

Swim Bladder Hypoinflation is Associated with Gut Dysfunction and Increased Mortality
in Larval Zebrafish (*Danio rerio*)

by

Kadri Pearce-Lawrence

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Supervisor: Timothy Erickson, Ph.D., Department of Biology

Examining Board: Shawn MacLellan, Ph.D., Department of Biology, Chair
Aurora Nedelcu, Ph.D., Department of Biology
Yang Qu, Ph.D., Department of Chemistry

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Dean of Graduate Studies

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Abstract

The swim bladder is a vital gas-filled organ in most bony fishes that allows them to maintain neutral buoyancy. Despite its significance, the consequences of inadequate initial inflation of this organ remain poorly understood. Previous research has suggested a connection between hypoinflation and mortality, but the underlying mechanisms remain unclear. Contrary to the theory that physostomes can inflate their swim bladders at any point after 4 dpf, I found significant increases in mortality associated with non-inflation, suggesting inflation must occur within a critical developmental period. Additionally, analysis of exogenous feeding in hypoinflated larvae challenges the contention that feeding is limited in this phenotype. I do however observe significant gut barrier degradation, gross morphological differences, and compromised lysosome activity in hypoinflated larvae. These discoveries offer crucial insight into the effects of hypoinflation on larval fish.

Dedication

For Joachim von Ledebur

“A life that touches others goes on forever.” - Unknown

Acknowledgements

I would like to begin by acknowledging that the land on which the University of New Brunswick resides is the territory of the Wolastoqey, Mi'kmaq, and Peskotomuhkati peoples. To recognize the land is an expression of my gratitude to those whose territory we are on, and a way to honour those who have been living on this land from time immemorial. I would also like to note that land acknowledgements do not exist in past historical tense: colonialism is a current and on-going process, and decolonizing extends beyond looking only to the past, but also the present and the future.

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List of Symbols, Nomenclature or Abbreviations

ApoE: apolipoprotein E

CTF: corrected total fluorescence

Dpf: days post-fertilization

Dph: days post-hatch

E3: a standard embryo rearing medium (See Appendix 1 for recipe)

EDTA: ethylenediaminetetraacetate acid

GFP: green fluorescent protein

GI: gastrointestinal

HPf: hours post-fertilization

kD: kiloDalton

LMA: low melting-point agarose

PBS: phosphate buffered saline

PFA: paraformaldehyde

Shha: sonic hedgehog signaling molecule a

TNF- α : tumor necrosis factor-alpha

Chapter 1: Introduction

1.1 Investigating the Swim Bladder Hypoinflation Phenotype Reveals Insights into Survival and Developmental Pathology

Most bony fish have a swim bladder, which are sac-like organs that aid in controlling buoyancy. The swim bladder is filled with gas and is located in the abdominal cavity of the fish. Depending on the species of fish, the swim bladder can vary in size and shape, although it is normally elongated and cylindrical in form. When the organ filled with gas, it brings the specific gravity of the fish closer to that of the ambient water, resulting in neutral buoyancy (Steen, 1970). The organ also plays secondary roles in respiration and as a sensory organ (Jones & Marshall, 1953). The ability to maintain buoyancy and swim effectively is beneficial for being able to find food, avoid predation, and eventually reaching reproductive age (Steen, 1970).

Swim bladder research has progressed significantly over time, from initial investigations into its basic anatomy to exploring its diverse functions in fish biology. Historically, scientists delved into evolution of the swim bladder, and its role in buoyancy regulation and behaviour. Recent research has focused on themes such as swim bladder function in fish behaviour, the influence of environmental factors on its development, and the role of gene expression. Exploring swim bladder pathologies has not only provided insights into its form and function but also offered treatments for fish swim bladder illnesses (Sirri et al., 2020) and contributed to broader knowledge about fish biology and aquatic environments (Strecker et al., 2011). This research has practical applications to the thriving aquaculture industry worldwide.

The overarching objective of my research has been to investigate the pathology of swim bladder hypoinflation in larval zebrafish, focusing on the mechanistic relationship between hypoinflation and mortality. As a note, the terms ‘hypoinflation’ and ‘uninflated’ are used interchangeably throughout to describe this phenotype. Goolish and Okutake (1999) posit starvation as the cause of death in hypoinflated zebrafish larvae, but this hypothesis remains uninvestigated. I addressed this problem by defining three research objectives (Figure 1). First, I investigated whether zebrafish have a critical developmental period during which they must initially inflate their swim bladders. Next, I tested the hypothesis that starvation is the cause of hypoinflation-associated mortality by quantifying survival of fed and unfed larvae with or without inflated swim bladders. Lastly, I tested whether gut form and function was compromised in hypoinflated larvae. Contrary to the current belief, I discovered that zebrafish do have a critical period of initial inflation. Also contrary to prevailing theories, I discovered hypoinflated larvae do consume the same amount of food as inflated larvae. Lastly, both gut form and function are compromised in hypoinflated larvae. The culmination of these experiments presents a novel understanding of an important aspect of fish development that has thus far been underrepresented in the literature. Further characterization of the pathological consequences and underlying mechanisms should be fruitful avenues of research.

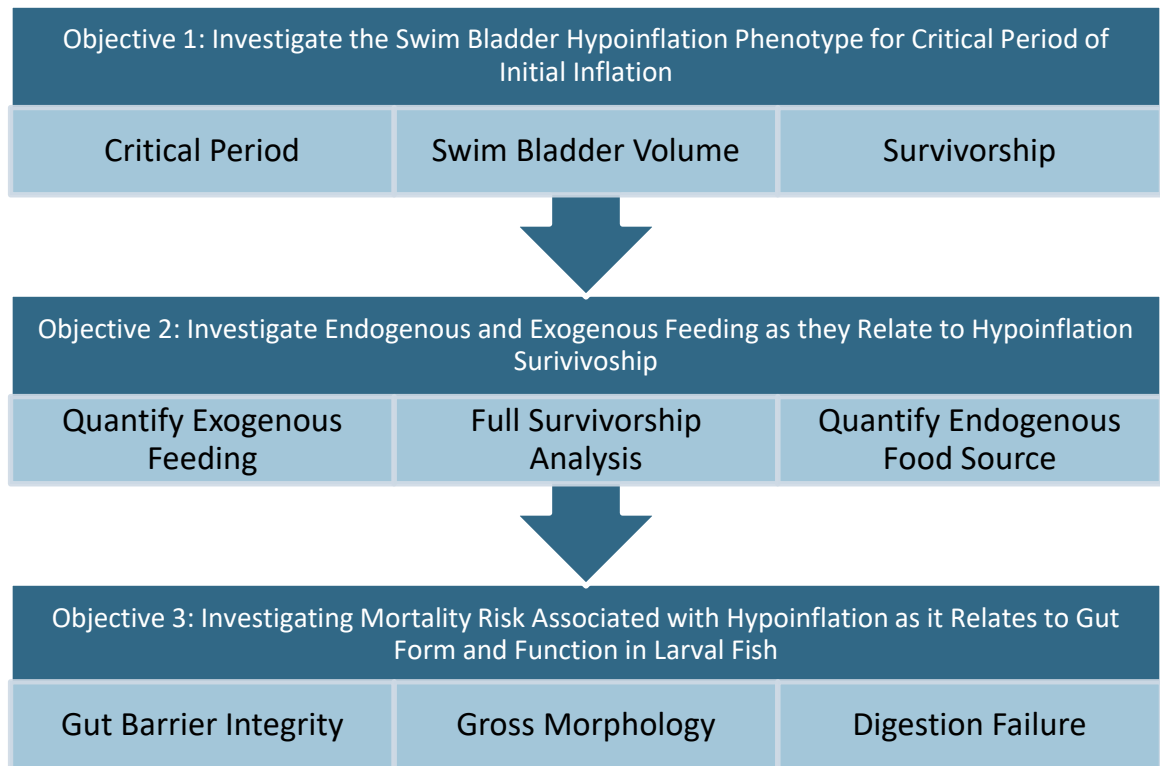


Figure 1: Outline of the research objectives and the experiments used to investigate them.

In this chapter, I review the current state of knowledge of the swim bladder and its abnormalities in relation to hypoinflation. The most detailed research into swim bladder development and genetics has been performed on zebrafish, and as such this review focuses heavily on what is known about this species. However, relevant examples from other fish species are also discussed throughout.

1.2 Swim Bladder Development & Initial Inflation

1.2.1 Gross morphology of the developing swim bladder and surrounding organs

In most teleost fish, the swim bladder is a sac-like organ that is filled with air and located in the abdominal cavity of the fish. The size and shape of the swim bladder can vary depending on the species of fish, but it is typically elongated and cylindrical in shape. The outer surface of the swim bladder is covered by an elastic layer of connective tissue and is surrounded by other organs in the abdominal cavity. Although it starts as a single chambered sac, the adult swim bladder is often double chambered organ with specialized functional regions (Pelster, 2004).

In most fishes, the swim bladder originates as a dorsal outgrowth of the posterior foregut (Pelster, 2004). Recognizing this common developmental origin of both the swim bladder and the gut holds pivotal significance, particularly in exploring the potential connection between proper swim bladder development and mortality due to starvation in hypoinflated larvae. Considering that proper digestion and nutrient uptake is dependent on the gut and that the gut and swim bladder are initially linked, it is possible that proper gut function is dependent on proper swim bladder function as well.

While smooth muscle is not present within the swim bladder until the early double chambered stage, striated muscle is present in the esophagus and pneumatic duct before initial inflation (Robertson et al., 2007). In zebrafish, the digestive tract opens and digestive enzymes begin to be secreted at 5-6 dpf, a day or so after initial swim bladder inflation (Wallace et al., 2005). The muscle within these regions is consistent with the larval ability to both initially inflate and to begin feeding.

The swim bladder and intestinal tract share developmental and physical connections, but their functional relationship is not well understood. A significant portion of this thesis focuses on the underappreciated relationship between swim bladder inflation and gut function.

1.2.2 Swim bladder ontogeny includes three distinct phases culminating with initial inflation of the organ

The cellular and tissue structure of the swim bladder and surrounding organs is an important area of research in the field of fish biology. In zebrafish, the early development of the swim bladder occurs in three phases: budding, growth/elongation, and inflation (Winata et al., 2009) (Figure 2):

Budding (36-65 hpf): This phase involves the initiation of the swim bladder bud as an epithelial evagination of the foregut. During this stage, and into the beginning of the growth stage, there is strong expression of the secreted signaling molecules *shha* (*sonic hedgehog signaling molecule a*) and *ihha* (*Indian hedgehog signaling molecule a*) in the swim bladder epithelium, pneumatic duct, and gut (Winata et al., 2009). The transcriptional regulators *motor neuron* and *pancreas homeobox 1* (*mnx1 / hb9*) and *SRY-box transcription factor 2* (*sox2*) are also expressed in the swim bladder epithelium (Korzh et al., 2011; Yin et al., 2011).

Growth/elongation (65-96 hpf): This phase involves the elongation of the bud to form the pneumatic duct, and additional growth of the swim bladder itself. At this stage, there are now three distinct tissue layers of the swim bladder: the epithelial layer (from the bud), the mesenchymal layer, and the outer mesothelium (Finney et al., 2006; Winata et al., 2009). Each of these layers has their own set of marker gene expression patterns,

coordinated growth, and organization. The Wnt and hedgehog signalling pathways are central in establishing the patterning and interactions between these tissue layers (Winata et al., 2010; Winata et al., 2009; Yin et al., 2011).

Inflation (96-120 hpf): The final phase of early swim bladder development is marked by initial inflation of the bladder, usually by the oral intake of air from the surface (Goolish & Okutake, 1999; Sawada et al., 2021).

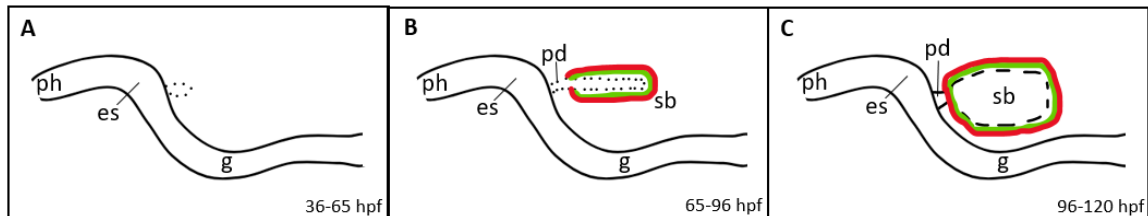


Figure 2: Schematic representation of the three phases of swim bladder development in zebrafish. A: Budding phase. B: Elongation phase. C: Inflation stage. Abbreviations: es, esophagus; g, gut; pd, pneumatic duct; ph, pharynx; sb, swim bladder. The swim bladder epithelium (dotted line) is surrounded by the mesenchymal tissue (green) and outer mesothelium (red).

1.3 Inflation and Regulation of Gas Exchange in the Swim Bladder

The timing of the first filling of the swim bladder differs from species to species, but typically coincides with yolk sac absorption as part of normal larval development (Clayton & Summerfelt, 2013). To initially fill the organ, larval fish will make their way to the air-water interface in a behaviour known as ‘swim-up’ or ‘surfacing’. This behaviour begins when the larva has approximately 50% of its yolk sac remaining and concludes with successful inflation, with complete reabsorption of the yolk occurring simultaneously or following soon after (Doroshev et al., 1981). To initiate surfacing, larvae perform tail-flicks to propel themselves upward and adhere to available substrates before repeating the process (Lindsey et al., 2010). Upon reaching the surface, the larvae penetrate the interface to gulp

air into the esophagus (Clayton & Summerfelt, 2013). The bubble of air then passes through the pneumatic duct and into the swim bladder (Woolley & Qin, 2010). At the junction of the pneumatic duct and the esophagus, there may be a sphincter formed by myocytes to help control the passage of gas (Finney et al., 2006). The process of initial swim bladder inflation is a vital developmental step for larval fish.

1.3.1 Critical period of initial swim bladder inflation

The pneumatic duct is critically important for initial inflation of the swim bladder, allowing gas to pass from the esophagus to the bladder. Fish who retain the pneumatic duct into adulthood are called physostomes, a group that includes zebrafish and other cyprinids (Evans & Damant, 1928). Conversely, fish where the pneumatic duct degenerates shortly after first filling are called physoclists (Pelster, 2004). As the swim bladders of both types of fish develop in a similar way, but differing in the persistence of the pneumatic duct, physoclistous fish are sometimes referred to as ‘transient physostomes’ (Trotter, 2005). Fishes that lose their pneumatic duct in early development have a finite temporal window in which they must inflate their swim bladders. This finite window is known as the critical period of initial inflation. Failure to initially inflate is associated with several pathologies, including physical deformities, growth retardation, and death (Bennett et al., 1987; Egloff, 1996; Goolish & Okutake, 1999; Kurata et al., 2012; Tsuji et al., 2016).

Interestingly, it appears that physoclistous larvae who fail to inflate their swim bladders will continue to attempt to reach the surface until their pneumatic duct has naturally degraded. After this point, they only appear to expend energy in attempts to catch food (J. F. von Ledeber & Wunder, 1937), seemingly knowing that the critical period of inflation has come and gone. Some species of physostomes, however, will continue to

attempt to inflate, even with surgical closing of their pneumatic duct rendering it unusable (von Ledebeur & Wunder, 1937). This continued effort to inflate would further divert energy stores away from developmental processes toward the futile attempt at inflation.

1.3.2 Other mechanisms of inflation allowed for the expansion of fishes to greater depths and ranges

Most teleosts have a swim bladder, however there are some exceptions. Pleuronectids (including the flounders) and angler fish (*Lophius*), for example, possess a swim bladder as juveniles but lack the organ as adults. Some bathypelagic (deep-sea) fish lack or have regressed swim bladders. Instead, buoyancy is achieved by the presence of large, oily livers that help offset the body's density in the case of sharks, or have decreased ossification of their bones in the case of teleosts lacking swim bladders (Corner et al., 1969; Denton & Marshall, 1958; Ragnar Fänge, 1983). In deep sea fishes that do have swim bladders, the organ is typically large and well-developed, allowing them to maintain their position at great depths where the pressure is much higher. The wall of the swim bladder of deep-sea fishes is much thicker than in shallow water fish, and is composed of a thick, fatty layer that helps resist oxygen diffusion (Wittenberg et al., 1980). These adaptations allow deep sea fishes to live and thrive in the extreme conditions of the deep sea.

The mackerel (*Scomber scombrus*), a teleost without a swim bladder, uses hydrodynamics to maintain their position in the water column, requiring constant expenditure of energy (Denton & Marshall, 1958). For these types of teleosts, it is posited that the less ossified skeletons, thin muscle layers and reduced scales helps to reduce their body weight thus reducing the amount of exertion required to swim (Denton & Marshall, 1958). In some cases, such as the cichlid *Tilapia mossambica*, fish have a swim bladder

but never possess a pneumatic duct, so they initially fill their swim bladders via the gas gland as larvae (Doroshev & Cornacchia, 1979).

Evolution of the swim bladder enabled fishes to diversify their habitats, behaviours, and physical forms. Subsequently, the evolution of an oxygen secretion mechanism removed some fishes' requirement for surface access to inflate their swim bladders. Instead, these fish release oxygen from hemoglobin in their blood using both Root-effect hemoglobins and the already established choroid *rete mirabile* (Berenbrink et al., 2005). This network of parallel, countercurrent venous and arterial capillaries also only evolved once, about 250 million years ago, primarily as an ocular support system allowing improved eyesight. The other *rete mirabile*, that supports swim bladder inflation, appeared 100 million years later (Berenbrink et al., 2005). These evolutionary changes allowed the emergence of teleost fishes that occupy greater depths.

1.3.3 The *rete mirabile* facilitates efficient gas exchange and buoyancy regulation in most swim bladders

The *rete mirabile* is a specialized network of blood vessels found in some fish species. The *rete mirabile* is located near the swim bladder and is involved in the regulation of gas exchange between the swim bladder and the blood. It consists of a series of capillaries that are arranged in a parallel fashion and surrounded by a network of smooth muscle cells that can contract or relax to control the flow of blood through the capillaries (Zaccone et al., 2012). Once filled, the volume of the swim bladder can be managed through internal gas exchange (Stewart & Hughes, 2014) that is reliant on the countercurrent gas exchange system in the *rete* (Pelster & Scheid, 1992). In most teleosts, the *rete mirabile* is responsible for this exchange (Wittenberg et al., 1964). The *rete*

mirabile is an important adaptation in fish that allows them to control their buoyancy and maintain their position in the water.

Both physostomes and physoclists regulate gas exchange to their swim bladders via gas glands and the *rete mirabile*. Since physoclists lack the pneumatic duct connection in adulthood, they remove gas via a modified area of the swim bladder that can control gas resorption by modifying the flow of blood to the region and using a muscular sphincter to regulate gas flow (Stewart & Hughes, 2014). In addition to using the gas gland to remove gas, physostomes may ‘burp’ gas from their swim bladder via the pneumatic duct (Franz, 1937).

1.4 Form and Function of the Swim Bladder

1.4.1 Form: Early stages reveal intricate epithelial and neural structures

1.4.1.1 Tissue structure of the swim bladder

While the inflated swim bladder is still in its single-chambered stage, the basal wall of the swim bladder epithelium consists of a thin, squamous cell lining along the lumen with a connective tissue layer (Robertson et al., 2007). The lateral wall also contains capillaries, fibroblasts, and the lateral nerve. Along the cranial aspect of the early swim bladder, there are blood vessels arranged in a systematic pattern resembling the *rete mirabile* of the swim bladder in adults, consistent with findings in other teleosts (Finney et al., 2006; Govoni & Hoss, 2001).

The proximal region of the pneumatic duct has similar structure to that of the swim bladder. Thick nerve bundles arising from the celiac ganglion accompany the length of the duct. At the esophageal end of the duct, the lumen of the duct becomes narrower with the

muscular wall becoming thicker. The duct itself is lined by squamous epithelial cells in a single thin layer (Zaccone et al., 2012).

1.4.1.2 Chambers

The swim bladder begins as a single-chambered organ. After initial inflation, its volume increases as body size increases until a second chamber develops by evagination, creating a second sac (Robertson et al., 2007). As both chambers then increase in size, the *ductus communicans* constricts between them, further separating them into distinct chambers. This developmental progression occurs at different times for various species. In zebrafish, initial inflation occurs around 5 dpf, with the double-chambered stage beginning around 21 dpf (Robertson et al., 2007).

1.4.1.3 Innervation

The swim bladder receives innervation from a complex network of nerves that control the muscles and surrounding tissues. This intricate innervation involves both the sympathetic and parasympathetic branches of the autonomic nervous system, which play crucial roles in regulating the swim bladder's functions. The autonomic nervous system, responsible for involuntary bodily functions, consists of three subdivisions: the sympathetic, parasympathetic, and enteric systems. In the context of the swim bladder, the sympathetic division of the autonomic nervous system extends neural pathways to the head, forming close associations with cranial nerves. Vagal fibers in the intestinal branch may

exhibit either parasympathetic characteristics or combine extra-cranially with the vagus nerve, forming a vago-sympathetic trunk (Nilsson, 2009).

In most fish species, the vagus nerve, a cranial nerve stemming from the brainstem to the viscera, is a main source of innervation for the swim bladder (Nilsson, 2009; Robertson et al., 2007). This nerve sends branches to the muscles and tissues surrounding the swim bladder, enabling the fish to regulate the bladder's volume and buoyancy. In addition, the swim bladder receives innervation from both sympathetic and parasympathetic nerves, further contributing to its control mechanisms within the autonomic nervous system.

Four key effectors within the swim bladder, subject to autonomic innervation, include resorbent blood vessels, the smooth muscle of the mucosa (*muscularis mucosae*), gas gland cells, and the blood vessels supplying them. The intricate vascular structure comprises parallel afferent and efferent capillaries of the *rete mirabile* (Nilsson, 2009). Both vagal and sympathetic stimulation contribute to the innervation of the swim bladder, with the primary supply originating from the *ramus intestinalis* of the vagus nerve (Nilsson, 2009). This dual innervation allows for precise control over blood flow, gas exchange, and overall function of the swim bladder, demonstrating the collaborative and complementary roles of the sympathetic and parasympathetic systems in regulating this essential organ.

1.4.1.4 Debate settled: The swim bladder and lung share homologous origins as evidenced by shared genes and evolutionary adaptations

If organs of two different species share similarities in function or structure and the similarity is due to having arisen from a common ancestral structure, the organs are 'homologous'. If the similarities are from independent origins, the organs are 'analogous'

(Owen, 1843). There is a long-standing debate among biologists about whether the swim bladder and the lung are homologous.

There is a growing body of evidence that suggests that the swim bladder and the lung are homologous as they share some similarities in their anatomy and development. For example, while the lung and the swim bladder have differences in function and morphology, the last common ancestor of tetrapods and teleosts had a simple lung-like organ that gave rise to the lung in tetrapods and the swim bladder in teleosts (Laurin, 2010) (Figure 3). These primitive aquatic lungs evolved as an adaptation to hypoxic stress in the Paleozoic era (Graham et al., 1978) which later diverged into the teleost swim bladder and the tetrapod lung. Recent investigations have provided compelling molecular evidence to support the swim bladder – lung homology hypothesis. For example, co-expression of several genes previously thought to be only in the tetrapod lung are also co-expressed in the zebrafish swim bladder. The *FoxA2* and *GATA6* genes are involved in early-acting endoderm specification and are expressed in the same relative location – spatially and temporally – in both types of animals (Cass et al., 2013). Additionally, several specific Hox genes have been identified in common between the developing human lung, mouse lung, and fish swim bladders (Cui et al., 2014; Zheng et al., 2011).

