# An analysis of ecological conditions and release procedures to guide reintroduction efforts for the Eastern Sand Darter (*Ammocrypta pellucida*) in Canada

by

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## Declaration of Authorship

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

The key ideas, primary contributions, experimental design, data analysis, interpretation, and writing of each chapter of this thesis were performed by myself, Adam Gouge, under the supervision of Christine Madliger.

In Chapter 1 (General Introduction) and Chapter 4 (General Discussion), I present unpublished material written by myself, Adam Gouge, under the supervision of Christine Madliger.

I have prepared Chapter 2 and Chapter 3 as manuscripts with intention for publication. For Chapter 2, I have prepared the manuscript with input from Andrew Drake and Christine Madliger, who will be included as co-authors. The ecological and community data that I analyzed from 2018 was collected independently by Fisheries and Oceans Canada. I collaboratively collected the ecological and community data in 2023 in partnership with Fisheries and Oceans Canada and I analyzed the data under the supervision of Christine Madliger. For Chapter 3, I have prepared the manuscript with input from Andrew Drake, Trevor Pitcher, and Christine Madliger, who will be included as co-authors. In collaboration with Trevor Pitcher and Andrew Drake, Christine Madliger recorded the behavioural videos from 2022, and I analyzed the videos to produce the data. I recorded and analyzed the behavioural videos from 2023 under the supervision of Christine Madliger. Audrey Verra and Anton Peter collected data from a sub-set of the substrate preference and swimming performance videos, respectively, and will also be included as co-authors on the publication produced from Chapter 3.

I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

I hereby certify that no part of this thesis has yet been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone’s copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Office of Graduate Studies, and that this thesis has not been submitted for a higher degree to any other University or Institution.

## Abstract

To address recent losses in freshwater biodiversity, reintroductions are becoming increasingly common as a tool for the conservation and rehabilitation of target species. As historically underrepresented approaches for fishes, many reintroduction projects have been unable to establish self-sustaining populations, due at least in part to an underappreciation of the stressors associated with the reintroduction process. Two elements that have a measurable influence on the probability of successful outcomes in reintroduction programs are the ecological conditions of the release habitat and the transport and release methods employed. The Eastern Sand Darter (*Ammocrypta pellucida*) is a small, benthic freshwater fish species that is threatened in parts of its range in Canada, and reintroduction has been proposed as a key component of its recovery strategy. To support the reintroduction of this species to Big Otter Creek in Ontario, Canada, I first assessed the environmental conditions and fish community assemblage along the length of Big Otter Creek to characterize reintroduction habitat. By considering factors known to influence Eastern Sand Darter persistence, including substrate composition and the presence of invasive species, I was able to identify a set of release sites that are likely to provide the best conditions for establishment. I then analyzed the influence of a simulated transport event on behaviour, and investigated whether the provision of in-river acclimation (i.e., soft-release) enclosures can promote recovery from transport stress. I found that transport stress did not influence behaviours related to activity level, space use, substrate preference, burrowing activity, response to a simulated predator, or swimming performance. I also found no differences in behaviour between control (non-transported) fish and those held in soft-release enclosures for 24- or 48-hours after transport. Some behaviours, however, differed between seasons (summer vs. fall). I conclude that Eastern Sand Darter may be behaviourally resilient to the effects of transport, particularly when conditions are ideal, and that traditional release methods with no acclimation period (i.e., hard-release) may be suitable for this species. Overall, my thesis contributes knowledge directly to reintroduction planning for the Eastern Sand Darter, while also more generally providing information on behaviour and responses to transport stress in conservation-relevant, small-bodied fishes.

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## Land Acknowledgement

This research was conducted in Robinson-Huron Treaty territory, on the traditional lands of the Anishnaabeg, and within the homelands of the Garden River and Batchewana First Nations and Métis People. The field studies contained herein were conducted on the traditional lands of the Mississaugas of the Credit First Nation, Six Nations of the Grand River, and Neutral peoples in the Haldimand Tract as well as on the traditional territory of the Haudenosaunee, Attiwonderonk (Neutral), and Mississauga Nation in land of the McKee Treaty. We acknowledge the deep history and ongoing contributions of these communities to this land and our collective responsibility to respect and uphold their rights, histories, and knowledge systems.

## Declaration of Environmental Impact

I would like to acknowledge the environmental impact of my thesis project and the specific measures deliberately taken to attempt to lessen it. My project involved travel from Sault Ste. Marie, Ontario to Brantford, Ontario over multiple occasions. As a research team, we used car pooling during these travel events, as well as to and from our field site to our accommodations. We similarly stayed in a shared accommodation to reduce our overall footprint. My project also required the use of natural and synthetic materials, such as the plastic containers used for behavioural arenas and the wooden enclosures we constructed for our soft-release experiments. As a lab, we reuse materials as much as possible, cleaning and storing our equipment for future projects. Finally, my project required me to complete sampling within the Grand River and Big Otter Creek. We minimized our impact by only accessing sections of the river necessary for sampling, releasing non-target species as quickly as possible, and ensuring that we left no trace of our experimental set-up on the shorelines. There is still considerable room to reduce the overall impact that science has on the natural environment, and I will continue to strive towards lessening my impact throughout the rest of my career.

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## Ethical Compliance Statement

This research was conducted in compliance with ethical animal use standards. Institutional animal care permits were granted by the University of Windsor (AUPP: #22-03) and Algoma University (AUP: 2024-CMSandDarter-01). The work was also permitted under the *Species at Risk Act* (22-PCAA-00042; 24-PCAA-00013) and completed under Ontario Licences to Collect Fish for Scientific Purposes (2022: #1101308; 2024: #1105959). The field studies were also registered with the Ontario Ministry of the Environment, Conservation and Parks in 2022 (M-102-4476058785) and 2024 (M-102-4562244892).

# Table of Contents

## List of Figures

## List of Tables

# Chapter 1 - General Introduction

## Freshwater ecosystems

Ecological disturbances produced by changing environmental conditions have recently led to a precipitous decline in biodiversity across a wide variety of taxa, including plants, animals, and fungi (Dirzo et al. 2014). Ecosystems can differ in their vulnerability to these disturbances as well as in the extent to which they experience the resulting biological consequences, leading to differential rates of decline among some classes of organisms and ecosystems (Dirzo et al. 2014). Freshwater ecosystems which support a wide variety of life across all kingdoms are highly susceptible to ecological disturbances and have experienced declines in biodiversity more substantial and at a greater rate than nearly all others (Dudgeon et al. 2006; Darwall et al. 2018). The importance of these ecosystems is evident in the reliance of nearly 6% of all known species on areas of freshwater that cover less than 1% of earth's surface (Dudgeon et al. 2006) The proportion of species supported by freshwater ecosystems are even greater when considering groups of vertebrates like fish, of which freshwater species comprise 40% (Dudgeon et al. 2006).

Numerous stressors primarily related to anthropogenic activity are introduced to aquatic ecosystems through habitat destruction, introduction of invasive species and contaminants, as well as overharvesting (Miller et al. 1989). The ecological repercussions to aquatic ecosystems are apparent, resulting in some of the greatest losses in global biodiversity ever recorded (Dudgeon et al. 2006). This trend in the loss of biodiversity and the increasingly large number of threatened and endangered species have caused common concern at a global level (Darwall et al. 2018; Desforges et al. 2022). There is a growing consensus that immediate action is needed to halt and reverse declines, consisting of increased research, threat delineation, rehabilitation, and international collaboration (Dudgeon et al. 2006; Comte et al. 2013; Sayer et al. 2025).

### Conservation and recovery

Worldwide concern about rapid loss of biodiversity has led to the United Nations declaring 2021-2030 as the UN Decade on Ecosystem Restoration (United Nations General Assembly Res. 73/284). In Canada, the Species at Risk Act (SARA) provides a framework for the protection, recovery, and management of imperiled species to support restoration and prevent further loss of biodiversity. The Committee on The Status of Endangered Wildlife in Canada (COSEWIC), a group of independent experts and researchers, assess and recommend if a Designatable Unit (i.e., species, subspecies, population) should be considered at risk and receive protection under SARA. Protection and recovery of imperiled species and their habitat involves the development and implementation of action plans that use various strategies and mitigation measures such as research and educational programs, the creation and enforcement of guidelines and regulations, as well as more active strategies such as supplementation or planned species reintroduction (SARA 2002).

### Reintroduction

Reintroduction is the planned repatriation of a species to an area from which it has been extirpated (Armstrong and Seddon 2008). Reintroduction efforts typically involve releasing individuals that have been captively bred or relocated from a source population in hopes of re-establishing viable breeding populations (Armstrong and Seddon 2008; IUCN/SSC 2013).

Reintroduction efforts have led to the recovery of populations of species such as Elk (C*ervus elaphus*) in Ontario, Canada (Rosatte et al. 2007), Grey Wolves *(Canis lupus)* in Yellowstone National Park, USA (Macdonald and Sillero-Zubiri 2004), Arabian Oryx (*Oryx leucoryx)* in the middle-east and northern Africa (Primack 2018), Bald Eagle (*Haliaeetus leucocephalus)* in the Lake Erie region of Canada (McKeane and Weseloh 1993), and African Wild Dogs (*Lycaon pictus*) in Gorongosa National Park, Mozambique (Bouley et al. 2021). Although some repatriation efforts have been successful, these projects have historically lacked comprehensive background research, ecological modelling, and the development of specific protocols tailored to each focal species, due in part to a scarcity of reintroduction-related data and knowledge of effective strategies (Seddon et al. 2007). As a result of this information deficiency, projects historically relied to some extent on trial and error and did not involve extensive monitoring or adaptive management throughout the reintroduction effort (Armstrong and Seddon 2008; Lamothe and Drake 2019).

Now, Reintroduction Biology has become an emerging field concerned with research and analysis into the biotic aspects of reintroduction and the development of more effective practices (Seddon et al. 2007). Although more reintroduction projects have included theory-based elements and integration of multiple disciplines, increased use of deduction and theoretical approaches (Seddon et al. 2007) and coordinating efforts between organizations and research fields (Lipsey et al. 2007) will lead to improved outcomes and a greater likelihood of successful reintroduction. In particular, rigorous research and analysis have the potential to provide information on all components of the reintroduction process, including choice of source populations, captive breeding considerations, transport and release techniques, release locations, and post-release monitoring protocols.

Despite rapid declines in populations, only approximately 4% of published reintroductions involve freshwater fishes, with more charismatic species in taxonomic groups such as mammals and birds being more typically represented (Seddon et al. 2005). By analyzing historical reintroductions of imperiled freshwater fishes in Canada, Lamothe and Drake (2019) highlighted the need for increased efforts towards repatriation of freshwater fish species. Of nearly 70 conservation strategies created for SARA-listed freshwater fishes, only 20% of plans include repatriation, relocation, or supplementation as a potential recovery strategy (Lamothe and Drake 2019) with many of these projects yet to be initiated (Lamothe et al. 2019). Further, due to the underrepresentation of freshwater species, Lamothe et al. (2019) suggested that progression of planned actions for freshwater species at risk in Canada, including reintroduction efforts, are necessary to ensure long-term stability of these species.

Fish reintroduction projects that have experienced setbacks and limited success have largely been attributed to oversight in one or more key areas of planning such as proper habitat assessment or logistical aspects of reintroduction protocols such as release methods (George et al. 2009). In some cases, the rate at which fish disperse from reintroduction sites following release has been cited as one of the factors influencing mortality in recently released individuals (Tetzlaff et al. 2019). In the case of reintroduction attempts for Greenback Cutthroat Trout (*Oncorhynchus clarki stomias*) in Colorado, USA, a failure to properly identify individuals captured for reintroduction purposes led to the release of the wrong trout species at release sites (Metcalf et al. 2007). In contrast, the success of other projects such as the reintroduction of the Red Citico Darter (*Etheostoma sitikuense*), Smoky Madtom (*Noturus baileyi*), and the Yellowfin Madtom (*Noturus flavipinnis*) to Abrams Creek in the Great Smoky Mountains National Park, USA have been credited to the careful strategy applied (George et al. 2009; Cochran-Biederman et al. 2015). In these cases, thorough background research on biology and life history and rigorous analysis of environmental conditions and release strategy were crucial steps in the development of successful reintroduction protocols (George et al. 2009; Cochran-Biederman et al. 2015). Given that there are many opportunities for reintroduction to support SARA-listed freshwater fish populations, addressing these elements will be of crucial importance to the design of successful reintroduction programs in Canada (Lamothe et al. 2019). Two components of the reintroduction process for freshwater fishes that have historically been under-investigated are: i) biotic interactions (i.e., community structure of reintroduction sites), and ii) stocking approaches (e.g., methods of capture, transport, and release).

### Reintroduction habitat and community composition

The novel ecological conditions of the reintroduction habitat pose some of the greatest challenges facing fish that are released for the purposes of reintroduction (Hunter-Ayad et al. 2021). Local adaptations are often considered when selecting the appropriate source population for translocation (Stockwell and Leberg 2002; Lamothe and Drake 2019). Fish belonging to populations that inhabit areas with ecologically similar conditions to the release site typically make ideal candidates as these individuals are most likely to express phenotypes that will allow them the greatest likelihood of survival within this setting (George et al. 2006; Turko et al. 2021). Although the biotic and abiotic aspects of the reintroduction habitat may be similar to those of the source population, they may differ in others that may be relevant to the species being reintroduced (IUCN/SSC 2013; Van Liefferinge et al. 2019). For example, the community composition of the areas to which fish are being reintroduced often differs from that of the source location. As a result, the collection of community assemblage data through surveys as well as subsequent analysis can provide insight into some of the challenges that individuals will likely face upon release into the reintroduction habitat, such as predation and interspecific competition (Lamothe and Drake 2019).

Another integral consideration in the composition of the release site community is the presence of invasive alien species, which are recognized as one of the greatest threats to imperiled freshwater fish in Canada (Dextrase and Mandrak 2006). Invasive species can pose challenges to native fish through increased competition and predation (Dudgeon et al. 2006). In particular, as these predators and competitors may not have been present within the ecosystem prior to extirpation, they can represent novel threats to which the native fish being reintroduced may not be adapted to handle (McAllister et al. 2022). Wedderburn et al. (2020) explored the use of reintroduction as a recovery strategy for Yarra Pigmy Perch (*Nannoperca australis*), a small-bodied percid of Australia, recognizing that competition from the invasive Eastern Gambusia *(Gambusia holbrooki*) was one the primary factors leading to population declines, as well as one of the greatest challenges facing recovery of the species. Such work emphasizes the importance of thorough assessment of ecological interactions between invasive and native fish populations during the development of reintroduction programs (Dextrase and Mandrak 2006). Indeed, an analysis of the outcomes of reintroduction projects involving freshwater fish globally by Cochran-Biederman et al. (2015) led to the conclusion that the consideration of the presence of invasive species at the release site is one of the greatest predictors of project success.

In addition to thorough understanding of the community assemblage of the reintroduction habitat, non-biotic attributes of these areas should also be assessed during development of a reintroduction strategy (IUCN/SSC 2013). Abiotic factors relating to water chemistry such as pH, temperature, and dissolved oxygen, along with other physical characteristics like substrate or vegetation structure, can provide an accurate description of current ecological conditions (Fisk II et al. 2014; Cochran-Biederman et al. 2015; Van Liefferinge et al. 2019). Overall, thorough habitat assessment prior to release has been shown to provide some of the best indicators of spawning success (i.e., a necessary component of the establishment of a self-sustaining population) for reintroduced freshwater fishes (Cochran-Biederman et al. 2015).

### Determining ideal release protocols: Soft vs. hard-release strategies

Reintroduction events involve capture, handling, and transport, all of which represent stressors for fish (George et al. 2006; IUCN/SSC 2013; Monk et al. 2020). A stressor can be defined as any stimulus that elicits a physiological response from an animal in an attempt to maintain homeostasis (Romero et al. 2009). Stress responses involve activation of neurological and endocrinological systems and subsequent changes in behaviour (Dickens et al. 2010). However, the severity, frequency, and duration of the stressor as well as co-occurrence with other stressors are key determinants in whether responses to stressors are acute or chronic (Dickens et al. 2010). Acute responses to stressors include the production of increased levels of catecholamines and corticosteroids (i.e., cortisol in fishes) through activation of the HPI (hypothalamic-pituitary-interrenal) axis, thereby allowing the individual to better overcome and recover from the stressful conditions (Sampaio and Freire 2016). However, this response can have detrimental effects if it is expressed chronically (Lynn et al. 2010), as persistence of cortisol at elevated levels can lead to increased susceptibility to disease, reduced long term fight or flight response, declines in growth, inhibition of reproduction, and other neurological dysfunctions (Levine 2005). Exposure to multiple sources of stress simultaneously or persistent stressors can therefore have cumulative negative effects on an individual (Teixeira et al. 2007), with consequences that scale to influence population-level processes (Bergman et al. 2019).

As a result of handling, percids experience a dramatic increase in cortisol levels which can remain high for several hours, while increased blood glucose levels in response to transport can persist for weeks (Acerete et al. 2004). In addition, changes in water quality and density are common sources of stress faced by fish during transport (Sampaio and Freire 2016). Temporary changes in behaviour, such as reduced ability to forage or evade predators, are often exhibited by fish experiencing physiological responses to these stressful conditions (Teixeira et al. 2007; Dickens et al. 2010) which can require several hours or days for recovery (Monk et al. 2020). As predation risk is a significant factor that can determine the success of reintroduction, retention of predator evasion capabilities of released individuals is imperative (Moseby et al. 2014).

Although pre-predator exposure or training has been explored within the scope of reintroduction protocols as a means of decreasing mortality due to predation, this method has typically been assessed with captive bred individuals and would therefore have limited application to translocation (i.e., wild-sourced) projects (Tetzlaff et al. 2019). Therefore, when fish are being moved between wild locations, promoting survival of the greatest proportion of released individuals by minimizing the risk of mortality due to predators must be accomplished through the use of alternate methods. If maximum survival is to be achieved, strategies must allow for any negative consequences of transport to subside before release.

Translocated individuals have traditionally been released immediately following arrival at the reintroduction site, termed hard-release (Resende et al. 2021). This method provides the transplanted individuals with no opportunity to recover from exposure to the numerous stressors associated with the capture and transport process before being released (Tetzlaff et al. 2019). Alternatively, soft-release methods involve placing transplanted individuals in a protective enclosure within the release area for a period of time to allow acclimation to their new environmental conditions (Tetzlaff et al. 2019). This acclimation period is cited as an opportunity for any effects from stress exposure to subside and generally promotes survival of released individuals when compared to hard-release alternatives (Resende et al. 2021). For example, a meta-analysis conducted across taxa indicates that soft-release is more effective than environmental enrichment or anti-predator training at increasing the likelihood of survival for released individuals (Tetzlaff et al. 2019). Overall, the limited knowledge available for fishes indicates that soft-released fish show less behavioural disruption and higher rate of survival compared to hard-released fish. For example, Atlantic Salmon (*Salmo salar*) that were soft-released were significantly more effective at dispersing from release sites, showing a tendency to migrate downstream earlier than hard-released fish (Mokdad et al. 2022). However, the potential for soft-release strategies to promote natural behaviours related to predator avoidance, foraging, and habitat establishment have not been investigated in fishes.

Uncertainty surrounding efficacy of soft-release protocols has been suggested as an issue facing conservation authorities and the potential reason for its exclusion from reintroduction programs (Resende et al. 2021). The use of soft-release as a prospective tactic in mitigating the effects of transport stress during reintroduction of freshwater fishes has the potential to increase post-release survival, and the effectiveness of this strategy should be further explored. Indeed, the development of soft-release tactics for reintroduction protocols that are tailored to the specifics of the target species are needed to determine whether such protocols improve the likelihood of reintroduction success (Moseby et al. 2014). Overall, while the individual effects of stressors are often considered during the planning of reintroduction projects, the effects of stress persistence or the cumulative effects of multiple stressors for behaviour have not often been accounted for (Dickens et al. 2010). Taking an approach that considers these effects and how to mitigate them during release has significant potential to improve the likelihood of successful reintroductions (Dickens et al. 2010).

### Eastern Sand Darter

Eastern Sand Darter (*Ammocrypta pellucida*) is a freshwater species of fish belonging to the family Percidae that has been suggested as a potential candidate for reintroduction in Canada due to its threatened status and extirpation from some waterways, such as Big Otter Creek in southwestern Ontario. It is a benthic fish with a nearly translucent, golden-brown, elongate body measuring between 45-70 mm in total length (Figure 1.1). The species can be found throughout regions of the United States such as Ohio, Indiana, Illinois, Kentucky, West Virginia, New York, Vermont, and Pennsylvania, as well as Ontario and Quebec within Canada (Figure 1.2), where it burrows in fine gravel or sandy substrate of streams, rivers, and lakes (Scott and Crossman 1973; O'Brien and Facey 2008). Eastern Sand Darter have been classified as threatened since 1994 following assessment by COSEWIC (COSEWIC 2009). COSEWIC assigned Quebec and Ontario populations as separate units in 2009 due to their geographic isolation from one another, indicating the need for independent recovery strategies tailored to the needs of each population (Figure 1.3; COSEWIC 2009). Individuals from the Grand River in Ontario are considered to belong to the same genetic population as those extirpated from Big Otter Creek (Figure 1.4), providing a potential source population from which to draw individuals for translocation (Edwards et al. 2007). Eastern Sand Darter spawn in the spring and summer months, and like many small bodied non-migratory species, the dispersal potential of juveniles post-hatch is limited (Johnston 1989). Due to the benthic lifestyle of Eastern Sand Darter, substrate composition is an important environmental factor that can influence the suitability of a habitat (O'Brien and Facey 2008). Siltation of substrate as a result of human activities such as agriculture has been cited as one of the primary drivers in the decline of Eastern Sand Darter from their native range (Spreitzer 1979; Holm and Mandrak 1996). For prey, Eastern Sand Darter rely primarily on benthic organisms belonging to invertebrate groups such as

Chironomidae, Cladocera, Ostracoda, Oligochaeta, and Ephemeroptera, at varying rates depending on availability (Burbank et al. 2019). As a result, substrate destruction and other factors resulting in decreases in prey availability have impacted the number of invertebrate larva available to Eastern Sand Darter, factors that may have contributed to population declines through parts of their native range (Lamothe and Drake 2019).

Competition for food and other resources between Eastern Sand Darter and other small-bodied fishes that occupy a similar isotopic niche is an important element that has the potential to drive population dynamics within community assemblages (Burbank et al. 2019). The level of competition from both native and non-native populations must be considered during the planning of reintroduction projects to maximize the likelihood of successful reintroduction. Due in part to the limited isotopic niche occupied by Eastern Sand Darter, this species is relatively susceptible to environmental disturbances relative to some co-occurring species, and places even greater importance on considering these competitive interactions (Burbank et al. 2019). As Round Goby (*Neogobius melanostomus*) are present in Big Otter Creek (Lamothe and Drake 2019), the planned reintroduction of Eastern Sand Darter to this body of water must specifically account for the presence of this invasive competitor. The translocation of this species will further require capture, holding, and a 2-hour transport event, providing the opportunity to test whether soft-release techniques can promote recovery from transport stress. This translocation represents one of the first reintroduction efforts for a small-bodied freshwater fish in Canada.

