

“...It is really a marvel, the living being. The fact alone that the thing can exist with the three- or four fold chromosome number is extremely remarkable. Most peculiar, however, for me is the fact that, in spite of the enlarged single cell, the size of the animal is not correspondingly increased. It looks as if the importance of the cell as ruling element of the whole had been overestimated previously. What the real determinant of form and organization is seems quite obscure.”

Albert Einstein, PhD

(From Fankhauser, 1972. Memories of Great Embryologists)

**TISSUE-SPECIFIC COMPENSATORY MECHANISMS AND
ERYTHROPOIESIS IN TRIPLOID ZEBRAFISH**

by

Christopher David Small

Bachelor of Science, University of New Brunswick, 2012

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

Doctorate in Philosophy

in the Graduate Academic Unit of Biology

Co-Supervisor: Tillmann Benfey, PhD, Biology
Co-Supervisor: Bryan Crawford, PhD, Biology

Examining Board: Yang Qu, Ph.D., Chemistry
Michael Duffy, Ph.D., Biology
Aurora Nedelcu, Ph.D., Biology

External Examiner: Martin Flajšhans, Ph.D., Biology, University of South Bohemia
České Budějovice

This dissertation is accepted by the
Dean of Graduate Studies

THE UNIVERSITY OF NEW BRUNSWICK

February, 2021

©C.D. Small, 2021

ABSTRACT

In eukaryotes, cell size is proportional to the size of the nucleus and to the amount of genomic DNA housed within. Ploidy transitions, or Whole Genome Duplications (WGDs), double the amount of genomic DNA in the nucleus and cell size is increased consequently. Genome duplication is a driver for the evolution of novel traits by producing genetic redundancy that buffers the negative fitness consequences of mutations in highly conserved genes. WGDs have been discovered in nearly every major taxon of multicellular eukaryotes, but are more common in plants than in animals for reasons that are not clear. In plants, changes in ploidy and genome size changes the size of the organism as the number of cells allocated to tissues does not compensate for larger cells. In contrast, cell size and body size are decoupled in vertebrates so polyploids are no larger than concomitant diploids. Research on the evolution of polyploids usually focuses on the fate of duplicated genes rather than on the developmental and physiological consequences of changing cell size and the cellular granularity of tissues (the degree to which a tissue is subdivided into discrete cells). Perhaps the relative scarcity of WGDs in animals pertains more to how these developmental and physiological consequences affect fitness rather than the evolutionary consequences that occur in later generations after polyploids have successfully reproduced.

Ploidy can be experimentally manipulated in most teleosts, but diploid/triploid comparisons are most common as triploids are reproductively sterile making them useful for the aquaculture industry. Triploids consistently underperform compared to diploids with reduced ability to tolerate stressful conditions and higher mortality rates when

raised in conditions optimized for diploids. Triploids typically struggle to tolerate aerobic challenges leading many to suggest a cardiorespiratory limitation, but there is no clear mechanism explaining this difference between ploidies. The zebrafish is a popular model vertebrate that has been a boon for developmental biologists and, increasingly, physiologists. In this thesis, I have taken advantage of the many mutants and transgenic strains with tissue-specific fluorescent reporters to answer questions about the biology of polyploid vertebrates that would not be possible in classic, non-model species such as in Atlantic salmon.

To do so, I developed a novel ploidy determination technique to non-lethally assess the amount of DNA in the nuclei of zebrafish embryos *in vivo* using confocal microscopy and image processing (Chapter 2). I demonstrated for the first time that the compensatory mechanisms maintaining organ and body size in polyploids are tissue-specific by comparing the morphology of the blood, muscle, and the vasculature at single cell resolution (Chapter 3). Focusing in on the blood and erythropoietic system, I quantified hematopoietic stem cells in diploid and triploid embryos to show that triploids have fewer of these blood progenitor cells in their hematopoietic tissue. Triploids also produce erythrocytes at a slower rate compared to diploids suggesting that there are more aged, senescent erythrocytes in circulation perhaps contributing to the well-documented physiological limitations of triploids compared to diploids (Chapter 4). The utility of the zebrafish system makes it an ideal model organism for studying the biology of polyploids not only to improve the performance of triploids in aquaculture, but also to better understand why polyploidy is rarer in animals than in plants.

DEDICATION

To my grandparents (James (Herb) and Doris Small, and George and Alice Costello) for setting a stage that valued education.

To my parents (David Small and Laurie Costello) for infinite patience and encouragement.

To Jennifer Anderson and Isaac McGrath for hiking this path alongside me and helping me through.

ACKNOWLEDGEMENTS

I owe a deep debt of gratitude to my co-supervisors Dr. Bryan Crawford and Dr. Tillmann Benfey for their willingness to take a chance on me as a student. The opportunity to work in Dr. Crawford's lab as an undergraduate student after taking his Embryology lab was a defining moment for me and his support for my application to graduate school was necessary and (thankfully) sufficient. Dr. Benfey's support of my transition from the Master's program to the Ph.D. program allowed me to expand my project and I truly appreciate his support for each step of this degree. Thank both of you for your patience whenever I barged into your office to ramble about whatever crazy idea I was thinking about that week. Thank you for your critical comments that helped shape ideas into experiments. I haven't stopped learning new things since the day I joined the labs and it has been a pleasure. I appreciate your knowledge about all subjects ranging from science to beer - thank you again for your support and for your friendship. I would also like to thank Dr. Jim Kieffer for agreeing to be on my supervisory committee. I always enjoyed our chats whenever we crossed paths at conferences and I wanted to thank you for coming to see my talks even if there were no sturgeon talks in my section! As a new graduate student speaking at CSZ for the first time especially, it was truly appreciated.

I would like to thank all of the lab members of the Benfey lab and Crawford lab who I was able to work with over the years. I'm indebted to all of the fish care workers at the UNB aquatic facility – thank you for teaching me how to take care of my fish and thank for you for helping me keep my fish healthy. Thank you especially to Robyn O'Keefe who taught me everything I know about raising zebrafish and for teaching me

what a happy zebrafish looks like! Thank you to Robyn Shortt for her endless work maintaining the zebrafish database and for helping raise my larvae when I had too many fish growing up. I was fortunate to be able to work with many undergraduate students during my time here at UNB and I would like specifically thank James Davis and Megan El-Khoury for their immense efforts during their honour's projects. Thank you to all of the graduate students I've worked with over the years. I would like to thank Krista MacRae specifically for her help collecting data for Chapter 4 and for all of her help with fish care. Thank you especially to Rachael Wyatt for your insight and critical thinking about whichever tangent we happened to be on that day. The rigour you demand from your experiments is the standard I hope to emulate, and I would not have made it through without our venting sessions in the office at the white board.

Thank you to NSERC and the NBIF for helping to fund my work and thank you to the UNB Microscopy facility (especially Steve Cogswell and Susan Belfry) for teaching me about the instruments while you solved my issues so that I could solve them myself the next time!

This has been a long journey and I need to thank my entire family for their support and patience. Their understanding was instrumental in my success and their sacrifice should be noted. Far too many visits, holidays, and special occasions were adjusted to allow me to "feed the fish" and take care of experiments and I can't thank you all enough. Thank you to my brother Andrew for tolerating me over the years, for always letting me rant about the frustrations of lab work and science stuff, and for always being there.

Above all else, I need to thank Jennifer Anderson and Isaac McGrath. Every step felt easier with you guys beside me regardless of whether it was in the fish wing with

ugly rubber shoes on or literally climbing a mountain. I am endlessly thankful to have had you both with me on this journey.