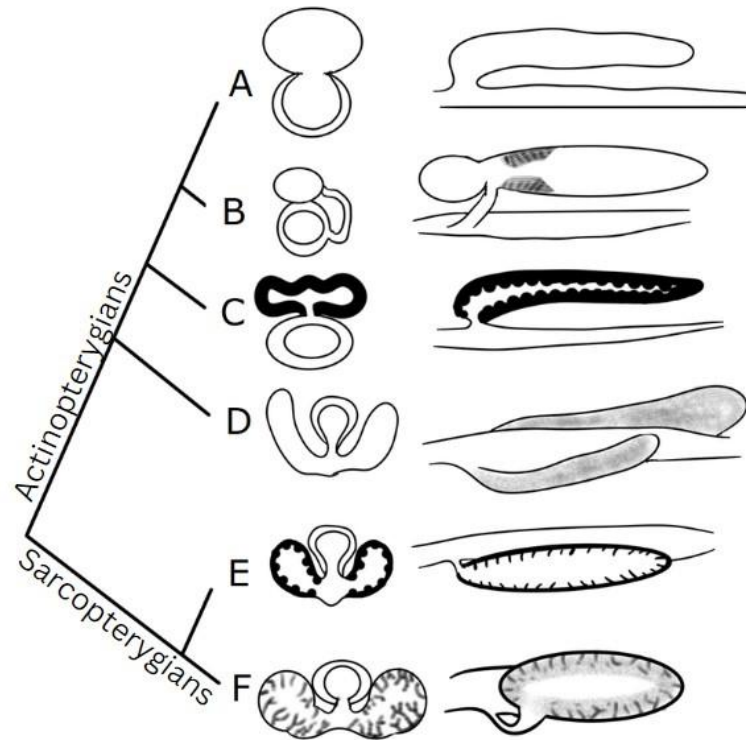


Figure 3: Phylogeny of the swim bladder with drawn depictions of the respective structure for each taxon. A. Typical teleost. B. The teleost *Erythrinus*. C. *Lepisosteus* and *Amia*. D. *Polypterus* and *Erpetoichthys*. E. The dipnoans (lungfishes) *Lepidosiren* and *Protopterus*. F. The tetrapod lung. Recreated from Figure 1.18 of (Laurin, 2010).

Conversely, lungs and gills are analogous structures. While they do both perform the same function – gas exchange – they have different evolutionary origins. Evidence of this is seen in dipnoans, which possess both gills and lungs (Laurin, 2010). As they appear together, they cannot be homologous. Developmentally, they also differ in that gills are outgrowths of the aortic arches while lungs are outgrowths of the pharynx (Laurin, 2010).

1.4.2 Function: Swim bladders provide fish with buoyancy regulation, efficient swimming, and house unique microbiomes

1.4.2.1 Dynamic control of buoyancy

Swim bladders can be classified as having either a respiratory function, as seen in lungfish, or as non-respiratory as seen more commonly. The non-respiratory swim bladders allow neutral buoyancy for the fish (Pelster, 2004; Steen, 1970). Physostomes regulate the volume of their swim bladders by gas secretion and resorption as well as by intake and expulsion of gas via the pneumatic duct. This pneumatic duct burping is performed by the opening of the pneumatic duct sphincter, contracting the smooth muscles of the swim bladder, and forcing the expulsion of gas from the mouth (Franz, 1937).

By adjusting the amount of gas in the swim bladder, fish use less energy to maintain position in the water column or swim at different depths. In addition, the swim bladder plays a role in the fish's ability to maintain its orientation and stability in the water, which is important for swimming horizontally. Volume maintenance is required to maintain neutral buoyancy as the hydrostatic pressure changes (Stewart & Hughes, 2014). The volume of a gas is inversely proportionate to the pressure (Power, 1664), such that as a fish descends the water column, the increase in pressure causes the volume of the swim bladder to decrease, resulting in the fish becoming negatively buoyant. To counteract this, the fish secretes gas into the swim bladder. The inverse is true for ascending the water column, and the fish resorbs gas from the swim bladder to counteract positive buoyancy.

1.4.2.2 Microbiome reservoir

The microbiome reservoir is a term used to describe the diverse community of bacteria that live in the gut of fish species. The specific role of bacteria in the gut can vary depending on the species of fish and the types of bacteria present, but they are generally involved in processes such as the breakdown of complex sugars, the production of vitamins and other beneficial compounds, and the suppression of harmful pathogens (Dyková et al., 2021; Khurana et al., 2020). How this reservoir is related to swim bladder function is largely unknown. However, a recent in-depth analysis of rainbow trout (*Oncorhynchus mykiss*) revealed that the swim bladder hosts a distinct microbiota to that of the gut in both composition and metabolic function (Villasante et al., 2019). This revelation highlights yet another aspect of the swim bladder that remains under-researched to date.

1.5 Swim bladder Disorders and Associated Pathologies

1.5.1 Types and timing of swim bladder disorders

Swim bladder disorders can manifest as under-filling (hypoinflation) or over-filling (hyperinflation) of the organ. In either case, the affected fish is unable to maintain buoyancy without excessive energy input, with hypoinflated fish sinking to the bottom and the hyperinflated fish floating at the surface. Hyperinflation disorders typically occur in adulthood due to illnesses such as rete vasculitis, gas supersaturation, bacterial infections, and other ailments (Boylan et al., 2017; Sirri et al., 2020), though it can occur upon initial inflation in larvae with lacking lateral line function (Venuto et al., 2023), or through filling with non-air substitute such as oil, as seen in Chapter 3.

Hypoinflation commonly occurs because of a failure to initially inflate the bladder during larval stages, or due to injury or infection as adults (Boylan et al., 2017; Clayton & Summerfelt, 2013). There can be environmental causes of non-inflation of the swim bladder. For example, hypoinflation has been attributed to the ingestion of bacteria, oils, and other debris from the surface of the water (Clayton & Summerfelt, 2010; Marty et al., 1995; Tsuji et al., 2016). As the larva breaks the air-water interface, this debris is ingested and pushed through the pneumatic where it embeds within the swim bladder lumen, preventing successful inflation (Marty et al., 1995). Increased turbulence at the air-water interface can also decrease the rate of successful inflation, as larvae are unable to breach the surface to gulp air (Schwebel et al., 2018), though the opposite has been shown in some cases (Doroshev & Cornacchia, 1979). Cultured striped bass (*Morone saxatilis*) have anomalous swim bladder inflation 20-70% of the time which can be a significant problem for hatcheries (Bennett et al., 1987). Successful swim bladder inflation rates can also be attributed to larval rearing conditions, with high success and increased overall growth favouring high intensity artificial lighting in species that are positively phototaxic (Woolley et al., 2014).

Failure to properly inflate a swim bladder can result in serious consequences to the fish, described below. In some cases, the pathology of non-inflation may be reversible, but in other cases, it may be permanent. Further research is needed to fully understand the pathology of non-inflation of the swim bladder in fish.

1.5.2 Physiological pathologies of buoyancy disorders include growth retardation, skeletal malformations, and death

1.5.2.1 Death

Non-inflation of the swim bladder is a risk factor for mortality in larval fish (Table 1). Difficulty with swimming can impede a fish's ability to catch prey for food, resulting in death due to starvation as well (Doroshov et al., 1981). This is not always the case, however, as uninflated yellow perch (*Perca fluiatilis*) can survive upwards of 10 months if provided with food in abundance (Egloff, 1996), for example. In zebrafish, uninflated larvae do show increased mortality with one study reporting just 27% survival by 14 dpf (Goolish & Okutake, 1999). Although given food, these zebrafish are presumed to have starved to death as an absence of stomach contents was observed in the uninflated larvae. In other cases, mortality in hatchery-reared gilthead sea bream (*Sparus aurata* L.) is observed primarily among larvae without inflated swim bladders, but potential causative factors were not investigated (Modica et al., 1993). In all these cases, death is a common outcome of hypoinflation across fish species except in the case of Pacific bluefin tuna (*Thunnus orientalis*) (Kurata et al., 2012). In this case, mortality was not different between inflated and hypoinflated tuna, though analysis of mortality did not begin until 18 days post-hatch which may have been an oversight for reporting on the link between hypoinflation and death. The consistency with which increased mortality is seen in hypoinflated fish highlights the importance of understanding the pathological consequences of this phenomenon.

Table 1: The result of swim bladder hypoinflation on survival in various fish species, including their cause of death and method of observation/type of assay.

Location	Species	Method of Observation	Result of Hypoinflation on Survival	Cause of Death	Source
Field	Yellow perch	Direct observation while snorkeling in various lakes	Reduced relative abundance	Presumed predation	(Egloff, 1996)
Field	Yellow perch	Catching samples using lift nets in various lakes	Reduced relative abundance	Presumed death	(Egloff, 1996)
Lab	Zebrafish	Direct counts of experimental larvae in laboratory	27% survival of uninflated larvae compared to 76% of inflated larvae	Presumed starvation	(Goolish & Okutake, 1999)
Lab	Yellow perch	Direct counts of larvae undergoing starvation in laboratory	Uninflated larvae show increased starvation mortality sooner than inflated larvae	Starvation	(Czesny et al., 2005)
Field	Yellow perch	Counts of inflated vs uninflated in samples of wild population in Lake Michigan	Relative occurrence of uninflated fish reduced over the season until no uninflated fish were collected	Presumed predation	(Czesny et al., 2005)
Lab	Pacific bluefin tuna	Direct counts of experimental larvae in oceanic net pens	No significant difference between inflated and uninflated fish long-term	n/a	(Kurata et al., 2015)
Lab	Gilthead sea bream (<i>Sparus aurata</i>)	Daily direct observation of deceased larvae in laboratory rearing tanks	Uninflated larvae makeup ~80% of the deceased fish until 50 dpf, then the deceased larvae are ~50% uninflated or 50% presenting with kidney calculosis	Non-inflation	(Modica et al., 1993)
Lab	Three-spined stickleback (<i>Gasterosteus aculeatus</i>)	Direct observation of larvae with induced hypoinflation	8/10 induced hypoinflated larvae perished early in development. The 2 survivors were cared for and lived for 10 months before being euthanized.	Presumed malnutrition/ Starvation from increased energy consumption	(J. F. von Ledeber & Wunder, 1937)
Lab	Black Sea turbot (<i>Scophthalmus maeoticus maeoticus</i>)	Direct observation during larval egg hatching and larval rearing	Larvae with uninflated swim bladders had severe swimming deficits and fin deformations but guts full of food. By 17 dpf, mass mortality began even with observed feeding still on-going.	The inability to initially inflate the swim bladder due to laboratory rearing conditions	(Spectorova & Doroshev, 1976)
Lab	Japanese medaka (<i>Oryzias latipes</i>)	Direct counts during an initial inflation analysis where air-water interface access was restricted	Larvae that were previously restricted from the surface during the normal initial inflation period had increased mortality.	Presumed lack of feeding behaviour	(Pandelides et al., 2021)

1.5.2.2 Feeding, starvation & growth

Swim bladder inflation is correlated with higher rates of growth. The swim bladder is an important organ for controlling buoyancy, and a fish with a non-inflated swim bladder will have difficulty maintaining its position in the water. This can affect the fish's ability to swim and forage for food, leading to increased energy expenditure and reduced growth. For example, California yellowtail (*Seriola dorsalis*) with uninflated swim bladders have a higher feed conversion ratio and are slower to grow than yellowtail with inflated swim bladders, resulting in lower body mass, shorter length, and smaller girth at the end of the grow-out period, but they do survive to adulthood (Schwebel et al., 2018).

Uninflated bluefin tuna (*Thunnus orientalis*) that survived the early larval stage showed smaller lengths and body weights as juveniles (22 dpf) but after 37 dpf, these differences were no longer significant indicating hypoinflation only contributed to growth retardation temporarily (Kurata et al., 2015). Uninflated yellow perch (*Perca flavescens*) also grow more slowly both in the lab and in the field (Czesny et al., 2005). In another study, uninflated perch larvae sampled from the wild were, on average, shorter and had a smaller height to length ratio (corpulence) than inflated larvae of the same sampling groups (Egloff, 1996).

Some research has noted a lack of food in the guts of uninflated larvae (Goolish & Okutake, 1999), while others specifically note intestinal food in uninflated larvae (Czesny, 2005; Hashimoto, 2012; Modica et al., 1993; Ribi, 1992; Spectorova & Doroshev, 1976). Regardless, fish that are uninflated appear to be affected by starvation faster than those with inflated swim bladders. In a study on yellow perch larvae, those with inflated swim bladders began to suffer increased mortality from starvation starting at 110 hours after

withholding food, but uninflated larvae began dying at just 60 hours. The inflated group did not reach 100% mortality from starvation until 160 hours, whereas the uninflated group perished completely by 136 hours (Czesny et al., 2005). Given that both groups of larvae were unfed, these results suggest that the premature death of uninflated perch is not due to starvation *per se*, but due to some other unidentified pathology associated with hypoinflation.

1.5.2.3 *Lordosis & locomotor behaviour*

Larvae of various species that fail to properly inflate their swim bladders often present with lordosis – a deformity in the spinal curvature of the fish. (Kitajima et al., 1994). The spinal curvature is caused by deformation of two or three sequential vertebrae. The extent of the deformation occurs on a spectrum, with more severe lordosis observable externally, and minor defects only distinguishable via X-ray. The vertebral damage seems to be induced by the high pressure exerted on the spine from the compensatory upward swimming required of negatively buoyant larvae (Egloff, 1996). Up to 40% of yellowtail larvae with uninflated swim bladders exhibit significant lordosis by the end of their 32 week grow-out (Schwebel et al., 2018). Cultured striped bass (*Morone saxatilis*) with uninflated swim bladders exhibit deficits in swimming behaviour (Bennett et al., 1987). The lack of inflation appears to cause an increased demand for energy expenditure when swimming, resulting in larval exhaustion with the fish settling at the bottom of the culture tanks. Uninflated striped bass fingerlings have survived to reach 15 cm in body length, however, 100% of these fish show spinal deformities and swim anomalously with a ‘head up’ swimming posture. It is unclear if these fish survive to adulthood, as they are removed from hatcheries during regular grading sessions. These studies highlight the effect to which

negative buoyancy can result in lordosis due to the altered swimming pattern in hypoinflated fish.

1.5.2.4 Metabolism, energy expenditure & respiration

As the swim bladder helps control buoyancy, a fish without a properly inflated bladder will expend more energy during swimming. For example, uninflated California Yellowtail larvae (*Physoclisti*) required 40% more food to gain the equivalent mass as their normally inflated counterparts (Schwebel et al., 2018) potentially indicating energy conversion deficits in uninflated larvae. Yellow perch and California yellowtail were also analyzed with respirometers for measurement of oxygen consumption. Yellow perch oxygen usage was measured while the fish were stationary (Czesny et al., 2005) while the California yellowtail fish were measured in a swim tunnel (Schwebel et al., 2018). In both studies, the uninflated fish consumed more oxygen, suggesting increased energy expenditure compared to inflated fish. These findings underline the critical role of swim bladder functionality in efficient energy utilization for fish.

1.5.3 Pathologies of uninflated swim bladders include hyperplasias, inflammatory responses, and cell death

In early larval stages, the swim bladder wall consists of columnar epithelium that develops into squamous epithelium under typical circumstances, along with squamous endothelium with fibroblasts and connective tissue (Goodsell et al., 1996; Robertson et al., 2007). The pathologies associated with swim bladder non-inflation encompass a range of morphological abnormalities, often involving hyperplasia of the epithelium and is sometimes accompanied by cell death, fibrosis, and signs of an inflammatory response

(Table 2). Inflammation is a complex response that is triggered by tissue damage or infection and is characterized by the recruitment of immune cells to the affected area. It is not clear whether non-inflation of the swim bladder causes an inflammatory response in fish with mixed reports across species. For example, Turquoise Killifish (*Nothobranchius furzeri*), Amberjack (*Seriola dumerili*), and Sea Bass (*Lateolabrax japonicus*), showed varying degrees of hyperplasia without clear indications of immune cell infiltration or microbial infection (Žák et al., 2023; Imai et al., 2011; Makino et al., 1995). Medaka (*Oryzias latipes*) develop swim bladder adenomas when they were restricted from the air-water interface (Furukawa et al., 2022) and turbot (*Scophthalmus maximus*) retain a columnar epithelium in noninflated swim bladders, show hyperplasia of gas gland cells and *rete mirabile*, and exhibit bacterial infection with ‘intense necrosis’ in neighboring tissues (Padrós & Crespo, 1995; Padrós et al., 1993). Additionally, striped bass larvae with non-inflated swim bladders show cellular degeneration and necrosis, as well as fibrotic degeneration, but again inflammation was not noted (Bennett et al., 1987). Alternatively, in larval walleye (*Sander vitreus*), ingestion of bacteria and debris from the air-water interface into the swim bladder resulted in macrophage invasion (Marty et al., 1995), though it remains unclear whether this ingestion caused the non-inflation or whether comorbidity of unknown pathology exacerbated the infection in hypoinflated larvae. Histological evaluation of these swim bladders suggests the extent of the damage, regardless of cause, appears to be irreversible.

In the case of the striped trumpeter (*Latris lineata*), non-inflation is associated with the maintenance of columnar epithelial morphology and epithelial folding (Goodsell et al., 1996; Trotter et al., 2001; Trotter et al., 2004). The presence of bacteria was noted, and fish

were unable to inflate their swim bladders even though the pneumatic duct was open (Trotter et al., 2001; Trotter et al., 2004). Similarly, in the angel fish cichlid (*Pterophyllum scalare*), the epithelium of an uninflated swim bladder exhibits a hypertrophic, cuboidal morphology but with no signs of bacteria or inflammation (Perlberg et al., 2008; Sanabria et al., 2009; Zilberg et al., 2004). Sea bream (*Sparus aurata*) display hypertrophic swim bladders in the absence of inflation with invasive growth of epithelial tissue in the lumen, but with no report of inflammation or infection, consistent with the angel fish cichlid findings (Paperna, 1978; Soares et al., 1994). Likewise, uninflated swim bladders in the Giant grouper (*Epinephelus lanceolatus*) and pikeperch (*Sander lucioperca*) also exhibit disorganized hyperplasia and fibrosis (Canepa et al., 2023; Demska-Zakêoe et al., 2003; Iwasaki et al., 2016), with hyperplasia of the gas gland also in the latter. The intricate array of pathologies observed in swim bladder non-inflation across various fish species underscores the complexity of this phenotype.

Table 2: The known pathologies associated with swim bladder non-inflation of various fish species, including whether hyperplasia or inflammation were noted.

Common Name (Species)	Hyperplasia	Inflammation	Other Pathologies	Source
Striped trumpeter (<i>Latris lineata</i>)	No	No	Columnar epithelium and epithelial folding; bacterial presence	Goodsell et al., 1996; Trotter et al., 2001; Trotter et al., 2004
Angel fish cichlid (<i>Pterophyllum scalare</i>)	Yes	No	Cuboidal epithelium	Perlberg et al., 2008; Sanabria et al., 2009; Zilberg et al., 2004
Sea bream (<i>Sparus aurata</i>)	Yes	No		Paperna, 1978; Soares et al., 1994
Giant grouper (<i>Epinephelus lanceolatus</i>)	Yes	No	Disorganized hyperplasia; fibrosis	Canepa et al., 2023; Iwasaki et al., 2016
Pikeperch (<i>Sander lucioperca</i>)	Yes	No		Demska-Zakêoe et al., 2003
Killifish (<i>Austrolebias nigrofasciatus</i>)	Yes	No	Bleeding into the swim bladder	Fonseca et al., 2018
Turquoise killifish (<i>Nothobranchius furzeri</i>)	Yes	No		Žák et al., 2023
Amberjack (<i>Seriola dumerili</i>)	Yes	No		Imai et al., 2011
Sea bass (<i>Lateolabrax japonicus</i>)	Yes	No		Makino et al., 1995
Striped bass (<i>Morone saxatilis</i>)	No	No	Fibrosis; cellular degeneration; necrosis	Bennett et al., 1987
Tilapia (<i>Sarotherodon mossambica</i>)	Yes	No	Developmental arrest and disorganization of columnar epithelium; tissue degeneration	Doroshev and Cornacchia, 1979; Doroshev et al., 1981
Medaka (<i>Oryzias latipes</i>)	No	No	Swim bladder adenomas	Furukawa et al., 2022
Turbot (<i>Scophthalmus maximus</i>)	Yes	Yes	Columnar epithelium; necrosis in neighbouring tissues	Padrós & Crespo, 1995; Padrós et al., 1993

Walleye (<i>Sander vitreus</i>)	No	Yes	Macrophage invasion	Marty et al., 1995
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1.6 Remaining Unknowns in Relation to Hypoinflation Pathologies

1.6.1 The swim bladder – gut relationship in fish remains poorly understood

The potential impact of swim bladder pathology, including inflammation and/or infection, on the gut is a critical aspect that requires further exploration. The existing literature does not agree on whether fish with uninflated swim bladders die mainly from starvation caused by increased energy use or if underlying gut or metabolism issues play a role in this condition. The specific nature of these concomitant factors and their precise contribution to mortality is yet to be fully revealed, but a link to the gut is likely. For example, bacterial infection of the swim bladder in hypoinflated turbot is associated necrosis of neighbouring tissues (Padrós et al., 1993). This spreading of cellular degradation to adjacent tissues could be causing damage to the gut of hypoinflated larvae.

Supporting this notion of a connection between the swim bladder and the digestive tract, the pneumatic duct may serve as a portal for foreign particles or microbes to travel between the two organ systems. African annual killifish (*Nothobranchius*) with secondary loss of swim bladder function due to mycobacterial infection (Dykova et al., 2021) were infected via infiltration through the pneumatic duct. The infection is initially localized to the swim bladder in juveniles but becomes systemic in adults with mycobacteria present in various organs, including the gut. This evidence supports the idea that swim bladder pathologies, including infections, may have repercussions on the health and integrity of the gut in fish. This thesis aims to address these knowledge gaps and contribute to a more comprehensive understanding of swim bladder hypoinflation and its associated pathologies, with a focus on the zebrafish model system.

Chapter 2: Critical Period of Initial Swim Bladder Inflation

2.1 Critical Period of Initial Swim Bladder Inflation Background

A critical period for larval fish is associated with a temporal window when mortality risks are high, and often associated with depletion of the yolk and starvation-related incidences (Ehrlich, 1974). Initial inflation is also a crucial point in development for survival that also coincides with yolk depletion and the onset of exogenous feeding (Clayton & Summerfelt, 2013; Goolish & Okutake, 1999; Tsuji et al., 2016; Woolley et al., 2014). A critical period in terms of initial swim bladder inflation for physostomes is a temporal window within which the fish must gulp air to complete the developmental process of initial inflation, after which inflation is no longer possible. The endpoint of this period is the time in which 50% of the fish are no longer able to inflate (May, 1974). The critical period for swim bladder inflation has not been described in zebrafish and reveals a gap in our understanding of the development of this widely studied and important model organism.

Functional degradation of the connection between the gut and swim bladder lumen marks the time when a swim bladder can no longer inflate in physoclists (Rieger & Summerfelt, 1998). For example, in the physoclistous walleye (*Sander vitreus*), larvae that fail to inflate their swim bladder exhibit normal cellular differentiation of the foregut but the pneumatic duct atrophies, resulting in a loss of ability to inflate their swim bladders. Subsequently, uninflated walleye suffer from degradation of the swim bladder along with a concomitant invasion of immune cells and bacteria (Marty et al., 1995). However, as zebrafish are physostomes and retain their pneumatic duct into adulthood (Dumbarton et al., 2010), theory would predict that zebrafish do not possess such a critical period.

However, literature (Goolish & Okutake, 1999) and our own observations show that non-inflation correlates with high levels of mortality at a timepoint coinciding with the completion of yolk resorption; a critical timepoint for survival in larval fish (Ehrlich, 1974). Physoclistous Japanese medaka (*Oryzias latipes*) exhibit this critical window and must inflate within a 5-day window or the ability is lost. Failure to do so within this window results in increased mortality (Pandelides et al., 2021). I initially hypothesized that zebrafish do not possess a critical period of inflation as they are physostomous fish. However, non-inflation may be increasing their mortality risk nonetheless, perhaps due to inflammatory responses, degenerative tissue damage, or other insurmountable developmental challenges faced by uninflated larvae.

To test whether zebrafish larvae are subject to a critical period for initial swim bladder inflation, I restricted air access to larval zebrafish at the time they would normally initially inflate their swim bladders. After allowing them air access for a period of 24 hours, I analysed them for successful inflation with a buoyancy test and microscopic confirmation. These restricted larvae were compared to those with unrestricted access to the surface. This experiment revealed that, contrary to theory, there is a critical window of initial inflation competency that ends after 7 dpf, though the demarcation is tempered by the associated increased mortality.

2.2 Methods

2.2.1 Fish husbandry

Adult zebrafish (Tübingen strain) were maintained at the University of New Brunswick's Aquatic Facility at 10 Bailey Drive, Fredericton, New Brunswick, Canada. Fish were maintained on a 14:10h light:dark cycle and cared for according to standard

conditions (Westerfield, 2000). Embryos for experimentation were obtained from natural mating events and reared in E3 medium (Appendix 1) at 28.5°C under the same photoperiod conditions as the adults.