## Thesis overview

Planned species reintroduction projects require extensive research, analysis, and planning to optimize the chances of success (George et al. 2009; IUCN/SSC 2013; Cochran-Biederman et al. 2015). Some aspects of reintroduction, such as the biotic and abiotic conditions of the reintroduction habitat, as well as the release methods used during translocation require careful consideration as these factors contribute heavily to the likelihood of survival and establishment of released fish (Teixeira et al. 2007; Cochran-Biederman et al. 2015). In the context of a planned translocation of Eastern Sand Darter to Big Otter Creek in Ontario, Canada, I first assessed the hydrological parameters and community ecology of sites along Big Otter Creek, with emphasis on variables known to relate to Eastern Sand Darter habitat suitability (Chapter 2). I then determined whether alternative release methods (i.e., soft-release) can promote behavioural recovery following transport (Chapter 3). Collectively, the goal of my thesis was to support the repatriation of this fish to parts of its native range, while also contributing to our knowledge of reintroduction biology for small-bodied freshwater fishes more generally.

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Figure 1.1 - Photo of Eastern Sand Darter (*Ammocrypta pellucida*) on sand substrate (Photo Credit: Rob Criswell).

Figure 1.2 - Distribution of Eastern Sand Darter in North America (Image: Environment and Climate Change Canada).

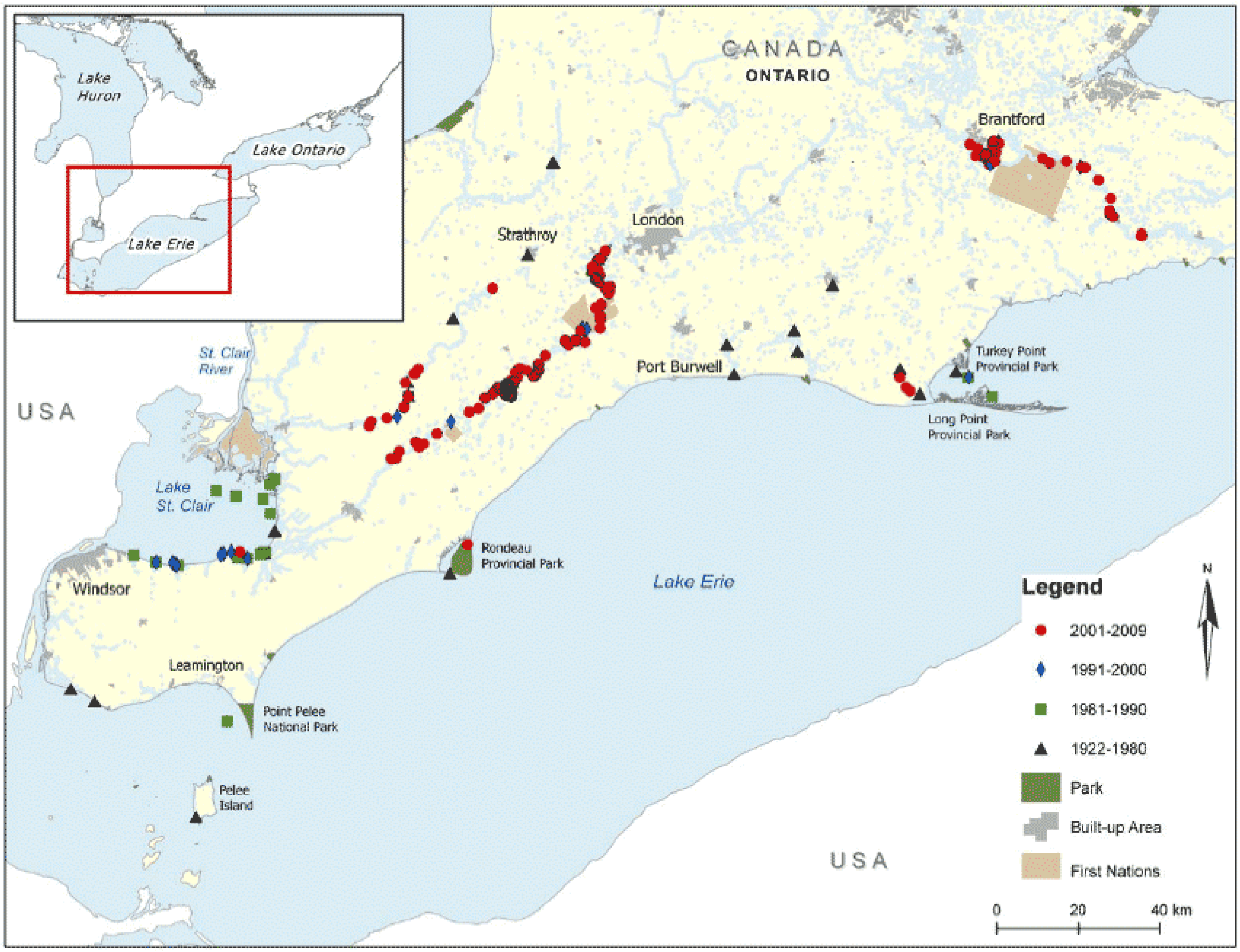

Figure 1.3 – Ontario distribution of the Easter Sand Darter (Fisheries and Oceans Canada 2012).

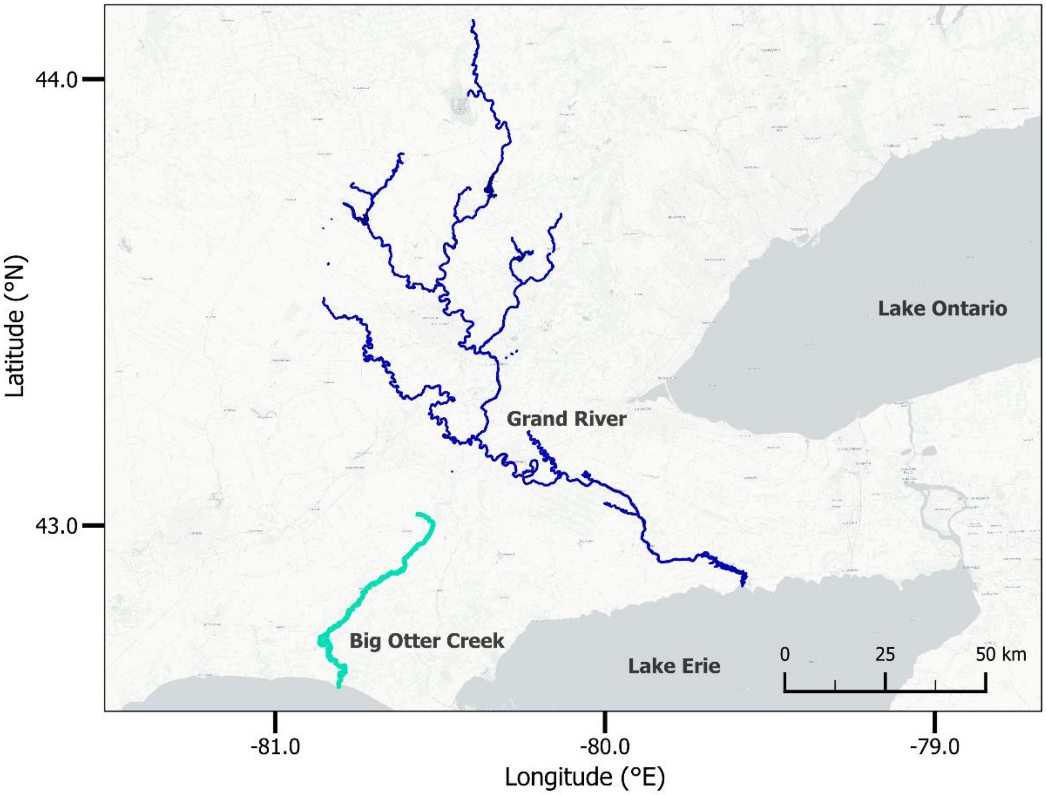

Figure 1.4 - Map of the Grand River and Big Otter Creek in southern Ontario, Canada. The Grand River population of Eastern Sand Darter is being accessed as the source for transfers of individuals to Big Otter Creek, where the species has historically been extirpated, as part of a reintroduction program led by Fisheries and Oceans Canada.

# Chapter 2 - An analysis of the ecological conditions of reintroduction habitat for the Eastern Sand Darter (*Ammocrypta pellucida*) in a Great Lakes tributary

## Abstract

To combat the increasing rate of loss of freshwater fishes worldwide, a variety of conservation and rehabilitation tools and techniques will be necessary. Reintroduction involves re-establishing populations of a species by releasing individuals into areas of their range where they have previously been extirpated. While historically less commonly used for fishes, reintroduction is increasingly being suggested as a viable recovery tool, including for freshwater fishes in Canada. However, to improve the potential success of reintroduction projects, careful attention must be paid to the conditions of the release habitat, which can have substantial influence on survival and therefore the ability of a population to become established. The Eastern Sand Darter (*Ammocrypta pellucida*) is a small, threatened freshwater fish species that is being reintroduced to a part of its range in Ontario, Canada, specifically to Big Otter Creek. Here, I characterized the reintroduction habitat of Big Otter Creek based on conditions known to influence Eastern Sand Darter persistence, including substrate composition, presence of invasive Round Goby (*Neogobius melanostomus*), and overall fish community composition, by comparing conditions between two sample years leading up to the reintroduction process. I found that the proportion of areas with fine, sandy substrate did not differ between years. I also found that Round Goby abundance and the number of sites at which it was recorded was lower in 2023 compared to 2018. By identifying areas that have overlapping metrics of suitability (sandy substrate, low Round Goby abundance, and fish communities that do not negatively

interact with Eastern Sand Darter), I also identified three sites (up- and downstream from Tillsonburg, Ontario) that represent promising potential release locations for translocated individuals. My results highlight the importance of considering both abiotic and biotic conditions of release sites and support the ongoing reintroduction efforts for Eastern Sand Darter in Ontario.

## Introduction

Nearly one-third of freshwater fishes are facing the immediate threat of extinction (Reid 2019). Environmental disturbances such as degradation of habitat, flow alteration, climate change, and the introduction of invasive alien species are among the greatest factors leading to population declines of many freshwater fishes within North America (Dextrase and Mandrak 2006; McAllister et al. 2022). Conservation and rehabilitation can involve multi-faceted approaches, including reintroduction of a species to parts of its native range from which it has been extirpated (Seddon et al. 2014). Reintroductions are becoming increasingly important to the conservation of freshwater fish, as this group is highly susceptible to reductions in population connectivity and fragmentation of suitable habitat (Olden et al. 2011). Several fishes in Canada have been suggested as candidates for reintroduction efforts, with some stages of planning or implementation being initiated (Lamothe and Drake 2019). These projects require detailed planning, with species-specific considerations in relation to delineating source populations, employing captive breeding and/or movement of wild individuals, choosing release sites, and monitoring outcomes (Lamothe et al. 2019; Lamothe et al. 2021). Although modern reintroduction efforts often include some level of habitat assessment, a review of freshwater translocations by Olden et al. (2011) concluded that taking more concerted data- and theory-driven approaches to these projects will increase chances of success.

The conditions of the habitat to which a species is going to be reintroduced is one of the most important elements to consider during reintroduction planning. Environmental conditions have the propensity to affect post release survival and, therefore, overall chances of success (Minckley 1995; George et al. 2009; Ayres et al. 2012; IUCN/SSC 2013; Fisk II et al. 2014; Cochran-Biederman et al. 2015). Both biotic and abiotic attributes such as assemblage and physicochemical conditions determine how suitable a habitat may be for a species and whether or not a population is likely to thrive within a release environment (Hirzel and Le Lay 2008). Assessment of both the living and non-living components of an area can be integrated to gain a more complete understanding of current ecological conditions, while the use of historical data and repeated surveys are important for tracking how these conditions have changed over time (Oberdorff et al. 2001; Fisk II et al. 2014; Santorelli et al. 2014). The required characteristics of a habitat, including access to nutrition, as well as other physical conditions, including water quality, differ depending on life history characteristics (Greenberg 1991; French and Jude 2001; Carlson and Wainwright 2010; Burbank et al. 2019; Firth et al. 2021). Important behaviours related to the acquisition of food, predator evasion, or mating can necessitate that a habitat possess specific characteristics (Minckley 1995). For example, while investigating the feeding and habitat behaviours of several benthic fish species, Greenberg (1991) characterized water depth as a primary predictor for which species would be present in a particular section of habitat. For benthic species, substrate composition is also especially important to consider because it can influence the effectiveness of behaviours related to nutrition acquisition, predator evasion, and mobility (Greenberg 1991; Sullivan et al. 2004). Further, levels of non-organic materials such as silt, suspended particles, and turbidity can play a substantial role in water chemistry, influencing both physiology and behaviour of fishes (Kerr 1995). For example, Australasian Snapper (*Pagrus auratus)* were significantly less efficient at foraging when placed in tanks with greater levels of turbidity (Lowe et al. 2015). During subsequent field sampling, snapper abundance was also negatively correlated with increasing turbidity (Lowe et al. 2015). Overall, analysis of the abiotic attributes of an area can provide insight into the factors that may be influencing important aspects of ecological health, such as abundance and diversity, thereby supporting the assessment and monitoring of release site locations for reintroductions (Berkman and Rabeni 1987; Smith et al. 2016; Wedderburn et al. 2020).

In addition to considering key abiotic characteristics of reintroduction environments, a holistic approach also considers community ecology to understand the larger interactions between both the biotic and abiotic components of a specific habitat (Jackson and Blois 2015). While background research and analysis during planning of conservation strategies such as reintroduction have typically been concentrated on a focal species with limited attention being given to a community-based approach, there is growing evidence that optimizing and predicting outcomes for reintroduction requires collecting data on a wider variety of species (Fisk II et al. 2014). For example, assessment of community assemblage can indicate the relative diversity and abundance of a particular taxonomic group of interest such as birds or fish of an area, and provides insight into important ecological relationships such as predator-prey interactions or intra- and inter-specific competition for resources (Santorelli et al. 2014). Knowledge of species co-occurrence patterns, as well as assemblage structure, are valuable ecological details that allow assessment and comparison of the conditions of multiple locations considered as release sites, and how favourable they may be for reintroduced species (Jaureguizar et al. 2003; Cochran-Biederman et al. 2015; Smith et al. 2016). The selection of suitable habitat for planned translocations of Atlantic Whitefish (*Coregonus huntsmani*) in Canada involved analysis of conditions of various locations, considering factors such as species interactions (Fisheries and Oceans Canada 2018). Modelling of species distribution has also been used in Abrams Creek (Great Smoky Mountains National Park, USA) to determine the suitability of habitat across reintroduction sites for three small fishes: Greenside Darter (*Etheostoma blennioides*), Banded Sculpin (*Cottus carolinae*), and Mottled Sculpin (*Cottus bairdii*) (Malone et al. 2018).

As part of community assessments, the specific influence of invasive species can also be informative for understanding potential dynamics within reintroduction sites. The spread of non-native invasive species is one of the greatest threats to global biodiversity and this threat is particularly prevalent for freshwater fishes (Jelks et al. 2008; Burkhead 2012). Within the context of translocation, invasive species can represent barriers to establishment directly (e.g., predation) or indirectly (e.g., competition, disease susceptibility). In mesocosm experiments testing potential establishment of the critically endangered Tequila Splitfin (*Zoogoneticus tequila*) in different communities, higher abundance occurred when fish shared habitat with established native species (Suárez-Rodríguez et al. 2023). In contrast, the presence of invasive Twospot Liverbearers (*Pseudoxiphophorus bimaculatus*) led to negative consequences in behaviour, namely reduced activity level (Suárez-Rodríguez et al. 2023). Assessing the level of pressure from invasive species was also an integral component of the translocation feasibility assessment tool developed by Kalogianni et al. (2024) to determine release waterbodies for the Corfu Killifish (*Valencia letourneuxi*). For many freshwater fish species, knowledge of invasive species and their abundance within the reintroduction habitat will be required for the greatest chance of successful reintroduction, and for adequate post-release monitoring (Dextrase and Mandrak 2006; Kalogianni et al. 2024).

One imperilled freshwater fish species that has been proposed for reintroduction is the Eastern Sand Darter (*Ammocrypta pellucida*), which inhabits streams, rivers, and lakes throughout parts of Canada and the United States (Putnam 1863; Ontario Ministry of Natural Resources 2012). This small, slender, translucent fish burrows within sand and fine gravel substrate and feeds primarily on invertebrates (Burbank et al. 2019). It is listed as threatened and is protected under the Species at Risk Act (SARA). To support the Ontario populations of Eastern Sand Darter, a reintroduction effort in Big Otter Creek, from which they have been extirpated, began in the spring of 2025, sourced by individuals from a stable population in the nearby Grand River (Andrew Drake; *personal communication*) (Figure 2.1). Given that reintroduction is a complex procedure requiring significant time, labour, collaboration, and resources to realize success in re-establishing a viable population, many details of the project must be specifically tailored to the habitat requirements of the Eastern Sand Darter (Seddon et al. 2007). Eastern Sand Darter are considered a habitat specialist requiring substrate of a specific size range for behaviours such as feeding and mating, with a strong preference for small to medium size sand and fine gravel (<0.5mm in diameter) (Daniels 1993; O'Brien and Facey 2008; Ginson et al. 2015; Thompson et al. 2017). In preference tests using simulated river environments, greater than 90% of Eastern Sand Darter burrowed in substrate composed of small or medium sized sand particles (Thompson et al. 2019). In the wild, capture of Eastern Sand Darter often occurs in areas exclusively containing sandy substrate (Thompson et al. 2019). The substrate specificity of Eastern Sand Darter limits the availability of suitable reintroduction habitat and also makes them vulnerable to environmental disruptions. Indeed, reduction of appropriate substrate as a result of agricultural land use in surrounding areas is recognized as one of the greatest contributing factors to the decline of this species throughout its range (Berkman and Rabeni 1987; COSEWIC 2009).

Invasive species also represent a potential threat to the Ontario population of Eastern Sand Darter. Round Goby (*Neogobius melanostomus*) is small freshwater fish species, invasive to the Laurentian Great Lakes, and poses various threats to small- bodied fish species depending on life stage (Dextrase and Mandrak 2006; B.L. Firth et al. 2021). In a tributary of Lake Erie, Round Goby abundance has been inversely correlated with the abundance of Eastern Sand Darter (Raab et al. 2018). As adult Round Goby may be predators of juvenile Eastern Sand Darter as well as competitors for food and space with adults, this invasive species represents a substantial concern for both reintroduction efforts and extant populations of the Eastern Sand Darter (Dextrase and Mandrak 2006; Lamothe and Drake 2019). In Big Otter Creek, Round Goby has been detected in the greatest abundance in areas most downstream and closest to Lake Erie (McAllister et al. 2022). Knowledge of Round Goby abundance across potential release sites in Big Otter Creek, as well as emphasis on monitoring over time, will be crucial to achieving successful reintroduction (Cochran-Biederman et al. 2015).

As part of the ongoing recovery strategy for the Ontario population of Eastern Sand Darter, Fisheries and Oceans Canada have completed multiple surveys of Big Otter Creek, the Grand River, and Ausable River in southwestern Ontario over several years leading up to the reintroduction efforts (Barnucz et al. 2020). My objective is to use the data collected from these efforts to characterize current ecological conditions within Big Otter Creek to support selecting the most appropriate sites for reintroduction. Using community assemblage data, as well as other measures of physical site characteristics such as substrate composition, I will characterize whether the ecology of Big Otter Creek has changed over time (between 2018 and 2023), and determine which areas in this waterway may be most favourable to Eastern Sand Darter. More specifically, I will compare measures of ecological relevance such as diversity indices, proportion of substrate composed of sand, the presence and abundance of Round Goby, and other physicochemical parameters across sample years to determine any significant changes over time. Additionally, I will pair known co-occurrence relationships between Eastern Sand Darter and other fish species from waterways in southwestern Ontario with fish community survey data from Big Otter Creek to estimate likelihood ratios across sites. I will subsequently determine any patterns of spatial convergence of attributes favourable to Eastern Sand Darter in Big Otter Creek, thereby supporting broader reintroduction planning and release for this species in future years.

## Methods

### *Community assemblage sampling*

To determine fish community assemblage in Big Otter Creek, sampling was performed from 9 – 19 July and 24 – 26 September in 2018, and 18-21 and 25-27 September and 3-5 October in 2023. Community samples were collected in downstream direction at each site using a bag seine net (length 9.2 m; height 1.8 m; 3 mm mesh). Samples were collected from 3 sub-sites (riffle, pool, and run) of wadeable depth (<1.2 m) (Gaspardy and Drake 2021) at n = 14 reaches. Three hauls were completed in each sub-site. Hauls from each pass were emptied in separate containers of river water, allowing approximately 5-minutes to elapse between each seine haul for the substrate to settle and fish to repopulate the sample area. Following the completion of the seining process at a given site, all captured individuals were classified to genus and species and measured for length (mm). The number of individuals and size range of each species at a given site was calculated. In circumstances where species identification was not possible in the field, samples were kept in formalin for subsequent identification from Fisheries and Oceans Canada personnel. Identification and processing of the three holding containers at each site took approximately 10 minutes total, after which we began habitat assessment. To avoid disturbing areas of the reach yet to be sampled, samples were collected in order from the most upstream segment of the reach to the most downstream segment. Catch per unit area (CPUA) was calculated by dividing the number of individuals of each species at each site by the seined area (80 $m^2$), and was used as a metric of relative abundance (McAllister et al. 2022). Animal Use Protocols for community sampling were approved by Fisheries and Oceans Canada.

### *Physicochemical characteristics*

Following the collection of community samples at each reach of the river, we measured environmental parameters to determine key physical characteristics of the habitat (Table 2.1). A YSI-EXO2 multiparameter sonde (YSI Incorporated, Yellow Springs, OH 45387 USA) was placed approximately 0.1m under the surface of the water to measure turbidity (NTU), dissolved oxygen (mg/L), temperature (°C) and conductivity (µS). A 120 Secchi tube was also used to measure water clarity and air temperature was measured using a Kestrel 3000 wind meter. We then measured stream velocity (m/S) using a Swoffer 2100 Current Velocity meter with the probe faced in the upstream direction, positioned at half the stream depth from three locations (fastest, slowest, mid-velocity) within the area covered by the seining process. The substrate composition was classified by sampling handfuls of riverbed and recording the mean diameter of particles: clay (0–0.002 mm), silt (0.002–0.02 mm), sand (0.02–2 mm), gravel (2–40 mm), cobble (40–256 mm), and boulder (>256 mm excluding bedrock).

### *Statistical analyses*

All statistical analyses were performed in R (version 4.4.1) within RStudio (version 2024.12.1+563). Due to differences in calendar dates over which sampling occurred in 2018 and 2023 and the potential for associated inherent seasonal variations in some of the physicochemical parameters such as temperature, dissolved oxygen, turbidity, and pH (Braaten and Guy 1999), contrasts of these metrics were not made using inferential analysis but provided as environmental descriptions. We assessed the proportion of sand across sites between sample years (2018 vs. 2023), with a Wilcoxon Rank Sum Test using the ‘wilcox.test’ function.

