EXPLORING THE EFFECTS OF SIZE AND AGE ON VULNERABILITY TO PREDATION OF LARVAL ZEBRAFISH (*DANIO RERIO*)

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Abstract

Predation is a leading cause of mortality in the yolk sac larval stage of teleost fish. Size and age are known to impact larval vulnerability to predation, but how they do so have been debated. The bigger-is-better hypothesis that proposed large larvae have fitness advantages over their smaller counterparts has come under scrutiny as most studies that support this hypothesis only consider proxies for probability of capture. A less explored hypothesis suggests that the benefits of size vary with each day, where it could be beneficial to be small or large depending on external conditions. This study seeks to expand on this hypothesis by exploring the effects of size and age on proxies of probability of encounter and probability of capture during the yolk sac stage of zebrafish larvae (*Danio rerio*). Direct yolk sac manipulation attempted to create larvae of distinct size groups at hatch, but it was found unsuccessful as the control and reduction groups were not significantly different at hatch ($p = 0.878$). Instead, larvae were split into large and small groups from the top and bottom 25% from standard length that varied in size from 8 to 22. Probability of encounter was estimated from free swimming trials which measured distance travelled (mm) and mean velocity (mm/s). Probability of encounter was estimated from escape response experiments, where larvae experienced a tactile stimulation. It was found that large larvae moved significantly further distances on day 4 ($p = 0.00835$), and significantly faster on day 3 ($p = 0.00813$). Escape response was measured as distance travelled (mm), mean velocity (mm/s), max acceleration (mm^2/s), and latency to respond. It was found that small larvae travelled significantly further on day 2 ($p = 0.00216$), but large larvae responded quicker to a threat than small larvae on day 2 ($p = 0.00399$). The estimates of probability of encounter and probability of capture in this study do not indicate that age or size effect the vulnerability to predation of yolk sac zebrafish larvae. This suggests small larvae are not at a fitness disadvantage to large cohort members, potentially providing the beginning steps to a counter argument of the bigger-is-better hypothesis.

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Chapter I: Literature review investigating the vulnerability to predation of teleost larvae and the effects of micromanipulation on development

Mortality during the Yolk Sac Stage

Mortality rates due to predation are high in the yolk sac stage of teleost larvae, resulting in most not reaching adulthood (Bailey and Houde 1989). Those that survive this vulnerable stage serve an important role in determining the genetic makeup of future generations. Exploring if mortality occurs at random, where all members of the population are equally likely to die at any time, or if there are factors that contribute to survival, have become a major subject of interest. Factors believed to affect survival include morphological, physical, and behavioral characteristics. It is known that growth rate has major effects on survival in early life history of fishes (Takasuka et al. 2021). It is well understood that environmental factors like temperature, pH and salinity can affect growth and development (Sawant et al. 2001; Kwong et al. 2014; Delomas and Dabrowski 2018). Body size is thought to be a major component of larval survival when facing predation, but to what effect is heavily debated. The leading hypotheses are the 'bigger is better' hypothesis, the stage duration hypothesis, and the (Litvak and Leggett 1992) approach.

Vulnerability to Predation

$$V_g = P_e \times P_a \times P_c$$

Gross vulnerability (V_g) is the probability of a prey being consumed by a predator at any given time; It is the product of three probability components (O'Brien 1979; Fuiman 1989). First, probability of encounter (P_e) is the likelihood the prey meets a predator. Second, probability of attack (P_a) is the likelihood of a predator attacking the prey item. Probability of attack is the predator's decision to attack the prey based on foraging theory which suggests that predators are more likely to pursue prey of larger size to optimize cost benefit ratios (Greene 2015). Lastly,

probability of capture (P_c) is the likelihood the predator can capture the prey. This factor is the success of the attack, and a proxy of this is the probability of prey escape.

Gross vulnerability is further influenced by size. At a larger body size, the probability of capture may decrease for a larva because of an increase in strength and speed. Bailey and Batty (1984) have found a decrease in larval capture success of larger and older flounder (*Platichthys flesus*), plaice (*Pleuronectes platessa*), herring (*Clupea harengus*), and cod (*Gadus morhua*) larvae when preyed on by medusa *Aurelia aurita* (Bailey and Batty 1984). Additionally, Bailey and Houde (1989) graphed the percent of larvae escaping attack versus standard length of six species and found similar effects of size on percent escaping attack (Figure 1.1). However, as body size increases probability of attack may increase since larvae are more visible and travel further distances (Bailey and Houde 1989). Additionally, the peak window when gross vulnerability is greatest changes with size of the larvae and the predation method. Vulnerability to filter feeding and invertebrate predation decrease rapidly once a size threshold is met. This occurs when larvae are no longer of size to be filtered by certain filter feeding species or have increased motility to escape a passive invertebrate predation attempt. Vulnerability to ambush predators act within size ranges as larvae are sensed chemically, visually, or physically by size-dependent predators (Bailey and Houde 1989). Thus, vulnerability to predation changes over time with size and predation style.

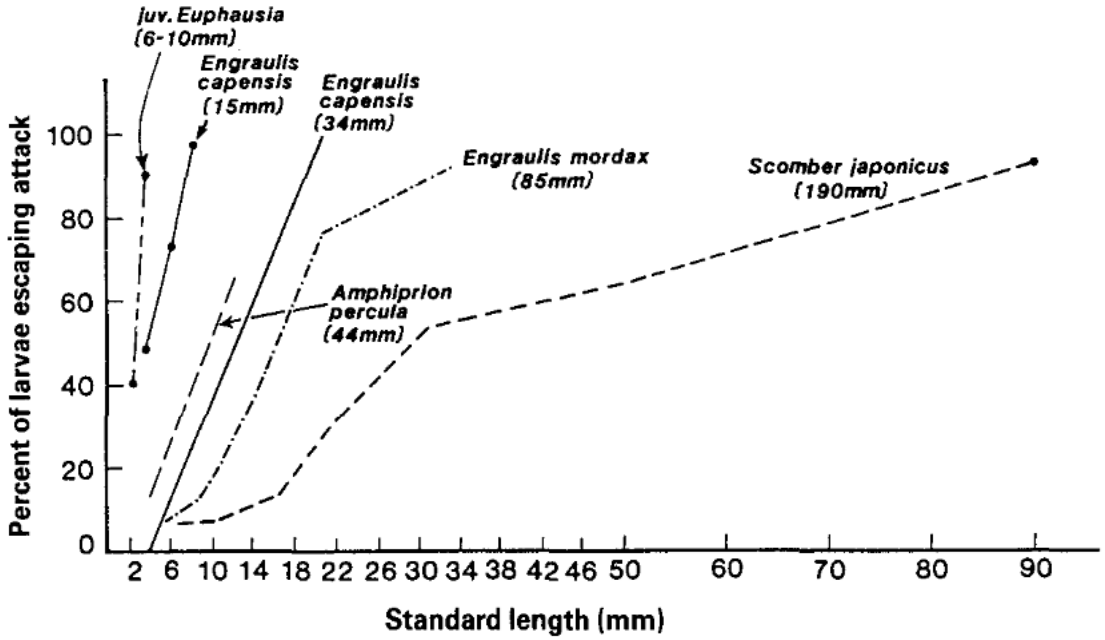

Figure 1.1 The percent of larvae escaping an attack versus standard length (mm) taken from Bailey and Houde (1989). These studies were not choice experiments, and no predators were present to choose larvae to attack. Data for *Engraulis* capensis feeding was taken from Brownell (1985); juvenile *Euphausia* from Theilacker and Lasker (1974); *Engraulis mordax* from Webb (1981); others from Fokvord and Hunter (1986).

'Bigger-is-Better' Hypothesis

The 'bigger-is-better' hypothesis suggests that the largest individuals of a cohort have a higher probability of survival. The prediction is that larvae which hatch larger or grow to be the largest at a given age are less vulnerable to predation (Bailey and Houde 1989). Literature continues to be published in support of this hypothesis. Fontes et al. (2011) found that pelagic growth of the temperate reef fish *Coris julis*, positively correlated with recruitment magnitude and that early growth has a disproportionally large effect on larval survival. Generally, the literature supporting this hypothesis is focused on the relationship to size and the ability of a larvae to detect, react, or avoid a predator (Leggett and Deblois 1994). It emphasizes the probability of escape; however, the outcome of predatory events is dependent on the combined probabilities of encounter, attack, and capture (O'Brien 1979). Studies of the 'bigger-is-better'

hypothesis fail to consider larger larvae are more likely to encounter prey due to increased speed, potentially increasing distance travelled, and increase in detection because of greater size in the predator's reactive field of view (Gerritsen and Strickler 1977). This suggests larger prey are more likely to be targeted then their smaller counter parts (Litvak and Leggett 1992).

Stage Duration Hypothesis

The most widely accepted hypothesis is the stage duration hypothesis. The stage duration hypothesis suggests that larvae experiencing more favorable feeding and environmental conditions will grow quickly, accelerating growth through vulnerable life history stages (Leggett and Deblois 1994; Fortier et al. 1995). This allows the larvae to experience less vulnerability overall during its life. Similarly, to the 'bigger-is-better' hypothesis, the larvae which grow the quickest will be the largest of their cohort which may be beneficial for predatory evasion and reproduction efforts later in life (Miller et al. 1988). They found that selected mortality acts upon the smallest and slowest growing individuals of a cohort. Their study looked at individuals over a long developmental period, but vulnerability is not consistent with time. Survival depends on daily selective pressures which can change rapidly, and vulnerability changes rapidly with it.

Litvak and Leggett challenged the "Bigger is Better" hypothesis

Contrary to the 'bigger-is-better' and stage duration hypotheses, Litvak and Leggett (1992) suggest increased survivorship of small larvae under certain stages or conditions during larval development. They suggested that the predator/prey system is not simple, and that examination of probabilities of encounter and attack need to be assessed to fully understand selective pressures of predation on larval sizes. Further, they found that on occasion, smaller is better, and at others, bigger is better, accounting for the variety of size at hatch of larvae. Small larvae may experience a lower probability of encounter and attack compared to larger counterparts. They are not as motile or visible due to their size, making them less likely to attract attention of a predator. Additionally, foraging theory suggests that a predator is less likely to

attack smaller prey due to the reduced energetic benefits (Greene 2015). To test this hypothesis, Litvak and Leggett (1992) offered capelin larvae, *Mallotus villosus*, of varying sizes to 3-spined stickleback, *Casterosteus aculeatus*, and jellyfish, *Staurophora mertensi*. They found that both predators preferentially selected larger larvae, suggesting that smaller larvae may experience an overall reduced risk of predation at that stage. Further studies investigating egg size and development rate in Arctic char, *Salvelinus alpinus,* found that embryos from smaller eggs develop faster (Valdimarsson et al. 2002). The size at hatch from these smaller eggs will increase probability of escape due to advanced development and a decrease in probability of attack due to the small size. Litvak and Leggett (1992) suggested that we need to examine all three components of gross vulnerability of a larva to predation to assess the impact of size and age. They concluded that it may be as simple as being the right size and the right time would be most beneficial, avoiding the predators when young, small, and vulnerable, and being larger when escape response has improved. Although these results are promising, few studies have yet to build on this hypothesis.

Predator Evasion

There are numerous mechanisms for predator avoidance including various behavioral and morphological adaptations. One behavioral adaptation is the C-type fast-start escape response seen in fish in response to an unexpected stimulus (Weihs 1972). The C-type escape is a short-latency response in which the fish rapidly accelerates from the position at the time of attack (Eaton and Didomenico 1986). The behaviour gets its name due to the characteristic C-shape the body makes during the initial phase of the escape response. This behaviour is one of the earliest seen in larval development of *Danio rerio,* occurring 44hr after fertilization. The early development of the C-type escape response suggests the importance of predator interactions in early life history.

Sensing predator

Larval fish escape response is triggered by both visual and physical cues (Blaxter and Fuiman 1990). Blaxter and Fuiman (1990) found that herring larvae (*Clupea harengus*) respond best with mechanosensory and visual cues. There are often limitations with visual sensing, as vision can be hindered by darkness or turbidity of the surrounding environment which could delay the escape response (Cerri 1983). Sensory cells, neuromasts, associated with the acoustic-lateralis system are used to sense predators in the surrounding environment (Harvey et al. 1992; Stewart et al. 2013). Upon sensing movement of a predator's approach or a suction-feeding strike, the fast start behaviour is instigated. A study by Stewart et al. (2013) compared larval zebrafish response to adult zebrafish predation with functional lateral lines and severed lateral lines. They found that those with functioning lateral line systems were 14 times more likely to escape. This finding indicates that lateral line's ability to sense water flow is a crucial defence.

