The Role of Physical Movement in Spinal Cord Repair Using an Adult Zebrafish Model

by

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Abstract

Spinal cord injury (SCI) is a debilitating medical condition that can lead to lifelong paralysis. Failure to regain mobility and restore neurons can be attributed to the non-permissive microenvironment within the mammalian spinal cord that is characterized by prolonged inflammation and glial scarring. Unlike mammals, the zebrafish has a remarkable ability to regenerate neurons in the spinal cord following a complete transection. Aside from the activation of chemical pathways that promote growth, zebrafish also have ependymoglial (stem) cells surrounding the central canal that can proliferate and differentiate into all cell types of the spinal cord. Previous studies have investigated factors that promote neuronal regeneration, yet the contribution of locomotion remains unevaluated. Thus, we aimed to explore the role of physical movement in spinal cord repair by developing a swim column that elicited swimming activity (exercise treatment) from injured fish. This apparatus was developed using an aquarium pump to direct flow into a PVC tube placed within an acrylic tank. Adult male zebrafish from motor neuron reporter line, *Tg(hb9:eGFP)*, were used. Results showed that the exercise treatment slowed the rate of recovery and diminished normal swimming behaviour. Downstream analysis of distance travelled, mean velocity, and mobility state duration revealed that SCI fish treated with exercise had delayed recovery compared to SCI fish that were untreated. Contrary to previous studies using mammalian models, we found that functional mobility was hindered, and swim restoration was limited. Furthermore, our findings contrast other zebrafish exercise studies that demonstrated increased neurogenesis in larvae, and attenuation of age-related diseases such as sarcopenia and cardiac failure. These contradictions may be attributed to sample size, experimental timeline, treatment duration, and stress levels. Although the current study did not yield statistically significant results, these preliminary findings lay the groundwork for deeper exploration of movement in regenerative models

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List of Acronyms and Abbreviations

BDNF	brain-derived neurotrophic factor
CC	central canal
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycans
Dpf	days post-fertilization
Dpi	days post-injury
ECM	extracellular matrix
EZ	ependymal zone
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
Hh	Hedgehog
iPSCs	induced pluripotent stem cells
IL-6	interleukin-6
NgR1	nogo receptor 1
PVC	plastic polyvinyl chloride
PTPσ	protein tyrosine phosphatase sigma

SCI	spinal cord injury
SEM	standard error of the mean
SEZ	subependymal zone
TNF-α	tumor necrosis factor alpha

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1. Introduction

1.1 Background on SCI

Spinal cord injury (SCI) is a complex medical condition that arises from damage to the spinal cord, often leading to losses in sensory, motor, and autonomic function (Tran et al., 2018; Alizadeh et al., 2019). While a range of incidents can cause a SCI, traumatic events such as motor vehicle accidents and falls account for 90% of the cases worldwide (World Health Organization [WHO], 2013). Lifelong tetraplegia, paralysis in all four limbs, is experienced by 16% of SCI patients as a result of neurotrauma in the cervical region of the spinal cord (WHO, 2013). In 2019, approximately 30,000 Canadians (804/million) were living with SCI, with over half these cases exhibiting tetraplegia (Thorogood et al., 2023).

The spinal cord consists of nerve bundles that facilitate communication between the central nervous system (CNS) and the rest of the body, aiding in coordinated responses to external stimuli (Alizadeh et al., 2019; Bican et al., 2013). Sensory information travels to the CNS where it is processed and able to inform motor commands (Birch, 2011; Leijnse & D'Herde, 2016). Behaviours and reflexes are coordinated by signalling pathways and networks that link neurons to various sensory receptors and muscle effectors (Bican et al., 2013). This organization of sensory-motor networks allows the body to acclimate to external and internal stimuli, enabling the maintenance of homeostasis, execution of movements, and coordination of complex behaviours (Nógrádi & Vrbová, 2013). Consequently, damage to the spinal cord can disrupt neural communication, making the performance of regular daily activities difficult.

1.2 Physiological Responses Following SCI in Mammals

Following SCI, at the cellular level, the physiological response occurs in two phases, termed primary and secondary injury. Primary injury is characterized as direct damage to the spinal cord resulting from mechanical forces that displace bone fragments (Alizadeh et al., 2019). The extent of the primary injury determines the overall severity downstream as initial impacts disrupt blood vessels, integrity of cell membranes, ionic balances, and oxygen levels (Alizadeh et al., 2019). The secondary injury begins minutes after SCI and can persist for months, involving a cascade of changes to the microenvironment (Yuan & He, 2013; Lima et al., 2022). Secondary responses can differ widely among vertebrates, due to their unique cell types, cellular responses, and physiologies.

During secondary injury in mammals, the microenvironment becomes compromised, affecting the homeostatic functions of resident cell populations (Alizadeh et al., 2019). In mammals, the cellular component (grey matter) of the spinal cord consists of both neurons and glial cells (Bican et al., 2013). Neurons populate the inner grey matter of the spinal cord where they are organized into two dorsal roots and two ventral roots (Nógrádi & Vrbová, 2013). To generate motor commands, upper motor neurons from the motor cortex in the brain transmits signals to the lower motor neurons of the spinal cord, which are then relayed to other regions of the body (Stifani, 2014). Each spinal segment is joined together by interneurons and glial cells to form spinal tracts and nerves that transmit signals toward or away from the spinal cord (Bican et al., 2013).

Although neurons are the fundamental unit of the nervous system, glial cells such as astrocytes, oligodendrocytes, microglia, and ependyma are more abundant (Rasband, 2016). Astrocytes support neuronal function by modulating neurotransmitters, regulating energy metabolism, and balancing ion levels, which are essential roles for maintaining homeostasis (Rasband, 2016). Oligodendrocytes are responsible for axon myelination within the CNS, allowing for fast and efficient neuronal communication (Li et al., 2024). Microglia are the resident macrophages of the CNS that mediate the immune response through phagocytic activity (Liddelow et al., 2020). Lastly, ciliated ependymal cells line the central canal of the spinal cord, producing cerebrospinal fluid, and can act as neural stem cells (NSCs) post-injury (Barnabé-Heider et al., 2010). These cells originate from radial glia during development (neurogenesis) and are essential for maintaining the functional integrity of the spinal cord (Kriegstein & Alvarez-Buylla, 2009).

With secondary injury in mammals, SCI is accompanied by axon degradation, the formation of a heterogenous tissue mass (glial scar), and prolonged inflammation (Cigliola et al., 2020). Distal axons that have been severed will dissolve through a process termed Wallerian degeneration (Rotshenker, 2011). The proximal axon tracts do not survive, resulting in widespread neuron death (Rotshenker, 2011). Additionally, upon injury, astrocytes undergo a dramatic change in phenotype, altering the extracellular matrix (ECM) composition by releasing excess chondroitin sulfate proteoglycans (CSPGs) (Buss et al., 2009; Bilalova et al., 2024). Increased CSPGs in the ECM promotes inhibition by interacting with multiple signalling pathways (e.g., NgR1, PTPσ) that suppress axonal regeneration (Sabelström et al., 2013; Hellenbrand et al., 2021). Reactive astrocytes also contribute to the formation of a heterogenous tissue mass which produces a physical barrier that prevents axonal regrowth (Alizedah et al., 2019; Cigliola et al., 2020). Moreover, prolonged inflammation can damage intact spinal cord tissue through activation of microglia and migration of peripheral macrophages and neutrophils to the lesion site (Shields et al., 2020; Li et al., 2024). These responses in mammals during secondary injury produce a non-permissive microenvironment, limiting functional recovery post-SCI and exacerbating the initial damage (Alizadeh et al., 2019).

1.3 Current Approaches to Treat SCI

Surgery, cell-based therapies, and physical rehabilitation are common methods that have been explored to help facilitate repair post-SCI. Decompression of the spinal cord through surgical realignment of the vertebrae can help relieve pressure and promote blood flow to the injury site (Ball & Sekhon, 2006). More recently, cell-based therapies have been developed to mitigate cell loss following secondary injury. These approaches include induced pluripotent stem cells (iPSCs), stem cell transplants, and activation of endogenous stem cells (Hu et al., 2023). Other non-invasive treatments such as physical rehabilitation, has also been extensively used for promoting joint mobility, systemic blood circulation, and bone strength (Richings et al., 2023). However, despite these research efforts and therapeutic advancements, restoring any level of function remains an ongoing challenge. The main issue being the inability to regenerate lost neurons with complete reliability, as the injured mammalian CNS does not promote repair (Hu et al., 2023).

1.4 Regenerative Models for Studying Repair

The use of animal models with successful regeneration, especially neurorepair, can lead to promising insights for understanding the regrowth of severed axons, repair of neuronal circuits, and restoration of full mobility (Sharif-Alhoseini et al., 2017). Researchers employing adult salamanders (eg. Axolotls; *Ambystoma mexicanum*), larval lampreys (eg. Sea lampreys; *Petromyzon marinus*), tadpoles (eg. African clawed frogs; *Xenopus laevis*), and teleost fish species can further our knowledge on neurorestorative approaches post-SCI (Cohen et al., 1986; Becker et al., 1997; Edwards-Faret et al., 2017; Sharif-Alhoseini et al., 2017; Walker et al., 2022). Numerous genetic tools have been applied to these models for exploring the signalling pathways involved in disease and injury recovery (Edwards-Faret et al., 2017). In particular, experimental manipulation of the zebrafish (*Danio rerio*) has shown the capacity to repair various body parts including the heart, brain, and spinal cord (Becker et al., 1997).