We used a Wilcoxon Rank Sum Test using the ‘wilcox.test’ function to compare Round Goby abundance between years. Additionally, the proportion of sites containing Round Goby were compared between years using a Fischer’s Exact Test.

We calculated Simpson’s and Shannon diversity indices for 2018 and 2023 using the ‘diversity’ function of the vegan package in R (Oksanen et al. 2001; R Core Team 2024). We used a Wilcoxon Rank Sum Test to compare diversity index values between years. Due to the lack of consensus on the efficacy of spatial autocorrelation adjustments to complex ecological data, and limitations of use with binomial data such as species presence-absence, this factor was not accounted for in our analyses (Dormann et al. 2007).

We determined the likelihood ratio (hereafter called occurrence probability) associated with the potential presence of Eastern Sand Darter based on fish community composition in 2023 Big Otter Creek sample sites. We used naive psi values, which were calculated using positive and negative occurrence patterns between Eastern Sand Darter and other fish species at sites in both Ausable Creek and the Grand River (as per Lamothe et al. 2019). This method uses the proportion of sites at which species occur together, or do not occur together, to determine how positively or negatively correlated they are. These values can then be used to make inferences about how likely a species is to occur based on the positive or negative associations with other species detected at that location. We were therefore able to estimate the probability that Eastern Sand Darter could be expected to be found at each site in Big Otter creek based on the other species present (Veech 2013; Lamothe et al. 2019).

## Results

### *Physicochemical characteristics*

Values for conductivity (µS), dissolved oxygen content (mg/L), turbidity (NTU), water temperature (°C), pH, and velocity (M/s) for 2018 and 2023 are summarized in Table 2.2. We found no significant differences in the proportion of sand substrate between years across sites ($W = 1067.5$, $p = 0.85$; Figure 2.2). In 2023, sites varied in their proportion of sand, ranging from 0-100% (Figure 2.3).

### *Round Goby*

Round Goby abundance was significantly greater in 2018 compared to 2023 ($W = 1414.5$, p-value $< 0.01$; Figure 2.4). Round Goby was also present at a greater proportion of sites in 2018 compared to 2023, although this difference was not significant ($p= 0.06$; odds ratio = 0.29; 95% CI, 0.93–5.79; Figure 2.4). In 2023, sites varied in terms of Round Goby abundance, with a mean CPUA of 0.03 (SE: ± 0.008) (Figure 2.5).

### *Diversity indices*

Mean Shannon diversity index was 0.041 (0.004 – 0.173; SE: ± 004) in 2018 (Figure 2.6A) and 0.138 (0.005 – 0.505; SE: ± 0.019) in 2023 (Figure 2.6B). The Simpson’s diversity index for 2018 ranged from 0.001-0.051 with a mean of 0.011 (SE ± 0.001) (Figure 2.6C). In 2023, the Simpson’s Diversity Index ranged from 0.001 to 0.26 with a mean of 0.05 (SE: ± 0.001) (Figure 2.6D). Values were greater in 2023 than 2018 for both the Shannon index ($W = 437$, $p<0.01$) and the Simpson diversity indices ($W = 400$, $p<0.01$) (Figure 2.7).

### *Community based occurrence probability*

Occurrence probability ranged from 0 – 1 with a mean of 0.278 and a standard error of ± 0.043. These values fluctuated across sites with no consistent gradient of change; however, sites closest to Tillsonburg indicated relatively low values, with many of the higher values occurring at sites furthest from the mouth of the river (Figure 2.8).

## Discussion

To promote establishment and population persistence, thorough assessment of release habitat is required during the planning of freshwater fish reintroduction projects (Cochran-Biederman et al. 2015). Understanding the physicochemical conditions and community composition of release waterways can provide insight into the feasibility of repatriation efforts (e.g., capacity of a habitat to support a reintroduced population) as well as pinpoint locations within these areas that represent the most appropriate release sites (Claireaux and Lefrançois 2007; Fisk II et al. 2014; Cochran-Biederman et al. 2015). To support the reintroduction of Eastern Sand Darter in southern Ontario, Canada, we characterized biotic and abiotic components of the release waterway that are known to be important to the ecology of this species.

Parameters related to water quality such as pH, temperature, and dissolved oxygen are vital to fish establishment and long-term survival (Alabaster and Lloyd 1982; Firth et al. 2021; Gui et al. 2023). Although there was variation in a number of parameters between years in Big Otter Creek, surveys were performed during slightly different times of the year, so some differences are most likely explained by seasonal variation (e.g., temperatures being warmer in 2018 compared to 2023), rather than anthropogenically-induced changes between sampling periods (Braaten and Guy 1999). However, valuable insights can still be gained from comparing parameters that are more stable seasonally and are likely to have the greatest influence on the Eastern Sand Darter. Taxon-specific protocols for assessing habitat suitability place importance on the habitat parameters which are of more ecological importance to the focal species (Johnson 2007).

The preferences related to substrate selection of Eastern Sand Darter have been well-documented. For example, combining survey data from two rivers with laboratory experiments, Obrien and Facey (2008) analyzed the size of substrate that Eastern Sand Darter chose for resting and burrowing. Small sediment size was preferred, with 39 of 44 fish being found on substrate <1.0 mm during the lab trials (O'Brien and Facey 2008). In Ontario, decrease in appropriate substrate material as a result of agricultural land use in the areas surrounding Big Otter Creek during the mid 20th century has been cited as one of the primary causes for the extirpation of Eastern Sand Darter from this waterway (COSEWIC 2009; Bouvier and Mandrak 2010). We found no differences in the percentage of sandy substrate between 2018 and 2023, indicating that the presence of habitat with this key ecological component is remaining stable over time. Under typical conditions, substrate composition is relatively constant with minimal fluctuations and only gradual changes over years (Galay 1983). Therefore, without drastic changes in land use in the surrounding areas of Big Otter Creek in the study period, it would be unlikely that the proportion of sandy substrate in the river would have changed by a significant margin, and therefore the result of no significant difference in sand proportion between our sample years was expected. Along the waterway, sites varied markedly in their proportion of sand. Sites with some of the lowest proportions of sand were grouped around the midpoint of our sample sites from downstream to upstream (i.e., Tillsonburg area) (Figure 2.3).

Although substrate size and material may be one of the greatest indicators of habitat suitability for Eastern Sand Darter, this metric alone cannot be used as a predictor of abundance or establishment. Biotic attributes such as the presence and abundance of predators and competitors are key determinants in the suitability of habitat (Greenberg 1991; Smith et al. 2016). Invasive species often outcompete native fishes for resources, rapidly expanding their range and abundance in the non-native ecosystems they invade (Morissette et al. 2018; Blair et al. 2019). Round Goby display a competitive advantage over many native benthic species in the Great Lakes (McAllister et al. 2022). By placing Round Goby in artificial streams with three native fish species from the Great Lakes [Logperch (*Percina caprodes*)*,* Slimy Sculpin (*Cottus cognatus*), and Spoonhead Sculpin (*Cottus ricei*)], Bergstrom and Mensinger (2009) showed that both Logperch and Spoonhead Sculpins lost significant mass while mass significantly increased in Round Goby. The spread of the invasive Round Goby is cited as a prominent concern for the persistence of Eastern Sand Darter in this region (McAllister et al. 2022; McAllister et al. 2022). Decreased Round Goby presence and abundance in 2023 compared to 2018 contradicts the expectation that this invasive species would be able to further spread in Big Otter Creek from their invasion front in Lake Erie and grow in numbers. Round Goby expanded to all of five of the Laurentian Great Lakes within five years of initial detection in these waterways in Lake Michigan in 1990, and has increased its range substantially over the following years (Blair et al. 2019). In Canada, the spread of aquatic invasive species has been a major concern to numerous stakeholders, leading to the development of action plans, including educational programs and legislation to help address this issue (Canadian Council of Fisheries and Aquaculture Ministers 2022). It is possible that significant efforts to control the spread of Round Goby may have been effective in reducing the abundance within Big Otter Creek. Comparison of historical surveys of data collected from other waterways would provide insight as to whether this is a local phenomenon and to what extent efforts to control the spread of Round Goby in Great Lakes waterways may have contributed to our results.

Tracking the distribution of Round Goby in a more targeted way would also provide valuable insight into what level of fluctuation in abundance may be explained by life history characteristics such as seasonal upstream and downstream migration (Blair et al. 2019). Specifically, the abundance of Round Goby in Great Lakes tributaries has been shown to fluctuate temporally, with differences in the number of adults and juveniles varying with upstream distance from the rivermouth depending on season (Blair et al. 2019). In Cobourg Creek, a Lake Ontario tributary, electrofishing surveys showed that the number of Round Goby captured per month ranged from 71 in May to 561 in August to 533 in September (Blair et al. 2019). In our study, 2018 sampling in Big Otter Creek occurred during July – September, while 2023 sampling occurred during September and October. As a result, seasonal fluctuation in abundance due to outmigration to Lake Erie may have had some influence on the difference in Round Goby abundance and presence between sampling years; systematic characterization of this migration process in Big Otter Creek has not yet been completed. However, several sites in 2023 contained very low goby abundance CPUA <0.015. Although these sites were dispersed throughout the total areas sampled, many of these occurred further upriver with several occurring consecutively, and only one site with a CPUA exceeding this value upriver from Tillsonburg (Figure 2.5). To fully understand the potential for regions of the river to be viable release locations in terms of minimizing Round Goby interaction throughout the full lifecycle, targeted seasonal assessment of Round Goby would be beneficial.

Along with investigating the presence of key species, broader statistical methods have been developed to quantify important community-based indicators of ecological health such as diversity and abundance (Heip et al. 1998; Rice 2003). Although the use of metrics such as Shannon and Simpson's indices have drawn criticism for oversimplification of biological conditions (Karr 1981), these can still be effective tools for community assessment, particularly when used as comparative values within a study, as well as in combination with other methods to bolster more specific analyses (Kwak and Peterson 2007). Both Shannon and Simpson's values were greater in 2023 than in 2018. Our model, however, did not account for spatial autocorrelation and therefore should only be used in support of more conclusive findings. As high levels of habitat degradation, loss of diversity, and presence of invasive species are correlated factors in many waterways (Braaten and Guy 1999; Früh et al. 2012), we expected that characteristics important to Eastern Sand Darter may overlap spatially. The Shannon and Simpson's diversity indices we calculated were highest in some of the areas that were most suitable for Eastern Sand Darter based on our other metrics (percentage of sandy substrate and Round Goby abundance), indicating an alignment of these broad metrics of ecological health and preferred habitat conditions of Eastern Sand Darter (Braaten and Guy 1999; Früh et al. 2012).

To determine whether the sites that we identified as having suitable sand, low Round Goby abundance, and high diversity also had overall fish community composition that is known to neutrally or positively co-occur with Eastern Sand Darter, we employed likelihood ratios to represent occurrence probabilities. Knowledge of which fishes typically occur together can provide valuable insight into predicting where a species might thrive (Claireaux and Lefrançois 2007). Occurrence probability ranged from 0-1, indicating that there would be a far greater likelihood of detecting Eastern Sand Darter at some sites than others based on the other species present at those sites (Lohr and Fausch 1997; Lamothe et al. 2019). At a number of sites, greater occurrence probability occurred concurrently with greater percentage of sand as well as lower Round Goby CPUA.

Although Round Goby abundance is low in Big Otter Creek in the sections that flow through Tillsonburg, the level of sand sections of Big Otter Creek areas directly surrounding this municipality is relatively low, making these sections potentially less than ideal for release. However, the agreement of relatively high (suitable) metrics (i.e., high proportion of sand, low Round Goby abundance, and positive community interactions) in multiple locations suggests several potential ideal release sites. Specifically, upstream from Tillsonburg near Otterville (42.940920, -80.584218), downstream between Carson Line and Bayham Drive (42.820779, -80.757532), and upstream from Vienna (42.698657, -80.829600) are favorable in all three of the characteristics we assessed. The agreement of multiple metrics at these sites suggests that there are locations that are likely to be most ecologically favourable to the persistence of Eastern Sand Darter upon release, in comparison to other regions of the river we assessed. Although these areas are not dispersed evenly across Big Otter Creek or across one contiguous stretch of river, these sites are moderately spaced from each other, which should allow for release that promotes dispersal of Eastern Sand Darter to multiple regions. Use of these sites will likely provide release individuals with a reasonable chance of survival and establishment, based on the ecological information available.

While our results can help determine which sites have the conditions most favorable to Eastern Sand Darter, increased reliability and accuracy would likely be achieved through expansion of our data and further statistical analysis. Only assemblage data was used for the calculation of the occurrence probability of Eastern Sand Darter using the likelihood ratio test. The integration of all of the habitat parameters into one suitability index, along with other aspects of the water quality data, could be used as a more comprehensive method for determining the most ideal sites for Eastern Sand Darter reintroduction (Braaten and Guy 1999; McAllister et al. 2022). Expert knowledge would be useful in determining how to weight the different metrics for establishing this type of suitability index. Overall, our results aim to support Eastern Sand Darter reintroduction to Big Otter Creek as well as provide a case study for future freshwater fish reintroduction planning.

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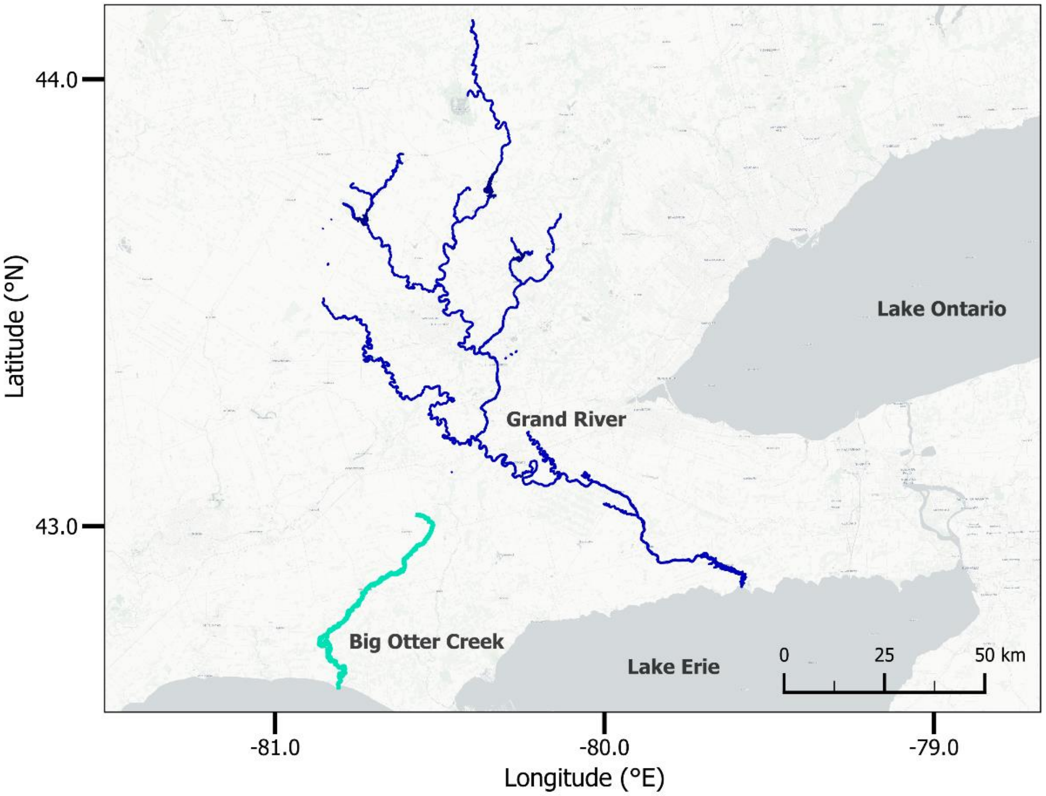

Figure 2.1 - Map of the Grand River and Big Otter Creek in southern Ontario, Canada. Big Otter Creek is the proposed reintroduction site for Eastern Sand Darter, with individuals being sourced from the Grand River. Community assemblage and environmental quality data were collected from Big Otter Creek in 2018 and 2023.

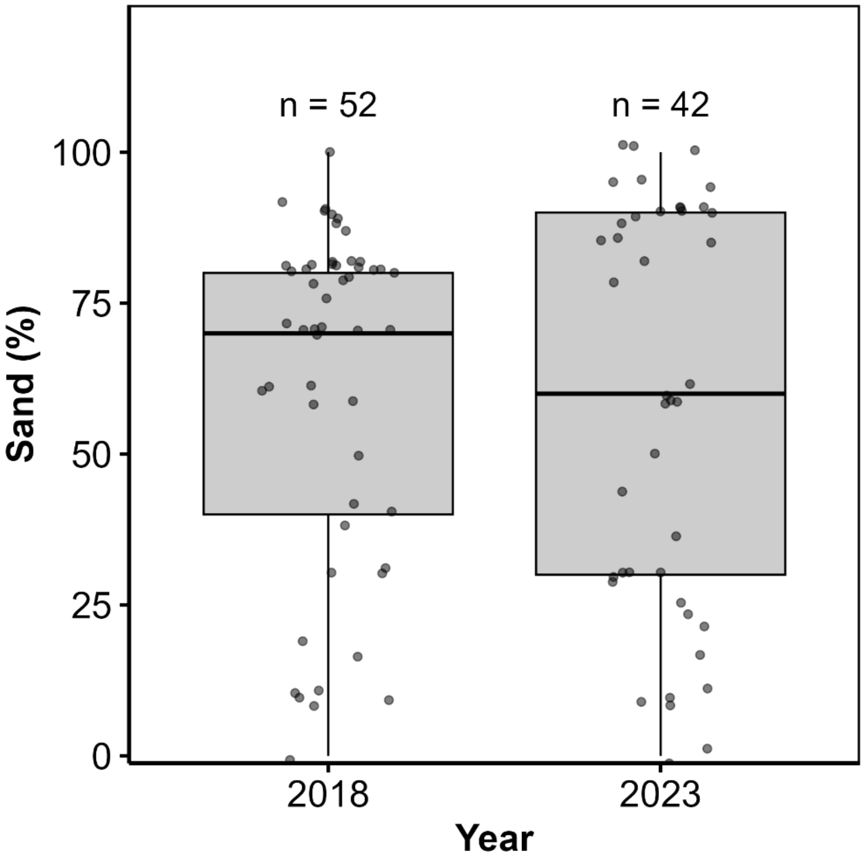

Figure 2.2 – Average proportion of sand across sites sampled in Big Otter Creek in 2018 versus 2023.

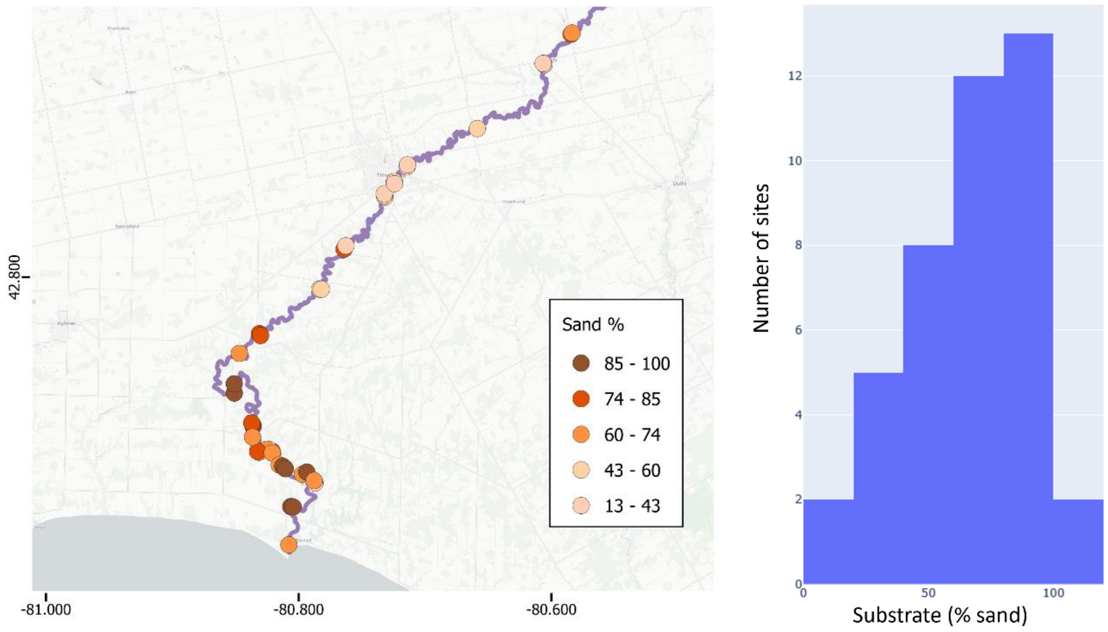

Figure 2.3 – Substrate composition (percent sand) at sites samples along Big Otter Creek in 2023. The histogram indicates the number of sites with certain percentages/ranges of sand.

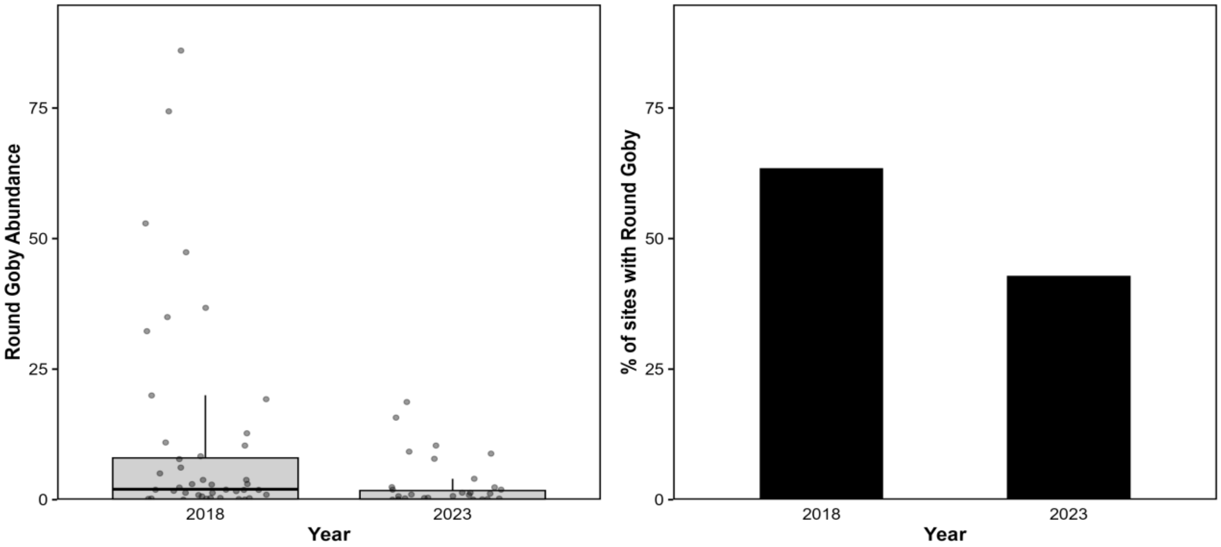

Figure 2.4 – Round Goby abundance (left) and the proportion of sites with Round Goby (right) along Big Otter Creek in 2018 versus 2023.