The C-type escape response is initiated by Mauthner cells (M-cells) which develop as early as 40 hr post fertilization in the hindbrain (Eaton et al. 1977). M-cells respond to tactile-vibrational stimulation and instigate a directional response. The escape response occurs as a series of strong tail flexures. The muscular contractions occur in a programmed sequence, creating an explosive acceleration. Stage-1 of the C-start, the head and tail bend the same way, preparing the animal for the second kinematic stage. In Stage-2 the fish propels itself in any direction away from the source stimulus, contributing randomness to the escape. Lastly, in stage-3 the fish continues to swim periodically laterally, more like steady swimming. The C-start is an important evolutionary behaviour adaptation that optimizes motion to maximize the escape response (Gazzola et al. 2012).

As mentioned, larvae display startle or escape responses from hatch, however there are other factors that affect the refinement and efficiency of the response. As the larvae age, their escape response becomes more refined (Colwill and Creton 2011). The C-start can undergo long-term habituation, indicating possibility of learning in larvae particularly as it relates to predator escape responses (Roberts et al. 2016). Furthermore, size can contribute to speed and strength of

larvae, improving the acceleration and distance travelled of the larvae as found in salmonids (Hale 1999).

Introduction to Zebrafish

Zebrafish (*Danio rerio*) are a small teleost species of the cyprinid family. Zebrafish have a fusiform and laterally compressed body with a protruding lower jaw. Their reflective bodies have a unique diagnostic feature of five to seven dark blue lateral lines that extend from the operculum to caudal fin (Barman 1991). They have unpaired anal fins, dorsal fins, tail fins, and paired pelvic fins (van Eeden et al. 1996). As adults, *Danio rerio* reach a maximum standard length from snout to caudal fin of 40 mm (Spence et al. 2008). Males and females are similar in morphology; however, the males tend to have more yellow coloration and larger anal fins (Spence et al. 2008). Additionally, males have a longer caudal peduncle compared to females (Duff et al. 2019).

This freshwater tropical fish is native to South Asia where it inhabits vegetated waters of slow-moving streams, ponds, ditches, and rice-fields (Engeszer et al. 2007). In the wild they experience a wide range of temperatures from as low as 24.6°C to over 38.6°C (Spence et al. 2008). *Danio rerio* are omnivorous in their natural habitat, consuming zooplankton, insects, phytoplankton, algae, plant material, spores, and invertebrate eggs as determined through gut content analysis (Spence et al. 2008). They are a social fish in the wild, aggregating in shoals and schools of varying populations. In fast-flowing they collect in groups up to 2000 fish whereas in slow-flowing they are an average of 11 fish per group (Shelton et al. 2020).

Zebrafish have become heavily domesticated and are an important model organism used to study vertebrate genetics, development, behaviour, toxicology and more (Meyers 2018). There are several features that have increased their use in research such as their ease of care, rapid development, high fecundity, small size, and ease of manipulation. They can spawn at intervals as little as 1.9 days year-round (Eaton and Farley 1974). In the context of my study, their embryo development and breeding are key factors as to why they are the optimal model organism. They

can breed when required and have a transparent chorion, allowing for complete visualization of the yolk sac for manipulations. Furthermore, they develop quickly from fertilization to larvae, allowing for efficient trials

Breeding

A significant advantage of using zebrafish is the wealth of knowledge on breeding behaviour and domestic breeding processes (Niimi and LaHam 1974; Nasiadka and Clark 2012; Tsang et al. 2017; Castranova and Wang 2020). Zebrafish are oviparous with external fertilization. They provide no parental care to offspring once hatched. They are highly fecund, and females may release multiple clutches of hundreds of eggs in a single spawning event (Nasiadka and Clark 2012). The presence of males stimulates oviposition of eggs. Females scatter the non-adhesive eggs for external fertilization (Nasiadka and Clark 2012). Generally, matting events occur at dawn when females appear to be less aggressive. Olfactory cues produced by male and female zebrafish play a role in synchronizing the reproduction event (van der Hurk and Lambert 1983; van der Hurk and Resink 1992). Olfaction assists in inducing breeding conditions; however, it is not an attractant of a mate. There is evidence of visual stimuli and behavioral cues leading to mate selection. Females demonstrate a preference for larger males when mating by releasing a larger quantity of eggs per mating event (Skinner and Watt 2007). Males that are more visually appealing, with brighter coloration, stripes, and symmetrical caudal fins, have also been suggested to have effects on mate choice.

Zebrafish reach sexual maturity after three months; however, egg quality and fecundity increase with age (Nasiadka and Clark 2012). Egg quality in females begins to deteriorate around 1.5 years, indicating that the ideal age for mating is between 6 months to a year old. Zebrafish can continually mate throughout the year at a frequency up to two or three times a week (Eaton and Farley 1974). Over-spawning, however, can occur if fish are bred too frequently resulting in a decrease in egg quality and quantity. To avoid this, breeding every 10 days is optimal (Niimi and LaHam 1974).

When setting up a mating event in the laboratory, environmental conditions, water quality, and ratio of males and females should be considered. Breeding is linked with photoperiod (14L:10D) as spawning events are triggered by the onset of light. It is crucial that fish are placed in the breeding tank before dusk to ensure that breeding occurs at desired time in the morning (Nasiadka and Clark 2012). To breed, zebrafish prefer a gravel like substrate. To replicate this in lab, many choose to use marbles. It both replicates gravel and prevents the fish from consuming the embryos. Furthermore, imitation plastic plants or green mesh structures are also used to simulate artificial spawning sites. The health of the fish is imperative and maintain appropriate temperature, oxygen concentrations, nitrogenous waste concentrations, and minerals in the water are vital for producing viable offspring. Zebrafish are group spawners however they may also reproduce in pairs (Spence et al. 2008). Females mean capita egg production decreases at higher densities, likely because of aggression from territorial males disrupting spawning attempts (Spence and Smith 2005).

Embryo development

Zebrafish embryo structure and development are factors that have led to the popularity of *Danio rerio* as model organisms. The transparent chorion allows for scientists to view embryonic development from zygote to larvae with a compound microscope. The fertilized zygote develops quickly, hatching in 72 hours into an early larva. Rate of development can be affected by temperature. The ideal incubation temperature is 28°C in a concentration of 5-10 embryos/ml (Kimmel et al. 1995). The newly fertilized zygote will begin dividing cells by two in the cleavage period as early as 0.75 hours post fertilization (hpf). The embryo will reach the blastula period approximately 2 ¼ hpf at the 128-cell stage. During the blastula stage, the yolk sac bulges towards the animal poll as epiboly begins. The gastrula period begins 5 ¼ hpf with the germ ring becoming visible from the animal pole, and continuing development to the bud of the tail and rudiment notochord formation. The mesoderm gives way to somite formation in the segmentation period 10 ¼ hpf. By 24 hpf, the development has reached the pharyngula period in which most vertebrate shared systems develop such as the venous system, digestive system, nervous system, musculature, retina pigmentation and movement occur. Lastly, 48 hpf the

hatching period begins as the larvae undergo the final stages of development such as fin formation, mouth protrusion, gill slits, and body coloration. Roughly 72 hpf the larvae hatch at an average length of 3.5 mm. The time of development and the size at hatch do vary within a clutch, however there is more variability seen in development between clutches than within a clutch.

Larval Development

Zebrafish hatch 3 days post fertilization (DPF) and continue their rapid development external to the chorion. Their swim bladder inflates, and the mouth continues to protrude as they begin to swim and perform predatory evasion responses (Wilson 2012). After the embryonic period, zebrafish development does not have determined stages. They remain developing as larvae for approximately six weeks, during this phase fish convert from feeding on yolk sac nutrients to external food sources, triple their size, and undergo a series of morphological changes in fins, pigments, and body morphology (Singleman and Holtzman 2014). During zebrafish development, the rate of development can be affected by temperature, water quality, food availability, genetics, and population density. Fish of the same cohort can develop at different rates, even if environmental factors remain consistent due to within cohort differences in size and yolk sac volume (Nasiadka and Clark 2012).

Generally, at 4 to 5 DPF the swim blather inflates, allowing the larval fish to swim in the water column (Wilson 2012). At 5 to 6 DPF, the digestive tract opens, and digestive enzymes are secreted, allowing the larvae to begin exogenous feeding (Holmberg et al. 2004). The yolk sac is not yet depleted of nutrients upon opening, and the larva will continue endogenous feeding until 7 DPF. The yolk will be completely absorbed, and survival of the larva relies on exogenous feeding at this point forward. Larvae that do not successfully feed exogenously will succumb to starvation roughly 10 DPF which can result in lower survival rates and death. This is a critical point in larval development since the failure of this transition results in mortality. Furthermore, neuromasts develop 4 DPF along with lateral lines (Raible and Kruse 2000). Lateral lines are sense organs on the surface of aquatic vertebrates that can detect movement and pressure in the

surrounding environment from mechanosensory hair cells (Suli et al. 2012). The development of this sensory organ is vital to predatory escape responses.

Micromanipulation

Micromanipulation is a growing experimental technique which allows researchers to manipulate living embryos. It is commonly used to inject DNA, RNA, proteins, and toxins into the yolk sac of developing zebrafish (Xu 1999; Zhao et al. 2019). In the context of this study, micromanipulation is used to reduce the yolk sac volume in the developing embryo based on a technique described by Jardine and Litvak (2003). Yolk sac volume affects body size of the larvae and accounts for most inter-brood variation in early life history (Ojanguren et al. 1996). Size is an important component of larval fitness as it increases swimming stamina, speed, and foraging abilities (Jardine and Litvak 2003). Applying this novel technique to study fitness and survival is unique. Researchers have previously investigated offspring fitness at size by breeding different sized females. This introduces a parental genetic factor that could contribute to the results rather than the measured parameter. Controlling for size within a cohort using microinjection eliminates the confound of parental genetics by comparing siblings (Jardine and Litvak, 2003).

The experimental procedure uses a nano-liter injection system with a pulled micropipette tip to perform small scale manipulations. The apparatus uses a small plunger within the micropipette tip filled with mineral oil to create pressure, allowing the rate of injection and reduction to be controlled. This automated system controls speed and volume of the manipulation which are important for survival and precision.

The optimal developmental stage to perform microinjections is debated in the literature. Jardine and Litvak (2003) suggest that 4-16 cell stage is optimal as the manipulation is less likely to interfere with the development of the blastoderm. This is within the first hour post fertilization as the embryo rapidly develops. A later study by Roustaian & Litvak (2007) suggests that micromanipulation at later stages is less likely to interfere with signaling for mesoderm

formation and microtubule array during cleavage stage. They compared survivorship of zebrafish injected throughout embryo development and found evidence that teleost yolk is vital for early development and is particularly vulnerable prior to the cleavage stage.

My Project

For my project, I investigated the relationship between age and size on predatory evasion behaviours of teleost larvae. I used zebrafish, *Danio rerio*, as my model organism. By using novel micromanipulation techniques, I created two size groups. I used silhouette collimation to track larvae using a high-speed Fastec camera. I collected latency of response, maximum acceleration, mean velocity, and total displacement using NOLDUS ethovision technology to estimate probability of encounter and probability of escape. Due to clutch size limitations a group spawning approach was used in this study.

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Chapter II Exploring the effects of size and age on vulnerability to predation of larval zebrafish (Danio rerio)

Introduction

Predation is one of the leading causes of mortality during the yolk sac stage of teleost larvae (Purcell and Grover 1990). Ichthyoplankton are a crucial diet component of a wide array of predators like cnidarians, zooplankton, and larger fish at various life history stages (Bailey and Houde 1989). Predation pressure serves an important evolutionary role in genetic determination for future generations, as Hjort's (1914) seminal work suggests recruitment depends on survival during early life history stage. The factors effecting this relationship are widely debated and still unclear. Investigating predation pressures has become an important topic of interest, exploring if mortality occurs at random, where all individuals are equally likely to survive, or if other factors influence their survival. It is believed that physical and behavioural characteristics can influence the success of ichthyoplankton when attacked (Johnson et al. 2014). Physically, growth rate and body size are known to have significant effects on survivorship in early life history of fishes (Takasuka et al. 2021). Body size is influenced by genetics, age and environmental factors like temperature, pH, and salinity which, in turn, effect survival rate (Sawant et al. 2001; Kwong et al. 2014; Delomas and Dabrowski 2018). Body size also contributes to swimming speed and strength of larvae which can influence their escape responses. Age affects larval size and is predicted to have effects on larval predatory evasion success as escape behaviours develop over time (Colwill and Creton 2011). Larvae have the capability to learn and improve predator escape responses, increasing likelihood of surviving an attack with age (Roberts et al. 2016). Together, size and age are driving forces in larval survival but to what extent is still unknown.

