large role. Phenotypic plasticity is generally defined as the ability of an individual to produce different phenotypes, or change the trajectory of phenotypic development, when exposed to different environmental conditions. This thesis proposes that by understanding the environmental factors and mechanisms that shape the phenotype, conservation biologists and managers alike can alter the rearing conditions and release protocols of captive-bred animals destined for stocking to increase the success of reintroduction efforts.

Throughout this thesis I examine the effects conditioning tactics – methods aimed at manipulating the rearing environment and release protocol to counteract the negative effects of captive-rearing on fitness-related behaviours in Atlantic Salmon (*Salmo salar*). Specifically, I investigated the effects of manipulating the early developmental rearing via enrichment (sensory enrichment via alarm cue exposure and physical enrichment via increased structure in the rearing

environment) on anti-predator related behaviours. Alarm cue exposure during early development had no significant effect on anti-predator related behaviour but fish exposed to alarm cue showed significant plastic changes to regional brain volumes (smaller olfactory bulbs), suggesting the potential for alarm cue to affect post-release behaviour. Physical enrichment during early development resulted in more wild-like behavioural phenotypes but did not ameliorate behavioural effects associated with stressful transport. I also investigated the effect of soft-release tactic – providing an in-stream acclimatization period prior to release– on the movement behaviour of fish released to the wild. Soft-released fish significantly differed from conventionally released (hard-release) fish in movement patterns, more closely matching wild-like movement pattern for this species. Finally, I investigated the effects of embryonic exposure to an artificial odorant (morpholine) and tested for behavioural evidence of imprinting at later developmental stages using two separate analytical approaches. Fish exposed to morpholine during the embryonic stage showed evidence for imprinting (a phenotypic plastic response) at the smolt stage but not the parr stage. This suggests that imprinting can be detected at a stage relevant for reintroduction efforts and provided support for the use of time-sensitive analyses when testing for behavioural evidence of imprinting.

This thesis provides supports for the use of conditioning tactics to manipulate phenotypically plastic responses to aid in the successful establishment of reintroduced animals to the wild. It also provides insight into the mechanisms involved in phenotypically plastic responses to changing environments.

DEDICATION

To my family –

We did it

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To Trevor: I can't thank you enough for your support, guidance, and all the hard work you've done to help me get here. From you I've learned to face challenges with optimism and how to better learn and grow from my experiences. You allowed me to explore creative ideas and dive deep into my curiosity. I have become an adaptive and rigorous scientist through your guidance. I appreciate our endless discussions – they mean a lot to me. You hold true to the idea of "reciprocal altruism" and that is really well reflected in the lab environment you've created. I am grateful for all the wonderful people I have had the pleasure to work with and do fun science with in your lab.

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CHAPTER 1
GENERAL INTRODUCTION

The world is currently facing a biodiversity crisis – extinction rates are climbing and projected to increase over the coming century (Ceballos et al. 2017). Anthropogenic stressors such as habitat degradation and over-exploitation are amongst the dominant drivers of global biodiversity loss (Jaureguiberry et al. 2022). Habitat restoration science has seen an increasing role in the fight against biodiversity loss (Lewis 2022). However, in the case that native species abundance levels decline to a point when populations can no longer sustain themselves, captive breeding becomes a necessary lifeline to prevent extinction (Fraser 2008). Captive breeding programs serve to shelter and maintain a subset of individuals from threats they are unable to survive in the wild, with the goal of reintroducing these individuals or subsequent progeny to parts of their historically occupied native range from which they were extirpated; a process known as 'reintroduction.' Reintroduction of captive-reared animals into the wild for conservation is a long-standing practice, dating back more than a century in the Western world (Kleiman 1989), but the success of these efforts is often poor or uncertain (Fischer and Lindenmayer 2000; Armstrong and Seddon 2008). Reintroduction success is often hindered by negative effects associated with a wide range of biotic and abiotic factors (reviewed in Cochran-Biederman et al. 2015) including: stress (Teixeira et al.2007), foraging issues (Reading et al. 2013), and interspecific competition (Houde et al. 2017). A recent review of reintroduction literature, however, has identified animal behaviour issues to be the most reported-upon problem contributing to the lack of success of reintroduction efforts (Berger-Tal et al. 2020). Captive-breeding programs tend to produce domesticated, behaviourally-

compromised animals that are less fit in natural settings, compared to their wild conspecifics due to differences in ecological conditions, such as predation (e.g., Fritts et al. 2007; Salvanes 2017; Solberg et al. 2020), disease and parasites (Cheng et al. 2015), and habitat complexity (Tetzlaff et al. 2018). The captive-rearing environment differs significantly from the natural environment that animals would be exposed to in the wild and differences in environment are likely to affect the development of particular phenotypes of individuals within each environment (Johnsson et al. 2014). An area where significant improvement for reintroduction of captively bred individuals can potentially be garnered is via a better understanding of the environmental factors in captive-rearing settings and release procedures, and the role that phenotypic plasticity plays in the development and manifestation of phenotypes.

1.1 The captive phenotype – the role of plasticity in reintroduction efforts (or biology)

Pigliucci et al. (2006) define phenotypic plasticity as the ability of individual genotypes to produce different phenotypes when exposed to different environmental conditions. In the most general sense, phenotypic plasticity can be separated into two basic categories, developmental plasticity – phenotypic change that is non-reversible and is a response to some environmental condition setting an individual down a particular phenotypic trajectory (Callahan et al. 1997) and phenotypic flexibility – reversible phenotypic change that can variably occur

within a single individual (Beever et al. 2017). While genetic adaptation can occur as quickly as over a single generation (Christie et al. 2012), growing evidence shows that captive animals differ from wild conspecifics in ways that are linked to plastic and flexible responses to the environment to which they are exposed in captivity (Adriaenssens and Johnsson 2013; Crates et al. 2022). The behavioural traits of captive individuals that may differ from those of wild conspecifics due to phenotypic plasticity are well-documented (reviewed in Crates et al. 2022). Generally, traits that are necessary but costly in the wild, such as anti-predator behaviours, are lost or wane if they provide no current utility within the environment to which they are exposed (e.g., anti-predator behaviour in the absence of predation pressure within a captive setting), through adaptive genetics or plastic responses (Pigliucci et al. 2006). Solberg et al. (2020) demonstrate, empirically, that for domesticated Atlantic Salmon (*Salmo salar*), directional selection for increased growth presents a trade-off with susceptibility to predation when exposed to a predator – domesticated Atlantic Salmon were less likely to survive exposure to predators (brown trout) in an artificial stream compared to wild conspecifics. Numerous studies of captive-reared animals (reviewed in Huntingford 2004) report reduced antipredator responses when exposed to predators. For example, the behavioural differences between hatchery-reared and wild salmon seem to be partially genetically based (Houde et al. 2010; Jackson and Brown 2011), however, anti-predator behaviour has been shown to be phenotypically flexible (Vilhunen 2006) and developmentally plastic (Poisson et al. 2017). Another well-documented behavioural category related to reintroduction

efforts is movement behaviour – particularly captivity induced changes in migratory patterns. For example, captive-bred monarch butterflies (*Danaus plexippus*) have been shown to lose their natural southward orientation and migratory behaviour altogether (Tenger-Trolander et al. 2019). With these differences in mind, there has been in recent years an increased effort to understand and guide the behavioural development of captive-reared individuals in an effort to produce more 'wild-like' released animals (Näslund 2021). In other words, there has been an increased effort to reduce the phenotype-environment mismatch experienced by captive-animals introduced into novel wild environments (Crates et al. 2022) (see Figure 1.1A).

1.2 Improving captive phenotypes – conditioning tactics

To improve the phenotypic development of captive-reared individuals, certain tactics have shown some promise - what Tetzlaff et al. (2019) refer to as conditioning – aimed at countering the negative effects associated with captive-rearing and reintroduction events (Figure 1.1B). Conditioning, both "animal-focused" (environmental enrichment) in the captive setting, as well as "environment-focused" (soft-release) in the field at reintroduction release sites, can offset some of these detrimental effects (Huntingford 2004; Jonsson and Jonsson 2014) with a goal to produce more wild-like behaviours (Hyvarinen and Rodewald 2013). Animal-focused environmental enrichment can be divided into different categories depending on the desired outcome of the enrichment (Teixeira et al.

2007; Naslund and Jonsson 2016). Among these categories are 1) physical enrichment, which includes the addition of structural complexity to the captive setting; 2) dietary enrichment, which refers to the type and/or delivery of food; and 3) sensory enrichment which concerns stimulation of sensory organs or the brain (including predator training). Studies of physical enrichment, in the form of added structural complexity, have produced a slew of mixed results, particularly in terms of ecologically relevant effects (reviewed by Johnsson et al. 2014). In white seabream (*Diplodus sargus*), physical enrichment (albeit with predator training) increased post-release survival and decreased dispersal rates from the point of release (D'Anna et al. 2012). Similarly, conditioned Atlantic Salmon showed higher survival rates in the wild (Hyvärinen and Rodewald 2013) and occupied more optimal habitat than unconditioned counterparts (Roberts et al. 2014). Specific to movement, increased shelter in the rearing tanks of burbot (*Lota lota*) resulted in reduced movement activity, but this study was conducted in a laboratory setting (Wocher et al. 2011). On the other hand, a number of studies fail to demonstrate any ecologically relevant effects of physical enrichment (see Berejikian et al. 1999; Tatara et al. 2008; Brockmark and Johnsson 2010; Näslund and Johnsson 2016). While it is unclear how enrichment helps post-release survival, it appears to produce more natural behaviours in semi-natural environments (Brown et al. 2003). Enrichment effects may be species-specific, and outcomes may depend on the life-history, and stage of the reintroduced animals in question (Naslund and Johnsson 2016). Additionally, the benefits of physical enrichment may be counteracted by other aspects of the captive rearing

environment (Johnsson et al. 2014). For example, food availability prior to release of Atlantic cod affected the activity level and risk-taking behaviour whether or not physical enrichment was provided (Moberg et al. 2011). Thus, it is important to consider other forms of enrichment in attempts to condition captively reared animals to produce ecologically relevant behaviour.

Environment-focused conditioning tactics are those that aim to condition animals in wild or semi-wild settings – the most common of these being "soft-release" (Tetzlaff et al. 2019). Soft-release generally refers to the practice of providing reintroduced animals with an acclimatization period, free of predators, at or near the release site prior to release (Brown and Day 2002). In their meta-analysis, Tetzlaff et al. (2019) found that soft-release had the largest effect on survival, movement, and site fidelity across taxa. Results from studies of the effects of soft-release tactics on survival and movement behaviour, however, remain inconsistent. This inconsistency may be due to species-specific effects or life-history dependent effects of soft-release. For example, no difference in dispersal rates was observed between two-year-old European grayling (*Thymallus thymallus*) smolt that were directly released into streams and those that were acclimatized in fenced-in pools at the release site prior to release (Thorfve 2002). On the other hand, acclimatized Brown trout (*Salmo trutta*) fingerlings showed a decrease in dispersal and a higher level of survival compared to directly released fish (Cresswell et al. 1983). Similar effects were observed for other salmonids in early life (pre-smolt) (Jonsson et al. 1999; but see Rosenberger et al. 2013). For natural subyearling anadromous (and potamodramous) fishes, time spent lingering

in riverine feeding sites is crucial for maintaining high growth rates and survival prior to smolting and migration downstream (Connor et al. 2003). If the goal of conditioning is to produce more wild-like behaviours that increase the chances of survival (Brown et al. 2003), then it would be beneficial to understand the effects of soft release on movement behaviour and activity levels for captive-bred salmonids.

Tetzlaff et al. (2019) consider predator training as a conditioning tactic separate from other forms of enrichment because it usually entails brief, infrequent conditioning sessions – this usually includes active attempts to condition specific behaviours by simulating predators or exposing animals to live predators or predator-related chemical cues. Here, however, I consider predator training to be included as a form of sensory enrichment. Nonetheless, anti-predator training has broadly positive effects on survival across taxa (taxa: actinopteryii, aves, mammalia, but not reptilia) and negative effects on movement behaviour (taxa: aves, mammalia, reptilia, but not actinopterygii) (Tetzlaff et al. 2019). As it relates to specifically to fishes, predator training has produced mixed results in terms of post-release success (Brown et al. 2013a). For example, Chinook salmon (*Oncorhynchus tshawyscha*) conditioned to recognize predator cues demonstrated increased anti-predator behaviour in a laboratory setting but this did not translate into enhanced post-stocking survival (Berejikian et al. 1999). Conversely, D'Anna et al. 2012, found that a combination of both physical enrichment and anti-predator training led to an increase in post-release survival and a decrease in movement behaviour of seabream (*Diplodus sargus*). Similarly, brook trout (*Salvelinus*

fontinalis) trained to recognize predators in a laboratory setting gained a survival benefit both in laboratory and field settings (Mirza and Chivers 2000). Although movement behaviour patterns are closely linked to avoidance of predators in the wild (Reading et al. 2013), few predator-training studies with fish as study subjects report on movement behaviour as a measure of post-release success (Tetzlaff et al. 2019). One study, however, does explicitly link predator training to increased exploratory behaviour and activity level in a laboratory setting (Panamanian bishop fish; Archard and Braithwaite 2011). Non-fish studies have generally demonstrated an effect of predator training on movement behaviour. For example, female captive-reared Greater Rheas (*Rhea americana*) trained to recognize a predator (puma) exhibited smaller home ranges and occupied more favourable habitat than non-trained conspecifics (the same was not true for males) (Cortez et al. 2018). It seems, then, important to consider not only species-specific responses to conditioning, but also the effects of multiple conditioning tactics on the movement behaviour of the species of interest (Naslund and Johnsson 2016).

In terms of aquatic species studies and reintroduction practices, alarm cue exposure has become a potentially useful tool for predator-training (Mirza and Chivers 2003; Kopack et al. 2016; Poisson et al. 2017). The presence of conspecific alarm cues, alarm chemicals released by mechanical damage to the skin of many aquatic taxa (reviewed in Chivers and Smith 1998), elicits innate antipredator responses (alarm reaction) in the absence of live predators (Brown and Smith 1997; Kopack et al. 2015; Brown et al. 2016) and strengthens the response and increases survival rates of hatchery-reared fishes under direct threat from live

predators (Berejikian et al. 1999; Gazdewich and Chivers 2002; Mirza and Chivers 2003). The response to alarm cues, however, is significantly reduced in conventionally reared hatchery fish compared to wild conspecifics (Jackson and Brown 2011).

Two general overlapping approaches to improving the alarm-related behaviour of captive-reared aquatic animals that have been recently tested are: (1) predator training i.e., pairing the alarm cue with a predator cue to allow hatchery fish to learn to associate the alarm reaction with a particular predator cue (e.g. Brown and Laland, 2001; Mirza and Chivers 2000; Vilhunen 2006) and (2) exposure of individuals to background predation risk via alarm cue during early development, which can increase survival, the baseline alarm reaction, and strength of anti-predator behaviour (e.g. Ferrari et al. 2015; Joyce et al. 2016; Brown et al. 2016). Animals exposed to an increase in perceived predation risk (via alarm cues) during early development may develop distinct anti-predator behavioural phenotypes in as little as 4 days (Ferrari 2014; Ferrari et al. 2015). For example, juvenile convict cichlids (*Amatitlania nigrofasciata*) exposed to alarm cues for 5 days exhibited higher levels of antipredator behaviours when exposed to either a predator model or a novel predator smell (Brown et al. 2016). Still, the benefit of these methods (predator training and exposure to predation risk) for post-stocking success have not proven particularly successful (Brown et al. 2013b). There are two main problems with these approaches; first, in regards to predator training, fish do not apparently retain predator training information long enough to gain benefits useful to post-stocking conditions (Brown et al. 2013a)

and second, ontogenetic stage exposure may affect the expression of anti-predator behaviour and reactivity to alarm cue signals (Brown et al. 2013b).

Taken together, a better understanding of conditioning, both animal-focused and environment focused, may help to better reduce the negative effects of captive-rearing and release, and has the potential to produce more wild-like behaviour for captive animals destined for reintroduction.

1.3 Imprinting in captivity

The olfactory imprinting hypothesis (Hassler and Wisby 1951) proposes that juvenile migratory animals imprint to unique chemical signatures during sensitive developmental periods and use the imprinted odour memories to return to sites with the chemical signatures that they were imprinted to. A major concern for conservation and recovery programs that reintroduce captive-bred migratory species (anadromous and potamodromous) is the risk of straying (Brenner et al. 2012) and the capacity to influence the location of return of individuals that home (otherwise known as targeted return sites) (Dittman et al. 2015). Straying from intended spawning sites, particularly for salmon, can have negative effects on wild salmon populations and can be an impediment to the successful establishment of a self-sustaining population in the wild (Brenner et al. 2012). Though some low level of straying is normal in both hatchery and wild populations, hatchery

program guidelines have been proposed to curtail levels of straying to below 10% (Paquet et al. 2011).

The majority of studies exploring the timing of imprinting in salmonids identify the parr-smolt transformation stage as a critical period for successful olfactory imprinting (reviewed in Bett and Hinch 2016). These studies have focused on species, such as coho salmon (*O. kisutch*) and Atlantic Salmon (*S. salar*), that rear for one or more years in natal stream systems before beginning the parr-smolt transformation (McCormick et al. 1996). However, a newer embryonic imprinting paradigm (Dittman et al. 2015) suggests a sensitive window for imprinting for salmon as early as during hatching, for species with life histories that require earlier imprinting (Tilson et al. 1994; Bett et al. 2016; Havey et al. 2017). For example, Pink salmon (*O. gorbuscha*) and Sockeye salmon (*O. nerka*) leave their natal streams and swim towards the ocean, or to a lake, respectively, shortly after emerging from the redd. For these species, imprinting presumably occurs before the fish leave their natal streams. Indeed, sockeye salmon (Tilson et al. 1994; Havey et al. 2017) and Pink salmon (Bett et al. 2016) hatchlings exposed to artificial odourants showed an attraction to the imprinting odour as adults, lending support to the embryonic imprinting paradigm. The success of Atlantic Salmon reintroduction into Lake Ontario will rely, in part, on the homing fidelity of released fish. The OMNRF (Ontario Ministry of Natural Resources and Forestry) has, in recent years, emphasized the stocking of parr stage fish in an effort to reduce the time these fish spend in hatchery settings as a best practice

(Johnsson et al. 2014). As such, it is important to determine whether the embryonic imprinting paradigm applies to Atlantic Salmon imprinting.

The specific chemical cues that are being imprinted upon by young salmon is an active area of research, and is critical for reducing straying in salmonids that are reintroduced for conservation. Evidence suggests that the primary chemical signal used by homing salmon in the wild is the unique and natural amino acid signature present in each stream (Ueda 2012). Early artificial imprinting studies, using odorants not normally found in natural water or municipal water systems (e.g., morpholine), demonstrate that these artificial odorants can be used to lure imprinted salmon to unfamiliar streams scented with these artificial odorants (reviewed in Hasler and Scholz 1983). Morpholine and PEA (phenylethyl alcohol) have since become the most common artificial odorants for imprinting partly because they serve as effective, safe, and economically feasible options for imprinting hatchery reared salmon (Dittman et al. 2015). While electrophysiological responses (using an electro-olfactogram), and more recently, genetic and molecular responses to imprinted odourants are typically used to detect evidence of imprinting (Yamamoto et al. 2010). Bett and Hinch (2016) suggest that these approaches should be paired or followed up with behavioural assays because behavioural responses are more ecologically valid and can be much more sensitive to detecting evidence of imprinting cues.

1.4 Study species – Atlantic Salmon (*Salmo salar*)

Atlantic Salmon (*Salmo salar*) were once an abundant top predator in Lake Ontario and represented one of its most valuable fisheries, but were extirpated from the lake a century ago (COSEWIC, 2016). The successful reintroduction of Atlantic Salmon into Lake Ontario is currently a top priority for management agencies (e.g., OMNRF: Ontario Ministry of Natural Resources & Forestry) and conservation groups (e.g., OFAH: Ontario Federation of Anglers & Hunters). A self-sustaining population of Atlantic Salmon may also provide ecosystem services through their role as a top predator, increasing the resiliency of food webs and stabilizing fishery yields (Myers and Worm 2003). Atlantic Salmon in Lake Ontario represent a unique opportunity to evaluate multiple factors that affect reintroduction outcome (e.g., genetics, rearing, environment), which will contribute to the development of a comprehensive reintroduction framework that will aid other restoration programs around the globe. Previously, it was found that high mortality and poor growth during the in-stream life stage is a major barrier to Atlantic Salmon reintroduction for Lake Ontario (e.g., Houde et al. 2016), although the causes have only been partially resolved. Efforts to reintroduce captive-bred Atlantic Salmon into Lake Ontario have been largely unsuccessful (Ontario Ministry of Natural Resources and Forestry 2020). More recently, since the 1990s, the OMNRF has been reintroducing Atlantic Salmon to Lake Ontario with an emphasis on stocking parr stage (<1 year old) fish to reduce the amount of time these fish spend in the hatchery setting (Ontario Ministry of Natural Resources and Forestry 2020). Still, fishes stocked for reintroduction have

relatively low survival rates during early periods post-release, presumably in part due to maladaptive behavioural responses to threat of predation (Brown and Laland 2001; Solberg et al. 2020). Resolving the causes of poor in-stream performance and evaluating potential mitigation strategies through a better understanding and modifications to the hatchery rearing environment and release protocol are essential steps to successfully reintroducing Atlantic Salmon into Lake Ontario.

1.5 Thesis overview

This dissertation focuses on the captive-breeding and release of Lake Ontario Atlantic Salmon from the perspective of exploring and understanding the role that behavioural plasticity plays in generating phenotypes conducive to the successful reintroduction of these fish to their historic range. The central question to my thesis is: how does the rearing environment and release protocol of hatchery-reared Atlantic Salmon affect behaviour relevant to the reintroduction of this species? Throughout the thesis, I test the working hypothesis that manipulating the rearing environment and/or release protocol can alter the behaviour of captive-bred Atlantic Salmon via phenotypically plastic responses to produce behaviours that are potentially beneficial in the wild environment (divided among four data chapters). In Chapter 2, I explore the behavioural and neuromorphological effects of early developmental exposure to alarm cue on captive-reared Atlantic Salmon. I investigate whether embryonic exposure to alarm cue leads to observable plastic

changes to anti-predator behaviour and evidence for plastic responses of gross brain morphology. In Chapter 3 I investigate the post-release movement behaviour of juvenile Atlantic Salmon released to a tributary of Lake Ontario. I compare the upstream and downstream movement patterns of conventionally released fish to soft-released using passive integrated transponder systems. In Chapter 4 I reared fish with structural enrichment in the attempt to condition a more wild-like antipredator behavioural response to a novel environment. I also document the effects of transport stress on antipredator-related behaviour and examine the role that enrichment and recovery from transport have on the behavioural stress response of juvenile Atlantic Salmon. Finally, in Chapter 5 I expose embryonic stage Atlantic Salmon to a synthetic odorant (morpholine) to investigate the developmentally plastic response to natal water cues. I test for evidence of imprinting (a developmentally plastic response) at the parr and smolt stage of fish that were exposed to the odorant during the embryo stage.