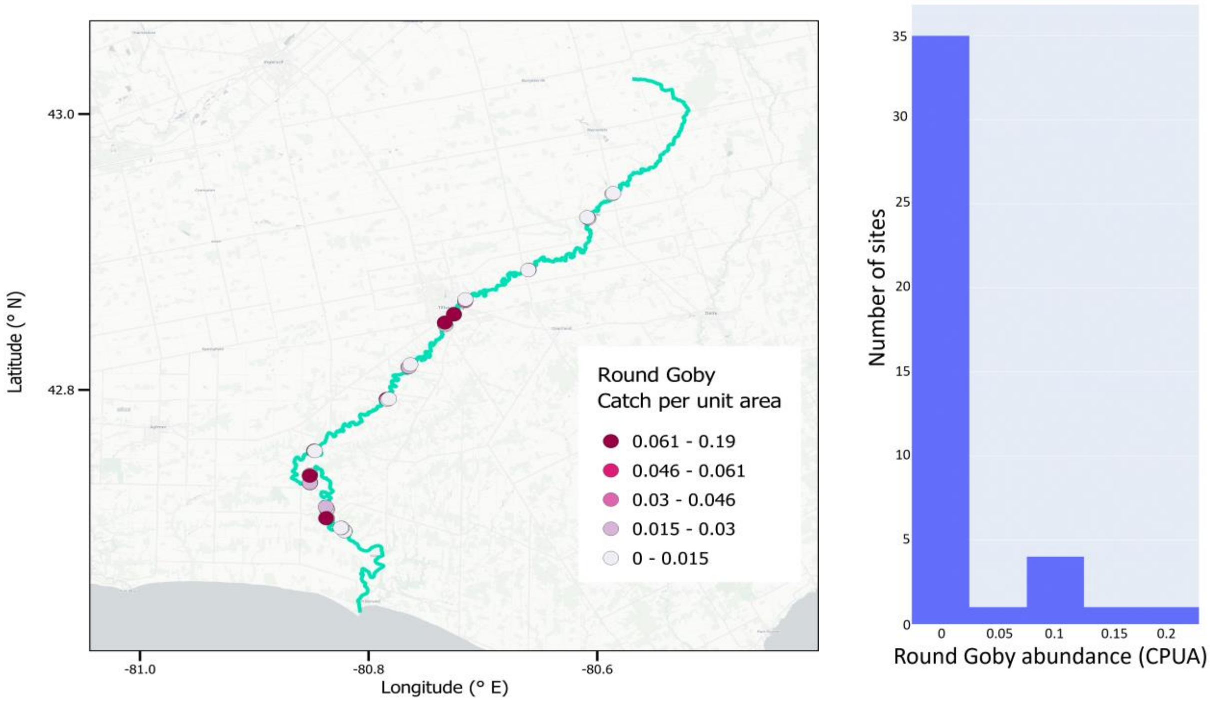

Figure 2.5 – Distribution of sites along Big Otter Creek in terms of Round Goby abundance (as indicated by catch per unit area – CPUA) in 2023. The histogram displays the number of sites that have varying levels of Round Goby abundance.

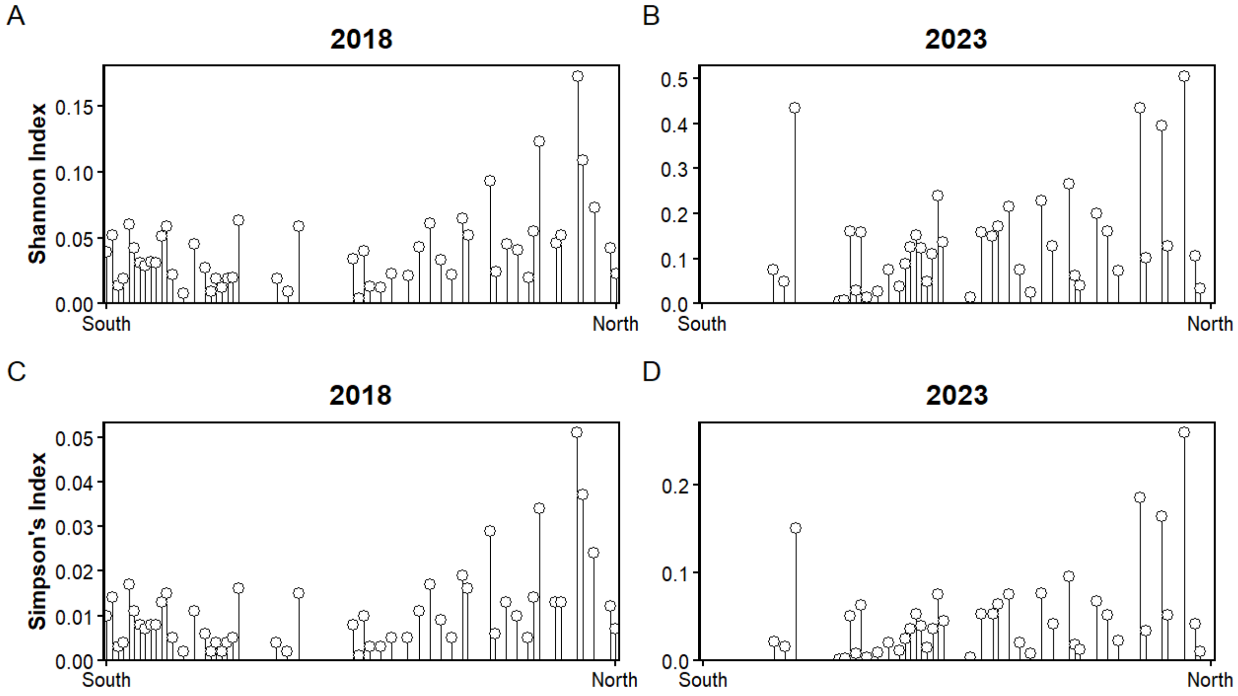

Figure 2.6 – Shannon and Simpson's diversity indices of individual sites samples along Big Otter Creek in 2018 and 2023.

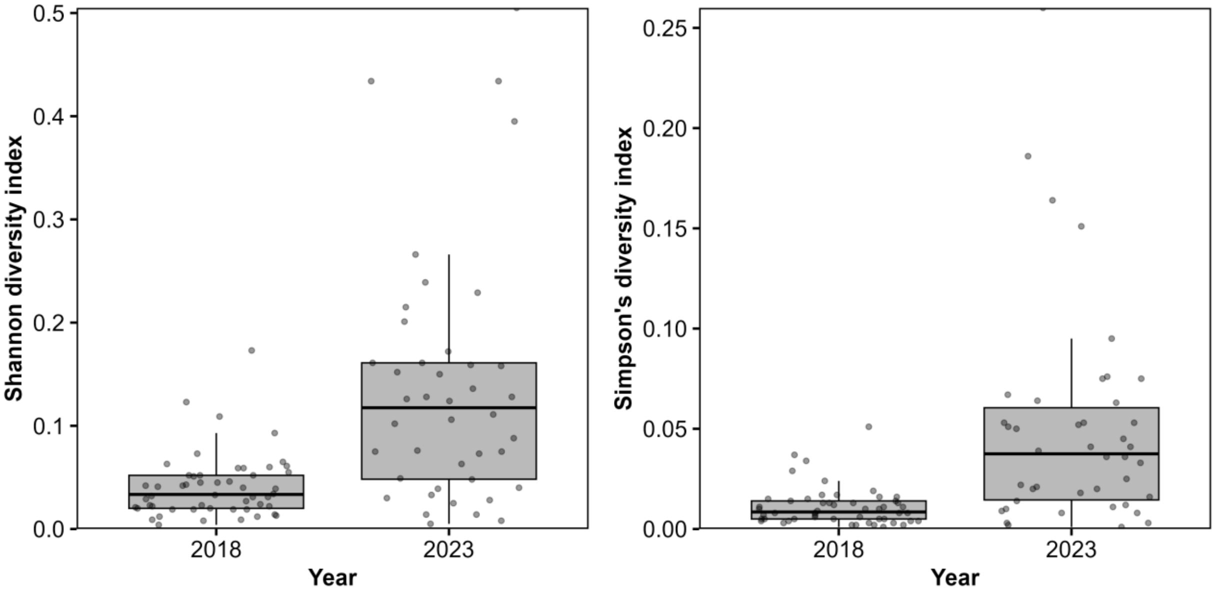

Figure 2.7 – Shannon (left) and Simpson's (right) diversity indices averaged over sites sampled in Big Otter Creek in 2018 and 2023.

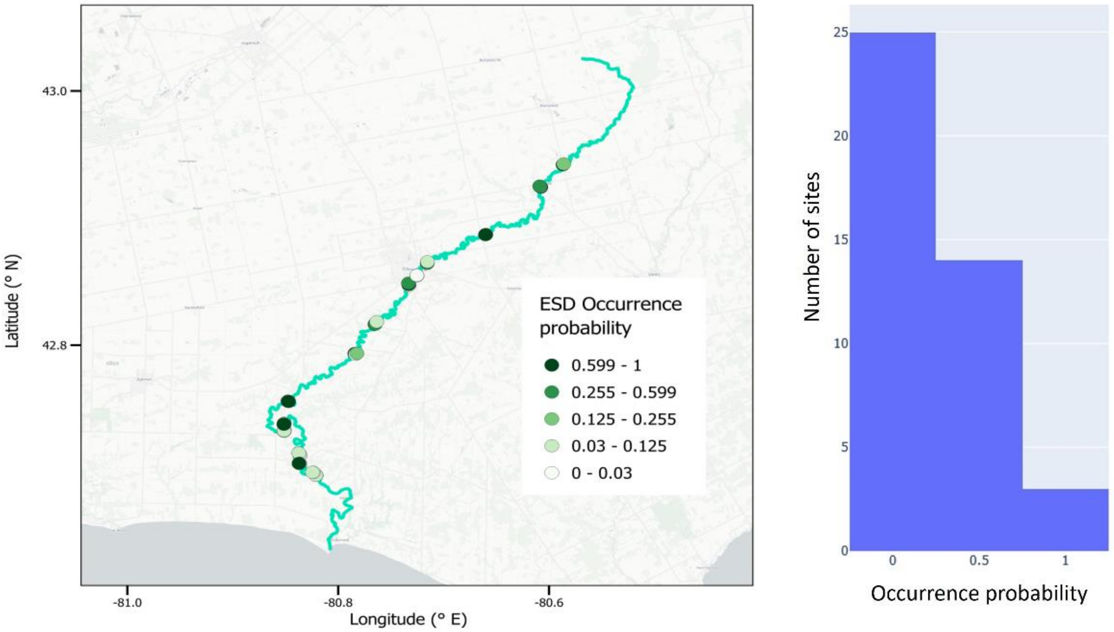

Figure 2.8 – Occurrence probability of Eastern Sand Darter, as calculated based on known community relationships with other fish species found along sites in Big Otter Creek in 2023. The histogram displays the variation in the number of sites falling into difference categories/ranges of occurrence probability.

Table 2.1 - Measurements recorded during habitat sampling in Big Otter Creek and their ecological relevance to Eastern Sand Darter.

| **Parameter Measured** | **Ecological relevance** |
|---|---|
| Water temperature (°C) | Affects numerous metabolic processes such as respiration. |
| Conductivity (µS) | Measure of concentration of dissolved salt other inorganic compounds that can be related to certain pollutant concentrations |
| Dissolved oxygen (mg/L) | Influence on respiration |
| pH | Important for biochemical reactions |
| Turbidity (NTU) | Influence on gill function |
| Velocity (m/s) | Affects substrate composition |
| Sand (%) | Preferred substrate of Eastern Sand Darter, crucial to performing behaviours such as mating and prey consumption, and likely important for station holding and predator evasion |
| Shannon diversity index | Indicator of overall ecological health |
| Simpson diversity index | Indicator of overall ecological health |

Table 2.2 - Mean environmental characteristics of Big Otter Creek in 2018 and 2023, averaged across sites. Values in parentheses represent ± standard error.

| | 2018 | 2023 |
|---|---|---|
| Water temperature (°C) | 20.06 (0.44) | 15.80 (0.18) |
| Conductivity (µS) | 551.78 (7.95) | 518.88 (6.30) |
| Dissolved oxygen (mg/L) | 9.28 (0.21) | 10.43 (0.19) |
| pH | 8.53 (0.02) | 8.48 (0.02) |
| Turbidity (NTU) | 18.40 (1.40) | 8.31 (1.07) |
| Velocity (m/s) | 0.20 (0.02) | 0.25 (0.02) |
| Sand (%) | 61.83 (3.81) | 57.74 (5.22) |
| Shannon diversity index | 0.04 (0.00) | 0.14 (0.02) |
| Simpson diversity index | 0.01 (0.00) | 0.05 (0.01) |

# Chapter 3 - Behavioural responses to transport stress in Eastern Sand Darter *(Ammocrypta pellucida)*: a comparison of release methods for informing reintroduction strategies

## Abstract

Reintroduction is becoming an increasingly important component of the conservation and rehabilitation of aquatic species. With the aim of establishing self-sustaining populations, the reintroduction process involves the transport and release of fish to habitat from which they have been extirpated. Throughout this process, fish are exposed to numerous stressors that can negatively impact the ability of these individuals to orient, find food resources, and evade predators following transport. The traditional method of hard-release involves releasing individuals immediately following transport, affording them no period of recovery before having to cope with the challenges of the reintroduction environment. An alternative strategy of soft-release involves temporarily holding individuals in an enclosure within the reintroduction site, providing them the opportunity to acclimate to their environment and recover from the transport event. Eastern Sand Darter (*Ammocrypta pellucida*) is a small species of benthic fish that is threatened in Ontario and has been suggested as a potential candidate for reintroduction to some of its historical range. Using open field behavioural tests and an assessment of swimming performance, we aimed to determine whether soft-release promotes the recovery of ecologically-relevant behaviours following transport. Specifically, we assessed activity time, space use, burrowing propensity, swimming performance, and response to a simulated predator in fish exposed to hard or soft-release protocols in comparison to non-transported (control) fish. We found no significant differences between treatment groups for most of the behavioural

metrics we assessed. While swim performance differed between groups in both summer and fall, the groups that differed from each other and the direction of the difference was inconsistent across seasons. We did find significant differences in some other behaviours between seasons, or between time periods (e.g., before vs. after introduction of the simulated predator). On the basis of the limited differences across the measured behaviours, we conclude that hard-release may be appropriate for reintroduction of Eastern Sand Darter, but that additional research is likely needed to explore this topic further. Our findings can support the ongoing development of effective reintroduction protocols for Eastern Sand Darter in Ontario, as well as provide information that can be considered during the reintroduction of other small-bodied, at-risk fishes.

## Introduction

As integral components of the biosphere, freshwater ecosystems support vast amounts of life across a diversity of taxonomic groups (Strayer and Dudgeon 2010). Recently, freshwater ecosystems have become some of the most imperiled on the planet (Dudgeon et al. 2006). Freshwater fishes are experiencing global losses at rates greater than any previously recorded, with many species experiencing extirpation and extinction (Miller et al. 1989; Dudgeon et al. 2006; Comte et al. 2013; Desforges et al. 2022). The precipitous decline of freshwater fishes in North America has been documented and identified as an area of major concern warranting appropriate conservation attention (Jelks et al. 2008; Burkhead 2012). In the wider toolbox of conservation approaches, reintroduction is becoming an increasingly important technique for the conservation and rehabilitation of freshwater ecosystems (Minckley 1995; Seddon et al. 2014). Reintroduction is the process of strategically releasing individuals of a species to parts of their native range from which they have been extirpated with the goal of re-establishing viable populations (IUCN/SSC 2013). Reintroduction can be achieved through the release of captive-raised individuals or by capturing and transporting individuals from a nearby source population (Dickens et al. 2010). Although there are inherent advantages to relocating fish from wild populations, the targeted species is often considered at-risk, placing limitations on the number of individuals that can be relocated. In such situations, ensuring the survival of as many translocated individuals as possible becomes increasingly important. As fish experience shifts in behaviour and physiology throughout different lifecycle stages, as well as in response to shifting environmental conditions like the change of seasons (Brown 2011; Wingfield 2013; Firth et al.

2021), adequate consideration must be given not only to whether a given population is appropriate for use as a source population, but when it is most appropriate to move individuals (Lamothe et al. 2021). Despite the high number of imperiled species, research regarding reintroduction of freshwater fishes, especially small-bodied groups, is sparse (Seddon et al. 2005; Olden et al. 2011; Lamothe et al. 2019).

For most fish, the reintroduction process will require a transport event that will expose individuals to several stressors associated with capture, restraint, vehicle movement, and release (Acerete et al. 2004; Dickens et al. 2010). Although these transport procedures may only last a few hours in duration, this step has the potential to disproportionately influence reintroduction success because of the associated disruptions to physiological processes and behaviour that can persist following release, particularly when an individual is exposed to numerous simultaneous or concurrent stressors (Barton 2002). For example, transport is known to lead to changes in blood levels of ions, respiration rate and oxygen availability, and various hormones such as stress hormones (i.e., cortisol) and energetic metabolites (Sopinka et al. 2016). Roberts et al. (2024) investigated the effects of various loading and release methods on the stress physiology of Atlantic Salmon (*Salmo salar*), documenting significant increases of both cortisol and lactate in fish that were subjected to transport methods when compared to non-transported fish. Similarly, Acerete et al. (2004) found significant increases in cortisol levels following transport in Eurasian Perch (*Perca fluviatilis, L.*). The correlation between stress, hormone levels, and altered behaviour as a result of procedures typical of translocation was also highlighted by Portz et al. (2006), who measured stress hormones and behaviour of juvenile Chinook Salmon that were captured and loaded into a transport vehicle, reporting significant decreases in both swim performance and startle response of fish with greater levels of circulating stress hormones.

The negative consequences following exposure to stress can be delayed, and persist for varying lengths of time, as well as be compounded depending on the magnitude and continuity of the stressors (Romero et al. 2009). In addition to the transport-related factors associated with reintroduction, fish may also experience stress when being introduced to the novel environment at their release site (Monk et al. 2020). The physiological and behavioural changes that are compounded and/or persistently associated with the stress of reintroduction may lead to an inability of fish to successfully perform important behaviours such as foraging, orienting, evading predation, and seeking shelter upon release (Mokdad et al. 2019). Ultimately, there is concern that such impairment leads to a decreased likelihood that an individual becomes established within their new environment (Davis 2010; Monk et al. 2020). Release-related stress, disorientation, and predation were cited as factors contributing to the limited establishment and high mortality rate of Razorback Sucker (*Xyrauchen texanus*) within some river sections of the United States (Mueller and Foster 1999). In an assessment of correlates of successful reintroduction projects involving freshwater fishes, physiological changes and subsequent behavioural consequences associated with transport were identified as one of the most important factors to consider to improve reintroduction outcomes (Cochran-Biederman et al. 2015).

Improving transport efforts has often focused on the maintenance of water quality (e.g., oxygen content, temperature) in transport containers as well as adjustments to fish density (Harmon 2009; Sampaio and Freire 2016). These approaches can assist in minimizing physiological disruption of fish during the transport, but fish may ultimately still arrive at their release location with elevated stress levels as indicated by plasma cortisol levels, concentrations of energetic metabolites, and metabolic rate (Roberts et al. 2024; Watt et al. 2024). Traditional conservation efforts involve the release of fish to the reintroduction waterway immediately following transport. This method, known as hard-release, affords individuals no time to recover from the effects of transport stress. In contrast, soft-release allows transported individuals an opportunity to acclimate to their new surroundings within a protective enclosure at the release site (Resende et al. 2021). This method eliminates the risk of predation while exposing fish to the conditions of their new environment (Tetzlaff et al. 2019; Resende et al. 2021) and has the additional potential to promote recovery from transport stress (Harmon 2009). In a comparison of hard and soft-release methods for Atlantic Salmon, Roberts et al. (2024) found significantly lower levels of glucose and lactate in individuals provided a period of acclimation in an in-river enclosure for both 48- and 96-hour periods, when compared to those individuals that were transported and provided no acclimation. Further, Mokdad et al. (2022) found that soft-release approaches can also promote more natural behaviours, with Atlantic Salmon given a six-day acclimation period showing greater probability of orienting and dispersing downstream in comparison to their hard-released counterparts.

Despite some experimentation on the topic of soft-release in fishes, a number of knowledge gaps persist regarding its potential to promote physiological and behavioural recovery, particularly for small-bodied fishes. Likely, species-specific assessments of release methods will be required to allow tailoring to the project in a way that improves the overall likelihood of reintroduction success (Moseby et al. 2014). One species of conservation concern in Canada that has had reintroduction suggested as a potential means of rehabilitation is the Eastern Sand Darter (*Ammocrypta pellucida*). This small benthic species of freshwater fish lives in lakes, rivers, and streams where it burrows in sand and fine gravel substrate (Scott and Crossman 1973; Spreitzer 1979). Adults are goldish brown in color and nearly translucent with an elongated body between 45-70 mm in length (Williams 1975). Burrowing is an integral behaviour in several aspects of the life history of Eastern Sand Darter, playing a role in functions such as mating and prey consumption, and possibly station-holding and predator evasion(Simon 1991). As a result, appropriate substrate composition (i.e., the availability of fine sand) is a key habitat requirement (Daniels 1989). The native range of this fish encompasses regions of the eastern United States, as well as Ontario and Quebec within Canada (Holm and Mandrak 1996). In Canada, the Ontario population of Eastern Sand Darter is classified as threatened under the *Species at Risk Act* and has been suggested as a potential candidate for reintroduction to promote reestablishment in areas where they have been extirpated (Holm and Mandrak 1996; Edwards et al. 2007).

Beginning in the spring of 2025, individuals from a stable Eastern Sand Darter population in the Grand River will be transported to Big Otter Creek, where the species has been extirpated. Due to their threatened status, steps must be taken to avoid negative consequences to the source population and ensure the greatest likelihood of survival of each reintroduced individual (Moehrenschlager and Lloyd 2016). Extensive assessment and statistical modelling were used to determine the number of individuals from the Grand River population that should be translocated while maintaining the stability of the source population (Lamothe et al. 2021). To further bolster success, there is an opportunity to assess the choice of release methods for the translocation process (Moseby et al. 2014; Resende et al. 2021). In particular, the use of soft-release has the potential to promote recovery from behavioral changes induced by transport stress, but research on the use of this tactic with small-bodied fish is extremely limited.