1.5 Zebrafish Spinal Cord Regeneration

In stark contrast to mammals, zebrafish possess the remarkable ability to undergo regeneration and full recovery after a complete transection of the spinal cord at all stages of development (Becker et al., 1997). These differences are largely attributed to the unique cell populations that are present within the zebrafish CNS (**Fig. 1a**). A non-mammalian cell type, the ependymoglial cells are present within 2-3 layers around the central canal of the spinal cord (Hui et al., 2015). These cells are considered to have intermediate characteristics between mammalian radial glia and ependymal cells (Hui et al., 2015). In all vertebrates, radial glial serve as neural precursor cells that can give rise to neurons and glia during development (Kriegstein & Alvarez-Buylla, 2009). Ependymoglia persist throughout adulthood in the zebrafish, and certain populations share some features with radial glia such as expression of glial fibrillary acidic protein (GFAP) and of aquaporin-4 (Barnabé-Heider et al., 2010; Cigliola et al., 2020). They also possess morphological similarities, such as long radiating processes that extended laterally from the canal, and NSC properties (Zambusi & Ninkovic, 2020). When the zebrafish is in homeostasis, ependymoglia are in a reversible cell cycle arrest, referred to as quiescence (Becker & Becker, 2008). However, with injury-induced cues from SCI, these cells can re-enter the cell cycle to proliferate and act as NSCs (Barnabé-Heider et al., 2010).

Zebrafish ependymoglia have been observed to undergo non-gliogenic (rather neuronal) cell division in response to injury, producing neural progenitors. This non-gliogenic cell division is attributed to activation of various signalling pathways that promote regeneration of neurons (Becker & Becker, 2020). Similar to development, during spinal cord regeneration, many of these pathways are re-engaged, with a variety being implicated in motor neuron regeneration (Dias et al., 2012; Goldshmit et al., 2018). Among these pathways, is the hedgehog (Hh) pathway and fibroblast growth factor (Fgf) signalling for promoting the development of motor neurons (Goldshmit et al., 2018; Cigliola et al., 2020). Other pathways are conversely downregulated, such as the Notch signalling pathway, which was shown to halt the differentiation of *hb9*-expressing motor neurons (Dias et al., 2012). These tightly regulated pathways allow for ependymoglia to successfully regenerate damaged neuron populations.

Alongside the regenerative abilities of ependymoglia, zebrafish also have other mechanisms that support spinal cord repair (Goldshmit et al., 2012; de Sena-Tomás et al., 2024). The infiltration of immune cells is followed by glial bridging 2-3 weeks post-SCI in adult zebrafish (**Fig. 1b**) (Sambur et al., 2024). A scaffolding is produced for axons to reconnect on either side of the lesion (**Fig. 1c**) (Cigliola et al., 2020). Upon migration of nearby glial cells to the lesion site, elongation occurs along their anteroposterior (Goldshmit et al., 2012). Axon regeneration is further supported by a permissive microenvironment, characterized by a controlled inflammatory response (de Sena-Tomás et al., 2024). This regulation prevents glial hypertrophy at the injury site, which in mammals leads to the formation of a glial scar (Tran et al., 2018). The chemical environment of the zebrafish spinal cord following injury thus remains favourable for axonal regrowth, allowing reinnervation of the appropriate targets (Reimer et al., 2008; Hui et al., 2014, 2015). By 2-6 weeks post-SCI, complete functional recovery is observed in adults, as evidenced by analyses of swimming behaviour and swim endurance against increasing water current velocities (Burris et al., 2021). Zebrafish typically display greater exploratory behavior and prolonged swim times (before exhaustion) at 2-weeks post-SCI (Burris et al., 2021).

1.6 Factors Influencing on Zebrafish Repair Mechanisms

Investigating the mechanism of regeneration and neurorepair in the zebrafish can offer valuable insights that may be translated to human SCI patients. Extensive research has been focused on understanding the different genetic factors, signalling pathways, and environmental conditions that enable zebrafish to achieve functional recovery (Reimer et al., 2008; Hui et al., 2014; Tsaraouchas et al., 2018; Becker & Becker, 2020; Klatt Shaw et al., 2021). These studies demonstrate the relationship between cellular plasticity and the zebrafish's permissive microenvironment that allows for restoration of spinal cord function. Evidently, researchers have dedicated their efforts into investigating the molecular and genetic drivers of regeneration. However, the potential contribution of mechanical factors such as physical movement remains poorly understood.

Physical movement in other animal models has been shown to positively influence gene expression and alter various pathways throughout the body (Sleiman et al., 2016). Studies have shown that important neuroprotective molecules such as brain derived neurotrophic factor (BDNF) increases with exercise, promoting axon growth (Sleiman et al., 2016). Beyond the accumulation of these molecules, physical movement can also influence SCI recovery by dampening the inflammatory response. Reduction of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) has been observed in SCI patients post-exercise (da Silva Alves et al., 2013; Paulson et al., 2013). Therefore, this regulation of the spinal cord microenvironment can ultimately mitigate secondary injury progression (da Silva Alves et al., 2013). These cellular changes may aid in driving increased recovery in SCI rodent studies subjected to various exercise treatment and rehabilitation paradigms (Jesus et al., 2021).

This evidence serves as a foundation for investigating potential exercise effects on regeneration processes present in the zebrafish. Thus raising an important research question: Does physical movement influence functional recovery in zebrafish post-SCI? Understanding this relationship could provide novel insight into the external factors that permit normal swim restoration following SCI.

1.7 Zebrafish a Leading Regenerative Model

The zebrafish is a good model to investigate this research question as previous literature has highlighted the positive effects of increased physical movement in larval neurogenesis. Notably, one study found that movement is a stimulates adult neurogenesis in the healthy spinal cord of zebrafish (Chang et al., 2021). Other studies in zebrafish have also highlighted the benefits of exercise for attenuating age-related diseases. For instance, Suniaga et al. (2018) demonstrated that four weeks of swim tunnel exposure significantly increased osteoblast activity, which can alleviate bone loss associated in conditions such as osteoporosis. Similarly, Rovira et al. (2018) reported that exercise shows cardioprotective effects by increasing cardiomyocyte proliferation following a heart injury. Another study by Sun et al. (2022) found that exercise intervention alleviated symptoms of sarcopenia in 21-month-old zebrafish by increasing protein synthesis. These findings suggest that exercise exerts profound physiological effects across various organ systems in zebrafish, influencing cell proliferation and tissue repair. Building on this evidence, exploring the role of movement, on zebrafish spinal cord repair could reveal whether similar processes contribute to restoration of normal swim behaviour. This research can potentially address outstanding gaps in zebrafish spinal cord studies, leading to better understanding of the factors that create optimal conditions for functional recovery.

The zebrafish is one of the most promising models in regenerative medicine with its ability to regain full motor function post-SCI at all stages of development (Klatt Shaw et al., 2021). Additionally, the zebrafish also possesses a 71.4% genetic similarity with human protein-coding genes, providing a strong basis for translating findings from this animal model to human biology (Howe et al., 2013). Compared to other regenerating species, the zebrafish is the most suitable model for this project as its spinal cord possesses cell populations and molecular processes comparable to those seen in mammals (Becker & Becker, 2008). Additionally, their ability to regenerate various organ systems from larval to adult stages makes them a highly generalizable model across different age groups (Gemberling et al., 2013). The zebrafish is, therefore, an invaluable tool for investigating spinal cord regeneration and producing translational findings.

2. Research Objectives and Hypotheses

This project focused on evaluating the post-SCI swimming behaviour of adult zebrafish after subjecting them to an increased movement condition. I hypothesized that the level of locomotor activity following SCI would influence the rate at which normal swim behavior is restored post-SCI. I predicted that increased locomotion would lead to faster restoration of normal swim behaviour post-SCI. The hypothesis was addressed through two research objectives. **Objective 1** aimed to develop a functional swim column and a standardized protocol (***experiment 1***). **Objective 2** quantified whether an environment with increased movement opportunities altered the rate of locomotor recovery (***experiment 2***).

3. Methods and Materials

3.1 Zebrafish Husbandry

All zebrafish were raised and maintained in the Rady Biomedical Fish Facility at the University of Manitoba, Rady Faculty of Health Sciences. Zebrafish were kept at a 12-hour light:dark cycle and housed in a standalone recirculating system with ultraviolet and carbon filters (Tecniplast, Italy). Fish facility water was regulated at 28°C, 900 µS conductivity, and pH 7.4. Feeding occurred three times a day using a 300 µm pellet food (Skretting, Gemma Micro 300) and live brine shrimp (Brine Shrimp Direct).