The zebrafish larvae were fed commercial zebrafish larvae food (GEMMA Micro 75, Skretting, Westbrook ME), a live *Paramecium aurelia* culture, or both. The *Paramecium* culture was maintained with wheat berries and E3 medium, with the culture split every two weeks to maintain a clean and stable population density. Each tube containing up to 25 larvae received 3 mL of filtered culture, approximately equivalent to 1000 *Paramecia*. This feeding regimen was administered twice daily, starting from 5 dpf.

2.2.2 Experimental design

The research objective of this experiment was to determine if there was a temporal window of inflation competence in larval zebrafish. To induce hypoinflation in larval zebrafish, I restricted the ability of larvae to access the surface by using a custom-built apparatus to restrict larval zebrafish from accessing the air-water interface (Figure 4). The apparatus was inspired by the glass tube method utilized by researchers in 1937 (J. F. von Ledeber, 1937). To modify the conical tubes, the conical end was cut off and 70 µm mesh was glued to the opening to allow free water exchange. The screw cap end was fitted with a 70 µm removable cell strainer (VWR, Radnor, PA) to allow for water flow-through as well as to allow larval feeding. These tubes were then submerged within the water column to ensure the larvae could not access the air-water interface. Control larvae were placed within similarly modified tubes that were positioned to allow for larval access to the surface. Control and restricted larvae were raised within the same apparatus to eliminate experiment-to-experiment variations in water conditions as confounding factors. An air

stone in the tank ensured proper oxygen saturation within the media and 1-2 L of the media was exchanged for fresh media daily.

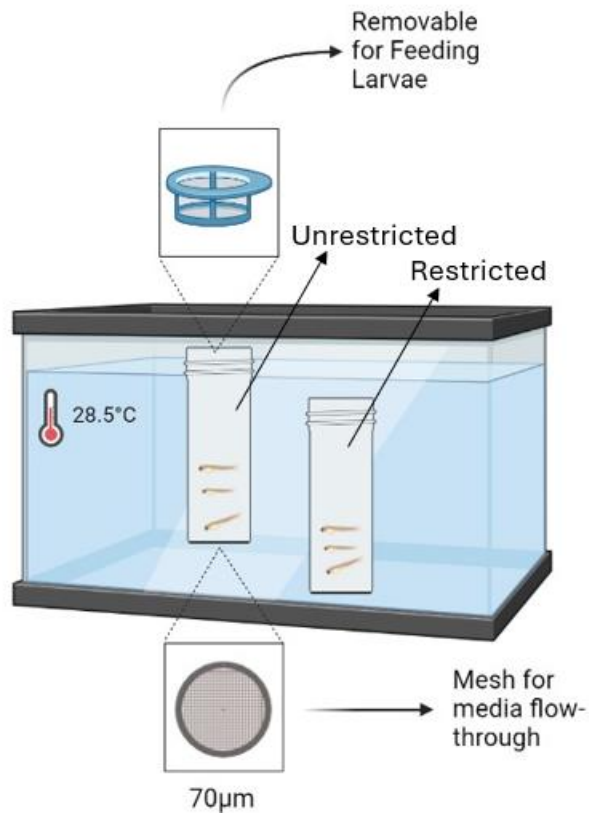


Figure 4: Depiction of the air-water interface restriction apparatus and larval tube construction. The E3 medium in the apparatus was maintained at 28.5 °C and the pH and dissolved oxygen levels were monitored daily. Image created using BioRender.com.

Since zebrafish initiate their pre-inflation surfacing behaviours after 3 dpf, the larvae were placed in the apparatus at 3 dpf. I used a total of 900 larvae in this investigation, split between 3 trials. Each trial consisted of six timepoints with 50 larvae per timepoint (25 restricted and 25 control), totaling 300 larvae per trial. At 4 dpf, one tube from the restricted group was allowed access to the surface for 24 hours. The next day, at 5 dpf, these larvae were anesthetized with 0.016% MS-222 (ThermoFisher #50-208-3317, Waltham, MA) (Appendix 2). To assess for successful inflation, the larvae were placed

individually at the 25mL mark in a 50 mL conical tube filled with E3 medium. Larvae that remained where they were placed in the column after a period of 20 seconds were considered inflated, and those that sank were considered uninflated. These larvae were then imaged using light microscopy (Zeiss Stemi SV11, Zeiss, Oberkochen, Germany) fitted with a Basler camera (Basler PowerPack, Ahrensburg, Germany) controlled using the Basler Microscopy Software 2.1. Successful or unsuccessful inflation was noted for each larva. The next tube of larvae at 5 dpf was then allowed access to the surface for the next 24 hours, again being imaged at 6 dpf to quantify inflation. This series of experiments continued until 10 dpf larvae from the last timepoint were imaged. Alongside the restricted larvae, a tube of control larvae were also imaged at each timepoint for comparison.

In addition to recording the inflation status of each larva, I used ImageJ (Schneider et al., 2012) to estimate the volume of the successfully inflated swim bladders to see if there was any difference in volume between previously restricted larvae and unrestricted larvae. Using the formula for an ellipsoid (Figure 5A), I estimated the volume of the inflated swim bladders in both treatment groups. Using ImageJ, I measured the horizontal (red of Figure 5B) and the vertical (yellow of Figure 5B) diameters of the inflated swim bladders. Larvae that failed to inflate their swim bladders were excluded from this analysis. These measurements are equal to '2b' and '2c' using the ellipsoid volume formula. Since I am assuming the swim bladder takes an ellipsoid shape, I can then assume that the value of 'a' is equivalent to 'c', which I have from the ImageJ measurements. With these data, I used R (R Core Team, 2023) to perform an ANOVA and post-hoc Z-test for significance. Overall, this design allowed for a comprehensive evaluation of if and when, a critical window for initial swim bladder inflation occurs.

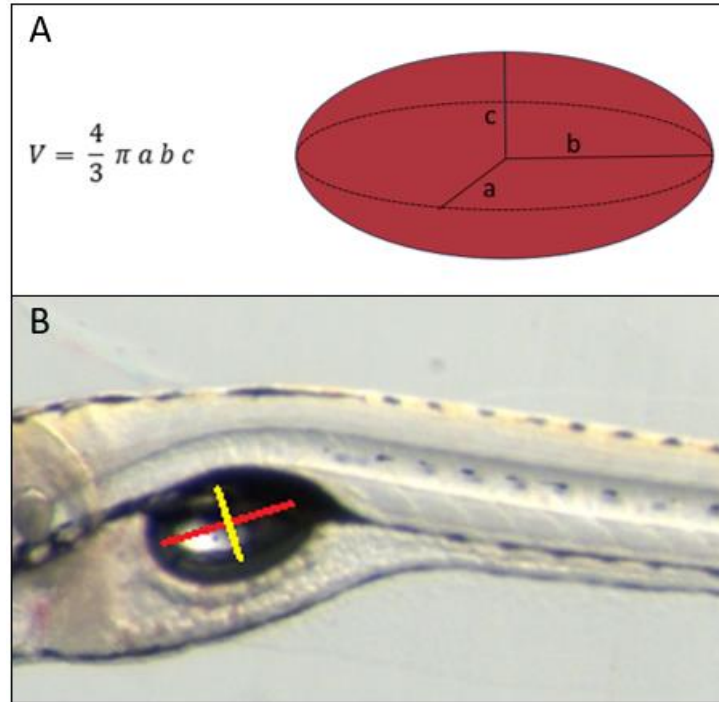


Figure 5: The larval swim bladder is shaped like an ellipsoid. Measuring the x-axis and y-axis diameters allows for the calculation of an approximate volume. A: The formula for the volume of an ellipsoid (left) with the visual representation of the values a, b, and c in the context of an ellipsoid (right). B: A larval zebrafish with an approximation of the measurements made with the yellow line as the x-axis diameter and the red line as the y-axis diameter.

2.3 Results & Data Analysis

2.3.1 The critical window for initial inflation is closed by 8 dpf

Zebrafish normally inflate their swim bladders between 4-5 dpf. To determine if there is a critical period during which zebrafish larvae must initiate swim bladder inflation, I restricted pre-inflated larvae from the air-water interface for successively later timepoints and quantified successful inflation after a 24-hour period of access to the surface. The visual confirmation of swim bladder inflation from the images was corroborated by the floating or sinking in a conical tube as described in the Methods. The data are summarized as the proportion of successfully inflated larvae (Table 3, Figure 6A). As not all the larvae

survived to be analyzed in this way, the total number of datapoints for this analysis is 719 rather than 900. I performed a two-way ANOVA to test for the effect of restriction status on inflation ability at different timepoints. There is a statistically significant difference in inflation ability between at least two groups indicating a significant main effect of age of the larval fish. Additionally, restriction status also had a significant effect on inflation ability. The interaction between age and restriction status was also statistically significant (Table 4). A post-hoc Z-test to determine at which age the significance is at was performed using R (Table 3) (R Core Team, 2023). The results indicate a significant decrease in the ability of zebrafish larvae to initiate swim bladder inflation at 8 dpf, 9 dpf and 10 dpf (Figure 6A).

Table 3: Proportion of successful inflation for restricted and unrestricted larvae from 5 dpf to 10 dpf, based on whether they were neutrally buoyant (successful inflation) or negatively buoyant (uninflated) (n=719).

	Unrestricted Control						Restricted						
Age	Trial 1		Trial 2		Trial 3		Trial 1		Trial 2		Trial 3		P=
	Proportion	n	Proportion	n	Proportion	n	Proportion	n	Proportion	n	Proportion	n	value
5 dpf	0.955	22	1.000	24	1.000	24	0.810	21	1.000	25	1.000	24	0.528
6 dpf	0.905	21	0.960	25	1.000	25	0.682	22	0.960	25	1.000	24	0.539
7 dpf	1.000	23	1.000	24	1.000	23	0.7823	23	1.000	22	1.000	24	0.424
8 dpf	1.000	20	0.860	22	1.000	24	0.350	20	0.630	19	0.500	16	0.014
9 dpf	1.000	16	0.960	23	1.000	25	0.190	21	0	10	0.125	8	0.003
10 dpf	1.000	15	1.000	24	1.000	23	0.222	9	0	3	0	0	0.006

Table 4: Two-way ANOVA output for the effect of restriction status and age on inflation ability.

Source of Variation	SS	df	MS	F	p-value
Age	1.2148	5	0.2430	25.76	6.48e-09
Restriction Status	1.5150	1	1.5150	160.59	4.01e-12
Age:Restriction Status	1.2720	5	0.2544	26.97	4.10e-09
Residuals	0.2264	24	0.0094		

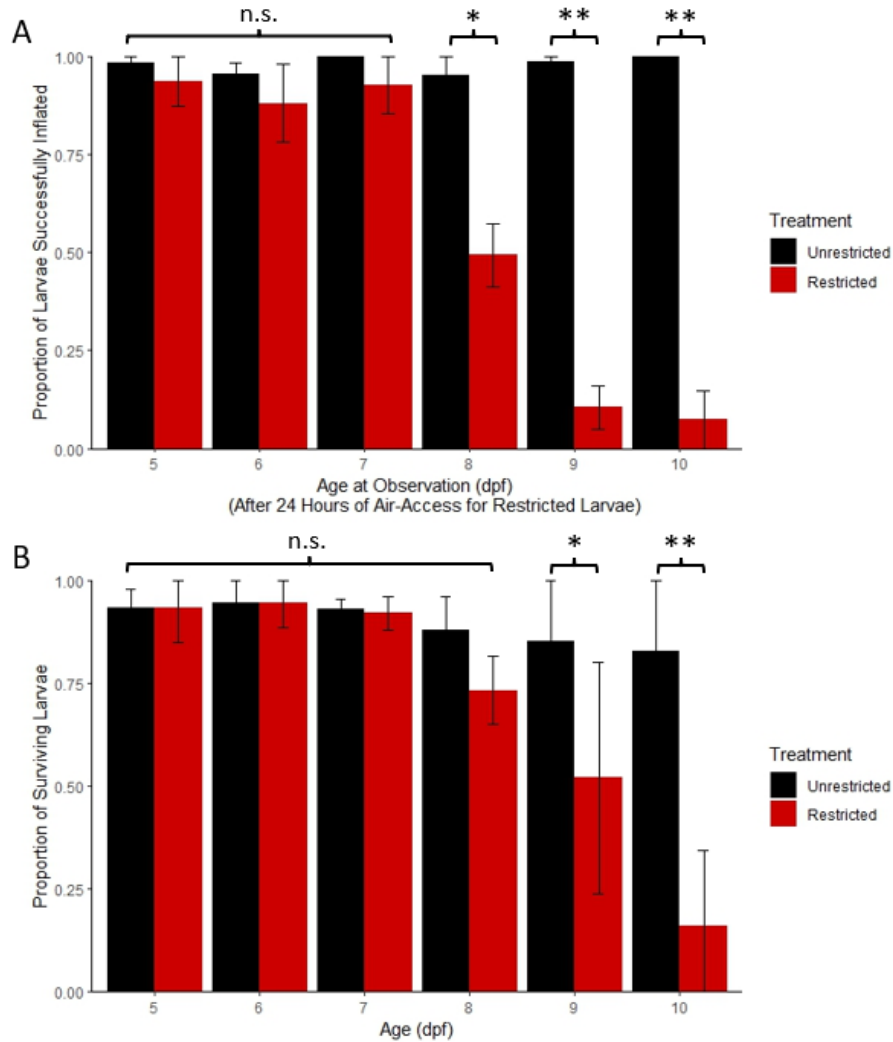


Figure 6: Zebrafish larvae fail to initially inflate if restricted from the air-water interface up to 7 dpf. A: Proportion of larvae that successfully inflated their swim bladders after being restricted from the surface until 24-hours before imaging (n = 70, 71, 69, 55, 39, 12 at 5, 6, 7, 8, 9, and 10 dpf, respectively), compared to control larvae whose access to the surface was not restricted (n = 70, 71, 70, 66, 64, 62). Error bars represent standard deviation. B: The proportion of survival for restricted and unrestricted larvae from the critical period of inflation experiment in A. Significance indicated as: n.s. = no significance, * = $P < 0.05$, ** = $P < 0.01$.

2.3.2 Swim bladder volume does not differ between previously restricted and unrestricted larvae from 5 dpf to 10 dpf

For larvae that were successful in inflating their swim bladders, I estimated their swim bladder volumes using microscopic imaging and measurements using ImageJ

(Schneider et al., 2012) (Figure 7). The two-way ANOVA revealed that there is no difference in swim bladder volume between restricted and unrestricted larvae across timepoints (Table 5).

Table 5: Two-way ANOVA output for the effect of restriction status and age on swim bladder volume in the case of successful inflation. Total n = 445.

Source of Variation	SS	df	MS	F	p-value
Age	6146517	5	8306745	28.565	<2e-16
Restriction Status	136316	1	136316	2.344	0.12651
Age:Restriction Status	1430826	5	1027112	3.532	0.00385
Residuals	25183512	433	58161		

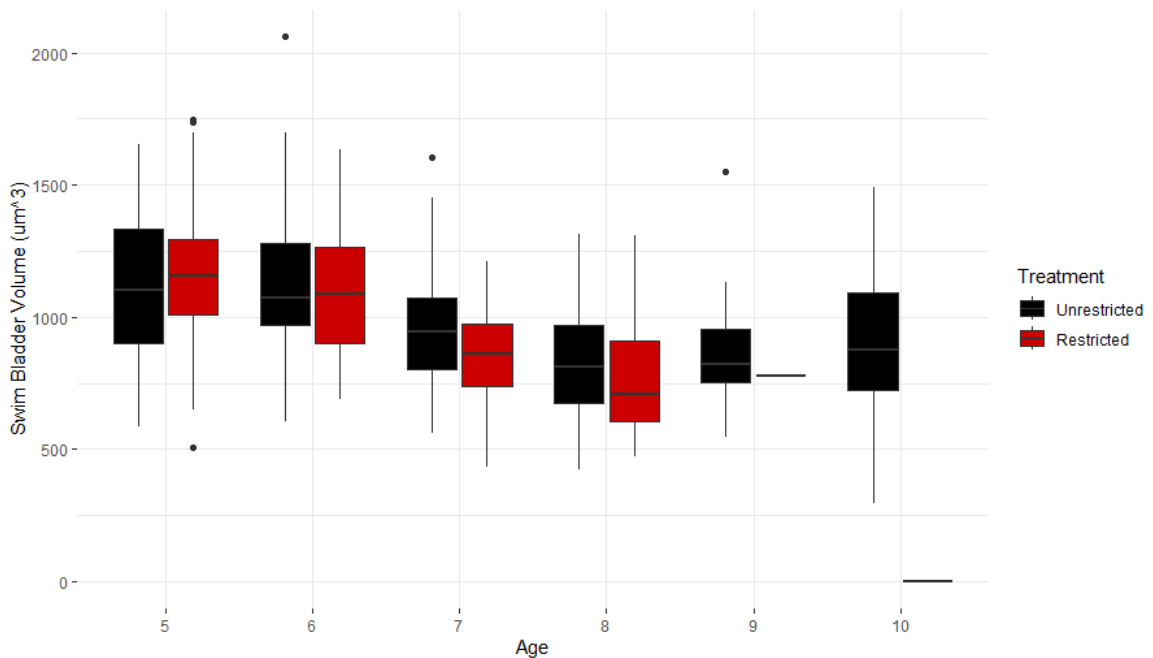


Figure 7: The volume of inflated swim bladders is not different between unrestricted and previously restricted larvae from 5 dpf to 10 dpf. The previously restricted larvae were measured 24 hours after they were permitted access to the surface to initiate initial inflation. n= 445 (Table 5).

2.3.3 Larvae that have not inflated their swim bladders by 9 dpf exhibit increased mortality

Coincidentally with the data gathered in Section 2.3.1 for successful swim bladder inflation, I noticed that there was a significant decline in survival of the restricted larvae over time. Decreased survivorship in uninflated larvae has been noted in a wide variety of fish species in both field and laboratory settings as described in Section 1.5 (Table 1). In zebrafish larvae, a direct analysis of survivorship has not been performed until now (Figure 6). I analyzed the effect of age and inflation status, and their interaction on survival using a two-way ANOVA. There is a statistically significant difference in survival between at least two groups indicating a significant main effect of age of the larval fish. Additionally, inflation status also had a significant effect on survival. The interaction between age and inflation status was also statistically significant (Table 6). Further statistical analysis shows the significant difference in survivorship between restricted and unrestricted larvae is at 9 dpf and 10 dpf (Figure 6B).

Table 6: ANOVA output comparing the effect of age and inflation status on each other and on survival of larval zebrafish.

Source of Variation	SS	df	MS	F	p-value
Age	0.274	5	0.274	26.16	0.00914
Inflation Status	0.112	1	0.112	10.65	0.011474
Age:Inflation Status	0.143	5	0.143	13.62	0.006119
Residuals	0.084	8	0.010		

2.4 Discussion

2.4.1 Zebrafish larvae have a finite window to initially inflate their swim bladder

I hypothesized that zebrafish larvae, being physostomes that retain their pneumatic duct into adulthood, should not have a critical period during which initial swim bladder inflation must occur. In contrast to this prediction, initial inflation of the swim bladder can proceed as normal in zebrafish larvae that have been restricted from accessing the surface up until 6 dpf. However, among larvae restricted until 7 dpf, given 24-hours access to surface, and analyzed at 8 dpf, there is a significant ($p=0.014$) decrease in initial inflation success (49.0% success in previously restricted larvae vs. 95.3% in control larvae) (Figure 6A). The following day, when larvae have been restricted to 8 dpf, allowed 24 hours of access to the surface, and analyzed at 9 dpf, the decrease in inflation ability is even more pronounced (11% vs. 98.7%) ($p=0.003$). As previously discussed, the temporal endpoint to the critical period is when 50% of the fish are no longer able to perform the task. Based on this definition and my results, I conclude that there is indeed a critical period of inflation for zebrafish larvae and that it ends at 7 dpf. If larvae are prevented from inflating up until 7 dpf timepoint, they lose the ability to do so even if given access to the surface. This contradicts commonly assumed ability of physostomes to inflate their swim bladders at any time after the organ is anatomically mature. The caveat to this conclusion is that, due to the increased mortality observed in the hypoinflated larvae, I cannot discern the cause of the loss of ability to inflate; this may be due to degradation of the pneumatic duct, or some other morbidity arising from hypoinflation.

2.4.2 Previous air-water interface restriction does not affect swim bladder volume in larvae that successfully inflate

Swim bladder volumes were estimated for all individuals that were successful in inflating to ascertain if there was a difference in volume between the unrestricted and restricted groups at each timepoint. There was no significant difference in swim bladder volume indicating that as long as the larvae was able to inflate, their ability to regulate the volume was not hindered. This finding aligns with the notion that successful inflation is a robust and effective process, demonstrating the resilience of the swim bladder in compensating for prior restrictions, emphasizing that once larvae overcome the initial challenges, their ability to regulate swim bladder volume remains unaffected. This resilience is noteworthy, as it adds nuance to our understanding of how environmental factors, in this case, air-water interface restrictions, may influence the developmental trajectory of larval fish.

2.4.3 Hypoinflation is a major risk factor for mortality early in fish development

As the critical period experiment progressed, I noticed a trend of decreasing survivorship in the previously restricted group of larvae culminating with fewer datapoints for that treatment group in the latter days of the experiment. Based on the data and observations from the critical period experiment, it is evident that the inability to initiate inflation precedes the decrease in survival. The decrease in survival becomes dramatic and statistically significant at 9 dpf onward. This critical period likely represents the time window during which successful swim bladder inflation must occur for optimal survival chances.

Failure to properly inflate a swim bladder can result in serious consequences to the fish, including death (Bennett et al., 1987; Doroshov et al., 1981; Egloff, 1996; Goolish & Okutake, 1999; Tsuji et al., 2016; Woolley & Qin, 2010) but the mechanisms of these consequences in zebrafish are unclear. Other consequences of hypoinflation in fishes include lordosis, increased energy expenditure, and reduced mobility (Egloff, 1996; Goolish & Okutake, 1999; Schwebel et al., 2018; Tsuji et al., 2016; Woolley et al., 2014). Cellular consequences in physoclists include immune responses and atrophy of the swim bladder (Marty et al., 1995) but it is unclear if these occur in physostomes like zebrafish as well. In some larger species, surviving fish that fail to initially inflate the swim bladder have smaller, often malformed bodies and organs, decreased lifespans, and complications with organ function and food conversion (Bennett et al., 1987; Egloff, 1996; Schwebel et al., 2018). Consistent with my findings, Goolish and Okutake reported that zebrafish with uninflated swim bladders have ~25% survivability by 10 dpf (Goolish & Okutake, 1999). They also observed a lack of food in the digestive tract of uninflated larvae, leading to the speculation that mortality was due to starvation (Goolish & Okutake, 1999). However, food consumption by uninflated larvae has not been rigorously quantified and the mechanistic link between food intake and mortality in uninflated larvae has not been investigated. The design of my study is unable to account for any of the more nuanced timings of initial inflation, and future research could include measuring timepoints in hours opposed to days. Additionally, as the critical period is defined by the end of the ability to inflate the swim bladder, this could also be investigated internally. Histological examination for the presence of the pneumatic duct over this period could potentially provide a better understanding of the presumed duct degradation. There has also not been a direct survival

analysis of hypoinflated zebrafish over time. Because of the results of these experiments as well as the questions left unanswered from the literature, an in-depth analysis of the mechanisms of mortality in these fish is warranted, as well as testing the hypothesis that starvation is the cause of their deaths.

Chapter 3: Investigating Endogenous and Exogenous Feeding as it Relates to Survivability in Hypoinflated Larvae

3.1 Background

3.1.1 Quantifying food intake in hypoinflated larvae

It has long been recognized that failure to initiate swim bladder inflation is associated with high levels of mortality in larval fish (Table 1). Previous theories have posited that starvation is a causative factor, the assumptions being that hypoinflated larvae are unable to capture food due to swimming deficits and/or that energy consumption outpaces food intake (Czesny et al., 2005; Goolish & Okutake, 1999; J. F. v. Ledeber & Wunder, 1937). However, mechanistic support for starvation-induced mortality in hypoinflated larvae is lacking and quantification of feeding has not been done in hypoinflated zebrafish. If an inability to consume food is the cause of the increased mortality in uninflated larvae, then there should be significantly less food in the guts of uninflated larvae as compared to inflated larvae.