To determine the effectiveness of soft-release as a strategy to promote recovery from transport stress in Eastern Sand Darter, we used open field tests to assess a number of ecologically-relevant behaviours. We compared activity level, space use, substrate preference, response to a simulated predator, and swimming ability of individuals captured directly from the river, transported individuals (i.e., hard-release), and those permitted an acclimation period in the river in an enclosure (i.e., soft-release). As transport is known to represent a stressor for fishes, we predicted that hard-release individuals will behave least similarly to controls, showing decreased swimming activity, lower swim performance, less propensity to burrow, lower preference for sandy substrate, and greater affinity for the edge of the arenas (i.e., thigmotaxis). As the effects of stress related to reintroduction processes are potentially mitigated by soft-release, we predicted that the use of this technique will promote recovery of behaviour to more closely resemble control fish, with longer acclimation periods resulting in greater recovery. We completed a number of the behavioural assessments in two seasons (summer and fall) to further determine whether the benefits of soft-release differ based on the time of year the translocation is performed. Understanding the ways in which Eastern Sand Darter behaviour is affected by the stress of handling and transport, as well the extent to which soft-release acclimation promotes recovery of ecologically-relevant behaviours, can help guide best practices for the reintroduction of this species and other small bodied fishes.

## Methods

Experimental procedures were completed from 16 – 19 June 2022, 19 – 22 September 2022, and 21 – 26 May 2024. We repeated the trials in both the summer and fall of 2022 to determine the potential role that seasonal changes may play in response to transport stress and recovery of behaviour. We modified the experimental design for the 2024 trials to assess additional behavioural responses, as well as add an additional treatment group that investigated a longer soft-release time. As there is no consensus on a single best behavioural bioassay for the assessment of stress or performance, our experimental designs allowed us to maximize the amount of data we could collect from each captured subject (Beitinger 1990). All experimental protocols were approved by either the University of Windsor (2022) or Algoma University (2024) Animal Care Committees, and were completed under federal *Species at Risk* permits and provincial *Ontario Fish Collection* permits.

### *Fish capture*

We collected fish from wadeable sections of the Grand River in Brantford, Ontario, Canada at the Cockshutt Bridge access point (43°06'37.2"N 80°14'42.7"W; Figure 3.1) using a bag seine (9.14 m x 1.80 m, 3 mm mesh; Gaspardy and Drake 2021). Fish were placed into a cooler containing fresh river water for sorting and identification, with individuals belonging to non-target being species being immediately released upon identification. Eastern Sand Darter measuring less than 37 mm in total length were classified as juveniles and also released (Drake et al. 2008), ensuring retention of only adult fish. We repeated the seining and sorting process until the number of collected Eastern Sand Darter required for each treatment group for the day was reached (up to n = 21 fish total per day in 2022; up to n = 32 fish total per day in 2024). Eastern Sand Darter were assigned to treatment groups at random. In 2022, fish were split into three groups of n = 7 fish each per sampling day: control (no transport), hard-release (transport), and 24-hour soft-release (transport followed by 24-hour holding in an in-river enclosure). In 2024, we included an additional soft-release group, resulting in fish being divided across four groups of n = 8 individuals each per sampling day: control (no transport), hard-release (transport), 24-hour soft-release (transport followed by 24-hour holding in an in-river enclosure), and 48-hour soft-release (transport followed by 48-hour holding in an in-river enclosure). On days on which we were unable to capture the target number of Eastern Sand Darter, subjects were divided evenly into groups. Fish allocated to the control group were immediately transferred individually into behavioural arenas (see behavioural assay sections below). All other groups were transferred to transport containers as described below.

### *Transport*

To simulate the transport and handling associated with a translocation, fish assigned to hard and soft-release groups were placed in 10-L coolers containing fresh river water with stress coat water conditioner at a concentration of 0.1 mL/L and aerated by battery powered air stones. The number of coolers used was adjusted to maintain fish at a density of 1.4 g/L. In accordance with suggestions for at-risk fish translocations, the density used for this procedure was substantially lower than the commercial animal care guidelines for maximum density for transport of live fish (Ayres et al. 2012; Whiterod 2019; Mokdad et al. 2022). Coolers were placed in the interior of a vehicle which was driven in the surrounding areas of the capture site for 2 hours to mimic the maximum transportation length typical of what would be involved with translocations of Eastern Sand Darter. Temperature and oxygen levels of water in the coolers were consistent with river water at the capture site and monitored using a YSI multiparameter probe. The mean temperature and dissolved oxygen content of water in coolers during transport during the summer 2022 trials were 22.45 ± 0.16°C and 105.08 ± 1.63% respectively, while the mean temperature and dissolved oxygen were 20.43 ± 0.14 °C and 94.01 ± 0.95 % saturation, respectively, during the fall trials. For 2024, the mean temperature and dissolved oxygen were 20.55 ± 0.26°C and 99.65 ± 1.70%, respectively.

Following transport, behavioural assays involving fish in the hard-release group occurred immediately upon return to the field site. To simulate soft-release procedures, prior to behavioural assays, fish were placed in enclosures composed of a 0.75 x 0.75 m wooden frame covered in Vexar screen with a removable lid for acclimations periods of either 24 hours (2022 and 2024) or 48 hours (2024 only). In 2024, fish in each soft-release group were placed in separate enclosures to minimize disturbance. The frame of the enclosures was anchored to the riverbed using metal t-posts and tied to the shoreline using rope in a secluded section of the river. This type of enclosure allowed flow-through of fresh river water while protecting fish from predation and other potential threats. Because disease testing between the Grand River and Big Otter Creek had not yet been completed at the time of our experiments, movement of fish between waterways was not permitted. As a result, soft-release enclosures were placed near the initial capture sites in the Grand River.

### *Behavioural assays*

To assess the effects of different release scenarios, we measured non-invasive behavioural variables that are correlated with performance in small-bodied fishes, as well as other behaviours important to Eastern Sand Darter specifically, including activity level, space use, startle response, substrate preference, swimming performance, and burrowing (Simon 1991; COSEWIC 2009). Table 3.1 provides an overview of each behavioural metric and its relevance to fish performance and/or ecology. All behavioural trials were video-recorded using GoPro (GoPro HERO9, Inc. San Mateo, CA) cameras mounted above the arenas.

#### Open field test

Open field tests were performed in summer (June; n = 63) and fall (September; n = 59) of 2022. In general, open field tests provide a means to assess ecologically-relevant behavioural metrics in fishes such as exploration tendency, boldness, foraging success, predation risk, and proxies of stress physiology (Wilson and Godin 2009; Toms et al. 2010; Sopinka et al. 2016). Following capture (control groups), transport (hard-release group), or transport and 24-hour holding in the in-river enclosure (soft-release group), we placed each subject in a separate behavioural arena comprised of a 25 × 32 cm opaque plastic aquarium (Figure 3.2). Arenas were filled with river water to a depth of 20 cm and located in the shade to avoid fluctuations in water temperature. Each arena contained a petri dish (15 cm diameter) filled with sand and gravel to mimic natural substrate. Fish were transferred from the sorting container, transport cooler, or enclosure to the behavioural arena and given 10 minutes to acclimate before the video trial was recorded. Fish were then left in the arena for 10 minutes before a novel object (metal washer tethered to a length of flagging tape and wire) was dropped from above. Fish were recorded for an additional 3 minutes. Videos were analyzed for total activity time (time spent swimming) and the time spent in each area of the arena (edge, interior, refuge) before and after the introduction of the novel object, as well as the subject's response to the object (i.e., whether a fish showed a flee response).  We considered a fish to have displayed a flee response if it swam away from the trout lure immediately following its insertion to the tank (Pleizier et al. 2015; Wilson et al. 2015; Latchem et al. 2021; Table 3.1). The mean daily water temperature of the Grand River during 2022 open field tests was 23.73 °C (SE ± 0.226) in the summer trials and 21.34 °C (SE ± 0.131) in the fall trials.

<u>Swimming performance</u>

Following the open field tests in both summer and fall of 2022, fish were transferred individually to annular arenas (91 cm diameter) filled with fresh river water to record their crude swimming performance (Katapodis and Gervais 2016). Fish were chased clockwise in the annular container by hand for 30 seconds, with overall distance covered by swimming being calculated by tracking movement within the arena using behavioural software based on video recordings of each trial (BORIS: Friard and Gamba 2016). These data were then scaled to calculate an estimate of actual distance each fish travelled. This value can be used to estimate relative swim performance between groups (i.e., treatment comparisons) (Portz et al. 2006).

### Substrate preference and burrowing

In late May of 2024, we performed behavioural trials aimed at assessing substrate preference, propensity to burrow, and response to a simulated predator. Fish (n = 94) were divided into four groups following capture: control (no transport), hard-release (transport), 24-h soft-release (transport with subsequent 24-hour holding in in-river enclosures), and 48-h soft-release (transport with subsequent 48-hour holding in in-river enclosures). Control fish were behaviourally assessed after capture, while all other groups underwent a 2-hour transport as described in the 2022 trials. We placed fish individually in small arenas (33 cm x 19 cm) divided into two sections: half containing tan aquarium sand and half containing rocky substrate (river rocks approximately 2.5 – 5 cm in diameter; Figure 3.3). Fish were permitted a 10-minute acclimation period and were then filmed for 18 minutes to assess behaviours. At 9 minutes, a simulated predator on a clear fishing line (“Tommy the Trout” Rainbow Trout lure, Westin) was dipped into one end of the arena and pulled through to the other side to simulate a predator event. Little is known about the full range of predators of Eastern Sand Darter (COSEWIC, 2009) and Rainbow Trout (*Onchorhynchus mykiss*) represent a reasonable proxy based on size and shape of piscivorous predators found in southern Ontario river systems. We subsequently assessed whether fish displayed a burrowing response to the simulated predator, as well as swimming activity, time spent on sand vs. rocky substrate, time spent in the edge versus open area of the arena, and time spent buried both before and after the predator encounter. The mean daily water temperature during 2024 substrate selection and burrowing assays was 22.89 °C (SE ± 0.134).

### *Elastomer tagging and release*

Following behavioural assay, fish were weighed, measured for total length, and tagged with Visible Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc.) on the ventral surface to avoid recapture of individuals already included in the experiment in subsequent trials. In 2022, fish in the summer trials were tagged with orange elastomer, while fish from the fall trials were tagged with green elastomer. In 2024, we used green elastomer tags. Fish were released into the same location they were captured.

### *Video analysis*

To track and record behaviours on all video recordings, we used BORIS (Behavioral Observation Research Interactive Software), an open-source computer application used to track defined parameters during live or recorded observations (Friard and Gamba 2016). We delineated the edge of the arena as the area encompassing one fish length from the edge of the tank. We considered a fish to be in a certain area of the arena (i.e., edge vs. interior, sand vs. rocky substrate) if more than half of the length of the fish was contained in that area. Ethograms containing descriptions of each of the tracked behaviours for both the open field tests and substrate preference and burrowing trials can be found in Table 3.2 and Table 3.3, respectively.

We also tracked each subject’s reaction to the novel object (2022) and simulated predator (2024), recording whether the individual fled, burrowed (2024 only), or showed no response.

### *Statistical analyses*

All statistical analyses were computed using R (version 4.4.1) within RStudio (version 2024.12.1+563) for Microsoft Windows 10 (Microsoft Corporation 2015; R Core Team 2024; RStudio Team 2024). We used the ‘DHARMa’ package to perform diagnostics on residuals and confirm homogeneity of variance (Hartig 2024).

#### Open field test

We used the *glmmTMB* package, which supports zero-inflated data analysis, to fit separate generalized linear mixed-effect models analyzing response variables of time spent swimming, time spent in the edge portion of the arena, and time spent in the refuge both before and after the introduction of the novel object (Brooks et al. 2017; R Core Team 2024) to determine whether these behaviours different among treatment groups (control, hard-release, soft-release). We used treatment group and season (summer [n = 63] vs. fall [n = 59]) as fixed factors and included date and fish ID as random factors (Bates et al. 2015; Brooks et al. 2017). We used a Gamma distribution with a log link function for all models (Brooks et al. 2017). We performed likelihood ratio tests using the ‘anova’ function in the glmmTMB package to determine significant models ($p < 0.05$), to which post-hoc analysis was using applied using the ‘emmeans’ function of the emmeans package (Lenth 2025).

### Substrate preference and burrowing assay

To account for the zero inflated behavioural data, we used the *glmmTMB* package to fit generalized linear mixed-effect models analyzing the response variables of time spent swimming, amount of time spent on the sandy side of the arena, time spent along the edge of the arena, and time spent buried in the sand. We included treatment group (control, hard-release, 24-h soft-release, 48-h soft-release), time (before vs. after the predator event), and the interaction of treatment and time as fixed factors. We included date and fish ID as random effects (Bates et al. 2015; Brooks et al. 2017). We used a Gamma distribution with a log link function for all models with the exceptions of time spent buried before the introduction of the novel object for which a Poisson distribution was used with a log link function, and overall time spent buried for which a Gaussian distribution was used with an identity link function. Distributions and link functions were selected based on best fit and convergence with our data. We performed likelihood ratio tests using the 'anova' function within the glmmTMB package to determine significant models ($p < 0.05$), to which post-hoc analysis was using applied using the emmeans function of the emmeans package (Brooks et al. 2017; Lenth 2025). Additionally, we used 'glmmTMB' to compare edge time to open time using a generalized linear mixed-effects model.

### Flee responses

We used a Fisher's exact test using the 'fisher.test' function to analyze whether the proportion of fish displaying an evasion response to the novel object in 2022 differed between treatment groups, using separate analyses for summer and fall. We also used a Fisher's exact test to analyze whether the proportion of individuals fleeing from the simulated predator different between treatment groups in 2024.

### Swimming performance

We compared swim performance (i.e., distance traveled under chase) between treatment groups in 2022 in the summer and fall using separate generalized linear mixed effects models. Using the *glmmTMB* package, we included distance as the response variable, treatment group and fish mass as fixed effects, and day as a random factor. To compare overall swim performance across seasons we used a Mann-Whitney U test.

## Results

### *Open field test*

### Activity time

We found a significant difference in the amount of time spent swimming between seasons (i.e., summer [n=63] vs. fall [n=59]) ($\beta = 0.53$, $z = 2.53$, $p = 0.01$; Figure 3.4; Table 3.4), with fish showing greater swimming activity in summer compared to the fall. During summer trials, we found no significant effect of treatment on time spent swimming either before (LRT: $\chi^2 = 1.32$, df = 7, $p = 0.52$) or after (LRT: $\chi^2 = 1.98$, df = 7, $p = 0.37$) the introduction of the novel object (Figure 3.4). Similarly, we found no significant differences in time spent swimming between treatment groups either before (LRT: $\chi^2= 2.4$, df = 7, $p = 0.30$) or after (LRT: $\chi^2= 4.7$, df = 7, $p = 0.10$) the novel object was introduced in the fall trials (Figure 3.4).

Space use

We found significant differences in the amount of time that each area of the arena (edge, open, and refuge) was occupied overall for both summer (LRT: $\chi^2$= 0.0, df = 6, p < 0.001) and fall (LRT: $\chi^2$= 105.53, df = 6, p<0.001). During summer trials, fish spent significantly greater time in the edge area than the refuge ($\beta$ = 2.82, SE = 0.02, z = 143.03, p = 0.01) or open area ($\beta$ = -0.82, SE = 0.01, z = -96.79, p < 0.001), with significantly less time being spent in the open area than the refuge area ($\beta$ = 2.00, SE = 0.02, z = 98.17, p < 0.01) (Figure 3.5). Similarly, during the fall trials, edge areas were occupied for a significantly greater amount of time than the open area ($\beta$ = -1.34, SE = 0.24, z = -5.66, p = < 0.001) or refuge ($\beta$ = 2.73, SE = 0.24, z = 11.54, p = < 0.001), with the refuge area being occupied for a significantly greater time than the open space ($\beta$ = 1.39, SE = 0.24, z = 5.88, p = < 0.001) (Figure 3.5).

For summer trials, we found no significant effect of treatment group on the amount of time fish spent in the edge of the arena before (LRT: $\chi^2$= 0.26, df = 6, p = 0.88) or following the introduction of the novel object (LRT: $\chi^2$= 0.1, df = 6, p = 0.95) (Figure 3.5). Similarly, we found no significant differences in amount of time spent in the edge between groups during the fall trials before (LRT: $\chi^2$ = 0.95, df = 6, p = 0.62) or after (LRT: $\chi^2$ = 0.09, df = 6, p = 0.96) the introduction of the novel object (Figure 3.5). We also found no significant differences in total time spent in the perimeter of the arena between seasons (LRT: $\chi^2$= 0.01, df = 5, p = 0.99).

We did not detect a significant difference in time spent in the refuge between seasons (LRT: $\chi^2$= 2.72, df = 5, p = 0.10). During summer, we did not detect a significant effect of treatment group on time spent in the refuge before introduction of the novel object (LRT: $\chi^2$ =

1.63, df = 6, p = 0.44) or after (LRT: $\chi^2 = 0.40$, df = 6, p = 0.82). For fall trials, we similarly did not detect any significant effect of treatment group on refuge time before (LRT: $\chi^2 = 0.38$, df = 6, p = 0.83) or after (LRT: $\chi^2 = 1.89$, df = 6, p = 0.39) introduction of the novel object.

Flee response

In both summer and fall, the majority of the fish in the control, hard-release, and soft-release groups displayed a flee response to the introduction of the novel object. Specifically, the proportion of individuals that fled when the novel object was dropped into the arena during summer trials was 80% for the control group, 71% for the hard-release group, and 62% for the soft-release group (Figure 3.6). During the fall trials, the proportion of individuals that fled was 57% for the control group, 76% for the hard-release group, and 71% for the soft-release group (Figure 3.6). There were no significant differences between treatments in the proportion of the individuals that fled in either season (summer: Fisher's Exact Test, p = 0.48; fall: Fisher's Exact Test, p = 0.42).

### *Substrate preference and burrowing*

Activity time

We did not find a significant difference between treatment groups (control, hard-release, 24-h soft-release, 48-h soft-release) in the amount of time spent swimming either before ($\chi^2 = 1.3$, df = 7, p = 0.73) or after ($\chi^2 = 0.76$, df = 7, p = 0.86) the simulated predator introduction (Figure 3.7). We also found no overall effect of time frame (before vs. after predator introduction) on swim time (LRT: $\chi^2 = 2.50$, df = 8, p = 0.11). There also was no significant interaction between treatment group and time frame (LRT: $\chi^2 = 0.06$, df = 11, p = 0.99) on time spent swimming.

### Space use

Across groups, fish spent significantly more time in the edge of the substrate preference arena compared to the open space (log-odds = 2.23, SE = 0.26, z= 19.9, p < 0.001). We did not find any significant differences in the amount of time spent in the edge between treatment groups either before (LRT: $\chi^2$= 1.56, df = 7, p = 0.67) or after (LRT: $\chi^2$ = 0.37, df = 7, p = 0.95) the predator introduction. There was no overall effect of time frame (before or after predator) on amount of time spent in the edge of the arena (LRT: $\chi^2$ = 0.07, df =8, p = 0.79). There also was no significant interaction between time frame and treatment group (LRT: $\chi^2$ = 0.43, df = 11, p = 0.93). Fish in all groups spent similar amounts of time in the sandy side of the arena both before (LRT: $\chi^2$= 2.82, df = 7, p = 0.42) and after (LRT: $\chi^2$= 2.93, df = 7, p = 0.40) the predator was introduced (Figure 3.8). Additionally, there was no significant effect of treatment group on time spent on sand between time frames (LRT: $\chi^2$= 3.49, df = 8, p = 0.32), time frame across all groups (LRT: $\chi^2$ = 0.15, df = 8, p = 0.7), or any significant interaction between treatment group and time frame on overall time spent on sand (LRT: $\chi^2$ = 2.26, df =11, p = 0.52).

### Burrowing

In total, 18 of 188 (i.e., 9.57%) fish burrowed across all groups, with no significant differences between treatment groups in the amount of time spent burrowed before (LRT: $\chi^2$ = 0, df = 6, p = 1.00) or after (LRT: $\chi^2$ = 3.64, df = 7, p = 0.30) the simulated predator introduction. The percentage of individuals that burrowed during trials for each group was 8.3% for the control, 18.2% for the hard-release, and 20.8% and 29.2% for the 24- and 48-hour soft-release groups, respectively. There was no significant interaction between treatment group and time period (before vs. after) in the amount of time spent burrowed in the sandy side of the arena. There was, however, a significant effect of time period on the time spent buried (LRT: $\chi^2 = 5.62$, df = 5, $p = 0.02$; Table 3.5), with fish spending significantly greater time burrowed following the introduction of a predator.

### Predator response

The majority of subjects fled in response to the introduction of the predator in the substrate selection and burrowing assays (control: 79%; hard-release: 82%; 24-hour soft-release: 88%; 48-hour soft-release: 83%; Figure 3.9). There were no significant differences in the proportion of fish that fled in response to the predator between groups (Fisher's exact test; $p = 0.93$)

### *Swim performance*

We found significant differences in swim distance in (cm) between groups during both summer and fall trials. During summer performance tests, soft-release fish had significantly greater swim distances than control fish ($\beta = 78.78$, SE = 29.00, $z = -2.72$, $p = 0.02$), and hard-release fish ($\beta = -104.50$, SE = 29.90, $z = -3.5$, $p < 0.01$) (Figure 3.10). During fall trials, control fish swam significantly shorter distances than both the hard-release ($\beta = -92.44$, SE = 26.57, $z = -3.48$, $p = 0.002$) and soft-release groups ($\beta = -73.02$, SE = 28.23, $z = -2.59$, $p = 0.03$) (Figure 3.10; Table 3.6). Additionally, distance traveled was significantly greater in summer compared to fall trials ($W = 1303$, $p < 0.01$).

## Discussion

The reintroduction process exposes fish to numerous stressors, with associated physiological and behavioural consequences that can ultimately be detrimental to post-release survival and establishment (Nomura et al. 2009; Roberts et al. 2024). Soft-release procedures allow fish an acclimation period within a protective enclosure at the release environment and may promote recovery from transport-related stress (Tetzlaff et al. 2019). To investigate the potential for soft-release strategies to promote recovery from transport stress for Eastern Sand Darter, we assessed a variety of ecologically-relevant behaviours of fish from the Grand River population in Ontario, Canada. We predicted that transport would alter behaviours in comparison to control (non-transported) fish and that a 24-48-hour recovery period in soft-release enclosures would promote recovery, leading to behaviours similar to control (non-transported) fish. Overall, our data did not support these predictions, and we found no significant differences between groups in most of the behavioural metrics we assessed. We did, however, find significant differences in swim performance between groups during both summer and fall, but these differences were not consistent across seasons nor did they indicate a recovery-related benefit of soft-release.