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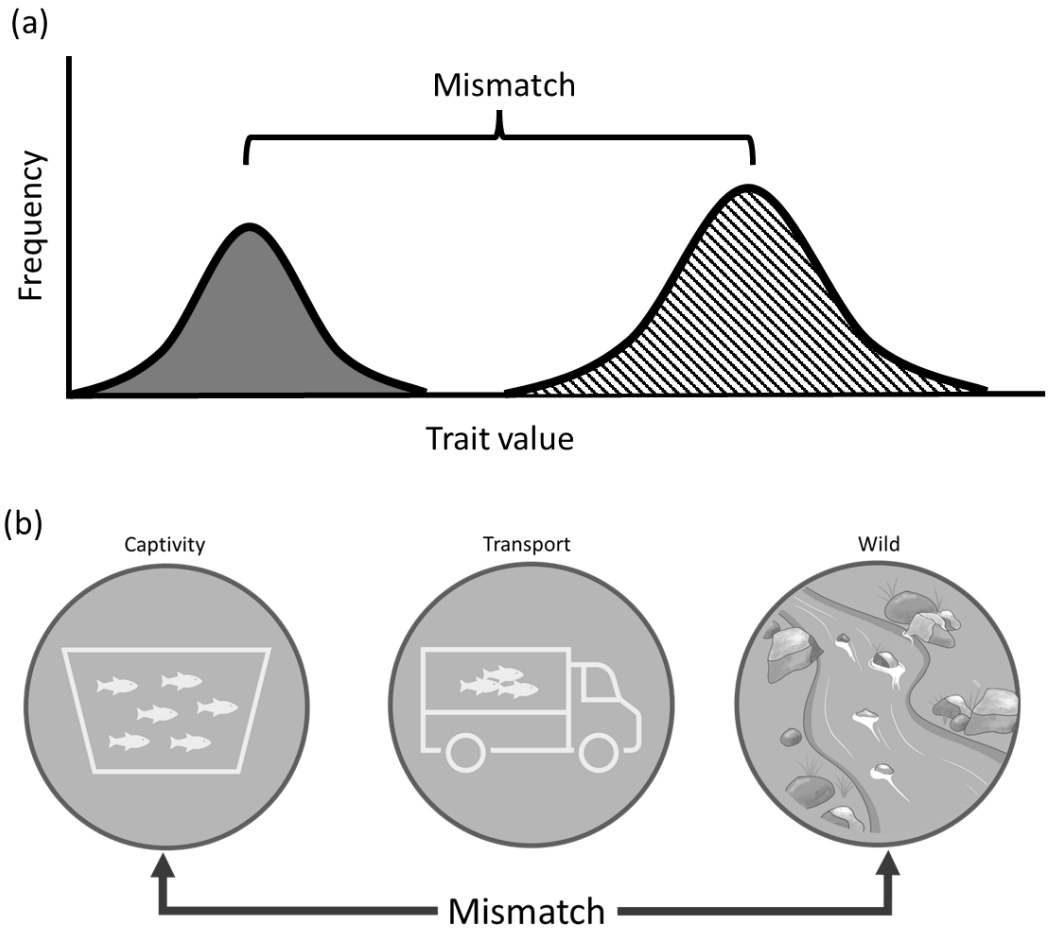

Figure 1.1 Schematic representation of the phenotype-environment mismatch (adapted from Carroll et al. 2014). Panel (a) represents a hypothetical mismatch between the current phenotype (dark gray distribution) and the phenotype best suited for a particular environment (line-patterned distribution). The mismatch is represented by the non-overlapping space between the two distributions. Panel (b) represents the three stages of reintroduction: captive breeding (captivity), transport, and the wild environment. Tactics aimed at reducing the mismatch include enriching the captive environment (animal-focused) or manipulating the transport and release protocol (environment-focused).

CHAPTER 2

NEUROMORPHOLOGICAL AND BEHAVIOURAL EFFECTS OF EARLY DEVELOPMENTAL EXPOSURE TO ALARM CUE ON CAPTIVE-REARED ATLANTIC SALMON (*SALMO SALAR*)

Mokdad, A. I., M. Elsheikh, O. M. Sulja, and T. E. Pitcher. 2022. Neuromorphological and behavioural effects of early developmental exposure to alarm cue on captive-reared Atlantic Salmon (*Salmo salar*). Canadian Journal of Fisheries and Aquatic Sciences:cjfas-2022-0100.

2.1 Introduction

The capacity of an organism to alter its behaviour in response to environmental conditions is referred to as behavioural plasticity (Stamps 2016). Behavioural plasticity can be beneficial to organisms that are reared in captivity because it allows those organisms to adjust to novel features of the captive-setting (reviewed in Johnsson et al. 2014). However, captive breeding conservation programs tend to produce domesticated, behaviourally compromised animals that are less fit, in natural settings, compared to their wild conspecifics due, in part, to differences in ecological conditions, such as an absence of predation and predator cues (e.g., Fritts et al. 2007; Salvanes 2017; Solberg et al. 2020). Traits that are necessary for survival and reproduction but costly in the wild, such as anti-predator behaviours, are lost or wane if they provide no current utility within the environment to which they are exposed, often via plastic responses (Pigliucci et al. 2006; Johnsson et al. 2014). This is especially problematic for reintroduction efforts, particularly those efforts using hatchery-reared fishes, because predation is a leading cause of reintroduction failure (Tetzlaff et al. 2019). For example, in domesticated Atlantic Salmon (*Salmo salar*), directional selection for increased growth rate in hatcheries presents a trade-off with susceptibility to predation (Solberg et al. 2020). In other words, while Atlantic Salmon grow at a faster rate in hatcheries, they exhibit lower survival rates when exposed to live predators (brown trout, *Salmo trutta*) in an artificial stream compared to wild conspecifics (Solberg et al. 2020). The authors suggest that domestication is accompanied by a reduction in predator recognition and anti-predator related behaviour, leading to increased

predation susceptibility. Numerous studies of hatchery-reared salmonids (reviewed in Huntingford 2004) and particularly in Atlantic Salmon (e.g., Houde et al. 2010), echo these results and report reduced antipredator responses when exposed to predators.

Efforts to study and improve antipredator behaviour in hatchery settings often utilize alarm cues as signals of predation risk in lieu of live predators (reviewed in Jackson & Brown 2011).The presence of conspecific alarm cues, alarm chemicals released by mechanical damage to the skin of many aquatic taxa (reviewed in Chivers & Smith 1998), elicits innate antipredator behaviour (alarm reaction) in the absence of live predators (Brown et al. 2016; Brown & Smith 1997; Kopack et al. 2015) and strengthens the response and increases survival rates of hatchery-reared fishes under direct threat from live predators (Berejikian et al. 1999; Gazdewich & Chivers 2002; Mirza & Chivers 2003). The alarm response is generally characterized by decreased activity (i.e., decreased movement and increased shelter use) (Chivers & Smith 1998). The response to alarm cue (alarm response), however, is significantly reduced in conventionally reared hatchery fish compared to wild conspecifics (Jackson & Brown 2011). Behavioural differences between hatchery-reared and wild salmon seem to be partially genetically based (Houde et al. 2010; Jackson & Brown 2011), however, anti-predator behaviour has been shown to be behaviourally plastic (Vilhunen 2006; Poisson et al. 2017).

One approach to improving the alarm-related behaviour of hatchery-reared fish that has gained recent attention is to increase hatchery background predation risk via alarm cue exposure during early development (Tetzlaff et al. 2019). This

approach has been shown to increase survival during manipulated predator interactions and strengthen baseline alarm reaction and anti-predator behaviour (e.g., Brown et al. 2016; Ferrari et al. 2015; Joyce et al. 2016). Animals exposed to cues that simulate an increase in perceived predation risk (via alarm cue exposure) may develop distinct anti-predator behavioural phenotypes in as little as four days (Ferrari 2014; Ferrari et al. 2015). For example, juvenile convict cichlids (*Amatitlania nigrofasciata*) exposed to alarm cues for 5 days exhibited higher levels of antipredator behaviours when exposed to either a predator model or a novel predator smell (Brown et al. 2016). Furthermore, Poisson et al. (2017) provide evidence that embryonic exposure to alarm cue generates a plastic response in anti-predator related behaviour in rainbow trout (*Onchorhynchus mykiss*). It is, however, unclear if the plastic predator-related response is retained to an ecologically relevant stage, and whether the exposed fish exhibit a difference in sensitivity to the alarm cue itself. The retention of learned predator-related cues varies widely and can be diminished through a process of adaptive forgetting (Brown et al. 2013; Ferrari et al. 2010). It is thus important to investigate whether alarm cue exposure can produce long-term plastic changes in the developmental trajectory of captive-reared animals – referred to as 'developmental plasticity'. A single study, to our knowledge, has demonstrated a developmentally plastic response to alarm cue exposure (Poisson et al. 2017). Embryonic rainbow trout exposed to alarm cue during a sensitive period of neural development (during the alevin stage), develop differential behavioural phenotypes as fry. Rainbow trout fry in that study displayed developmentally plastic variation in several behavioural

measures linked to anti-predatory behaviour and cognitive ability. However, behavioural measures in that study were observed between five and ninety days after exposure to alarm cue, raising the question of whether these responses correspond to short-term, reversible changes (referred to as 'flexibility', Stamps 2016) or developmental plasticity.

Behavioural plasticity is influenced, in no small part, by variation in neural investment (reviewed in Ebbesson & Braithwaite 2012). Teleost fish exhibit a high degree of neurogenesis and cell proliferation occurring continuously throughout life (Zupanc 2008) which sets the stage for high levels of plasticity in brain morphology (Eifert et al. 2015). Predation is a key factor in shaping the brain through evolution via natural selection (Walsh et al. 2016; Samuk et al. 2018), but given the close link between brain and behaviour, the role of plasticity on brain morphology is gaining attention (Gonda et al. 2013; Reddon et al. 2018). Laboratory studies manipulating perceived predation risk demonstrate variation in overall brain size and investment to specific region size that correspond to anti-predatory related behaviour (Gonda et al. 2012; Reddon et al. 2018; Joyce and Brown 2020). For example, in a study on nine-spined stickleback (*Pungitius pungitius*), Gonda et al. (2012) found that the presence of predators in housing tanks affected the volume of the olfactory bulb (OB), as well as the hypothalamus – a significant increase in the relative (to body size) OB size and a decrease in relative hypothalamus size when exposed to increased predation risk. In a semi-natural experiment, Atlantic Salmon and redbelly dace (*Phoxinus eos*), exposed to alarm cue for a two-week period, showed changes in brain structure (Joyce and

Brown 2020). Atlantic Salmon developed an overall different brain shape, including a smaller optic tectum compared to non-exposed conspecifics and northern redbelly dace (*Phoxinus eos*) developed larger brains, accounted for by a larger olfactory bulbs and optic tecta.

The extremely plastic nature of the fish brain and behaviour (Gonda et al. 2013; Joyce & Brown 2020) and the ability for fish olfactory systems to function at early embryonic stages (Dittman et al. 2015; Hara & Zielinski 1989) allow for the testing of hypotheses pertaining to developmental plasticity and perceived risk of predation. In this study, we test the hypothesis that early developmental exposure to alarm cue (i.e. increased background predator risk perception) leads to a developmentally plastic response in anti-predator behaviour in yearling Atlantic Salmon. First, we test the assumption that there is an innate behavioural response to alarm cue exposure then we test two predictions that follow from the hypothesis – (1) if early developmental exposure to alarm cue (pre-exposure) influences antipredator behaviour in a developmentally plastic manner then antipredator-related behaviours should differ between developmentally exposed fish (henceforth referred to as 'pre-exposed') and non-exposed fish (referred to as 'non-exposed') at a later developmental stage, and (2) pre-exposure, should affect the behavioural response to acute exposure to alarm cue (a change in environment) as yearling. Behaviour was measured at the yearling stage to provide an ecologically relevant assessment of anti-predator behaviour given that the fingerling/yearling stages are the predominant life-stages that Atlantic Salmon are reintroduced by hatcheries (e.g., Ontario Ministry of Natural Resources and

Forestry 2020). Finally, to better understand the link between perceived predation risk and neural investment, we examine the relative size of the whole brain and five brain regions related to anti-predator behaviour and survival in the wild (olfactory bulb, telencephalon, optic tectum, hypothalamus, and cerebellum) (reviewed in Ebbesson & Braithwaite 2012) between pre-exposed and non-exposed fish (at the fry stage). Relative investment in brain development was measured at the fry stage to increase our chance of detecting effects of pre-exposure given that environmental effects on brain morphology are known to disappear over time and after transfer to environments lacking a given stimulus (Näslund et al. 2012).

2.2 Materials and methods

Alarm cue Preparation

The alarm cue was extracted from 2-year-old Atlantic Salmon, reared at the Freshwater Restoration Ecology Centre (FREC), located in LaSalle, Ontario, Canada. The alarm cue extraction followed the protocol of a previous study (Brown & Smith 1997). Briefly, fish for skin extraction were administered a lethal dose of anaesthetic (buffered tricaine mesylate (MS222)), skin was removed, subsequently homogenized using a mortar and pestle, and filtered through a cotton filter. Dechlorinated water was added to the homogenate to produce a final skin homogenate (i.e. alarm cue) stock concentration of 487.5cm^2/L . The alarm cue

was divided into 20mL aliquots and then frozen. Dechlorinated water was also frozen in 20mL aliquots and eventually (see below) served as the control for the untreated group.

Experimental crosses

Atlantic Salmon gametes (eggs and sperm) were collected from hatchery-reared fish, of the Sebago strain, maintained at the Ontario Ministry of Natural Resources and Forestry (OMNRF) Harwood Fish Culture Station since 2006. This strain is being used for reintroduction efforts to Lake Ontario (Ontario Ministry of Natural Resources and Forestry 2020). Original lines were collected in 2006 from Sebago Lake, Maine and reared at Harwood Fish Culture station. Gametes were collected from fish who had been reared in captivity for two generations (parents collected from the wild but raised exclusively in captivity). Eggs from a haphazardly chosen female were fertilized using one haphazardly chosen male. Batches of fertilized eggs were divided in half to produce duplicate family replicates and each replicate was reared in a separate recirculating vertical incubator that used dechlorinated municipal water, that was aerated and maintained at 7-8°C throughout the experiment. Alarm cue exposure began 51 days post-fertilization (dpf), during early post-hatch (when ~50% hatch was occurring for each of the family replicates) and continued until the fry stage, 103 dpf (when ~50% of the fish had absorbed the yolk-sac). The timing of alarm cue administration followed Poisson et al. (2017) to induce developmentally plastic

responses and supported by evidence suggesting that the olfactory system is functional immediately after hatch (Hara and Zeilinski 1989). The alarm cue was administered to the 'pre-exposed' incubation stack by adding a single frozen 20ml aliquot to the recirculation reservoir once every 3 days until the end of the administration period – a total of 16 alarm cue administrations took place during this period. The final concentration of skin (alarm cue) that pre-exposed fish were exposed to, per administration, was $0.032cm^2/L$. This concentration is in line with developmental exposures necessary to increase perceived predation risk and elicit a response in salmonids (Brown et al. 2011; Mirza & Chivers 2003). Non-exposed fish received 20mL administrations of frozen dechlorinated water only, with the same method and timeframe as the pre-exposed group. Non-exposed fish, therefore, experience a relatively lower level of perceived predation risk compared to the pre-exposed fish. For post-hatch rearing, replicate groups of each family were transferred to and housed separately in 35 L tanks connected to a recirculating system using dechlorinated municipal water (absent of alarm cue) that was aerated, filtered and kept between 10-16°C, to mimic wild river temperatures.

Neuromorphology

In January of 2018, once fish had absorbed their yolk sacs (~103 dpf) and before transfer into separate rearing tanks (see above), 60 fish (n = 30 pre-exposed and n = 30 non-exposed, see above), intended for our brain measure study, were

euthanized in 100mg/L of MS-222 (tricaine methanesulfonate), and subsequently weighed (to 0.01g). Following body mass measurements, fish heads were removed and drop-fixed in 4% neural buffered paraformaldehyde for 24 hours. Heads were then transferred into vials containing phosphate buffer solution and refrigerated for later analysis. Brains of the fish were excised from the skull, and the optic nerves and brain stem were severed at a standard position at the brain stem (at the entrance of the vertebral column) (Fraser et al. 2012). Once removed, the whole brain was weighed (+- 0.001 g) and placed on a wax dissection tray such that all hemispheres of the brain were proportionate (Fraser et al. 2012). Photographs of the dorsal, ventral, and lateral views of the brain were taken using a digital microscope (following Pollen et al. 2007). For the left and right lateral photographs, the brain was sectioned along the midsagittal plane before positioning on the dissection tray. Samples were hydrated with PBS every minute and immediately before photographs were taken. Samples that were damaged during dissection were noted and photographed but excluded from the photographic analysis, resulting in a total of 56 brains analyzed (28 pre-exposed, 28 non-exposed). All individuals were identified by a haphazard identification number, and thus dissections and image analyses were performed blindly. The methods of Pollen et al. (2007) and Gonda et al. (2013) were used to determine the volume of the various brain regions. Briefly, the photographs taken were imported into ImageJ, and the length, width, and height of the olfactory bulb, telencephalon, optic tectum, cerebellum, and hypothalamus were measured, as well as the total length, width, and height of each brain. The width of a structure was defined as the

greatest distance enclosed by the structure perpendicular to the midline of the brain. The widths of the olfactory bulb, telencephalon, optic tectum, and cerebellum were taken from dorsal images, and the width of the hypothalamus was taken from the ventral image. In accordance with Gonda et al. (2012), for paired structures (olfactory bulb, telencephalon, optic tectum, and hypothalamus), the width of the two sides were measured together. The length of all regions were taken from lateral images, with the exception of the hypothalamus. The length of the hypothalamus was taken from the ventral image due to difficulties viewing the horizontal boundaries of this structure in the lateral image. The length of the olfactory bulb, telencephalon, cerebellum, and hypothalamus was defined as the greatest distance enclosed by the given structure parallel to the estimated projection of the brain. For the optic tectum, the length was defined as the greatest distanced enclosed by this structure (Gonda et al. 2012). The heights of all regions were taken from lateral images. The height of a structure was defined as the greatest distance enclosed by the structure perpendicular to the estimated projection of the brain, with the exception of the optic tectum. The height of the optic tectum was defined as the greatest distance enclosed by this structure perpendicular to the length measurement (Gonda et al. 2012). Finally, the volume (V) of each brain structure was calculated according to the ellipsoid mode: $V = (L \times W \times H) \times \pi/6$ (van Staaden et al. 1995). Total brain volume was determined by adding the volume of the five major subregions (Fong et al. 2019). To assess the potential for observer bias, a subsample (n = 20) of brains were measured and coded (for all five brain regions mentioned above) blindly by two separate

individuals. A two-way mixed model intraclass correlation found high reliability between the two observers for total brain volume measures (ICC = 0.95, $p < 0.001$)

Behavioural trials

A total of 120 individuals (n=60 pre-exposed and n = 60 non-exposed) were used for the behavioural experiment. Behavioural trials were conducted on yearling Atlantic Salmon when parr marks were visible, between 31st December, 2018 and 14th January 2019 (approximately 1 year after pre-exposure, see above). The mean mass of pre-exposed fish was (4.65g ± 2.19) and the mean mass of non-exposed fish was (4.50g ± 2.63). Behavioural trials were conducted in a test-tank which was a 43 L plastic bins (30cm x 40cm x 60cm) filled with approximately 20L of water from the home tanks (approximately 20cm depth), with a single layer of gravel on the bottom of the bin, and a 10cm-long PVC tube (used as shelter) with an internal diameter of 1.27cm (referred to henceforth as test-tank; see Figure 2.1). Each test-tank was fitted with an external pump to create a recirculating low flow (~0.3m/s at the inflow and 0.05m/s at the outflow) into the testing area – this would allow for circulation of the alarm cue within the test-tank. Dye-tests were conducted between trials to ensure that the alarm cue would distribute throughout the entire testing arena. Fish were not fed for 24 hours prior to the start of the experiment as part of an unrelated experiment and to control for variability in behaviour related to hunger (Näslund et al. 2017). A previous experiment found that food deprivation prior to behavioural trials did not affect the alarm response in

Atlantic Salmon (Lau et al. 2021). At the beginning of each trial, two fish were placed into a test-tank (to provide social ecological context) – resulting in a total of 60 trials with 120 unique individuals – and left to acclimate for 30 minutes. The behaviour of the fish was recorded for the final 5 minutes of acclimation to establish a baseline rate for the focal behaviours (referred to as baseline measures). Following the baseline recordings, 2 mL of distilled water was injected into each tank immediately followed by either another 2 mL of distilled water or 2 mL of alarm cue using 2mL syringes fixed to the side of the test-tank above the water level (depending on the treatment, see below; referred to as post-stimulus measures). Juveniles in the post-stimulus trials were either exposed to 0.032cm^2/L alarm cue (matching the pre-exposure concentration) or distilled water (control) resulting in four treatment groups as follows: (1) pre-exposed fish acutely exposed to alarm cue, (2) pre-exposed fish that received distilled water, (3) non-exposed fish acutely exposed to alarm cue, and (4) non-exposed fish that received distilled water during behavioural trials. Pilot tests using this concentration of alarm cue showed it to be sufficient to elicit behavioural responses in fry compared to control water. Fish movements and interaction were video recorded for 5 minutes after the addition of the alarm cue or the control water (post-stimulus).

Each five-minute test period (baseline or post-stimulus) was scored for behavioural measures (using Solomon Coder (https://solomon.andraspeter.com), by an experimenter blind to the specific treatment) including; total time spent motionless (secs), total time spent associated with the shelter (secs), and number of aggressive acts – these behavioural metrics have been correlated with antipredator

responses in other studies using juvenile Atlantic Salmon (de Mestral & Herbinger 2013; Jackson & Brown 2011). Total time spent motionless is defined here as the total time the salmon spent not moving wherein movement is defined as a change of location by at least half a body length (Jackson & Brown 2011). A fish was considered to be associated with the shelter when the head was one body length or less away from the PVC shelter (Clark & Moore 2018; Salvanes 2017). An individual was associated with the shelter when the head of the fish was within one body length of the shelter. Aggressive acts were calculated as number of biting motions or rapid approaches towards the other fish. Four of the 60 behavioural video recordings were corrupted and so ultimately 56 trials (n = 112 fish) were analyzed (n = 26 non-exposed/control; n = 30 non-exposed/alarm stimulus; n = 28 pre-exposed/control; n = 28 pre-exposed/alarm stimulus).