Table of Contents

Abstract.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Table of Contents.....	vii
List of Figures.....	xii
List of Symbols, Nomenclature or Abbreviations.....	xiv
1.0 Introduction.....	1
1.1 Why is polyploidy more common in plants than in animals?.....	1
1.2 Genome duplication and polyploidy.....	2
1.3 Polyploidy in evolution.....	3
1.4 Polyploid physiology.....	6
1.5 Triploid hematology.....	8
1.6 Why zebrafish?.....	11
1.7 Thesis goals and approaches.....	12
1.8 Conclusion.....	14
1.9 References.....	16
2.0 Early, non-lethal ploidy and genome size quantification using confocal microscopy in zebrafish embryos.....	29
Abstract.....	29
2.1 Introduction.....	31
2.2 Methods.....	36
2.2.1 Animal husbandry.....	36

2.2.2 <i>In vitro</i> fertilization and triploid induction.....	36
2.2.2.1 Gamete collection.....	36
2.2.2.2 Fertilization and triploid induction.....	37
2.2.3 Determining ploidy with Tg(h2a.f/z:H2A.f/z:eGFP) embryos.....	38
2.2.3.1 Confocal microscopy.....	38
2.2.3.2 Image processing.....	39
2.2.3.3 Validation of ploidy assignment.....	40
2.2.4 Optimizing triploid induction using hydrostatic pressure.....	40
2.2.5 Survival rate in diploid and triploid embryos.....	41
2.2.6 Measuring heart rate in diploid and triploid embryos.....	42
2.3 Results.....	42
2.3.1 Nucleus volume can be measured non-lethally in zebrafish embryos at 48 hours post fertilization.....	42
2.3.2 Embryos with larger nuclei that develop from pressure-treated zygotes are <i>bona fide</i> triploids.....	43
2.3.3 Optimal triploid yield is achieved by pressure-treating zygotes at 75 or 90 spf	43
2.3.4 Survival rate during early development is reduced in triploid embryos	44
2.3.5 Embryonic heart rate is not affected by ploidy.....	44
2.4 Discussion.....	45
2.5 Conclusion.....	50

2.6 Acknowledgments.....	51
2.7 References.....	52
3.0 Tissue-specific patterns of compensation for increased cell size maintains organ size and body size in triploid zebrafish.....	72
Abstract.....	72
3.1 Introduction.....	73
3.2 Results.....	78
3.2.1 Early cleavage rate and blastomere volume are not significantly different in diploid and triploid zebrafish.....	78
3.2.2 Triploid embryos have fewer, larger erythrocytes in circulation.....	78
3.2.3 Myofiber length is constrained by myotome size and N:C ratio is maintained by reducing myonuclear count in triploids.....	79
3.2.4 Triploid blood vessels have thicker vessel walls and lumen diameter is not increased.....	80
3.3 Discussion.....	89
3.3.1 Early development.....	90
3.3.2 Blood.....	92
3.3.3 Muscle.....	95
3.3.4 Blood vessels.....	95
3.3.5 Conclusions and future directions.....	97
3.4 Experimental procedures.....	99
3.4.1 Animal husbandry.....	99
3.4.2 Triploid production and identification.....	100
3.4.3 Blastomeres.....	100

3.4.3.1 Cleavage rate.....	100
3.4.3.2 Blastomere volume.....	101
3.4.4 Erythrocytes.....	101
3.4.5 Muscle.....	102
3.4.5.1 Myotome morphology.....	102
3.4.5.2 Rate of somitogenesis.....	103
3.4.5.3 Myofiber morphology.....	103
3.4.5.4 Sarcomere length.....	104
3.4.5.5 Myonuclear count.....	104
3.4.6 Vasculature.....	105
3.4.6.1 Vascular patterning.....	105
3.4.6.2 Intersomitic vessel cellularity.....	105
3.4.6.3 Vessel wall thickness.....	106
3.5 References.....	106
3.6 Supplementary figures.....	118
4.0 Triploids have fewer hematopoietic stem cells and slower steady-state rate of erythropoiesis.....	120
Abstract.....	120
4.1 Introduction.....	120
4.2 Methods.....	127
4.2.1 Animal care.....	127
4.2.2 Hematology.....	128
4.2.3 Counting hematopoietic stem cells in 36 hpf embryos.....	131
4.2.4 Measuring erythropoiesis using Tg(gata1a:dsRed) zebrafish.....	132

4.3 Results.....	134
4.3.1 Hematology.....	134
4.3.2 Hematopoietic stem cells in 36 hpf embryos.....	135
4.3.3 Measuring rate of erythropoiesis using Tg(gata1a:dsRed) zebrafish	135
4.4 Discussion.....	136
4.5 Conclusion.....	142
4.6 References.....	152
5.0 General discussion.....	169
6.0 Appendix 1: Stimulating erythropoiesis using [10]-gingerol as a feed additive in adult zebrafish.....	178
6.1 Introduction.....	178
6.2 Methods.....	180
6.3 Results.....	181
6.4 Discussion.....	183
6.5 References.....	183
7.0 Appendix 2: Measuring tissue turnover in diploid and triploid brook charr (<i>Salvelinus fontinalis</i>) using a diet shift experiment with stable isotopes.....	187
7.1 Introduction.....	187
7.2 Methods.	189
7.3 Results.....	191
7.4 Discussion.....	192
7.5 References.....	195
8.0 Curriculum Vitae	

List of Figures

Figure 1.1: Diagram of ploidy manipulation in teleost oocytes.....	12
Figure 2.1: Pressure-treated zebrafish embryos have myofibers with larger nuclei.....	67
Figure 2.2: Pressure-treated zebrafish embryos with larger nuclei are <i>bona fide</i> triploids.	68
Figure 2.3: Start time of hydrostatic pressure treatment affects fertilization rate, survival rate, triploid induction rate, and triploid yield.....	69
Figure 2.4: Survival until 120 hpf is significantly affected by ploidy.....	70
Figure 2.5: Heart rate is affected by temperature but not ploidy at 56 hpf.....	71
Figure 3.1: Cell cycle and cell volume are not affected by ploidy in pre-gastrula zebrafish embryos.....	81
Figure 3.2: Erythrocytes are larger, but fewer in number in triploid zebrafish embryos	82
Figure 3.3: Ploidy does not affect myotome size, sarcomere size, or the rate of somitogenesis in zebrafish embryos.....	83
Figure 3.4: The size and number of myofibers in each myotome are not affected by ploidy.....	84
Figure 3.5: The number of nuclei per myofiber is significantly reduced in triploid embryos.....	85
Figure 3.6: Vascular patterning is not significantly affected by ploidy.....	86
Figure 3.7: Triploid ISVs are not comprised of fewer cells than diploid ISVs in 48 hpf zebrafish embryos.....	87
Figure 3.8: Vascular endothelial cells are thicker in triploid zebrafish.....	88
Figure 3.S1: DIC micrograph of streaking blood cells in dorsal aorta.....	118

Figure 3.S2: Cartoon model of a multi-nucleated myofiber spanning a myotome.....	119
Figure 4.1: Hematocrit can be reliably measured in small volumes ($\geq 1 \mu\text{L}$) of blood.	144
Figure 4.2: Triploid zebrafish have fewer, larger erythrocytes than diploids.....	145
Figure 4.3: Triploids have fewer GFP-positive cells in the caudal hematopoietic tissue of Tg(c- myb:eGFP) zebrafish embryos than diploids at 36 hpf.....	146
Figure 4.4: Triploid zebrafish embryos have fewer hematopoietic stem cells than diploid embryos at 36 hpf.....	147
Figure 4.5: Erythropoietic recovery can be measured using Tg(gata1a:dsRed) zebrafish.....	148
Figure 6.1: Erythropoiesis is impaired in Tg(gata1a:dsRed) zebrafish fed methanol- treated pellets, regardless of addition of [10]-gingerol.....	182
Figure 7.1: $\delta^{13}\text{C}$ increases in blood cells over time after a diet-switching to an isotopically distinct diet in triploid and in diploid juvenile brook charr, <i>Salvelinus</i> <i>fontinalis</i>	192

List of Symbols, Nomenclature or Abbreviation

The following standard abbreviations are found throughout this thesis:

2n/3n:	diploid/triploid
ATP:	adenosine triphosphate
A.U.:	arbitrary units
BSA:	bovine serum albumin
c-myb:	c-myb transcription factor (gene name)
DA:	dorsal aorta
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dsRed:	a red (546 nm) fluorescent protein
eGFP:	a green (488 nm) fluorescent protein
ERM:	Embryo Rearing Media
Flk1:	Flk1 (or kdlr) transcription factor (gene name)
gata1a:	gata1a transcription factor (gene name)
h2a.f/z:	histone subunit 2a.f/z (gene name)
hpf:	hours post fertilization
hsc:	hematopoietic stem cell
ISV:	intersomitic vessel
ivf:	<i>in vitro</i> fertilization
mCherry:	a red (546 nm) fluorescent protein
MS-222:	tricaine methanesulfonate
myl7:	myosin light chain 7 (gene name)