As I determined during the critical period experiments, the optimal time for initial swim bladder inflation ends by 7 dpf (Figure 6A). However, I also observed nearly 90% mortality among 10 dpf larval zebrafish that were restricted from the surface, compared to just 10% in their unrestricted counterparts (Figure 6B). Since I observed food in the guts of uninflated larvae, I predict that the ability to ingest food was not hindered in hypoinflated larvae as previously thought. To arrive at a more comprehensive understanding of starvation as it relates to inflation, I performed an experiment to quantify food intake by hypoinflated larvae and measure how failure to initiate swim bladder inflation affects survivorship during this critical developmental period.

3.1.2 Analysis comparing the effects of starvation and hypoinflation on survival

The survivorship analysis from Chapter 2 indicated that non-inflation correlates with increased mortality, possibly related to starvation. There were limitations to that analysis though, including an endpoint of 10 dpf and hypoinflated larvae having access to the air-water interface in the last 24 hours before analysis. Regardless, if there is an interaction between hypoinflation and death solely due to starvation, I predicted that the uninflated, unfed treatment group will have decreased survivorship compared to the uninflated, fed treatment group. If starvation is not affected by inflation status, I expect there would be no difference in survivorship between those groups.

3.1.3 Quantifying endogenous feeding

To determine how hypoinflation relates to starvation, it is also important to examine both exogenous food intake as well as the yolk sac's role as the endogenous food source. During the initial phases of a fish's life, its digestive system is not fully functional, making it unable to utilize external sources of nutrition (Wilson, 2012). The yolk sac of a larval fish supplies crucial nutrients before exogenous feeding begins (Jardine & Litvak, 2003). In zebrafish, exogenous feeding can begin at 5 dpf when the developing gut is able to function for the first time. If exogenous feeding does not begin by the time the yolk is completely resorbed, larval fish face starvation (Jardine & Litvak, 2003). In zebrafish, the yolk sac volume is positively correlated to body size at the time of exogenous feeding, and negatively correlated to death from starvation (Jardine & Litvak, 2003). Under normal circumstances, most of the yolk is utilized prior to initial inflation, yet delaying the onset of initial feeding does not impact survival until after 8 dpf (Hernandez et al., 2018), so it is possible there is some yolk reserve remaining during that time to be utilized before

starvation occurs. For that reason, it is reasonable to presume there is yolk that remains between 5 dpf and 8 dpf for utilization as a redundancy measure for survival should external food not be available immediately. With this in mind, I wanted to determine if the usage of yolk sac lipid reserves differed between inflated and hypoinflated larval zebrafish. I hypothesize that hypoinflated larvae will utilize their yolk stores faster than their inflated counterparts due to increased energy expenditures resulting from deficits in locomotion and exogenous feeding.

3.2 Methods

3.2.1 Quantification of food intake as a control to allow interpretation of fed and unfed uninflated survivorship curves

To determine whether uninflated larvae consume less food than their inflated counterparts under laboratory conditions, I fluorescently labelled *Paramecia aurelia* with a lipophilic tracer (Shimada et al., 2012) and presented them to fasted groups of larvae with inflated and uninflated swim bladders. The fluorescence intensity in the gut was imaged using fluorescent microscopy and quantified for further data analysis.

To ensure the larvae were being fed an adequate, standardized quantity of paramecia, I measured the density of the paramecia culture by adding six drops of Detain II (Ward's Natural Science Establishment, Rochester NY) to 1 mL of the filtered culture and performing random counts of transects in a Sedgewick Rafter Counting Cell (#S50, Graticules Limited, Tonbridge, England). I recorded an average of ~333 *Paramecia* / mL. This means that in the 25 mL stock centrifuged to 1 mL, there are approximately 8000 *Paramecia* / mL.

I collected the *Paramecia* culture and filtered it twice through 70 μm mesh to minimize the presence of debris. Two 50 mL conical tubes were filled halfway with the culture and centrifuged at 4000 RPM for five minutes. The pellets were then resuspended in 1 mL of E3 in separate 1.5 mL Eppendorf tubes. To each tube, 1 μL of 3 mM FM 1-43 (ThermoFisher, Waltham, MA) was added and the tubes were briefly vortexed and then left on an agitator (ThermoFisher) at 120 RPM for 30 minutes. After 30 minutes, the tubes were centrifuged at 4000 RPM for 5 minutes. The pellets were then resuspended to rinse the dye, and the centrifuge and resuspension process was repeated for a second and third wash to remove unincorporated FM 1-43 dye.

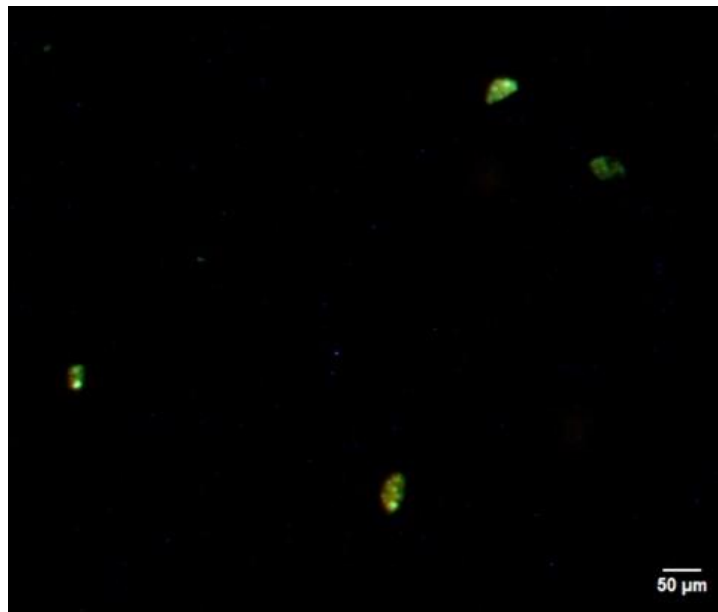


Figure 8: Live *Paramecia aurelia* fluorescently labeled with FM 1-43 dye, incubated for 30 minutes, and rinsed three times. Scale bar = 50 μm .

Because they are negatively buoyant, uninflated larvae tend to remain at the bottom of the water column. To ensure that both inflated and uninflated larvae enjoyed similar access to the paramecia, I standardized the column height by using shallow 100 mm petri dishes with 20 mL E3 media for feeding. Each inflated and uninflated group consisted of 10 larvae at 8 dpf. This age was chosen because survivorship past 8 dpf is very poor (Figure

6B). Each dish of larvae was allowed to feed on ~4000 fluorescently labeled paramecia for a period of one hour. The paramecia density and duration of feeding was determined through a series of preliminary tests to determine the ideal feeding standards for this experiment. For each trial, five unfed larvae from the clutch were imaged to serve as negative controls to account for autofluorescence in the gut. After one hour of access to the food, both sets of larvae were anesthetized with 0.016% w/v MS-222 (ThermoFisher), mounted on glass slides with 1.2% low melting-point agarose (LMA), and imaged with a microscope (Zeiss) fitted with a GFP-filter, a camera (Basler) operated by the Basler Microscopy Software 2.1, and connected to a fluorescence light source (X-Cite Series 120, Excelitas Technologies Corp., Waltham, MA).

To quantify fluorescence intensity, I analyzed the anterior bulb of the gut in each image using ImageJ (Schneider et al., 2012). A non-fluorescent region in the background was also selected and measured for background subtraction. From these values, the corrected total fluorescence (CTF) was calculated using the following formula:

$$CTF = Integrated\ density - (Area\ of\ bulb\ selected \times Mean\ fluorescence\ of\ the\ background)$$

I then performed a t-test in R (R Core Team, 2023) to determine if hypoinflation significantly hinders food consumption.

3.2.2 Investigating the relationship between inflation status and feeding status on survival

To gain a deeper understanding of the connection between starvation and swim bladder inflation, I conducted an experiment to measure the interaction of these factors on

survival during this crucial developmental phase. To do this, I compared the survivorship between four treatment groups (Figure 9).

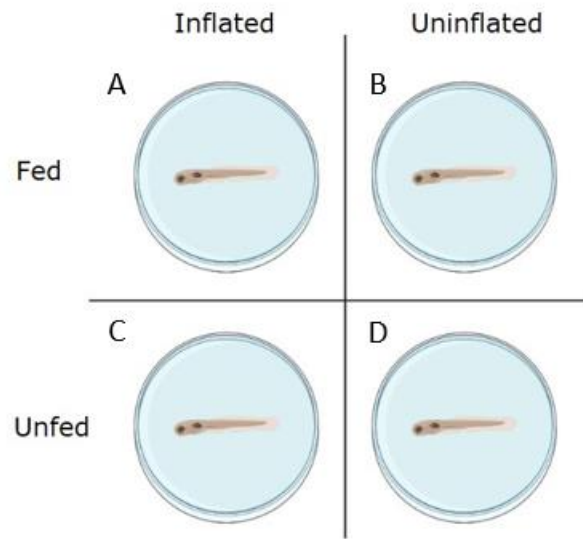


Figure 9: Depiction of the four treatment groups comprising the survivorship experiment: A. fed, inflated; B. fed, uninflated; C. unfed, inflated; and D. unfed, uninflated.

In addition to these four treatment groups, survivorship of a fifth group was also analyzed to determine the effect of mechanical usage of the organ on the mortality risk to larval fish. To test this, I had the larvae ‘inflate’ their swim bladders with food-grade coconut oil (President’s Choice, Brampton ON Canada) that is modified to remain liquid at room temperature. I filled a 250 mL plastic beaker with 150 mL E3 embryo medium, placed 3 dpf larvae into the medium, and added a thin layer of coconut oil so the air-water interface became an oil-water interface. The beaker was incubated at 28.5°C until the larvae were 5 dpf and had filled their swim bladders with oil instead of air. The larvae were then removed from the beaker for the duration of the survivorship experiment alongside the other four groups in the apparatus to ensure equal parameters between all five groups.

The embryos were all reared under standard conditions, as outlined previously, until 3 dpf. At this point, the larvae were separated into one of two restriction apparatuses with the difference between the two being food restriction or not. This was to ensure any residual food that passed through the strainers of the tubes could not then pass back into the tube of a group that was meant to be starved.

Each tube had 20 larvae in it. Across the five treatment groups, there were 300 larvae in the study (n = 240 for the original four groups, and n = 60 for the oil-filled larvae). Each morning and afternoon, surviving larvae were counted and any deceased larvae were removed. Those in the fed groups were fed at these times. Water conditions, including pH, temperature and dissolved oxygen, were also recorded daily to ensure consistency (Table 7). The primary outcome of interest was larval survivorship over time within the four main treatment groups. Using R, I generated survivorship curves for each group to visualize differences in mortality rates between the groups over the critical developmental period. I performed a two-way ANOVA with Tukey's post-hoc tests using R to determine the effects of age and treatment group (inflation and feeding status) on survival.

Table 7: Water quality parameters for the tanks housing either the fed or unfed larvae analyzed for survivorship.

	Fed Tank			Unfed Tank		
	Trial 1					
Larval Age (dpf)	pH	Temperature (°C)	O ₂ (mg/mL)	pH	Temperature (°C)	O ₂ (mg/mL)
5	6.87	27.9	5.1	6.86	27.4	4.8
6	6.85	28.2	5.1	6.88	28.2	4.7
7	6.84	28.5	5.0	6.87	28.0	4.6
8	6.83	28.8	4.9	6.86	27.8	4.5
9	6.82	28.1	4.8	6.85	28.6	4.4
10	6.81	28.2	4.7	6.84	28.4	4.3
11	6.80	28.2	4.6	6.83	27.9	4.2
12	6.79	28.1	4.5	6.82	28.3	4.1
13	6.78	28.0	4.4	6.81	27.8	4.0

14	6.77	28.9	4.3	6.80	27.7	3.9
Trial 2						
Larval Age (dpf)	pH	Temperature (°C)	O ₂ (mg/mL)	pH	Temperature (°C)	O ₂ (mg/mL)
5	7.55	28.0	5.3	7.80	28.2	4.6
6	6.85	27.6	5.2	6.85	27.9	5.3
7	6.87	27.9	5.1	6.86	27.4	4.8
8	6.82	27.8	4.8	6.84	27.8	5.0
9	6.81	28.2	4.7	6.83	27.6	4.9
10	6.80	28.2	4.6	6.82	27.4	4.8
11	6.79	28.1	4.5	6.81	27.3	4.7
12	6.78	28.0	4.4	6.80	27.3	4.6
13	6.77	27.9	4.3	6.79	27.3	4.5
14	6.76	27.8	4.2	6.78	27.3	4.4
Trial 3						
Larval Age (dpf)	pH	Temperature (°C)	O ₂ (mg/mL)	pH	Temperature (°C)	O ₂ (mg/mL)
5	6.90	27.9	5.1	6.84	27.4	4.8
6	6.81	28.2	5.1	6.84	28.2	4.7
7	7.02	28.5	5.0	7.11	28.0	4.6
8	6.79	28.8	4.9	6.86	27.8	4.5
9	6.81	28.1	4.8	6.93	27.6	4.4
10	6.81	28.2	4.7	6.85	27.4	4.3
11	6.87	28.2	4.6	6.83	27.8	4.2
12	6.80	28.1	4.5	6.85	27.8	4.1
13	7.00	28.0	4.4	6.97	27.9	4.5
14	6.91	27.9	4.3	6.92	27.3	4.8

3.2.3 Measuring the reserves of endogenous yolk lipids in larval fish

Wild-type zebrafish embryos were reared to 3 dpf as previously described. At 3 dpf, the larvae were separated into groups of 25 and placed into the restriction apparatus either with or without access to the air-water interface. Each day, 1 L of embryo medium was replaced in the chamber. Feeding did not commence at 5 dpf as usual, as the presence of food could have been a factor that changed yolk sac usage.

To prepare the solution for *in vivo* labeling of neutral lipids, I dissolved 1.25 mg of Nile Red (ThermoFisher) in 1 mL of acetone (ThermoFisher) (Flynn et al., 2009). This

solution was then stored in a dark environment at -20°C, ensuring its stability and potency for subsequent usage. Starting at 5 dpf, 25 larvae from each of the treatment groups (inflated and hypoinflated) were divided into the wells of a 6-well plate with 3 mL of embryo medium. I added the Nile Red solution to create a final concentration of 0.5 µg/mL. The larvae were placed in the dark for 30 minutes, enabling complete staining.

After the staining process, I removed the larvae from the dye solution and fixed them in a 4% formaldehyde solution overnight. The next day, I rinsed the fixed larvae and stored them at 4°C in 1X PBS. This process was repeated at 6 dpf and 7 dpf for a total of 150 larvae, minus 9 larvae removed from the investigation due to incidental mortality for a total of 141 larvae. Once all the larvae for each treatment at each timepoint were stained and fixed, I mounted them using 1.2% LMA and imaged them using a fluorescent stereomicroscope fitted with the GFP filter. Using ImageJ (Schneider et al., 2012), I measured the visible area of the remaining yolk lipids, if any, and then used R for subsequent ANOVA analysis and data visualization.

3.3 Results

3.3.1 Inflated and hypoinflated larval zebrafish consume equal amounts of food

To determine the effect of hypoinflation on food consumption, the data generated from the image analysis were analyzed in R with a Welch's two sample t-test and visualized with a boxplot (Figure 10C). Both groups of larvae (inflated and uninflated) exhibited a spectrum of food intake; however, there was no significant difference in means between the two groups (Table 8). A total of 44 larvae between the two groups were quantified.

Table 8: Welch's Two Sample t-test output testing the effect of inflation status on fluorescence intensity of the gut following feeding with fluorescently labeled *Paramecia*.

t	df	p-value	Upper CI	Lower CI
1.3075	41.963	0.1981	-2041.119	9552.809

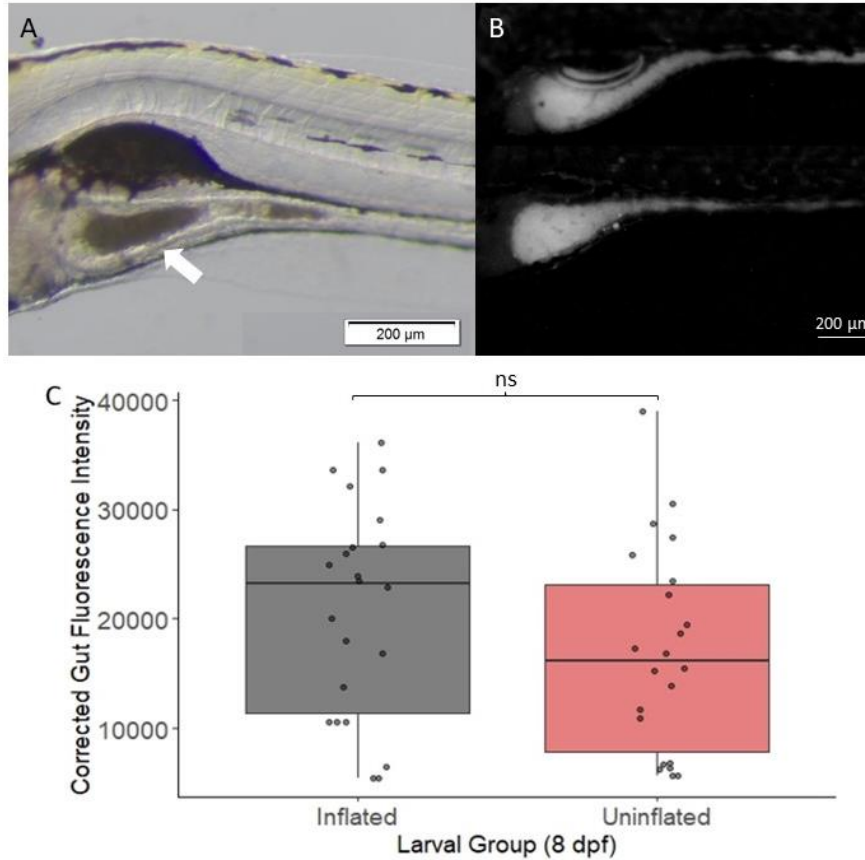


Figure 10: Swim bladder inflation status does not affect food intake. A: An 8 dpf larva with hypoinflated swim bladder with food in its gut (white arrow). B: Fluorescence in the guts of 8 dpf inflated (top) and uninflated (bottom) larvae exposed to fluorescently labeled *Paramecia*. Scale bar represents 100 μ m. C: Corrected gut fluorescence of 8 dpf zebrafish larvae with inflated or uninflated swim bladders following feeding with fluorescently labeled *Paramecia*. There is no difference in fluorescence intensity, and therefore food intake, between the two test groups (n=44).

3.3.2 Investigating the relationship between inflation status and feeding status on survival reveals significant interactions

To examine the effect of swim bladder filling, regardless of the substance filling it, I had larvae fill the organ with coconut oil to be analyzed for survival alongside the other four groups (Figure 9). Once filled with oil, the swim bladder volumes were compared to the data previously gathered in Chapter 2 for the same age group of inflated (i.e., unrestricted access to the air-water interface) larvae of the same strain (Tübingen) ($n = 69$). I plotted these volumes on a boxplot (Figure 11C) to visualize the volume differences, and a Welch's t-test resulted in a statistically significant p-value of $7.45e-08$, indicating that larval zebrafish fill their swim bladders with oil, typically to greater volumes than with air (Table 9).

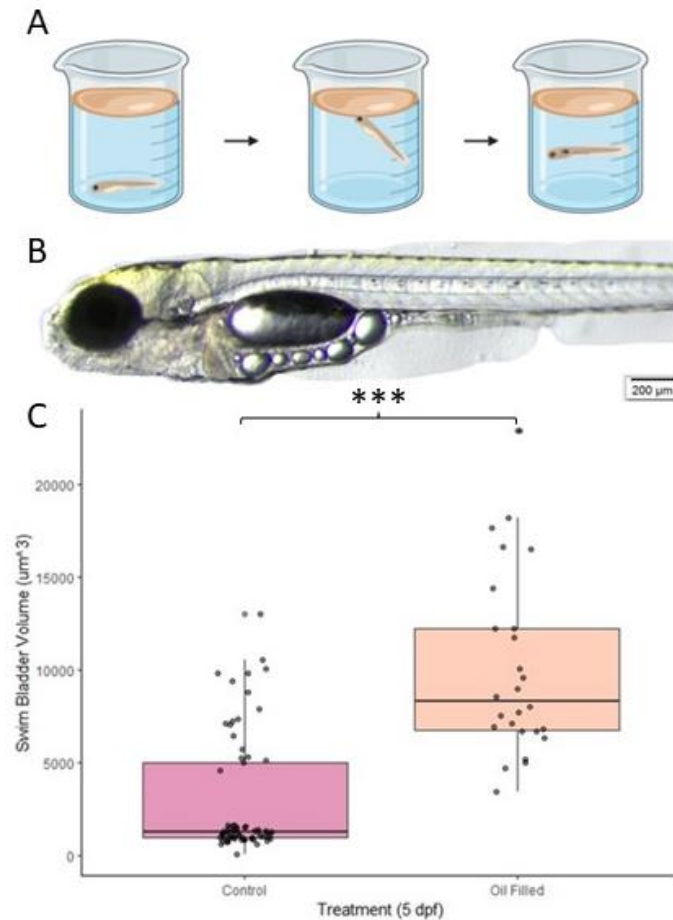


Figure 11: Zebrafish larvae that fill their swim bladders with coconut oil instead of air over-fill the organ. A: Depiction of the process of encouraging oil 'inflation' in zebrafish larvae at 3-5 dpf. B: Light microscopy image of a 5 dpf larva that has filled its swim bladder with coconut oil. C: Volume estimations of air-inflated (n=69) and oil-filled (n=25) swim bladders in 5 dpf larvae.

Table 9: Welch's Two Sample t-test output comparing the effect of oil-filling of the swim bladder to typical air filling on swim bladder volume.

t	df	p-value	Upper CI	Lower CI
-6.866	33.225	7.45e-08	-9272.747	-5034.594

Analysis of the survivorship experiment yielded interesting insight into the effects of swim bladder inflation status and feeding status on larval zebrafish survival during this early and critical developmental period up to 14 dpf. I created a survivorship curve of the five treatment groups to provide a visual representation of mortality trends over time

(Figure 12). These curves were statistically compared using a two-way ANOVA, revealing statistically significant effects of age, treatment, and their interaction on survival (Table 10). Post-hoc tests show that all treatment groups were significantly different from one another except for two: unfed, uninflated compared to fed, uninflated, and unfed, inflated compared to oil-filled (Table 11).

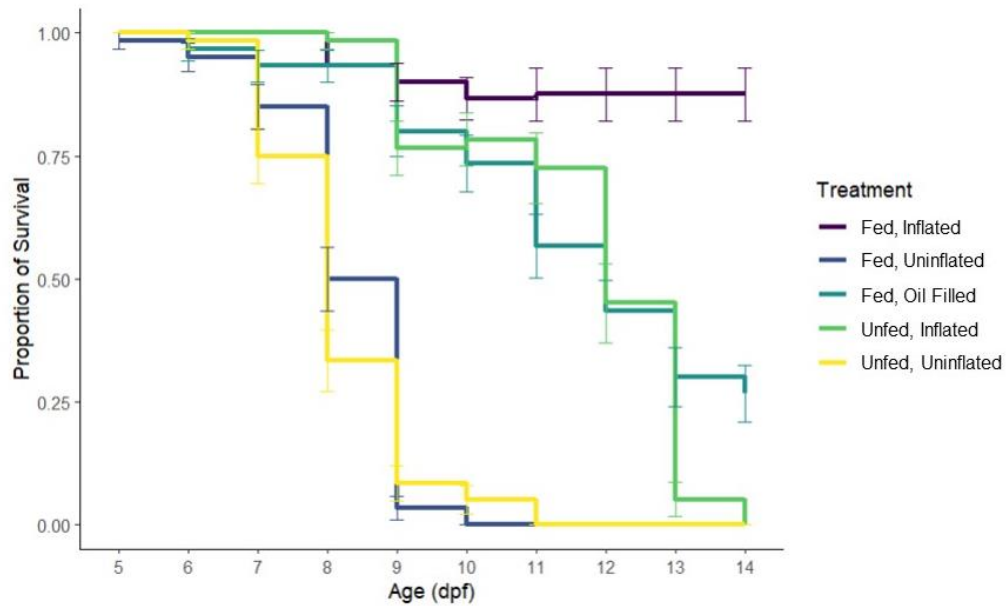


Figure 12: Inflation status is a better predictor of survival than feeding status. Survivorship curves for 5 to 14 dpf larvae (n=300) separated into five treatment groups with varying inflation and feeding status. The fed, uninflated (dark blue) and unfed, uninflated (yellow) groups were not significantly different from one another ($p=0.2$). The unfed, inflated (light green) and fed, oil-filled (dark green) groups were not significantly different from one another ($p=0.3$). All other group-group comparisons were significantly different (Tables 9 and 10).