Statistical Analyses

Neuromorphology

We used general linear models to investigate the effect of pre-exposure on the size of the brain and five brain regions. All measures used in these analyses were $\log_{10}$ transformed prior to analyses (Kotrschal et al. 2012). To investigate the role of pre-exposure on total brain volume, we fitted a linear model with total brain volume as the response variable, treatment (pre-exposed, non-exposed) as fixed factor, and body mass (excluding the mass of the brain) as a covariate (Kotrschal et

al. 2017). Preliminary analysis included an interaction term between treatment and body mass, but the interaction term was not statistically significant and so was removed from subsequent analyses to preserve degrees of freedom (Beck & Bliwise 2014). To investigate the role of pre-exposure on the relative brain region volumes we used separate linear models for each brain region with the volume of the brain region of interest as a response variable, treatment (pre-exposed and non-exposed groups) as a fixed factor, and the total brain volume, excluding the volume of the brain region of interest (referred to as 'rest of brain'), as a covariate (Fong et al. 2019). Similar to the previous analysis, an interaction term between the covariate and the fixed factor was tested and subsequently removed from analysis. The assumption of homoscedasticity was analysed using Levene's test, and differences were found to be nonsignificant ($p > 0.05$) and normality of the data was tested with Shapiro-Wilk test, and no significant deviations from normality were detected.

Behaviour

The three behaviours measured were indexed as combined z-scores to increase sensitivity for analysis and producing a variable of measure representing overall activity score (Labots et al. 2018). We followed the methods used by a previous study measuring Atlantic Salmon anti-predator behaviour in the presence of alarm cue administration (Lau et al. 2021). Briefly, a Z-score was calculated for each behavioural measure for each observation period, the measures were then

combined into an index of activity scores as follows: Z-score (log [number of aggressive acts + 1]) – Z-score (time spent motionless) – Z-score (time spent sheltering). The minimum Z-score was subtracted from each Z-score to produce positive Z-scores across measures. General linear mixed models were used to analyze activity scores for baseline (pre-acute exposure to alarm cue) and post-acute-exposure observations. For baseline analysis, activity score was predicted by pre-exposure as a fixed factor with body mass as a covariate and trial number as a random factor to account for experimental tank effects. A developmentally plastic response to alarm cue on baseline behaviour (Poisson et al. 2017) would be indicated by a significant main effect of pre-exposure (e.g. if developmentally pre-exposed and non-exposed fish differ in activity score prior to acute exposure of alarm cue). For post-stimulus observations, activity score was predicted by developmental pre-exposure and acute alarm exposure as fixed factors as well as an interaction term between these two factors, with body mass and pre-stimulus activity score as covariates and trial number and fish ID as a random factor. An innate response to alarm cue would be indicated by a significant main effect of acute alarm exposure. A significant interaction effect between pre-exposure and acute alarm exposure would indicate a differential response to alarm cue between developmentally pre-exposed and non-exposed fish. All statistical analyses were conducted in R Studio version 1.4.1103. Linear mixed-effects models were run using the lme4 package (Bates et al. 2015).

2.3 Results

Neuromorphology

We found no significant difference in body mass between the two treatment groups (pre-exposed and non-exposed) ($t(54) = 0.006$, $P = 0.093$). Pre-exposed fish had a mean body mass of 0.17g ± 0.022 and non-exposed fish had a mean body mass of 0.18g ± 0.23.

We found no significant effect of pre-exposure on total brain volume (beta = 0.031, $t(53) = 1.071$, $P = 0.29$). For the five brain regions measured, we found a significant effect of pre-exposure on olfactory bulb volume (beta = 0.18, $t(53) = 2.06$, $P = 0.045$; Table 2.1 and Figure 2.2). Pre-exposed fish had significantly smaller olfactory bulb volume ($0.017mm^3 \pm 0.005$) compared to non-exposed fish ($0.02mm^3 \pm 0.007$) We found no significant effect of pre-exposure on the volume of the remaining four brain regions (see Table 2.1 and Figure 2.2).

Behaviour

A linear mixed-effects model found no significant effect of pre-exposure on baseline measures of activity score in the pre-stimulus observations (beta = -0.34, $t(107) = -0.92$, $P = 0.36$). A linear mixed-effect model for activity score for the post-stimulus observations found no significant main effect of pre-exposure on activity score (beta = 0.52, $t(104) = 1.02$, $P = 0.31$), no significant main affect of

acute exposure (beta = 0.082, t(104) = 0.15, P = 0.88), and no interaction between pre-exposure and acute exposure to alarm cue (beta = -0.45, t(104) = -0.60, P = 0.55).

2.4 Discussion

This study was designed to test whether early developmental exposure to conspecific alarm cue (pre-exposure) leads to developmentally plastic changes in anti-predator related behaviour (time spent motionless, sheltering, and aggressive acts) and corresponding neuromorphological (regional brain volume) investment in Atlantic Salmon destined for stocking to the wild for reintroduction efforts. We found no evidence to suggest that pre-exposure had an effect on baseline anti-predator behaviour or the behavioural response to acute alarm cue exposure (alarm response) for yearling hatchery-reared fish. We did, however, find differences in neural investment to select regional volumes. Of the five brain regions measured – telencephalon, optic tectum, olfactory bulb, hypothalamus, and cerebellum – we found a significant and negative effect of pre-exposure on the relative volume of the olfactory bulb of pre-exposed fish with no significant effect on the remaining brain regions. Interestingly, the difference in olfactory bulb volume did not translate to differences in behaviour for the behavioural metrics we observed.

Perceived predation risk is known to elicit differential neural investment in fishes including changes in overall size and size of various brain structures

(reviewed in Gonda et al. 2013). Studies of predator-mediated brain variation have primarily focused on brain sizes differences as metrics of comparison but the results across studies often conflict (see Reddon et al. 2018; Walsh et al. 2016). Under natural settings, population-level comparisons in environmentally induced structural changes to the brain are often dependent on species (Joyce & Brown 2020), sex (Reddon et al. 2018), and life-history (Gonda et al. 2012). For example, male guppies (*Poecilia reticulata*) who are experimentally exposed to cues of predation risk develop larger brains for their body size than non-exposed males (Reddon et al. 2018). By contrast, wild guppies (*Poecilia reticulata*) that were translocated from high to low predation sites evolved relatively larger brains compared to those from low to high predation sites (Mitchell et al. 2020) Similarly, three-spine stickleback (*Gasterosteus aculeatus*) experimentally exposed to increased predation risk develop smaller rather than larger brains (Samuk et al. 2018). These contrasting effects of predation risk on brain size may represent a trade-off between neural tissue investment and other fitness-related traits (Dunbar and Shultz 2017) or the employment of different anti-predator responses (Samuk et al. 2018). Samuk et al (2018) suggest that fish that employ a change in habitat as an antipredator response will experience a different suite of cognitive challenges compared to fish that employ increased vigilance, leading to differential investment to neural tissue (for cognitive and sensory tasks) and other tissues (such as swimming muscles).

In the context of our results, perceived predation threat during early development (pre-exposure) did not affect overall size of the brain but led to a

developmentally plastic response in neural investment (i.e., pre-exposed fish developed smaller olfactory bulbs as fry) with no corresponding change in behavioural activity level when exposed to alarm cue as yearling. Variation in olfactory bulb size has been demonstrated under experimental manipulation of perceived predatory risk in nine-spined sticklebacks (Gonda et al. 2012). In that study, fish whose parents originated from pond environments (characterized by low levels of predation) developed larger olfactory bulbs in the presence of perceived predation but fish whose parents originated from marine environments (characterized by high levels of predation) showed no plastic response in olfactory bulb size under perceived predation risk manipulation. Similarly, Northern redbelly dace (*Phoxinus eos*) exposed to perceived predation (in the form of alarm cue) developed larger olfactory bulbs compared to non-exposed fish (Joyce and Brown 2020). In contrast, an observational study found a significant negative relationship between predator biomass and olfactory bulb volume in guppies (*Poecilia reticulata*) (Kotrschal et al. 2017). In that study, variation in olfactory bulb size was associated with biomass of only one of the four predator species measured, suggesting that variation in olfactory bulb size is at least indirectly dependent on predator-prey dynamics. It is difficult to draw conclusions about olfactory bulb size variation given the scarce literature pertaining to the topic. And, given our experimental design – brain measurements were collected at the fry stage while behavioural measures were collected at the parr stage – we were unable to directly link variation in predation-related behavioural measures to neural correlates.

Given that we did not find a significant main effect of acute alarm cue exposure on behaviour– the pre-exposed and non-exposed fish did not exhibit an innate alarm reaction – it is possible that the alarm cue concentration used in this experiment (0.032cm^2/L) was below the behavioural-response threshold for alarm response in Atlantic Salmon. However, hatchery-reared Atlantic Salmon have been shown to exhibit innate behavioural responses to alarm cue at similar concentrations to the ones used in the current experiment (Lau et al. 2021). Additionally, juvenile rainbow trout (*Oncorhynchus mykiss*) were found to consistently exhibit overt fright reactions to concentrations of alarm cue at 1 cm^2 of skin in 134 255 L of water – far below the concentrations used in the current study (Mirza & Chivers 2003). Interestingly, rainbow trout that had been pre-exposed to alarm cue but showed no overt behavioural response to subsequent acute exposure to alarm cue still exhibited an increase in survival during live predator encounters (Mirza & Chivers 2003). Moreover, glowlight tetras (*Hemigrammus erythrozonus*) exposed to subthreshold concentrations of alarm cue only exhibited overt antipredator responses in the presence of secondary visual predator cues (Brown et al. 2004). It is possible that the behavioural measures we observed were not sensitive to alarm cue exposure at the concentrations we provided and that studies that include secondary cues would aid in our understanding of how alarm cue exposure during early development affects behavioural responses at later developmental stages (Brown et al. 2004).

We found no effect of early developmental pre-exposure to alarm cue on behavioural plasticity in our study. In other words, pre-exposure to alarm cue did

not affect the baseline or post-stimulus activity of hatchery-reared salmon. These results are in contrast with an earlier alarm cue study that suggests embryonic exposure to alarm induces behavioural plasticity in rainbow trout (Poisson et al. 2017). That study, however, found the effect of pre-exposure on activity level significantly interacted with time throughout the behavioural trial. It is possible that effects of pre-exposure on activity level in our current experiment were not captured in the two 5-minute recording periods and that behaviour should be compared across a wider range of time. In addition, rainbow trout showed immediate differential responses in activity between exposed and non-exposed fish when a secondary cue (a novel object) was present (Poisson et al. 2017). As noted above, secondary cues may be necessary or aid to elicit certain alarm responses in fishes (Brown et al. 2004).

Taken together, the results from our study are inconclusive as to whether early developmental exposure to alarm cue leads to a developmentally plastic response in brain morphology or behaviour. A developmentally plastic response would be one that produces long-term plastic changes in developmental trajectory. In fact, previous studies have demonstrated phenotypically flexible (i.e., reversible, rather than developmentally plastic) responses to external stimuli (Donaldson & Brown 2022; Näslund et al. 2012). Atlantic Salmon reared during early development in structurally enriched tanks developed differences in brain size as alevin compared to non-enriched counterparts, however, those effects disappeared over time when the enrichment was removed at the fry and parr stages (Näslund et al. 2012). The results from that study suggest no critical early developmental

period for enrichment in determining brain growth trajectory. Similarly, juvenile convict cichlids exposed to alarm cues for only 14 days showed significant changes in brain size compared to non-exposed counterparts, but those effects were diminished in the absence of alarm cue after only 11 days (Donaldson & Brown 2022). The results from that study suggest a flexible neuroplastic response to alarm cue exposure, however, it is important to note that the initial alarm cue exposure in that study was administered at the juvenile stage, outside of the potential critical window for olfactory development (Knudsen 2004; Hara & Zielinski 1989). Experiments comparing the effects of early developmental alarm cue exposure on neuromorphology across time and over developmental periods are necessary to establish whether a critical period for exists for alarm cue exposure to lead to developmentally plastic responses.

It remains unclear whether pre-exposure would provide an advantage to hatchery-reared animals in the wild. Experiments comparing the alarm response and survivability of pre-exposed and wild fish during live predator exposure would be informative in this regard. Our results highlight the importance of and ability to exploit plastic responses to generate differences in brain structures and the potential role of those changes to behaviour in later developmental stages.

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Table 2.1 Results from the general linear models showing the effect of pre-exposure on the total brain volume and regional volume for each of the five brain regions studied in juvenile Atlantic Salmon (*Salmo salar*). The model for the total brain volume included body mass (excluding the mass of the brain) as a covariate and pre-exposure as a fixed effect. The models for each brain region (volumes in mm3) included the total brain volume, excluding the volume of the brain region of interest (referred to as 'rest of brain'), as a covariate and pre-exposure as a fixed effect. All variables were log10-transformed prior to analysis (see Methods for details).

	Estimate	SE	df	t value	*P*
Total brain					
Body mass	0.43	0.10	53	4.10	<0.001
Pre-exposure	0.031	0.029	53	1.07	0.29
Olfactory bulb					
Rest of brain	0.67	0.37	53	1.83	0.073
Pre-exposure	0.18	0.089	53	2.06	0.045
Telencephalon					
Rest of brain	1.21	0.19	53	6.36	<0.001
Pre-exposure	-0.036	0.045	53	-0.81	0.42
Optic tectum					
Rest of brain	0.49	0.082	53	5.99	<0.001
Pre-exposure	0.021	0.026	53	0.822	0.415
Cerebellum					
Rest of brain	1.02	0.27	53	3.83	<0.001
Pre-exposure	0.049	0.063	53	0.78	0.44
Hypothalamus					
Rest of brain	0.15	0.25	53	0.60	0.55
Pre-exposure	0.056	0.062	53	0.90	0.37

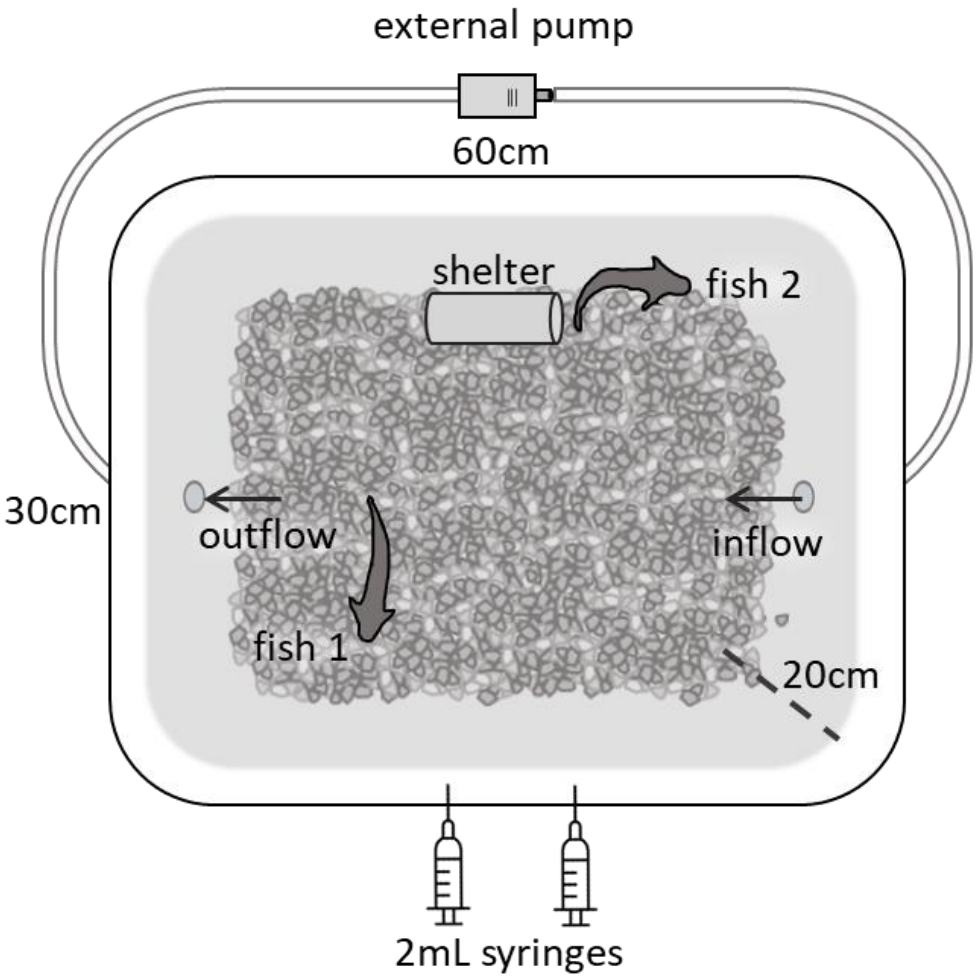

Figure 2.1 Schematic top-down view of behavioural test-tank with two fish per trial. Overall dimensions of the tank are 60cm length, 30 cm width, and 40 cm height (filled to a height of 20cm). The bottom of the test-tank was covered by a single layer of substrate. The shelter was constructed of a 10cm-long PVC tube with an internal diameter of 1.27 centimeters. The test-tank was fitted with an external pump to create recirculating flow. Alarm cue and control water for the post-stimulus observations were administered through 2mL syringes through openings on the side of the test-tank just above the water level (see Methods for more detail).

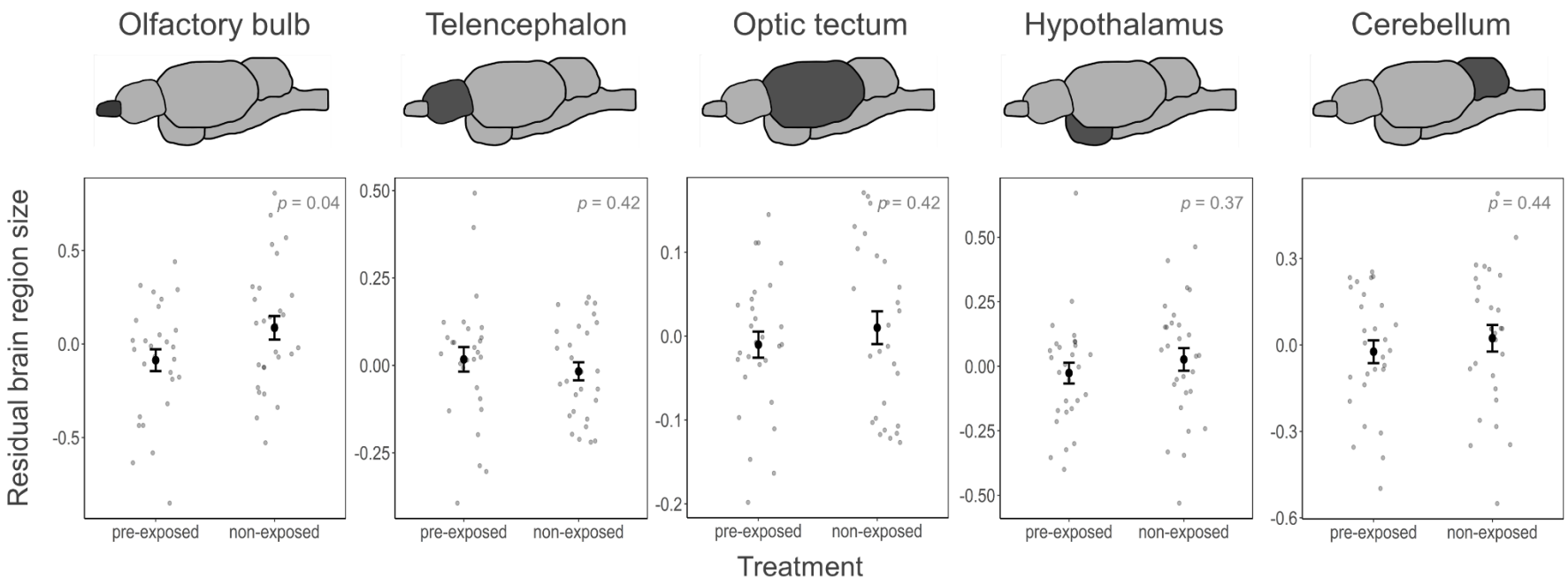

Figure 2.2 Panels depict the size of each brain region in the pre-exposed and non-exposed treatments of Atlantic Salmon (*Salmo salar*) juveniles (see Methods for details). Pre-exposed fish are those that received early developmental exposure to alarm cue, and non-exposed fish are those that received distilled water during early development. Light grey points represent relative brain region sizes of individuals from each treatment group. Dark grey points represent means and error bars represent 95% confidence interval.

CHAPTER 3

UPSTREAM AND DOWNSTREAM DISPERSAL BEHAVIOUR OF HARD- AND SOFT-RELEASED JUVENILE ATLANTIC SALMON

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3.1 Introduction

The success of reintroduction efforts is generally measured in terms of the establishment and persistence of a self-sustaining population (Dickens et al. 2010) but successful outcomes are limited in number and scope (reviewed in Fischer & Lindenmayer 2000; Seddon et al. 2007). The period immediately following release of reintroduced animals - the establishment phase - is a precarious and sensitive period for survival (Dickens et al. 2010); the failure of reintroduced animals to survive and persist in the wild is thought to be linked to captively reared animals exhibiting maladaptive behaviours during this phase (Einum and Fleming 2001; Jule et al. 2008). These maladaptive behaviours include poor foraging (Einum & Fleming 1997), reduced anti-predator responses (de Mestral and Herbinger 2013), and atypical dispersal behaviour (Swaisgood 2010). The rising number of unsuccessful reintroductions has been met with a set of approaches – what Tetzlaff et al. (2019) refer to as 'conditioning' – aimed at countering the negative effects associated with captive-rearing and reintroduction events. Conditioning can be 'animal-focused', which involves various forms of environmental enrichment while in the captive setting or 'environment-focused', which involves enriching the release environment (Tetzlaff et al. 2019). Conditioning can offset some of these detrimental effects experienced by captively-reared organisms (Huntingford 2004; Jonsson and Jonsson 2014) with a goal to produce more "natural-like" behaviours (Hyvärinen and Rodewald 2013).These approaches have been gaining in popularity among wildlife management as well as conservation biologists (Hutchison et al. 2012; Reading et al. 2013; Jonsson and Jonsson 2014).

Environment-focused conditioning tactics are those that aim to expose captive-bred organisms to wild or semi-wild settings – the most common of these tactics being ‘soft-release’ (Tetzlaff et al. 2019). Soft-release generally refers to the practice of providing reintroduced animals with an acclimatization period (fish that experience soft-release are referred to as ‘acclimatized’), free of predators, at or near the release site prior to release, compared to conventional ‘hard-release’ wherein reintroduced organisms receive little to no acclimatization prior to being released into the wild (Brown and Day 2002). In their meta-analysis, Tetzlaff et al. (2019) found that soft-release, compared to other tactics (environmental enrichment and predator training), had the most significant impact on stress-related post-release survival, dispersal, and site fidelity across the range of taxa studied. Furthermore, Swaisgood (2010) argues that the most important behavioural concept in reintroduction programs is dispersal behaviour – a topic which, until recently, has received little attention in reintroduction research.