N:C ratio:	Nuclear:Cytoplasmic
PBStx:	Phosphate-buffered saline (with triton X-100)
spf:	seconds post fertilization
Tris:	Trisaminomethane, Tris(hydroxymethyl)aminomethane
WGD:	Whole Genome Duplication
ZGA:	Zygotic Genome Activation

1.0 Chapter 1: Introduction

1.1 Why is polyploidy more common in plants than in animals?

One of the most paradoxical aspects of scientific endeavour is that great discoveries and achievements tend to generate more questions than satisfying answers. The sequencing of the human genome was correctly lauded as an enormous accomplishment for science and medicine, but among the many surprises that arose from this success was the observation that humans had no more genes than many organisms considered to be “simple” or “lower” on the tree of life. In fact, the classic laboratory mouse has more genes than the scientists studying them with an estimated ~30000 genes in mice (*Mus musculus*) compared to only ~20000-25000 genes in humans (*Homo sapiens*) (Pray, 2008). Before next-generation sequencing technologies rose to prominence as the standard tool for studying taxonomy and evolutionary biology, many biologists were captivated by an idea known as the C-Value Paradox that states that there is no correlation between genome size and complexity in eukaryotes (Gregory and Hebert, 1999; Gregory, 2002; Gregory, 2005). Genome size varies by nearly five orders of magnitude across the metazoan tree of life (Cavalier-Smith, 1978; Gregory, 2001) and gene number varies by at least one order of magnitude (Pray, 2008; Choi et al., 2020). The causes and consequences of these differences, with respect to an organism’s size, morphology, longevity, or other aspects of its biology that affect fitness and evolution are still unclear (Gregory, 2002; Choi et al., 2020). Simply comparing the number of genes or size of the genome among organisms is clearly insufficient for explaining the incredible diversity and complexity that arose during metazoan evolution, so why is there so much genome size variability in eukaryotes and how does it occur.

1.2 Genome Duplication and Polyploidy

In 1970, Dr. Susumo Ohno proposed in his book “Evolution by Gene Duplication” that gene and genome duplication was a significant factor in the evolution of genome size variation and hypothesized that these duplication events would be drivers of evolution by relaxing selection on highly-conserved genes (Ohno, 1970). Extra copies of genes could act as buffers to loss-of-function mutations in critical genes thereby reducing their negative consequences on fitness and increasing the likelihood that beneficial mutations could evolve and persist. This idea is encapsulated in Dr. Ohno’s statement that “Natural selection merely modified, while redundancy created” (Ohno, 1970). Specifically, he predicted that two rounds of whole genome duplication (WGD) had occurred at the base of the vertebrate lineage despite only having data on relative genome size, karyotypes, and allozymes (often referred to as the 2-R hypothesis). The advent of genome sequencing technologies as a tool for evolutionary biologists has revealed that many vertebrate genes are present as four copies with high sequence similarity convincingly refuting dissenting opinions regarding the 2-R hypothesis (Dehal and Boore, 2005). Genes that are homologous due to whole genome duplication rather than other mechanisms that yield copies of specific segments of the genome are referred to as ‘ohnologues’. Further to this, in cases where all four ohnologues are maintained in the genome, there are typically two sets of ‘ohnologue-pairs” and each member of the ohnologue-pair is more similar to each other (in terms of both sequence similarity and synteny) than to the members of the sister ohnologue pair indicative of two sequential WGDs rather than a single event that created four copies of each (Conant, 2020). Since Dr. Ohno’s prescient ideas regarding the potential of duplication as a driver of evolution, scientists have discovered WGDs and polyploidy in

nearly all eukaryotic taxa (Masterson, 1995; Gregory and Mable 2005; Braasch and Postlethwait, 2012; Mueller, 2015; Kenny et al., 2016; Schwager et al., 2017).

1.3 Polyploidy in Evolution

Scientists have been curious about the presence and prevalence of polyploids for nearly a century. In 1925, Dr. Hermann Muller published his paper “Why polyploidy is more common in plants than in animals” in which he discusses the bias among taxa regarding the frequency of polyploids using *Drosophila* as his animal model (Muller, 1925). Plants have been called the “Champions of Polyploidy” (Soltis et al., 2009) as there have been many more WGDs and ploidy transitions in plants compared to animals, (Masterson, et al., 1995; Aversano, 2012; Conant, 2020). We now have evidence for WGDs throughout the animal kingdom as well, including both invertebrates (Gregory and Mable, 2005; Kenny et al., 2016; Schwager et al., 2017) and vertebrates (Dehal and Boore, 2005; Braasch and Postlethwait, 2012; Conant, 2020). As discussed above, there were two WGDs at the base of the vertebrate lineage (as predicted by Dr. Ohno, 1970), one WGD at the base of the teleosts (Hoegg et al., 2004) after their divergence from ancestral *Acipenseriformes* and *Holostei*, and several more within the teleost clade (Braasch and Postlethwait, 2012). Early developmental biologists were fascinated by the discovery of polyploid amphibians (Fankhauser 1939; Griffiths et al., 1940) and fish (Swarup, 1959) noting that polyploid vertebrates (with more DNA in each cell) had larger cells, but that the size of the various organs and tissues was not affected by ploidy so the animals were no different in size. In contrast, polyploid plants are significantly larger than individuals of the same species at lower ploidy levels as the number of cells allocated to each tissue is not reduced to compensate for increased cell size (Aversano et al., 2012; Zhang et al., 2019). Dr. Muller’s (1925) conclusions regarding the relative

scarcity of polyploidy in animals using *Drosophila* could be specific to invertebrates rather than to all animals as it is not yet clear how most invertebrate taxa respond to changes in ploidy. Sea urchins increase the size of their cells as ploidy and genome size increases (Laubichler and Davidson, 2008; Scheer, 2018), but there is little data regarding differences in overall body size for urchins within a species (likely due to the technical challenges of lab-rearing urchins to maturity, and the dramatic effects of the environment on adult size and morphology in echinoderms) (Böttger, et al., 2011). Body size in molluscs (Nell, 2002) and nematodes (Clarke et al., 2018) is positively correlated to ploidy, (i.e. as in plants, polyploid mollusks and nematodes are larger) but there is some dispute among researchers as to how arthropods respond to changes in ploidy (Bridges, 1921; Muller, 1925; Dobzhansky, 1939; Orr-Weaver, 2015). There is a clear link between ploidy and cell size in *Drosophila* (Dobzhansky, 1939; Gregory and Mable, 2005; Nandakumar et al., 2020) but it is difficult to tease apart body/organ size difference due to ploidy from size differences due to sex in a species that uses chromosome balancing for sex determination (Bridges, 1921; Muller, 1925; Gregory and Mable, 2005). Genome size does correlate to body size in crustaceans (Jeffrey et al., 2017), and the recent discovery of a WGD in the evolution of spiders (Kenny et al., 2016) emphasizes the need to understand the morphological consequences of increased ploidy in arthropods as the idea of spiders suddenly doubling in size due to a WGD is unsettling. Using a single species to make inferences regarding the prevalence and consequences of polyploidy in animals will not suffice given that the responses to ploidy changes varies across animal taxa. Understanding how and when evolution favours polyploidy will require knowledge of the consequences of ploidy and cell size changes within that clade specifically.

Most research on polyploidy in animals has focused on understanding the fate of ohnologues after WGD (Postlethwait, 2007). Many polyploid lineages have undergone a process known as “rediploidization” in which ohnologous genes diverge from one another. Although pseudogenization was once thought to be the most common fate of an ohnologue, recent studies are showing that subfunctionalization of ohnologues (splitting the functions that were once performed by a single gene) and neofunctionalization (gain of novel function) are far more common than originally thought (Braasch et al., 2016; Lien et al., 2016; Conant, 2020). Indeed, Ohno’s (1970) idea that duplication would be a driver for the evolution of novelty seems to hold true as WGDs seem to correlate to bursts of speciation in polyploid lineages (Braasch and Postlethwait, 2012). Furthering this idea, we now have evidence linking some incredible physiological innovations in teleosts to the divergence of ohnologues such as ionoregulation in anadromous salmonids facilitated by subfunctionalization of gill Na^+/K^+ -ATPases (Richards et al., 2003; Bystrianski et al., 2006; Bystrianski et al., 2007) and extreme hypoxia tolerance in goldfish and crucian carp due to neofunctionalization of a critical member of the pyruvate dehydrogenase complex, which evolved into a pyruvate decarboxylase that can produce ATP from lactic acid without oxygen (Fagernes et al., 2015). Studying the genetic consequences of WGD has provided support for Ohno’s hypothesis (1970), but have not yet answered Muller’s (1925) question: why is polyploidy more rare in animals than in plants? Perhaps the scarcity of polyploids in animals pertains more to the developmental and physiological consequences of being made up of fewer, larger cells than to the genetic consequences of having duplicate copies of every gene.