When investigating vulnerability to predation, it is important to consider the model of gross vulnerability to predation (O'Brien 1979; Fuiman 1989). Gross vulnerability to predation is made up of three factors predicting the chance a prey item is consumed. First, probability of encounter (P_e) is the likelihood of a prey being detected by a predator. Second, probability of attack (P_a) is the probability of a predator attacking its prey based on foraging theory. Foraging theory suggests that the most energetically rewarding prey item is the best choice for a predator to attack (Townsend and Winfield 1985). Lastly, probability of capture (P_c) is the likelihood the

predator will be successful in its attempt to eat the prey after encountered and attacked. Together these components are predictive of prey survival and must all be considered to understand the influence size and age. Furthermore, body size of prey covaries with vulnerability. As size increases, probability of capture decreases due to faster acceleration and increased motility (Bailey and Batty 1984). However, probability of encounter and attack may simultaneously increase. Probability of encounter increases because large larva travel further distances and, thus, have an increased chance of predator interaction and detection (Bailey and Houde 1989). Moreover, when body size increases so does vulnerability to attack because, as foraging theory suggests, larger prey are more nutritionally beneficial (Townsend and Winfield 1985). In contrast, as size decreases, probability of attack and encounter also decrease, although the probability of capture increases due to reduced strength. When studying vulnerability to predation, consideration must be given to not only probability of capture, but also the probabilities of encounter and attack.

The Effect of Size on Survival Leading Hypotheses

The effect of body size on larval predation is heavily debated in the literature. The three leading hypotheses are the 'bigger-is-better' hypothesis, the stage duration hypothesis, and the 'smaller-is-better' hypothesis (Bailey and Houde 1989; Litvak and Leggett 1992; Leggett and Deblois 1994; Fortier et al. 1995). The 'bigger-is-better' hypothesis suggests that the largest larvae of a cohort at any given age are less vulnerable to predation because they have increased motility, speed, and strength due to their size (Bailey and Houde 1989). Literature in support of this hypothesis continues to be published; however, it mainly focuses on probability of capture alone which is not a good predictor of survival (Bergenius et al. 2002; Takasuka et al. 2004; Islam et al. 2010; Fontes et al. 2011). The stage duration hypothesis, the most widely accepted today, suggests that larvae in more favourable environmental conditions will outgrow vulnerable life history stages faster (Leggett and Deblois 1994; Fortier et al. 1995). It shares some similarities to the bigger-is-better hypothesis by proposing that the largest larvae of a cohort at a given time will be less vulnerable, and overall reduced vulnerability if the individual grows fastest. Lastly, Litvak and Leggett (1992) proposed that variation in success by size changes

daily, and certain sizes at certain life stages could yield survival benefits. Daily variability could attribute to increased survival for small larvae in a cohort, due to predation pressure on bigger siblings allowing for a larger window of opportunity to learn predator avoidance. Small larvae could experience reduced probability of attack and encounter as they are less motile, thus might not encounter or attract attention of as many predators, and are less nutritive than their larger siblings (Bailey and Houde 1989; Greene 2015). Focusing on size, developmental stage and changes in daily vulnerability, similarly to Litvak & Leggett (1992), takes into account aspects of all three hypothesis to determine gross vulnerability to predation (O'Brien 1979; Fuiman 1989).

Zebrafish Predatory Evasion Behaviour

Larval predatory evasion behaviours influence survivorship. Teleost larvae have evolved numerous behavioural and morphological mechanisms for predator avoidance. One common behaviour found throughout life history of the model organism zebrafish (*Danio rerio*) is the C-type fast escape response which gets its name from the characteristic C-shape made by the body during the initial phase of the response (Weihs and Lighthill 1972). The C-type escape response is a short-latency rapid acceleration from the position at the time of the stimulus (Eaton and Didomenico 1986). The C-type escape is one of the earliest seen in larval development of zebrafish, occurring 44 hours post fertilization (hpf). The early development of this response is indicative of its importance and the pressure of predation in early life history. The C-escape is stimulated by visual and physical cues. Visual appearance of predators can trigger the response; however, vision can be limited by darkness or turbidity of water (Cerri 1983). Physical cues like water movement are rapidly sensed by lateral lines that extend along the length of the body. The C-type escape response commences predator evasion responses. Predator evasion behaviours can undergo refinement with age, allowing for improvement with learning over time. Furthermore, size influences the distance, speed, and strength of the larvae and has been seen to improve acceleration of salmonoid larvae (Hale 1999).Tracking the C-escape and the subsequent evasion response to simulated tactile predation will allow a comparison swimming measures across age and size.

The focus of my research is to investigate the effects of age and size on predatory evasion behaviours of teleost larvae. I used zebrafish as a model organism in this study for their rapid development, transparent chorion, high fecundity, and small size (Meyers 2018). By using the novel micromanipulation technique, two size groups were created to estimate both normal swimming and response to tactile predation using silhouette collimation. Previous studies focused on probability of capture; thus this study will also consider probability of encounter. Swimming behaviour measures are acting as a proxy for probability of encounter, and predator evasion response measures as a proxy for probability of capture. I hypothesis that 1) that larger larvae will swim further than their smaller counterpart's overtime and that 2) larger larvae will respond faster, accelerate faster, and move further during their predation responses overtime.

Materials and Methods

Fish Husbandry

The brood stock of Zebrafish used for breeding were from a colony in the Litvak Lab at Mount Allison University with exception to breading group 3. The greater colony has been accumulated over the previous 2 years. Breading group 3 was acquired from PetSmart, Moncton NB and was bred three weeks after its arrival. The colony was held in an Aquabiotech zebrafish recirculation system (Pentair 18 tanks: 12 x 3.0L Tank part #: PCT3-D; 6 x 10.0L tank part #:10-7-A). The systems maintained a temperature of 27°C, a pH between 7 and 8, salinity of 1 ppt, and an ammonia level of 0. Water quality was checked daily during experiments to ensure optimal environmental conditions. The lights were on a 14 light:10 dark cycle. Artificial sunrise and sunset were produced by hanging lights (NOMA LED rope Christmas lights, pure white, product #151-0756-2) and began thirty minutes prior to the room lights in the morning, and 30 minutes after at night. Fish were fed three times a day with dried flake food (Nutrafin Max, A6700C) in the morning and evening, and freeze-dried blood worms (Hikari Bio-Pure, 33201) or *Artemia (*Brineshrimp Direct, 08929454GR), at noon.

Breeding

A total of six trials were performed using four different families of zebrafish. The families were comprised of two females and two males. The evening prior to a trial, the four fish were placed in a breeding tank as the final dusk lights turned off. The breeding tank was a standard 3.0L tank wich had two layers of glass marbles on the bottom and a plastic aquarium plant to simulate a reproductive environment. At the onset of simulated dawn, fish were allowed to breed for 30 minutes. The fish were then removed and returned to their original tank. The breeding tank was moved to the sink were the marbles and plastic plant were carefully removed. The water in the tank was gently mixed to suspend the embryos and was pored over a stainless-steel mesh sieve (Adaptive Science Tools). Embryos were rinsed with 0.0005% aqueous bleach solution that was warmed to 28°C. The sieve was flipped over a large petri dish and rinsed with warmed embryo medium to collect the embryos (Westerfield, 2007; 10% Hanks Balanced Salt Solution with calcium and magnesium 1x, Hyclone Labortaotries Inc, SH3003002; 0.15 g/L penicillin-G potassium Fisher BioReagents; and 0.004% methylene blue). Debris was removed from the petri dish with a sterile glass Pasteur pipette.

Micromanipulation

Larvae of varying sizes from the same cohort were created for this study using micromanipulation techniques. Micromanipulation is an experimental technique that allows living embryos to be altered. In this experiment, the yolk sac volume was reduced precisely, decreasing body size of the larvae in a controlled manner (Jardine and Litvak 2003). This reduction results in less nutrients during early development, causing the size difference needed for experimentation. Looking at size differences in this way avoids possible interference from genetic differences which could skew results when trying to determine relationships between age and size when facing predation. In this study I used group breeding instead of pairs to ensure a large clutch size, thus the benefit of reducing genetic variability by comparing within siblings was lost.

Custom micropipette tips were made from glass capillaries (WPI, Glass capillaries for Nanoliter 2010 fire polished 2, Item no. 504949) and pulled using a Sutter P-1000 Flaming/Brown micropipette puller (Heat: 803, Pull: 100, Vel: 30, Time: 250, Press: 500). Before use, micropipette tips were pierced through a tissue wipe to create a jagged tip. This improves the tip's ability to pierce the chorion and yolk sac. The tips were then backfilled with mineral oil to prevent air from interfering with the backpressure in the micro-injector. The tip was secured to a Nanoject 2000 injector (WPI instruments) connected to a Micro4 micro syringe pump controller (WPI instruments).

The petri dish containing the embryos was held on a heated stage (Tokai Hit ThermoPlate TP-V12) at a temperature of 28°C. A second standard sized sanitary petri dish with a glass microscope slide was placed on the microscope stage. The second petri dish was used as the micromanipulation arena. Embryos were pipetted using a sterile glass Pasteur pipette into the petri dish along the long side of the glass slide. Embryo medium was removed until the embryos were half exposed to the air and held along the slide with hydrostatic tension.

From the clutch, three treatment groups were created: control with no micromanipulation, sham with a pierced chorion, and the reduction group with a 24 nl reduction (Jardine and Litvak 2003). The embryos were viewed at 10x magnification under the microscope (Zeiss SteREO Discovery V.20, with Zeiss Axiocam 506 Colour camera). Embryos were separated into groups of six and imaged directly before and after microinjections. Embryos were rotated using fine-tip forceps, so the vegetal pole was facing out, away from the slide. The micropipette tip was positioned on the microinjector using a joystick manipulator (Narishige, Joystick Micromanipulator model no. MN-151) perpendicular to the chorion. Then the tip was slowly inserted through the chorion into the yolk sac to withdraw 24 nl of yolk, or to puncture both membranes depending on desired treatment. All embryos were manipulated within their first two hours of fertilization to remain in the cleavage developmental period (Litvak and Leggett 1992; Roustaian and Litvak 2007).

After injection procedures, each embryo was placed individually into a sterile test tube using a glass pipette. Each test tube was filled halfway with embryo medium **(**30 ml**).** The tubes

were held in racks and placed in a hot water bath at 28°C. Racks were positioned at an angle to increase surface area to volume ratio to maximize oxygen saturation. The photoperiod was the same as the brood stock, 14:10 light to dark with a 30-minute dawn and dusk period generated by string lights.

Imaging

Every day after hatch, yolk sac larvae were imaged using the Zeiss stereo discovery V.20 system at 25x magnification. This was performed for the first four days post hatch during experimental trials until fish began to starve due to complete yolk-utilization on day 5. Larvae were removed from their individual test tubes using the wide end of a glass pipette, and placed into a small petri dish of 2% methylcellulose gel preheated to 28°C. The gel inhibited excess movement while allowing larvae to respire. Larvae were positioned dorsally with fine tip forceps for imaging. During imaging, the embryo medium was changed for each test tube before the larvae was returned. The larvae were returned to the test tubes via the glass pipette.

Image analysis

Images of embryos before and after injection, and of larvae were measured using Image-J. Embryos vertical and horizontal axis were measured to calculate the yolk volume before and after injection and of the sham and control groups. The volume was calculated using $V= 4/3 \times \pi \times (\text{small axis radius})^2 \times (\text{large axis radius})$. Lateral body area, yolk sac area, eye area, fin area, and standard body length were measured. Standard body length was measured from the tip of the protruding snout to the end of the notochord. The total lateral area was the total body area excluding the fins.

Silhouette collimation predation simulations

Predation simulation trials occurred from day one to day four post hatch. The technique of silhouette collimation was used to track larvae during escape and swimming trials. A highspeed camera (FASTEC TS5) that was sensitive to infrared light was suspended on a metal frame above a glass pane that supported the arena. Below the glass pane, an infrared LED light panel (Amazon) with 60 lights at 920nm were positioned in line with the camera and a convex lens that dispersed the light evenly (similar to Litvak and Leggett 1992). The experimental arena that housed the larvae was made from a petri dish with the walls blacked out with electrical tape to prevent visual reactivity of the larvae when moving around the room. The water in the arena maintained a temperature of 28°C and was changed frequently during experimentation. The camera was connected to a laptop with an ethernet chord which mirrored the camera and controlled capturing the video. The swim trial videos commenced 10 seconds after larvae entered the arena. The larvae then rested for 30 seconds and were subjected to the tactile predation stimulation. Larvae were tapped lightly on their lateral lines with a hair super glued to jewelry wire and the reaction was recorded for 7s.

The suctorial predation had to be dropped from the experiment that used Families 1 and 2. This was due to the strength of the suction being too great and resulted in the larvae becoming swept up in the current rather than eliciting the C-type escape response that was used as an identifier that the predator evasion behaviour began. Furthermore, clarity issues with collimation image from ripples was challenging and disrupted tracking. The set up was similar with exception to the arena. The arena was made of 4.5 PVC pipe in grey adhered with aquarium silicone to a large petri dish. The PVC was beveled at the top to allow the meniscus of the water to flatten evenly in the arena. A glass Pasteur pipette with the tip sanded off was adhered through the side of the PVC with aquarium silicone and plastic tubing extended from it. The tubing was connected to a controlled pipette that produced a controlled suction. During experimentation, larvae were placed in a plastic tube in the arena in front of the Pasteur pipette to adapt for 30 seconds to 2 minutes. As the plastic tube was removed, a petri dish was placed on the surface of the water as a wave depressor. The larvae would remain were the plastic tube had been and the controlled pipetted would suck 1 ml of water. It appeared during experimentation that the larvae

were reacting properly, but upon review the current was too strong and larvae were not eliciting the C-type escape response needed. As a result, these trials were dropped from the final analysis.