Dispersal behaviour is an area in which soft-release tactics might produce more appropriate behaviours that increase the chance of survival. Dispersal rate and timing are commonly measured metrics for movement related to survival of reintroduced organisms (Tetzlaff et al. 2019). To date, studies of the effects of soft-release on survival and dispersal rate have shown inconsistent results. For example, no difference in dispersal rates was observed between two-year-old (smolt) European grayling (*Thymallus thymallus*) that were directly released into streams and those that were acclimatized in fenced-in pools at the release site prior to release (Thorfve 2002). In contrast, soft-released Brown trout (*Salmo trutta*)

fingerlings showed a decrease in dispersal and a 10-18% higher level of survival compared to hard-release fishes (Cresswell and Williams 1983). Similar effects – a decrease in dispersal from release site and increased survival rates for soft-released fish – have been observed for other salmonids in early life (pre-smolt) (Jonssonn et al. 1999; but see Rosenberger et al. 2013). The understanding of dispersal from release sites is beneficial to reintroduction efforts in the context of salmonid growth and ultimate survival; for wild subyearling salmonid fishes, time spent lingering in riverine feeding sites is crucial for maintaining high growth rates and survival prior to smolt and migration downstream (Connor et al. 2003).

Another common measure of movement behaviour, as it relates to soft-release benefits, specifically for stream fishes, including salmonids, is the pattern of dispersal and spatial distribution of reintroduced organisms (Egglishaw and Shackley 1973, 1980; Beall et al. 1994; Crisp 1995; Teichert et al. 2011; Foldvik et al. 2012; Eisenhauer et al. 2020), such analysis can involve simply measuring movement in a single dimension (upstream and downstream) (Eisenhauer et al. 2020). While a downstream monitoring bias appears to exist in the literature pertaining to salmonids, as reviewed by Eisenhauer and colleagues (2020), these authors suggest downstream dispersal is proportionally higher (mean of 87.6% across studies) compared to upstream dispersal. Additionally, results from a study of the differential dispersal of wild and captive-bred juvenile masu salmon (*Oncorhynchus masou*) showed that captive-bred salmon were caught in upstream traps at a significantly higher proportion compared to wild counterparts (Nagata et al. 1994). Those authors suggested that the difference in dispersal direction was

due to differences in swimming behaviour brought on by the environments in which the individuals develop (Nagata et al. 1994).

Another important way in which movement behaviour can be understood is through the daily cycle of diurnal and nocturnal activity (Metcalfe et al. 1998). Most animals are adapted to consistent diurnal or nocturnal lifestyles (Metcalfe et al. 1998). Adaptation to captive-rearing settings can alter the temporal activity pattern of fishes (Álvarez and Nicieza 2003). For example, Álvarez and Nicieza (2003) found that captive-bred brown trout were predominantly active during the day whereas wild brown trout were predominantly active at night (see also Alioravainen et al. 2020 for semi-natural conditions). For wild Atlantic Salmon (*Salmo salar*), diel activity patterns are season- and age-dependent (Johnston et al. 2004). Young-of-year (YOY) Atlantic Salmon were observed to be more active during the day in early summer and shift to a more nocturnal lifestyle in late summer and into the colder seasons – the opposite was found for older post-YOY salmon in the same study (Johnston et al. 2004). It remains unclear whether captive-rearing or soft-release affect the temporal activity patterns of Atlantic Salmon released into the wild.

Atlantic Salmon were once an abundant top predator in Lake Ontario and the target of a valuable fishery until their extirpation in the late 19th century (Dunfield 1985; Hawkins et al. 2019). While the Lake Ontario habitat has been restored and many of the factors leading to extirpation have been alleviated (Beeton 2002), reintroduction efforts have yet to restore a self-sustaining population, possibly due to a failure of captive environments to prepare the fish for

the natural environments in the tributaries where they are released (Stewart et al. 2014) In this study we reintroduced captive-bred Atlantic Salmon to a tributary of Lake Ontario using two tactics – conventional 'hard-release', and 'soft-release' wherein fish were allowed to acclimatized for 6 days in specially designed enclosures within the stream prior to release. We used passive integrated transponder (PIT) tags to monitor dispersal patterns of these fish during the study period. We compare the effects of hard- and soft-release tactics on the spatial and temporal dispersal of these fish – specifically the timing of up- and downstream dispersal. We also examine day- and night activity patterns between the groups of fish.

3.2Materials and methods

Fish stock and husbandry

Atlantic Salmon were from the Sebago Lake strain (ME, USA; 43.9°N, 70.6°W), which has been maintained for two generations at the Ontario Ministry of Natural Resources and Forestry (OMNRF) Harwood Fish Culture Station (44.18.06°N, 78.14.73°W). In the fall of 2017, gametes were collected from adults housed at the Harwood Fish Culture Station and transported to a hatchery facility at Western University, London, Ontario. At the Western University facility, eggs and milt were crossed using a 2 by 2 design, wherein each block in the cross consists of two females, with half of each female's eggs fertilized by each of two males. A total

of 14 blocks were created (28 females, 28 males). Fertilized eggs were incubated in vertical incubation stacks with a circulating water system maintained at 7 ± 1°C. After hatch, on February 18th 2018, fry were moved to mixed-family tanks with a single layer of loose gravel at the bottom of each tank. Tank temperatures were maintained at 7 ± 1°C until March 7th 2018, at which point the temperature was transitioned over a 3-week period to 11 ± 1°C. Tanks were kept at this temperature until mid-April when temperature was again increased over a 3-week period to 15 ± 1°C.

PIT tagging and measurements

During the second week of September 2018, we haphazardly selected 610 fall fingerling Atlantic Salmon and individually marked them with a passive integrated transponder (PIT) tag (Biomark TX1411SST; 12.5 × 2.07 mm, 0.102 g). We followed Cook et al. (2014) for PIT-tagging procedure – briefly, fish were anesthetized in 50mg/L of MS-222 (tricaine methanesulfonate) and tags were injected into the body-cavity with pre-loaded syringes. A subset of the tagged fish (192 hard-release and 167 soft-release) were weighed and measured for fork length for comparison between groups. Fish were monitored, in their home tank, for tag-rejections, condition, and mortality for 1 month prior to release to ensure that PIT tags remained inside the body of the fish.

Study site, enclosures, and PIT-tag antennas

Our hard and soft release experiment was conducted in the East Duffins Creek, Ajax, Ontario, Canada within the Greenwood Conservation Area (43° 53' 55.9"N 79° 03' 54.2" W). East Duffins Creek is a 32 km tributary that empties into Lake Ontario and is part of the larger Duffins Creek watershed with a drainage area of 283 km^2.

To assess upstream and downstream dispersal of tagged fish, 1m × 3m long PIT-tag antennae (Biomark, Boise, Idaho) were anchored at narrow chokepoints of the stream 350m apart. The antennae were anchored parallel to and across the stream bed in the middle of the water column to detect PIT-tagged fish swimming throughout the water column. The detection range of the antennas were set to 45cm above and below the antennas to maximize detection of fish passing across the antennae. To prevent fish from swimming around the arrays, avoiding detection, mesh panels were installed on either side of the arrays to cover the remaining width of the stream. The dominant substrate (>50%) as defined by grain size (Wentworth 1922) at each of the arrays was rubble (54 – 179 mm). The 350 m stretch of stream between the two arrays was the designated "release site" – this is where the soft release enclosures (see details below) were installed and where the fish from both hard and soft treatments were released. Fish detected at the upstream antenna were considered to be dispersing upstream while fish detected at the downstream antenna were considered to be dispersing downstream. Fish that were not detected on either

of the antenna were considered to have not dispersed and remained at the release site.

In total, nine soft release enclosures were anchored to the stream bed within the release site in three sets of three at a depth of 0.30 m - each group 50 m apart starting 100 m downstream of the upstream PIT-tag antenna. The three enclosures per set were arranged staggered downstream from one another (with no more than 1 m distance apart) so as to prevent flow obstruction from one enclosure to the next. The enclosures were constructed by connecting four 1 m x 1 m wooden frames covered with a 5-mm mesh net to create a 1 m^3 enclosure with no top or bottom panel. Steel rods were anchored into the stream bed at each of the four corners of the enclosure to provide stability and prevent the enclosures from washing away. The top of each enclosure was then covered with a twine grid to prevent avian predators from feeding on the salmon within.

Transport and Release

On October 9 approximately half of the fish (n = 300, soft-release treatment) were transported to the release site in 100L live-well coolers at a density of 11.5g/L. Water temperature was continuously monitored during transport and sealed bags of ice were added every half hour to the cooler to maintain water temperature within 2°C of the home tank temperature on the day of release (14.8°C). The overall trip duration was approximately 3 hours. Upon arrival at

Greenwood Conservation Area, at 15:40 local time, fish were transported to the release site from the cooler to the stream in 19L plastic pails. The process of transporting all of the fish from the vehicle to the enclosures took a total of 30 minutes. Fish were haphazardly and evenly distributed between the nine possible soft-release enclosures. Approximately 33 fish were housed in each enclosure, resulting in a density of approximately 9g/L. Water temperature at the middle three enclosures was 14.5°C at the time the fish were placed in the enclosures. The outsides of the enclosures were cleaned of debris and checked daily for mortalities. No mortalities were observed over the 6-day acclimatization period within the soft-release enclosures. On October 15, after 6 days of soft-release acclimatization, the remaining fish (n = 310, "hard-release" group) were then transported to the release site in the same manner as previously described for the soft-release group (see above). Fish arrived at Greenwood Conservation Area at 15:15 local time. The hard release fish were transported in 19L plastic pails (approximately 33 per pail) next to each of the nine soft-release enclosures and released simultaneously with the lifting of the enclosures such that the hard release and soft release groups were released simultaneously. Fish dispersal was then monitored for a total of 57 days post-release, until the beginning of the river freeze-up stage.

Monitoring upstream and downstream dispersal

To detect and log PIT tags passing the antennas, each of the antennae was connected to an individual PIT tag reader (Biomark IS1001 Data Logger Board). Scan time for each reader was set to 75 msec and idle time to 120 msec. A pilot study on detection was carried out to ensure the reliability and detectability of passing pit tagged fish. A 'test-fish' (rectangular foam piece injected with a PIT tag) floated above each antenna three times simultaneously three sets of times throughout the day – once set in the morning, one set in the afternoon, and a final set of times before sunset. The PIT tag reader recorded, with 100% detection efficiency, the test-fish floating above. The readers ran continuously for all 57 days of the experiment. Once a week the readers were turned off briefly (<1 minute) to allow for one of the experimenters to replace batteries that powered the system.

Fish that were not detected were assumed to have not dispersed away from the release site. For those fish that were detected on an antenna, multiple detections per fish were possible and logged, however, only unique first detections were used for analyses in this study. Unique first detections on the upstream antenna were assumed to represent upstream dispersal and unique first detections on the downstream antenna were assumed to represent downstream dispersal.

Environmental Data

Water discharge data were obtained from National Hydrological Service (https://wateroffice.ec.gc.ca/search/real_time_e.html, accessed 4 April 2019).

Water discharge (m^3 s^{-1}) was measured at 5-min intervals at Duffins Creek at Ajax hydrometric station (station number: 02HC049; 43°50'56" N, 79°03'22" W), 8.5 km downstream of the release site. During the experimental period (October 9, 2018 to December 11, 2018), mean water level was 0.83 m (range over study period = 0.71m – 1.88m) and mean discharge within the same period was 2.41 m^3/s (range = 1.88 m^3/s - 35.5 m^3/s). Daily averages were used for analysis in the linear models. Daylight timings were obtained from National Research Council Canada (https://nrc.canada.ca/en/research-development/products-services/software-applications/sun-calculator/).

Statistical Analysis

We used a logistic regression to develop a generalized linear model for testing the likelihood of fish detection on either array based on size (for the subset of fish we had size measures) and treatment simultaneously. To examine the relationship between treatment (hard- vs. soft-release fish) and dispersal from the release site (as measured by number of fish detected on either array) we performed a chi-square test of independence with alpha value set at .05. We assumed for these analyses, and the analyses that follow, that undetected fish remained at the release site. The relationship between up- and downstream dispersal, for those fish that were detected on the arrays, and treatment were also tested using a chi-square test of independence.

The effect of treatment (hard vs. soft-release) on latency to dispersal for those fish that were detected was examined. Latency to dispersal was measured as the time (in days) from the date of release that a fish was detected on either array. Our ANOVA model was set up with latency to detection as the response variable and treatment (as a dummy variable – with 0 representing hard-release and 1 representing soft-release) as the predictor variable. We then ran the same model separately for downstream and upstream data exclusively to examine the effects of treatment on latency to dispersal for each direction of dispersal.

The diurnal timing of dispersal across both treatments was tested using a Chi-Square contingency test with expected frequencies for day and night set at 53% and 47%, respectively, as the average daylight and darkness hours throughout the study period (October 15 to December 11, 2018). A Chi-Square test of independence was used to compare day and night detections between treatment (Dodd et al. 2018).

Data analysis was performed in Microsoft Excel and R language for statistical computing (ver. 4.1.0; R Foundation for Statistical Computing) with RStudio version 1.4.1106 (RStudio Team, 2021).

3.3 Results

Based on the subsample of fish that had mass measured at the time of tagging, hard-release fish weighed 3.83g (SD ± 1.27) and soft-release fish weighed

3.84g (SD ± 1.50). There was no significant difference in log odds ratio of fish detection between treatment group (B = -19.10, SE = 0.12, P = 0.99) or as a result of mass (B = -0.018, SE = 0.12, P = 0.33). In total, 232 of the 610 tagged Atlantic Salmon (38%) dispersed from the release site, as measured by detection at either antenna. Dispersal from the release site was significantly more likely for the hard-release fish (42%, n = 131 of 310 released) than for the soft-release fish (34%, n = 101 of 300 released) (X^2 = 4.77, d.f. = 1, n = 610, P = 0.029). Of the 232 fish detected, 189 were detected at the downstream antenna and 43 were detected at the upstream antenna. Hard-release fish were significantly more likely to move upstream (11%, n = 33 of 310) than soft-release fish (3%, n = 10 of 300) (X^2 = 12.44, d.f. = 1, n = 610, P < 0.001). Downstream dispersal did not differ significantly between hard-release fish (32%, n = 98 of 310) and soft-release fish (30%, n = 91 of 300) (X^2 = 0.12, d.f. = 1, n = 610, P = 0.73).

Latency to disperse either upstream or downstream ranged from 0 to 56 days post-release (see Figure 3.1). On average, soft-release fish were detected ~15 days earlier (mean detection latency = 10.34 days) than hard-release fish (mean detection latency = 25.79 days) (Figure 3.1a). A linear model showed a significant difference between detection latency and treatment (hard-release or soft-release) group ($F_{1,230}$ = 113.1, P < 0.01). Next, we ran these same analyses separately for downstream detections (n = 189) and upstream detections (n = 43). On average, soft-release fish were detected downstream significantly sooner (~18 days earlier:mean detection latency = 10.75 days) compared to hard-release fish (mean detection latency = 28.89 days) ($F_{1,187}$ = 154, P < 0.01). For upstream detections

soft-release fish were detected upstream ~10 days earlier (mean detection latency = 6.6 days) compared to hard-release fish (mean detection latency = 16.61 days). A linear model showed a significant difference between detection latency upstream and treatment ($F_{1,42} = 6.32$, $P = 0.016$).

Atlantic Salmon dispersed significantly more often during the night (73%, $n = 170$ of 232) than during the day (27%, $n = 62$ of 232), even when accounting for the proportion of daylight (47%) and darkness (53%) hours ($X^2 = 38.29$, d.f. = 1, $n = 232$, $P < 0.001$)). Hard-release fish dispersed at a higher proportion during the day (31%, $n = 40$ of 131) compared to soft-release fish (22%, 22 of 101) but the difference between daylight and darkness detections was not significant ($X^2 = 1.80$, d.f. = 1, $n = 232$, $P = 0.18$).

3.4 Discussion

In this study we found that spatial (upstream and downstream) and temporal (time to disperse after release and time of day detected) dispersal patterns of juvenile Atlantic Salmon were affected by release tactic. We found that soft-release fish were less likely to move away from the release site, and when they did move, they moved downstream earlier and were less likely to move upstream compared to hard-release fish. We found that juvenile Atlantic Salmon were more likely to be detected moving both up- and downstream during the night compared to during the day – we did not, however, find a significant difference between the

two release groups in the time of day when they were detected. Taken altogether, these results suggest that the effects of soft-release may indeed reduce or diminish the mismatch between the captive and wild environment and ultimately aid in the success of reintroduced animals.

The observed stronger site fidelity – less dispersal away from release site – for acclimatized fish is in line with previous acclimatization studies in salmonids (Cresswell and Williams 1983; Kaya and Jeanes 1995; McCormick et al. 1998; Jonssonn et al. 1999; Eisenhauer et al. 2020). Previous studies have found that wild European grayling (*Thymallus thymallus*) are less likely to disperse from release sites than hatchery-reared conspecifics (Turek et al. 2010). Additionally, acclimatization prior to release was found to reduce dispersal rates for a number of salmonids (Kaya and Jeanes 1995; Jonssonn et al. 1999). Benefits of acclimatization have been attributed mainly to the recovery from stressful handling and transportation which can affect swimming performance (Maule et al. 1988), orientation (Kruzynski et al. 1994) , predator avoidance (Gadomski et al. 1994; Olla et al. 1995), and feeding efficiency (Pickering et al. 1982). Recovery from stress depends on the type, intensity, and duration of stressor (Olla et al. 1995; Zhang et al. 2020). Recovery from transport stress can take hours (Iversen et al. 1998) to weeks (Vieira Madureira et al. 2019). Enrichment can, however, reduce the stress response of fish (Näslund et al. 2013; Rosengren et al. 2017; Zhang et al. 2020) and time needed for recovery after stress in laboratory experiments. These effects, combined, could explain both the smaller number of soft-released fish moving away from the release site as well as the earlier downstream dispersal

exhibited by soft-release fish in our study. It is possible that the soft-release fish in our study benefit in terms of energy consumption and use from remaining at or near the release site, which could ultimately lead to greater survival and the earlier downstream dispersal could be explained as an earlier dispersal to find more suitable habitat when faced with competition for territory near or at the release-site (Höjesjö et al. 2016). More targeted studies should investigate growth rates, survival, microhabitat use and competition between hard- and soft-release fish in the wild.

We found a significantly higher proportion of hard-release fish dispersed upstream compared to soft-release fish. The main direction of dispersal for 0+ and 1+ year stage Atlantic Salmon in the wild is downstream (Foldvik et al. 2012). Atlantic Salmon parr do exhibit upstream dispersal in early summer which correlates to downstream movement of smolt, freeing up suitable feeding territory (Armstrong et al. 1997). However, upstream dispersal is more energetically costly than downstream dispersal, and so should be a less probable choice for fish avoiding unfavourable environments or in search of favourable environments (Nemeth et al. 2003). It is possible that stress-induced disorientation (Kruzynski et al. 1994) caused a proportion of the hard-release fish to swim upstream. Another possibility is that the early downstream dispersal of soft-release fish served as a stimulus for the hard-release fish to move upstream similar to the natural occurrence during the downstream summertime dispersal of smolts mentioned above (Armstrong et al. 1997). The results suggest that soft-release tactics may

engender, upon salmonids, more appropriate movement behaviour after release compared to conventional hard-release tactics.

Activity levels of salmonid fishes are highly responsive to predation risk and food availability (Metcalfe et al. 1999; Orpwood et al. 2006) and vary with season and water temperature (Roy et al. 2013). The findings from the current study support previous findings that during the autumn season Atlantic Salmon parr were more active during the night than during the day (Roy et al. 2013; Dodd et al. 2018). Although Atlantic Salmon parr predominantly forage during daylight, relying heavily on vision to successfully forage on drifting prey (Keenleyside 1955), the autumnal nocturnal activity can be explained by reduced energy requirement and increased predation pressure (Roy et al. 2013). We predicted here that the hard-release fish, constrained to suboptimal habitat, would shift to diurnal feeding to secure sufficient energy sources. We did not, however, find any significant difference in activity levels relating to photoperiod between the two release groups. Although overall day/night activity levels did not differ between release groups, further investigations with higher spatial acuity are required to more accurately address whether release tactic or stress levels affect foraging behaviour in relation to photoperiod. Since a large portion of fish from both release groups remained at the release site, it is possible that the spatial distributions of feeding overlap but that the temporal aspect of feeding differs – benefiting one group over another.

Benefits of large-scale implementation of ‘soft-release’, or in-stream acclimatization prior to release, tactics have yet to be fully established. Some of

the most common difficulties relating to reintroduction work for management and conservation are a lack of funding, a lack of post-release monitoring, and maladaptive behaviours exhibited by reintroduced organisms (Berger-Tal et al. 2020). We demonstrate, here, by monitoring a sample of reintroduced fish, a clear difference in dispersal behaviour between conventionally released (hard-release) and conditioned (soft-release) captive-bred Atlantic Salmon juveniles in the wild. While we do not compare dispersal behaviour between captive and wild salmon, we suggest that the behavioural differences exhibited by soft-release fish could be a step towards a less maladaptive and more 'wild' phenotype, conducive to better success for reintroduction.