1.4 Polyploid physiology

Most studies comparing the physiology of polyploids in teleosts have focused on diploid/triploid comparisons as triploids are reproductively sterile in many teleost species (Piferrer et al., 2009) making them useful for aquaculture as they do not invest energy into sexual maturation focusing instead on somatic growth improving flesh quality (Poontawee et al., 2007). More importantly, farming triploids prevents gene flow between farmed and wild populations preventing introgression of alleles that are favourable in the farm into wild fish where they may be less advantageous (Piferrer et al., 2009; Benfey, 2016). Research has focused on identifying physiological differences between diploids and triploids that affect performance and survival in an aquaculture setting as triploids are more vulnerable to sub-optimal conditions and die more frequently than diploid fish (Ojolick et al., 1995; Maxime et al., 2008). When reared in conditions optimized for raising diploid fish, triploids tend to be less tolerant to high temperature (Verhille et al., 2013; Sambraus et al., 2018; Riseth et al., 2020), low oxygen (Scott et al., 2014; Hansen et al., 2015; Sambraus et al., 2017; Benfey and Devlin, 2018), and exercise (Hyndman et al., 2003; Bernier et al., 2004). Riseth et al. (2020) pose the question: “Is it advantageous to be triploid at lower temperature?” and conclude that there are negligible differences in aerobic scope and swimming performance at low temperature. The authors point out that this does not mean that there are no environments that favour polyploids, only that there is more to it than simply temperature, aerobic metabolism, and swimming performance. Indeed, the inverse correlation between genome size (and therefore cell size) and temperature in marine environments suggests that colder temperatures should favour polyploids over diploids but the mechanism is not yet clear. Given the consistent link between aerobic stress and

high triploid mortality, there have been many studies testing for differences in hematology (Benfey and Sutterlin, 2004; Bernier et al., 2004; Leal et al., 2019) and cardiorespiratory physiology (Verhille et al., 2013; Fraser et al., 2015; Sambaous et al., 2017). Verhille and colleagues' experiments on diploid and triploid rainbow trout (*Oncorhynchus mykiss*) demonstrated that triploids hearts become fatally arrhythmic sooner and more frequently than diploid hearts when the fish presented with an incrementally increasing thermal challenge (Verhille et al., 2013). This implicates a cardiorespiratory limitation in polyploids, but we do not have a mechanistic explanation for the failure of triploid hearts in tasks that diploid hearts can perform reliably. There have been several studies comparing cardiac morphology (Gamperl and Farrell, 2004; Fraser et al., 2013; Fraser et al., 2015) and function (Simonot and Farrell, 2009; Verhille, et al., 2013) between diploid and triploid hearts, but none have revealed an obvious mechanism that would explain this difference in physiological tolerances. Adult triploids tend to have larger hearts thought to be indicative of increased workload (Fraser et al., 2013; Fraser et al., 2015). If triploid hearts are working harder than diploid hearts under standard conditions, this could explain why triploids succumb sooner when they are challenged and raises another question: why are triploid hearts working harder than diploid hearts performing the same task? The primary function of the heart is to pump erythrocytes throughout the body to deliver oxygen (via hemoglobin) to the various tissues of the body, while simultaneously removing waste metabolites so perhaps the perceived increased workload on the heart was caused by some effect of ploidy on blood physiology.

1.5 Triploid Hematology

Blood is a tissue that conforms to the “fewer, larger cells” pattern of compensation for changes in ploidy, such that many hematological variables such as hematocrit (volume percentage of erythrocytes in total blood) and hemoglobin concentration are not significantly altered in polyploids, but other variables such as mean corpuscular hemoglobin concentration (hemoglobin per cell) and the number of circulating erythrocytes (cells per volume of blood) are affected by ploidy (Benfey, 1999; Benfey and Sutterlin, 2004). Many studies have focused on the consequences of increased erythrocyte size since this would affect surface area to volume ratio of the cell perhaps limiting transport across the membrane (Nikinmaa, 1990; Snyder and Sheafor, 1999). Furthermore, erythrocytes have a highly malleable cell membrane since they must traverse capillaries with a lumen that is narrower than the cross-sectional diameter of the cells, forcing them to squeeze and deform in order to deliver oxygen to tissues (Nash and Egginton, 1993; Bahrami and Childs, 2020). Larger cells would be harder to push through these small vessels and they would be more likely to be damaged in transit unless the microvascular system compensates by increasing lumen size. Little is known about the dimensions of the vascular system in triploids (and the microvascular system specifically) as it is challenging to measure vessel diameter *in vivo* and fixed sections are not representative of *in vivo* dimensions since the vessels are no longer “inflated” by blood pressure (Nash and Egginton, 1993), even if we assume that artifactual tissue shrinkage during fixation is consistent between ploidies.

What is rarely considered are the consequences that arise from packing the same amount of hemoglobin into fewer cells on the physiology of the fish. As is the case in information processing, imaging, and other contexts, there must exist an optimal packet-

size for oxygen delivery. Packing all of the hemoglobin in the blood into one cell would clearly be suboptimal for delivering oxygen to all of the tissues in the body simultaneously. There must be some minimum threshold of hemoglobin apportioning in order for an animal to be able to maintain efficient oxygen delivery. Making more, smaller erythrocytes increases the ability of an animal to distribute oxygen throughout the body, but at some point making more and more cells with fewer and fewer molecules of hemoglobin in each must become metabolically costly and present decreasing returns with respect to oxygen delivery. An optimized cardiorespiratory system must balance the advantages associated with improved oxygen delivery gained by smaller but more abundant hemoglobin packets versus the increased metabolic cost of having to produce and differentiate more erythrocytes. Further to this, since mean corpuscular hemoglobin concentration is increased and the total number of circulating cells is reduced, each erythrocyte that is damaged or destroyed has a larger effect on the total amount of circulating hemoglobin. Inducing triploidy would cause an abrupt shift in the distribution of hemoglobin in the blood perhaps affecting the ability to distribute oxygen optimally and would likely contribute to increased workload on the heart especially during an aerobic challenge. Damaged and aged erythrocytes are removed from circulation and replaced quickly in order to maintain hematocrit and hemoglobin concentration. In fish, the source of these new erythrocytes is a population of hematopoietic stem cells (HSCs) that reside in the kidney.

The effect of ploidy on the number of HSCs has not been reported, but Simonot and Farrell (2009) induced anemia in diploid and triploid rainbow trout and reported that triploids had a reduced capacity for erythropoiesis during recovery and were less likely to recover from the anemia. In a recent study by Hansen et al. (2015) comparing the

effects of low oxygen at high temperatures on the performance of diploid and triploid Atlantic salmon (*Salmo salar*), the authors noted that triploids had lower levels of bilirubin circulating in the blood indicative of reduced erythrocyte turnover possibly due to slower erythrocyte clearance. In vertebrates, erythrocytes differentiate from a population of HSCs that reside in a stem cell niche that is highly conserved at the molecular level, but highly variable anatomically (Martinez-Agosto, 2017; Kapp et al., 2018). Cell turnover is not widely studied from a physiological point of view in non-mammalian species, but erythrocyte turnover specifically is highly regulated and extremely rapid relative to other tissues with cells that circulate for only ~30 days in mice and ~120 days in humans (Franco, 2012; Franco et al., 2013; Higgins, 2015). Teleost erythrocytes retain their nucleus and maintain gene expression for most of their life (Götting and Nikinmaa, 2017) so teleost erythrocytes are thought to have much longer lifespans than mammalian erythrocytes. There is, however, very little data regarding absolute age and lifespan for erythrocytes in any species of fish (Morera and MacKenzie, 2011; Witeska et al., 2013). In teleosts, the primary site of erythropoiesis is localized entirely to the anterior region of the kidney (Wattrus and Zon, 2018; Kapp et al., 2018; Kobayashi et al., 2019) whereas, in mammals, hematopoietic stem cells are distributed throughout the body in the bone marrow. The difference in size of the hematopoietic tissues between mammals and teleosts suggests that the demand for erythropoiesis is lower in teleosts and the simplest explanation for this is that the cells survive longer before they are turned over. Erythropoiesis in teleosts was not well understood until recently with the rise of the zebrafish as a powerful model organism for the study of development and hematopoiesis specifically (Carradice and Lieschke, 2008).