Video analysis

Video analysis was successful for three of the four trials tactile predation trials. The footage of the first trial was dark, blurry, and the larvae were often touched aggressively in the head rather than along lateral lines in predation experiments. This resulted in successful analysis of three families from three trials.

Videos were analyzed using Noldus EthoVision XT software (v.16). Beginning with the swim trials, all videos were uploaded to the software in the same file. The arena size was calibrated in this software using the arena selection tool. Each arena was calibrated as 88mm in diameter and the outline was traced to generate arena area. Areas of light interference were removed from the arena by tracing around them. Once the arena area was set, automated tracking settings were used to differentiate the colour of the shadow made by the larvae and the light background. The settings were adjusted so that data collection began when the video began to play. Each video was played in the Noldus track viewer to record swimming metrics of the fish movement. Some videos had light obstructions in different places due to spills under the arena, or lint on the surface of the water. In these cases, additional arenas were created to remove the obstacles and allow for clean tracking. After the video footage was tracked for each larva, the analysis calculation tool calculated the distance traveled (mm) and the mean velocity (mm/s).

Predation trials were analyzed using Noldus EthoVision XT software (v.16), like the swim trials, with exception to removing the tactile stimuli. Since the wire occurred in different locations for each trial, every video required its own unique arena. The arena creation and calibration process resembled the swim trials, however, the area in which the wire entered the arena was outlined and removed. This allowed the tracking software to not confuse the dark shadow of the wire with the body of the larva. Occasionally, portions of the video could not have the wire removed. This was rare and dealt with individually, as some of the issues were unique to

each video. The most common issue was that the wire entered an area of the video after the larvae left, or ripples were made when it was removed. These small interruptions were often only a few milliseconds long and were edited out of the track. Occasionally, larvae swam underneath or too close to the wire to be differentiated by the software. This occurred in two videos, and they had to be dropped from the experiment. The calculation tool generated the total distance travelled (mm), mean velocity (mm/s), mean acceleration (mm^2/s), and max acceleration (mm^2/s). Each video was reviewed to estimate the latency to respond (s). Latency was calculated as the difference from when the larva was touched to when it first moved, and each track was divided into two milliseconds increments. Since the video is 2-dimensional and the larva exist in 3 dimensions, it was sometimes challenging to discern when it was touched. Generally, it was from when the body was moved by the wire, or when there was no light between the wire and the body.

Statistical analysis

In this study, there were 30 control embryos, 59 sham embryos, and 97 volume reduction (24 nl) embryos for a total of 186. Of these, only 59 survived through the experiment, dividing into 22 controls, 17 shams, and 20 reductions. All data was organized using Microsoft Office Excel 2007 and analysed in R (R Core Team 2021). All data sets were checked for assumptions first visually and then statistically using Levene's homogeneity of variance test, and a Shapiro-Wilk test of normality on the residuals of the models unless stated otherwise (Fox J, Weisberg S 2019). If data failed assumptions, a log10 transformation was applied. An alpha of 0.05 was used for survivorship, yolk-reduction, and size at hatch tests. A corrected alpha of 0.0125 was applied to t-tests for swimming and predator behaviour due to use of the data 4 times, once at each day.

Survivorship

Survivorship was calculated as the proportion of larvae surviving to hatch. This was calculated for each treatment and every trial. Treatment was nested within breading group, thus

an unreplicated block design was used. Breading group is the random factor, and treatment is a fix factor within it. The package lmerTest (Kuznetsova A, Brockhoff PB, Christensen RHB 2017) was used to generate a mixed model. An ANOVA with type II sums of squares tested the model. Emmeans performed post-hoc tests by treatment as a pairwise tukey test.

Yolk reduction

To determine if yolk volume was reduced as described, paired t-tests compared the yolk volume before and after reduction and sham treatments. A Shapiro-Wilk tested normality, and a Kolmogorov-Smirnov tested homogeneity of variance.

Determining effect of treatment at hatch

Although adult zebrafish were unable to be bred in pairs, I attempted to control the size of larvae at hatch using a micromanipulation technique that reduces yolk-volume to generate larvae of different sizes at hatch. To compare if treatment groups generated larvae of distinct sizes, a series of t-tests compared the standard length of larvae at hatch. First, a t-test of equal variance compared the control and sham group. The control and sham were pooled into one large control group and were compared to the size of the reduction group at hatch using a t-test of equal variance.

Generating fish at size

The micromanipulation was found to negatively impact the survival of embryos treated. Due to this complication, a quartile approach generated two distinct size groups from all larvae pooled together. A small and large group was created by selecting individuals with standard lengths in the top and bottom 25% of each day post hatch. This generated a data set for each day including individual larval ID, swim behaviour metrics, predation behaviour metrics, and size

group of large and small larvae based on standard lengths. A t-test of equal variance then compared the two size groups.

Probability of encounter

Measures of a free swim trial acted as a proxy for probability of encounter. Probability of encounter was estimated from swimming measures of distance travelled (mm) and mean velocity (mm/s). At each of the four days post hatch, t-tests of equal or unequal variance, depending on the outcome of the homogeneity test, compared the swimming measures of small and large larvae at each day. This was repeated for mean velocity. Due to repeated testing of the same individuals over time, an alpha correction was applied.

The probability of encounter was compared between larvae of the same size at different ages by performing a t-test comparing standard length of small and large larvae at different days. The swimming measures were compared between two days that standard length was not found be significantly different. Due to repeated testing, an alpha correction was applied.

Escape response

Measures of the escape response behaviour acted as a proxy for probability of capture. The escape response metrics were total distance (mm), mean velocity (mm/s), maximum acceleration (mm^2/s), and latency to respond (s). Testing followed the method used to estimate probability of encounter, performing t-tests of equal or unequal variance to compare the response of two size groups to one another each day.

The escape response of equivalent size but different ages were tested by first t-tests comparing standard length of small and large larvae at different days. Once the days when size was not significantly different between these groups was determined, a series of t-tests compared the escape response metrics between ages.

Results

The effect of treatment on survival was compared using an unreplicated block model. There was a significant effect of treatment on mortality (2.1, $F_{(4)} = 75.245$, $p = 0.000669$). Post-hoc testing revealed that control had significantly greater survival than both the sham ($t(4) = 9.548$, $p = 0.0015$) and reduction groups ($t(4) = -11.457$, $p = 0.0007$), but there was no difference in survival between micromanipulated groups ($t(4) = -1.908$, $p = 0.25$).

Yolk reduction

To determine the effectiveness of the manipulations, two paired t-tests compared sham and reduction embryo yolk volumes before and after microinjection (Figure 2.2). The paired t-test of the 24 nl reduction revealed a significant difference in yolk volume before and after treatment ($t(87) = 9.1275$, $p = 2.348e-14$). The paired t-test of sham yolk volume before and after was also significant ($t(53) = 3.818$, $p = 0.000354$).

Determining effect of treatment

To determine the success of treatment on size at hatch, a series of t-tests were performed. First, a t-test of equal variance compared the control and sham standard length at hatch. There was no significant difference in size at hatch ($t(30) = 0.300$, $p = 0.766$), thus the variables were combined to be one control group (Figure 2.3). The new total control group was compared to the size of the 24 nl reduction group using a t-test of equal variance, finding no significant difference in size at hatch (Figure 2.4, $t(54) = -0.154$, $p = 0.878$).

Generating fish at size

Since treatment was unsuccessful in creating groups of distinct sizes at hatch, a small and large group were generated each day for the escape and swim trials from the top and bottom 25%

of larvae by standard length (Figure 2.5). The t-test of equal variance found that this was a successful method to create distinct sized larval groups ($t(98) = 7.9118$, $p = 3.91e\text{-}12$).

Probability of encounter

The distance travelled (mm) was compared between small and large larvae generated from the upper and lower quartile of pooled standard length on each day post hatch in a series of t-tests. A t-test of equal variance was used on days 1, 2, and 4, and a t-test of unequal variance on day 3 (Figure 2.6). There was no significant difference in distance travelled on day 1 ($t(16) = 0.204$, $p = 0.841$), day 2 ($t(14) = -1.875$, $p = 0.0818$), and day 3 ($t(22) = 0.594$, $p = 0.0818$). The large group moved significantly further than small larvae on 4 days post hatch ($t(27) = 2.846$, $p = 0.00835$).

Mean velocity (mm/s) was compared between the small and large larvae on each day post hatch in a series of t-tests (Figure 2.7). The same t-tests were applied to each day as in the distance travelled analysis. There was no significant difference in mean velocity on day 1 ($t(16) = -1.004$, $p = 0.330$), day 2 ($t(14) = -0.197$, $p = 0.847$, or day 4 ($t(27) = 2.469$, $p = 0.0202$). The large group had a significantly greater mean velocity than the small group on day 3 ($t(18.157) = 2.9711$, $p = 0.008128$).

To compare ages of different sizes, the standard length of the small and large reduction groups was compared across days post hatch. The standard length of small larvae on day 4 and the large larvae on day 1 are not significantly different ($t(30) = -1.628$, $p = 0.114$). Of these larvae that were different ages, there was no significant difference in the distance travelled (mm) ($t(30) = -0.118$, $p = 0.907$), or mean velocity (mm/s) (Figure 2.8, $t(30) = -1.101$, $p = 0.280$).

Escape response

The distance travelled (mm) of the escape response was compared by size at each day post hatch (Figure 2.9). T-tests of equal variance found no significant difference in distance

travelled on day 1 ($t(20) = 0.714$, $p = 0.483$), day 3 ($t(24) = -0.154$, $p = 0.879$), or day 4 post hatch ($t(22) = -1.2393$, $p = 0.228$). The small larvae moved significantly further than the large larvae on day 2 post hatch ($t(26) = -3.404$, $p = 0.00216$).

The mean velocity (mm/s) of the escape response were compared by size at each day post hatch (Figure 2.10). T-tests of equal variance found no significant difference in mean velocity on day 1 ($t(20) = 1.826$, $p = 0.0828$), day 2 ($t(15.959) = -0.138$, $p = 0.8919$), day 3 ($t(24) = 0.983$, $p = 0.335$), or day 4 post hatch ($t(24) = 0.54283$, $p = 0.5922$).

The maximum acceleration (mm^2/s) of the escape response were compared at by size at each day post hatch (Figure 2.11). T-tests of equal variance found no significant difference in maximum acceleration between sizes on day 1 ($t(20) = 1.521$, $p = 0.144$), day 2 ($t(26) = -1.495$, $p = 0.147$), day 3 ($t(24) = 0.543$, $p = 0.592$), or day 4 post hatch ($t(22) = -0.525$, $p = 0.591$).

The latency to respond (s) to the stimulus was compared by size at each day post hatch (Figure 2.12). T-tests of equal variance found no significant difference in latency by size on day 1 ($t(20) = 0.837$, $p = 0.412$), day 3 ($t(24) = -0.0947$, $p = 0.9253$), or day 4 post hatch ($t(22) = -2.190$, $p = 0.039$). A t-test of unequal variance found large larvae reacted significant faster than small larvae on day 2 ($t(18.451) = -3.2872$, $p = 0.00399$).

To compare the escape response of larvae at the same ages but different sizes, the standard length of the small and large reduction groups was compared across days post hatch. The standard length of small larvae on day 2 and the large larvae on day 1 are not significantly different ($t(23) = 1.416$, $p = 0.170$). T-tests of equal variance compared the four escape response swimming metrics between different aged larvae (Figure 2.13). This found no significant difference in distance travelled ($t(23) = 0.753$, $p = 0.459$), mean velocity ($t(23) = 2.195$, $p = 0.0385$), maximum acceleration ($t(23) = -0.0878$, $p = 0.931$), or in latency to respond ($t(23) = -0.157$, $p = 0.877$).