Adult male zebrafish between 4-months to 8-months old (of ~2.5-3 cm total body length) from the *Tg(hb9:eGFP)* transgenic line were used for all experiments. Since zebrafish exhibit a male bias of approximately 1:3 in favor of males, a greater number of male zebrafish were available for the study (Liew et al., 2012). While our analysis focused on behaviour, *Tg(hb9:eGFP)* is a motor neuron marker that can be subsequently used to evaluate the effects of physical movement in this cell population (Arkhipova et al., 2012). Animal ethics and care were practiced in accordance with the University of Manitoba Animal Care Committee and the Canadian Council for Animal Care.

3.2 Experimental Groups

Exercise treatment was evaluated by testing the following experimental groups: ***(1)*** SCI-Exercised, ***(2)*** Sham-Exercised, ***(3)*** Uninjured-Exercised, and ***(4)*** SCI-Nonexercised. Groups 2-4 served as control groups and followed the same timeline as the experimental group ***(1)*** (**Fig. 2a, b**). The Sham-Exercise group ***(2)*** accounted for the potential effects caused by the injury. Additionally, to ensure that manual handling into the swim column did not impact the study, group ***(3)*** was also subjected into the forced exercise paradigm. To assess the effects of increased movement, the SCI-Nonexercised group ***(4)*** served as a baseline control.

3.3 Spinal Cord Injury and Sham Protocol

Zebrafish were anesthetized using 4.2 mL of 0.4% tricaine methanosulfonate (tricaine) for every 100 mL of fish facility water. Sedation was determined based on diminished response to tail pressure and weakened breathing, in line with plane 4-5 of anesthetization. Then, animals were transferred to a petri dish under a dissecting microscope (Leica EZ4 W) and positioned dorsally. To perform the SCI, fish were held using tissue forceps while continuous lateral incisions were made with surgical scissors through the 2nd quartile of the spinal cord between the hind brain and dorsal fin (**Fig. 1c**). Instruments were sterilized after every fish using 70% ethanol (EtOH). A complete transection was achieved once the spinal cord was severed, after penetrating the dorsal muscle tissue. To ensure a proper SCI procedure was performed, a touch tail response test was conducted with a pipette. Swimming impairment of the lower half of the body indicated a successful injury had occurred, confirmed by a negative (lacking) tail touch response. The sham protocol followed the same general procedure, but the incision did not go beyond the muscle layer, leaving the spinal cord intact.

The SCI or the sham procedures required 30 seconds-1 minute and afterwards fish were placed into individual recovery tanks with fish facility water (off-system). They were monitored for up to 15 minutes to ensure that normal breathing patterns were reestablished by observing regular opercular beats. To maintain consistency, all fish were not fed for 3 days post-injury (dpi) as SCI prevented injured fish from swimming to the surface to feed and leftover food could have led to infection at the injury site. After 3 post-procedural days elapsed, fish were returned to their normal on-system tanks. Individuals remained isolated for the remainder of the experiment and fed only brine shrimp until endpoint was reached. All groups, including the control fish, were treated the with the same post-procedural care, regardless of injury status.

3.4 Swim Column Development

To study the effects of exercise in zebrafish, development of a cost-effective swim column with accessible materials was required. Commercial swim columns are prohibitively expensive and were not in the scope of the current project. Multiple iterations of swim columns were designed and fabricated (**Fig. 3a, b**), predominantly inspired by the Loligo commercial swim column brand. The first model failed to produce a well-distributed flow, which led to various adjustments ensuring equal water dispersal was achieved.

The established swim column consisted of a plastic polyvinyl chloride (PVC) tube (40.6 cm length; 5.08 cm diameter). Water flow was generated using Hydor Pico 70 pump, as previously described in the Frankland and Thiele Lab (**Fig. S1**). Animals were subjected to a flow rate of 250L/hr in 7 L of fish facility water regulated at 26-28°C. Fifty-five straws were used at both ends of the tube to evenly disperse the flow of water from the pump, providing 28.2 cm free swim space. Straws ensured all column regions received an evenly distributed current. Four sponges were added to secure the tube to the bottom of the tank, while also preventing fish from swimming out of the entry port after being netted into the column (**Fig. 3b**).

3.5 Swimming-Induced Exercise Training

To analyze the effects of increased locomotion, exercise training conditions were developed for this experiment. At 14-dpi exercise fish were subjected to a swim column challenge for a total of 2-hours everyday for five days. This interval was selected based on preliminary data collected at the start of the study and was determined to optimize recovery and experimenter availability. Fish were placed in the column for a total time of 3-hours, which included a 30-minute habituation period followed by 30-minute increments of exercise with 10-minute breaks in between (**Fig. 2b**). Treatments began in the morning at 10:00am-11:00am and ended in the early afternoon at 1:00pm-2:00pm Physical exertion was confirmed by fish swimming against the current, known as rheotaxis (Olszewski et al., 2012).

3.6 Behavioural Tracking

All fish were studied starting at 2-dpi every second day excluding the weekends for the duration of the experiment (3-weeks), resulting in six recordings per fish (**Fig. 4**). Individual fish were netted into a glass tank (28 cm x 23.5 cm x 20 cm; Into the Blue Aquarium Services Inc., Winnipeg) containing 2L of 28°C system water and placed over a light box to illuminate the tanks and produce better contrast. Fish in each tank were isolated from each other using a cardboard divider, allowing two tanks to be simultaneously recorded using an open-field tracking system (Ethovision XT, Noldus) for n=2 per trial. Following a 30-minute acclimation period, fish were recorded for 5-minute sessions that were separated into 30-second time bins (**Fig. 6**).

To assess the impact of physical movement, we analyzed swim behaviour in zebrafish across six different timepoints; 2, 4, 9, 11, 16, and 18-dpi. Three parameters were chosen for downstream analysis; **(1)** mean velocity (cm/s^2), **(2)** distance travelled (cm), and **(3)** mobility state duration. The duration of each mobility state was determined by the percentage of the fish's body in motion, with immobility defined as <10% and high mobility as >50%. These values were chosen based on body movement threshold being exceeded. Considering no exercise-treated fish reached >60%, 50% was a relevant upper limit. Immobility (<10%) was selected based on the default settings (40% difference) between lower and upper movement thresholds.

3.7 Statistical Analysis and Graphical Representation

Statistical analyses were conducted to assess mean velocity and distance travelled. Behavioral data was collected over six timepoints and averaged for each group per experimental day. To account for the small sample sizes and confirm normality, a Shapiro-Wilk test was performed on R software (v4.3.2). A two-way repeated measures ANOVA was conducted for mean velocity and distance travelled over time per group. This statistical analysis and graphical representation were completed using GraphPad Prism version 10.3, with statistical significance set at $p < 0.05$. Mobility state duration had missing values, thus we instead opted to visualize trends using excel (v.2502).

4. Results

4.1 Swim Column Model 2 Improves Water Current Dispersion

In order to address the role of locomotion, we first needed to establish a reliable swim column where we could subject zebrafish to a water current. The first swim column model successfully generated a continuous flow and fish exhibited rheotaxis. However, based on multiple runs, fish frequently clustered near the bottom or back of the tube, avoiding full engagement with the current (**Fig. 3a, Video 1**). In model 1 of the swim column, more fish were stationary and not actively swimming against the current. We thus concluded that flow distribution was inconsistent, with stronger currents concentrated at the center of the tube and weaker currents at the top and bottom sections. These variations led to inconsistent swimming behavior, affecting the reliability of exercise-induced swimming treatments. Additionally, we set-up model 1 with a section of the column dedicated for habituation by separating it using a porous plastic divider. However, this introduced extra handling as manual removal was necessary to allow fish to freely swim in the column post-habituation. This manipulation during the experiment may have induced unintended stress and movement of the tube.

Modifications were made in the second model that improved flow stability and overall swimming consistency. The addition of fifty-five straws at both ends of the PVC tube helped evenly disperse water flow, creating a more uniform current throughout the entire column (**Fig. 3b, Video 2**). Therefore, even if fish clustered in specific areas, they would still experience the same flow. Additionally, removing the plastic dividers and mesh coverings further reduced handling and water disturbance, allowing for uninterrupted rheotaxis. The addition of three sponges helped to mitigate the lifting of the tube caused by air bubbles from the straws. These sponges secured the tube to the bottom of the tank and also maintained the fish within the intended swimming area. These refinements resulted in higher activity and engagement with the current. Based on these observations, model 2 was selected as the final design for swimming-induced exercise training as it improved flow regulation, minimized external handling, and enhanced data reliability.

4.2 Exercise Paradigm and Timeline Establishment

To analyze exercise intervention post-SCI, we had to determine an appropriate timeline and exercise paradigm for the zebrafish. After pilot runs of this experiment (data not shown), we found that zebrafish were able to tolerate exercise at 14-dpi. Given that previous studies in this lab reported the initiation of normal swimming behaviour at 7-dpi, our initial goal was to implement exercise treatments at this timepoint (Trzuskot, unpublished data). However, we opted to use later dates (14-dpi) as we observed that fish could not swim against the current at the start of their recovery (7-dpi). We also determined the feasible end-point for successful completion of the project was 21-dpi. This timeline allowed us to collect sufficient data for further analysis.