3.5 References

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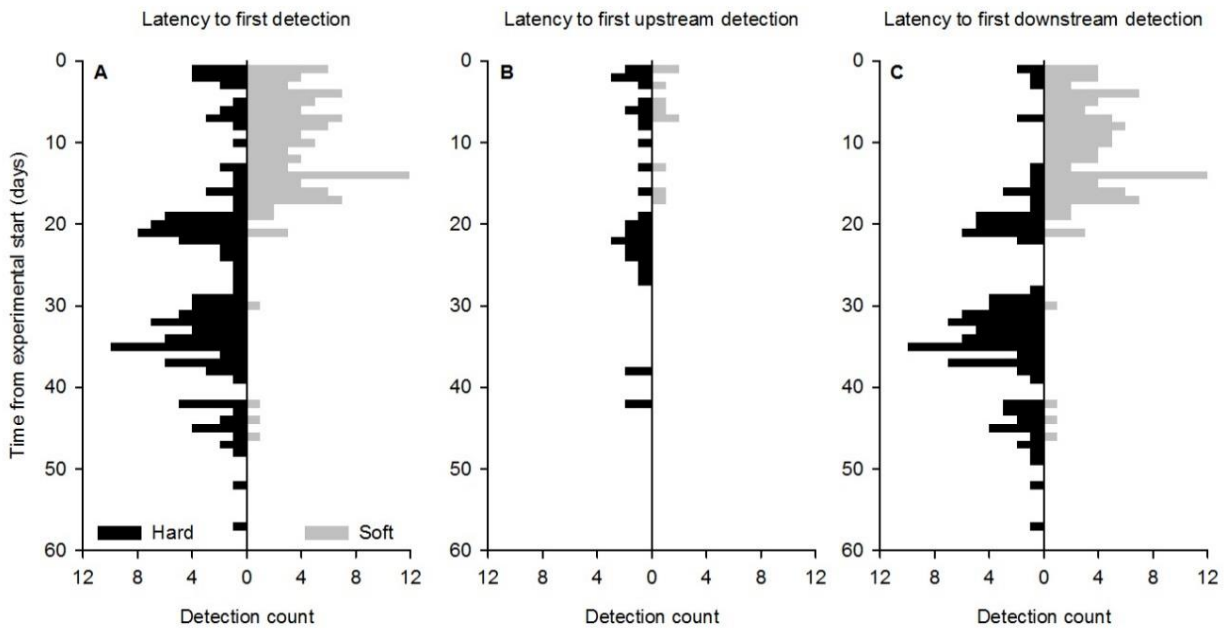

Figure 3.1 Plot of unique detection count (each count represents one unique fish detected) by latency (in days) of detection from time of release (a-c) and mean stream flow rate (m^3/s) by day post-release (d). Panel (a) shows detection count at up- and downstream

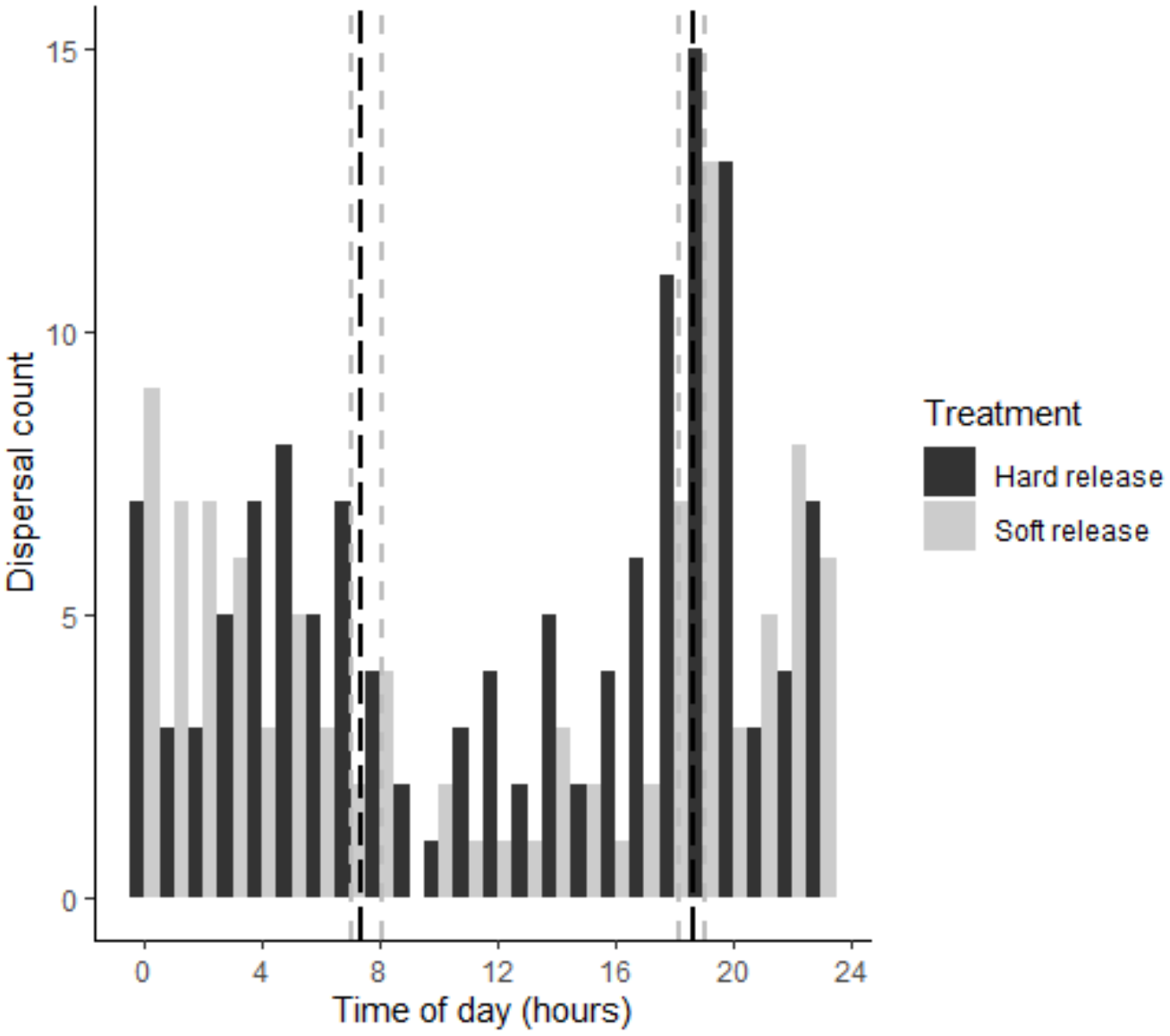

Figure 3.2 Histogram of unique dispersal detections summed for each hour of the day across the study period. Black dashed lines represent the median nautical twilight time, grey dashed lines represent the range of nautical twilight time surrounding the median across the study period (57 days). Black bars represent hard-release detections and grey bars represent soft-release detections

CHAPTER 4

THE EFFECTS OF STRUCTURAL ENRICHMENT, TRANSPORT, AND RECOVERY ON ANTI-PREDATOR BEHAVIOUR IN HATCHERY-REARED ATLANTIC SALMON (*SALMO SALAR*)

4.1 Introduction

The release of captive-reared fish to supplement or enhance wild populations, or as part of reintroduction efforts, is a common tool for the management and conservation of fishes (reviewed in Berger-Tal et al. 2020). The success of reintroduction efforts, however, has generally been low, with high mortality rates due to predation of released captive-reared animals following release (Teixeira et al. 2007; Seddon et al. 2012; Tetzlaff et al. 2019). High mortality rates of released captive-reared fish are thought to be linked to maladaptive post-release behaviour shaped by plastic responses to the captive-rearing environment (Johnsson et al. 2014). Behavioural phenotypes that are adaptive in the barren, predator-free environment of captivity likely differ from those that are adaptive in the wild (Álvarez and Nicieza 2003; Salvanes 2017; Alioravainen et al. 2020). Understanding behaviour, and particularly the interaction between fishes and their environment – in the hatchery and in the wild – can provide important insights and direction to produce more wild-like phenotypes and improve the success of reintroduction efforts (Näslund 2021; Cooke et al. 2022). There has been a recent focus amongst wildlife managers and conservation biologists to produce fish with more adaptive behavioural phenotypes by means of enriching the captive-rearing environment and ameliorating negative effects associated with the stress of transportation (Jones et al. 2021; Näslund 2021; Crates et al. 2022).

Captive-reared fish tend to perform poorly in natural environments, and exhibit reduced antipredator behaviours, compared to wild conspecifics (reviewed in Näslund 2021). In order to determine which antipredators behaviours correlate with increased survival in the wild, Miyamato and Araki (2020) matched masu salmon (*Oncorhynchus masou masou*) behaviours in open-field aquarium tests to survival in semi-natural streams. The authors found regular defensive behaviours (i.e., shelter association and low activity levels) to be critical determinants for the survival of fish against predation (Miyamoto and Araki 2020). Differences in these defensive behaviours have been demonstrated between captive and wild fishes. For example, in the presence of live predators, wild juvenile brown trout (*Salmo trutta*) increase sheltering behaviour while hatchery-reared conspecifics showed no behavioural response to the presence of a predator (Álvarez and Nicieza 2003). Similarly, in an open-field test, wild juvenile Atlantic Salmon (*Salmo salar*) displayed higher levels of risk-averse antipredator behaviours – they spent more time associated with shelter and immobile – compared to hatchery-reared counterparts (Salvanes 2017). These behavioural differences between hatchery-reared and wild fish have been linked to pronounced difference in the physical rearing environments that each group experiences (Johnsson et al. 2014; Clarke et al. 2016). Conventional, barren hatchery rearing might not provide fish with suitable stimuli for developing the behavioural repertoire to exploit the use of shelter and risk-aversion needed for survival in the wild (Solås et al. 2019). As such, structural enrichment (any form of physical complexity added to housing for captive animals), also referred to as physical enrichment, of the hatchery-rearing

environment has become increasingly used to plasticly alter the behaviour of fishes in an effort to improve post-release antipredator responses (Tetzlaff et al. 2019; Jones et al. 2021). Indeed, structural enrichment has been found to improve shelter-seeking behaviour and decrease stress responses across genera (reviewed in Jones et al. 2021)

Transport of animals from captive-reared settings to the wild is an unavoidable stressor during the reintroduction process imposed on animals to be released (Berger-Tal et al. 2020). While the documentation of behavioural response of fish to transport stress is scarce (but see Vieira Madureira et al. 2019), the physiological response to transport stress is well documented (e.g. Barton 2002; Cogliati et al. 2019; Vieira Madureira et al. 2019). Typically, post-transport stress response is linked to increased plasma cortisol and glucose levels (Barton 2000). Indeed, rearing with structural enrichment has been found, across a number of studies, to decrease baseline stress levels (Näslund et al. 2013), post-transport stress response (Cogliati et al. 2019), and promote recovery after stressful events (Pounder et al. 2016). In addition to physical enrichment, an increasingly common tactic used to promote recovery from stressful transport and to allow released animals to acclimatize to novel environments is the use of post-transport recovery tanks in the laboratory (Vieira Madureira et al. 2019) or soft-release pens in the wild (Tetzlaff et al. 2019). In the laboratory, behavioural and physiological recovery of juvenile brown trout from transport stress was demonstrated after at least 7 days in new recovery housing (Vieira Madureira et al. 2019). In the wild, the use of predator-free soft-release pens promoted more wild-like movement

behaviour of hatchery-reared juvenile Atlantic Salmon after 6 days of acclimatization to the wild setting (Mokdad et al. 2022a).

In this study we first test the hypothesis that structural enrichment leads to plastic changes in antipredator-related behaviour of juvenile Atlantic Salmon, producing more 'wild-like' phenotypes (Salvanes 2017; Latchem et al. 2021). We test the prediction that fish reared under a structurally enriched environment will exhibit behaviours indicative of increased risk-aversion – a decrease in movement activity, and an increased tendency to remain near the walls of the open-field arena (Salvanes 2017) – compared to fish reared under conventional barren rearing conditions. Second, we investigate the behavioural response to stressful transport in these fish. We test the hypothesis that rearing under structural enrichment mends potential negative behavioural effects of transport. Third, we investigate the effects of post-transport recovery on the behavioural response to stressful transport and we test the hypothesis that rearing under structurally enriched conditions promotes the recovery of Atlantic Salmon from stressful transport.

4.2 Methods

Experimental Fish

Atlantic Salmon fry were sourced from fourth-generation hatchery-reared stocks, used for the Lake Ontario reintroduction effort, from the Ontario Ministry of Natural Resources and Forestry (OMNRF) Normandale Fish Culture Station

(42.72.01°N, 80.33.99°W). On 21st April 2021, 800 Atlantic Salmon fry, weighing *ca.* 2g body mass, were transported from Normandale Fish Culture Station, *ca*. 270km by road, to the Freshwater Restoration Ecology Centre (FREC) in Lasalle, Ontario (42.23.67°N, 83.10.49°W). Upon arrival at FREC, fish were reared in 1200L circular fiberglass tanks with 400 fish per tank under one of two rearing conditions: one tank containing structure and substrate (enriched, see below and Figure 4.1 for details), and one tank without additional structure or substrate added (barren). Rearing tanks were connected to a temperature-controlled recirculating system using dechlorinated, aerated, and filtered municipal water. Temperatures ranged between 16°C and 18°C throughout rearing. Fish were fed using commercial aquaculture pellets (1% body weight per day). Rearing tanks were connected to the same water-circulation and filtration system and were thus exposed to similar water conditions. The enriched tank contained four custom-built physical enrichment structures placed evenly around the tank. Each structure was created by securing two 30 x 30cm PVC plates (painted green with marine paint) to a 100cm threaded stainless steel rod embedded into a large stone (*ca.* 20cm in length) (Figure 4.1). For substrate, *ca.* 75% bottom of the enriched tank was covered with a single layer of river rock (2-4cm in length) – the middle intersect of the tank was left without river rock to allow for proper water drainage.

Experimental overview

Experimental trials began on 23rd June 2021 and ran through 2nd July 2021. To evaluate the effect of rearing environment (structural enrichment), transport, and recovery on antipredator behaviour, our experiment was designed to behaviourally test fish from each rearing treatment (enriched v. non-enriched) prior to transport (*non-transport group*), immediately following transport (*transport group*), and seven days following transport while being reared in a novel 'recovery' environment (*recovery group*), absent of structural enrichment (see Figure 4.2). Behavioural trials were conducted as open-field trials in barren tanks (see below for details). Recovery groups included both transported and non-transported fish to assess for the effects of recovery on transport as well as the effects of novel environment (recovery tanks) on behavioural responses. The experimental process was repeated on two separate occasions, the first round of transports was conducted on 23rd June 2021, and the second round of transports was conducted on the 25th June 2021. Post-recovery behavioural trials were conducted on the 30th June 2021 and 2nd July 2021. All experimental procedures were approved by the University of Windsor Animal Care Committee (University of Windsor AUPP # 21-08).

Open-field trials

Behaviour was tested in open-field trials, similar to previous studies, and allows for the measurement of behavioural activity related to exploration and risk-taking/risk-aversion (see Salvanes 2017; Latchem et al. 2021). Behavioural arenas

were glass aquaria measuring 77cm by 32cm and filled to a water depth of 18cm. Water temperatures in the behavioural arenas were within 1°C of the original rearing tanks for the duration of behavioural analysis. We used eight arenas simultaneously for each round of trials. Arenas were placed side-by-side with 10-30cm spacing between each. Opaque black plastic sheets surrounding each arena up to the water line minimized visual disturbances from the surroundings and prevented any visual contact of fish from adjacent arenas. A black curtain surrounded the entire array of behavioural arenas to maintain constant light levels and prevent any outside visual disturbances. An eight mega-pixel camera was mounted above each behavioural arena (8 cameras total) – cameras were connected to a digital recording system (Swann, 16 Channel Digital Video Recorder) to allow for simultaneous recording across the eight trials.

To commence behavioural trials, three fish from the same experimental group were carefully and randomly netted into each test arena. Each round of behavioural trials consisted of twelve enriched fish and twelve non-enriched fish evenly spread across the eight behavioural arenas (four arenas containing only enriched fish and four arenas containing only non-enriched fish). Behaviour was recorded from when the fish entered the test arena and continued for 120 minutes. Two 1-minute video snap-shots were analyzed to quantify the proportion of time spent immobile (motionless) and the proportion of time spent near the wall of the arena (Salvanes 2017) (video analysis described in detail below): the first 1-minute video snap-shot was collected 5 minutes after the test fish entered the open-field test arena (5-minute timepoint) and the second 1-minute video snap-shot occurred

35 minutes after the fish entered the open-field test arena (35-minute timepoint). The two timepoints were used to compare the behavioural response immediately after transport (5-minute timepoint) and shortly after transport (35-minute timepoint). Following behavioural trials, fish were removed from test tanks, anesthetized with buffered tricaine methanesulfonate (MS-222), imaged with a digital camera, weighed, and fork length measured.

Transport stress

Beginning in the morning on each transport day, 12 fish from each rearing treatment (enriched and non-enriched rearing) were transferred in 20L buckets with water to the test arenas to commence behavioural trials – these fish were considered as 'non-transport' group fish (see Figure 4.2). Transfer of fish from rearing tanks here, and for all sampling points, was done carefully to minimize disturbances to the fish as we sampled repeatedly from the same tanks throughout the experiment. The four structures were removed from the enriched tank prior to netting to prevent potential bias in fish selection during the netting process. A similar disturbance was mimicked prior to netting from the non-enriched tank – the experimenter submerged their hand into the water and mimicked the act of removing structures from the tank prior to netting. We then transferred 36 fish from each rearing tank (enriched and non-enriched) into 45L-capacity recirculating live-well coolers for transport. Bilge pumps in the live-well coolers were powered by 12V marine batteries. Based on a subsample of fish mass measurements one

week prior to transport, live-well coolers transporting enriched fish were filled to 20L from the rearing water source and live-well coolers transporting non-enriched fish were filled to 35L for a final transport density of *ca.* 8-9g/L per live-well. Water temperature in each live-well cooler was monitored and maintained within 1°C of the rearing tank water temperature (17°C ± 1).

Once 36 fish were transferred to live-well coolers from each of their respective rearing tanks, the live-well coolers were loaded onto the cargo-bed of the transport vehicle and secured with straps to prevent any excess movement. Fish were transported for *ca.* 4 hours over a total distance of *ca.* 350km. The transport vehicle was driven at a speed of 100km/hr (highway driving) for the majority of time during the transport. Two stops were made during each transport to check water temperature – during each stop, 500mL of water was removed from each tank and replaced with 500mL of crushed dechlorinated ice to maintain water temperature within 1°C of the rearing tank temperature. Upon return to FREC, 12 fish from each live-well were carefully netted into 20L buckets of water (same source water as the experimental arenas) and transferred to the experimental tanks to commence the behavioural open-field trials – these fish were considered as 'transport' group fish.

Recovery from transport – novel environment

The remaining 24 fish from each live-well cooler (24 enriched, 24 non-enriched), post-transport, were transferred to separate recovery tanks per group (12 fish per recovery tank). An additional 12 fish from each rearing treatment (non-transported) were simultaneously transferred to separate recovery tanks. Recovery tanks were novel, barren environments– measuring at 58cm by 28cm and filled to a depth of 40cm. Recovery tanks temperatures were maintained within 1°C of the original rearing tanks. A plastic mesh was placed over the recovery tanks to prevent fish from jumping into adjacent tanks. Fish were left in the recovery tanks for 7 days and fed commercial aquaculture pellets at the same rate as the original rearing (1% body weight per day). On the morning of the 7th day post-transport, the non-transported recovery groups (12 enriched, 12 non-enriched) were transferred from their respective recovery tanks to commence behavioural trials. Timing of non-transported recovery behavioural trials was similar to those of the non-recovery groups (described above). On the afternoon of the 7th day post-transport, the transport recovery groups (12 enriched, 12 non-enriched) were transferred from their respective recovery tanks to commence behaviour trials.

Behavioural video analyses

Each 1-minute timepoint (5minutes and 35minutes) was scored for behavioural measures (using Solomon Coder (https://solomon.andraspeter.com), by an experimenter blind to the specific treatment) including: time spent near the wall of the tank and time spent motionless. To match fish from the 5-minute

timepoint to the 35-minute timepoint, we compared relative sizes of fish between the two timepoints using ImageJ software (Schneider et al. 2012). To facilitate behavioural coding, a transparent sheet was placed over the monitor to overlay the video recording and a grid was drawn to mark a 8cm perimeter in from test tank wall (Salvanes 2017) (see Figure 4.3). The area between the lined perimeter and the walls was denoted 'near wall'. Time spent near the wall was defined as the total time the fish spent with its entire body within the 'near wall' area, or when the fish had any part of its body in contact with the wall (Salvanes 2017; Latchem et al. 2021). Time spent motionless was defined as the total time the fish spent not moving – moving is defined as a change of location by at least half a body length (Mokdad et al. 2022b).

Statistical analyses

Proportional data for time spent near the wall and time spent motionless were analyzed using generalized linear models, assuming quasi-binomial distribution and logit as a link function (Salvanes 2017). Separate analyses were run for proportion of time near wall and proportion of time motionless at each of the two time-points (resulting in four separate analyses). Rearing treatment (enriched v. non-enriched), transport (transported v. non-transported), and recovery were fixed effects; mass and replicate were covariates; tank ID was considered a random factor. Fish in the enriched rearing treatment were significantly smaller (mean ± s.d. = 4.87g ± 1.92) than non-enriched fish (7.34 ±

2.71), which is a common finding throughout the enrichment literature (see Solås et al. 2019; Salvanes 2021) however, mass had no significant main effects or interactions between any of the fixed effects for any of the models and so was only included as a covariate. Three-way interactions between the fixed effects, and any two-way interactions were analyzed, followed by post-hoc tests for significant interactions – simple two-way interactions and pairwise comparisons were Bonferroni-corrected.

Data analyses was performed using R language for statistical computing (ver. 4.1.0; R Foundation for Statistical Computing) with RStudio version 1.4.1106 (RStudio Team 2021).

4.3 Results

Time spent near wall

5-minute timepoint

There were no significant main effects for any of the fixed factors or interaction for time spent near wall at the first timepoint (Figure 4.4a) – rearing treatment: $t(1,163) = -1.33$, $p = 0.19$; transport: $t(1,163) = 1.07$, $p = 0.29$; recovery: $t(1,163) = -1.66$, $p = 0.10$, rearing treatment x transport x recovery: $t(1,163) = 1.47$, $p = 0.14$.

35-minute timepoint

There was a statistically significant three-way interaction effect between rearing, transport, and recovery treatments (Figure 4.4b), $t(1,163) = 2.30$, $p = 0.023$. There was a significant two-way interaction between enrichment and transport for non-recovery group fish, $F(1,83) = 5.59$, $p = 0.02$ but not for the recovery group $F(1,78) = 0.82$, $p = 0.37$. There was a significant main effect of enrichment for non-transported, non-recovered fish $F(1,42) = 6.16$, $p = 0.017$, but not for transported, non-recovered fish $F(1,39) = 0.95$, $p = 0.34$. This analysis indicates that enrichment has a significant effect on time spent near wall for non-transported fish, non-recovered fish, but not for transported, non-recovered fish. Estimated marginal means for these groups indicate that enrichment led to an increase of 14.1% in proportion of time spent near wall. The proportion of time spent near wall (mean ± s.e.) for non-transported, non-recovery, enriched fish was 97.8% ± 0.036 and 83.7% +0.036 for non-transported, non-recovery, non-enriched fish.

There was also a significant two-way interaction between transport and recovery for non-enriched fish $F(1,81) = 7.32$, $p = 0.008$, but not for enriched fish $F(1,80) = 0.058$, $p = 0.81$. There was a significant main effect of transport for non-enriched, non-recovery fish $F(1,42) = 6.55$, $p = 0.014$, but not for non-enriched,

recovery fish $F(1,37) = 3.18$, $p = 0.083$. This analysis indicates that transport has a significant effect on time spent near wall for non-enriched, non-recovered fish, but not on non-enriched, recovery fish. Estimated marginal means for these groups indicate that transport led to an increase of 15% in proportion of time spent near wall. The proportion of time spent near wall (mean ± s.e.) for non-enriched, non-recovered, transported fish was 98.7% ± 0.035, and 83.7% ± 0.036 for non-transported counterparts.