1.6 Why zebrafish?

The zebrafish has become a popular model vertebrate for lab studies especially among developmental biologists, but it is also becoming more common in physiological studies. The availability of transgenic strains with tissue-specific reporter genes stably integrated into the genome provides unique opportunities to study the effects of polyploidy on development and physiology. Taking advantage of the extensive experimental toolkit available in zebrafish including the aforementioned transgenics, mutants, and immunohistochemistry, I have revisited some of the observations made by the original developmental biologists interested in polyploids (Dobzhansky, 1939; Fankhauser, 1939; Swarup, 1959) using new technologies to understand how polyploids compensate for having larger cells by reducing cell number to maintain body size. Ploidy can be manipulated in most species of teleost (Figure 1.1) if *in vitro* fertilization is possible by disrupting the extrusion of the second polar body after fertilization to produce a triploid (Kavumpurath and Pandian, 1990; Benfey, 1999; Piferrer et al., 2009) or by interrupting cytokinesis after DNA replication to make a tetraploid (Myers et al., 1986; Piferrer et al., 2009; Heier et al., 2015;) and the zebrafish is no exception making them the ideal model system for this work.

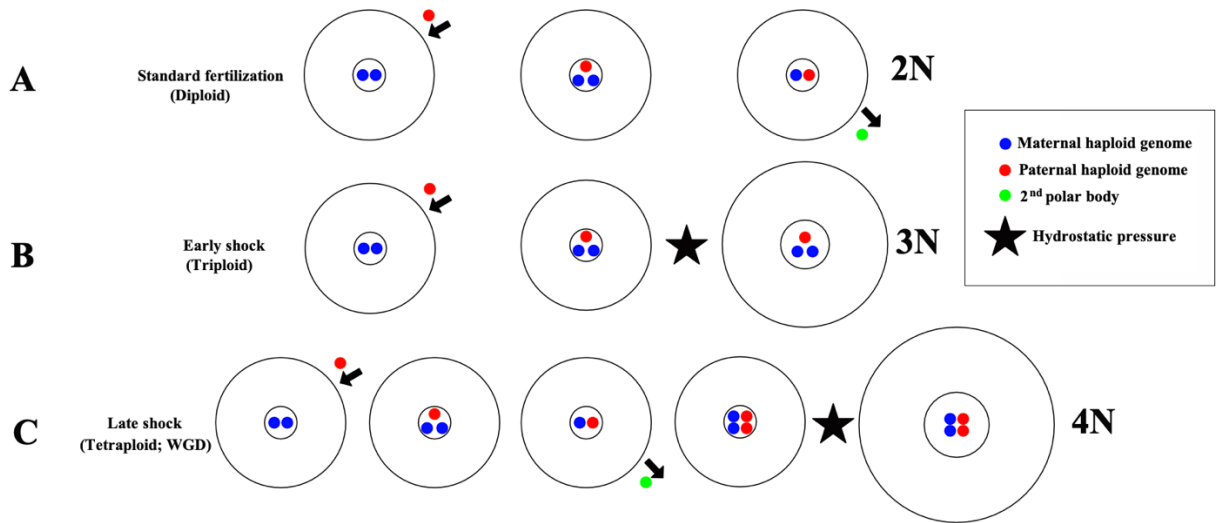


Figure 1.1 Ploidy can be manipulated in teleost fish. Oocytes contain two copies of the maternal genome (blue circles). **A)** Under standard conditions, the paternal genome (red circle) enters the nucleus during fertilization and one set of maternal chromosomes is extruded as the 2nd polar body (green circle). **B)** Treating embryos with hydrostatic pressure before extrusion of the polar body results in a triploid embryo. **C)** Treating embryos with hydrostatic pressure after extrusion of the polar body, but before cytokinesis results in a tetraploid embryo (ie: a whole genome duplication).

1.7 Thesis goals and approaches

In this thesis, I intend to quantify the number of HSCs in diploid and triploid zebrafish and subsequently determine if ploidy affects the rate of steady-state erythropoiesis. I predict that triploids will have fewer HSCs and that this will correlate to slower rates of steady-state erythropoiesis. Before I could target this overarching question about blood physiology, I needed a technique that would allow me to distinguish individual zebrafish embryos of different ploidy. Therefore, I developed myofiber nuclear volume analysis for non-destructive ploidy determination *in vivo* at two days old using Tg(h2a.f/z:H2A.f/z::eGFP) zebrafish that have fluorescent chromatin

(Chapter 2). I can now determine ploidy very early in development without having to sacrifice the animal allowing for further experimentation on animals confirmed to be triploid. This enabled me to perform high-resolution morphological comparisons of various tissues within the living embryo to determine how different tissues respond to ploidy/cell size changes (Chapter 3). Using transgenic lines that express fluorescent proteins in hematopoietic stem cells (Tg(c-myb:eGFP)) and in differentiating erythrocytes (Tg(gata1a:dsRed)) specifically, I show that triploids have fewer hematopoietic stem cells and that there are fewer young, newly differentiated erythrocyte in circulation in triploids suggesting reduced turnover in agreement with the studies by Simonot and Farrell (2009) and Hansen et al. (2015) (Chapter 4).

Recently, Ferri-Lagneau and colleagues discovered that erythropoiesis can be stimulated in zebrafish embryos simply by adding [10]-gingerol (a bioactive molecule found in the ginger plant) to embryo media (Ferri-Lagneau et al., 2011; Chen et al., 2013; Ferri-Lagneau et al., 2019). Other studies have demonstrated that many hematological variables increase in adults when fish are fed pellets with ground ginger in the feed mixture (Ho et al., 2013; Haghighi and Rohani, 2013; Kanani et al., 2014). To test whether this could be a strategy to boost erythropoiesis in zebrafish, I attempted to infuse standard zebrafish pellets with 10-gingerol (Sigma-Aldrich). Using the same erythropoietic assay described in Chapter 4, I determined that this protocol actually reduced erythropoiesis in adult zebrafish but the consistent effect in the treatment and the solvent control suggests that this was not due to the [10]-gingerol, but rather an effect of the infusion technique so repeating this experiment with a feed that includes either ground ginger or purified 10-gingerol in the formulation rather than as an additive could still be an effective way to stimulate erythropoiesis in fish (Appendix 1).

I also designed an experiment intended to empirically measure the rate of erythrocyte turnover (and therefore erythrocyte lifespan) between diploids and triploid brook trout (*Salvelinus fontinalis*). Briefly, a diet-shift experiment was undertaken using feed pellets spiked with L-Alanine ^{13}C (Sigma-Aldrich) and measuring the how long it would take for the isotopic signature of the blood to shift to that of the new diet. Preliminary data confirmed that the methodology was sound: the infused pellets were isotopically distinct from the baseline diet and 5 weeks into the experiment there was a measurable difference in the isotopic signature of the blood. Unfortunately, a catastrophic failure of the plumbing in the aquatic facility at UNB caused massive mortality in the experimental fish killing all of the triploids and more than 50% of the diploids. I designed this experiment based on previous work on erythrocyte turnover (using a different stable isotope marker) in brook trout that estimated that 50% turnover of the blood would occur ~8 weeks into the experiment (M. Charest M.Sc. Thesis 2010) and so I decided to run the experiment for 10 weeks. With all of the triploids dying approximately halfway through the experiment, there was no detectable effect of ploidy on the rate at which the isotopic signature of the blood was changing. The preliminary data demonstrating that the technique was effective at producing an isotopically distinct diet should be useful for future studies and this experiment would be worth repeating (Appendix 2).