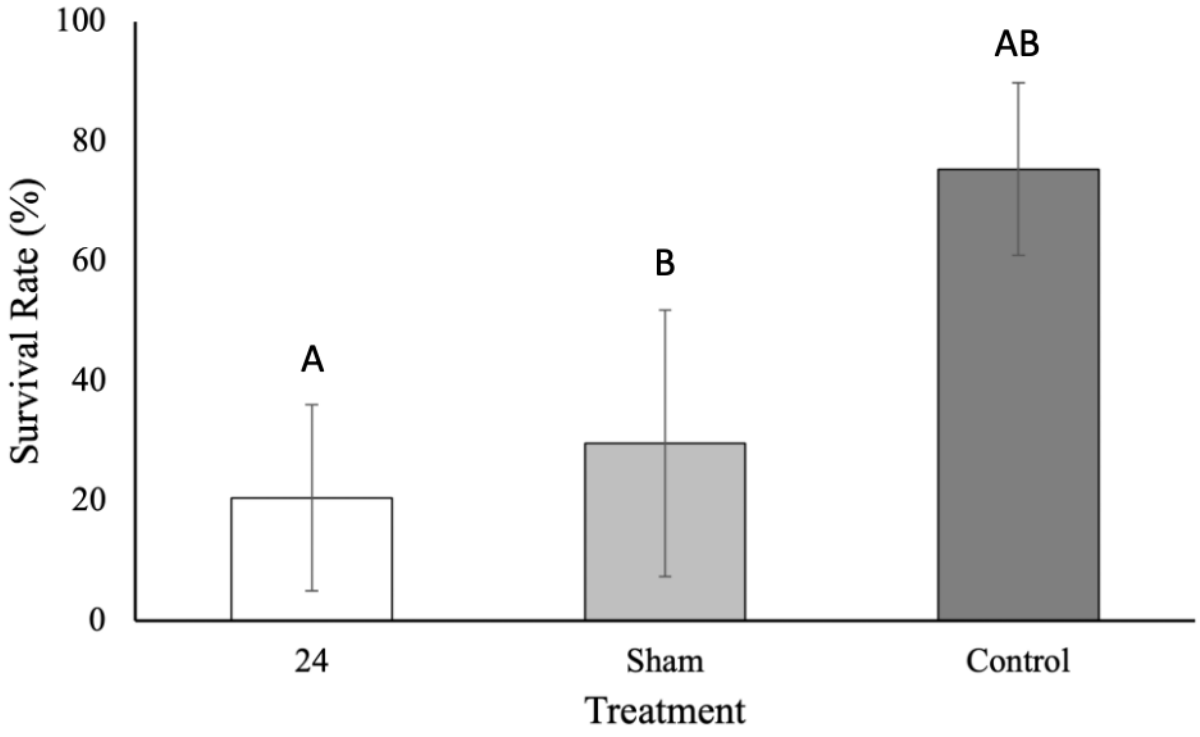

Figure 2.1 Mean ± SEM survival (%) of larvae surviving to hatch (n = 186). The white represents the 24 nl reduction group, the light grey represents the sham group, and the dark grey represents the control group. Survival rate was calculated as a proportion of those who survived to hatch over total selected. Significant differences are denoted with capital letters ($\alpha = 0.05$).

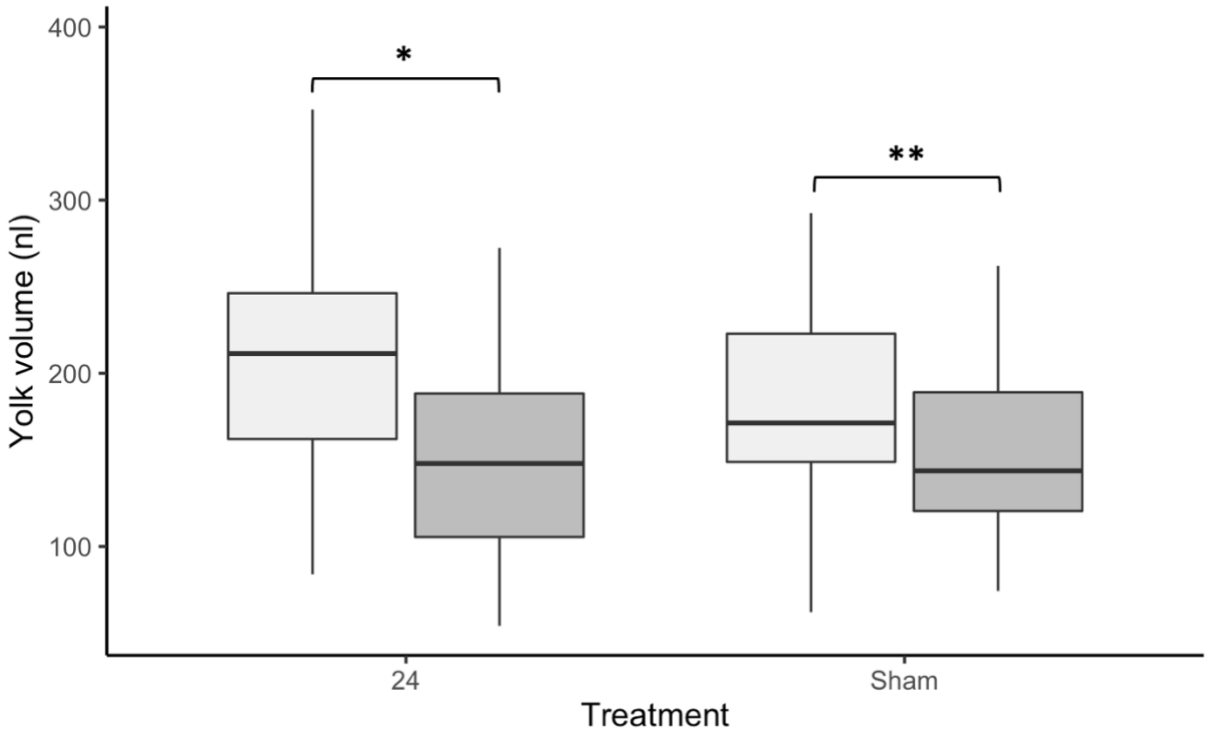

Figure 2.2 The yolk volume (nl) of the 24nl reduction group (n = 88) and the sham injection group (n=54) before and after microinjection. The light grey represents the before group, and the dark grey represents the after. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers. Significant differences in yolk-volume are denoted with asterisk ($\alpha = 0.05$).

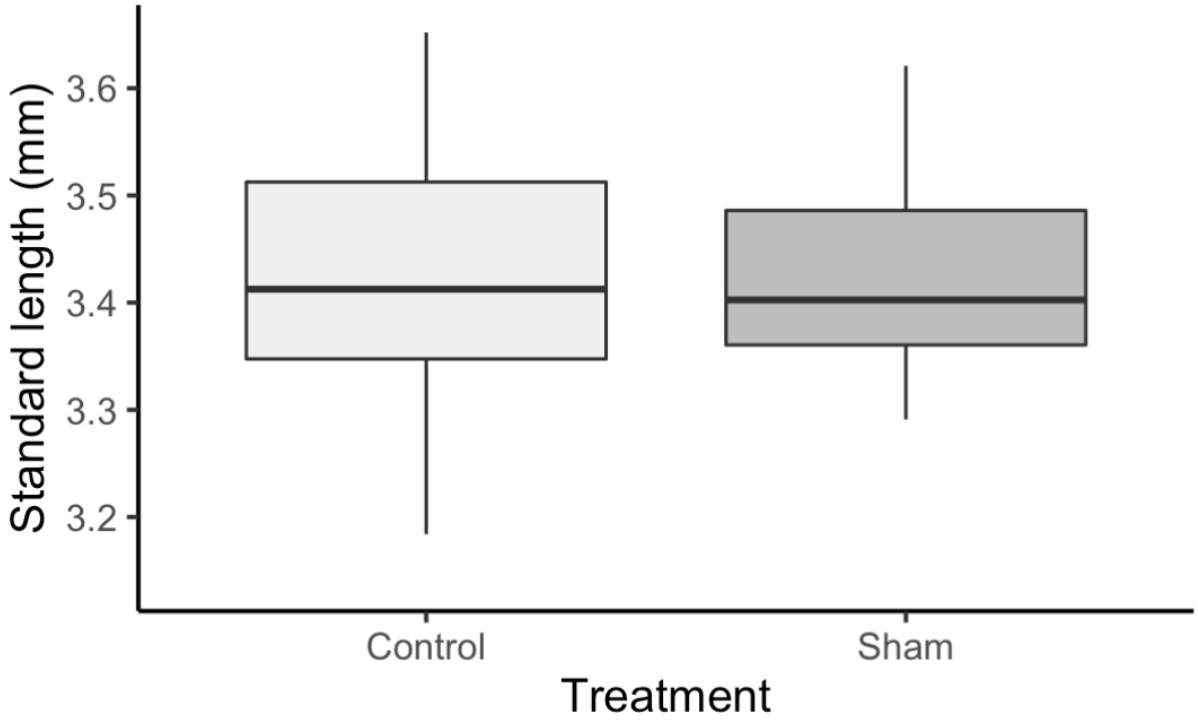

Figure 2.3 The pooled standard length at hatch (mm) of the control (n = 20) and sham (n = 12) treatment groups across all breading groups. The light grey represents the control group, and the dark grey represents the sham. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers.

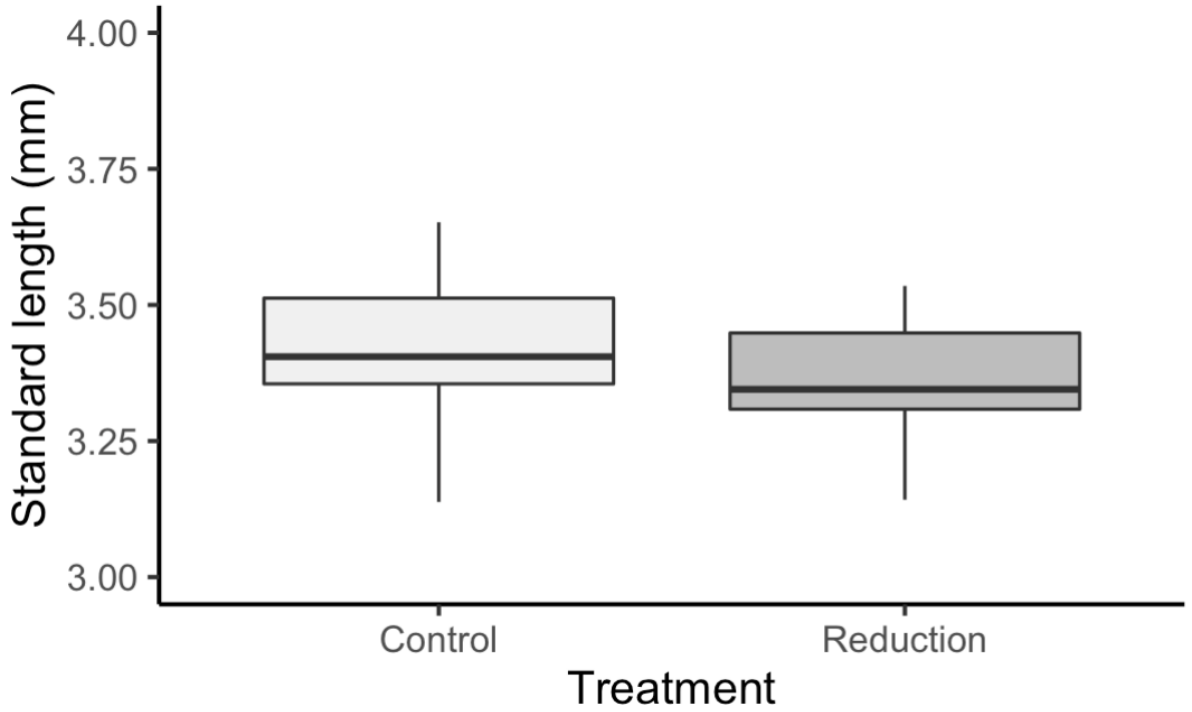

Figure 2.4 The pooled standard length at hatch (mm) of the control (n = 32) and 24nl reduction group (n = 24) treatment groups. The light grey represents the control group, and the dark grey represents the reduction group. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**.**