Additionally, we had to determine the duration of each exercise session. Since SCI significantly affects motor abilities and induces stress, we first tested fish with 10-minutes in the swim column, and gradually increased to 30-minute and then 1-hour trials. We found that ~45 minutes to 50-minutes, the injured fish were less active and involuntarily drifted to the back of the column, indicating that they could no longer swim against the current. Thus, to minimize the stress-induced effects, we chose to have 2-hour active exercise sessions separated into 30-minute intervals with 10-minute breaks in between (**Fig. 2b**).

4.3 Behavioural Recovery Post-SCI and Exercise Treatments

We investigated how exercise influences behavioral recovery of injured zebrafish, as previous studies suggest it aids in regaining functional mobility. To assess the impact of physical movement, we analyzed swim behaviour in zebrafish across six different timepoints following SCI; 2, 4, 9, 11, 16, and 18-dpi (**Fig. 2a**). Three parameters were chosen for analysis; **(1)** mean velocity, **(2)** distance travelled, **(3)** mobility state duration, with a two-way repeated measures ANOVA analyzing data collected for mean velocity and distance travelled. Missing values for mobility state prevented us from performing statistical analyses on this parameter. Additionally, heat map and tracking visualizations of these measurements provided additional insight into the potential influence of exercise in regaining mobility following SCI in zebrafish (**Fig. 5**).

4.3.1 Mean Velocity is Lower in SCI-Exercised Fish

Evaluating mean velocity allowed us to assess restoration of normal swimming behaviour and sustained movement post-SCI in zebrafish (**Fig. 6a**). At 2-dpi and 4-dpi, the SCI-Exercised and -Nonexercised fish were exhibiting similar movement patterns with one another. By 11-dpi, the SCI-Exercise fish show a slight increase from the SCI-Nonexercised fish, but following this, the movement levels remained low, averaging 0.799 cm/s^2. Surprisingly, the SCI-Nonexercised fish exhibited a higher mean velocity for the rest of the experimental timeline. Additionally, mean velocity for the Sham-Exercised group was lower than the Uninjured-Exercised group.

Overall, despite the results from the two-way repeated measures ANOVA not yielding statistically significant ($p>0.05$) results, the SCI-Exercised fish displayed the lowest mean velocity among all groups. These trends in the data suggest that exercise did not enhance velocity and may have instead negatively impacted recovery post-SCI.

4.3.2 SCI-Exercised Fish Exhibit Reduction in Distance Travelled

Total distance travelled was used to quantify swimming exploration of zebrafish post-SCI (**Fig. 6b**). A two-way repeated measures ANOVA test was performed to compare the differences in distance travelled between exercised and non-exercised groups. At 2-dpi, the SCI-Exercise and SCI-Nonexercised groups had similar movement patterns as visualized by the heat map (**Fig. 5**). By 11-dpi, the SCI-Exercised group show a slight increase from the SCI-Nonexercised group, but this trend does not continue. SCI-Exercised fish remained at a lower level between 16-dpi and 18-dpi compared to their untreated (nonexercised) counterparts (**Fig. 5, 6b**). Though this finding was not statistically significant ($p>0.05$), these patterns oppose the hypothesis of the study.

Unexpectedly, the Sham-Exercise fish, which served as a control for the injury treatment, were also not swimming at the control levels. Though the difference was not significant ($p>0.05$), we did not anticipate these groups to vary in locomotor capacity. These findings suggest that exercise does not aid in locomotor recovery and may have negatively affected distance travelled in zebrafish post-SCI.

4.3.3 Mobility State Duration Reveals Diminished Movement Levels for SCI-Exercised Fish

Mobility state duration was analyzed to quantify periods of active swimming versus inactivity. High immobility duration indicated more time spent inactive (**Fig. 7a**). At 9-dpi, both SCI-Exercise and SCI-Nonexercised fish predominantly remained in a low mobility state (immobile). By 11-dpi, both groups showed a reduction in time spent immobile (movement <10% of body). However, the SCI-Exercise fish exhibited a slower decline, indicating delayed recovery trajectory (**Fig. 7a**). This pattern was also reflected in the high mobility state measurement, which analyzed time spent in an active state (**Fig. 7b**). SCI-Nonexercised fish exhibited a noticeable increase in movement starting at 16-dpi (**Fig. 7b**). In contrast, SCI-Exercise fish did not spend any time in the highly mobile state (movement >50% of body), indicating that there are persisting motor deficits.

Notably, similar to previously stated results, the Sham-Exercise fish do not follow the same mobility state patterns as the Uninjured-Exercise fish. The high incidence of time spent immobile at 11-dpi was reflected in previous data that also found an observable decrease in distance travelled and mean velocity at this timepoint (**Fig. 6a, 6b, 7a**). Statistical analyses were not conducted on these data due to missing values. However, these trends reveal further insights into the movement patterns of zebrafish post-SCI across different treatment conditions.

5. Discussion

5.1 Project Summary

Researchers have investigated the benefits of physical rehabilitation as an intervention for human SCI patients and other mammalian models. However, its impact on regenerative species has yet to be explored. To this end, we aimed to determine if the positive effects of exercise translated to highly regenerative models such as the zebrafish. Our primary objective was to evaluate the role of physical movement by subjecting SCI fish to exercise treatments and subsequently analyzing their swimming behaviour. Inconsistent with the hypothesis that exercise improves functional mobility in zebrafish, the results of this study show that recovery is delayed across all locomotion parameters that were measured. When compared to SCI-nonexercised fish, SCI-exercised fish at 16-dpi maintained low levels of movement across all behavioural analyses (**Fig. 6a, 6b, 7a, 7b**). These patterns can be observed despite the results being statistically not significant. Contrary to our initial predictions, exercise may have imposed a physical strain, potentially disrupting normal recovery trajectories from taking place.

5.2 Swim Restoration is Reduced in SCI-Exercised Fish

For all three parameters that we measured, there was a consistent decrease in movement levels for the SCI fish subjected to exercise. Though we expected an environment with increased locomotion would facilitate faster repair, it is plausible that exercise treatment introduced stress, thereby reducing functional mobility. In the zebrafish, glial bridging produces a robust scaffold for axons to reconnect on either side of the lesion (Cigliola et al., 2020). This process occurs typically around 15-dpi and is completed at 30-dpi (Cigliola et al., 2020). Considering that we subjected the fish to exercise at 14-dpi, stress-associated movement could have negatively impacted glial bridging, reducing normal swim restoration. It is possible that our exercise application occurred during a sensitive period wherein external factors such as stress may have prohibited SCI recovery. These factors could potentially explain the discrepancies between our original hypothesis and prediction.

5.3 Diminished Functional Recovery Compared to Rodent Models

As this study poses a new question in the field, we primarily interpreted our findings by drawing conclusions from rodent models. Rodents are a widely recognized tool for investigating SCI, with various studies demonstrating the positive effects of exercise on their recovery. In contrast to observations in these models, our results indicate that exercise diminishes locomotor recovery in zebrafish post-SCI. A study performed by Jesus et al. (2021) reported increased running duration in mice that underwent an intensive six week forced exercise paradigm after a cervical spinal cord hemisection. Other rodent studies found exercise-associated improvements in limb mobility starting at two weeks post-SCI, with reported rehabilitation up to eight weeks following the procedure (Sandrow-Feinberg et al., 2009).

Variations in our findings may be attributed to the study design. Our experimental timeline only spanned 18 days, which may have prematurely terminated the study before we could observe significant results. Given that differences in mobility were seen at approximately two weeks post-SCI in rodent models, our shorter timeline did not allow us to investigate long-term effects. Notable differences were only observed starting at 16-dpi, but since the experiment was concluded at 18-dpi, we could not make further conclusions.

5.4 Limited Improvement Compared to Previous Zebrafish Studies

The results of this study also varied in comparison to previous literature investigating exercise effects on neurogenesis and age-related diseases in zebrafish. Notably, Chang et al., 2021 demonstrated that movement influenced spinal cord proliferation in the uninjured adult zebrafish spinal cord. After 10 experimental days consisting of 6-hour exercise sessions, increased DNA synthesis, a hallmark of cell proliferation was observed (Chang et al., 2021). Subsequently, they found that ~60% of these cells were differentiating into neurons, indicating that exercise positively influences neurogenesis (Chang et al., 2021). Moreover, other studies have also supported the benefits of physical movement in zebrafish for attenuating age-related diseases. For instance, Suniaga et al. (2018) reported substantial increases in bone-forming osteoblasts after subjecting zebrafish to a rigorous 6-hour exercise regime over the course of four weeks. These results were supported by another study that showed improved cardiac function in the regenerative state (Rovira et al., 2018). After heart cryoinjury, at 3-dpi, fish were subjected to exercise treatments for six hours over 13 experimental days, and the authors found that cardiomyocyte proliferation had significantly increased (Rovira et al., 2018). Another study supporting the positive effects of exercise reported that sarcopenia symptoms such as mitochondrial dysfunction was alleviated in zebrafish (Sun et al., 2023). After eight weeks of 4-hour exercise training sessions, biochemical pathways that reduce oxidative damage was observed (Sun et al., 2023).