1.8 Conclusion

In this thesis, I have developed a novel way to determine ploidy in zebrafish making them an invaluable tool for research on the developmental biology and the physiology of polyploids (Chapter 2). I show that there are distinct tissue-specific mechanisms whereby tissue architecture is adjusted to compensate for increased cell size

to maintain tissue function, organ size, and body size (Chapter 3). Secondly, by measuring the number of hematopoietic stem cells and comparing the relative rates of erythropoiesis between diploid and triploid zebrafish, I characterize yet another mechanism that may limit aerobic scope in triploids (Chapter 4). And finally, I discuss potential consequences of these effects on the physiology of triploids as well as some ideas for future directions in Chapter 5.

For polyploids to persist evolutionarily, they must be able to survive to maturity, find a polyploid mate, reproduce, and pass on their genes before Ohno's (1970) assertion about redundancy facilitating the evolution of novel adaptive traits has time to manifest. If the physiological limitations imposed by increased ploidy, reduced cellular granularity, and increased cell size prevents polyploids from surviving to pass on their genes, then ohnologues will not have the opportunity to mutate and evolve novel function. A detailed understanding of the morphological consequences of polyploidy and WGDs, and the physiological consequences of those changes, is necessary for understanding the conditions that will be most favourable for polyploids. This could be by improving the fitness of polyploids specifically, or by simply by minimizing their disadvantages allowing them to persist for long enough for the genetic advantages proposed by Ohno (1970) to manifest. Understanding the developmental and physiological constraints imposed by changes in ploidy will improve our ability to optimize rearing conditions for triploids in aquaculture and will hopefully bring us closer to being able to answer Muller's (1925) original question from nearly 100 year ago: "Why polyploidy is more common in plants than in animals?"

1.9 References

- Aversano, R.E., Ercolano, M.R., Caruso, I., Fasano, C., Rosellini, D., Carputo, D., 2012. Molecular tools for exploring polyploid genomes in plants. *Int. J. Mole. Sci.* 13, 10316-10335. <https://doi.org/10.3390/ijms130810316>.
- Bahrami N., Childs S.J., 2020. Development of vascular regulation in the zebrafish embryo. *Development*. 147, dev183061. <http://dx.doi.org/10.1242/dev.183061>.
- Benfey, T.J., 1999. The physiology and behavior of triploid fishes. *Rev. Fish.* 7, 39-67. <http://dx.doi.org/10.1080/10641269991319162>.
- Benfey T.J., Sutterlin, A.M., 2006. The haematology of triploid landlocked atlantic salmon, *Salmo salar* L. *J. Fish Biol.* 24, 333-338. <http://dx.doi.org/10.1111/j.1095-8649.1984.tb04804.x>.
- Benfey, T.J., 2016. Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (*Salmo salar*) as a case study. *Rev. Aquac.* 8, 262-282. <https://doi.org/10.1111/raq.12092>.
- Benfey, T.J., Devlin, R.H., 2018. Ploidy has minimal effect on hypoxia tolerance at high temperature in rainbow trout (*Oncorhynchus mykiss*). *Physiol. Biochem.* 91, 1091-1101. <https://doi.org/10.1086/700218>.
- Bernier, N.J., Brauner, C.J., Heath, J.W., Randall, D.J., 2004. Oxygen and carbon dioxide transport during sustained exercise in diploid and triploid chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat.* 61, 1797-1805. <http://dx.doi.org/10.1139/f04-110>.
- Böttger, S.A., Eno, C.C., Walker, C.W., 2011. Methods for generating triploid green sea urchin embryos: An initial step in producing triploid adults for land-based and

near-shore aquaculture. *Aquaculture*. 318, 199-206.

<https://doi.org/10.1016/j.aquaculture.2011.04.027>

Braasch, I.P., Postlethwait, J.H., 2012. Polyploidy in fish and the teleost genome duplication. In: Soltis, P.A., Soltis, D.E. (Eds.), *Polyploidy and Genome Evolution* (1st ed., p. 341-386). Berlin: Springer. https://doi.org/10.1007/978-3-642-31442-1_17.

Braasch, I.P., Gehrke, A.R., Smith, J.J., Kawasaki, K., Manousaki, T., Pasquier, J., Amores, A., Desvignes, T., Batzel, P., Catchen, J., Berlin, A.M., Campbell, M.S., Barrell, D., Martin, K.J., Mulley, J.F., Ravi, V., Lee, A.P., Nakamura, T., Chalopin, D., Fan, S., Weisel, D., Cañestro, C., Sydes, J., Beaudry, F.E.G., Yi, S., Hertel, J., Beam, M.J., Fasold, M., Ishiyama, M., Johnson, J., Kehr, S., Lara, M., Letaw, J.H., Litman, G.W., Litman, R.T., Mikami, M., Ota, Y., Saha, N.R., Williams, L., Stadler, P.F., Wang, H., Taylor, J.S., Fontenot, Q., Ferrara, A., Searle, S.M.J., Aken, B., Yandell, M., Schneider, I., Yoder, J.A., Volff, J.N., Meyer, A., Amemiya, C.T., Venkatesh, B., Holland, P.W.H., Guiguen, Y., Bobe, J., Shubin, N.H., Di Palma, F., Alföldi, J., Lindblad-Toh, K., Postlethwait, J.H. 2016. The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. *Nat. Genet.* 48, 427-437. <https://doi.org/10.1038/ng.3526>.

Bridges, C. Triploid intersexes in *Drosophila melanogaster*. *Science*. 54, 252-254.

Bystrianski, J.S., Richards, J.G., Schulte, P.M., Ballantyne, J.S., 2006. Reciprocal expression of gill Na⁺/K⁺-ATPase α -subunit isoforms α 1a and α 1b during sea water acclimation of three salmonid fishes that vary in their salinity tolerance. *J. Exp. Biol.* 209, 1848-1858. <https://doi.org/10.1242/jeb.02188>.

- Bystrianski, J.S., Frick, N.T., Richards, J.G., Schulte, P.M., Ballantyne, J.S., 2007.
Failure to up-regulate gill Na⁺,K⁺-ATPase α -subunit isoform α 1b may limit
seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*). *Comp.*
Biochem. Physiol., Part A Mol. Integr. Physiol. 148, 332-338.
<https://doi.org/10.1016/j.cbpa.2007.05.007>.
- Carradice D., Lieschke G.J., 2008. Zebrafish in hematology: Sushi or science? *Blood*.
111, 3331-3342. <https://doi.org/10.1182/blood-2007-10-052761>.
- Cavalier-Smith, T., 1978. Nuclear volume control by nucleoskeletal DNA, selection for
cell volume and cell growth rate, and the solution of the DNA C-value paradox.
Journal of Cell Science. 34, 247-278
- Charest, M.A., 2016. Turnover rates and trophic discrimination factors of $\delta^{34}\text{S}$ in brook
trout, *Salvelinus fontinalis*. Master of Science Thesis, University of New
Brunswick.
- Chen, H., Soraka, D.N., Haider, J., Ferri-Lagneau, K.F., Leung, T-C., Sang, S., 2013.
[10]-Gingerdiols as the major metabolites of [10]-gingerol in zebrafish embryos
and in humans and their hematopoietic effects in zebrafish embryos. *J. Agric.*
Food Chem. 61, 5353-5360. <https://doi.org/10.1021/jf401501s>.
- Choi, I-Y., Kwon, E-C., Kim, N-S., 2020. The C- and G-value paradox with polyploidy,
repeatomes introns, phenomes and cell economy. *Genes Genom.* 42, 699-714.
<https://doi.org/10.1007/s13258-020-00941-9>.
- Clarke, E.K., Rivera Gomez, K.A., Mustachi, Z., Murph, M.C., Schvarzstein, M., 2018.
Manipulation of Ploidy in *Caenorhabditis elegans*. *J. Vis. Exp.* 133, e57296.
<https://doi.org/10.3791/57296>.