### *Swimming activity*

Locomotion is a fundamental aspect of animal behaviour, with activity time being one of the most commonly assessed metrics in field tests (Brown 2011). Given that stress can modulate activity level in fish, understanding how an individual moves throughout their environment can provide insight into the overall physiological state of the animal (Cooke et al. 2022). During transport, fish generally experience increased production of cortisol, the mobilization of glucose, and an increase in metabolic rate (Roberts et al. 2024). As a result, we expected that hard-release fish, which were behaviourally assessed immediately following transport, would show more anxiety-like and energetically-stressed behaviours, including decreased activity levels in relation to non-transported (control) fish. Although we did not observe any difference in activity level between control and hard-release fish, it is possible that stress levels still differed, but were not manifested as differences in activity level. The 2-hour transport time used in our study was chosen to mimic a likely reintroduction event for this species. Relative to other transport studies, this time period is short, and while it may have still induced a stress response in the fish, they may not have experienced an elevation in stress hormone levels or metabolic rate that persisted long enough to subsequently impact locomotor activity. This finding is consistent with those of Nomura et al. (2009) where stress hormones and behaviour of Atlantic Salmon smolts were measured before, during, and after capture and transport periods. Although cortisol levels rose quickly due to capture and loading, an increase in erratic behaviours was only detected after 6 hours, with no significant changes in behaviour being reported for observations made before this time point (Nomura et al. 2009). As a result, it is also possible that some behavioural disruptions may be delayed, warranting further work that examines behavioural profiles over extended periods post-transport.

We also did not find any differences in swimming activity between fish experiencing the soft-release enclosure periods (24-hours or 48-hours) compared to control or hard-release fish in either of the behavioural trials. This finding contradicts our predictions, as we expected to see increasing similarity to baseline (control) behaviour with greater soft-release acclimation time. However, given that the transport event did not cause a change in swimming activity level, it is not surprising that fish held in the enclosures maintained activity levels similar to control fish. The enclosures were designed to provide an environment that would match appropriate natural conditions for this species in terms of depth, temperature, and substrate composition. While our study focused on the use of soft-release enclosures for promoting behavioural recovery, they also have the potential to support physiological recovery, provide an acclimation opportunity to new environmental conditions, and impart a predator-free environment for orienting in a new waterway (Roberts et al. 2024). Our findings indicate that, if soft-release enclosures were to be used for this species as an opportunity to support other components of reintroduction success, they will not compromise a key component of exploratory behaviour.

Although we found no significant differences in time spent swimming across treatment groups, all fish swam significantly less in the fall compared to the summer. Several factors such as photoperiod, breeding stage, and water temperature can influence background levels of stress and energetics and therefore may have led to the seasonal differences in swimming activity that we observed (Radabaugh 1989; Wingfield 2013; Schreck et al. 2016). As ectotherms, metabolic rate increases with temperature and can be accompanied by increases in activity level (Sullivan 1954; Hela and Laevastu 1962; Neubauer and Andersen 2019). Additionally, water temperature is crucial for fishes, as it directly influences physical and chemical properties such as pH and dissolved oxygen (Berka 1986; Harmon 2009; Firth et al. 2021). The negative consequences of suboptimal water temperature on fish were highlighted during behavioural trials by Firth et al. (2021) in which Eastern Sand Darter *(Ammocrypta pellucida)* temporarily exhibited behaviours including sporadic movements and compromised equilibrium when exposed to increasing water temperatures. During periods of stress, the efficiency at which fish are able to absorb oxygen through their gills may be compromised, placing even greater importance on the amount of dissolved oxygen in their environment during these times (Harmon 2009). The correlation between physiological and behavioural responses to different temperatures can differ across fish species (Halsey et al. 2015). Knowledge of how temperature affects the behaviour of a species is important for the development of transport and release methods that will promote behaviours leading to greatest likelihood of post-release survival and therefore chances of successful reintroduction.

There was a trend for fish in all groups to decrease their swimming activity following the brief introduction of a simulated predator, as well as spend more time along the edge of the arena compared to the open section. Limiting movement and open-space activity following a predator encounter may indicate hesitancy to explore in the face of increased risk perception (Lima and Dill 1990; Conrad et al. 2011). Indeed, limiting movement as well as concealment may be used by fish as predator avoidance strategies (Lima and Dill 1990). Regardless of the amount of food available, Three-spined Sticklebacks, (*Gasterosteus aculeatus*) decreased movement during foraging and spent greater time in a single sheltered location when placed in aquariums with predatory Brown Trout (*Salmo trutta*) (Fraser and Huntingford 1986). Black Carp (*Mylopharyngodon piceus*), a benthic species, also spent significantly less time swimming in the presence of a predatory Snakehead (*Channa argus*), during behavioural trials (Tang et al. 2017).

An additional explanation for a decrease in swimming activity following exposure to the simulated predator could be related to overall changes in exploratory behaviour over the trial time. Specifically, exploration may be occurring in the earlier period of the trial and, once the arena is fully explored, the subject may be more likely to rest to conserve energy. In the substrate and burrowing trials, fish spent a greater amount of time buried in the sandy substrate following the introduction of the simulated predator compared to the time period prior to its entry, which could support either explanation; fish may be burrowing to conserve energy or to avoid risk associated with perceived predation. Eastern Sand Darter are known to burrow when exposed to appropriate substrate, and it has been suggested that burrowing may be used as a potential resting state (Daniels 1989; Simon 1991). Further, a correlation between the size of a novel environment and activity level is believed to exist for mammalian behaviour, and has recently been suggested to potentially also influence fish behaviour (Stewart et al. 2012). While there were no differences between the size of the arenas in our trials, future studies implementing a size element would likely highlight whether or not spatio-temporal exploratory behavioural patterns exists for Eastern Sand Darter. Further, a trial that does not include a predator introduction could determine whether exploratory behaviour naturally decreases over the experimental time period, or was specifically related to predator introduction. Given that stress can also potentially influence other behaviours related to movement beyond total activity, future work could incorporate other aspects of fish locomotion. For example, the acceleration, number of movement events, and direction of movement could be influenced by stressors such as those associated with transport (Conrad et al. 2011) and have downstream consequences for success after release.

### *Space use*

Fish must effectively navigate their environment and occupy areas with favourable conditions that adequately provide access to resources such as food, as well as limit susceptibility to dangers such as predators (Monk et al. 2020; Cooke et al. 2022; O'Connor et al. 2023). Refuge seeking can be an important antipredator response (Ryer et al. 2004). During behavioural trials by (Nunes et al. 2019), individuals of the cryptic species Redlip Blenny (*Ophioblennius trinitatis*) were significantly more likely to seek areas providing the greatest refuge and avoid areas with less protection in response to a predator. For Eastern Sand Darter, we found no significant differences in space use between treatment groups before or after the novel object was added in either season. These results differed from our expectations that soft-release and control fish behaviours would be similar but differ from hard-release fish. There are a number of non-mutually exclusive explanations for the similar space use behaviours between treatment groups. Environmental complexity can influence the movement of fish (Mikheev et al. 2010). Our arenas were relatively simplistic with uniform edges and consistent water depths. Although treatment groups may have been experiencing different levels of stress, the uniformity of the environment may have led to similar behaviours overall. Additionally, we analyzed non-mutually exclusive space use variables as separate response variables and only considered the general placement in areas of the arena, and not aspects of spatial behaviour such as body positioning or how it entered the area. As we found with activity level, it is also possible that the transport event did not lead to disruptions to this type of behaviour. Adding additional structural complexity and assessing the correlation between multiple space use variables would provide greater insight into what level stress plays on space use behaviours.

We also did not find any significant differences between groups in the amount of time spent in the sandy, compared to the rocky, section of the arenas used for the substrate preference and burrowing assays. Previous research has indicated that fish show a significant preference for ideal substrate during some behavioural trials. For example, during behavioural testing by Bizzaro et al. (2016), when subjects of the benthic burrowing species of fish the Pacific Sand Lance (*Ammodytes personatus*) were placed in arenas containing equal amounts of two of a possible seven sediment sizes for four hours, a significantly greater number of fish were burrowed in course substrate when compared to the other six sizes. Fish also showed a significantly greater preference for the substrates most similar in size to coarse sand between the other six substrate sizes used in the trials (Bizzaro et al., 2016). As the ideal substrate for Eastern Sand Darter is sand and small gravel (Barnucz et al. 2020), our results were unexpected. To determine the substrate preference of benthic fish, trials have typically recorded the resting or buried location of fish at temporal or behavioural endpoints, and did not consider the amount of time spent in a given substrate (Bizzarro et al. 2016; Thompson et al. 2017). Our methodology in measuring substrate selection may have led to our results differing from previous research as well as our expectations. Expanded analysis of Eastern Sand Darter substrate preference of greater time length, analyzing not just total time in each area but how this time was spent (i.e., swimming or resting) would provide greater insight into the results we observed and behaviours related to habitat selection overall.

In both seasons of 2022 (open field test) and in 2024 (substrate preference and burrowing assay), fish spent a significantly greater of time in the edge section of the arenas compared to the open space. Exploration-avoidance axis can be used to measure fish behaviour, and describes the tendency to explore a novel environment (Conrad et al. 2011). Thigmotaxis refers to a behaviour where individuals remain close to an edge or wall and is indicative of anxiety-like behaviour, often the result of exposure to stressors or release to a novel environment (Lucon-Xiccato et al. 2022). In our trials, across all groups, a significantly greater amount of time was spent within the edge of the arena, and fish only entered the open space sections for short periods of time (i.e., a few seconds at a time). Horka et al. (2024) observed similar responses by Peter's Elephantnose Fish (*Gnathonemus petersi*) in open field tests, where fish spent greater than 83% of their time in the perimeter of the arena. Thigmotaxis may function in reducing time spent in open space and therefore susceptibility to predators (Lucon-Xiccato et al. 2022). We expected that fish would display greater thigmotaxis following introduction of the novel object or simulated predator, and that transported individuals would be more likely to avoid open space. As Eastern Sand Darter were no more likely to explore open space before the introduction of the predator, regardless of treatment group, it is possible that thigmotaxis is a default strategy when being faced with a novel environment, even in the absence of the immediate threat that a predator poses.

Behavioural responses differ depending on the certainty and magnitude of the impending threat (Conrad et al. 2011; Lucon-Xiccato et al. 2022). Fear is described as the behavioural response shown to immediate threats to safety (e.g., a predator in pursuit), while anxiety is used to describe the suite of behaviours that would accompany potential threats that are less certain, severe, or acute, such as the ambiguity of an unfamiliar environment(Silva et al. 2015). Regardless of the transport event, it appears that fish in our trials were displaying anxiety-related behaviours. During a reintroduction event, fish in soft-release enclosures will experience multiple characteristics of their release environment (e.g., water temperature, oxygen content, turbidity level, salinity, local substrate composition) within a protected space. Feasibly, initial tendencies to display anxiety-related behaviours, like thigmotaxis, will occur within the enclosure. On release, particularly if fish are permitted to leave the enclosure volitionally, fish may display greater exploratory behaviour leading to greater ability to find food and establish a habitat (O'Connor et al. 2023). Given that greater exploration following release can lead to discovery and access to areas with greater resources (Armstrong et al. 1997), soft-release enclosures may still prove beneficial overall.

*Response to simulated predator*

The ability of a fish to recognize and escape potential predators is crucial for survival (Baerends 1971; Brown 2011; Conrad et al. 2011; Nunes et al. 2019). This behavioural capacity, which includes unimpaired functioning of threat detection and evasion behaviours, is considered to be essential to reintroduction success, as it increases the likelihood of permanent establishment (George et al. 2009). As expected, the majority of fish fled in response to the introduction of a novel object or simulated predator. However, there were no significant differences between treatment groups in the proportion of fish that showed a flee response. Our results are in contradiction to Olla et al. (1995) who exposed Coho Salmon (*Oncorhynchus kisutch*) and Chinook Salmon (*Oncorhynchus tshawytscha*) smolts to one of three variations of handling and then released fish in tanks containing predators. A significantly greater number of smolts exposed to handling fell victim to predation when released to tanks with predators when compared to the number of control (i.e., non-stressed) smolts eaten, with mortality increasing significantly with frequency and duration of handling (Olla et al. 1995). Our results are in alignment with those of Nunes et al. (2019) where Redlip Blenny (*Ophioblennius trinitatis*) invariably fled in response to the introduction of a simulated predator across risk scenarios. These results suggest that, although this behaviour may be vulnerable to impairment in some cases due to stress, some species have the capacity to maintain at least some level of functioning during stressful events. It is also possible that our transport event was not stressful enough to lead to impairment of the predator response. Due to the sensitive nature of Eastern Sand Darter as a threatened species, we employed handling and transport methods designed to minimize stress as much as possible. For example, we avoided air exposure during netting/transfer, matched transport water temperatures to in-river temperatures, used extremely low transport densities, regularly monitored oxygen levels, and drove carefully to minimize jostling within transport containers. It is possible that, for species that cannot be afforded these types of transport considerations, transport may result in changes to predator avoidance capability. To gain a clearer overall picture of predator evasion capacity, future experiments could also measure reaction times in relation to predator capture capacities. Our field tests only considered whether or not an individual demonstrated a flee response, but not how long an individual took to respond or whether capture would be avoided. Specifically, analysis of the swim trajectory of subjects in response to the predator may indicate whether or not evasion performance is impaired by transport stress and the potential for mitigation by soft-release procedures.

### *Swim performance*

Swim performance can be used as an indicator of overall fitness in fish (Katapodis and Gervais 2016), and our relatively simple metric allows for comparison among treatment groups (Portz 2006). During swim performance tests in both summer and fall, we found differences among treatment groups. Interestingly, in both seasons, soft-release fish traveled greater distances than control fish. This finding is contrary to our prediction that, as control fish have not been exposed to transport, this group would have the lowest levels of background stress and energetic demand, and therefore be able to swim greater distances during trials (Dickens et al. 2010; Roberts et al. 2024). Unexpectedly, the hard-release group swam the shortest distance in summer, but the greatest distance in fall. It is therefore difficult to draw conclusions from our results. As with the results of our other behavioural trials, our findings suggest that temporal changes to behaviour such as season shifts may be relatively more pronounced and easily detectable, as we found that fish swam further distances overall in summer compared to fall, likely due to the higher associated water temperatures (Radabaugh 1989; Blair et al. 2019). The ambiguity of the results related to treatment stresses the importance of expanding our knowledge of how fish behave in response to stress and what methods can best be used to assess these responses.

### *Burrowing behaviour*

As darters are concealed once burrowed in the substrate, burrowing behaviour has been suggested as a predator evasion mechanism in Eastern Sand Darter (Simon 1991). During the substrate preference and burrowing assay, only one fish burrowed as an immediate response to the trout lure, with some individuals burrowing before this event. However, the time spent burrowed was significantly greater in all groups during the time period after the predator introduction compared to the time period prior. Fish that burrowed during the second half of the behavioural assay remained buried for the full extent of the trial. These results suggest that burrowing is likely not used as an acute behavioural reflex to escape a predator in pursuit, but instead may benefit individuals by visually concealing them or providing camouflage to prevent detection by a transient predator. These findings are in alignment with a study by Daniels (1989), where a burrowing response was not able to be elicited in any of the Eastern Sand Darter under study through startling. Similarly, Simon (1991) introduced a wooden bass to containers holding Eastern Sand Darter and did not observe any burrowing in response to this simulated predator, concluding that this behaviour is not a startle response. However, these studies did not record behaviour for any extended period after introduction of their stimuli. Similar to most of our other behavioural results, there were also no differences among treatment groups in the amount of time that fish spent buried in the sand. Burrowing is integral to multiple aspects of Eastern Sand Darter life history (Spreitzer 1979; Daniels 1989; Simon 1991), and should therefore be considered during planned reintroduction. It may be the case that this behaviour is maintained much like the predator flee response, and therefore may not ultimately be influenced by the stress typical of transport. Future work focused specifically on burrowing in this species when exposed to various stressors beyond predator introduction may provide insight into how fully resilient this behaviour is to transport stress.

### *Implications for reintroduction of Eastern Sand Darter*

Collectively, our results indicate that Eastern Sand Darter do not experience significant disruption to behaviour as a result of short-term transport, at least in the context of the behaviours we measured (activity level, exploration, burrowing, substrate preference, ability to flee a predator, and swimming performance). These findings are in contrast to much of the physiological literature, which records alterations to stress and energetic physiology as a result of handling and transport activities. For example, Roberts et al. (2024) reported increased levels of cortisol in Atlantic Salmon that had been transported for approximately 2 hours, with recovery to baseline levels of these indicators not occurring even after 24 or 48 hours in soft-release enclosures. Additionally, Tambaqui (*Colossoma macropomum*) required up to 96 hours to recover from the stress of being transported a distance of 400 km (Paixão et al. 2024). However, in Redside Dace (*Clinostomus elongatus*), another at-risk, small-bodied species, transport procedures did not lead to any changes to maximum metabolic rate or thermal tolerance, even under a manipulation to lower body condition (Watt et al. 2024). As susceptibility to different sources of stress can vary across species (Sopinka et al. 2016), it is possible that Eastern Sand Darter may be relatively resilient to the stress commonly associated with transport for reintroduction, especially under relatively ideal transport conditions.

Given the small size of Eastern Sand Darter, we were unable to assess the stress physiology of transported individuals, which may have differed from control fish without subsequent influence on the behaviours we assessed. Because survival in the release waterway will be related to multiple aspects of fish performance, we cannot conclude that soft-release enclosures have no potential benefit in the reintroduction of Eastern Sand Darter without additional endpoints. However, our results do not indicate that soft-release enclosures will be necessary for maintaining many ecological behaviours following transport and it is therefore possible that hard-release is a viable strategy for this species. Given the cost and logistical requirements of soft-release, performing a hard-release offers a more streamlined approach to the movement of fishes in translocations. As discussed above, the lack of difference between the control and transported individuals may be a result of optimal transport conditions. The maintenance of optimal conditions such as water quality and stocking density may have limited the amount of stress of this process overall, therefore reducing the contrasts in stress-induced behaviours between groups. These transport techniques could easily be replicated within a larger reintroduction context for this species.

It is important to note that the handling of the control subjects may have introduced some stress that could influence behaviour. Because of logistical constraints in the field, fish were regularly assessed for behaviour approximately 30 minutes following initial capture, when it is possible for physiological effects of capture to still be present. If the control group was demonstrating some stress-induced behaviours as a result of capture and handling during our experimental procedures, there is a possibility that, although the physiological states between subjects of each group may not be identical, the behavioural patterns exhibited may share some resemblance. Although the experimental process may have been stressful to all of our fish subjects at some level, the background levels of stress in each group were likely to have differed (Petitjean et al. 2019). For example, we did not observe differences between the control or transport groups in comparison to the soft-release groups. In those cases, fish were remaining in an enclosure for 24-48 hours prior to being carefully transferred to their respective behavioural arena. Further, the behaviours we assessed were not lost (i.e., acutely or chronically disrupted) in any group. As a result, while it is likely that experimental procedures induced a certain level of stress, we do not believe they are the driving mechanism behind our findings.

### *Conclusions*

We did not find any consistent differences in any of the behavioural metrics across groups for any of our trials. Although further research into several of these behaviours with modified experimental conditions would likely yield more conclusive results, our results did not indicate any benefit of the use of soft-release for transported Eastern Sand Darter. Hard-release may therefore be appropriate for the translocation of Eastern Sand Darter. We acknowledge that our soft-release enclosures were placed in the capture waterway (Grand River), and future work could investigate similar questions with releases in the reintroduction habitat (Big Otter Creek). Our results also support some growing evidence that small-bodied fishes, when transported under ideal conditions, may be resilient physiologically and behaviourally (e.g., Watt et al. 2024). Further research into a variety of strategies for mitigating negative effects of stress in various species will lead to better understanding of how fish react to and recover from transport stress. Overall, these results can help inform selection of the most appropriate transport and release methods, which can lead to greater survival of released individuals and yield greater success of reintroduction projects overall.

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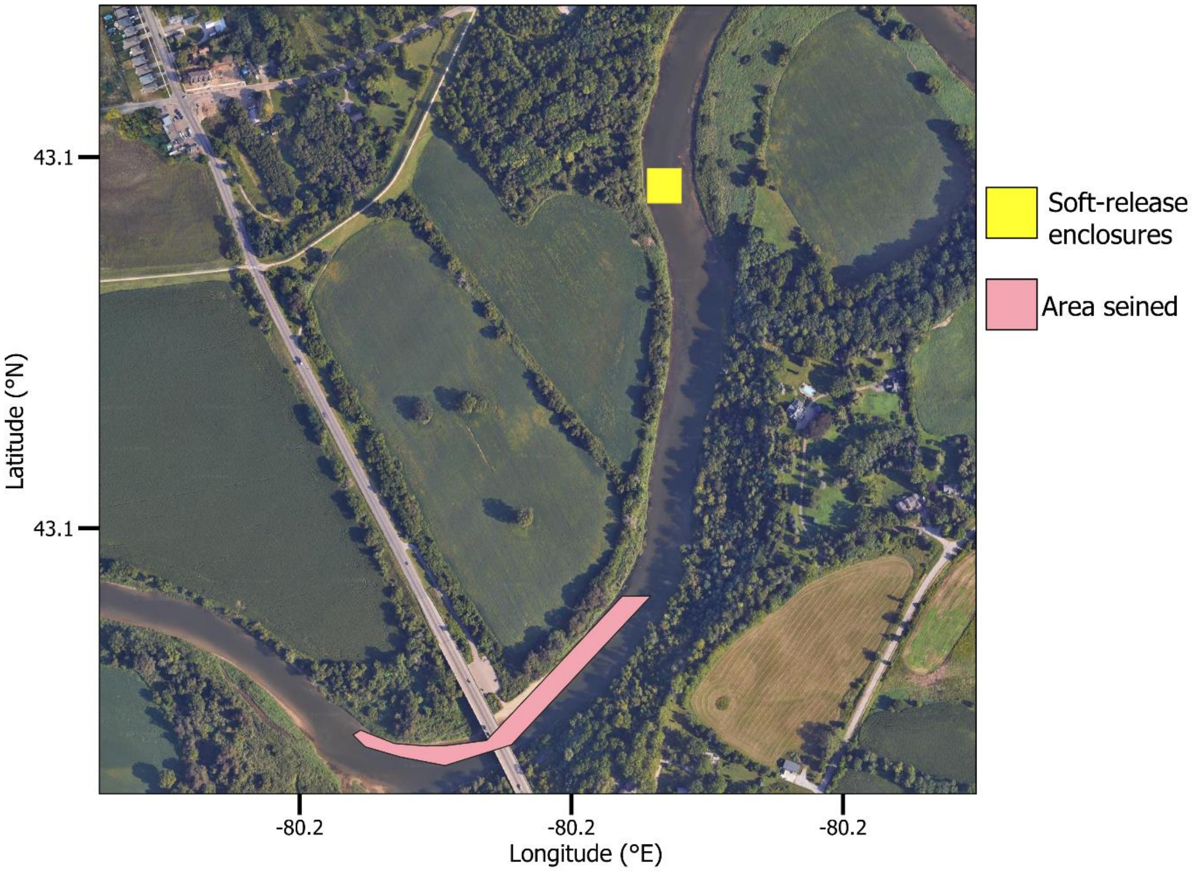

Figure 3.1 - Map of study area depicting area of the Grand River seined for collection of Eastern Sand Darter (red) and the location of soft-release enclosures (yellow).