Table 10: Two-way ANOVA output analyzing the effects of age and treatment (inflation status and feeding status) on survival. SS = sum of squares; df = degrees of freedom; MS = mean squares; F = F-statistic; p-value = probability value.

Source of Variation	SS	df	MS	F	p-value
Age	8.44	9	8.44	201.03	<2e-16
Treatment	4.92	4	1.64	39.08	<2e-26
Age:Treatment	2.02	4	0.675	16.07	1.52e-09
Residuals	4.03	96	0.042		

Table 11: The survivorship treatment group comparisons and their respective adjusted p-values from a post-hoc Tukey's test.

Treatment Group Comparison	Adjusted p-value
Fed, Uninflated – Fed, Inflated	0.0000000
Oil-filled – Fed, Inflated	0.0003435
Unfed, Inflated – Fed, Inflated	0.0002935
Unfed, Uninflated – Fed, Inflated	0.0000000
Oil-filled – Fed, Uninflated	0.0000000
Unfed, Inflated – Fed, Uninflated	0.0000000
Unfed, Uninflated – Fed, Uninflated	0.8134572
Unfed, Inflated – Oil-filled	0.9996547
Unfed, Uninflated – Oil-filled	0.0000000
Unfed, Uninflated – Unfed, Inflated	0.0000000

3.3.3 The rate of yolk lipid consumption is not affected by inflation status

To quantify the area of the Nile Red-stained yolk sac lipids, I used ImageJ. Representative images of each of the timepoints for each treatment group can be seen in Figure 13A. Using ggplot2 in R, I plotted the area of the Nile Red fluorescence on a bar chart with the 141 jittered data points, each representing an individual larva (Figure 13B). The ANOVA result reveals no significant difference in remaining yolk reserve with respect to inflation status (Table 12).

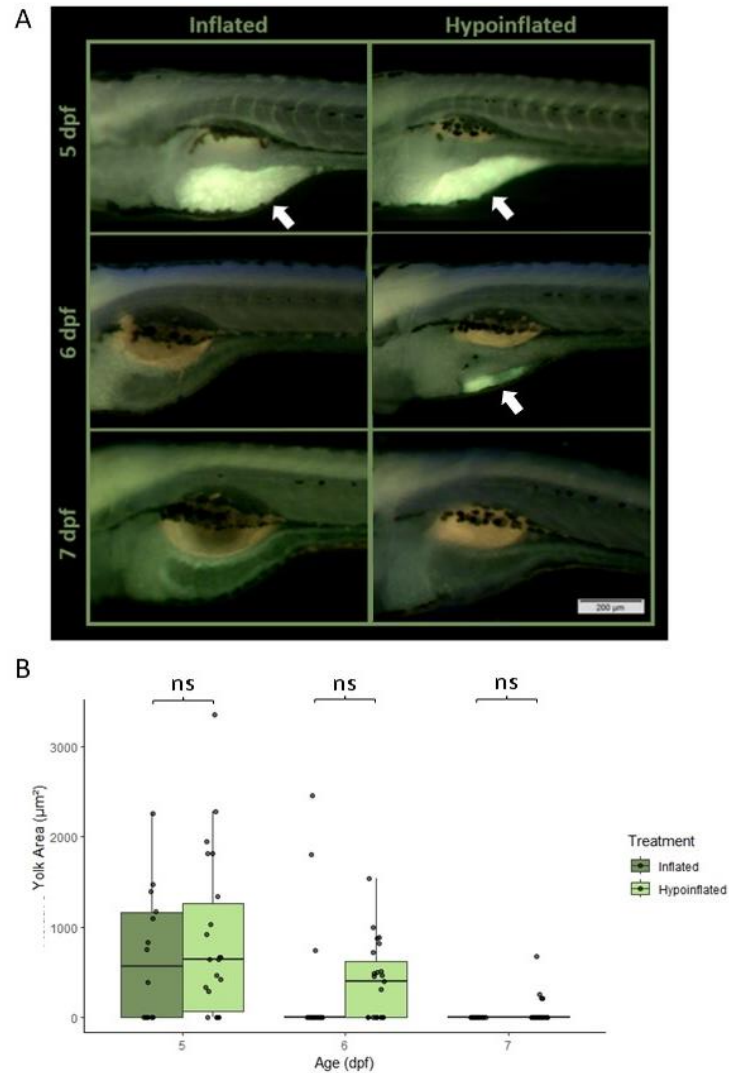


Figure 13: Inflation status does not affect yolk lipid consumption. A: Representative images of the six groups of larvae (inflated or hypoinflated, at 5, 6, and 7 dpf) stained with Nile Red and imaged using a fluorescent stereomicroscope fitted with a GFP-filter. At 5 dpf, there is notable yolk lipid reserve remaining in both treatment groups. At 6 dpf, there is a small amount remaining, and by 7 dpf the yolk sac is depleted. Arrows indicate the stained lipid. **B:** The area of yolk sac lipid reserve in regularly inflated and hypoinflated larval zebrafish at 5, 6, and 7 dpf (n=141).

Table 12: The two-way ANOVA output analyzing the effects of inflation status on remaining yolk area at different timepoints.

Source of Variation	SS	df	MS	F	p-value
Age	13206864	2	6603432	24.026	1.19e-09

Inflation Status	149521	1	149521	0.544	0.462
Age:Inflation Status	130100	2	65050	0.237	0.790
Residuals	37104525	135	274848		

3.4 Discussion

Earlier hypotheses regarding the correlation between hypoinflation and mortality have implicated starvation as the underlying cause, albeit without concrete mechanistic evidence. Starvation can result from various mechanisms, such as insufficient feeding or improper digestion. While anecdotal (Goolish & Okutake, 1999; Ribi, 1992) and quantified (Czesny et al., 2005; Hashimoto et al., 2012) accounts from different fish species have occasionally hinted at reduced feeding in hypoinflated fish, the prevailing belief is that they struggle to capture food effectively due to swimming impairments. In the specific case of zebrafish, there has yet to be a comprehensive and direct comparative analysis of their feeding abilities to support these predictions.

3.4.1 Contrary to popular belief, starvation in hypoinflated larvae is not due to a lack of feeding

For this assay, larvae that were either hypoinflated or normally inflated at 8 dpf were exposed to a standardized concentration of fluorescently labeled paramecia in shallow containers such that the depth of the media would not be a factor affecting access to food for the hypoinflated larvae. At 8 dpf, zebrafish larvae that are hypoinflated consume the same amount of food as their normally inflated counterparts ($p=0.198$). For decades, the assumption has been that hypoinflated larvae die due to starvation, with researchers posting this starvation is from a lack of food consumption (Goolish & Okutake, 1999). My results do not support the hypothesis that starvation is due to an inability to eat, and instead

suggests that under laboratory conditions, hypoinflation does not negatively impact food acquisition or ingestion.

The survivorship experiment aimed to decipher the relationship between swim bladder hypoinflation, starvation, and mortality during the critical developmental period. Five treatment groups were used: fed and inflated, unfed and inflated, fed and uninflated, unfed and uninflated, and fed and oil-filled. After analysis of the survival of these five treatment groups from 5 - 14 dpf, the results indicate several interesting conclusions:

1. There is no significant difference in survival between the fed- and unfed-uninflated larvae, indicating the presence or absence of food does not affect the survival of uninflated larvae.
2. All treatment groups with filled swim bladders, regardless of the contents, had increased survival rates compared to the uninflated groups. Especially interesting is the finding that larvae with oil-filled swim bladders outlive their unfilled counterparts. This suggests that filling the organ with either air or oil is sufficient to prolong survival relative to larvae with unfilled swim bladders.

Had the survival rate of the unfed-uninflated and the unfed-inflated groups been the same, then the prevailing theory of starvation being the primary driver of mortality in hypoinflated fish would be supported. However, my findings indicate that hypoinflation plays a more intricate and complex role in the observed mortality of these fish. While starvation may be a factor, particularly in the wild settings, it is not the sole or even primary driver of mortality in uninflated zebrafish larvae in controlled laboratory conditions.

While these findings do align with previous research that highlights the vulnerability of larval fish to starvation (Czesny et al., 2005), this is a novel perspective

that examines the specific interaction between swim bladder inflation status and starvation. These data are the first of their kind to provide empirical evidence that sheds new light on how these factors interact and impact survival of larval zebrafish.

The inclusion of the oil-filled treatment group is an interesting piece of the puzzle. The oil-filled group showed increased survival compared to both uninflated groups, suggesting the role of the swim bladder may extend beyond buoyancy control. Usage of the organ, regardless of normal inflation or over-filling, appears to provide some rescue effect to mortality. This seems to indicate that the mechanical forces associated with filling the swim bladder may impact survival. Further analysis may help further our understanding the swim bladder such as histological examination of the cellular structure in oil-filled and hypoinflated larvae.

3.4.2 Endogenous lipid reserves are not utilized differently between inflated and hypoinflated larvae

As for the yolk reserve, the results indicate that hypoinflation does not have a notable impact on the speed of yolk usage at any of the time points (Figure 13B). In relation to the overarching assumption that hypoinflated zebrafish larvae die due to starvation, it appears that hypoinflation is not related to any difference in absorption rate of the yolk sac. Future work to elucidate the mechanisms of how hypoinflation impacts survival is vital to expanding our understanding of this critical developmental time in fish. Towards this end, I investigated gut form and function in relation to mortality risk associated with hypoinflation in Chapter 4.

Chapter 4: Investigating the mortality risk associated with swim bladder hypoinflation as it relates to gut form and function in larval fish

4.1 Background

4.1.1 Hypoinflation is predicted to disrupt gut barrier integrity

Initial inflation of the swim bladder is a critical developmental milestone for most species of teleost fish. In natural environments, failure to inflate severely hinders swimming, the ability to catch prey, and the ability to evade predation, which all increase larval mortality in hypoinflated fish. In controlled laboratory settings, predation is not a factor, but increased mortality of uninflated zebrafish is still observed. The prevailing theory has been that uninflated larvae die due to starvation from an inability to feed. My results suggest that this is not the case, as uninflated larvae consume the same amount of food as inflated larvae, and uninflated, unfed larvae die at a more rapid rate than their inflated, unfed counterparts (Chapter 3). These discoveries suggest that the cause of death might not be connected to eating too little, though starvation is not off the table entirely. The mechanisms may be related to problems with their gut instead, as the swim bladder originates as an evagination of the developing foregut (Pelster, 2004).

Due to their shared developmental origins, the potential link between proper swim bladder inflation and gut function may have substantial implications for understanding the mortality patterns observed in hypoinflated larvae. Proper gut function, essential for digestion and nutrient uptake (Zhang et al., 2015), might be contingent on the functional state of and/or mechanical forces generated by the swim bladder, given their physical proximity. This may affect the fish's ability to process food efficiently, ultimately

contributing to increased mortality, despite apparent food consumption as evidenced in Chapter 3.

The evidence of abnormal cellular organization in hypoinflated swim bladders (Bennett et al., 1987; Marty et al., 1995), often accompanied by signs of an inflammatory response, suggests a profound connection between inflammation, the gut, and the swim bladder. The inflammatory response observed in hypoinflated swim bladders could further impair gut function (Marjoram et al., 2015), creating a vicious cycle in which compromised swim bladder functionality hampers nutrient absorption, potentially triggering an inflammatory cascade that exacerbates both swim bladder and gut dysfunctions. These findings underscore the need for a comprehensive investigation into the physiological interplay between the swim bladder and gut, shedding light on the complex mechanisms underlying larval fish mortality in instances of hypoinflation. I hypothesize that there is a link between hypoinflation and gut function, resulting in increased mortality due to starvation. If hypoinflation causes disruption to the gut barrier integrity of larval fish, then I would expect to see leakage from the gut in these fish. To test this theory, I performed an assay for intestinal gut barrier integrity analysis using the microgavage technique (described in Chapter 4.2.1).

4.1.2 Hypoinflation is predicted to impact gut morphology

Anatomically, the inflated swim bladder is a large organ that takes up a considerable amount of volume within the developing fish. In inflated larvae, the shape of the gut appears to be influenced by the size and placement of the filled swim bladder as the gut curves around the ventral side. While performing other investigations, I noticed that

uninflated larvae exhibited a change in gut morphology (Figure 14), but dedicated experiments were required to make a definitive statement.

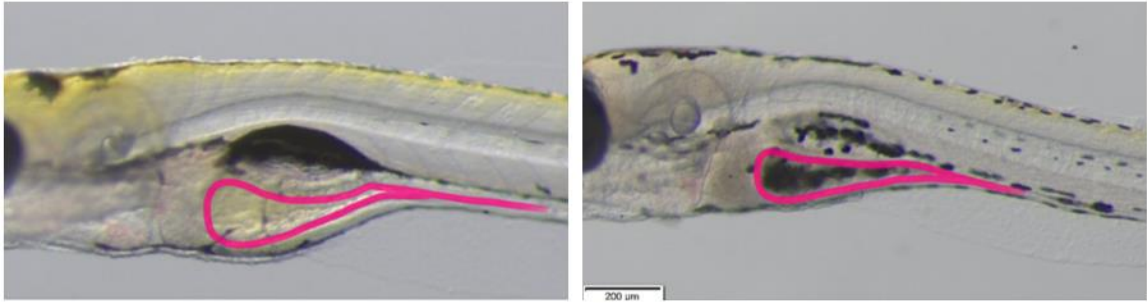


Figure 14: 6 dpf inflated (left) and hypoinflated (right) zebrafish larvae with the borders of their gut highlighted in pink.

I predict that hypoinflated larvae will have a malformed gut compared to their inflated counterparts. To determine the degree to which hypoinflation affects the gross gut morphology, I measured the gut angle in inflated and uninflated groups of fish.

4.1.3 Hypoinflation is predicted to negatively affect digestion ability in larval fish

The gut barrier plays a crucial role in controlling the exchange of substances between the lumen of the gut and the rest of the body. Enterocytes line the lumen of the developing gut in zebrafish and are responsible for absorbing nutrients from food and preparing them for circulation within the body (Zeituni et al., 2016). This process is crucial to proper growth and development of the larval fish. The integrity of the gut barrier relies on the proper functioning of the junctions between these enterocytes. These junctions prevent the unregulated passage of nutrients, water, ions and other substances between the gut lumen and the rest of the body (Shen et al., 2011). When these junctions become compromised, it can lead intestinal dysfunction.

If hypoinflation negatively affects gut barrier function as hypothesized in 4.1.1, it raises the intriguing possibility that the lysosome-rich enterocytes of the gut may also

experience compromised catabolic functionality. In other words, the very cells responsible for nutrient absorption and processing may not be working optimally under conditions of hypoinflation. If digestion and nutrient absorption are compromised, it helps support the starvation hypothesis and would provide mechanistic evidence on a cellular level. I predicted that hypoinflation results in compromised lysosomal ability.

4.2 Methods

4.2.1 Microgavage

The microgavage of zebrafish larvae utilizes a microinjector (PV850, World Precision Instruments, Worcester, MA) fitted to the manipulator (MM3301R, World Precision Instruments) attached to a microscope (#504-941, World Precision Instruments). I pulled the glass capillaries (#TW100F-4, World Precision Instruments) into needles using a needle puller (PUL-1000, World Precision Instruments) using the following settings: Heat index: 650; Force (g): 250; Distance (mm): 8.00; Delay (0.1 s): 0. The needles were clipped to a diameter of approximately 30 μm , then fire-polished to remove sharp edges using a microforge (MF-200, World Precision Instruments). The zebrafish larvae are anesthetized with MS-222, mounted on glass slides with LMA, and positioned at an angle to complement the angle of the needle in the microinjector. With microinjector settings at 2.0 PSI and an injection time of 0.1 seconds, this method allows for precise injections of $\sim 80 \mu\text{m}$ diameter droplets ($\sim 4.5 \text{ nl}$) of media (Cocchiario & Rawls, 2013).

4.2.2 Measuring gut angle to assess gut morphology differences between inflated and hypoinflated larval fish

Zebrafish were spawned and embryos collected as previously described. At 3 dpf, embryos were placed in the restriction apparatus with half having access to the air-water interface, and the remaining half being restricted from the surface to induce hypoinflation. To visualize the contours of the gut lumen, I microgavaged larvae with a 5% Texas Red-conjugated 3 kDa dextran solution (ThermoFisher) (Appendix 1) at 7 dpf and 8 dpf for a temporal progression analysis of gross gut morphology. These larvae were then imaged using confocal microscopy at 10× magnification. Using ImageJ (Schneider et al., 2012), I calculated the gut angle of these larvae by measuring from the midline of the anterior portion of the bulb to the midline of the anterior portion of the midgut, to the anterior portion of midline of the hindgut (Figure 15). This provided quantitative data on the gross morphology of this developing organ at 7 dpf and 8 dpf.

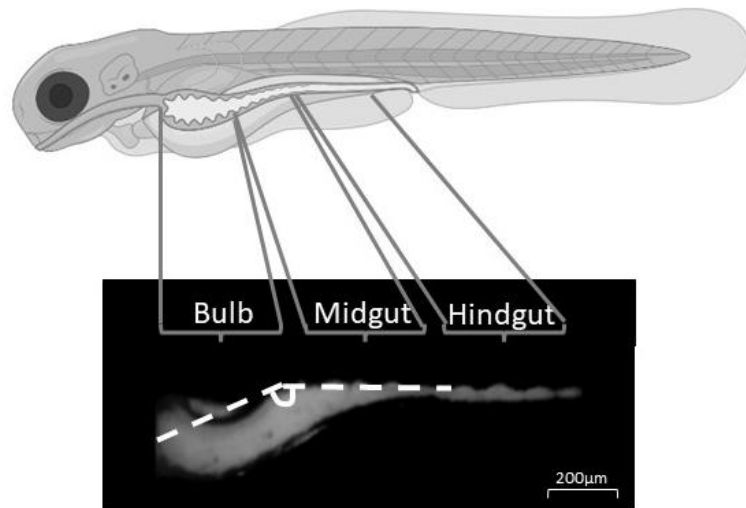


Figure 15: Depiction of the angle measurements used to calculate gut angle in the gross gut morphology assay of a 7 dpf larvae that have been microgavaged with 5% Texas Red dextran solution. The primary digestive functions of the intestinal regions are: mechanical digestion (bulb), chemical digestion (midgut), and ion absorption (hindgut) (Gao et al., 2022).

4.2.3 Microgavaging fluorescent dextran to measure gut leakage as an indicator of barrier integrity

For this assay, I used a transgenic zebrafish line *Tg(apoeb:LY-EGFP)* that expresses membrane-targeted GFP (green fluorescent protein) under control of the *apoeb* gene regulatory elements (Peri & Nüsslein-Volhard, 2008). Enterocytes are one of the cell types marked by GFP in this transgenic line, allowing me to visualize the lining of the gut and assay for deficits in gut barrier function (Figure 16).

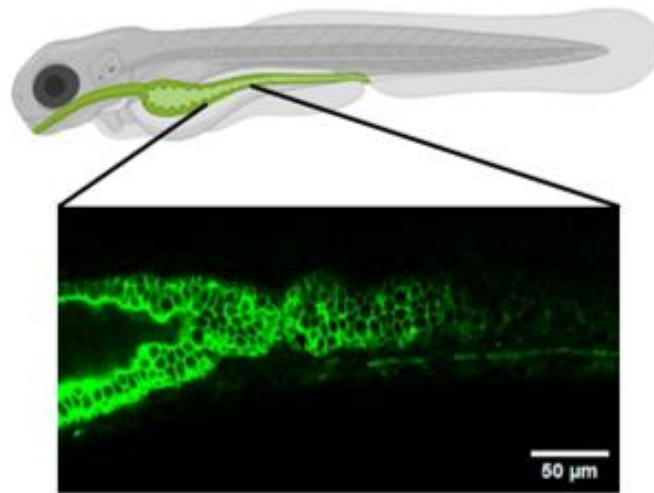


Figure 16: Confocal image of GFP-positive enterocytes in the posterior portion of the bulb of the gut and the anterior portion of the midgut of an 8 dpf *Tg(apoeb:LY-EGFP)* zebrafish larvae (bottom). Scale bar represents 50µm. Cartoon not to scale (top), and for anatomical location reference only.

The aim of this experiment was to investigate gut barrier integrity by quantifying gut leakage. I predict there will be a defect in gut barrier function and an increase in leakage in the hypoinflated larvae. For this assay, I spawned the *Tg(apoeb:LY-EGFP)* zebrafish and collected 45 fertilized embryos. At 3 dpf, the embryos were placed in the restriction apparatus with one third of them (n=15) prevented from accessing the surface and the

remaining two thirds (n=30) having normal access to the air-water interface. To reduce any confounding factors with relation to feeding, the larvae were fasted throughout the experimental process. At 7 dpf, the larvae were removed from the apparatus and separated into their three groups: inflated, hypoinflated, inflated-positive control (Figure 17). I microgavaged approximately 4-5 nl of 3 kDa dextran conjugated to Texas Red (#D1830, ThermoFisher, Worcester, MA) (Appendix 1) into the anterior intestinal bulb of each larva. As a positive control for leakage, I also gavaged larvae with a 50 mM EDTA dextran solution (Appendix 1) (ethlenediaminetetraacetate acid/EDTA; ThermoFisher) to disrupt Ca^{2+} -dependent tight junctions (Cocchiaro & Rawls, 2013).

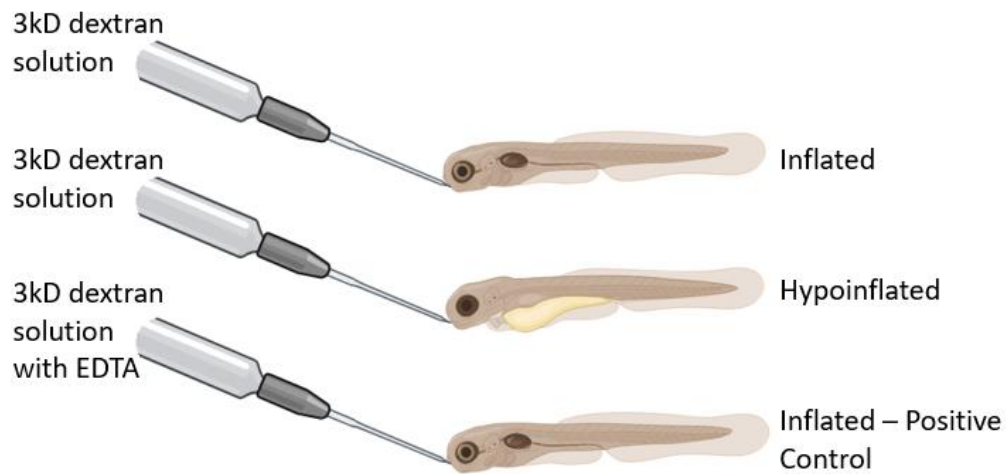


Figure 17: Depiction of the three treatment groups in the gut barrier integrity assay.

After gavage, the larvae were unmounted and left to recover in embryo medium. After a 1-hour recovery period, the larvae were re-anesthetized, remounted with LMA, and imaged using confocal microscopy using a 25×0.95 NA water immersion objective. Using ImageJ (Schneider et al., 2012), I analyzed the images to quantify dextran fluorescence present beyond the intestinal epithelium as a readout for loss of gut barrier integrity. To do this, I selected a square area approximately $15\text{ }\mu\text{m}$ across ($225\text{ }\mu\text{m}^2$), using visualization

of the GFP-positive enterocytes to ensure the measured area was outside the border to the gut. The captured area was measured for fluorescence intensity in the dextran channel. To help limit bias, I chose the measured area as the portion closest to the centre of each image. This was repeated for each image, and the data collected was analyzed with an ANOVA and subsequent t-tests.

4.2.4 Assessing digestion ability by analysing presence of acidified vacuoles within enterocytes of the gut between hypoinflated and inflated larvae

I microgavaged 7- and 8-dpf *Tg(apoeb:LY-EGFP)* larval zebrafish (n=18) with ~4 nl of 1 mM (0.4 mg/mL) LysoTracker Red (Invitrogen). After 1 hour of recovery, the larvae were re-anesthetized, mounted, and imaged using a confocal microscope (Leica TCS SP 8, Wetzlar, Germany) fitted with a 25× 0.95NA water immersion objective, and using the associated LAX software (Leica Application Suite X, Buffalo Grove, IL). I used ImageJ to quantify the normalized mean fluorescence intensity of the LysoTracker as an indicator of lysosome acidification; a proxy for the ability of enterocytes to break down complex molecules. I imported this data to R for data visualization and statistical analysis.