- Conant, G.C., 2020. The lasting after-effects of an ancient polyploidy on the genomes of teleosts. *PLoS ONE*. 15, e0231356.
<https://doi.org/10.1371/journal.pone.0231356>.
- Cunha, C., Doadrio, I., Abrantes, J., Coelho, MM., 2011. The evolutionary history of the allopolyploid *Squalius alburnoides* (Cyprinidae) complex in the northern Iberian Peninsula. *Heredity*. 106, 100-112. <https://doi.org/10.1038/hdy.2010.70>.
- Dehal P, Boore J., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol*. 3, 1700-1708.
<https://doi.org/10.1371/journal.pbio.0030314>.
- Dobzhansky, T., 1928. The influence of the quantity and quality of chromosomal material on the size of the cells in *Drosophila melanogaster*. *Wilhem Roux' Archiv für Entwicklungsmechanik der Organismen*. 115, 363-379.
- Fagernes, C.E., Stensløkken, K-O., Røhr, Ä.K., Berenbrink, M., Ellefsen, S., Nilsson, G.E., 2015. Extreme anoxia tolerance in crucian carp and goldfish through neofunctionalization of duplicated genes creating a new ethanol-producing pyruvate decarboxylase pathway. *Sci. Rep*. 7. <https://doi.org/10.1038/s41598-017-07385-4>.
- Fankhauser, G., 1939. Polyploidy in the salamander, *Eurycea bislineata*. *J. Hered*. 30, 379-388.
- Ferri-Lagneau, K.F., Moshal, K.S., Grimes, M., Zahora, B., Lv, L., Sang, S., Leung, T-C., 2011. Ginger stimulates hematopoiesis via BMP pathway in zebrafish. *PLoS ONE*. 7, e39327. <http://dx.doi.org/10.1371/journal.pone.0039327>.

- Ferri-Lagneau, K.F., Haider, J., Sang, S., Leung, T-C., 2019. Rescue of hematopoietic stem/progenitor cells formation in *plcg1* zebrafish mutant. *Sci. Reports.* 9, 244. <https://doi.org/10.1038/s41598-018-36338-8>.
- Franco, R.S., 2012. Measurement of red cell lifespan and aging. *Transfus. Med. Hemother.* 39, 302-307. <https://dx.doi.org/10.1159/000342232>.
- Franco, R.S., Puchulu-Campanella, M.E., Barber, L.A., Palascak, M.B., Joiner, C.H., Low, P.S., Cohen, R.M., 2013. Changes in the properties of normal human red blood cells during *in vivo* aging. *Am. J. Hematol.* 88, 44-51. <https://doi.org/10.1002/ajh.23344>.
- Fraser, T.W.K., Fleming, M.S., Poppe, T.T., Hansen, T., Fjelldal, P.G., 2014. The effect of ploidy and incubation temperature on survival and the prevalence of aplasia of the *septum transversum* in Atlantic salmon (*Salmo salar* L.). *J. Fish. Dis.* 37, 189-200. <https://doi.org/10.1111/jfd.12091>.
- Fraser, T.W.K., Mayer, I., Hansen, T., Poppe, T.T., Skjaeraasen, J.E., Koppang, E.O., Fjelldal, P.G., 2015. Vaccination and triploidy increase relative heart weight in farmed Atlantic salmon, *Salmo salar* L. *J. Fish. Dis.* 38, 151-160. <https://doi.org/10.1111/jfd.12216>.
- Götting, M., Nikinmaa, M.J., 2017. Transcriptomic analysis of young and old erythrocytes of fish. *Front. Physiol.* 8, 1046. <https://doi.org/10.3389/fphys.2017.01046>.
- Gregory, T.R., & Hebert, P.D.N., 1999. The modulation of DNA content: Proximate causes and ultimate consequences. *Genome Research.* 9, 317-324. <https://doi.org/10.1101/gr.9.4.317>.

- Gregory, T.R., 2001. Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biological Reviews*. 76, 65-101.
<https://doi.org/10.1017/S1464793100005595>.
- Gregory, T.R., 2002. Genome size and developmental complexity. *Genetica*. 115, 131-146. <https://doi.org/10.1023/A:1016032400147>.
- Gregory, T.R., Mable, B.K., 2005. Polyploidy in animals. In: Gregory, T.R. (Eds.). *The Evolution of the Genome* (1st ed., p. 427-517).
<https://doi.org/10.1016/B978-012301463-4/50010-3>.
- Griffiths, R., 1941. Triploidy (and haploidy) in the newt, *Triturus viridescens*, induced by refrigeration of fertilized eggs. *Genetics*. 26, 69-88.
- Haghighi, M., Rohani, M.S., 2013. The effects of powdered ginger (*Zingiber officinale*) on the haematological and immunological parameters of rainbow trout *Oncorhynchus mykiss*. *JMPHTR*. 1, 8-12.
- Hansen, T.J., Olsen, R.E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T., Fjelldal, P.G., 2015. Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*. 435, 354-360. <http://dx.doi.org/10.1016/j.aquaculture.2014.10.017>.
- Heier, J., Takle, K.A., Hasley, A.O., Pelegri, F., 2015. Ploidy Manipulation and Induction of Alternate Cleavage Patterns Through Inhibition of Centrosome Duplication in the Early Zebrafish Embryo. *DEV DYNAM*. 244, 1300-1312.
<https://dx.doi.org/10.1002%2Fdvdy.24315>.
- Higgins, J.M., 2015. Red blood cell population dynamics. *Clin Lab Med*. 35, 43-57.
<https://dx.doi.org/10.1016%2Fj.cll.2014.10.002>.

- Ho, S-C., Chang, K-S., Lin, C-C., 2013. Anti-neuroinflammatory capacity of fresh ginger is mainly attributed to 10-gingerol. *Food Chem.* 141, 3183-3191.
<http://dx.doi.org/10.1016/j.foodchem.2013.06.010>
- Hoegg, S., Brinkmann, H., Taylor, J.S., Meyer, A. Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. 2004. *J. Mol. Evol.* 59, 190-203. <https://doi.org/10.1007/s00239-004-2613-z>.
- Hyndman, C.K., Kieffer, J.D., Benfey, T.J., 2003. Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture.* 221, 629-643. [https://doi.org/10.1016/S0044-8486\(03\)00119-4](https://doi.org/10.1016/S0044-8486(03)00119-4).
- Jeffrey, N.W., Ellis, E.A., Oakley, T.H., Gregory, T.R., 2017. The genome size of ostracod crustaceans correlate with body size and evolutionary history, but not environment. *Jour. Hered.* 108, 701-706. <https://doi.org/10.1093/jhered/esx055>.
- Kanani, H.G., Nobahar, Z., Kakooli, S., Jafarian, H., 2014. Effect of ginger- and garlic-supplemented diet on growth performance, some hematological parameters and immune responses in juvenile *Huso huso*. *Fish Physiol. Biochem.* 40, 481-490. <https://doi.org/10.1007/s10695-013-9859-6>.
- Kapp, F.G., Perlin, J.R., Hagedorn, E.J., Gansner, J.M., Schwarz, D.E., O'Connell, L.A., Johnson, N.S., Amemiya, C., Fisher, D.E., Wölflle, U., Trompouki, E., Niemeyer, C.M., Driever, W., Zon, L.I., 2018. Protection from UV light is an evolutionarily conserved feature of the haematopoietic niche. *Nature.* 558, 445–448.
<https://doi.org/10.1038/s41586-018-0213-0>.
- Kavumpurath, S., Pandian, T.J., 1990. Induction of triploidy in the zebrafish, *Brachydanio rerio* (Hamilton). *Aquac. Res.* 21, 299-306.
<https://doi.org/10.1111/j.1365-2109.1990.tb00468.x>.