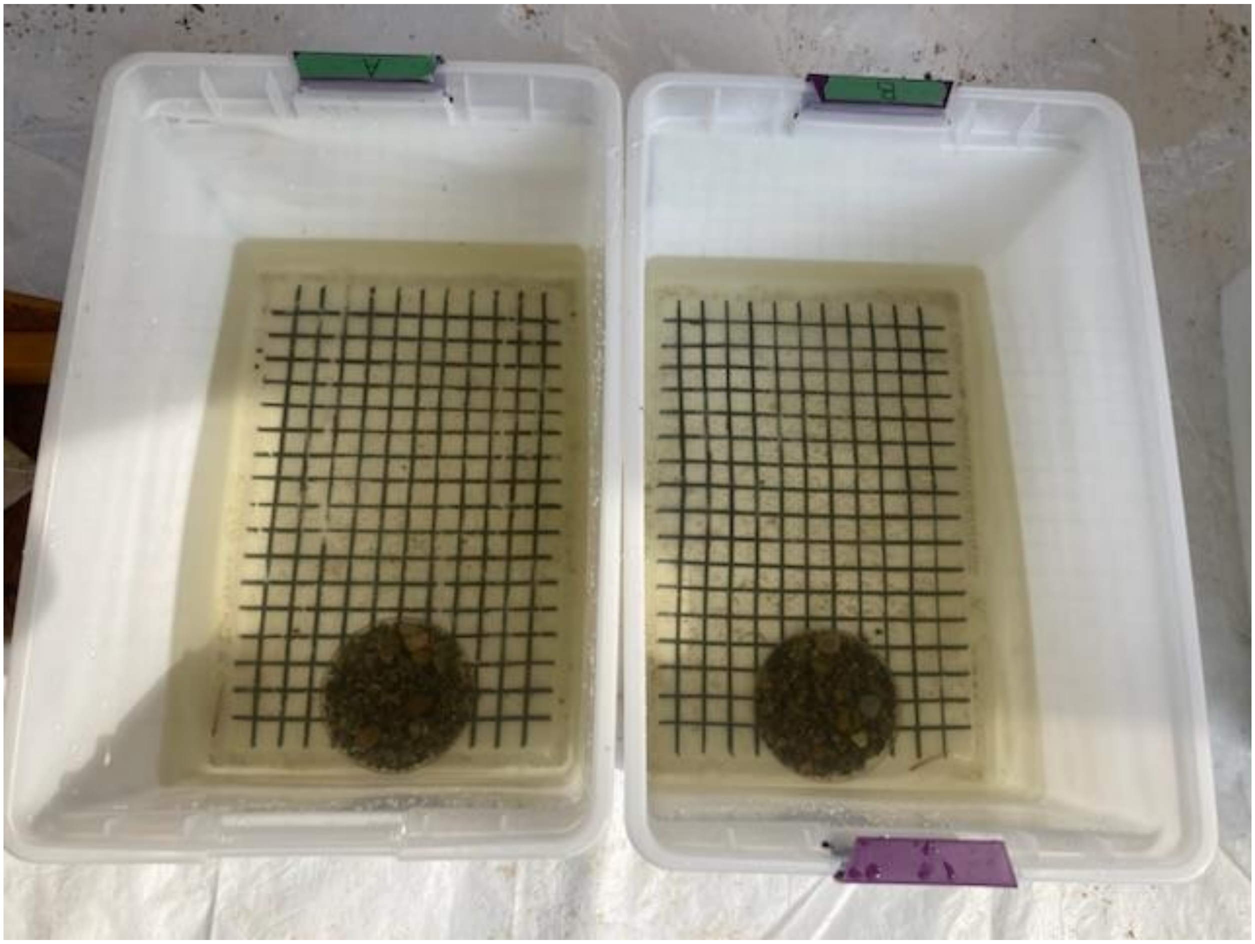

Figure 3.2 - Open field test arenas use to assess activity level, space use (edge vs. open vs. refuge), and response to a novel object in summer and fall of 2022.

Figure 3.3 - Substrate preference and burrowing behaviour arena, with one half containing rocks (~2.5 – 5 cm diameter) and the other half containing fine sand.

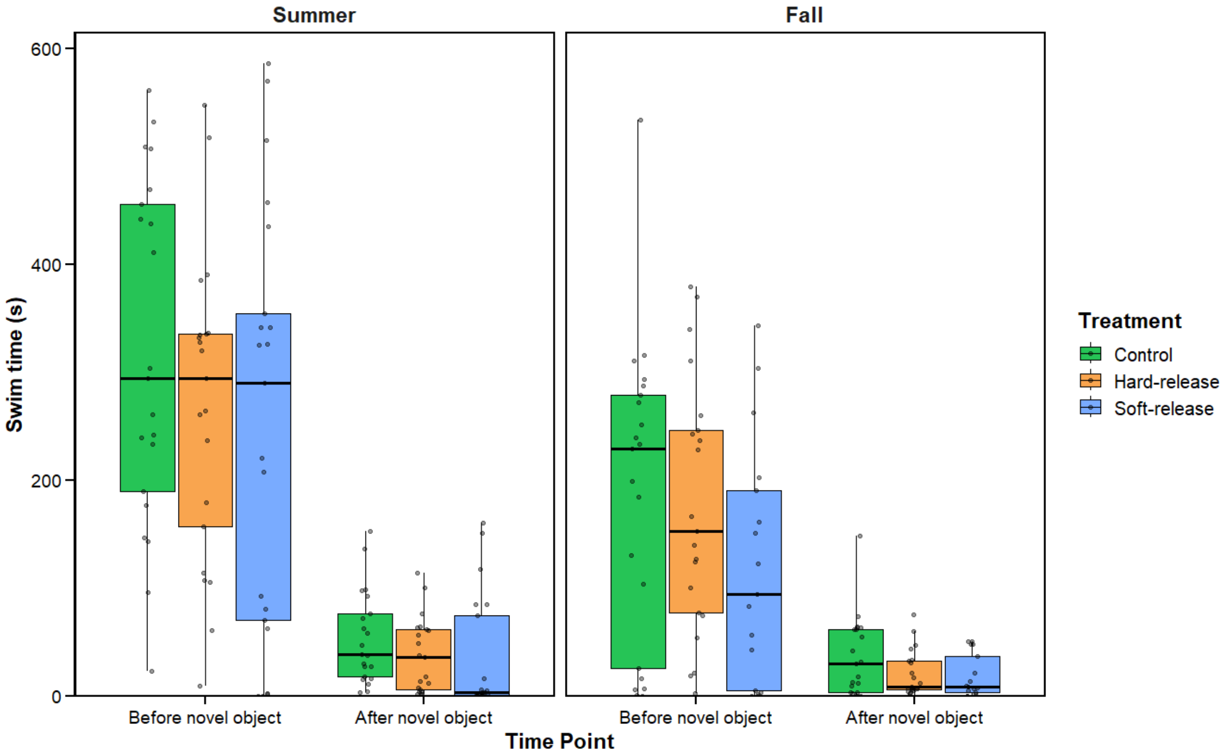

Figure 3.4 - Amount of time spent swimming during the open field tests before and after the addition of a novel object across treatments: control (non-transported), hard-release (2-hr transport), and soft-release (2-hour transport followed by 24-hour in-river acclimation). Trials were completed in summer and fall of 2022. Boxplots depict median and interquartile range, with whiskers representing the minimum and maximum.

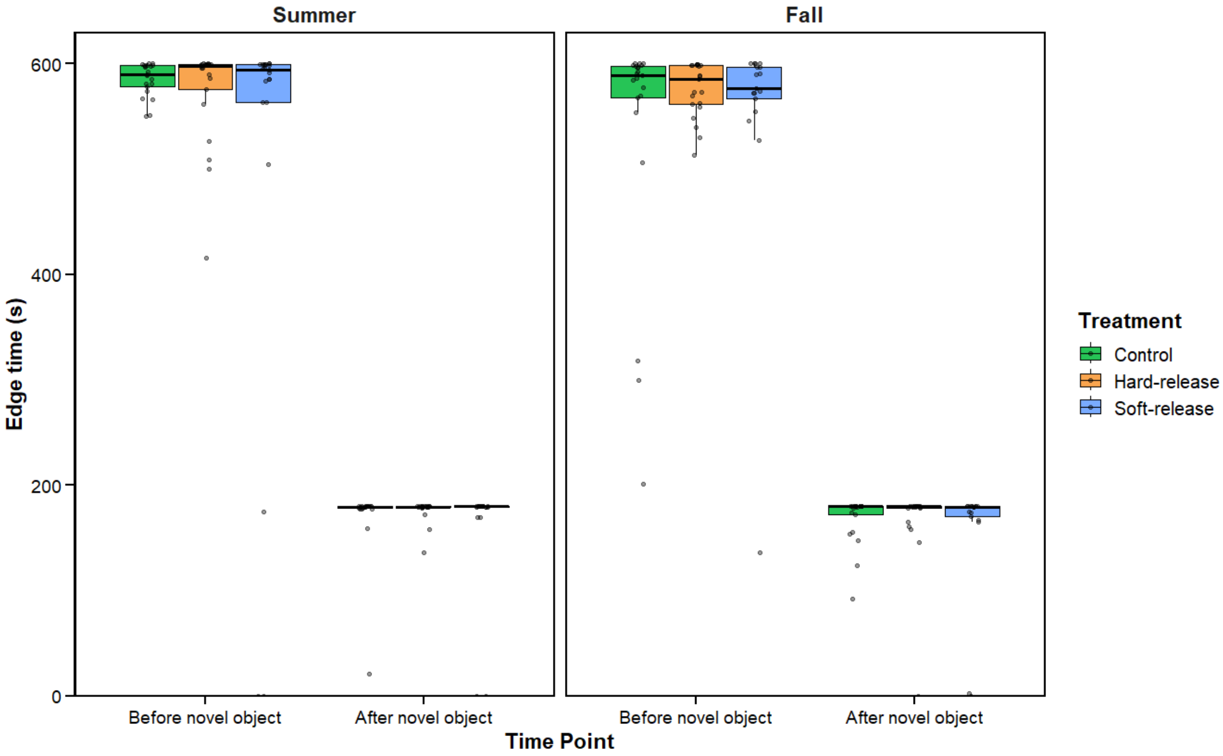

Figure 3.5 - Amount of time spent in the edge portion of the arena (i.e., within one body length of a wall) during the open field tests before and after the addition of a novel object across treatments: control (non-transported), hard-release (2-hr transport), and soft-release (2-hr transport followed by 24-hr in-river acclimation). Trials were completed in summer and fall of 2022. Boxplots depict median and interquartile range, with whiskers representing the minimum and maximum.

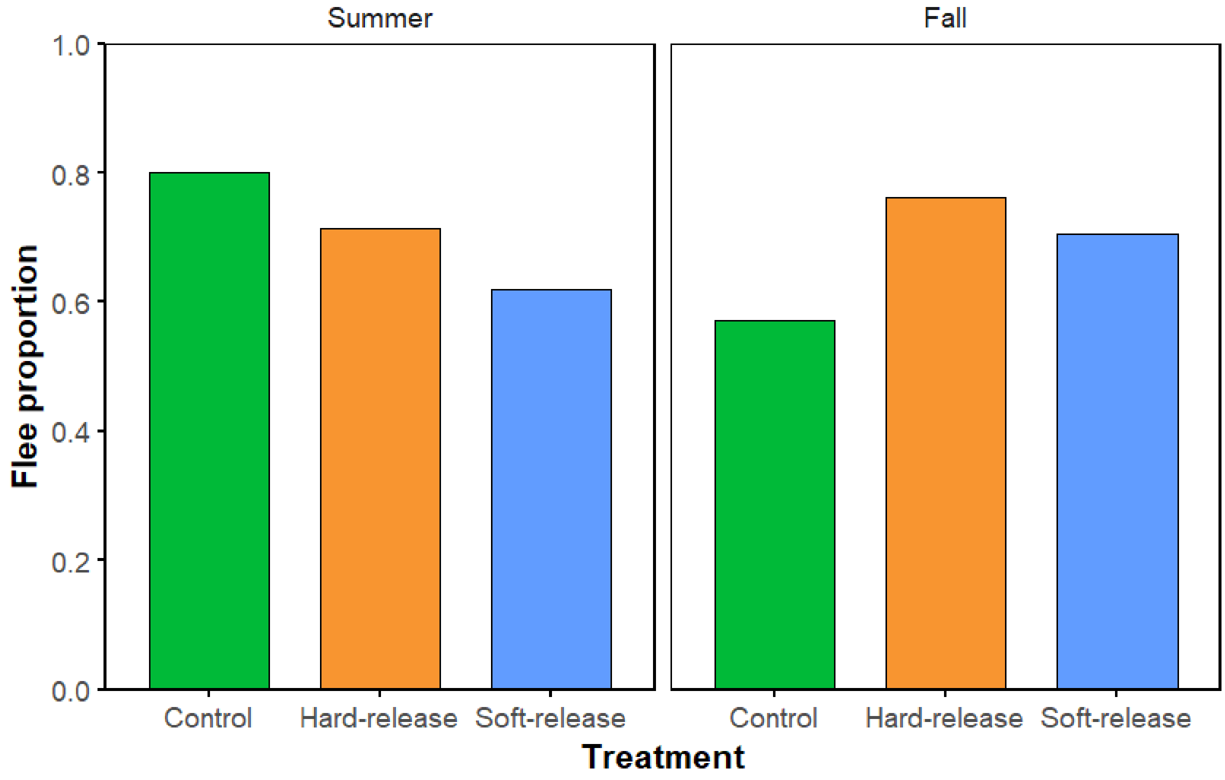

Figure 3.6 - Proportion of fish that showed a flee response to the introduction of a novel object during the open field tests in summer and fall of 2022 across treatments: control (non-transported), hard-release (2-hr transport), and soft-release (2-hr transport followed by 24-hr acclimation in in-river enclosures).

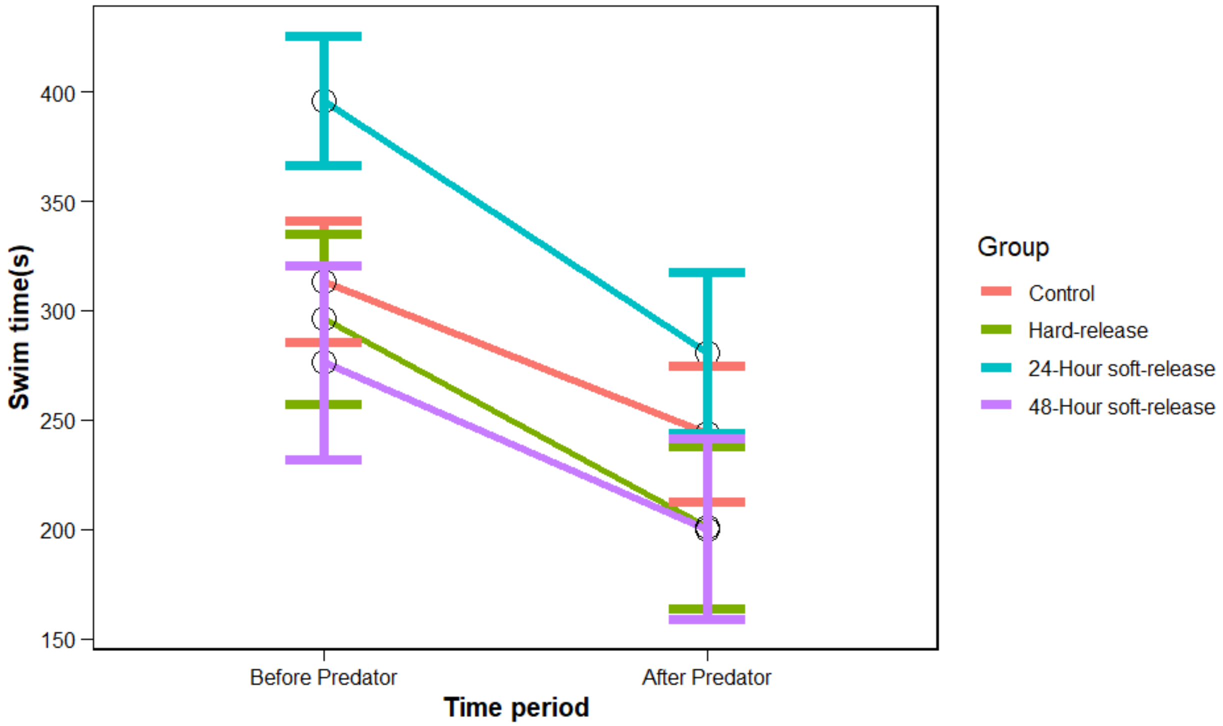

Figure 3.7 - Time spent swimming before and after the introduction of a simulated predator across treatment groups in the substrate preference and burrowing assay. Treatment groups included control (non-transported), hard-release (2-hr transport), and 24-hr soft-release (2-hr transport followed by 24-hr in-river acclimation), and 48-hr soft-release (2-hr transport followed by 48-hr in-river acclimation).

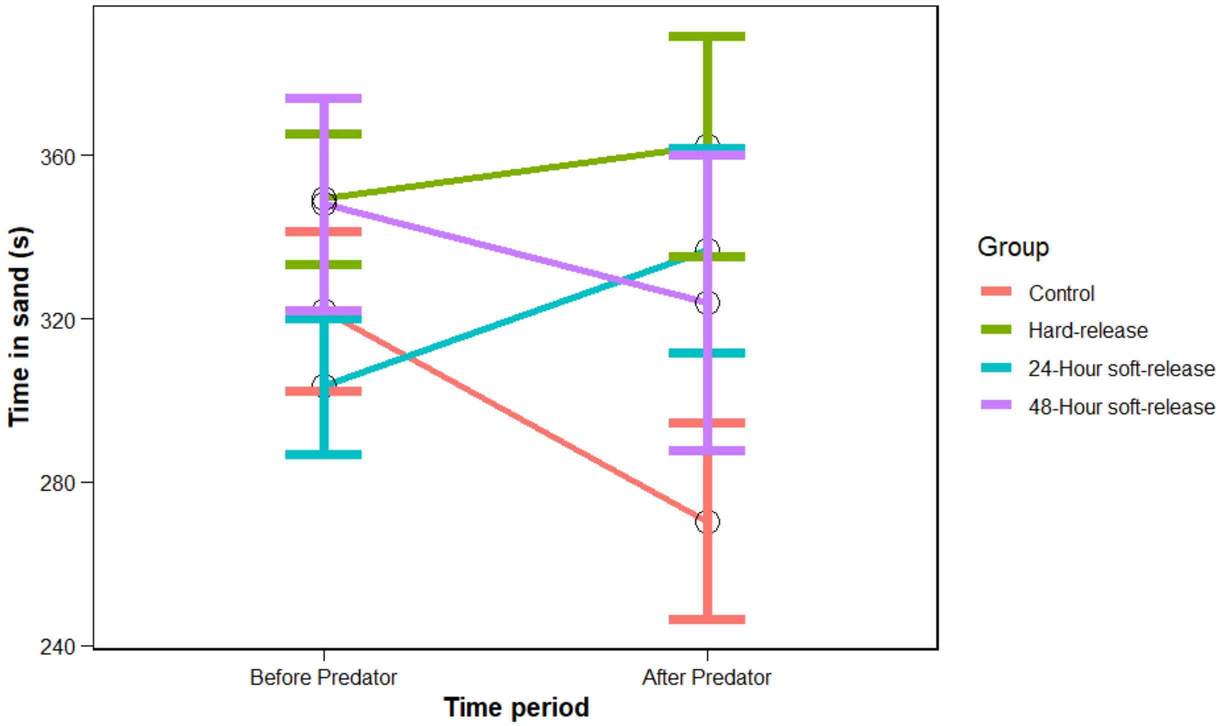

Figure 3.8 – Time spent in sandy side of arena before and after the introduction of a simulated predator across treatment groups in the substrate preference and burrowing assay. Treatment groups included control (non-transported), hard-release (2-hr transport), and 24-hr soft-release (2-hr transport followed by 24-hr in-river acclimation), and 48-hr soft-release (2-hr transport followed by 48-hr in-river acclimation).

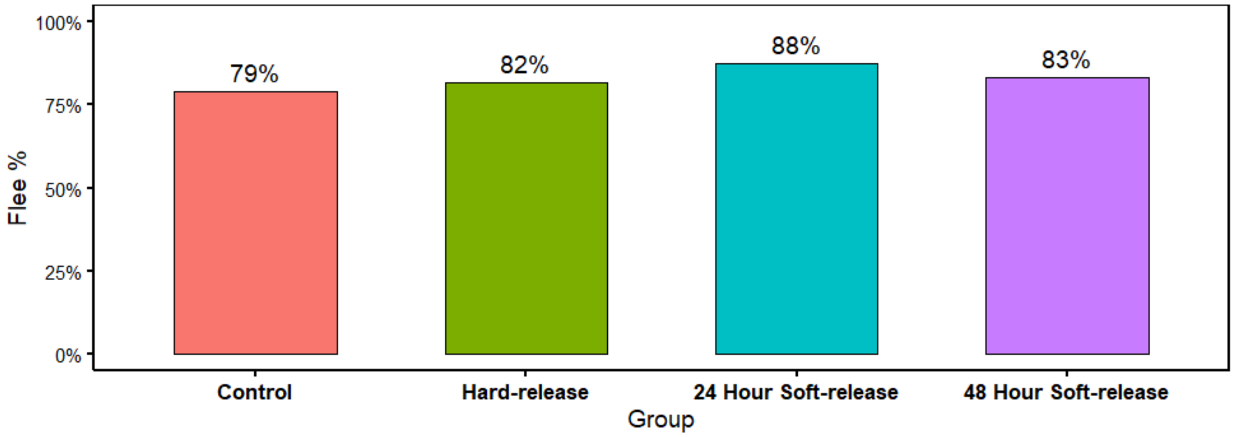

Figure 3.9 - Proportion of individuals in each group that fled in response introduction of predator.