Time spent motionless

<u>5-minute timepoint</u>

There was no significant three-way interaction between rearing treatment, transport, and recovery on proportion of time spent motionless (Figure 4.5a), $t(1,163) = -1.49$, $p = 0.14$, however, there was a significant two-way interaction between transport and recovery $t(1,163) = 2.09$, $p = 0.037$ at both levels of enrichment (enriched and non-enriched groups). There was a significant main effect of transport for non-enriched, non-recovery fish, $F(1,42) = 29.9$, $p < 0.001$ and for enriched, non-recovery fish $F(1,39) = 12.0$, $p = 0.001$, but not for non-enriched, recovery fish, $F(1,37) = 2.82$, $p = 0.10$. This analysis indicates that transport has a significant effect on time spent motionless for non-recovery fish at both levels of enrichment, but not for recovery fish. Finally, there was a significant simple main effect of recovery for non-transported, non-enriched fish, $F(1,43) =$

15.7, $p < 0.001$, indicating an effect of recovery on time spent motionless in the absence of transport. Estimated marginal means for these groups indicate that transport led to a 51% decrease in proportion time spent motionless for non-enriched, non-recovery fish and a 33.5% decrease for enriched, non-recovery fish. The proportion of time spent motionless (mean ± s.e.) for non-enriched, non-recovery, non-transported fish was 99.0% ± 0.075 and 48.4% ± 0.074 for transported counterparts. For enriched, non-recovery, non-transported fish, proportion of time spent motionless was 78.7% ± 0.075 and 45.2% ± 0.080 for transported counterparts. Recovery led to a 31.7% decrease in proportion of time spent motionless for non-enriched, non-transported fish. The proportion of time spent motionless for non-enriched, non-transported, non-recovery fish was 99.0% ± 0.075 and 67.3% ± 0.074 for recovered counterparts.

35-minute timepoint

There was a significant main effect of recovery (Figure 4.5b), $t(1,163) = -2.95$, $p = 0.0036$, and a significant main effect of rearing environment (enrichment), $t(1,163) = -2.21$, $p = 0.028$, on proportion of time spent motionless. Estimated marginal means show that, overall, fish in the recovery group spent a lower proportion of time motionless (mean ± s.e. = 18.3% ± 2.6) compared to non-recovery fish (28.9% ± 3.3). Enriched fish spent a lower proportion of time motionless (19.1% ± 2.9) compared to non-enriched fish (28.3% ± 3.2).

4.4 Discussion

Behavioural responses to stress are often dichotomized into pro-active ('fight-flight') – indicated by active attempts to counteract stressful stimuli, including increased movement activity– and reactive ('conservation-withdrawal) responses – indicated by attempts to avoid being detected by potential predators, including sheltering and reduced movement (Øverli et al. 2007). A study of masu salmon (*Oncorhynchus masou masou*), linking individual behavioural responses to survival in a semi-natural stream, found that reactive behavioural responses were the most critical determinants of survival from a live predator (Miyamoto and Araki 2020). Furthermore, a study comparing the behavioural response of wild and captive-bred Atlantic Salmon, found that wild fish were more reactive (i.e. they spent more time immobile and near the wall of the open field arena) compared to the more proactive captive-bred fish (Salvanes 2017). We hypothesized that rearing with structural enrichment would lead to more 'wild-like' behaviour for Atlantic Salmon parr– we predicted that enriched fish would be less active and spend more time near the wall in an open-field test (i.e., exhibit a reactive behavioural response). First, our results show that enrichment did indeed lead to a phenotypically plastic response – an increase in proportion of time spent near the wall for enriched fish compared to non-enriched fish – however, this effect was present for behavioural observations made at the 35-minute timepoint and not observations made at the 5-minute timepoint, and only for non-recovery groups.

Contrary to our prediction, enriched fish were more active (spent less time motionless) compared to non-enriched fish. These findings are in line with previous findings that enrichment leads to increased levels of exploratory behaviour in fish (Lee and Berejikian 2008) and a decrease in time spent immobile for Atlantic Salmon parr (Salvanes 2021). The increased activity exhibited by the enriched fish, compared to the non-enriched fish can potentially be explained by an increased tendency to avoid the stressful novel environment in search of shelter (Näslund et al. 2013). This might also explain the different timing of effects of enrichment on the two behaviours measured – enriched fish show an increased tendency to find structural shelter (less time spent motionless – more time spent active), followed by a resort to an increase in time spent near the wall as a reactive response in the absence of physical structure. The difference in behaviour of the enriched fish and the wild fish in the aforementioned study (Salvanes 2017) can further be explained by a difference in rearing environment of the wild fish in that study prior to behavioural testing. Salvanes (2017) reared wild fish in barren tanks (similar to the testing arena in our current study) for *ca.* one month prior to behavioural testing. Because the test arena in our current study was likely more similar to the barren rearing environment, fish from the structurally enriched environment may have perceived the test arena as a higher risk (and potentially a more severe stressor) than fish reared without structural enrichment (Watz 2019), leading to a difference in observed behaviour patterns between our enriched fish and wild fish from the previous study (Salvanes 2017). Future studies should aim to test enriched and non-enriched fish in barren and structurally enriched

conditions to determine the effects of environmental novelty on the behavioural responses from both groups of fish. Furthermore, comparing fish reared under structurally enriched environments with wild-fish under similar conditions could better test the hypothesis that rearing with structural enrichment produces fish with more 'wild'-like behavioural phenotypes. This has important implications for the generally regarded hypothesis that experience with rearing stimuli leads to an increased probability that an individual will select or show less of a stress response to habitats that contain similar stimuli (Davis and Stamps 2004).

Second, we explored the effects of transport on anti-predator-related behaviour and to our knowledge this is the first study to report on such behavioural responses to transport stress. Our results show that transport had a similar effect on behavioural response in the open-field test to enrichment – non-enriched fish: transported fish spent more time near the wall and less time motionless compared to non-enriched, non-transported fish. This would suggest that the stress-coping response of fish to transport is to increase activity and increase the use of shelter (staying near the wall). Indeed, an increase in swimming activity is a common acute-stress coping behaviour in fishes (Svendsen et al. 2021). We further wanted to test whether rearing with structural enrichment would affect the behavioural stress response to transport. A recent study of Chinook salmon (*Oncorhynchus tshawystscha*) demonstrated a reduced physiological stress response to transport for fish reared with structural enrichment compared to fish reared in barren tanks (Cogliati et al. 2019). We found no such effect of enrichment between the enriched and non-enriched fish in our study. In our current study, rearing in

structural enrichment and transport both had the effect of increasing activity level and time spent near shelter (near the wall) in the open-field test. This further supports the idea that the barren environment of the open-field test served as an acute stressor to fish reared with structural enrichment, bolstering the proposition to include an experimental manipulation of the open-field test arena (with and without structure) in future studies.

Third, and finally, we were interested in the effect of post-transport recovery on the behavioural response to stress and enrichment. Post-transport recovery has been a common tactic employed to improve post-release movement behaviour and survival of hatchery-reared fishes in the wild (Sandodden et al. 2001; Hutchison et al. 2012; Tetzlaff et al. 2019; Mokdad et al. 2022a). Furthermore, rearing environment has been shown to promote recovery and ameliorate the effects of acute stressors in laboratory settings (Pounder et al. 2016). In our current study, however, we found no such interaction between enrichment and recovery. We instead found that fish in the recovery group, particularly those that were not subjected to transport but were transferred from rearing tanks to recovery tanks for one week prior to testing, showed a decrease in time spent motionless (an increase in activity) during behavioural testing. This would suggest that the novel recovery environment or subsequent transfer to the testing environment served as an acute stressor to the fish. This is in line with previous studies showing post-transport recovery in new environments as stressful events for fishes (Nikinmaa et al. 1983; Vieira Madureira et al. 2019). In order to better assess the effects of post-transport recovery in the laboratory, future studies

might benefit from behavioural testing in the novel recovery environment, foregoing subsequent transfer to a new testing arena.

While we did find that enrichment promotes a phenotypically plastic behavioural response in hatchery-reared Atlantic Salmon – enrichment led to more reactive/risk-averse fish in the absence of external stressors – neither enrichment, nor post-transport recovery had a significant effect on the behavioural response to transport stress. Indeed, a previous study of structural enrichment of hatchery-reared Atlantic Salmon fry found that structural enrichment alone was not sufficient to improve the post-release survival (Solås et al. 2019). The authors suggest that the size of the released fish is an important trait to the survival of fishes, as they found a size-selective feeding pattern by predators. We found, in accordance with previous findings on Atlantic Salmon, enriched fish to be significantly smaller in size compared to non-enriched counterparts (Rosengren et al. 2017; Solås et al. 2019). There is a possibility that enrichment might lead to a preference for hiding instead of feeding if shelters are available. Future enrichment studies should monitor the feeding behaviour across rearing treatments to test this hypothesis. Finally, to better assess the effects of enrichment on wild release, and to potentially improve the survival of hatchery-reared fish, future wild-release studies should size-match fish from rearing treatments prior to release.

4.5 References

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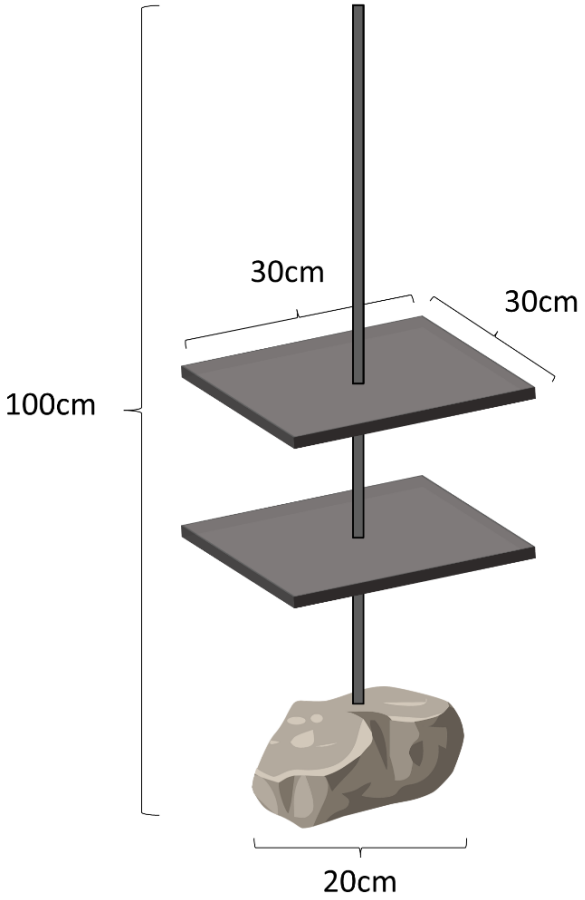

Figure 4.1 Schematic representation and dimensions of the physical enrichment structures used for the enrichment rearing treatment of Atlantic Salmon (*Salmo salar*). Four structures total were added to each of the enrichment tanks along with a single layer of rock substrate covering approximately 75% of the rearing tank floor. See Methods section for details.

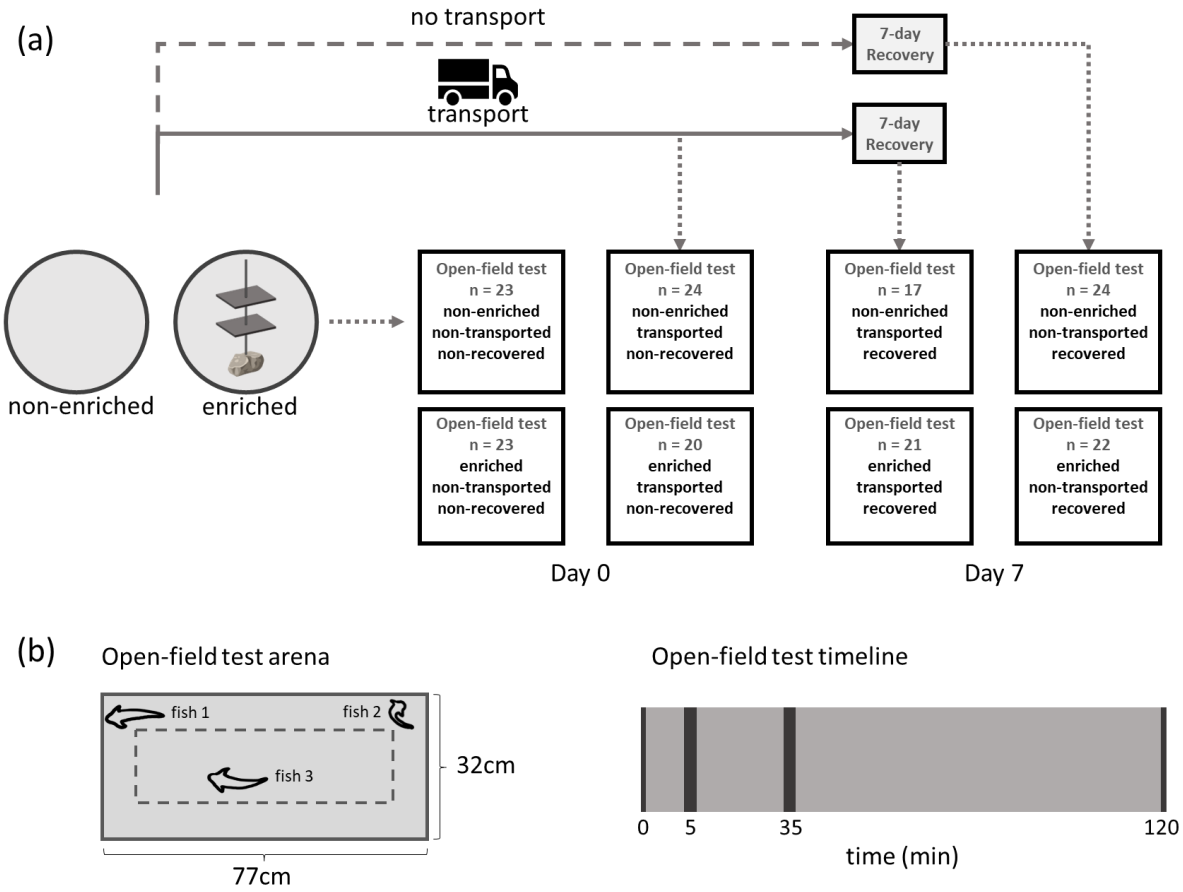

Figure 4.2 a) Schematic overview of enrichment, transport stress, and recovery phases of the experiment. Dotted arrows represent transfer of fish from each experimental group to open-field test arenas for behavioural testing. Dashed arrows represent transfer of fish from rearing environment to recovery environment without transport. Solid arrows represent fish transport. Sample size for each behavioural trial is represented. Treatment groups for each behavioural are described within each open-field test box. b) Schematic representation and dimensions of the open-field test arena and timeline used for behavioural trials. Open-field test arena: dashed line represents an 8cm inset boundary demarcating the border between the open area and the area near the wall. The area between the wall and the dashed line represents the area near the wall. Fish 1 and fish 2 represent fish that would be considered 'near the wall' and fish 3 represents a fish that would be considered in the open area. Open-field test timeline: timepoint 0 represents the time fish entered the test arena. Timepoints 5 and 35 (dark-shaded area) represent the two1-minute video snap-shots analyzed to quantify behaviour (5-minute timepoint and 35-minute timepoint). Video recording in the open-field test lasted 120 minutes.

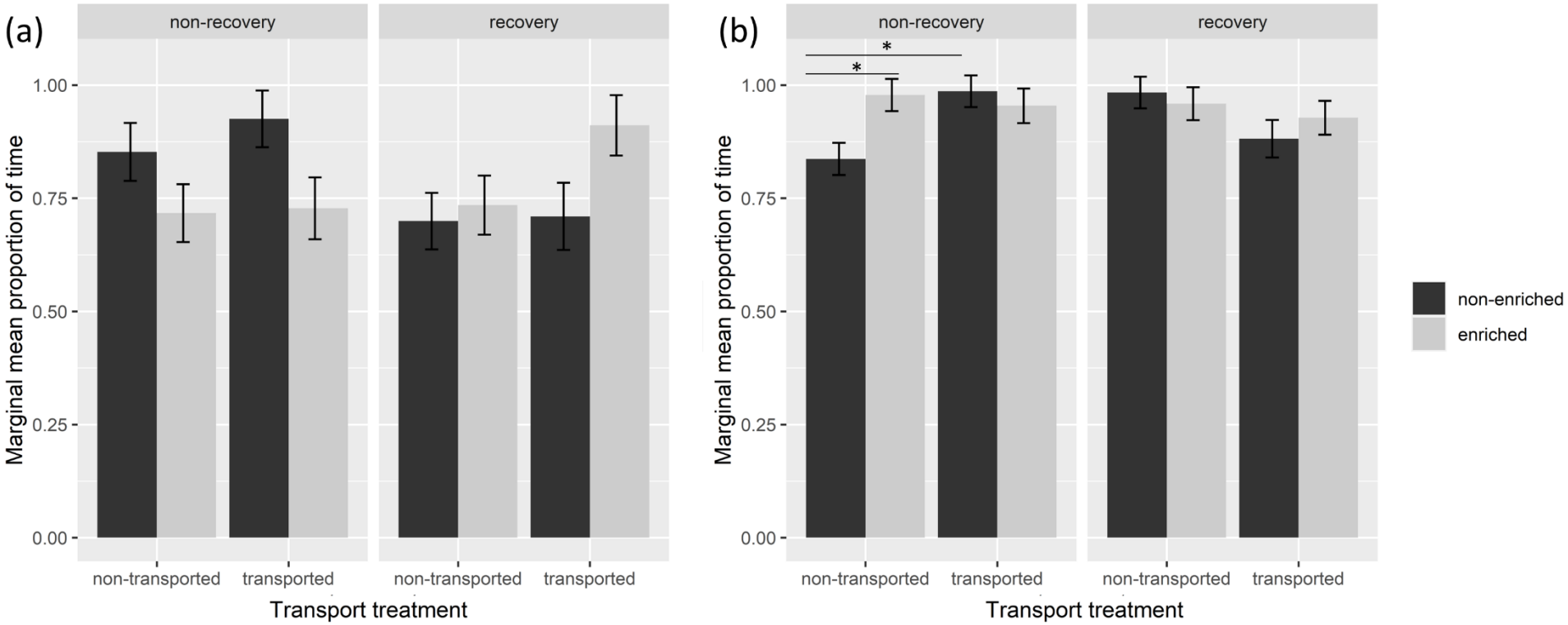

Figure 4.3 Estimated marginal means for the proportion of time spent near the wall for non-enriched (black bars) and enriched (grey bars) fish across transport treatment (non-transported and transported) and recovery group treatment (non-recovered and recovered). Error bars represent standard error of the means. Panel (a) represents data from the 5-minute timepoint (5 minutes after fish were introduced to the open-field test tank) and panel (b) represents data from the 35-minute timepoint (35 minutes after fish were introduced to the open-field test tank). Asterisks represent statistically significant differences between groups. Generalized linear models included mass and replicate as covariates, enrichment, transport, and recovery as fixed effects, and tank ID as random effect (see methods for details).

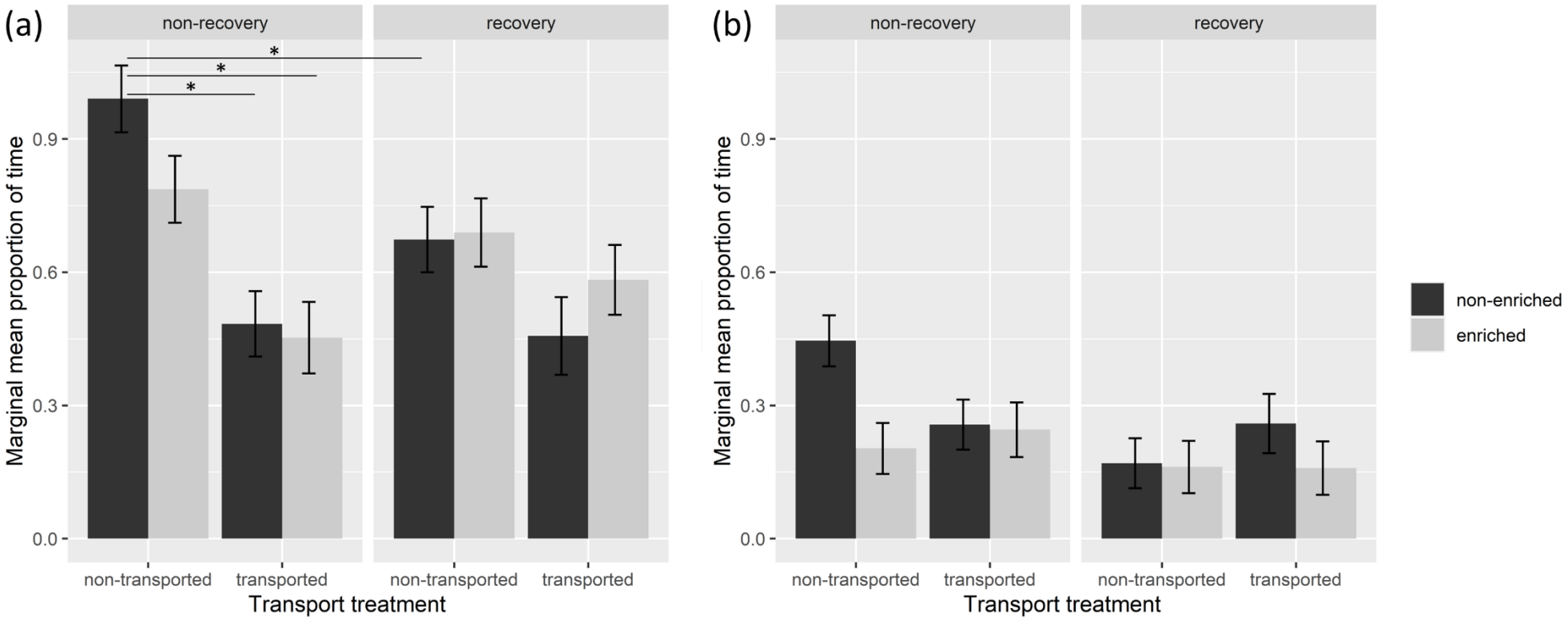

Figure 4.4 Estimated marginal means for the proportion of time spent immobile for non-enriched (black bars) and enriched (grey bars) fish across transport treatment (non-transported and transported) and recovery group treatment (non-recovered and recovered). Error bars represent standard error of the means. Panel (a) represents data from the 5-minute timepoint (5 minutes after fish were introduced to the open-field test tank) and panel (b) represents data from 35-minute timepoint (35 minutes after fish were introduced to the open-field test tank). Asterisks represent statistically significant differences between groups. Generalized linear models included mass and replicate as covariates, enrichment, transport, and recovery as fixed effects, and tank ID as random effect (see methods for details).

CHAPTER 5

TESTING FOR BEHAVIOURAL EVIDENCE OF OLFACTORY IMPRINTING AT PARR AND SMOLT STAGE OF ATLANTIC SALMON

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5.1 Introduction

Stocking of hatchery-reared salmon has become a common practice for conservation biologists and wildlife managers in the attempt to supplement wild populations, enhance fisheries, and, in particular, as part of conservation-based reintroduction efforts (Stewart et al. 2014; Prindle & Bishop 2020). While these practices have become common, many reintroduction efforts fail to produce self-sustaining populations in the wild (Cochran-Biederman et al. 2015). For instance, despite the release of millions of hatchery-reared Atlantic Salmon (*Salmo salar*) to the inner Bay of Fundy, the yearly return of adults to natal rivers to spawn is between 0 and 10 (reviewed in Lamothe et al. 2019). The leading causes of conservation translocation failure, reported by wildlife managers and researchers, have been issues related to movement and dispersal behaviour of translocated animals (Berger-Tal et al. 2020). A large body of study has focused efforts to understand migration behaviour, and to develop hatchery rearing and release practices to increase fidelity to targeted spawning locations (e.g. Bett & Hinch 2016a; Stewart et al. 2014).