These findings consistently demonstrate that increased physical activity has widespread positive effects across different organ systems in zebrafish. Although this evidence supports exercise intervention as a potential regenerative aid in zebrafish post-SCI, our results differed. A possible explanation for the discrepancies in our current study is the limited duration of exercise exposure. Specifically, our study only implemented 2-hour exercise sessions over five experimental days. Though it was the maximum feasible timeframe for successful completion, this was a substantially shorter timeline compared to those used in prior studies, potentially leading to variable results.

5.5 Recovery Outcomes are Influenced by Induction of Exercise Treatments

Another key observation in this study was the influence of exercise timing on recovery outcomes. We initiated the sessions at 14-dpi instead of 7-dpi, when normal swimming behaviour typically begins (Trzuskot, unpublished data). Though we already extended the recovery period, it may be necessary to apply exercise treatment at a later dpi as increased stimulation and mechanical stress could have played a role in the diminished swim restoration that was observed in this study. By 18-dpi, fish in the SCI-Exercise group demonstrated a reduced likelihood of swimming and exploring their environment compared to SCI-Nonexercised fish (**Fig. 5**). This lethargic behaviour, characterized by low activity, is indicative of the stress response in zebrafish, and can be further analyzed in a future experiment using cortisol measurements (plasma, skin mucous) (Demin et al., 2021). These observations oppose our predictions, and differ from previous literature (Sandrow-Feinberg et al., 2009; Jesus et al., 2021).

5.6 Differences in Swim Column Design May Have Influenced Outcomes

Variations in this study could have also stemmed from differences in methodological apparatus. In our experiments, we developed our own swim column using readily accessible materials (**Fig. 3b**), which may have resulted in some inconsistencies with previous studies. Employing commercialized swim columns (Loligo Brand) or using designs based on previously established literature could reduce discrepancies and increase reproducibility. Using swim column designs such as those described in Suniaga et al. (2018) could enhance validity and reduce instrumental errors in the study.

5.7 Study Limitations

In this study, we examined the behaviour of four experimental groups ranging from n=3 to n=4 per group. Using a low sample size made the study easily susceptible to high levels of variations and outliers. These low values were mainly attributed to high mortality rates post-SCI. Previous studies show that SCI survival rates are typically near 70% using wildtype fish (Fang et al., 2012), but in this study, we found that survival rates were considerably lower, often below

50%. Though there are studies evaluating SCI survival in other transgenic lines, we did not find studies evaluating SCI in *Hb9* fish which were used for our study. Notably, previous observations in the Lindsey Lab have found that there are line-specific survival rates, but this data has yet to be quantified. Additionally, because of this mortality, we had different sample sizes across the experimental groups and during the trial runs of the study. These inconsistencies could explain the high variabilities reported throughout the data. Therefore, it is possible that different transgenic lines and strains of zebrafish may respond differently to SCI, and thus may have varying sensitivities to exercise treatments.

Unexpected discrepancies were also observed in the Sham-Exercised and Uninjured-Exercised groups, suggesting that the sham procedure may have inflicted greater damage than intended. This difference could have led to impaired locomotion, compromising the comparisons with the injured groups. Additionally, exercise may have exacerbated this strain, affecting swimming performance. As a result, the sham may not have served as a reliable control. Thus, refining the sham procedure for use in exercise paradigms can help ensure valid comparisons for future studies.

We also aimed to perform statistics on three parameters, but behavioural recordings for mobility state could not be recovered. Technical issues with the Ethovision Software led to gaps in the data, particularly in the SCI-Exercised group. Therefore, we could only evaluate general trends for these measurements, rather than performing statistical analyses.

5.8 Future Directions

Future research should aim to further investigate the role of movement in spinal cord repair, particularly by incorporating molecular analyses. Given that our study focused on behavioural recovery after exercise treatment, evaluating molecular and cellular changes can provide further insight into the spinal cord microenvironment. Specifically, immunohistochemical analysis of motor neuron markers such as *Hb9* could clarify if physical movement is also diminishing regeneration of these neuronal populations. Additionally, investigating ependymoglia cell proliferation and differentiation using the S-phase marker, 5-Ethynyl-2′ deoxyuridine (EdU) and neuronal marker HuC/D can provide insight into the extent of regenerative neurogenesis. Another consideration is the stress response induced by exercise treatment, which can be addressed by performing cortisol assays.

Moreover, performing longitudinal studies and lengthening the experimental timeframe could improve the robustness and consistency of the findings. A significant variation in this study compared to previous research was the duration and frequency of the exercise treatment. Notably, most studies conducted at least a four hour increased movement paradigm lasting six to eight weeks (Rovira et al., 2018; Sun et al., 2023; Suniaga et al., 2018). Therefore, applying well-established timelines could increase comparability across other studies, allowing for greater confirmation of whether exercise diminishes functional recovery post-SCI.

5.9 Conclusion

This study explored a novel question for understanding the different factors that promote functional recovery post-SCI in zebrafish. While exercise is known to enhance motor function, promote neurogenesis, and mitigate disease progression, our findings suggest that its impact on zebrafish SCI recovery is more complex. The observed reduction in movement for all locomotion parameters suggests that physical stimulation could potentially induce stress and diminish regenerative abilities. As this study represents an emerging area of investigation, further research is necessary to confirm our findings and determine the optimal timeline, intensity, and duration for evaluating the effects physical movement in zebrafish post-SCI.

6. Figures

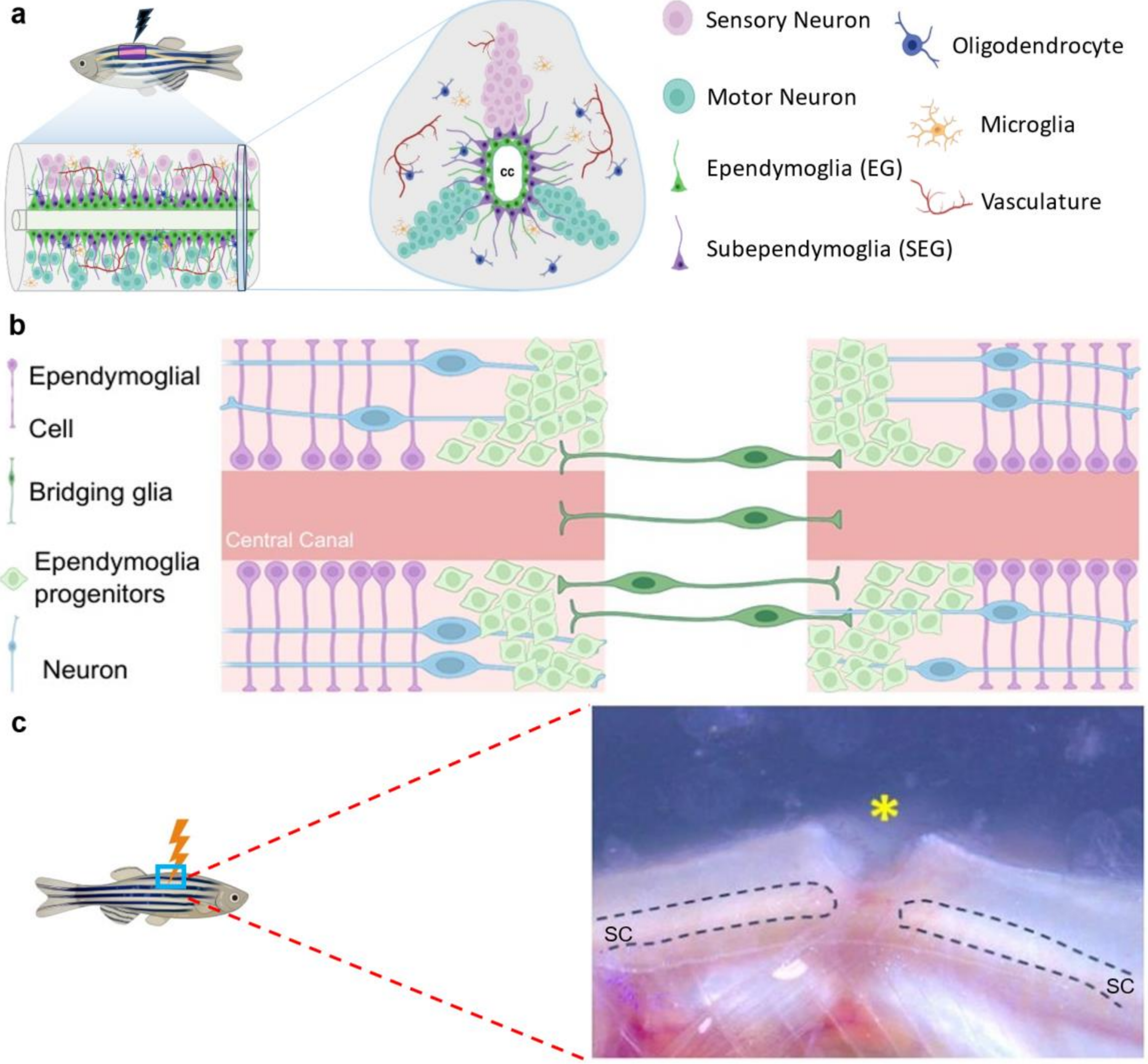

Figure 1. The uninjured and injured zebrafish spinal cord cell populations. (a) Coronal cross-section of the zebrafish spinal cord consisting of various cell populations. Motor neurons occupy the two dorsal horns. The ependymoglia (EZ) and subependymoglia (SEZ) are found 2-3 layers surrounding the central canal (CC). Oligodendrocytes and microglia also reside close to the central canal with vasculature found in the surrounding tissue (adapted from Trzuskot, 2022). **(b)** Sagittal cross-section of the zebrafish spinal cord 20-dpi. Spinal cord injury prompts

differentiation of ependymoglial cells, producing ependymoglia progenitors that will differentiate and replace various cell types in the spinal cord. Bridging glial provides scaffolding for reconnecting at either side of the lesion (modified from Riley et al., 2022). **(c)** A close-up image of the injury location (2nd quartile of the spinal cord) is indicated by the yellow asterisk. A successful injury is determined by a complete transection of the spinal cord (SC) as delineated by the black dashed lines.