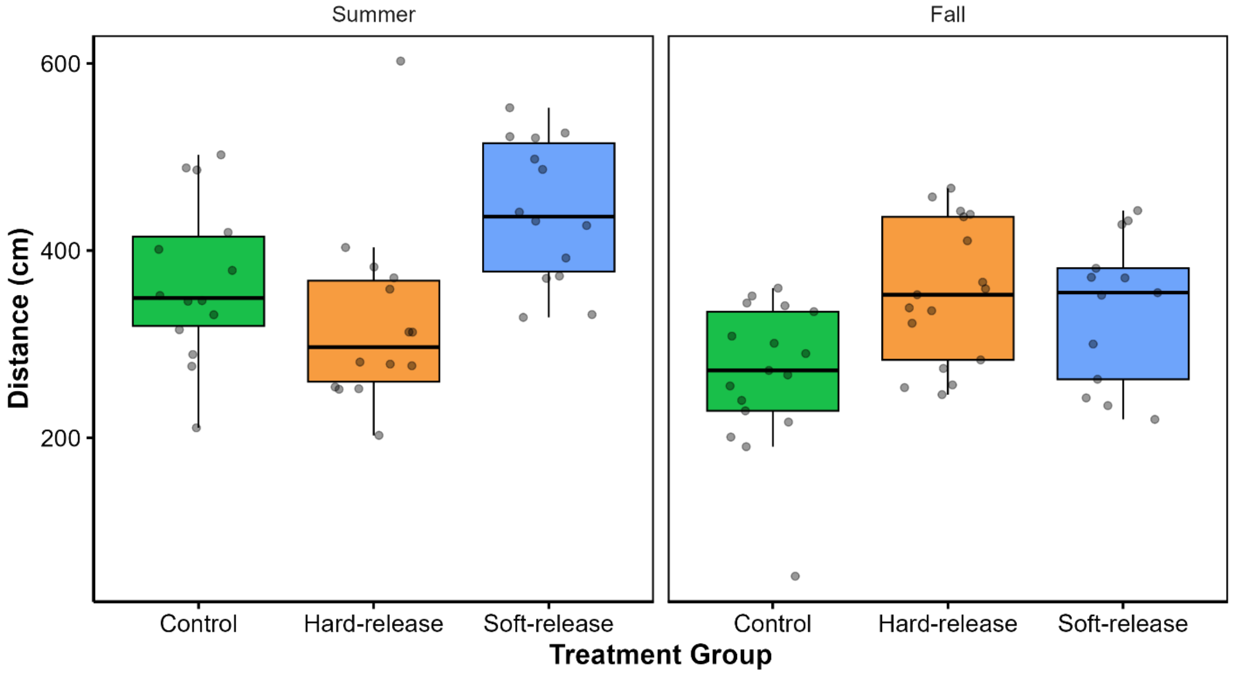

Figure 3.10 - Distance traveled during swimming performance trials where fish were chased for 30-seconds by hand in an annular arena. Treatment groups included control (non-transported), hard-release (2-hr transport), and soft-release (2-hr transport followed by 24-hr in-river acclimation). Trials were completed in summer and fall of 2022. Boxplots depict median and interquartile range, with whiskers representing the minimum and maximum.

Table 3.1 - Behavioural metrics assessed in Eastern Sand Darter and corresponding ecological relevance for fishes.

| **Behaviour measured** | **Ecological relevance** |
|---|---|
| Activity (swim) time | Effective motility important to survival (predator evasion, foraging for food and seeking shelter) (Schreck et al. 2016) |
| Substrate preference | Quality of substrate important to benthic organisms (Bouvier and Mandrak 2010) |
| Time spent buried | Burrowing behaviour potentially integral to survival substrate dwelling species (Daniels 1989; Simon 1991) |
| Edge / Open / Refuge time | Exploratory or risk-taking behaviour important for locating important resources (Jenkins et al. 2021) |
| Predator response (burrow, flee, or freeze) | Propensity to recognize and evade predators important for survival (Davis 2010) |

Table 3.2 - Ethogram indicating behaviours that were tracked in Eastern Sand Darter during open field trials.

| **Behaviour Type** | **Behaviour** | **Description** |
|---|---|---|
| Action | Swim | Any noticeable movements or undulations resulting in movement of the body in terms of location or orientation |
| Action | Flee | Rapid movement in response to novel object |
| Location | Refuge | Location of subject within the arena or petri dish (refuge) |
| Location | Edge / Open | Location of subject within the arena<br>Edge refers to perimeter (subject location < 1 body length from wall) of the arena; Open refers to space (> 1 body length from wall) of arena |

Table 3.3 - Ethogram indicating behaviours tracked in Eastern Sand Darter during substrate preference and burrowing trials.

| Behaviour Type | Behaviour | Description |
|---|---|---|
| Action | Swim | Any noticeable movements or undulations resulting in movement of the body's location or orientation |
| Action | Burrow | Active burrowing action into the substrate |
| Action | Flee | Rapid movement in response to trout lure |
| Location | Buried | Buried beneath sand following a burrowing event |
| Location | Sand / Rock | Location of subject within the arena, either on rock or sand substrate |
| Location | Edge / Open | Location of subject within the arena<br>Edge refers to perimeter (subject location < 1 body length from wall) of the arena; Open refers to space (> 1 body length from wall) of arena |

Table 3.4 - Output statistics for GLMM comparing Summer and Fall swim times overall, during open field tests.

| **Term** | **Estimate** | **Std error** | **Statistic** | **P value** |
|---|---|---|---|---|
| Intercept | 5.08 | 0.15 | 34.76 | > 0.01 |
| Season | -0.53 | 0.21 | -2.53 | 0.01 |

Table 3.5 - Output statistics for GLMM comparing buried time overall before and after predator introduction, during substrate selection and burrowing trials.

| **Term** | **Estimate** | **Std error** | **Statistic** | **P value** |
|---|---|---|---|---|
| Intercept | 7.58 | 34.46 | 0.22 | 0.83 |
| Time Period | 61.05 | 23.84 | 2.56 | 0.01 |

Table 3.6 - Output statistics for GLMM comparing distance covered between groups, during swim performance tests.

| **Term** | **Estimate** | **Std error** | **Statistic** | **P value** |
|---|---|---|---|---|
| Intercept | 265.21 | 18.90 | 14.03 | > 0.01 |
| Treatment: Hard-release | 92.44 | 26.57 | 3.48 | > 0.01 |
| Treatment: Soft-release | 73.02 | 28.23 | 2.59 | 0.01 |
| Season | 94.00 | 26.21 | 3.59 | > 0.01 |
| Treatment: Hard-release x Season | -118.14 | 39.16 | -3.02 | > 0.01 |
| Treatment: Soft-release x Season | 5.76 | 38.90 | 0.15 | 0.88 |

# Chapter 4 - General discussion and conclusions

## Summary

Reintroducing organisms to parts of their range in which they no longer persist can be used as a strategy in the larger toolbox aimed at the conservation and rehabilitation of species (Houde et al. 2015). Development of effective reintroduction strategies require rigorous planning and careful execution (George et al. 2009). As the field of Reintroduction Biology has become more experimentally based, the opportunity to delineate the factors that have the greatest influence on population establishment and overall project success has become more possible (Armstrong and Seddon 2008). While attention must be paid to every facet of reintroduction planning, the ecological conditions of the reintroduction site and the methods used for transport and release can both have large implications for individual survival and population persistence (George et al. 2009; Cochran-Biederman et al. 2015). Specifically, the condition of the habitat in which individuals are released will determine whether or not this area meets the ecological and life history requirements of the species being reintroduced (Minckley 1995). The transportation and release portion of reintroduction exposes animals to stress which can negatively influence the physiology and behaviour of individuals for hours or days following the event (Barton 2000; Nomura et al. 2009; Roberts et al. 2024); even with ideal release habitat, if individuals are unable to recover from the physical transport process, establishment will be compromised. As a result, transferring individuals to a protective “soft-release” enclosure in the reintroduction waterway following transport can afford fish a period of acclimation to their new environmental conditions as well promote recovery before they must face the challenges of becoming established in their new habitat (Tetzlaff et al. 2019).

While reintroduction has been used more extensively in mammalian species, it is becoming increasingly important for preservation and recovery of freshwater fishes (Minckley 1995; Seddon et al. 2005). As a group, these species have collectively seen extensive losses in biodiversity and represent one of the most imperilled groups on the planet (Dudgeon et al. 2006; Sayer et al. 2025). Eastern Sand Darter is an at-risk benthic freshwater fish species, which inhabits areas of sandy substrate in lakes, rivers, and streams (Scott and Crossman 1973; Daniels 1993). Eastern Sand Darter are threatened in Ontario, Canada but have been selected for reintroduction to parts of their native range from which they have been extirpated (i.e., Big Otter Creek) (COSEWIC 2009; Bouvier and Mandrak 2010). Successful reintroduction of Eastern Sand Darter through the translocation of individuals from the Grand River will require thorough analysis of the conditions of the reintroduction habitat as well as consideration of transport and release procedures used (George et al. 2009; Lamothe et al. 2019a; Lamothe and Drake 2019). Therefore, the objective of my thesis was to contribute to ongoing Eastern Sand Darter reintroduction plans by describing the conditions of Big Otter Creek and determine which areas may represent the most appropriate release sites, as well as refining the methods of transport and release that will promote the maintenance of ecologically-relevant behaviours.

In Chapter 2, I assessed the ecological conditions of Big Otter Creek using data primarily collected through surveys performed in 2018 and 2023, further informed by supplementary community assemblage data from surveys in the nearby Grand River and Ausable Creek

(Barnucz et al. 2020; Gaspardy and Drake 2021). When characterizing the habitat, I gave special consideration to parameters that have historically been correlated with Eastern Sand Darter prevalence and those that are most likely to influence the likelihood of Eastern Sand Darter survival, including substrate composition and abundance of a key invasive species (Bouvier and Mandrak 2010; Burbank et al. 2019; McAllister et al. 2022). I also used known positive, negative, and neutral relationships between Eastern Sand Darter and other members of the fish community to characterize sites along Big Otter Creek in terms of their probability of supporting Eastern Sand Darter (Lamothe et al. 2019b). The objective of this chapter was primarily descriptive in nature, but I was able to use empirically-founded analyses to identify several locations of Big Otter Creek in which conditions in some or all of these metrics converged, indicating potentially favourable release locations.

In Chapter 3, I assessed the effect of transport stress on Eastern Sand Darter behaviour and investigated whether soft-release techniques can promote more natural behaviour in relation to traditional hard-release procedures. I analyzed behavioural trials on Eastern Sand Darter consisting of open field, substrate preference, and swim performance tests, taking the potential for seasonal differences into consideration.  I found that transport procedures did not significantly result in disruptions to Eastern Sand Darter behaviour, including activity level, substrate use, burrowing propensity, ability to flee, or time spent in edge vs. open space. I did, however, find some differences in swim performance between treatment groups during both our summer and fall trials, but they were not consistently indicative of negative consequences of transport stress. Holding periods of 24- to 48-hours also did not alter behaviour in relation to control (non-transported) fish, apart from fish swimming further distances during swimming performance trials following soft-release in comparison to controls. Despite the lack of consistent measured behavioural differences between treatment groups, I found some differences in behaviours between seasons and between time periods prior to or after the introduction of a simulated predator. My results indicate that careful attention to transport conditions may mean that Eastern Sand Darter are behaviourally resilient to transport stress, and that reintroduction programs can use hard-release. My results also contribute valuable knowledge regarding the behaviour of an understudied at-risk freshwater fish.

Taken together, my results provide support to the overall feasibility of an Eastern Sand Darter translocation program in southern Ontario. As part of larger investigations that also have considered source populations, disease risk, transport conditions, and post-release monitoring, my results contribute to the development of an evidence-based plan for ensuring survival and establishment. As I outline in more detail below, my work emphasizes that collectively considering the full range stressors that fish face during reintroduction is important for detailed planning and execution. It further calls attention to the value of incorporating behavioural and physiological information (i.e., mechanism) into conservation science and Reintroduction Biology more specifically. Finally, I describe how future work can expand from my analyses and results to benefit both the ongoing Eastern Sand Darter reintroduction program, as well as our knowledge on supporting repatriation efforts for other small-bodied freshwater fish species.

## Integrating behaviour and physiology into reintroduction biology

For decades, the fields of Conservation Behaviour and Conservation Physiology have been contributing tools and techniques to support conservation, restoration, and rewilding (Lipsey et al. 2007; Armstrong and Seddon 2008; Tarszisz et al. 2014). The value of these fields lies in their capacity to measure mechanisms (i.e., cause-effect relationships) and therefore to help identify how animals respond to changes in their environment, as well as determine whether certain approaches are better than others when attempting to solve conservation challenges (Cooke et al. 2013; Cooke et al. 2014). In Chapter 3, I used assessments of metrics of behaviour for both of these types of applications; I investigated the effects of transport as a stressor and attempted to compare the success of alternative release methods. By far, the most common use of physiological and behavioural monitoring in the context of conservation has been to delineate whether organisms are under threat from certain environmental changes, or to more generally quantify “stress” (Cooke et al. 2014; Madliger et al. 2022). Fish display both physiological and behavioural responses to stressors when faced with environmental challenges (Sopinka et al. 2016). Depending on several factors such as the duration, timing, and frequency of stressors, both the behaviour and physiology of animals may be affected by stress in different ways (Barton 2002; Wingfield 2013; Petitjean et al. 2019). However, no single physiological or behavioural metric alone provides adequate information regarding the levels of stress or disruption that an animal is experiencing (Pottinger 2008; Petitjean et al. 2019). For example, Vanderzwalmen et al. (2021) showed that the behaviour of Variatus Platy (*Xiphophorus variatus*) changed as a result of transport, showing greater incidence of erratic swimming, biting, and freezing, despite no associated change in waterborne cortisol levels, highlighting that measuring only a single response could lead to ambiguous conclusions. While measures of glucose and corticosteroid levels in the blood, as well as other measures of physiological function such as metabolic rate, have traditionally been used to assess the effects of stress on the performance of animals, assessing behavioural responses can provide greater understanding of the downstream consequences that stress may have for the performance of fishes (Davis 2010; Archard et al. 2012; Sopinka et al. 2016). In my thesis, I measured multiple metrics of behaviour that are related to a variety of ecological functions to better characterize the effects of transport and release. Adding physiological metrics would provide even greater understanding of the effects of transport, as well assist in determining whether soft-release enclosures are beneficial to overall recovery in more capacities than just the maintenance of normal behaviours. For example, as the metabolism of fish can be influenced by anthropogenic and environmental stressors and is related to performance and fitness (Radull et al. 2002; Ling et al. 2019; Alfonso et al. 2021), assessment of metabolic rate could provide another useful metric for assessing transport stress and soft-release effectiveness (Pottinger 2008; Watt et al. 2024). Importantly, measurement of metabolic rate is relatively non-invasive and can be completed streamside (Treberg et al. 2016).

While determining stress and disturbance is a logical application for physiology and behaviour within the context of Reintroduction Biology, there are a number of other aspects of reintroduction projects where the consideration of mechanistic information can be worthwhile. For example, understanding physiological tolerances can assist in choosing appropriate source populations. By quantifying thermal tolerance (CTmax) of Redside Dace (*Clinostomus elongatus*), Turko et al. (2021) were able to predict the likely responses of different populations to reintroduction to a warmer, more urbanized habitat. Similarly, there are applications for risk assessment (i.e., pre-release planning), ensuring welfare in captive breeding programs, and post-release monitoring (Tarszisz et al. 2014).

## Appreciating the suite of stressors associated with fish reintroduction

Reintroduction projects inherently involve repeated stressors for the individuals being relocated, including capture and removal from a familiar environment, handling, transport, release, acclimation in a new waterway, establishment of a habitat, and environmental variability within the reintroduction environment (Dickens et al. 2010). As exposure to multiple stressors either simultaneously or successively can have cumulative effects (Beitinger 1990; Sopinka et al. 2016), both pre- and post-release conditions must be considered during reintroduction efforts; my thesis assessed components of both the pre- and post-release suite of stressors. Different species vary in their resiliency to particular stressors, and even within species, inter- and intra-individual responses to stressors have been documented (Beitinger 1990; Sopinka et al. 2016). Water quality parameters such as dissolved oxygen and temperature can directly influence the health and well-being of fish and incite a stress response when outside of certain thresholds (Alabaster 1982a; Alabaster and Lloyd 1982). Most species can tolerate minor fluctuations in some aspects of water quality to accommodate seasonal and daily variability (Petitjean et al. 2019). However, rapidly changing conditions or those outside the range which the animal can tolerate, will result in negative fitness consequences (Petitjean et al.

2019). Monitoring both water quality of release habitat and water quality of transport containers will ensure conditions meet the requirements of the species being reintroduced and remain relatively consistent throughout the process. As a result, considerations as simple as attention to water and air temperature on the dates of transport and release can have measurable improvements to outcomes for transported fish (Alabaster 1982b; Harmon 2009). Pairing this with a seasonal understanding of water quality in relation to species-specific habitat requirements in potential release sites can address this stressor across the full span of a reintroduction events.

In addition to the relatively predictable and cyclical changes of water conditions such as those that occur with annual progression of seasons, more permanent shifts in habitat conditions overall may also occur (Chu et al. 2005; Jackson and Blois 2015). Continual monitoring of physicochemical conditions can be incorporated to detect changes in environmental conditions and determine how these changes may be influencing the released populations within that habitat. Although the amount of time and resources required for community sampling may limit how often surveys occur, the monitoring of abiotic conditions is less complex. Technology such as remote temperature sensors, hand-held equipment capable of measuring and recording multiple physicochemical parameters rapidly and simultaneously, as well as openly accessible data from existing water quality monitoring stations provide the means to continually assess many of these abiotic conditions with limited effort. While I have used aspects of water quality here as an example of how a certain type of stressor can be relevant over multiple time periods of a reintroduction event, the same project life cycle considerations can be made for other parameters that occur repeatedly (e.g., fish handling). Ideally, a reintroduction program will also consider that different types of disruptions (e.g., water quality, handling, introduction to a novel environment) have the potential to represent repeated stressors for fish, thereby emphasizing the need for mitigation measures that consider cumulative or chronic stress.

The biotic components of the release environment can also be viewed as representing a potential stressor within the context of reintroduction projects. As predators or competitors can create challenging conditions in release sites, the level of predation pressure as well as competition for resources are important aspects of the habitat to consider. Knowledge of the diversity and abundance of species that both positively and negatively interact with the focal species across different areas of reintroduction habitat can help develop strategies that account for these interspecific interactions and plan accordingly to limit any negative consequences on reintroduction outcomes. In Chapter 2, I was able to incorporate known fish community relationships, ascertained from stable populations of Eastern Sand Darter, to gain knowledge of the potential interactions in a new reintroduction environment. Interaction with invasive species can also be particularly important to consider for freshwater fishes in the Great Lakes, as these alien invaders typically show competitive advantages over species native to the ecosystem and can simultaneously represent predators and competitors (Dextrase and Mandrak 2006; Bergstrom and Mensinger 2009). Access to food is one of the most important resource-related aspects of community ecology, and dietary overlap can create competition between species within the same ecosystem (Vadeboncoeur et al. 2002; Bergstrom and Mensinger 2009;

Firth et al. 2021). Incorporating the collection of data regarding available food resources, combined with knowledge of community assembly of habitat can be integrated to provide insight into what level of competition stress the reintroduced fish will face and what level of success they will have in acquiring the resources required for survival (Kalogianni et al. 2023). Frequent sampling and analysis of these conditions should be used to track any environmental changes over different time scales, and the resulting ecological consequences. Knowledge of any changes in habitat characteristics are likely to have a meaningful impact on Eastern Sand Darter and will aid in adapting reintroduction strategies to changing ecological conditions.

### Limitations and future directions

The length of time a fish may require to recover from a stressful event may exceed the length of time that has typically been used as an acclimation period in most published research involving soft-release methods (Nomura et al. 2009; Roberts et al. 2024). The application of soft-release methods involving time periods of greater length may yield greater recovery from the effects of stress which may persist for several days. However, as captivity can create stress for fish (Fisher and Romero 2019), longer soft-release may introduce new considerations and challenges, such as the level of resource (e.g., food) access available during acclimation and the negative consequences of prolonged captivity. Soft-release enclosures used during research are typically limited in size and complexity. These temporary enclosures are light weight and are easily installed and removed from the waterway. The use of larger enclosures that place less restriction on the movement of fish during the acclimation period may also allow greater access to important resources such as nutrition or shelter. The fossorial behaviour of Eastern Sand

Darter is an important aspect of their ecology (Daniels 1989). The use of soft-release enclosures designed to accommodate specific requirements of the species being translocated should be considered. For example, ensuring adequate depths of substrate to allow Eastern Sand Darter to burrow during acclimation periods could provide greater resting and recovery. These characteristics would also provide these individuals greater access to benthic invertebrates within the substrate on which Eastern Sand Darter rely for nutrition (Burbank et al. 2019). The use of acclimation periods of greater length would necessitate greater access to resources, but experimental investigation that measures all of the potential benefits of soft-release enclosures (e.g., physiological recovery) over a wide range of time periods would be worthwhile.

Our experiments were not able to determine whether soft-release enclosures provide any benefit to individual survival. The proportion of individuals surviving the translocation procedure will determine the number of individuals that can become successfully established within their new habitat, and contribute to the foundation of a viable population of Eastern Sand Darter. It can be difficult to estimate survival rate in translocations, and determining this with any accuracy is often not possible, particularly in small-bodied fishes where technologies such as telemetry are not available (Swarr et al. 2022). To better understand soft-release as tool for the reintroduction of freshwater fishes more generally, experiments could be completed in larger-bodied species where movement can be tracked (e.g., using RFID tags or telemetry tags).

While open field tests provide some insight into a variety of behaviours, interpreting the results is sometimes not straightforward. Factors such as inter- and intra-specific variations can result in a range of behavioural tendencies and inconsistent results when using traditional metrics. Behaviours such as refuge seeking, exploration, and predator evasion tactics are crucial for survival but can differ across species (Huckstorf et al. 2009). As two species may respond differently to the same stimuli, extensive knowledge of these behavioural attributes of a species are required to fully comprehend the results of behavioural trials (Beitinger 1990; Barton 2002). For Eastern Sand Darter, the results from my burrowing and substrate preference experiment suggests that burrowing behaviour is not an immediate predator evasion tactic, but that it still may function in limiting conspicuousness after a predator has been identified. In general, small-bodied fishes often do not have comprehensive accounts of life history, and therefore there is a need to gain greater understanding of behaviours and their ecological importance to better understand how different species or populations of fishes respond to stressors in their environment.

Finally, my thesis also used a detailed analysis of habitat parameters that are of greatest ecological relevance to Eastern Sand Darter to help determine release locations. Physicochemical parameters as well as substrate composition can provide some insight into the locations which have the abiotic conditions most suitable to Eastern Sand Dater, but selection of release sites should also involve an assemblage-wide assessment, with the most rigorous analysis focusing on community-based interactions such as pressure from invasive species. My analysis was able to identify broad areas that are likely more suitable for the ecological requirements of Eastern Sand Darter, but was ultimately based on a qualitative assessment of the overlap of the multiple individual characteristics I assessed. Following my work with an analysis that integrates these habitat components into a single likelihood metric would be even more informative and would allow weight to be given to the aspects of the habitat that are most important for persistence (e.g., based on expert knowledge).

### Conclusions and implications for the translocation of Eastern Sand Darter

My results were able to provide information on release locations and methodologies that can be used in an ongoing Eastern Sand Darter reintroduction program. I characterized a number of key environmental and community variables along Big Otter Creek, and identified broad areas that can likely support Eastern Sand Darter population establishment. I did not document any negative consequences of transport stress in the context of the behaviours I assessed. In accordance with animal care guidelines as well as to prevent mortality of a threatened fish, I used optimal transport conditions, potentially minimizing any transport related stress. As a result, potentially high levels of resiliency to transport stress coupled with ideal transport conditions may have resulted in limited differences in stress between groups and therefore negligible behavioural differences. As a result, I suggest that hard-release represents a viable method for this species under these conditions, but that additional physiological information would be worthwhile for fully assessing the potential benefit of soft-release. Overall, my results have contributed to our understanding of the behavioural ecology of Eastern Sand Darter and provide supportive knowledge for their reintroduction in Ontario. More broadly, they draw attention to key considerations for reintroductions of small-bodied freshwater fishes.

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