4.3 Results

4.3.1 Measuring gut angle of inflated and hypoinflated larval fish reveals significant differences in gross gut morphology

By microgavaging larval zebrafish with a fluorescent solution of dextran at 7 dpf and 8 dpf, the contours of the gut lumen can be easily visualized by fluorescence microscopy. These images were measured for gut angle and a two-way ANOVA was performed to determine the effect of hypoinflation on gut angle (Figure 18).

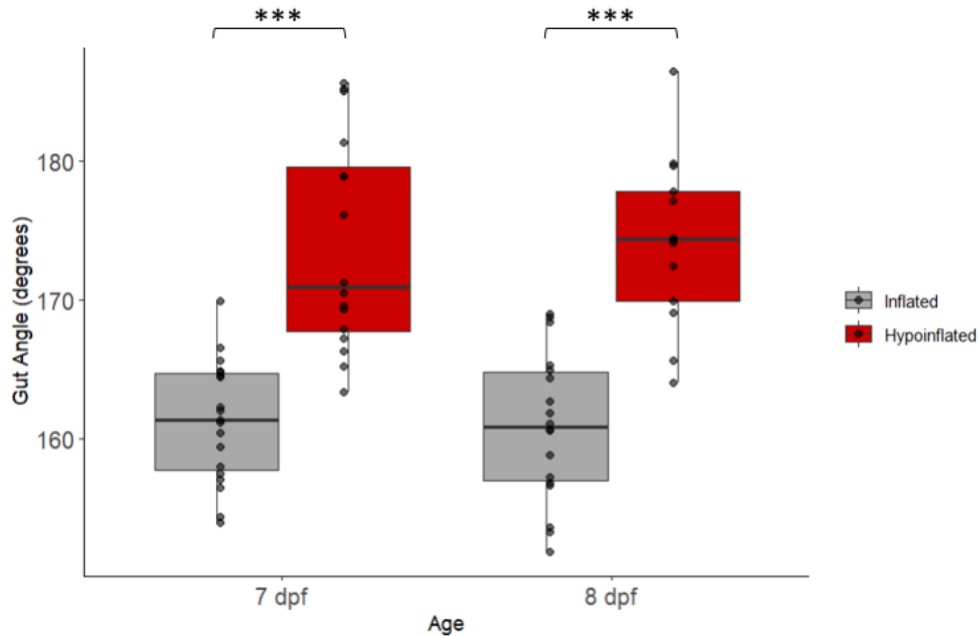


Figure 18: The gut angle comparison between hypoinflated and inflated zebrafish larvae at 7- and 8-days post-fertilization (n=66).

The ANOVA revealed a statistically significant difference in gut angle between treatment groups but not between timepoints (Table 13). Welch's Two Sample t-tests showed this difference in gut angle between treatment groups to be true at both 7 and 8 dpf (Table 14). This is consistent with the hypothesis that hypoinflation causes a disruption to the shape of the developing zebrafish gut. Interestingly, some of the hypoinflated samples had gut angles that were at or even greater than 180° when the average angle for the control inflated fish was just above 160° .

Table 13: Two-way ANOVA output to compare the effect of hypoinflation on gut angle in larval zebrafish at multiple timepoints.

Source of Variation	SS	df	MS	F	p-value
Inflation Status	2707	1	2707	77.811	1.51e-12
Age	0.1	1	0.1	0.003	0.958
Age:Inflation Status	2.4	1	2.4	0.068	0.795
Residuals	2156.9	62	34.8		

Table 14: Welch's T-test output to compare the effect of hypoinflation on gut angle in larval zebrafish at 7 dpf and at 8 dpf.

Source of Variation	t	df	p-value	Upper CI	Lower CI
7 dpf					
Inflation Status	5.81	22.80	6.69e-06	8.07	17.02
8 dpf					
Inflation Status	6.28	23.56	1.85e-06	8.93	17.69

4.3.2 Hypoinflated zebrafish larvae exhibit gut barrier defects

Experimental intestinal studies using the zebrafish model have frequently used the immersion method, which delivers substances to the gut by bathing larvae in a solution of the material. This method, however, is not very precise in dosage or timing of uptake of the material, leading to variability between individuals. Instead, microgavage of larval zebrafish offers a better way to deliver a precise dose of material directly to the anterior bulb of the developing gut with precise timing allowing for rigorous and reproducible studies of intestinal barrier function and motility (Cocchiario & Rawls, 2013). In this study, I microgavaged groups of inflated and uninflated larvae with a Texas Red (TR)-conjugated 3 kDa dextran to assay for gut barrier integrity. The 3 kDa dextran is large enough that will not be permitted to diffuse outside the gut lumen in larvae with intact barrier function. Conversely, TR-dextran fluorescence will be observed outside of the gut lumen if barrier function is compromised. As a positive control for leakage, I microgavaged larvae with an EDTA-solution mixed with the TR-dextran. EDTA is a Ca^{2+} -chelator that disrupts epithelial tight junctions (Cocchiario & Rawls, 2013), thus inducing a similar loss of barrier function predicted for hypoinflated larvae (Figure 19).

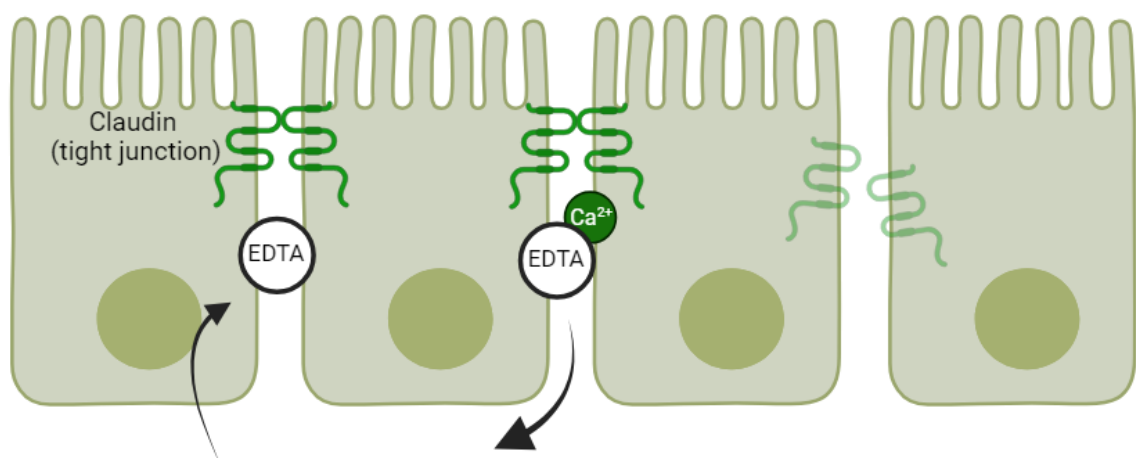


Figure 19: Disruption of the epithelial tight junctions using EDTA: The epithelial lining is created with enterocytes linked via tight junctions composed of claudin. When EDTA is introduced (left) it chelates the calcium from the junction (centre) which disrupts the integrity of the claudin junction resulting in gaps in the epithelial lining (right). Schematic created using BioRender.com.

As expected, larvae that were microgavaged with EDTA solution exhibited significant leakage of the 3 kD dextran from the lumen of the gut into the surrounding tissue. Interestingly, uninflated larvae exhibited statistically indistinguishable leakage ($p = 0.99$) to EDTA positive controls, and normally inflated larvae showed negligible leakage ($p = 0.02$ with respect to EDTA controls) (Table 16). Confocal microscopy images of these larvae taken at $25\times$ magnification showed an array morphological variation (Figure 20). These data helped distinguish between normal gut morphology and abnormal leakage beyond the epithelial barrier that was illuminated with the transgenic marker in these fish.

Table 15: The ANOVA result analyzing the effect of treatment on gut leakage.

Source of Variation	SS	df	MS	F	p-value
Treatment	1.369e+09	2	684704278	6.172	0.00966
Residuals	1.886e+09	17	110942057		

Table 16: Pairwise t-test output for the effect of treatment on gut leakage.

	Inflated	Uninflated
Uninflated	0.02	-

EDTA Positive Control	0.02	0.99
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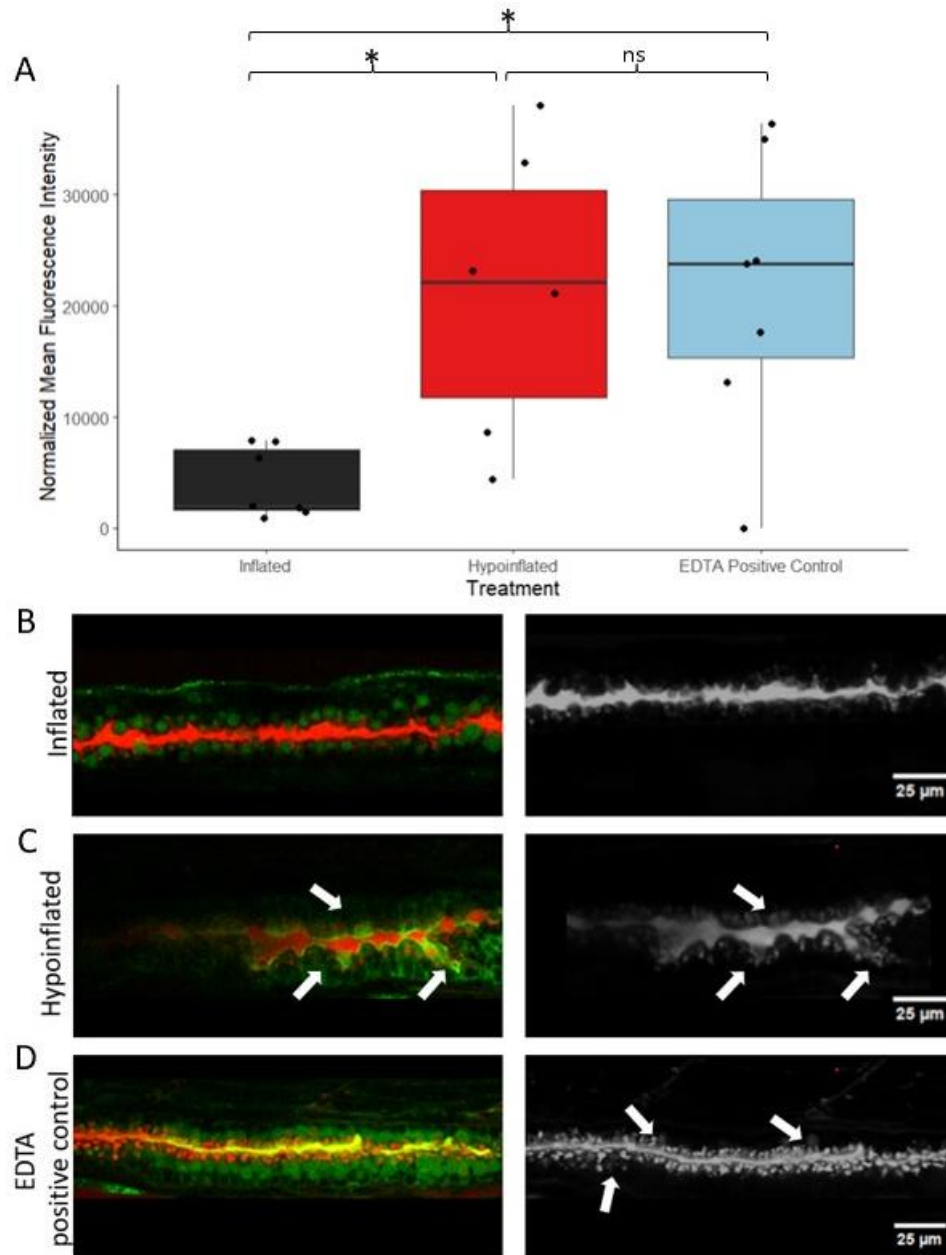


Figure 20: Hypoinflated larvae have leaky guts. A: The normalized mean fluorescence intensity of the spaces beyond the gut lining border in 7 dpf *Tg(apoeb:LY-EGFP)* larvae that were microgavaged with an EDTA-free, Texas Red-dextran solution (inflated and hypoinflated) or an EDTA-dextran solution as a positive control (n=21). * = $P < 0.05$; ns = no significant difference. **B-D:** Representative confocal images of the three treatment groups of 7 dpf *Tg(apoeb:LY-EGFP)* larvae microgavaged with their respective solution. The merged GFP and Texas Red channels (left) are contrasted with their respective Texas Red only channel shown in greyscale for clarity

(right). The white arrows show examples of diffuse leakage in the hypoinflated larva. The EDTA larvae show diffuse leakage of the Texas Red dextran. The inflated larva does not show leakage. The finger-like projections in the inflated larval gut are normal morphology and not leakage.

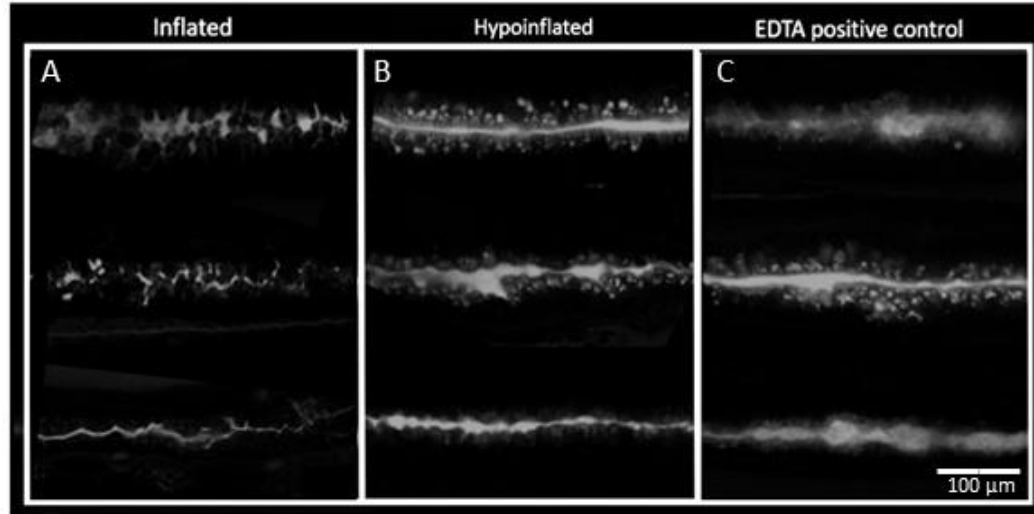


Figure 21: Hypoinflation causes increased permeability comparable to EDTA. Further representative confocal images of 7 dpf larvae microgavaged with a Texas Red-dextran solution (hypoinflated & inflated) or a dextran-EDTA solution (positive control). The diffuse and blurred edges of the positive control and hypoinflated larvae are indicative of gut barrier leakage. The inflated group shows differing normal morphology with smooth edges or finger-like projections but no leakage beyond the intestinal lumen. Scale bar = 50 μm.

4.3.3 Hypoinflation negatively impacts presence of acidified vacuoles within enterocytes of the larval gut

In stomachless fish like zebrafish, digestion is mediated, in part, by vacuolated enterocytes of the GI tract that contain a high density of lysosomes that help hydrolyse proteins and other nutrients endocytosed from their apical surfaces (Park et al., 2019). Lysosomes are membrane-bound organelles found primarily in animal cells that contain enzymes responsible for breaking down various biomolecules. They are acidified through a process called protonation, in which protons (H^+ ions) are actively pumped into the lysosomal lumen by a proton pump called the vacuolar-type H^+ -ATPase (V-ATPase)

against their concentration gradient, making the lysosomal lumen more acidic than the cytoplasm (Futai et al., 2019). Acidified, membrane-bound compartments in the cell can be visualized with Lysotracker Red (DND-99, Invitrogen, Eugene, OR). The posterior end of the midgut of larval zebrafish contains enterocytes that are lysosome-rich (Gao et al., 2022; Park et al., 2019), so this is the region of the gut that I focused on for my analysis.

In images of inflated larvae, acidified vesicles are visualized as punctate dots localized within the enterocytes (Figure 22). In images of hypoinflated larvae, the Lysotracker fluorescence is most often seen as broad and hazy as it has not been taken up by the vacuolated enterocytes or the compartments are not acidified properly. In instances where there were some punctate compartments in the hypoinflated larvae, their abundance was reduced compared to the inflated larvae at both 7 dpf and 8 dpf. I measured the fluorescence intensity of Lysotracker fluorescence with ImageJ and represented this data visually using R (Figure 23). Qualitatively, there is a decrease in Lysotracker fluorescence in uninflated larvae at 7 dpf, but a lack of datapoints means statistical analysis could not be performed. At 8 dpf, there is a significant difference in Lysotracker fluorescence intensity between inflated and hypoinflated larvae ($p=0.02$; Table 14). These results indicate a possible lysosomal acidification defect in the enterocytes of larvae who fail to inflate.

Table 14: T-test output testing the effect of inflation status on Lysotracker fluorescence intensity at 8 dpf.

t	df	p-value	Upper CI	Lower CI
-2.7985	9.3884	0.01996	-10653.93	-97655.33

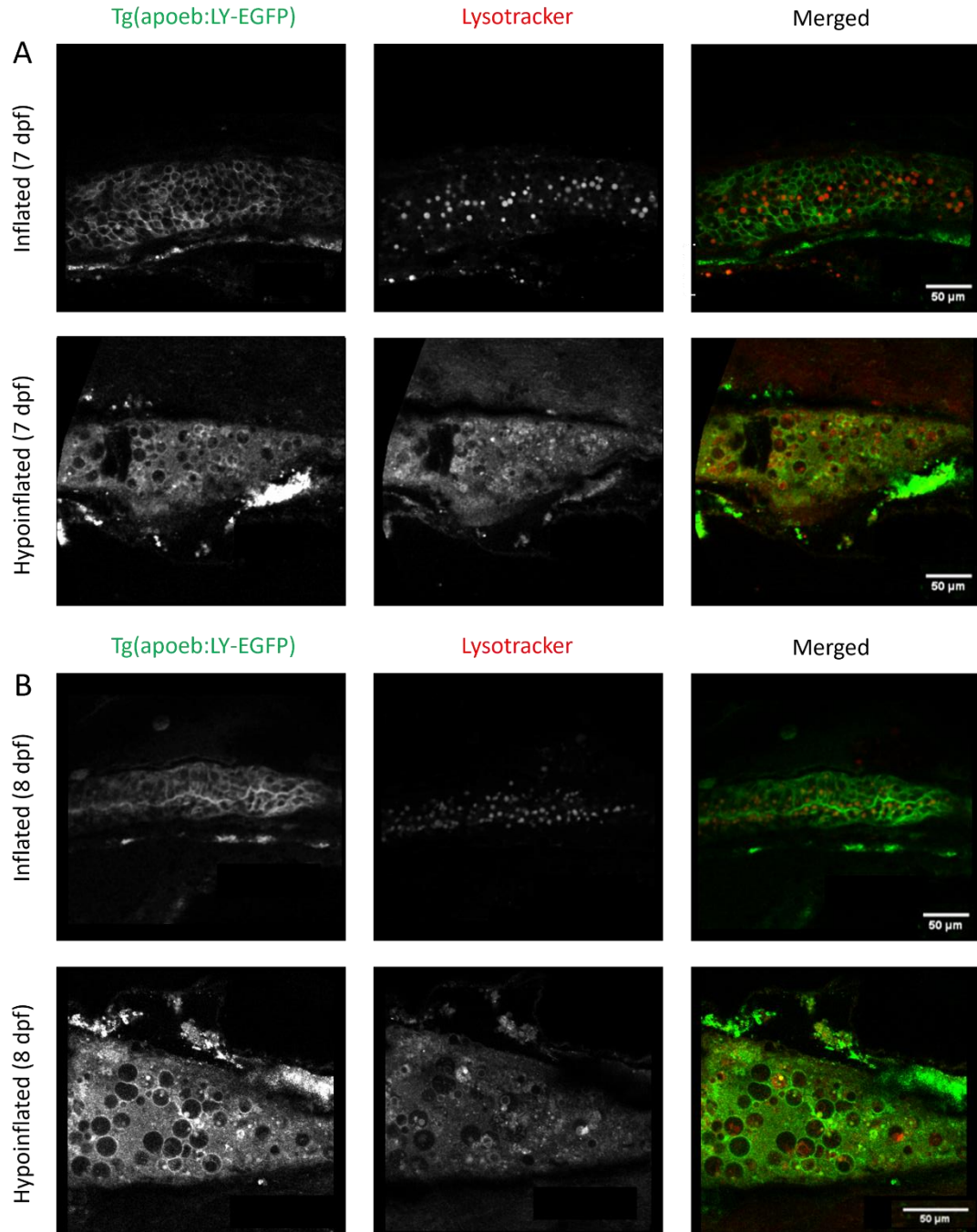


Figure 22: The guts of hypoinflated larvae exhibit acidification defects. A: Representative confocal images of 7 dpf *Tg(apoeb:LY-EGFP)* inflated and hypoinflated zebrafish larvae microgavaged with Lysotracker red and imaged 1-hour post-recovery. **B:** Representative confocal images of 8 dpf *Tg(apoeb:LY-EGFP)* inflated and hypoinflated zebrafish larvae microgavaged with Lysotracker red and imaged 1-hour post-recovery. Scale bars are 50 μm.

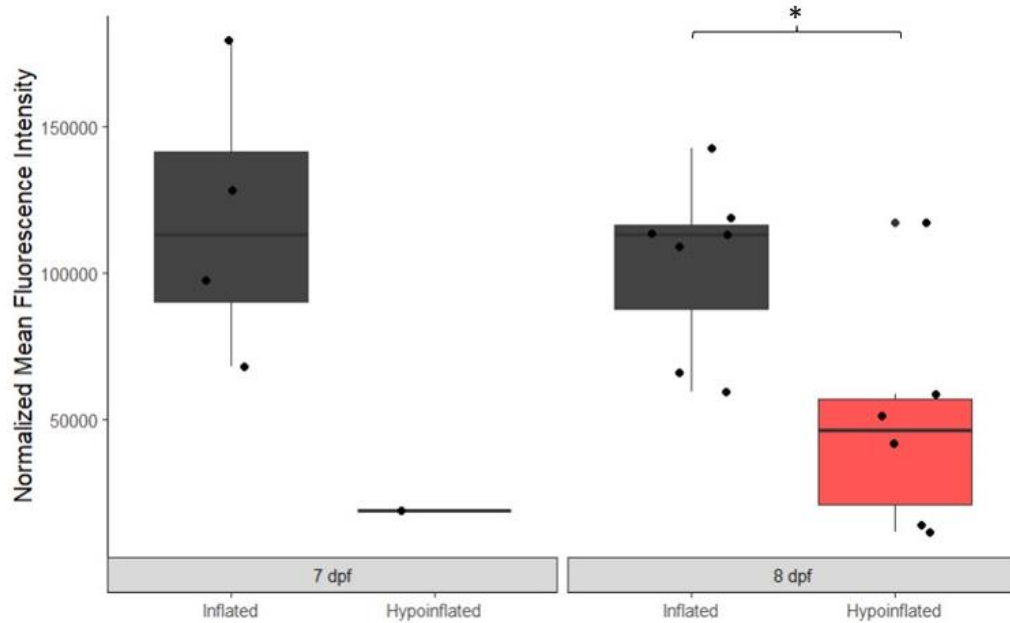


Figure 23: The normalized mean fluorescence intensity of lysosomes in the midgut of 7- and 8-dpf *Tg(apoeb:LY-EGFP)* larvae microgavaged with Lysotracker red and imaged 1-hour post-recovery. T-test p-value = 0.02 for 8 dpf data. Not enough data for statistical analysis at 7 dpf. (n=18).

4.4 Discussion

4.4.1 Hypoinflation significantly alters gross gut morphology in larval fish

This study explored the connection between swim bladder development and gut morphology in larval zebrafish. I observe significant malformations in the guts of hypoinflated larvae; some hypoinflated larvae exhibited gut angles exceeding 180 degrees, emphasizing the extent of deformations caused by swim bladder hypoinflation.