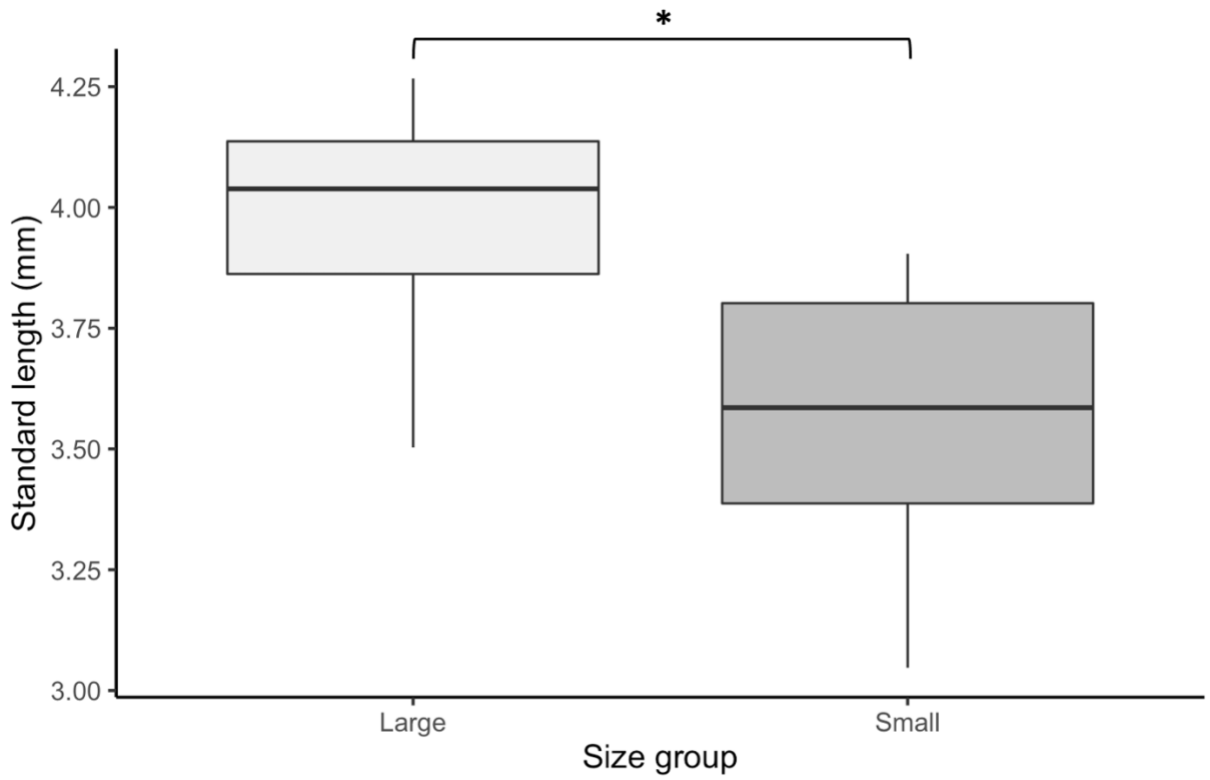

Figure 2.5 The pooled standard length (mm) of the large and small groups. The light grey represents the large group, and the dark grey represents the small group. Significance is denoted with an asterisk (p = 3.91e-12). The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**.**

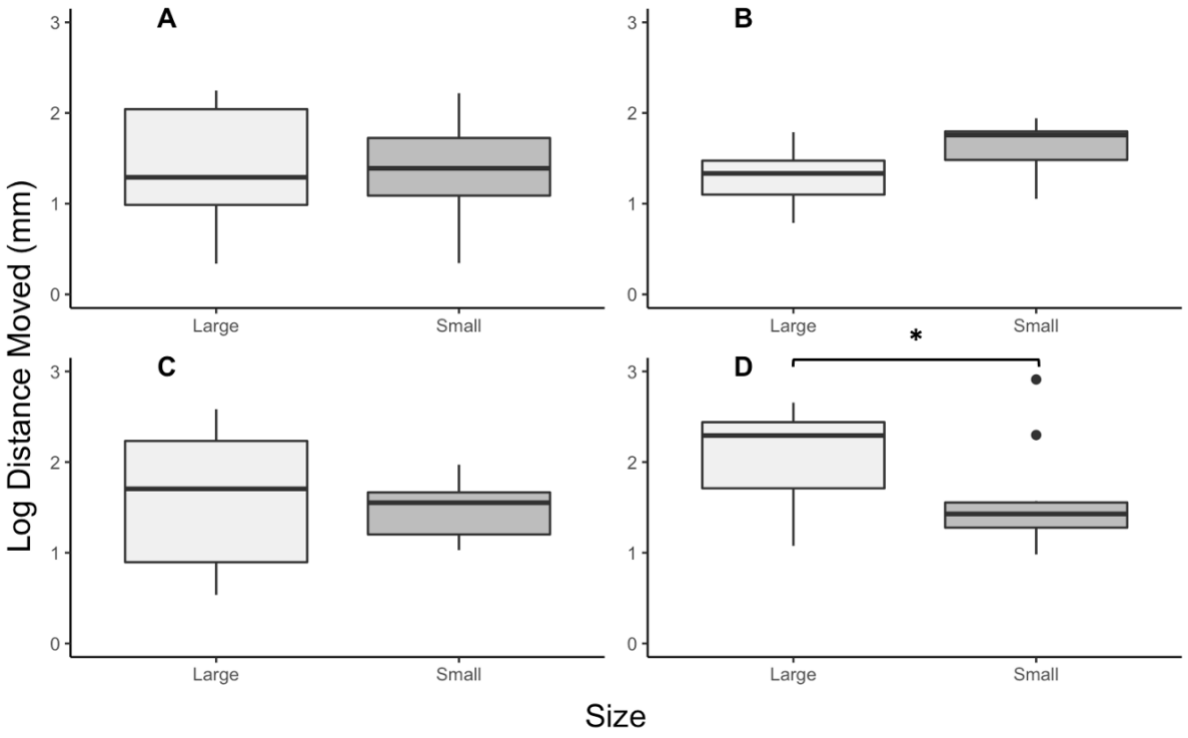

Figure 2.6 A boxplot of the Log10 transformed distance traveled (mm) of the swim response versus size group at each day post hatch. The light grey represents the large larvae, and the dark grey represents the small. Significance is denoted with an asterisk (α = 0.0125). The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**. A)** 1 day post hatch, small (n = 9), large (n = 9). **B)** 2 days post hatch, small (n = 8), large (n = 8). **C)** 3 days post hatch, small (n = 9), large (n = 15). **D)** 4 days post hatch, small (n = 14), large (n = 15).

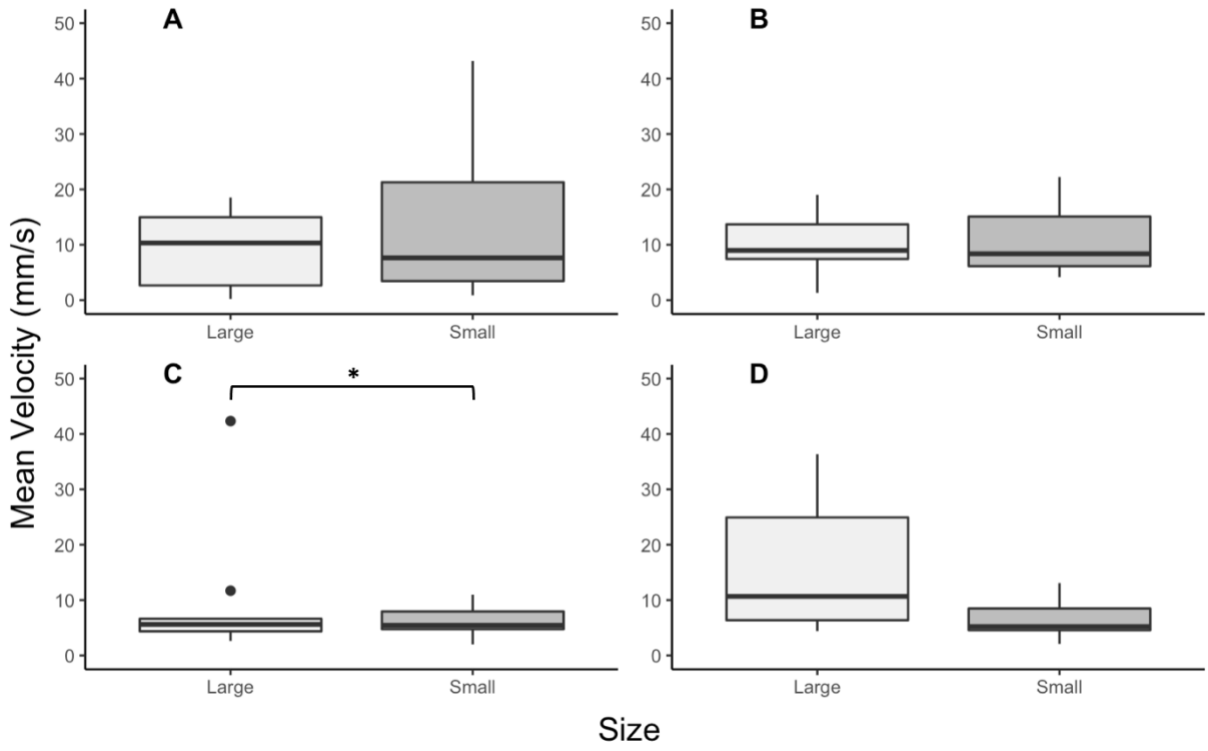

Figure 2.7 A boxplot of the mean velocity (mm/s) of the swim response versus size group at each day post hatch. The light grey represents the large larvae, and the dark grey represents the small. Significance is denoted with an asterisk ($\alpha = 0.0125$). The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**. A)** 1 day post hatch, small (n = 9), large (n = 9). **B)** 2 days post hatch, small (n = 8), large (n = 8). **C)** 3 days post hatch, small (n = 9), large (n = 15). Mean velocity was log10 transformed for analysis. **D)** 4 days post hatch, small (n = 14), large (n = 15). Mean velocity was log10 transformed for analysis.

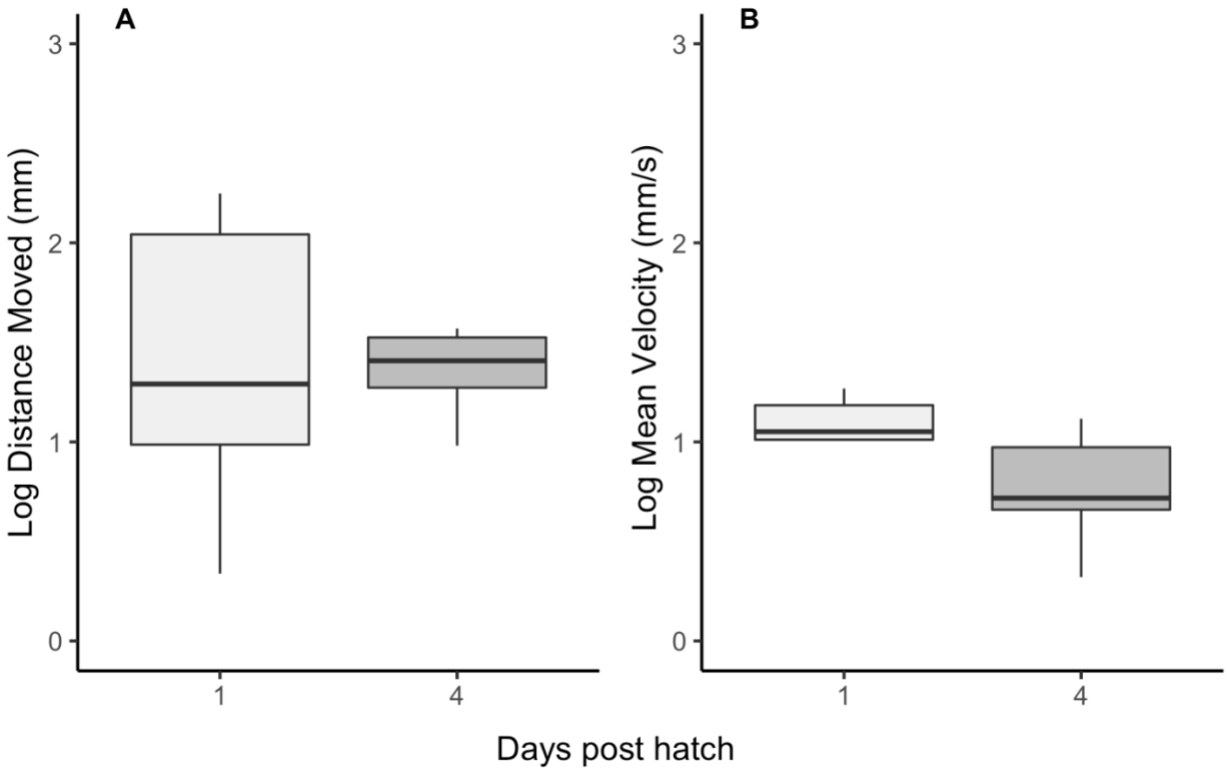

Figure 2.8 A boxplot of the **A)** Log10 transformed distance moved (mm) and **B)** Log10 transformed mean velocity (mm/s) of the swim response versus days post hatch. The light grey box represents the large larvae at 1 day post hatch (n = 10), and the dark grey represents the small larvae at 4 days post hatch (n = 22). These days were selected because the standard length of larvae was not significantly different. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**.**