Salmonids are one of the best-studied groups of migratory fish that are capable of natal homing – a return to natal waters to spawn after feeding at distant locations – a phenomenon that relies largely on olfactory directional cues for navigation (Wisby and Hasler 1954). To explain olfactory natal homing, Bett and Hinch (2016a) hypothesized that juvenile salmonids imprint to the chemical signature of their natal stream before migrating to feeding sites and retrieve this

olfactory information as the primary directional cue to home back to their natal stream to spawn. Olfactory imprinting is a form of unconditioned learning wherein olfactory information is acquired during a sensitive or critical period of development and then retrieved and used in a behavioural context later in life (Nevitt and Dittman 1999). Hasler and Scholz (1983) hypothesized that salmon imprint on a 'bouquet' of odours in their rearing environment, but a number of studies have demonstrated that fish are able to imprint to and distinguish between single imprinting odorants such as morpholine (Hara and Brown 1979), phenylethyl alcohol (Bett et al. 2016), and L-arginine (Armstrong et al. 2021) added to complex natural waters. Experiments in which a single imprinted odorant was added to natural water, in an attempt to increase salmonid returns, have produced mixed results. For example, hatchery-reared Coho Salmon (*Oncorhynchus kisutch*) that were exposed to morpholine at the pre-smolt and smolt stage of development returned at higher rates after wild release to a targeted stream that was artificially scented with morpholine, than individuals that were never exposed to morpholine (Cooper et al. 1976). In contrast, exposing juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) to morpholine did not affect the adult return rates to artificially scented streams compared to non-exposed salmon (Hassler and Kutchins 1990).

The sequential imprinting hypothesis posits that fish can imprint at different stages of juvenile development and way points along their natal river systems – beginning at emergence from natal gravel and continuing during their residence and downstream migration through natal river systems (Harden-Jones

1968; Ueda 2018). The majority of imprinting studies have focused on imprinting during the parr-smolt transformation stage of development (reviewed in Bett & Hinch 2016a; e.g. Havey et al. 2017), but a new paradigm for hatchery programs has been proposed to include embryonic imprinting as a tool to increase homing fidelity prior to release (Dittman et al. 2015). The embryonic imprinting paradigm suggests that if exposure to target spawning water chemistry or artificial odorants added to rearing water is sufficient to induce imprinting, then conservation managers should be able to imprint fish to odorants at early developmental stages and release fish into the wild prior to the parr-smolt transformation stage. Embryonic imprinting is supported by experimental evidence that at the time of their homing migration, adult Sockeye (*Oncorhynchus nerka*), Pink (*Oncorhynchus gorbuscha*), and Atlantic Salmon exhibit a preference for artificial odorants they experienced during their embryonic stage (Bett et al. 2016; Havey et al. 2017; Armstrong et al. 2021). While evidence suggest that salmonid fry, at the time of their emergence from gravel, exhibit a preference for water in which they were incubated (Bodznick 1978; Dittman et al. 2015), it remains unclear whether fish show preference for natal water at other developmental stages, prior to the homing adult stage.

Captive-bred salmonids, including Atlantic Salmon, exhibit maladaptive migratory behaviour and higher straying rates compared to wild conspecifics– possibly linked to a lack of imprinting to targeted return sites (Horreo et al. 2017). Atlantic Salmon in Lake Ontario was the most striking example of a landlocked freshwater population of the species in the world (Smith 1995; Webster 1982).

Fish migrated up the tributaries by the thousands, but the species was extirpated from Lake Ontario by ~1900. Recent efforts to restore Atlantic Salmon to the highest quality tributary in New York state has had some success (Prindle & Bishop 2020). However, poor returns of experimentally released fish and documented straying suggests that homing to release sites would enhance this restoration effort. The ability to detect evidence of imprinting in captive-bred salmon prior to release might be a useful confirmation tool for managers whose aims are to increase fidelity of homing fishes to targeted return sites.

In this study, we sought to determine whether we could detect behavioural evidence of imprinting at two juvenile life stages (parr and smolt) in Atlantic Salmon after embryonic imprinting to a single artificial odorant added to rearing water. Specifically, we exposed Atlantic Salmon to morpholine during the hatching and alevin stage, and behaviourally assessed the attraction to this odorant at the parr and smolt stage of development using a two-choice preference test (Jutfelt et al. 2017). Behavioural differences in the preference for natal cues will be tested and compared between these groups – evidence for imprinting will be assessed based on differences in preference for natal cues between these groups. Parr and smolt represent distinct juvenile riverine life stages prior to the lacustrine or oceanic adult feeding stage and Atlantic Salmon are commonly stocked at these stages (Stewart et al. 2014; Prindle and Bishop 2020). Most imprinting studies that utilize behavioural choice tests to demonstrate evidence of imprinting, analyze and report data as an average time spent in each water type across the entire trial (for examples of two-choice behavioural preference tests, see Bett and Hinch 2015;

Havey et al. 2017; Armstrong et al. 2021). However, Jutfelt et al. (2017) suggest that side preference should, instead, be analyzed over time because it is possible that by averaging time spent in each water type, preference for a particular cue can be masked by the dynamic activity of a fish in each trial. Analyzing behavioural preference data over time can also provide information on the time required for a fish to respond to a cue. In this study, we use a two-choice chamber to test for behavioural preference to a water source that either does or does not match rearing water chemistry – that is, a water source with or without the addition of morpholine. We analyze our behavioural imprinting data using the two methods – averaging time spent in each arm for each trial and analyzing time spent in each arm binned into one-minute intervals across the entire trial – and compare the response results for both parr and smolt stages.

5.2 Materials and methods

Experimental Animals and Odorant Exposure

Eggs and sperm used to generate the experimental Atlantic Salmon were collected from separate populations of wild males and females. On 7 November 2018, eggs were collected from wild spawning females (n=5) at the Cayuga Inlet (Ithaca, NY) at the NYSDEC fish ladder and sperm was collected from spawning wild males (n=6) at Salmon River (Altmar, NY) held at the New York State Department of Environmental Conservation Salmon River Hatchery. Gametes

were transported to the Tunison Laboratory of Aquatic Science (Cortland, NY), fertilized, and transferred to hatching trays. Fertilization crosses were made using a 1:2 (1 female to 2 males) breeding scheme. At approximately 29 days post-fertilization (dpf), eyed gametes were separated by treatment group (morpholine-treated or control) and transferred to triplicate cylindrical tanks (12.7cm x 55.88cm, with 28.3L volume). Tanks were set up as flow through systems with individual flow rates of 0.5L/min of source water (mixed spring and well water). A stock solution of 0.5M morpholine dissolved in source water was continuously delivered to the treatment group tanks via a peristaltic pump (Masterflex L/S model 07522–30, Cole-Parmer, QC, Canada) to achieve a final exposure concentration of $5x10^{-5}$M morpholine. Morpholine (Sigma Aldrich, MO, USA) treatment began at 49dpf (26 December 2018) and continued until 119dpf (3 June 2019) with a 27-day window (between 5 January 2019 and 1 February 2019; 59dpf and 86dpf, respectively) of no morpholine exposure due to a lack of supply of morpholine. After morpholine/control exposure, fish were moved to round tanks (84 x 41cm, at 189L) where they remained in communal treatment groups until behavioural trials. On 12 December 2019, the experimental fish were implanted with passive integrated transponder (PIT) tags which allowed for unique identification of individual fish. As fish outgrew enclosures, they were moved to larger round tanks (152 x 76cm, at 2000L) and finally into a single outdoor raceway (5.94 x 1.52 x 0.46m at 4118L). Throughout the experimental rearing, water temperatures fluctuated naturally and ranged between approximately 5°C

and 10°C. The fish were fed commercial salmon feed, ad libitum, throughout the experiment.

Behavioural trials

Parr Behaviour

Behavioural trials were first conducted on a subset of fish (20 morpholine-treated fish, and 20 control fish) at the parr stage between 12 February 2020 and 26 February 2020 (462dpf and 476dpf, respectively). This life stage and time of year is consistent with natal stream overwintering of wild counterparts (Hutchings et al. 2019). A two-choice chamber was used to test the behavioural responses of Atlantic Salmon to morpholine (see Figure 5.1). The design of the chamber was a modified version of those described by Jutfelt et al. (2017). The two-choice chamber was constructed from glossed acrylic and measured (20 x 20 x 98 cm). An opaque divider (66cm long) separated the upstream reach of the choice chamber into two separate arms. Each arm of the chamber was supplied by a head tank (40 x 50 x 40 cm) feeding into the arms. Source water was pumped into each arm of the maze at a rate of approximately 50L/min and dye tests were regularly run to ensure no water exchanged between the two arms. Water depth was maintained at 5 centimeters. Using the peristaltic pump, a stock solution of (stock concentration) morpholine was dripped into one of the two arms (randomly assigned arm for each trial) to achieve a final concentration of $5x10^{-5}$M. This

concentration is above the threshold of detection for salmonids (Cooper et al, 1976). The choice chamber was flushed with source water for five minutes between trials to limit the possibility of traces of morpholine affecting subsequent trials. The arm receiving morpholine was randomly alternated between trials such that there was an equal number of trials with morpholine in each arm.

At the start of each behavioural trial, a randomly chosen individual was transferred from either the treatment or control tank to the choice chamber and acclimated to the chamber for eleven minutes before morpholine was dripped into the designated arm. Fish activity was monitored with a camera (AKASO 4K action camera) positioned overhead such that the entire two-choice chamber was captured within the field of view. We coded the position of the fish at each 0.2 second intervals for the duration of recording using the behaviour coding software Solomon Coder (Version Beta: 17.03.22). From these raw data, we extracted the amount of time spent in each arm at each 0.2 second intervals.

After trials were completed (26 February 2020), each experimental fish was anesthetized with tricaine methanesulfonate (MS-222) and fork length and mass were recorded and the fish was returned to its home tank. The fork lengths and masses (mean ± standard deviation) of the parr at the time of the trials were 165.06 ± 14.60 mm and 40.69 ± 9.35 g for control (n=18) and 172.50 ± 16.54 mm and 46.88 ± 11.58 g for morpholine exposed (n=12) fish. No significant difference in mass ($t = -1.54$, $df = 20.18$, $p = 0.14$) or standard length ($t = -1.27$, $df = 21.62$, $p = 0.22$) was found between the two treatment groups (morpholine and control).

Smolt Behaviour

Smolt behavioural trials were conducted between 9 August 2021 and 17 August 2021 (1006 dpf and 1014 dpf, respectively). The life-stage and time of year are consistent with downstream migration away from the natal stream in wild Atlantic Salmon (Prindle and Bishop, 2020). A two-choice chamber was constructed from plywood and fish-safe epoxy, with similar design features as the one described for the parr experiments above, but with larger dimensions (81 × 33 × 300 cm) to accommodate larger fish. Source water was pumped into each arm of the maze at a rate of approximately 80L/min and dye tests were regularly run to ensure no water exchanged between the two arms. Using the peristaltic pump, a stock solution of morpholine was dripped into one of the two arms (randomly assigned arm for each trial) to achieve a final concentration of $5x10^{-5}$M. At the start of each behavioural trial, a randomly chosen Atlantic Salmon was transferred from either the treatment or control tank to the choice chamber and acclimated to the chamber for fifteen minutes before morpholine was dripped into the randomly designated arm. The fish were acclimated in the chamber for a further five minutes before recording was initiated. Activity was recorded for twenty minutes. Finally, we extracted the same metrics of behavioural activity as for the parr behavioural trials, described above. The fork lengths and masses (mean ± standard deviation) of the smolts at the time of the trials were 338.2 ± 19.3 mm and 327.36 ± 63.74 g for control (n=20) and 349.5 ± 28.8 mm and 378.02 ± 100.46 g for morpholine

exposed (n=13) fish. We found a significant difference in mass ($t = -2.3$, $df = 38.1$, $p = 0.03$) between the two treatment groups (morpholine and control) – morpholine-exposed individuals were significantly heavier than control individuals. We found no significant difference in standard length between the two groups ($t = -1.8$, $df = 39.5$, $p = 0.09$).

Statistical Analyses

Parr analyses

For the first method of analysis – what we henceforth refer to as 'time-averaged analysis' – we analyzed time spent in morpholine or control arm as a total combined (5 minute) time spent across the entire trial (Bett et al. 2016; Armstrong et al. 2021). First, for each treatment group, we compared time spent in each arm using a Wilcoxon signed rank test to (Bett et al. 2016). We then compared proportion of time spent in the arm with morpholine using a one sample t-test ($\mu = 0.5$, $\alpha = 0.05$) to determine whether fish spent a proportion of time in morpholine that differed from 0.5 (Bett et al, 2016). Finally, to test for a difference in preference for the morpholine arm, we compared the time spent in the morpholine arm between treatment groups using a Mann-Whitney U-test.

In contrast to the time-averaged analysis, we used a mixed-model to compare the time spent in the morpholine arm, between treatment groups, with the data binned into one-minute segments (a total of five one-minute bins) (as

suggested in Jutfelt et al, 2017). We refer to this type of analysis – including a continuous factor of time – as a time-sensitive analysis. We used time spent in the morpholine arm as the response variable, time-point (minutes after the acclimation period), treatment group (morpholine-treated or control), the interaction between trial time and treatment as fixed effects, and the fish ID of each test animal as a random effect. Mass was initially included as a covariate but was found to be non-significant and removed from the overall model.

Smolt analyses

Similar to the parr analysis, we conducted time-averaged analysis, comparing the total combined time spent in each arm (20 minutes total) using a Wilcoxon signed-rank test, for each treatment group, and the proportion of time spent in the arm with morpholine (across the entire 20-minute trial) using a one sample t-test ($\mu = 0.5$, $\alpha = 0.05$). We then compared the time spent in the morpholine arm between the two treatment groups using a Mann-Whitney U-test.

We then conducted a time-sensitive analysis, using a mixed model to compare the time spent in the morpholine arm, between treatment groups, with data binned into one-minute segments (with twenty, one-minute bins per trial period). We used time spent in the morpholine arm as the response variable, time-point, treatment group, the interaction between trial time and treatment group as fixed effects, and the fish ID of each test animal as a random effect. Because body mass differed between the two treatment groups, it was initially included in the

model as a covariable but was removed because it had no significant effect on the response variable.

All statistical analyses were conducted in R Studio version 1.4.1103. Linear mixed-effects models were run using the lme4 package (Bates et al. 2015).

5.3 Results

Parr behaviour

With the time-averaged analysis, we found no significant difference in time spent in either arm for parr exposed to morpholine as alevin ($W11 = 76$, $p = 0.83$) or fish that were never exposed to morpholine ($W17 = 187$, $p = 0.44$) (Figure 5.1A). Morpholine-treated parr did not spend a proportion of time in the morpholine arm that differed from 0.5 ($t11 = -0.12$, $p = 0.91$). Similarly, control parr did not spend a proportion of time in the morpholine arm that differed from 0.5 (or 50%) ($t17 = 0.79$, $p = 0.44$). The comparison of time spent in the morpholine arm resulted in no significant difference between morpholine-treated and control parr ($U = 109.5$, $p = 0.97$).

The time-sensitive analysis found similar results to those in the time-averaged analysis. We found no significant effect of treatment (beta = -3.18, df = 28, $p = 0.78$) or timepoint (beta = -1.40, df = 118, $p = 0.21$) on time spent in the

morpholine arm and no significant interaction between treatment and timepoint (beta = -0.75, df = 118, p = 0.67) (Figure 5.1C).

Smolt behaviour

With the time-averaged analysis, we found no significant difference in time spent in either arm for smolts exposed to morpholine in embryonic development (W12 = 73, p = 0.57) or fish that were never exposed to morpholine (W19 = 190, p = 0.79). Morpholine-treated smolts did not spend a proportion of time in the morpholine arm that differed from 0.5 (t12 = -0.37, p = 0.72). Similarly, control smolts did not spend a proportion of time in the morpholine arm that differed from 0.5 (t19 = -0.072, p = 0.94). The comparison of time spent in the morpholine arm found no significant difference between morpholine-treated and control parr (U = 138.5, p = 0.77).

From the time-sensitive analysis, we found no significant main effect of treatment on time spent in the morpholine arm (beta = 13.15, df = 31, p = 0.15). However, we did find a significant effect of timepoint (beta = 0.76, df = 625, p < 0.001) and a significant interaction between treatment and timepoint (beta = -1.45, df = 625, p < 0.001). Our data suggest a trend of avoidance to rearing water – that is to say, morpholine-exposed fish avoided the morpholine arm, and non-morpholine exposed fish avoided the control arm (see Figure 5.1D). At the beginning of the trial (first minute of analysis), morpholine-exposed fish spent an

average time (mean ± standard error) of 32.31 ±. 8.63 seconds in the morpholine arm and near the end of the trial (15th minute of analysis) spent 19.68 ± 6.64 seconds in the morpholine arm. For the first minute of analysis, non-exposed fish spent an average time of 24.00 ± 6.74 seconds in the morpholine arm and at the 15th minute of analysis spent 31.91 ± 6.34 seconds in the morpholine arm.

5.4 Discussion

The results from our study provide behavioural support for the hypothesis that olfactory imprinting can occur at the embryonic through alevin stage of Atlantic Salmon development, and that salmon may be able to detect changes to even a single component of the 'olfactory bouquet' they were reared in (Hasler and Scholz 1983). This is consistent with previous experimental studies demonstrating embryonic imprinting in salmonids (Bett et al. 2016; Armstrong et al. 2022), thus providing support to the hypothesis that multiple imprinting windows exist to facilitate homing back to natal sites (Dittman et al. 1996; Bett and Hinch 2015; Armstrong et al. 2022). We tested for behavioural evidence of olfactory imprinting at the parr and smolt stage of Atlantic Salmon development but found evidence only for the smolt stage and only when we included a time-sensitive analysis that accounted for temporal change in preference. These findings highlight the need to incorporate temporal aspects of behavioural measures, particularly for studies testing avoidance and preference to odorants (Jutfelt et al. 2017).

In contrast to the hypothesis that the critical window for imprinting occurs at the parr-smolt stage of salmonid development, the sequential imprinting hypothesis (Dittman et al. 2015; Bett and Hinch 2016) suggests that imprinting can occur at multiple life stages, including during the embryonic and alevin stage of development (Dittman et al. 2015). Indeed, experimental studies in salmonids have lent support to this hypothesis, for example providing evidence of imprinting at multiple life stages of Sockeye Salmon development (*Oncorhynchus nerka*) (Havey et al. 2017), and during the embryonic/alevin stage of Atlantic Salmon development (Armstrong et al. 2021). Our results for fish exposed to morpholine during the embryonic and alevin stage, support the embryonic imprinting hypothesis. Embryonic (and alevin) imprinting might be useful for managers whose aims are to reintroduce or supplement Atlantic Salmon populations prior to smoltification (Dittman et al. 2015). However, it remains unclear whether imprinting at the embryonic or parr-smolt transformation stage differs in strength of response to natal stream odorants. Studies comparing the strength of response to imprinting between these stages could help to guide management in the decision-making process as to when imprinting will be most beneficial to increase salmon returns.

The majority of reported behavioural studies of imprinting test for behavioural evidence of imprinting at the adult stage, when homing to natal streams is ecologically and physiologically relevant (reviewed in Dittman et al, 2015). These studies often report a preference for rearing water chemical composition at the adult (homing) stage. Some behavioural evidence for preference

for rearing water comes from studies at the fry stage for Sockeye Salmon (*Oncorhynchus nerka*) (Bodznick 1978) and steelhead salmon (*Oncorhynchus mykiss*) (Dittman et al. 2015). However, it is unclear from these studies whether fry prefer water to which they were imprinted or if they were attracted to non-imprinted olfactory cues present in the testing conditions (Bodznick 1978; Dittman et al. 2015). Imprinting is thought to rely on the brain-pituitary-thyroid system, wherein hormones such as thyroxine exert their effects to facilitate long-term memory formation of natal stream odours (Armstrong et al. 2021). Activation of the brain-pituitary-gonadal system, usually during homing migration, is in turn, thought to enhance the olfactory memory retrieval necessary for behavioural preference to imprinted cues and homing (Ueda 2019). Our study found no behavioural evidence for response to the imprinting cue at the parr stage (i.e., the fish showed no preference for rearing or non-rearing water conditions). This could be explained as a lack of initiation of the BPG system – in other words, Atlantic Salmon might not exhibit a preference to either familiar or unfamiliar water signatures. However, given the behavioural response to the imprinting cue at the smolt stage (outside the timeframe of homing migration), it is possible that we simply did not allow the parr in our experiment enough time to acclimate to the test environment in order to respond to the cue and make a choice of preference (Jutfelt et al. 2017). In our study, parr were acclimated to the test environment for only one minute and behavioural recording lasted only five minutes. In contrast, smolt were acclimated in the full test environment for five minutes and behavioural recording lasted 20 minutes. A more extensive acclimation period and

longer behavioural recording is necessary to discern whether Atlantic Salmon parr do, in fact, exhibit a behavioural response to embryonic imprinting cues.

Our two separate analyses (time-averaged and time-sensitive analysis) for smolt behaviour in response to a single altered odorant, morpholine — produced different results. From our time-averaged analysis, we found no behavioural evidence for imprinting. However, when we considered the temporal change in activity, by measuring time spent in the morpholine arm within one-minute bins – time-sensitive analysis – we found a significant effect of embryonic imprinting at the smolt stage. At the beginning of the behavioural trial, fish showed no preference for either odorant stream, but as the trial progressed, a preference was detected. These results are likely associated with a delayed response to the odorant streams, reflecting the time it takes for the fish to decide which water chemical signal it prefers. Imprinting studies generally employ the use of what we have called 'time-averaged analysis' to detect behavioural evidence of imprinting in choice studies (Dittman et al. 2015; Bett et al. 2016; Havey et al. 2017; Armstrong et al. 2021). While a time-averaged analysis may be sufficient to detect strong effects of imprinting, we recommend representing the data as a function of time (time-sensitive analysis) to detect effects that may be masked by variation in activity over time, as well as to provide information on response time and persistence in activity, as suggested by Jutfelt et al. (2017).