This assay contributes valuable information to the understanding of the interplay between swim bladder development and gut morphology in larval zebrafish. As starvation may contribute to increased mortality of hypoinflated larvae, this link between a malformed swim bladder and malformation of the gut is an important discovery. When the swim bladder is inflated, it occupies space within the body cavity, exerting pressure on adjacent

organs, including the gut. An inflated swim bladder, for instance, pushes the spine up and the digestive tract down (Canepa et al., 2023). Mechanical forces play a crucial role in the development of muscle tissues in organs like the gut (Durel & Nerurkar, 2020; Joshi et al., 2021). This is likely part of the reason why the oil-filled larvae from Chapter 3.3.2 exhibited partially rescued mortality effects compared to hypoinflated larvae as the filling with oil simulated the effects of filling with air. Mechanical forces of inflation and constant pressure from the filled or inflated swim bladder could influence the development and organization of muscles in the gut wall. Changes in muscle development may affect gut motility, potentially leading to altered peristalsis (the wave-like contractions that move food through the digestive tract). Altered gut motility can impact the efficiency of food digestion and nutrient absorption (Abrams et al., 2012). Assays to detect changes in peristalsis and smooth muscle development may shed more light on the consequences of this altered morphology and would provide beneficial information to better understand how hypoinflation affects the developing fish.

4.4.2 Hypoinflation is associated with a disruption in gut barrier integrity

In the context of teleost fish development, successful inflation of the swim bladder represents a pivotal milestone crucial for survival. Failure to inflate severely impacts essential functions like swimming, prey capture, and predator evasion in natural environments, likely leading to starvation and predation-related mortality in the wild. However, uninflated zebrafish larvae in controlled laboratory settings have also shown unexpectedly high mortality rates, despite consuming similar amounts of food as their inflated siblings (Chapter 3). Thus, my findings challenge the prevailing belief that mortality of uninflated larvae is attributable to starvation resulting from lack of food intake.

In this chapter, I evaluated impaired gut function as a possible contributor to the mortality of uninflated zebrafish larvae.

Given the shared developmental origins of the swim bladder and the gut, a comprehensive investigation into the interplay between the swim bladder and gut is imperative. I proposed that hypoinflation disrupts the gut barrier integrity of larval fish, compromising tight junctions between gut enterocytes. My results on 7 dpf hypoinflated larval zebrafish shows a significant deterioration in gut barrier integrity, which may be a mechanistic contributor to starvation in these fish. Further investigation is required to determine if starvation is a downstream consequence of gut degradation. However, degradation can be harmful in a number of ways regardless of the possible link to starvation.

Immune stimuli and inflammatory mediators like TNF- α influence the permeability of the tight junctions that link the epithelial cells lining the gut (Zhang et al., 2015). It is reasonable to hypothesize that the loss of gut barrier integrity in hypoinflated larvae is caused by an inflammatory reaction in the uninflated swim bladder. Additionally, intact tight junctions in the gut act as a barrier to bacterial infiltration (Zhang et al., 2015). In uninflated turbot (*S. maximus*) hyperplasia of gas gland cells and *rete mirabile* occurs, as well as bacterial infection with ‘intense necrosis’ in neighboring tissues (Padrós & Crespo, 1995; Padrós et al., 1993). Compromised barrier function could further enhance an inflammatory response as the infiltrating bacteria stimulate the influx of macrophages and neutrophils. Based on this information and the results from my gut barrier assay, I predict that the compromised gut barrier is caused by a damaging inflammatory response induced

by hypoinflation. Future research should focus on examining uninflated larval zebrafish for inflammatory markers to confirm this.

As intestinal enterocytes are responsible for absorbing nutrients and prepping them for circulation (Zhang et al., 2015), disruption of enterocyte function could also cause malnutrition. A cell morphology assay could be beneficial to assess cell structure and function for an indicator of malnutrition in uninflated larvae, as well as an assay for digestion ability. Additionally, a timeline of epithelial degradation from 5 dpf to 8 dpf would provide further insight into the effect of hypoinflation on gut barrier integrity.

4.4.3 Hypoinflation negatively impacts presence of acidified vacuoles within enterocytes of the larval gut indicating compromised digestion ability

In inflated larvae microgavaged with Lysotracker, presumptive lysosomes were clearly visualized as distinct dots, as expected within the enterocytes of the posterior midgut (Park et al., 2019). Contrary to this, the Lysotracker stain was indistinct and hazy within the guts of uninflated larvae. Where distinct lysosomal labeling was observed in hypoinflated larvae, there appeared to be fewer labeled compartments compared to the inflated larvae at both time points. These findings suggest deficiencies in lysosome biogenesis or acidification in the enterocytes of larvae that fail to initially inflate their swim bladders, possibly stemming from disrupted cellular processes caused by swim bladder hypoinflation. Since bacterial infiltration of non-inflated swim bladders can cause an inflammatory response (Padrós et al., 1993; Marty et al., 1995), and these infections are known to affect neighbouring tissues, it is possible an inflammatory response to hypoinflation is responsible for both the gut permeability disruption and lysosomal deficits. Another possibility is that disruptions in swim bladder development might be impacting

the blood supply and neural connections in the surrounding area, affecting the overall function of the gut. Additionally, the pressure exerted by the developing swim bladder on the neighboring gut could influence the mechanical forces experienced by the gut tissues. These mechanical cues play a crucial role in the development and organization of smooth muscles in the gut wall (Joshi et al., 2021), affecting gut motility and, consequently, the efficiency of food digestion and nutrient absorption.

While my results suggest differences in presumptive lysosome distribution between the two treatment groups (Figure 22), it is important to acknowledge the limitations of this experiment. Firstly, the research focuses on zebrafish larvae, and while they serve as a valuable model for understanding developmental processes, the specific outcomes observed may not directly translate to all other fish species, as some variation occurs between phylogenies. Additionally, the study does not delve deeply into the genetic or molecular mechanisms underlying these phenomena, leaving room for further investigation in these areas. For example, I had planned to perform another microgavage experiment to examine whether the lack of punctate Lysotracker stain in hypoinflated larvae was due to a lysosomal defect versus an endocytic defect. I was going to microgavage with FM-143 dye and image for uptake, however the absorption spectrums overlap making the imaging inconclusive. This could instead be performed with another dye in future analysis.

Nonetheless, this assay provides support for the hypothesis that hypoinflation disrupts the ability of the gut to either absorb or mobilize nutrients. Thus, while uninflated larvae may consume food, they may still “starve” to death due to gut dysfunction. To further this theory, an enzymatic activity assay could provide information to indicate compromised nutrient breakdown. To date, the effects of swim bladder hypoinflation on

gut form and function have been largely ignored. It is now clear that swim bladder hypoinflation causes severe damage to gut form and function, and overall survival in larval fish.

Chapter 5: Conclusion

In this thesis, I set out to unravel some mysteries surrounding swim bladder hypoinflation, specifically focusing on zebrafish larvae, a physostome species with retained pneumatic ducts. One of the fundamental questions driving this inquiry was whether zebrafish larvae, like many other teleosts, have a critical period for initial swim bladder inflation. Contrary to prevailing wisdom, my results demonstrate a distinct critical period for swim bladder inflation in zebrafish larvae, starting from 4 dpf, when the first larvae begin inflating their swim bladders, and ending at 7 days post-fertilization. Beyond this point, larvae lose the ability to inflate, highlighting a crucial developmental milestone.

To investigate this phenomenon, I conducted experiments restricting air access to larval zebrafish, demonstrating that the ability to initially inflate the swim bladder is lost before 8 dpf. Statistical analyses confirmed that zebrafish larvae lose the ability to inflate if restricted beyond 7 dpf, contrary to the prevailing physostome theory that there should not be a temporal limit. Importantly, the inability to initiate inflation precedes a decrease in survival, indicating the critical importance of successful swim bladder inflation during this temporal window for optimal survival chances. The observed decrease in survival rates becomes more pronounced after the critical period of inflation has passed, underlining the vital role of proper swim bladder development for larval viability.

Consequently, this temporal demarcation is limited with a caveat that the observed high mortality in hypoinflated larvae could skew the interpretation of the data. Is the critical period defined by the end of the period in which the larvae are able to initially inflate, as hypothesized, or is it defined by the death? Moving forward, internal investigations such

as histological examinations of the pneumatic duct, could provide valuable insights. can build upon these findings.

Contrary to the prevailing assumption, my research provided compelling evidence that hypoinflated larvae are not hindered in their ability to consume food. Quantitative analysis revealed that hypoinflated larvae at 8 days post-fertilization consumed a comparable amount of food to their typically inflated counterparts, dispelling the notion that starvation results from an inability to eat in hypoinflated larvae.

To comprehensively investigate the relationship between swim bladder hypoinflation, starvation, and mortality, I conducted survival experiments under controlled laboratory conditions. Surprisingly, my results indicated that the presence or absence of food did not significantly impact the survival of uninflated larvae. Furthermore, larvae with filled swim bladders, regardless of the contents (air or oil), exhibited increased survival rates compared to their uninflated counterparts. Notably, larvae with oil-filled swim bladders demonstrated enhanced survival to that of either of the hypoinflated groups, suggesting a potential rescue effect associated with swim bladder filling. This observation hinted at the possibility that mechanical forces related to swim bladder inflation could influence larval survival. Additionally, I explored the utilization of endogenous lipid reserves, represented by the yolk sac, in both inflated and hypoinflated larvae. The results indicated that hypoinflation did not affect the speed of yolk sac usage, suggesting that hypoinflation was not linked to differences in yolk absorption rates.

This is inconsistent with prevailing theory attributing high mortality rates among hypoinflated larvae to starvation, as my results demonstrated that hypoinflated larvae consumed similar amounts of food as their inflated counterparts, suggesting that mortality

was not solely linked to reduced food intake. Instead, I turned my attention to exploring potential disruptions in gut form and function as a contributing factor to increased mortality in hypoinflated larvae. My investigation into gut morphology and function provides a deeper understanding of the swim bladder-gut relationship.

Gut barrier assay performed on hypoinflated larval zebrafish reveals a significant deterioration in gut barrier integrity, potentially contributing to the mechanistic starvation observed in these fish. The compromised gut barrier integrity might be a result of an inflammatory reaction spurred by hypoinflation and potential swim bladder degradation. Future research should focus on examining uninflated larval zebrafish for inflammatory markers to confirm this hypothesis. As well, lysosome acidification defects in the enterocytes of hypoinflated larvae suggest intricate connections between these systems. I hypothesize that this lysosomal dysfunction is indicating a disruption in digestive ability leading to starvation, but more research is needed to confirm that. Future studies delving into the genetic and molecular underpinnings of these interactions could unveil crucial insights into fish physiology and health. Further research into the interplay between swim bladder hypoinflation, gut function, and immune responses holds promise for unraveling complex physiological networks.

In this thesis I challenge existing assumptions and shed light on previously under-researched facets of fish physiology. By bridging gaps in our knowledge, these findings serve as a robust foundation for future research endeavors. Exploring the interplay between swim bladder hypoinflation, gut function, and immune response holds promise for unraveling complex physiological networks, paving the way for a deeper understanding of

fish physiology. In conclusion, this thesis not only enriches our understanding of swim bladder development but also opens doors to exciting avenues of research.

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Appendix 1: Recipes

1. MS-222 / Tricaine (0.4% w/v ; 25X stock solution)
 - 200 mg MS-222 powder
 - 49 ml distilled water
 - 1 ml 1 M Tris pH 9.5
 - Diluted 1:25 (0.016%) for working solution
2. E3 embryo medium 50X (2L)
 - 1800 ml distilled water
 - 29.2 g NaCl
 - 1.3 g KCl
 - 4.8 g CaCl₂-H₂O
 - 6.8 g MgCl₂-6H₂O
 - pH to ~7 & top up to 2 L with dH₂O
3. E3 embryo medium 1X (16L) – 5 mM NaCl, 0.17 mM KCL, 0.33 mM CaCl₂, 0.33 mM MgCl₂
 - 15.68 L distilled water
 - 320 ml 50X E3
 - 67 ml 1 M sodium bicarbonate
 - i. 5.9 g sodium bicarb
 - ii. 67 ml dH₂O
4. 4% paraformaldehyde (PFA) in PBS (4 mL)
 - 500 ul 32% PFA
 - 400 ul 10X PBS
 - 3100 ul water
5. 1.25% Nile Red solution (1 ml)
 - 1.25 mg Nile Red
 - 1 ml acetone
 - Store in the dark at -20°C
 - Add to 3 ml E3 (0.5 µg/ml) for working solution
6. 3kD dextran solution (1 ml)
 - 200 ul 5% dextran conjugated to Texas Red
 - 100 ul 10X PBS
 - 100 ul 0.5% phenol red
 - 600 ul ddH₂O
7. 1:10 EDTA:3kD dextran solution (220 ul)
 - 200 ul 3kD dextran solution
 - 20 ul EDTA, pH 8, 500 mM

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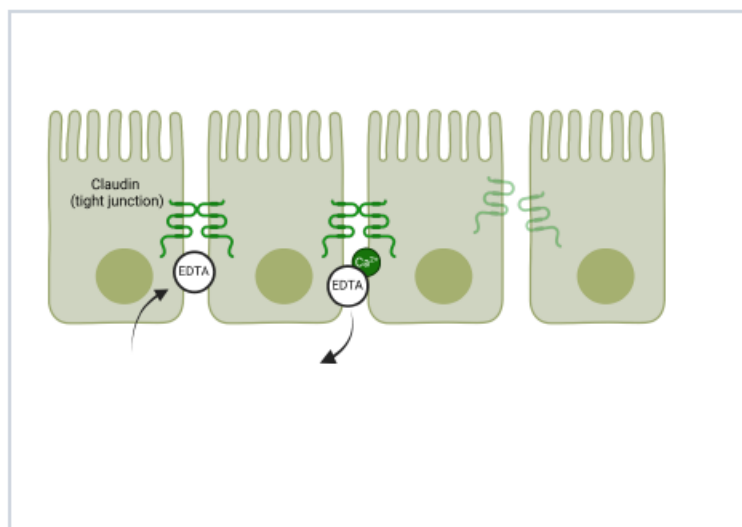
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Appendix 3: Scientists Connected Through Time

Trigger Warning

The following appendix discusses topics including, but not limited to, murder, genocide, the Holocaust, antisemitism, and general violence. Please read at your discretion and comfort level only. The contents of this appendix are not necessary to understanding the main body of this thesis; it is a historical passion project related to an aspect of the research.

Summary

Throughout my research for this thesis, I was guided by nearly 100 articles written by hundreds of scientists. As I looked deeper into just one of those scientists, I was overtaken by curiosity about his life, his work, and his death. Born in the early 1900s, Joachim von Ledebur's academic career started to take off just as the Nazi party came to power in Germany. Exactly what happened to him after 1938 remains lost to history. However, through genealogy records, Polish university archives, vital statistics records, grave marker records, Holocaust memorial records, and books, I have compiled a brief historical overview of the time along with my own speculation on the possible outcomes for von Ledebur. Peoples' stories matter, and peoples' stories are worth telling. Thank you for reading his.

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Chapter 1: The Apparatus

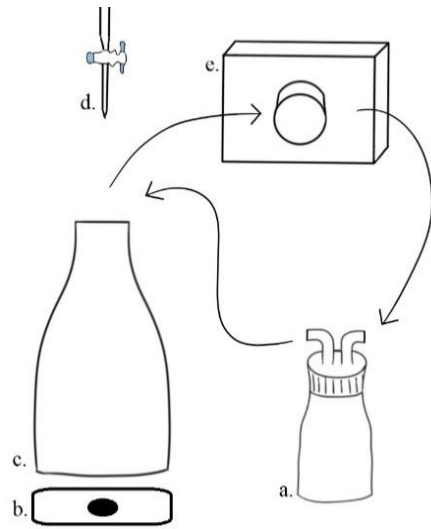


Figure 24: The first prototype for the modified air-water interface restriction apparatus schematic. A: Sealed test-chamber. B: Magnetic stirrer. C: 1L reservoir. D. Automatic feeder. E. Pump.

initially fill their swim bladder. These small bubbles that remained under the cap of the restriction bottle (Figure 24), were difficult to remove with consistency, resulting in many delays and failed trials.

Through near-frantic and semi-desperate deep diving into the literature, I

came across a single sentence in an older paper (S. I. Doroshev & Cornacchia, 1979) that mentioned an experiment that used a restriction apparatus to prevent swim

When designing the original prototype for the air-water interface restriction apparatus, inspiration was drawn from a similar experiment (Goolish & Okutake, 1999b) that showed a diagram for their working apparatus (Figure 25). In modifying it for my purpose, there were many months of trials and tinkering in an attempt to get it to work without flaw. Zebrafish larvae, as has been discussed in the body of this thesis, are very good at finding even the smallest of bubbles that they then use to

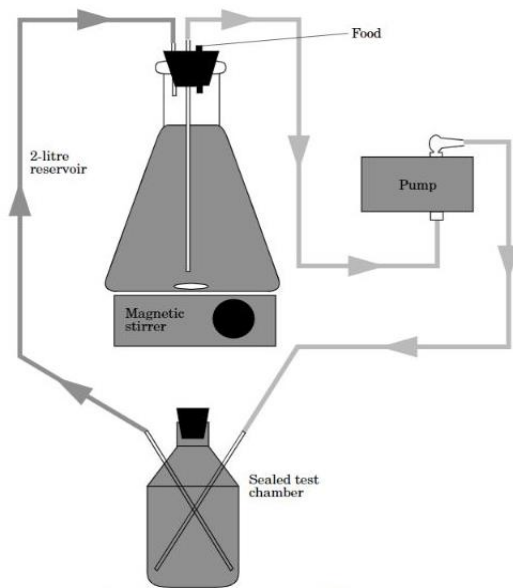


Figure 25: Schematic for the air-water restriction apparatus that was used as the inspiration for my first design of my own apparatus. This apparatus was referred to as the Goolish Apparatus, after the first author of the original publication.

bladder inflation. Back-tracking to find the original publication, I found J. v. Ledebur and W. Wunder's 1937 publication (J. F. von Ledebur & Wunder, 1937), written in German. This paper had exactly the information I needed, and their design became the basis for my final restriction apparatus that I continued to use for the duration of the experiments within this thesis.

As the information in the von Ledebur & Wunder paper proved so interesting and useful, I kept reading. Wilhelm Wunder was born in 1898 and authored dozens of research papers on fish and reptile biology as well as several textbooks, publishing his 58th and final work in 1988 at the age of 90 (WorldCat, 2023). When looking at the publication list for von Ledebur, he drops off the scientific radar in 1939. There is very little record of Joachim von Ledebur, except that he was born Lyck, Poland in 1902, submitted his dissertation titled 'Über Pancreacysten' (About Pancreatic Cysts (ZVAB, 2023)) in 1926, and co-authored several papers on physiology between 1930 and 1939. Then, World War II began in September 1939, and von Ledebur is not seen on the record again. Searches of both Holocaust records and German military records turned up no one by that name and year of birth.

Whether Axis or Ally, or one of the millions of Holocaust victims, or perhaps just a scientist doing his best to survive during a historic geo-political moment in time, Joachim von Ledebur's contribution to the scientific community is remembered through time. But, what happened to him?

Chapter 2: Joachim Oskar Hasso von Ledebur

Initially, I worried that the J. v. Ledebur I had been silently thanking for aiding my efforts had disappeared off the scientific radar because he had become a Nazi. A man fitting

this abbreviated name and age is a well-documented war criminal and Nazi who defected to the British MI6 (The British National Archives, 1945) in 1944, eventually making his way to Argentina. I struggled with crediting Ledebur the Nazi in my thesis because of this. When I discovered details of his early life, and noted how they did not mention science or anything remotely related, I dug a little further. The true J. von Ledebur who published the fish research was not easy to find, but I can confirm these are two different men.

On August 11, 1902, Joachim Oskar Hasso von Ledebur was born to Arthur and Anna von Ledebur (nee Walter) in Lyck, Poland (then Prussia). There is some indication that Anna's mother Minna Becke was an Ashkenazi Jew. Additionally, there are several people on her side of the family tree that have stereotypical Jewish names such as:

- Maria Steinbruek, Joachim's great aunt
- Paul Schwartz, Joachim's second cousin
- Margarethe Eckardstein, Joachim's aunt

On the other side of the tree, we have Joachim's father, Arthur. Arthur's father was Karl von Ledebur (Joachim's paternal grandfather), a highly decorated war veteran and prominent German theatre director. There are conflicting reports on whether Karl was married two or three times, however one of Karl's wives was actress Josephine



Figure 26: Karl von Ledebur (Bühnen-Angehöriger, 1908)

Birnbaum, who was an Ashkenazi Jew. Some records indicate that she was Arthur's mother, however they were not married until 1874: when Arthur was already 8 years old. By rational accounts, Elisabeth (Karl's first wife), was Arthur's mother. She unfortunately died when Joachim was just 12 days old.

The precise Ledebur heritage is unknown, but context clues lead me to believe they may have been ethnic Jews on Joachim's mother's side. On his father's side, the addition of 'von' in the name denotes ties to the German aristocracy which did not commonly include Jewish families, yet the density of the Jewish population where they were from, and the fact that inter-racial marriages were not common, it is possible that the von Ledeburs were a Jewish family as well. In families where interracial marriage did occur, they were later classified by the Nazis as *Judisch versippt* (related by marriage to Jews).



Figure 27: The grave marker of Karl von Ledebur in the Fricourt German Military Cemetery in Fricourt, France.

The von Ledebur lineage is linked to the noble families of the Kingdom of Westphalia, wherein in 1808, Napoleon passed the first laws granting Jews equal rights. The Kingdom was one of the most progressive regions of the time, being the first to enact new reforms. In the following 100 years, many new titles were conferred to Jewish families equating to about 9% of the total German aristocracy. It is possible that the von Ledeburs were one of these families and that this is where they gained their nobility titles.

To understand the other hints at the von Ledebur's heritage, first we must understand some of the academic social structure of Karl's time. Karl was a successful theatre director, and started his career by attending a Prussian cadet corps. These military schools for boys were notorious for *Mensur* (academic fencing) within *Burschenschaften* (the German equivalent of fraternities). Academic fencing is a traditional form of fencing

that originated in Germany and Austria in the late 19th century. This type of fencing uses a specific type of sword called a Mensur blade. The goal of the fencing is not to score points, but to gain experience and develop character, and often to solve disputes of honor. The duels lasted until one participant surrendered, and thus were nearly never fatal, though many participants were left with scars particularly on the face (Figure 28). These facial scars are called *schmiss* (dueling scars) and became a badge of honor. At the time of Karl's attendance of the cadet corps, these scars were considered very fashionable. Throughout this period and beyond, clean shaven looks were preferred so their scar could be proudly displayed.



Figure 28: Otto Skorzeny, a Waffen-SS member during World War II, proudly displaying his dueling scar.

Academic fencing was primarily practiced by students at universities and military training corps, and was seen as a way to build discipline, courage, and physical fitness. It was particularly popular in Germany and Austria, and many student fencing clubs were formed in the late 19th and early 20th centuries. As these fencing clubs grew in popularity, minority groups began to create or join, and as a result we see documentation of Jewish men displaying their dueling scars in the later portion of their era. In Karl's time, however, Jews would have been disallowed from joining the *Burschenschaften* within the corps. In the few photos of Karl that exist today, he is always displayed with his full and luscious beard, and no discernable facial scarring. Given the popularity of the duels and the display of the scars, the fact that Karl is not proudly displaying any leads me to believe that he did

not have them. He attended a cadet corps that certainly practiced *Mensur* so perhaps the reason he was not included was because he was Jewish.

But, back to Joachim.

The youngest of three, Joachim grew up with his parents and his siblings, Dora and Walter. His father Arthur joined the German army and was employed as a commander of the Husaren Regiment #12 in Oberst, Germany in 1911. He was promoted to cavalry commander in February 1915, just after World War I began in July 1914. He was promoted again to general major on October 31, 1918. The war ended less than 2 weeks later, on November 11, 1918. Arthur apparently died in 1945, but corresponding documentation cannot be found. It is unclear if this was because he was one of the many victims hastily sent to the gas chambers as the end of WWII drew ever closer, or if he died in an unrelated manner.

Joachim von Ledebur attended the University of Jena where he submitted his doctoral dissertation (Figure 29) in 1926 at the age of 24 entitled ‘Über Pancreacysten’ (About Pancreatic Cysts (ZVAB, 2023)) under the supervision of Professor Dr. Berblinger, a renowned pathologist and major contributor to the emerging discipline of endocrinology. Dr. Berbinger had accepted the position of Chair of Pathological Anatomy at the University of Jena in September 1922.