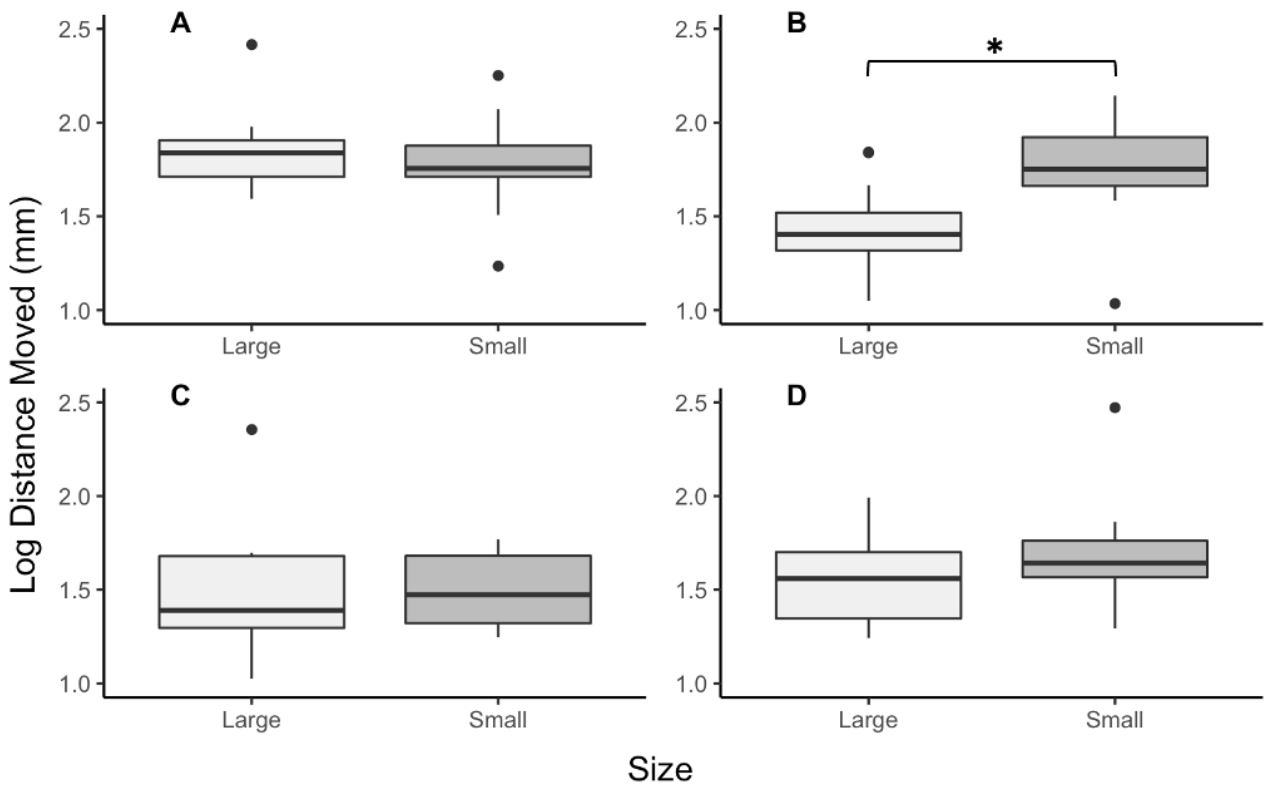

Figure 2.9 A boxplot of the Log10 transformed distance moved (mm) of the escape response versus size group at each day post hatch. The light grey represents the large larvae, and the dark grey represents the small. Significance is denoted with an asterisk ($\alpha = 0.0125$). The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**. A)** 1 day post hatch, small (n = 11), large (n = 11). **B)** 2 days post hatch, small (n = 14), large (n = 14). **C)** 3 days post hatch, small (n = 13), large (n = 13). **D)** 4 days post hatch, small (n = 12), large (n = 12).

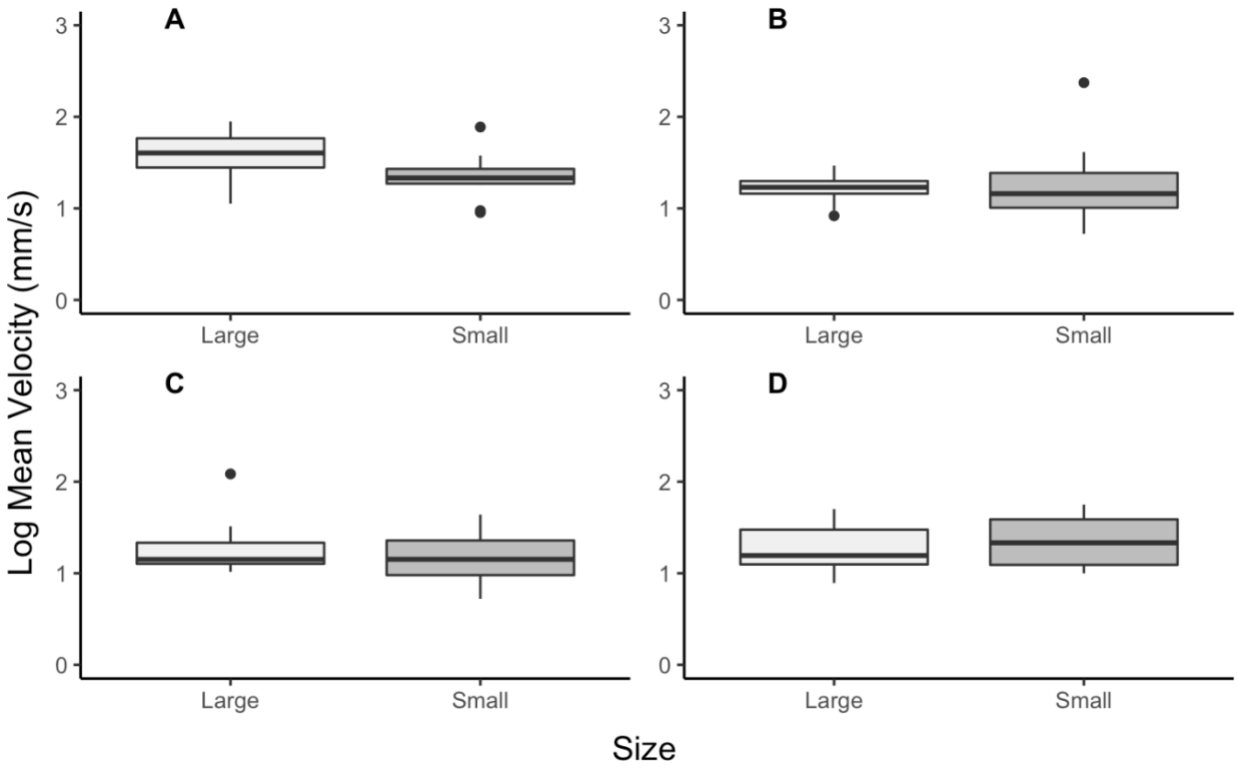

Figure 2.10 A boxplot of the Log10 transformed mean velocity (mm/s) of the escape response versus size group at each day post hatch. The light grey represents the large larvae, and the dark grey represents the small. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**. A)** 1 day post hatch, small (n = 11), large (n = 11). **B)** 2 days post hatch, small (n = 14), large (n = 14). **C)** 3 days post hatch, small (n = 13), large (n = 13). **D)** 4 days post hatch, small (n = 12), large (n = 12).

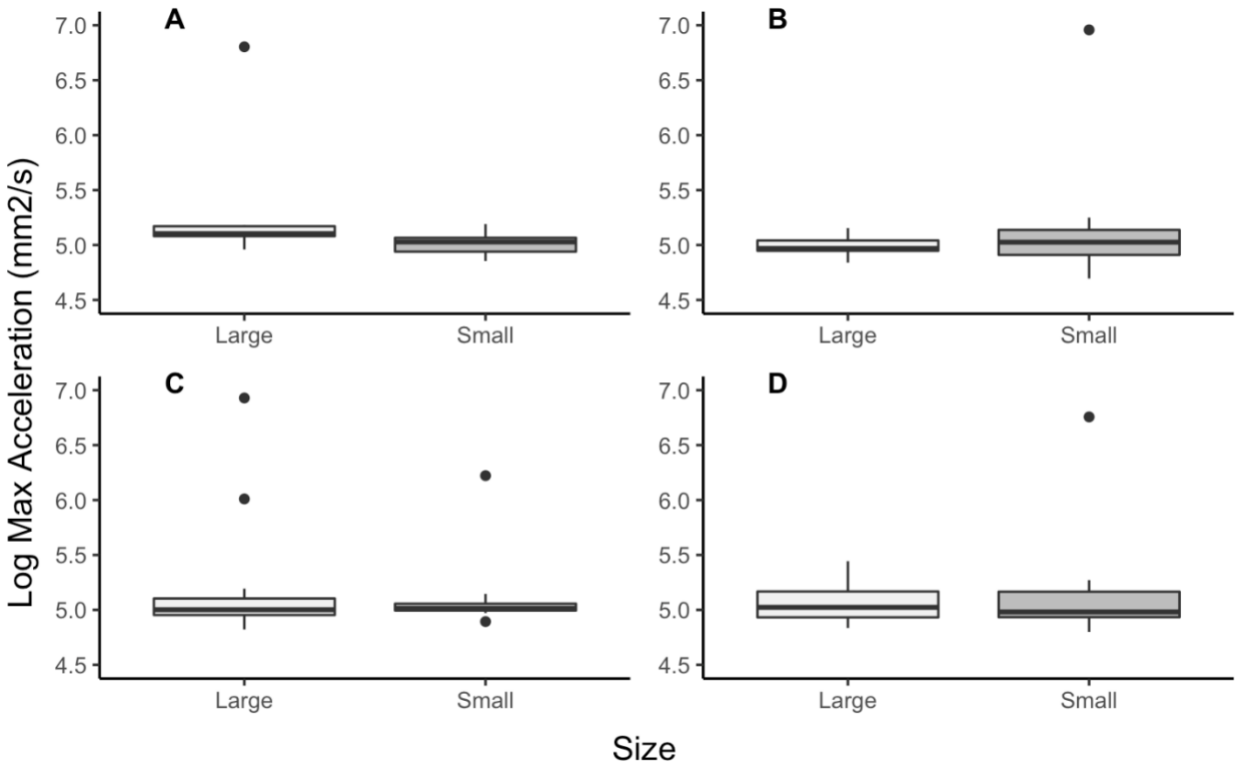

Figure 2.11 A boxplot of the Log10 max acceleration (mm^2/s) of the escape response versus size group at each day post hatch. The light grey represents the large larvae, and the dark grey represents the small. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25^{th} percentile and 75^{th} percentile, and the whiskers extend to the minimum and maximum excluding outliers**. A)** 1 day post hatch, small (n = 11), large (n = 11). **B)** 2 days post hatch, small (n = 14), large (n = 14). **C)** 3 days post hatch, small (n = 13), large (n = 13). **D)** 4 days post hatch, small (n = 12), large (n = 12).

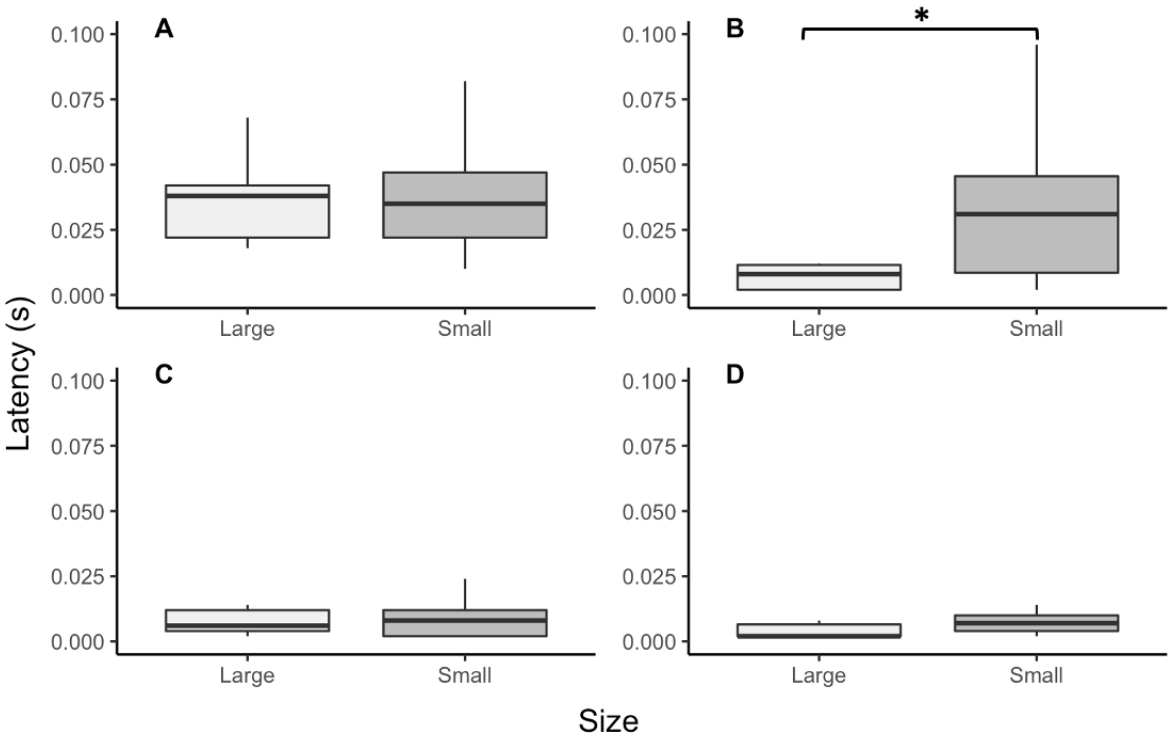

Figure 2.12 A boxplot of the latency to respond (s) of the escape response versus size group at each day post hatch. Statistical analysis was performed on log transformed latency. The light grey represents the large larvae, and the dark grey represents the small. Significance is denoted with an asterisk ($\alpha = 0.0125$). The dark band of the boxplot is the median, the upper and lower limits of the box are the 25^{th} percentile and 75^{th} percentile, and the whiskers extend to the minimum and maximum excluding outliers**. A)** 1 day post hatch, small (n = 11), large (n = 11). **B)** 2 days post hatch, small (n = 14), large (n = 14). **C)** 3 days post hatch, small (n = 13), large (n = 13). **D)** 4 days post hatch, small (n = 12), large (n = 12).