Interestingly, our data suggest that smolts may show avoidance to the chemical composition of the water they were reared in. That is to say, fish that had been exposed to morpholine in source water as embryos and alevin avoided

morpholine-treated source water as smolts, and fish that were reared in source water absent of morpholine, avoided source water void of morpholine. To date, behavioural studies that demonstrate evidence for imprinting have found fish to prefer water with chemical composition similar or identical to that in which they were reared (Armstrong et al. 2021; Bett et al. 2016a; Bett & Hinch. 2016b; Havey et al. 2017). This contrasts with the results from our current study. However, imprinting studies almost exclusively focus behavioural preference tests on adult fish, when fish would naturally begin their homing migration (Bett and Hinch 2016b). The smolt stage in Atlantic Salmon is characterized by physiological and morphological changes, and a response to 'external releasing factors' that initiation downstream migration (Fernandes et al. 2015). Our finding that Atlantic Salmon smolts avoid the rearing water to which they imprint could suggest that the rearing water 'olfactory bouquet' acts as an external releasing factor to signal an increased propensity for downstream migration. To test this hypothesis, future imprinting studies should include behavioural preference tests across different life stages and include other non-source water cues (Bodznick 1978).

Attempts to achieve successful imprinting and improve homing fidelity to targeted sites have produced mixed results (reviewed in Dittman et al. 2015). Managers and researchers who aim to increase site fidelity by adding imprinting odorants to targeted return sites might find it worthwhile to determine whether the fish to be released have, in fact, imprinted to the intended cues prior to release. Taken together, our study adds to the evidence that fish can imprint, detect, and respond to a single imprinting odorant (morpholine) added to a complex

background water signature. We show that imprinting can be detected at the smolt stage, and we highlight the importance of including temporal consideration to behavioural analysis to detect evidence of imprinting.

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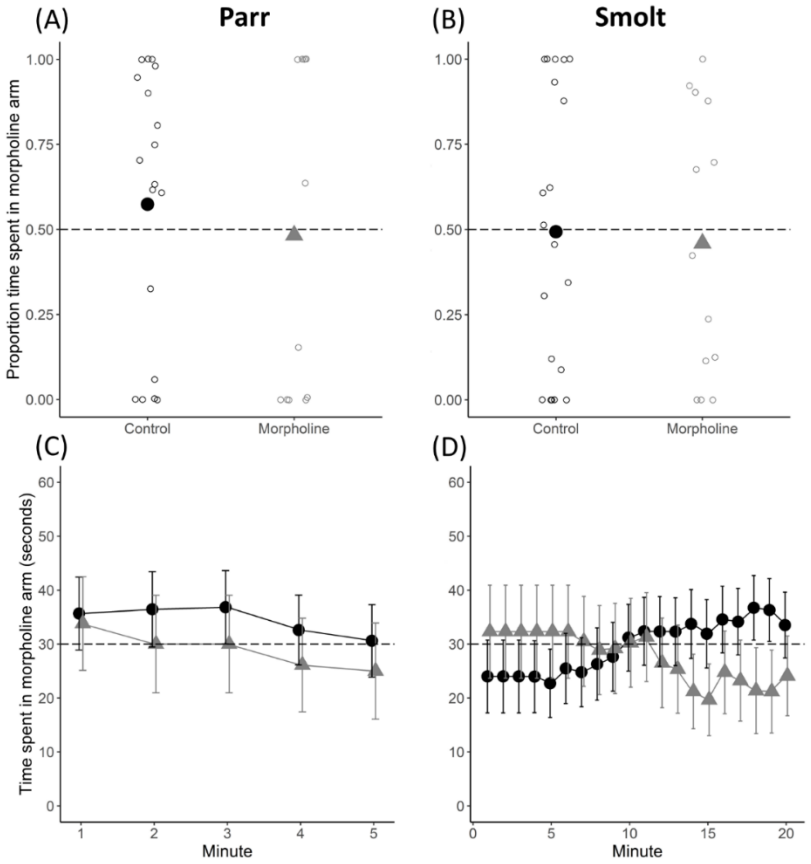

Figure 5.1 The proportion of time Atlantic Salmon (*Salmo salar*) parr (A, C) and smolt (B, D) spent in the two-choice chamber arm containing morpholine in the water source relative to the control arm, containing only source water. Fish in the control group were never exposed to morpholine and fish in the morpholine group were exposed to morpholine as embryos and alevin (embryonic yolk-sac stage). (A) and (B) represent results from the 'time-averaged' analysis, with time for each individual averaged over the entire trial and represented as a proportion of time (5-minutes total for parr, and 20-minutes total for smolt). Open circles represent results for individual fish, black filled circle represents the mean for control fish, and grey filled triangle represents the mean for morpholine-treated fish. The dashed horizontal line marks a proportion of 0.50 representing no side preference for either arm (see Methods for details). (C) and (D) represent results from the 'time-sensitive' analysis for parr and smolt, respectively. The value for each minute is the mean time spent, in seconds (from 0 to 60 seconds), in the two-choice chamber arm containing morpholine relative to the control arm and the error bars show s.e.m. The two treatments are slightly offset on the x-axis to improve clarity due to overlapping data points. Black-filled circles represent results for control fish and grey triangles represent results for morpholine-treated fish. The dashed horizontal line marks 30 seconds (half of each time bin) representing no preference for either arm.

CHAPTER 6

GENERAL DISCUSSION

The reintroduction of captive-reared species is an important tool for conservation biology in the effort to combat the increasing global biodiversity loss (Armstrong and Seddon 2008; Fraser 2008). Deficient or maladaptive behavioural traits of captive-reared aquatic animals has been a long-recognized problem hindering the success of reintroduction efforts and has been linked to differences between the captive-rearing environment and the natural environment to which animals are to be reintroduced (reviewed in Johnsson et al. 2014). Theory suggests that two non-mutually-exclusive differences in phenotypic variation could account for discrepancies between captive-reared and wild phenotypes – in other words, discrepancies in reaction norms between captive-reared and wild phenotypes (Stearns 1989; Johnsson et al. 2014). First, if environmental variation in the captive-rearing environment is lower than the natural environment, the associated phenotypic variation of captive-reared animals may in fact fall within the full range of natural phenotypic variation, albeit with a smaller variance (Piersma and Drent 2003).Second, if the captive-rearing environment exposes animals to rearing conditions outside the range of the natural environmental variation then the phenotypic variation of captive-reared animals may fall completely outside of the range of phenotypes expressed by their wild counterparts (Ghalambor et al. 2007). Some of the most common behavioural deficiencies identified from captive-rearing studies have been related to changes in neural development (Ebbesson and Braithwaite 2012, 2012; Hegab and Wei 2014; Mes et al. 2019), anti-predator related behaviour (Berejikian et al. 1999; Jackson and Brown 2011; Cogliati et al. 2022) and movement/migration behaviour (Aarestrup et al. 2005; Bett and Hinch

2016; Rosengren et al. 2017). Attempts at addressing behavioural deficiencies and improving post-release performance have exploited the phenotypic plasticity of captive-reared animals to close the gap between captive and wild phenotypes by manipulating the captive-rearing environment and release strategies (reviewed in Crates et al. 2022).

In an effort to guide the phenotypic development of captive-reared individuals, certain conditioning tactics aimed at countering the negative effects associated with captive-rearing and translocation events have showed some promise (reviewed in Tetzlaff et al. 2019). Animal-focused conditioning (i.e., or e.g., environmental enrichment) in the captive setting, aims at manipulating the rearing environment in order to produce more adaptive behavioural phenotypes (Huntingford 2004; Johnsson et al. 2014), while environment-focused conditioning (e.g., soft-release) aims at manipulating the release strategy in order to alleviate the negative effects associated with reintroduction release. The goal of these tactics is to produce more "wild-like" behaviours in an effort to increase post-release success (Hyvarinen and Rodewald, 2013). The goal of this dissertation was to explore the effects of environmental rearing environment and release protocol on key phenotypically plastic behavioural deficiencies exhibited by captive-reared Atlantic Salmon (*Salmo salar*). The results from the data chapters contained in this dissertation reinforce the hypothesis that manipulation of the rearing environment and release protocol can lead to phenotypically plastic changes in brain and behaviour. However, it is important to keep in mind that these data chapters consider only a very narrow range of possible behavioural variation under confined

environmental scenarios (for review of other behaviourally plastic traits associated with captivity see Näslund 2021; Crates et al. 2022). A more thorough approach would be to examine and compare reaction norms of conventionally reared fish, enriched fish, and wild fish across naturally occurring environmental variation instead of only select behaviours under strict laboratory environments. After all, diversity in trait expression among individuals and at the population level is important for post-release success as it provides more opportunity to express adaptive traits in varying environments (Watters and Meehan 2007).

Chapter 2 of this dissertation was designed to test the hypothesis that embryonic exposure to alarm cue would lead to improvements in anti-predator behaviour, via phenotypic plastic change. This hypothesis is not new and has considerable evidence to support it (e.g., Berejikian et al. 1999; Poisson et al. 2017; Lau et al. 2021; Crane et al. 2022). Typically, though, alarm cue enrichment studies test the effect of alarm cue enrichment in the absence of other cues (e.g. (Poisson et al. 2017; Lau et al. 2021) – to test baseline changes in behaviour – or in conjunction with predator-related cues (kairomones) – to test for effects of predator-training (Crane et al. 2021). Chapter 2 differed from those studies in that the aim was to determine whether embryonic exposure affects the strength of response to acute exposure to alarm cue. While the response to alarm cue is considered to be innate in most fishes (Mirza and Chivers 2001; Jesuthasan and Mathuru 2008), including Atlantic Salmon, (Lau et al. 2021) hatchery rearing is known to reduce the alarm response (Jackson and Brown 2011). The hypothesis of Chapter 2 predicted a strengthened response to alarm cue after enrichment. We

found no evidence of an innate behavioural response to alarm cue for the set of behaviours measured: number of aggressive acts, time spent motionless, and time spent near shelter. We also found no effect of alarm cue enrichment on the subsequent behaviour in response to acute alarm cue exposure. A possible explanation for the discrepancy between the results from Chapter 2 and similar studies with Atlantic Salmon (e.g., Lau et al. 2021) is that the concentration of alarm cue used to elicit a response in the particular behaviours studied was below the activation threshold level. Indeed, varying levels of alarm cue exposure may elicit differential behavioural responses (Mirza and Chivers 2003b). This highlights the need for future alarm cue enrichment research to not only consider the dose of alarm cue (and strength of stimuli in general) and the responses to enrichment that are being measured. Furthermore, the 'context hypothesis' of alarm cue function posits that alarm cues prime an individual to respond to subsequent ambiguous cues (Stephenson 2016). This would suggest that the alarm cue enrichment alone is necessary but not sufficient to elicit a heightened behavioural response. Alarm cue might provide the context for a heightened response and secondary cues are necessary to trigger the response (Brown et al. 2004). Alarm cue enrichment was found to have a significant negative effect on the size of the olfactory bulb, which would suggest that plastic changes had occurred and lends support to the claim that behavioural responses other than those tested in the study may have also changed. The effect of a smaller olfactory bulb – due to alarm cue exposure – on behavioural outcomes remains unclear but could be elucidated by exploring a wider range of behaviours to link form to function.

Overall, future studies should aim to include secondary ecologically relevant stimuli, such as simulated predator attacks, and test a wider range of anti-predator related behaviours.

Chapter 3 of this dissertation compared the post-release movement pattern of hatchery-reared Atlantic Salmon parr between conventionally released (hard-release) non-acclimatized fish with soft-release counterparts. Soft-release fish displayed higher levels of site-fidelity compared to hard-released fish, in line with previous acclimatization studies (Cresswell and Williams 1983; Kaya and Jeanes 1995; Eisenhauer et al. 2020). Higher site-fidelity is often associated with increased post-release survival as animals that remain near the release site incurs lower energetic cost associated with movement and release site fidelity could suggest that fish have established suitable territory in the release area (Berger-Tal and Saltz 2014). When fish were detected moving away from the release site, soft-release fish were detected moving sooner than hard release fish and were more likely to move downstream compared to an increased tendency of hard-release to move upstream. The post-release dispersal patterns of hatchery-reared and wild Atlantic Salmon parr has not been previously compared, however, studies examining the dispersal of fry from artificial redds (Eisenhauer et al. 2020) and wild parr and smolt distribution patterns (Foldvik et al. 2012) suggest the more 'natural' or wild-like behaviour to be downstream movement. This would suggest that soft-release may potentially lead to a phenotypically flexible response more closely matched to wild phenotypes, in terms of movement patterns, compared to conventional hard-release. This hypothesis could be tested using a very similar

methodology to that used in Chapter 3 – by comparing the post-release movement between hard-released, soft-released captive-reared, and wild fish. In fact, a recent study comparing migration patterns between hatchery-reared and wild Atlantic Salmon found that hatchery-reared fish displayed abberant movement patterns compared to wild conspecifics (Iden Nilsen et al. 2022). The potential for soft-release to flexibly change the behavioural phenotype of hatchery-reared fish has been clearly demonstrated in Chapter 3 –the question of how to incorporate soft-release with other conditioning tactics and the potential role of soft-release in the success of establishment of released stock is discussed in detail below.

Chapter 4 of this dissertation was designed to test the effect of structural enrichment on risk-taking behaviour of Atlantic Salmon parr in a context relevant to reintroduction efforts (in conjunction with transport stress). Because structural enrichment is known to reduce stress response (Cogliati et al. 2019) and promote recovery from stressful events (Pounder et al. 2016), it was predicted that structural enrichment would ameliorate the negative behavioural effects associate with stressful transport and improve behavioural recovery after transport. Consistent with previous structural enrichment studies, enriched fish developed what was considered reduced risk-taking behaviour (Roberts et al. 2011; Rosengren et al. 2017). Interestingly, transport stress similarly led to reduced risk-taking behaviour for both enriched and non-enriched treatment groups. It is noteworthy that the behavioural test environment was a barren tank more closely matching the barren rearing environment than to the enriched rearing environment. This fact considered, the similarity between the behavioural response to

enrichment and transport stress could suggest that rearing with structural enrichment resulted in a familiarity with complex environments and the test environment represented a stressful unfamiliar novel environment for enriched fish, resulting in a stronger behavioural stress response (similar to transport stress) for enriched fish compared to non-enriched fish. One way for future research to resolve this potentially confounding effect is to include preference tests across different ecologically relevant environments for each rearing treatment (Johnsson and Näslund 2018). Finally, recovery groups (fish that received 7 days of acclimation to a novel environment) also showed a decrease in risk-taking behaviour. Here, again, the problem of unfamiliar novel environments likely played a role in the observed results. The aim of recovery after transport stress in Chapter 4 was to simulate the soft-release tactic of Chapter 3 and to investigate the effect of enrichment on behavioural recovery after transport. Admittedly, the methodology used in Chapter 4 introduced novel environment as a potentially confounding variable. Fish were allowed to acclimate to a novel tank environment for one week before behavioural testing in yet another novel test tank. In retrospect, the test tank should have served as the recovery tank for acclimation and behavioural test should have been conducted in the same tank in which they were recovered in order to remove the effects of handling and tank novelty on the behavioural response. Again, the role that testing environment plays is emphasized here, and should be considered in future testing of behavioural responses to enrichment.

Chapter 5 of this dissertation was designed to answer the question: can behavioural evidence of embryonic olfactory imprinting be detected at the parr and smolt stage of captive-bred Atlantic Salmon. Results from two-choice preference tests indicated that Atlantic Salmon smolt, but not parr, show behavioural evidence of imprinting to morpholine. Importantly, the behavioural tests conducted at the parr and smolt stage used individuals from the same cohort and treatment groups – that is, fish used in the tests were from the same imprinting groups tested at different time point (as parr and then smolt). It is likely that the lack of evidence of imprinting at the parr stage reflected the different methodologies used to assess behavioural evidence between the two stages (discussed in more detail in Chapter 5). Even so, from a conservation management perspective, the results from Chapter 5 provide a tool for managers and conservation biologists to test for evidence of imprinting prior to reintroduction. Interestingly, the results from smolt preference tests suggest that smolts might show an avoidance to the chemical composition of the water they were reared in – in contrast to most adult salmonid preference tests showing an affinity for rearing water (Bett and Hinch 2016; Havey et al. 2017; Armstrong et al. 2021). The smolt stage in Atlantic Salmon represents a life-stage characterized by physiological and morphological changes in preparation for downstream migration (Fernandes et al. 2015). The results here might suggest that the imprinted cue act as an attractant at certain life-stages (adult stage) when migration towards the olfactory cue is ecologically relevant, and as a deterrent or an external cue to initiate downstream migration when ecologically relevant (smolt stage). This hypothesis has yet to be tested but could lead to

improving the fundamental understanding of how olfactory imprinting shapes the behaviour of migratory animals.

An important lens through which to view the results from the data chapters contained within this dissertation is what Näslund (2021) refers to as the 'post-release survival time course'. Post-release mortality is generally highest immediately following release, then decreases before reaching a hypothetical breakpoint at which time it stabilizes at a relatively low level (e.g., Poh et al. 2018; Long et al. 2018; Larocque et al. 2020). The post-release survival time course is linked to three identified phases of establishment of reintroduced animals (Henderson 1980; Näslund 2021): (1) recovery of normal movement behaviour, (2) experience and familiarization with the novel environment, and (3) establishing a feeding regime. Experimental attempts for improving behavioural performance of captive-reared animals should take care to identify and consider the effects of the particular conditioning tactic used (e.g., antipredator training, environmental enrichment, and soft-release) in the context of the post-release survival time course and the phases of establishment of reintroduced animals. This can potentially help to focus the scope of conditioning studies and link conditioning tactics to specific goals within the post-release survival time-course or to a particular phase of establishment. For example, in Chapter 3 of this dissertation, soft-release net pens prevented the potential immediate post-release mortality of the reintroduced fish (post-release survival time course) and led to a recovery of normal movement behaviour compared to hard-released fish (phase 1 of establishment). Chapter 2 (related to alarm cue enrichment) and Chapter 4 (related to structural enrichment

and transport stress) might be important in shortening the post-release survival time-course by shifting the breakpoint closer to the release timepoint. The potential effect of enrichment in these cases could be to enhance the familiarization with the novel release environment in terms of readiness to avoid predation and use structure for shelter (phase 2 of establishment) (Näslund 2021). In Chapter 2, enrichment with alarm cue did not lead to observed plastic changes to anti-predator related behaviour but did affect the development of regional brain morphology. Given that changes in brain structures are closely linked to behavioural variation (Gonda et al. 2012; Reddon et al. 2018), it is possible that alarm cue enrichment in captivity could lead to behavioural modification post-release. In Chapter 2, structural enrichment did lead to plastic changes in behaviour but did not ameliorate the behavioural effects of transport stress. This could explain previous findings that structural enrichment alone does not increase post-release survival (e.g. Brockmark et al. 2007; Rosengren et al. 2017; Solås et al. 2019). It is possible that structural and alarm cue enrichment together can help to increase familiarity to the release environment (phase 2 of establishment) but not with survival early in the post-release survival time course. If reintroduced animals do not survive the initial stages of release, the potential benefits of captive-rearing enrichment may not have a chance to be expressed. This point highlights the need to consider the post-release survival time course in conditioning and release studies. If soft-release can help to reduce the immediate high mortality rates during the initial stages of establishment, then research on the effects of enrichment should incorporate this tactic (soft-release) in wild release studies to determine whether enrichment can

provide a benefit to reintroduced animals during the establishment phase. Studies should also consider the combined effects of other tactics simultaneously. For example, a study of the combined effects of alarm cue and structural enrichment on instream survival of cutthroat trout (*Oncorhynchus clarki*) found that structural enrichment or alarm cue conditioning alone had a negative effect on survival against predation but a positive effect when combined (Berejikian et al. 1999). The consideration of post-release survival time course and the phases of establishment, and the combination of different conditioning tactics could provide benefit to reintroduction efforts and facilitate comparisons of the efficacy of conditioning tactics across studies. It should be noted that imprinting studies (such as in Chapter 5) do not necessarily fit into the framework of post-release survival time course and establishment phases. The general goal of imprinting studies for conservation is to increase fidelity of stocked individuals to targeted return sites (usually targeted spawning locations) assuming the released animals successfully establish after release and survive long enough to make return migrations (Dittman et al. 2015).

The ability of captive-reared animals to express behavioural plasticity under different environmental conditions and experiences is strongly linked to changes in neural circuitry (neuroplasticity) (Ebbesson and Braithwaite 2012). The degree to which environmental variation will impact the behavioural phenotype of an animal is dependent on the sensitivity of the brain (more specifically the neural circuits) to respond to experience (Knudsen 2004). The effect of environmental variation (or experience) can differ in strength and influence on neural circuits

(and so behaviour) across the lifespan of an organism due to a property of neural circuits termed ‘sensitive windows’ or ‘sensitive periods’. Sensitive windows are described as developmental periods or stages in which experience or stimuli shape phenotypic development to a greater degree than in other periods or stages (Fawcett and Frankenhuis 2015). Indeed, responsiveness to specific conditioning stimuli has been shown to change across ontogeny (Näslund and Johnsson 2016; Salvanes 2021; Jones et al. 2021; Hammond et al. 2022). For example, structural enrichment during the fry stage of Atlantic Salmon had no effect on exploratory behaviour but the same enrichment during the parr stage produced individuals that were more exploratory compared to non-enriched counterparts (Salvanes 2021). Similarly, environmental enrichment had a stronger positive effect on post-release survival of sub-adult compared to adult mountain yellow-legged frogs (*Rana muscosa*). Studying the timing and duration of species-specific (and potentially population-specific) sensitive windows might help to better target the timing of enrichment and conditioning to produce more pronounced and potentially more adaptive post-release phenotypes. Importantly, sensitive windows are prone to plastic change – the duration (Frankenhuis and Panchanathan 2011) and responsiveness (Joyce et al. 2016) of sensitive windows can be shaped by experience earlier in an individual’s development (Fawcett and Frankenhuis 2015) or via parental (intergenerational) or ancestral experience (transgenerational effects) (Crane et al. 2021). For example, the retention of conditioned behavioural response to predator training was enhanced among juvenile convict cichlids (*Amatitlania nigrofasciata*) that had prior experience with elevated levels of

background risk of predation (Joyce et al. 2016). In terms of transgenerational effects, offspring of fathead minnows (*Pimephales promelas*) that were exposed to simulated predation risk (via alarm cue) showed a heightened anti-predator behavioural response to alarm cue compared to offspring of parents who were never exposed predation risk (Crane et al. 2021). Finally, in terms of imprinting, the number and duration of olfactory imprinting windows is known to be species-specific ('sequential imprinting hypothesis' Bett and Hinch 2016; Armstrong et al. 2021), suggesting an evolutionary or even plastic potential for imprinting windows to be modified. The possibility of manipulating olfactory imprinting windows should be further investigated and could lead to improved imprinting strategies to increase the success of reintroduction efforts. The potential to change or enhance the phenotypic response to enrichment and release tactics via prior experience or through parental/ancestral effects could be an instrumental leap towards producing captive-bred stock that more closely match their wild counterparts and are more equipped to deal with the complexities faced in the wild.

Throughout this thesis I have considered the phenotype-environment mismatch that exists as a consequence of captive-rearing – a critical component of reintroduction efforts. Each data chapter considered at least one key aspect of the reintroduction effort and demonstrates the potential of using conditioning tactics – enriching the captive environment and/or manipulating the release protocol – to alter plastic behavioural traits to make them better suited for the wild environment to which they are reintroduced.

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