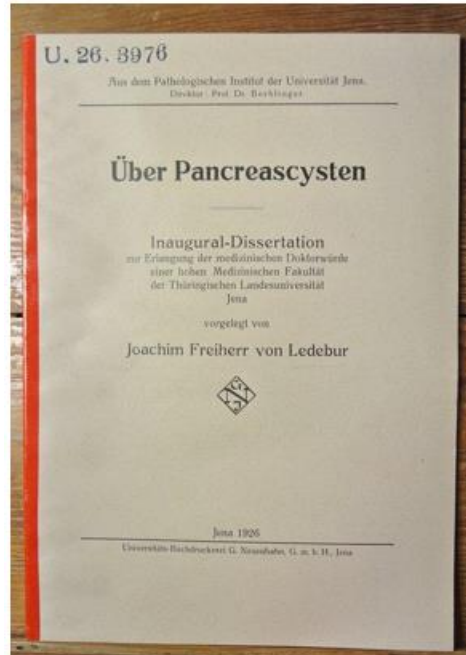


Figure 29: Joachim von Ledebur's doctoral dissertation.

The year following his dissertation, Ledebur published his first journal article while at the University of Rostock, in Rostock, Germany. He then moved to Breslau, Germany (now Wrocław, Poland) where he published his second article in 1928. Below is a list of the dates and locations of his subsequent publications:

- 1929: May & June, Breslau (Frhrn Ledebur, 1929; J. von Ledebur, 1929).
- 1930: June, Breslau (Wachholder & von Ledebur, 1930).
- 1931: January, May & October, Breslau (Joachim Frhr von Ledebur, 1931; Kurt Wachholder & von Ledebur Mi, 1931; J. F. von Ledebur, 1931).
- 1932: January, March, May, August (2), & October, Breslau (Joachim Frhr von Ledebur, 1932a, 1932b; Kurt Wachholder & Freiherr von Ledebur, 1932; J. F. von Ledebur & Hurt, 1932; Wachholder & Freiherr von Ledebur, 1932).
- 1933: March & April, Breslau (Joachim Freiherr von Ledebur, 1933; J. F. von Ledebur & Wachholder, 1933).

- 1934: June, Breslau (J. von Ledebur & Wunder, 1934).
- 1937: October, Breslau (J. F. von Ledebur & Wunder, 1937)
- 1939: Two chapters in the book *Ergebnisse der Biologie*, Berlin.

After 1939, Ledebur drops off the publication radar except where his works are cited in hundreds of publications from then until now. It is unclear when his chapters in the 1939 book were submitted, but, as I will detail later, I am not convinced von Ledebur was still alive by the time the book was published.

I have parsed out a few theories of what may have happened to Joachim von Ledebur, and each one breaks my heart in a different way. I will detail these theories for you as we take a brief journey through the life and times of Eastern Europe in the 1930s and '40s.

Chapter 3: Universities in Nazi Germany

While WWII would not officially begin until September 1939, the anti-Semitic sentiments that lead to the Nazi regime had long been growing in Eastern Europe. The National Socialist Party came to power with Hitler at the helm in 1933. By this time, von Ledebur was an accomplished researcher and instructor (Schlesische Friedrich-Wilhelms-Universität zu Breslau, 1934) at the University of Breslau in Breslau, Germany (now Wrocław, Poland). Universities across Germany immediately underwent a massive wave of dismissals of Jewish faculty members in 1933 (Grüttner, 2022). As early as February, instructors were being dismissed on antisemitic grounds at Breslau after multiple instances of armed protests called for their dismissal and shut down the universities with their blockages (Jewish Telegraphic Agency, 1933). By late 1935, the *Volksbildung*

(Thuringia's Ministry of People's Education) reported there were no remaining Jewish employees at the University of Jena where Dr. Berblinger (Figure 30), Joachim's former doctoral supervisor, was working at the time. In an analysis of these dismissals across the different universities, it was determined that 80% of those dismissed were done so on the grounds of antisemitism, yet less than a third were actually Jewish (Grüttner, 2022). Later, Hitler ordered the elimination of Polish 'intelligentsia' and in 1939 there was a wave of arrests of professors in Krakow and elsewhere. Within a few months, most of them were dead – or 'liquidated', as crudely described by Nazi leader Hans Frank (Aurthur



Figure 30: Dr. Walther Berblinger (UAJ Photo Collection, no. 311)

Allen, 2014). Of those who were released, some bravely defied the regime further by returning to teaching at an underground university that was attended by students such as Karol Wojtyla, the future Pope John Paul II (Aurthur Allen, 2014).

Though Berblinger was of "Aryan" ancestry, he was married to Hedwig Elstaetter, the daughter of a Jewish factory owner. In 1937, Berblinger and one other man were the only remaining employees described as *Judisch versippt* (related by marriage to Jews). In the subsequent seven years, the University of Jena withdrew dozens of doctoral degrees from Jewish alumni (Grüttner, 2022). Through his exemplary record and reputation, Berblinger was able to hold onto his position longer than most despite the discrimination against his wife's heritage.

As Nazi rhetoric infiltrated every nook and cranny of German life, Berblinger's reputation would not be enough to keep him from facing discrimination forever. The enforcement of the *Gesetz zum Schutze des deutschen Blutes und der deutschen Here* (the Law for the Protection of German Blood and Honor) in 1937 would classify Berblinger as "Jewish-like", and those in power insisted he divorce his wife or face consequences. Hedwig's sister also faced similar circumstances. Her husband obliged, and they were divorced in 1938 at the behest of the regime. When Berblinger refused the ultimatum, he was forced to retire his position and immediately took a position in Switzerland where he and his wife lived the rest of their lives together. He would never return to his homeland, even after the war had ended.

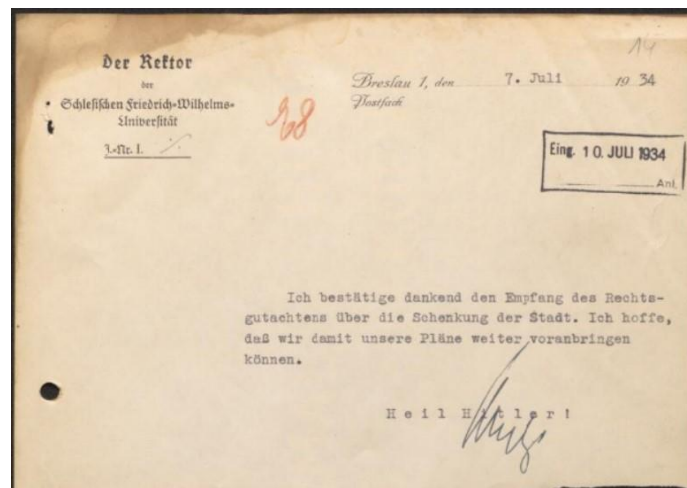


Figure 31: A university of Breslau thank you letter regarding a recent donation, 1934. Note the 'Heil Hitler' salute at the end.

In the seven years between Ledebur's dissertation and Hitler's rise to power, Ledebur published 13 articles, including six in 1932. After 1933, Ledebur only published two papers (one in 1934 and one 1937). While the nuances of von Ledebur's life at the University of Breslau are unclear, the university underwent similar expulsions as Berblinger's university. At the time, and before, it was not uncommon for professors and scientists to spend long hours at cafes where they could meet, network, and discuss their

work outside the scrutiny of the universities(Aurthur Allen, 2014). Official university correspondences have signatures featuring ‘Heil Hitler’, indicating political integration within the university by this point (Figure 31). Between 1933 and 1945, the University of Breslau deprived and revoked more than 250 doctoral degrees from Jewish students and alumni. Across Germany, conservative estimates of upwards of 2000 degrees were revoked, often following the withdrawal of German citizenship from those of Jewish heritage (Grüttner, 2022).

Throughout the Nazi tyranny, many universities required genealogical records of parents and grandparents, military service records, photographs, and more to approve new students/staff, and for the right to remain employed for current staff. The decision was often influenced by reputation and letters of recommendation from members in good standing with the Reich. This process of subjective argument may have been what allowed von Ledebur to remain at the University of Breslau after 1933, just as it had allowed Berblinger to remain at Jena, but would put a damper on the extent of his work post-1933. Not only did von Ledebur’s publications slow down, but his teaching schedule was also reduced. In the earlier years of his attendance at Breslau, he is listed as being on different oversight committees, teaching several physiology courses each year, and listed as an assistant professor of physiology. By the academic year 1937/38, he is listed as an instructor on leave, and he is no longer teaching any courses (Schlesische Friedrich-Wilhelms- Universitat zu Breslau, 1937). The following year, he is off the record entirely.

In addition to the removal of Jewish students, staff, and faculty, the University of Breslau used forced labor between 1933 and 1945. The Act on the Prevention of Genetically Diseased Offspring was enacted in 1933, emboldening the eugenics movement

to subject 1200 Jewish women and forced laborers to reproductive sterilization at the university's own Women's Clinic (Finzsch et al., 2014).

Chapter 4: *Polenaktion*

A few years later, in early 1938, Germany annexed Austria causing a geopolitical ripple throughout the region. With tens of thousands of Polish Jews living abroad, the Polish government feared this progression of events from Germany would cause an unmanageable influx of Polish Jews to return to Poland in hopes of avoiding anti-Semitic persecution. As Poland was still recovering from the depression, fears of this mass emigration pushed Poland to pass a law in early October 1938 that allowed the denationalization of Polish citizens if they had been living abroad for more than five consecutive years. This new law required that Polish passports issued abroad would only be valid with a special stamp of approval. This law would come into effect on October 30, 1938.

Germany took this new law as a direct offense against their own attempts to remove foreign Jews from Germany and offered an ultimatum to Poland: rescind the law, or Germany would deport everyone before it came into effect, essentially making Poland's new law ineffective before it even came to pass. Without waiting for a response to the ultimatum, the Reich instructed police offices across the country to take all Polish Jews into 'protective custody' and transport them to the border to be deported immediately.

Throughout October 28th and 29th 1938, upwards of 17 000 Jews with Polish citizenship were detained from their homes, everywhere from large cities to rural villages. They rounded up everyone they could find, including entire families to young unaccompanied children. They were instructed to pack only one suitcase after which they

were transported via train to the border. Poland was not expecting such an act and was woefully unprepared when the first trains began to cross. In short time however, Poland stopped allowing them to cross and sent the subsequent trains back to Germany before they could cross. This created a stalemate in which the people subject to this back and forth were left stranded in the cold at border towns.

Finding refuge in these border towns would not last long, however, as Poland quickly closed access to them. Makeshift refugee camps popped up to give shelter, but conditions were not favorable in a desolate no man's land for these now stateless people. Most habitants of these camps remained in limbo for near a year before they were finally granted access to their homeland of Poland, just in time for the infamous German invasion of Poland in 1939.



Figure 32: Polish Jews expelled from Germany during *Polenaktion* at a refugee camp at the border town of Zbaszyn, Poland in late 1938/early 1939 (Jewish Museum Berlin, 2023).

In the initial chaos of the deportations, one family managed to send a distressing postcard about the terrifying ordeal to their 17-year-old son Herschel Grynszpan who was living in Paris, France. He received the postcard a few days later, on November 3rd, 1938.

The following Monday, he purchased a revolver and made his way to the German embassy in Paris and calmly asked to see an embassy official. Ernst vom Rath greeted Herschel in his office, where Herschel then open fire, shooting five bullets at the diplomat, killing him. When French police arrested Herschel without a fight, they found a postcard addressed to his parents with the words, “May God forgive me ... I must protest so that the whole world hears my protest, and that I will do”.

And hear his protest, they did. Germany retaliated against the news of the assassination swiftly and cruelly. They immediately banned Jewish children from attending school, stopped all Jewish owned newspapers, and enacted harsh penalties to any Jew found with a weapon, including sentences of 20 years confinement in a concentration camp. Two days after the assassination, a catastrophic pogrom against Jews across the nation would be enacted. This pogrom would come to be known as *Kristallnacht* – Crystal Night, or the Night of Broken Glass.

Chapter 5: *Kristallnacht*

The assassination of vom Rath on November 7th, 1938 spurred a major wave of anti-Semitic violence across Germany, Austria, and Sudetenland. November 9th and 10th, 1938 saw a major pogrom against Jews that would later be known as *Kristallnacht* – Night of Broken Glass – named for the shards of glass that littered Jewish neighbourhoods in the aftermath of the wreckage. The pogrom was carried out primarily by the Nazi Party’s paramilitary forces with aid from civilians and the Hitler Youth at the command of Heinrich Müller, a high-ranking SS commander. His order stated that the “most extreme measures” be taken against Jewish people across the region. During the destruction, it was not only tolerated by non-Jewish Germans, but actively welcomed and celebrated. Hugh

Greene, a British journalist in Germany, wrote of “fashionably-dressed women clapping their hands and screaming with glee, while respectable middle-class mothers held up their babies to see the ‘fun’”.

As Nazi sanctioned attacks were mounted across the country on the first night, Jewish homes, schools, businesses, cemeteries, and synagogues were demolished by violent militarized rioters wielding sledgehammers and other instruments of brutality.



Figure 33: The remnants of a Boerneplatz synagogue in Frankfurt, Germany after the *Kristallnacht* pogrom. (Holocaust Encyclopedia, 2023)

An estimated 7 500 businesses were damaged, but property was not the only target of these riots. Brutal beatings and murders were prevalent these nights, and while the victims of the concentration camps, and later the death camps, have been quite well documented, those who perished during *Kristallnacht* were rarely made mention of by name in the annals of historical documents. Upwards of 30 000 Jewish men were detained at this time and sent to what were concentration camps at the time, most of which would quickly devolve into death camps. In the brief time between the arrests and the looming transport to the camps, hundreds of these men died by suicide.



Figure 34: Jewish men being marched through the town of Baden-Baden, Germany on November 10, 1939, on their way to a concentration camp. Based on the proximity to camps at the time, these men were likely led to Dachau. (Holocaust Encyclopedia, 2023)

Kristallnacht was a turning point in the pre-war geopolitical landscape. The persecution of Jews in Germany up to this point had been primarily economic and political in nature, with social exclusion as a tactic for enhancing anti-Semitic rhetoric and response. The ruthless and unapologetically cruel events of November 9th and 10th marked what historians now often refer to as the beginning of the Holocaust as we know it today, shifting the narrative for onlookers to that of outward violence sanctioned by the Reich. Foreign opinion altered drastically as well, with other nations realizing the extent to which hateful leadership was causing incomprehensible violence to its victims. In the year following *Kristallnacht* right before WWII would officially begin, more than 115 000 Jews would flee Germany. In the coming years, more than 11 million victims would never have the opportunity to do so.

Brief Summary Timeline

1920s

- 1926: Joachim von Ledebur (JVL) publishes his dissertation (age 24) in Jena, Germany at *Thüringische Landes universitat Jena*. He begins at Rostock later that year.
- 1927: JVL publishes a paper in April.
- 1928: JVL publishes paper in August.
- 1929: JVL publishes paper May and June. He starts his position at Breslau.

1930s

- 1930: JVL publishes a paper June. He is listed as an assistant physiologist but does not teach any courses yet.
- 1931: JVL publishes 2 papers January and May. He teaches one physiology course.
- 1932: JVL publishes 6 papers Jan, March, May, August (2) and October. JVL teaches two physiology courses.
- 1933: Dachau established in March. JVL publishes two papers in March, April and teaches two physiology courses. Hitler comes to power.
- 1934: JVL publishes in June. He teaches 3 physiology courses.
- 1935-36: JVL still listed as an assistant physiologist at Breslau but no courses taught and no publications.
- 1937: JVL publishes paper in October. He is now listed as on leave from his lecturer position (Schlesische Friedrich-Wilhelms- Universität zu Breslau, 1937).
- 1938: *Polenaktion* 28 October. *Kristallnacht* November 9-10. JVL is not listed at Breslau any longer.

- 1939: WWII starts September 1. Poland invaded by Germany (Sept 1) and Soviet Union (Sept 17). A textbook (*Ergebnisse der Biologie*) with 2 chapters authored by JVL is published. Polish students are expelled from Breslau university in June.
- 1940: Auschwitz-Birkenau & Bergen-Belsen established.

Chapter 6: Theories

Though his parents were German born, Joachim von Ledebur was of Polish birth – it was Prussia when he was born, but Poland by the time of WWII – and thus likely classified as a Polish Jew in the eyes of the regime, if we are to believe the von Ledeburs were Jewish. The addition of ‘von’ in their name is not a saving grace to Nazi tyranny regardless, as can be witnessed by the over 4000 von- names visible in the digitized volumes of the Holocaust victim records. It is also known that the Nazis were very strict on who was considered Aryan, but less descript on who classified as the ‘other’, and their hate knew little bounds when targeting their victims.

Of the Nazi’s 11 million victims, almost half were not Jewish. The yellow star that Jews were forced to wear was just one of many wearable concentration camp badges forced on victims, including the pink triangle for gay men, the black triangle for those deemed *asozial* (asocial) or *arbeitsscheu* (work-shy) including Romanis, alcoholics, lesbians, and those with disabilities. The red triangle was for political prisoners, liberals, communists, and those who assisted Jews against the regime. If von Ledebur was not considered or identified as Jewish, that does not protect him from victimization.

I have three main theories for what may have happened to Joachim von Ledebur, starting with 1938’s *Polenaktion*.

Theory 1: *Polenaktion*

It is possible that von Ledebur was one of the 17 000 people rounded up on the cold autumn days of October 28th and 29th, 1938 during the *Polenaktion* event, wherein Nazi forces loaded trains of people and sent them to the Polish border for deportation. By this time, he has no more published articles on his research and disappears from public record. His book chapters are not published until 1939, however it is possible that these chapters could have been submitted in advance to the final publication and that he was deceased by this time.

If he had been one of the victims of *Polenaktion*, there are a couple possible outcomes for him. He could have died en-route to the border, or in one of the refugee camps. This would explain why there is no further record of him and no known gravestone as the decedents at this time were not able to have been given proper burial or rites due to the circumstances. In comparison to the other atrocities at the time, few victims died during the expulsion to Poland, but it is not impossible that von Ledebur was one of them.

Alternatively, he could have survived the deportation and crossed into Poland after spending the year in limbo. If he survived and then escaped the wrath of the German invasion, he no doubt would have shown back up on the historical record somewhere though. Considering these reasons, I am inclined to think von Ledebur was not involved directly with *Polenaktion*.

Theory 2: Escape

Even less likely than these possibilities is that of a ‘happy’ ending for von Ledebur. In the grand scheme of anything being possible, perhaps he escaped persecution in 1937-1938, moved abroad and died peacefully in his sleep many years later as an old man.

Though wishful thinking, it is almost entirely just a wish. Had he escaped, there would be record of his emigration as is seen in the countless now-digitized ship records of emigrants to the United States at Ellis Island, and other ports across the world. There would also be an eventual death certificate or grave marker, which is also nowhere to be found. Trust me, I *scoured* the records.

Even more compelling of an argument against an escape by von Ledebur is that his publications stop. He was not just a scientist to have a quiet career on the sideline. He was an eager, thoughtful, and motivated scientist, publishing up to six articles in a single year while also teaching. Had von Ledebur survived, he surely would have resurfaced elsewhere to continue his work as his colleagues Wunder, Berblinger, Wachholder, and others did.

Theory 3: Sachsenhausen

Most likely, Joachim von Ledebur was one of the 30 000 men captured during *Kristallnacht* and sent to their impending deaths. Based on the time point in the existence of the concentration camps as well as von Ledebur's last known location immediately prior to *Kristallnacht*, he was probably taken to Sachsenhausen concentration camp either in November 1938 or in September 1939.

Chapter 7: Sachsenhausen Concentration Camp

Sachsenhausen was established in 1936 and remained operational until 1945. It was primarily a labor camp that also had a gas chamber and a medical experimentation area. Tens of thousands of people died at Sachsenhausen from typhus epidemics and other disease outbreaks, malnutrition, execution, and medical experimentation. Executions were commonplace in the early stages of its existence, but the methods varied as the guards

experimented with different ways to optimize for the greatest number of people killed paired with the least effort or time.

Some of the first executions were done by forcing prisoners into a small room under the pretense of having their height measured. They were instead shot in the back of the neck through a sliding trap door. The guards decided this took too much time to orchestrate, so they tried other methods, such as having the prisoners dig a trench, enter the trench, and wait to be shot by firing squad. While this method proved effective for mass casualties, it created panic among the prisoners making them difficult to control. Sachsenhausen later became one of the first camps to trial the gas chamber and was notorious for shuttling prisoners to Auschwitz, Dachau, and Buchenwald in the later years. Unlike some of the other situations where records were taken strictly, those who were shuttled onto the cattle-cars, taken to one of the death camps, and then led directly to the gas chambers have no records. The names of those victims are lost, save for those remembered by witnesses. Grynszpan, the boy who killed the German diplomat in retaliation for his family, was eventually sent to Sachsenhausen. What happened to him remains unknown, but he was declared dead after liberation as he was never found.

If von Ledebur somehow managed to dodge arrest during *Kristallnacht* and was still free and alive for the publication of the book he contributed to in 1939, he was likely still sent to Sachsenhausen. The book was published that year in Berlin, Germany, and Sachsenhausen saw a massive increase of Jewish prisoners in September 1939 as the Nazis held a campaign of targeting Polish Jews, primarily in the greater Berlin area, and sending them to the camp. Targets at that time specifically included priests, professors, doctors, and teachers in an attempt to eliminate educated Jews in the hopes of preventing organized

resistance (Aurthur Allen, 2014). The exact number of victims at Sachsenhausen is unknown as documentation was poor, especially in the early years (and immediately before liberation, as documents were destroyed as the Allies drew closer), but it is estimated that 200 000 people made their way through the camp. Most of the 200 000 inmates were later transferred to Auschwitz – the most famous of the Nazi death camps. At the time of the Sachsenhausen liberation in 1945, only 1300 of the 200 000 inmates remained alive behind its walls – that is just 0.65% surviving Sachsenhausen. From everything we now know about the timeline, locations, and local events, Joachim von Ledebur was most likely sent to Sachsenhausen concentration camp sometime between November 1938 and September 1939, never to be seen from or heard from again.



Figure 35: Inmates of Sachsenhausen concentration camp on December 19, 1938. Is one of these men von Ledebur? Did they know him in his final days? (The Associated Press, 2022)

Chapter 8: Zikhrono livrakha

In the singular decade of Joachim von Ledebur's known academic career, he contributed 16 scientific journal articles and two chapters of an academic book. His works

have been cited hundreds of times since then, furthering his contribution to science. Had he been able to continue his work, he would likely have gone on to be a pioneer in his field of physiology, and the emerging discipline of endocrinology. His mentor, Dr. Berblinger, as well as Wilhelm Wunder and several other scientists he worked alongside went on to live long, full lives and have accomplished careers, though not all unscathed from the tyranny of the Reich. Wunder managed to hold his position in Breslau until 1945 when he was forced to flee, mere months before the end of the war, for example. Regardless of what happened to von Ledebur, and we may never truly know for sure, he deserved to live a long, full life, too.

In Jewish teachings, the phrase, “May their memory be a blessing” is used when someone dies. It means that it is up to those who bare the memories to keep the goodness of the person’s life alive. It means those memories are a continued gift of their life, blessing those who remember beyond just their mortal years. While they are no longer with us, they are never truly gone while there is still someone who remembers them. The entire goal of the Nazi’s campaign of extermination was based on eliminating the lives of those they did not deem worthy of life, and on eliminating all those who knew them. This effort was a deliberate act to wipe the existence of any memories of the individuals. In doing so, the blessings their lives gave would be gone forever.

We have no photos of Joachim von Ledebur. We have no records of his life beyond what is outlined here. There is no known headstone. No military records. No immigration records. No name in the Holocaust memorial records. He has no known descendants.

His family line ended with him.

His family line ended because of the Nazis.

His family line ended because of intentional hate and cruelty and intolerance.

Yet, in this small, singular act of defiance against the Nazi regime all these years later, I remember.

I remember *him*.

I remember him every time I walk into the laboratory at my university, and I remember how his work was the basis from which I have drawn inspiration to be able to perform my own research, nearly 100 years later.

I remember him every time I see a photograph of victims of the Holocaust, and I wonder if one of the unknown faces in the crowd could be his. And I remember that each one of those faces also has a story worth telling, just like him.

I remember, and because I remember him, I keep his goodness alive.

And now *you* will remember him, too.

May his memory be a blessing.

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Curriculum Vitae

Candidate's full name: Kadri Pearce-Lawrence

Universities attended: Carleton University, BA Biology 2019

Conference Presentations:

Atlantic Regional Developmental Biology Symposium 2023, Halifax, NS. **Kadri Lawrence**, Timothy Erickson. Lost in Inflation: Exploring Mortality Factors among Hypoinflated Zebrafish Larvae (*Danio rerio*). 23 June, 2023.

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