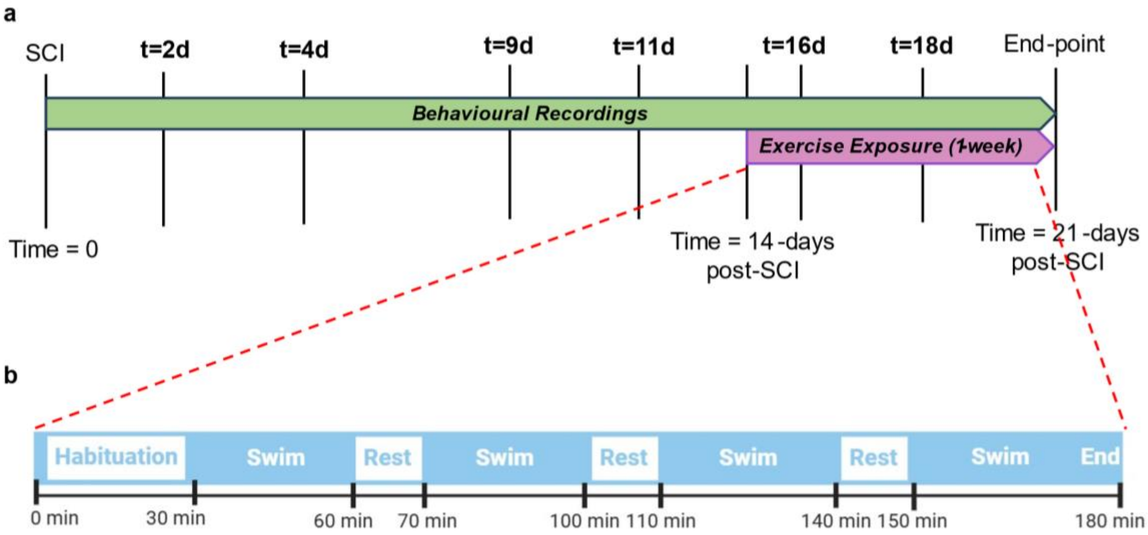

Figure 2. Experimental timeline for spinal cord injury (SCI) and subsequent analyses. **(a)** SCI commenced at time 0 (t=0). By 14 days post-SCI, fish from groups 1-3 were exposed to exercise treatments for one week. Behavioural recordings were carried out in parallel beginning at t=2 days post-SCI and conducted at the bolded time points. At the experimental endpoint (21 days post-SCI), fish were sacrificed. **(b)** Timeline of swimming-induced exercise sessions for zebrafish lasting 180-minutes. The 30-minute habituation period was followed by 30-minute exercise intervals separated by 10-minute rest periods. White boxes indicate rest phases when the pump was off, allowing free movement.

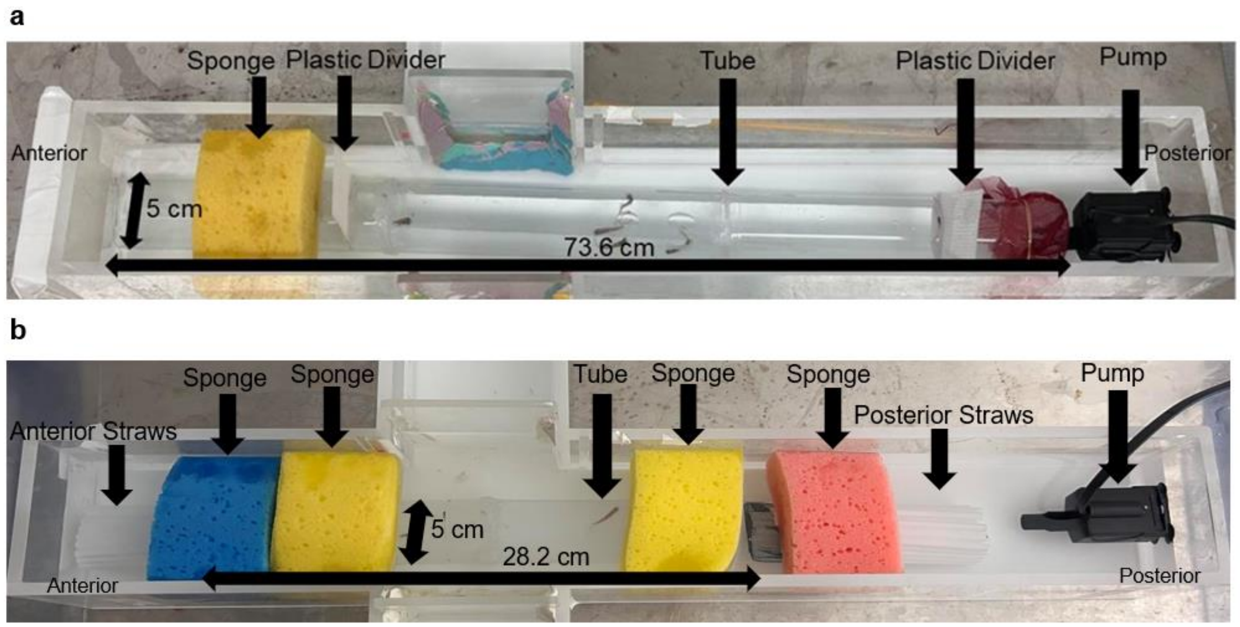

Figure 3. Representative images of the exercise swim tunnel set up. (a) Model 1 of swim column set-up used for pilot studies of the experiment. Tubing was placed in the middle of the tank to resemble a tunnel system. The aquarium pump was placed on the posterior end of the tank. Fish subjected to exercise were placed at the opening on the anterior end and blocked from the rest of the tunnel by a plastic divider. Once the fish were in the tunnel, a sponge was added to block the entry port. **(b)** Model 2 of the swim column set-up used for the exercise paradigm. Straws were added to the anterior and posterior end of the tube to evenly distribute the water flow from the pump. Removal of the plastic divider provided 28.2 cm of free swim space. Additional sponges were added throughout the column to secure the tube to bottom of the tank.

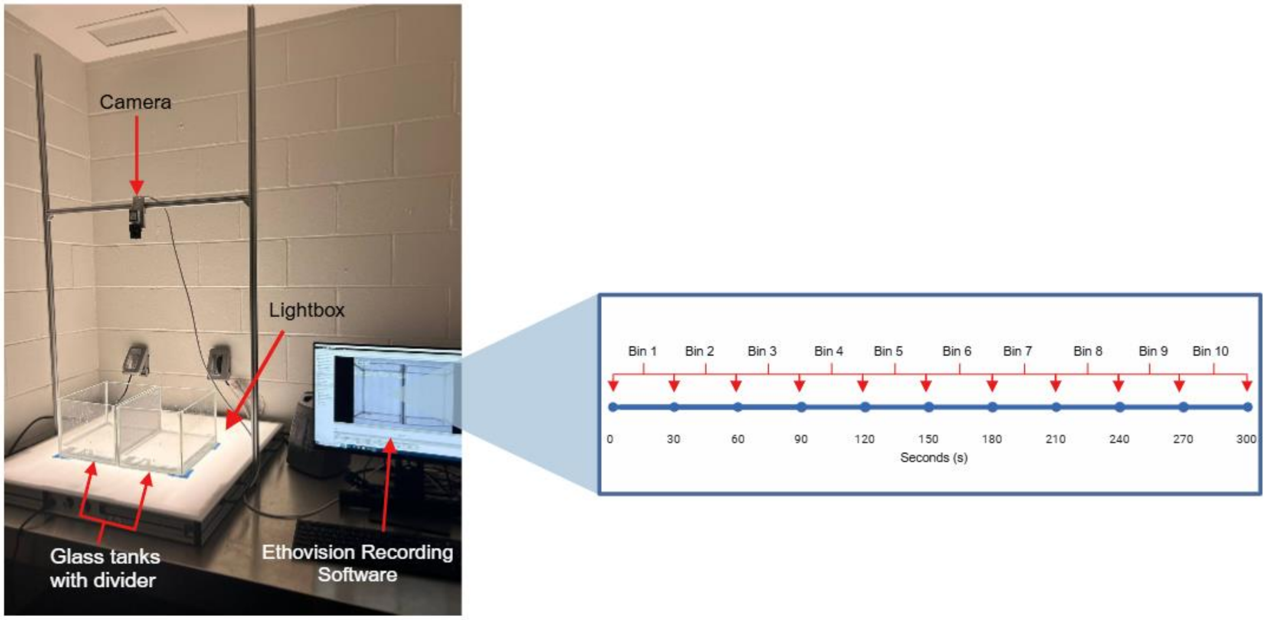

Figure 4. Behavioural recording set-up with bolded points indicating recording sessions. Fish were netted into glass tanks positioned on a light box and separated by a divider. Data output of overhead camera recordings was visualized using Ethovision Recording Software. Depending on the sample size, recordings were conducted with one (n=1) or two (n=2) fish per trial. A single session consisted of a 5-minute (300-second) recording with behavioural measurements separated into 30-second time bins.