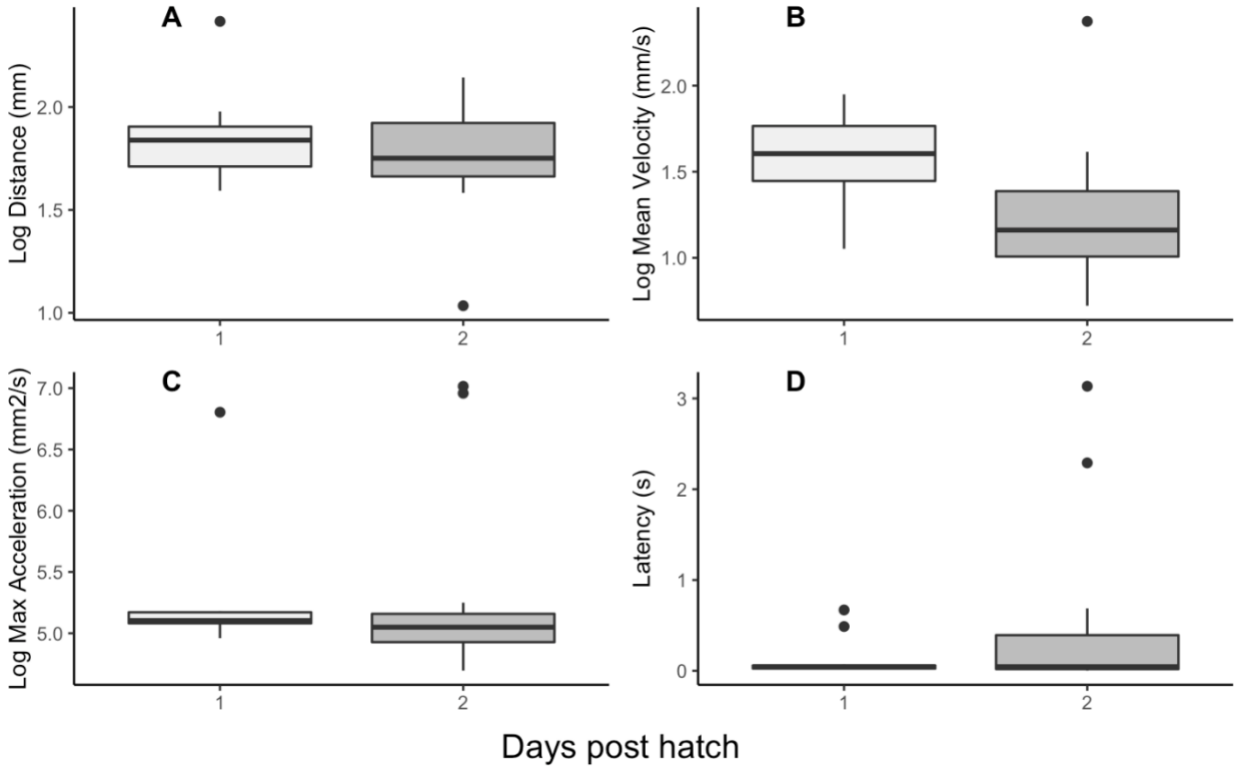

Figure 2.13 A boxplot of the **A)** Log10 transformed distance moved (mm), **B)** Log10 transformed mean velocity (mm/s), **C)** Log10 maximum acceleration, **D)** Latency to respond (s) of the escape response versus days post hatch. Statistical analysis was performed on log10 transformed data. The light grey box represents the large larvae at 1 day post hatch (n = 11), and the dark grey represents the small larvae at 2 days post hatch (n = 14). These days were selected because the standard length of larvae was not significantly different. The dark band of the boxplot is the median, the upper and lower limits of the box are the 25th percentile and 75th percentile, and the whiskers extend to the minimum and maximum excluding outliers**.**

Discussion

Yolk sac larvae experience high rates of mortality, often driven by predation (Purcell and Grover 1990). The purpose of my study was to investigate the effects of size and age on vulnerability to predation of larval zebrafish. Historically, the bigger-is-better hypothesis dominated the field and proposed that larger larvae experience fitness benefits over their smaller counterparts (Bailey and Houde 1989). Studies that support this hypothesis only consider vulnerability of capture in their work. Litvak and Leggett (1992) contested this hypothesis, suggesting that the effect of size on vulnerability to predation changes daily, where it could be better to be big or small depending on the environment at a given time. I sought to further investigate this hypothesis, by comparing proxies of probability of capture and probability of encounter of yolk sac zebrafish larvae of different sizes. I predicted that larger larvae would swim further in free swimming experiments than their smaller counter parts overtime. I also predicted that larger larvae would exhibit stronger predator escape responses overtime.

Micromanipulation

Survival rate

Unlike in Jardine and Litvak (2003), I found a reduction in survival of embryos subjected to micromanipulation. The control group had a significantly higher survival rate than both the sham and reduction groups, which did not significantly differ in survival between each other. Additional comparisons of yolk volume before and after treatment found that both the reduction and sham groups experienced a decrease in yolk-volume after treatment. Consequently, it is not clear if decline in survival was due to the manipulation itself, or the removal of yolk. These findings contradict the results described by Jardine and Litvak (2003), as they found no significant difference in survival between control and manipulated embryos, and no significant difference in yolk volume in the sham group before and after treatment. The low survival rate seen across groups could be the result of lower quality eggs since the breeding stock was a year old (Nasiadka and Clark 2012). The lower egg quality could have also been caused by poor diet, as fish did not always maintain feedings 3 times a day and were not fed nutritious meals like

Artemia often. Furthermore, it is unclear if the clutch was from one female or both females in the breeding tank. Clutch sizes were greater than 200 in every trial. Had this high clutch size come from one female there could have been a trade-off between number of eggs and the success of the egg (Vance 1973; Smith and Fretwell 1974), but since they were in the same tank the mother could not be determined. Human error performing the injections could have had adverse effects on survival. Teleost yolk is shown to be intrinsically linked to successful development, with embryos early in ontogeny being particularly to vulnerable negative effects of yolk disruption (Roustaian and Litvak 2007). In this study, the micromanipulation occurred during the cleavage period when embryos could have been susceptible to the negative effects of yolk disruption. Although the treatment impacted survival, all surviving embryos developed normally post hatch, so it was assumed that treatment did not affect behaviour.

Size at hatch

The goal of the micromanipulations was to generate larvae of distinct sizes at hatch, but this did not occur as predicted. As expected, based on the work of Jardine and Litvak (2003), there was no significant difference in size of control and sham larvae at hatch. Contrarily, there was no significant difference in size at hatch of the control and reduced group. As mentioned, the sham group also experienced an accidental yolk reduction during manipulation which could contribute to the lack of size difference observed. The difference in size at hatch could also been overshadowed by the difference within and between clutches from different females. It is well documented that egg size changes with female size and age (Hulata et al. 1974; Kamler 2005; Nasiadka and Clark 2012). Furthermore, egg size and subsequent offspring size varies within clutches from the same mother. A within population meta-analysis of 102 species found that the average coefficient of variation of offspring size within populations is 5%, with some showing additional variation of up to 15% (Marshall and Keough 2007). In this study, I found that the coefficient of variation of all fish at hatch was 5.9%, which is very comparable to their findings for within offspring variation. Marshall and Keough (2007) suggests the variability in size seen in a clutch has fitness benefits, but to what extent is yet to be determined. There is potential that greater variation in offspring size is adaptive in more heterogeneous environments as the success of larvae based on size can vary greatly. In combination, both within and between variance could

have overshadowed the micromanipulation treatment, contributing to the lack in size difference at hatch.

Probability of encounter

This study weakly supports the hypothesis that larger larvae will move further distances, but the findings were not consistent over time. I found that larger larvae moved significantly further on day 4, and on average swam faster on day 3, than the small larvae. Additionally, I found no significant difference in distance and swimming speed of larvae of the same size at different ages. Previously size has been shown to increase swimming speeds of larval largemouth bass, yellow perch, walleye, Pacific herring, lake whitefish, Atlantic herring, plaice, northern anchovy, and Pacific mackerel (Blaxter 1986; Masuda 2006). Although all species experience an increased swimming speed at size, the rate of this increase is unique to the species. The average difference in standard length between the small and large group each day was 0.875 mm or a 27% difference in length. It is possible changes in swimming speed are low in this age range, or that this difference in size is not great enough between groups. Further studies should investigate the development of swimming speed overtime of larval zebrafish to understand the rate of improvement. Moreover, this difference was only seen in each swimming metric on one day of the four and could suggests a daily variance in swimming performance. This is somewhat like Jardine and Litvak (2003) who suggest this daily variance contributes most to gross vulnerability to predation of a larvae.

Escape response

I found little support of the hypothesis that larger larvae will have a stronger escape response than smaller larvae over time. Small larvae moved a significantly further distance than larger larvae of the same age at day 2 post hatch. Also at 2 days post hatch, large larvae responded significantly quicker than small larvae. Comparing larvae of different sizes at the same age found no significant differences in any of the four escape measures. Although the

latency of response supports the original hypothesis, the method of calculating this metric was prone to error. The wire and larvae existed in three-dimensional space, but a video is only two dimensions. As a result, it was challenging to determine if the hair had touched the larvae upon its lateral lines or went beneath the body of the fish. Therefore, the accuracy of this measure is low, and this may not be a true representation of latency. The suggestion that larval size does not impact vulnerability of capture was also found in yolk sac capelin larvae (Litvak and Leggett 1992). This study investigated capture directly, rather than through a proxy like escape response, by exposing 150 large or small larvae of the same size and age to stickleback and jellyfish in a closed arena. They found that there was no significant difference in probability of capture between the large and small size groups when predators were given the choice.

Most studies that investigate probability of capture use escape response as a proxy. Overall, the finding that size has no effect on any of the four-escape metrics contradicts the literature (Webb 1978, 1981; Bailey and Houde 1989). A possible explanation of this difference is that standard length might not be the best body measure to represent size. A previous study compared the escape response at size of seven teleost species found that maximum velocity and distance varied with length and that the relationship was unique to species (Webb 1978). In addition to this, they suggested that rather than just considering total length, the success of an escape response is best predicted by a compromise between muscle and body mass, and the profile of the lateral body and fins. Looking at measures like the profile of the lateral body and fins would not be challenging to measure and should be considered for use in future studies.

Limitations estimating gross vulnerability

The estimates of probability encounter and probability of capture were not strongly indicative that either are affected by larval age or size. Possible reasons why the effects of age and size were not seen in this study are the small sample sizes and the exclusion of predator choice. First, the sample size was small in this study due to trials removed for poor video quality, high mortality rates of micromanipulated embryos, and no significant difference in standard length at hatch. To create two size groups post-hoc, the sample size each day was cut in half. The large and small group were created from the highest and lowest 25% of larvae by standard

length. The small sample size becoming smaller during statistical analysis reduced the power to detect a meaningful difference in behaviour metrics. Moreover, this experiment was a highly controlled manipulation and did not consider the vulnerability to attack. Predator choice and predator density are known to impact the survival of different larvae size. The argument Litvak and Leggett (1992) made against the bigger-is-better hypothesis was supported by exhibiting the power of predator choice on survival. Probability of capture was not significantly different between size groups, but when a predator had a choice it would attack larger larvae, suggesting benefits for being a small size in high predation densities. Introducing predator choice into a laboratory study is challenging due to animal care guidelines limiting studies that could harm vertebrates. To partially replicate the findings of Litvak and Leggett (1992), using a cnidarian and other invertebrates could aid in exploring the effect of predator choice.

Conclusion

This study suggests that age and size do not significantly impact the probability of encounter or probability of capture of zebrafish yolk sac larvae. This finding is not strongly supported due to issues with a low sample size. Future studies should optimize micromanipulation techniques to alter the size at hatch of larvae from the same breeding pair. This could help reduce mortality and error that resulted in post-hoc methods of generating size groups. Micromanipulation could also limit the potential confounding effects of genetic variability on behaviour and size at hatch. If size and age do not impact probability of encounter and capture, then future studies should investigate how size and age impact vulnerability of attack.

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