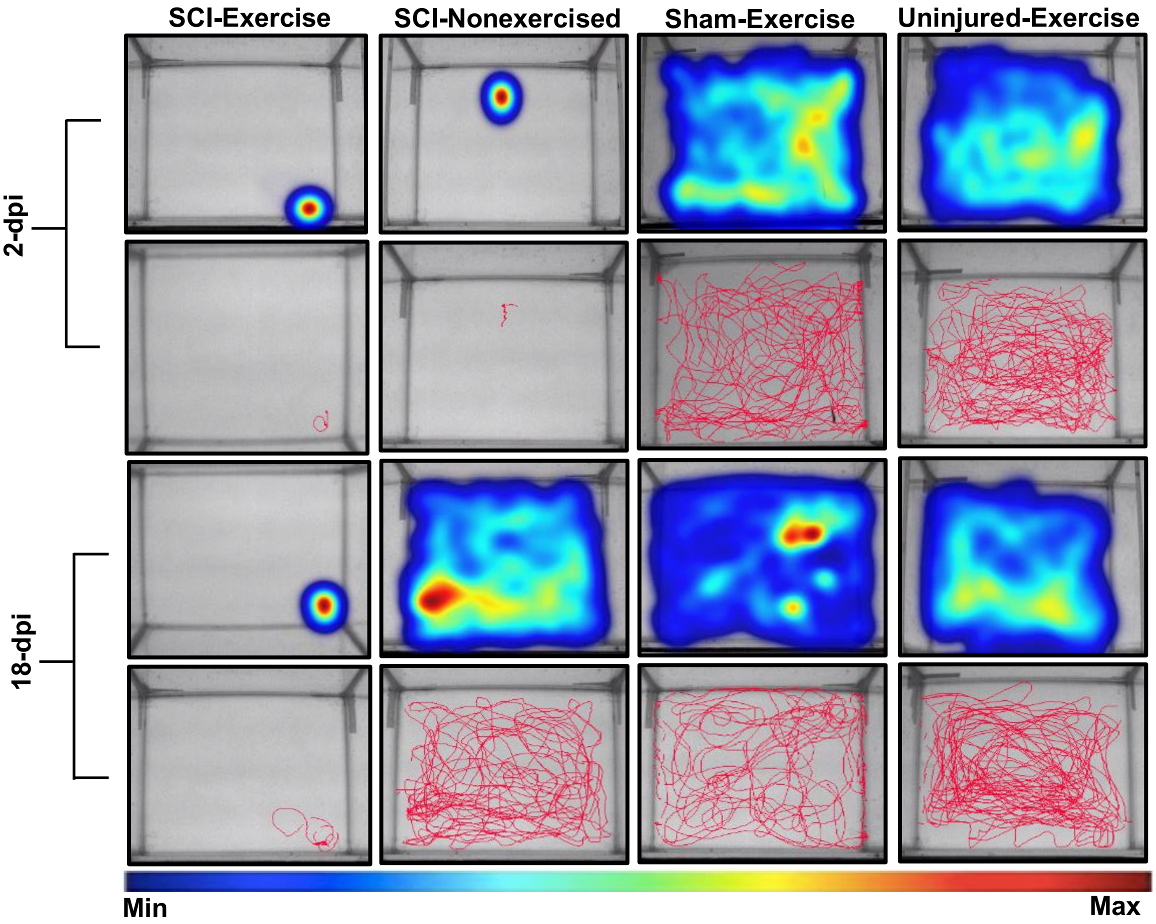

Figure 5. Heat map activity and track visualization. Heat map activity and track visualization on first day of recordings (2-dpi) and last day of recordings (18-dpi). Columns represent different conditions from left to right: SCI-exercise, SCI-nonexercised, Sham-exercised, Uninjured-exercised. The colour scale (bottom) indicates time spent in region of arena from low (blue) to high (red).

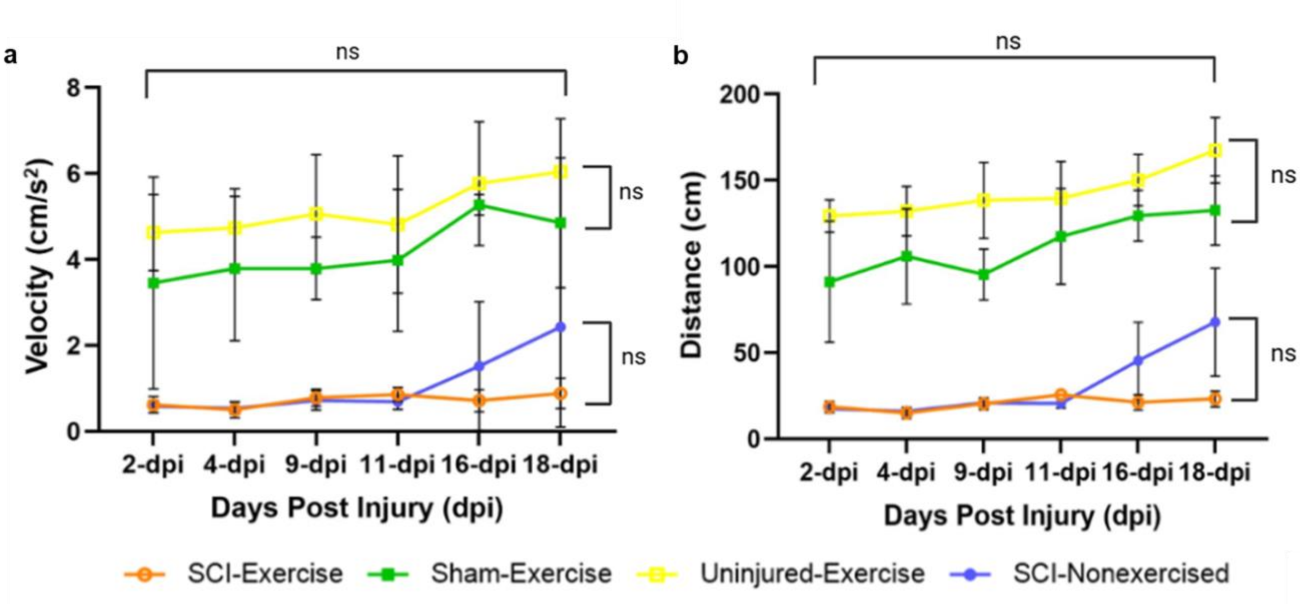

Figure 6. Behavioural measurements for velocity and distance across days post-injury (dpi). (a) Comparison of mean velocity (cm/s^2) over six recording sessions. **(b)** Total distance traveled (cm) analyzed across dpi. Two-way repeated measures ANOVA with Tukey's multiple comparisons test; $*p < 0.05$; SCI-Exercise (n=3), Sham-Exercise (n=3), Uninjured-Exercise (n=4), and SCI-non-exercised (n=4); error bars indicate SEM. No significant differences were found across all comparisons.

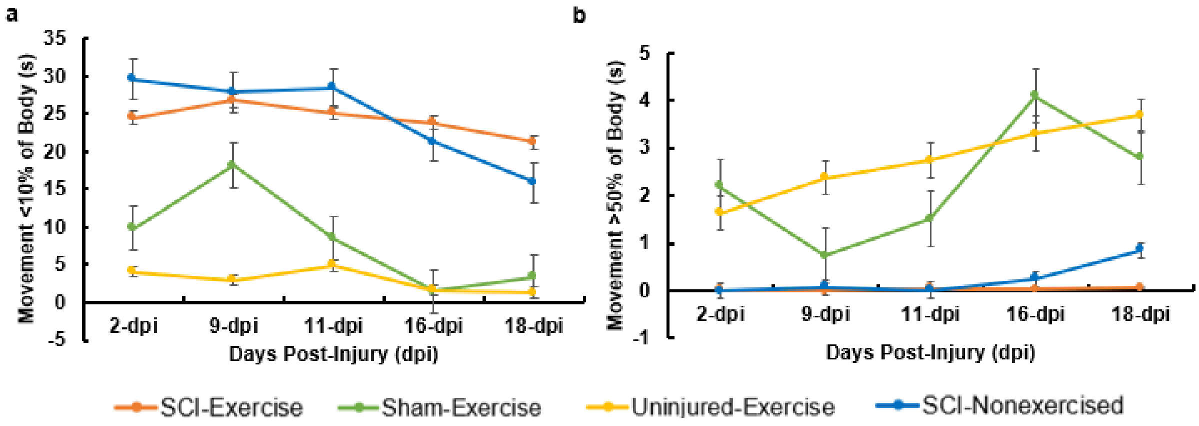

Figure 7. Behavioural observations for time spent in two mobility states. (a) Time spent in low mobility (immobile state), defined as moving <10% of the entire body across experimental groups over time. Greater values represent longer time spent in a state of immobility. **(b)** Time spent in high mobility state, defined as moving >50% of the entire body across six recording sessions for all experimental groups. Greater values indicate more time spent in a mobile (active) state. Error bars indicate standard error of the mean (SEM). No statistical analyses were performed on these data.

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8. Appendix

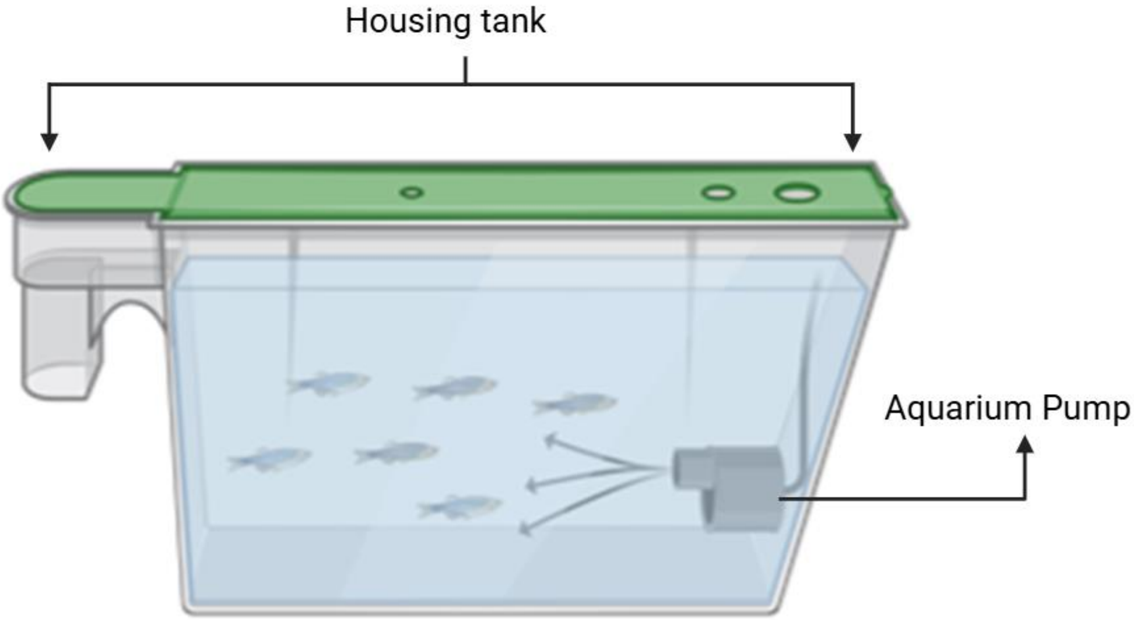
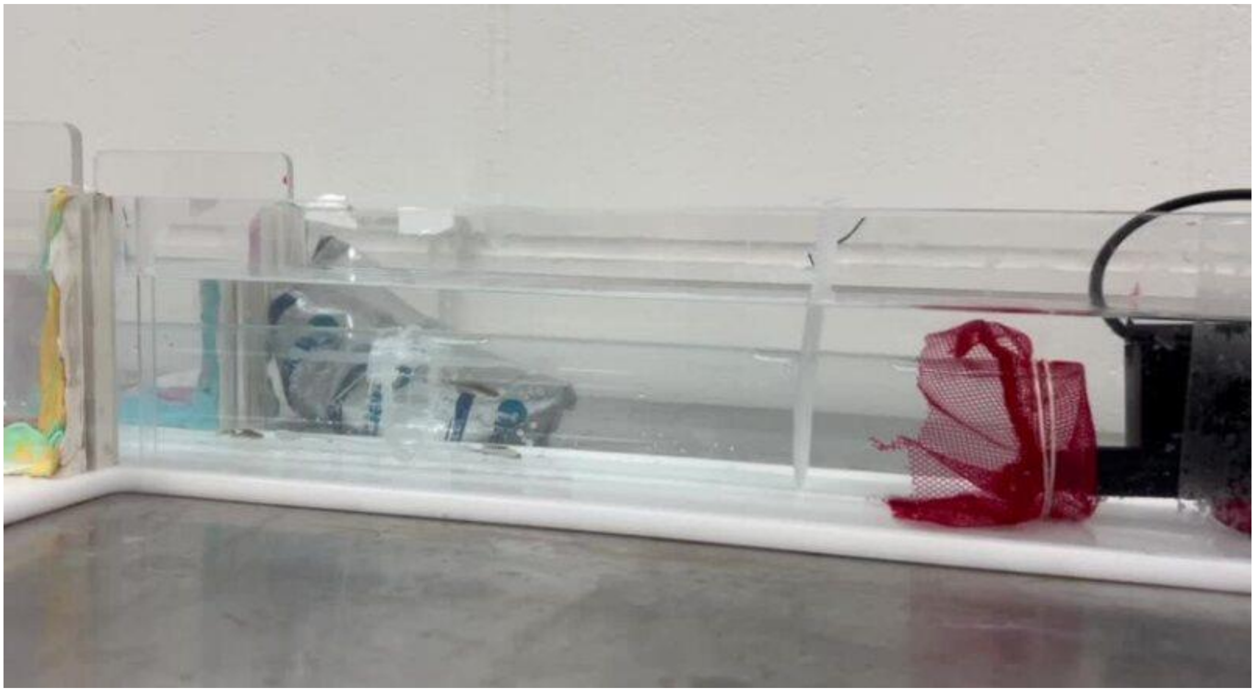
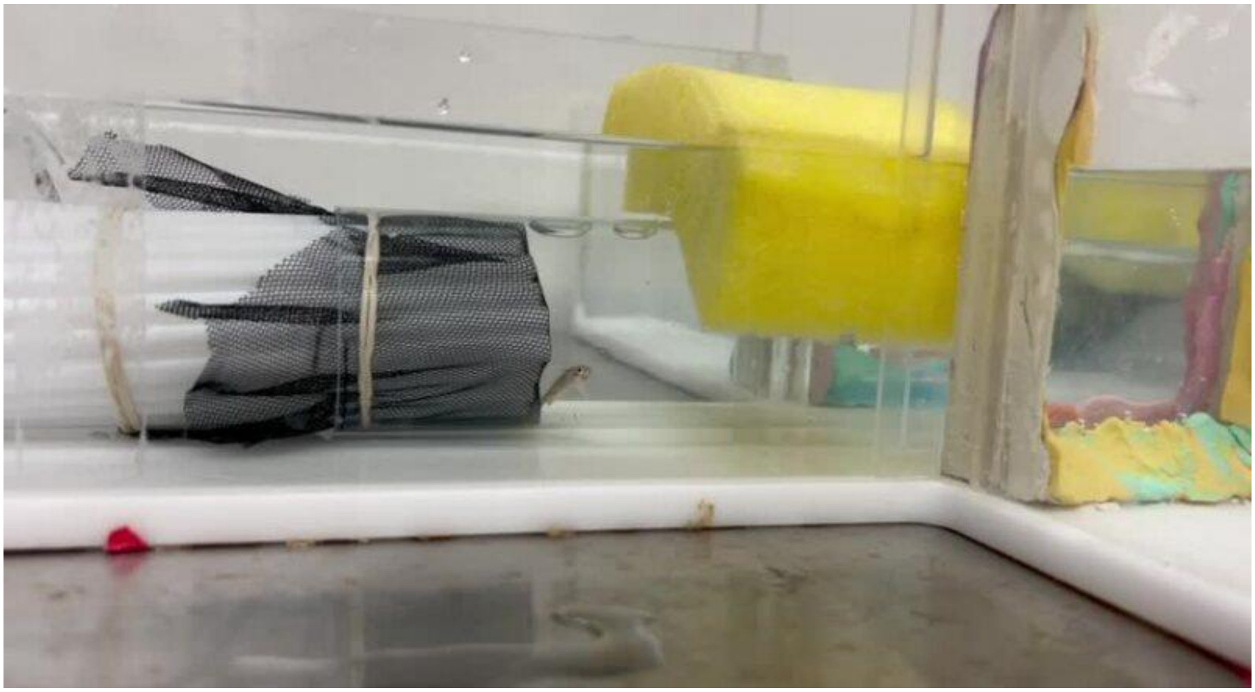


Figure S1. Illustration of exercise chamber design from by the Frankland and Thiele Lab.

A Hydor Pico 70 pump was used to produce a current for zebrafish to swim against.



Video 1. Recording of zebrafish 9-days post injury in exercise chamber. Model 1 of swim column was used in this set-up with flow concentrated in the center of the column.



Video 2. **Recording of zebrafish 16-days post injury in exercise chamber.** Model 2 of swim column was used in this set-up with greater flow distribution.