- Kenny, N.C., Chan, K.W., Nong, W., Qu, Z., Maeso, I., Yip, H.Y., Chan, T.F., Kwan, H.S., Holland, P. W.H., Chu, K.H., Hui, J.H.L. (2016). Ancestral whole-genome duplication in the marine chelicerate horseshoe crabs. *Heredity*. 116, 190-199. <https://doi.org/10.1038/hdy.2015.89>.
- Kobayashi, I., Kondo, M., Yamamori, S., Kobayashi-Sun, J., Taniguchi, M., Kanemaru, K., Katakura, F., Traver, D., 2019 Enrichment of hematopoietic stem/progenitor cells in the zebrafish kidney. *Sci. Rep.* 9, 14205. <https://doi.org/10.1038/s41598-019-50672-5>.
- Laubichler, M.D., Davidson, E.H. 2008. Boveri's long experiment: Sea urchin merogones and the establishment of the role of nuclear chromosomes in development. *Dev. Biol.* 314. <https://doi.org/10.1016/j.ydbio.2007.11.024>.
- Leal, M.J., Eenennaam, J.V.P., Schreier, A.D., Todgham, A.E., 2019. Triploidy in white sturgeon (*Acipenser transmontanus*): Effects of acute stress and warm acclimation on physiological performance. *Comp. Biochem. Physiol. A*.
- Licht, L.E., Bogart, J.P., 1990. Comparative rates of oxygen consumption and water loss in diploid and polyploid salamanders (genus *Ambystoma*). *Comp. Biochem. Physiol.* 97, 569-572. [https://doi.org/10.1016/0300-9629\(90\)90129-G](https://doi.org/10.1016/0300-9629(90)90129-G).
- Lien, S., Koop B.F., Sandve, S.R., Miller, J.R., Kent, M.P., Nome, T., Hvidsten, T.R., Leong, J.S., Minkley, D.R., Zimin, A., Grammes, F., Grove, H., Gjvusland, A., Walenz, B., Hermansen, R.A., von Schalburg, K., Rondeau, E.B., Di Genova, A., Samy, J.K.A., Vik, J.O., Vigeland, M.D., Caler, L., Grimholt, U., Jentoft, S., Våge, D.I., de Jong, P., Moen, T., Baranski, M., Palti, Y., Smith, D.R., Yorke, J.A., Nederbragt, A.J., Tooming-Klunderud, A., Jakobsen, K.S., Jiang, X., Fan, D., Hu, Y., Liberles, D.A., Vidal, R., Iturra, P., Jones, S.J.M., Jonassen, I.,

- Maass, A., Omholt, S.W., Davidson, W.S., 2016. The Atlantic salmon genome provides insights into rediploidization. *Nature*. 533, 200-205.
<https://doi.org/10.1038/nature17164>.
- Liu, X-L., Jiang, F-F., Wang, Z-W., Li, X-Y., Li, Z., Zhang, X-J., Chen, F., Mao, J-M., Zhou, L., Gui, J-F., 2017. Wider geographic distribution and higher diversity of hexaploids than tetraploids in *Carassius* species complex reveals recurrent polyploidy effects on adaptive evolution. *Sci. Reports*. 7, 5395.
<http://dx.doi.org/10.1038/s41598-017-05731-0>.
- Martinez-Agosto, J.A., Mikkola, H.K.A., Hartenstein, V., Banerjee, U., 2017. The hematopoietic stem cell and its niche: A comparative view. *Genes Dev*. 21, 3044-3060. <http://www.genesdev.org/cgi/doi/10.1101/gad.1602607>.
- Masterson, J., 1994. Stomatal size in fossil plants: Evidence for polyploidy in majority of angiosperms. *Science*. 264, 421-424.
<https://doi.org/10.1126/science.264.5157.421>.
- Maxime, V., 2008. The physiology of triploid fish: Current knowledge and comparisons with diploid fish. *Fish Fish*. 9, 67-78. <https://doi.org/10.1111/j.1467-2979.2007.00269.x>.
- Morera, D., MacKenzie, S.A., 2011. Is there a direct role for erythrocytes in the immune response. *Vet. Res*. 42, 89. <http://www.veterinaryresearch.org/content/42/1/89>.
- Mueller, R., 2015. Genome biology and the evolution of cell-size diversity. *Cold Spring Harb. Perspect. Biol*. 7, a019125. <https://doi.org/10.1101/cshperspect.a019125>.
- Muller, H.J., 1925. Why polyploidy is rarer in animals than in plants. *AM NAT*. 59, 346-353.

- Myers, J.M., Hershberger, W.K., Iwamoto, R.N., 1986. The Induction of Tetraploidy in Salmonids. *J WORLD AQUACULT SOC.* 17:1-4. <https://doi.org/10.1111/j.1749-7345.1986.tb00546.x>.
- Nandakumar, S., Grushko, O., Buttita, L.A., 2020. Polyploidy in the adult *Drosophila* brain. *eLife.* 9, e54385. <https://doi.org/10.7554/eLife.54385>.
- Nash, G.B., Egginton, S., 1993. Comparative rheology of human and trout red blood cells. *J. Exp. Biol.* 174, 109-122.
- Nell, J.A. (2002). Farming triploid oysters. *Aquaculture.* 210, 69-88. [https://doi.org/10.1016/S0044-8486\(01\)00861-4](https://doi.org/10.1016/S0044-8486(01)00861-4).
- Nikinmaa, M. (1990) Vertebrate red blood cells: Adaptations of function to respiratory requirements. Berlin: Springer Berlin Heidelberg. <https://doi.org/10.1007/978-3-642-83909-2>.
- Ohno, S., 1970. *Evolution by gene duplication*. Berlin: Springer Berlin Heidelberg. <https://doi.org/10.1007/978-3-642-86659-3>.
- Ojolick, E.J., Cusack, R., Benfey, T.J., Kerr, S.R., 1995. Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture.* 131, 177-187. [https://doi.org/10.1016/0044-8486\(94\)00338-O](https://doi.org/10.1016/0044-8486(94)00338-O).
- Orr-Weaver, T.L., 2015. When bigger is better: the role of polyploidy in organogenesis. *Trends Genet.* 31, 307-315. <http://dx.doi.org/10.1016/j.tig.2015.03.011>.
- Piferrer, F., Beaumont, A., Flaguère, J-C., Flajšhans, M., Haffray, P., Colombo, L., 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture.* 293, 125-156. <http://dx.doi.org/10.1016/j.aquaculture.2009.04.036>.

- Poontawee, K., Werner, C., Müller-Belecke, A., Hörstgen-Schwark, G., Wicke, M., 2007. Flesh qualities and muscle fiber characteristics in triploid and diploid rainbow trout. *J. Appl. Ichthyol.* 23, 273-275. <https://doi.org/10.1111/j.1439-0426.2007.00843.x>.
- Postlethwait, J.H., 2007. The zebrafish genome in context: ohnologs gone missing. *J. Exp. Zool.* 308B, 563-577. <https://doi.org/10.1002/jez.b.21137>.
- Pray, L. 2008. Eukaryotic genome complexity. *Nature Education.* 1:96.
- Richards, J.G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na⁺/K⁺-ATPase α -isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J. Exp. Biol.* 206, 4475-4486. <https://doi.org/10.1242/jeb.00701>.
- Riseth, E.N., Fraser, T.W.K., Sambraus, F., Stien, L.H., Hvas, M., 2020. Is it advantageous for Atlantic salmon to be triploid at lower temperatures? *J. Therm. Biol.* 89, 102548. <https://doi.org/10.1016/j.jtherbio.2020.102548>.
- Saccucci, M.J., Denton, R.D., Holding, M.L., Gibbs, H.L., 2016. Polyploid unisexual salamanders have higher tissue regeneration rates than diploid sexual relatives. *J. Zool.* 300, 77-81. <https://doi.org/10.1111/jzo.12339>.
- Sambraus, F., Olsen, R.E., Remen, M., Hansen, T.J., Torgersen, T., Fjelldal, P.G., 2017. Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture.* 473, 1-12. <https://doi.org/10.1016/j.aquaculture.2017.01.024>.
- Sambraus, F., Remen, M., Olsen, R.E., Hansen, T.J., Waagbø, R., Torgersen, T., Lock, E.J., Imsland, A., Fraser, T.W.K., Fjelldal, P.G., 2018. Changes in water temperature and oxygen: the effect of triploidy on performance and metabolism

in large farmed Atlantic salmon. *Aquac. Environ. Interact.* 10, 157-172.

<https://doi.org/10.3354/aei00260>.

Scheer, U. 2018. Boveri's research at the Zoological Station Naples: Rediscovery of his original microscope slides at the University of Würzburg. *Mar. Genomics.* 40.

<https://doi.org/10.1016/j.margen.2018.01.003>.

Schwager, E.E., Sharma, P P., Clarke, T., Leite, D.L., Wierschin, T., Pechmann, M., Akiyama-Oda, Y., Esposito, L., Bechsgaard, J., Bilde, T., Buffry, A.D., Chao, H., Dinh, H., Doddapaneni, H., Dugan, S., Eibner, C., Extavour, C.G., Funch, P., Garb, J., Gonzalez, L.B., Gonzalez, V.L., Griffiths-Jones, S., Han, Y., Hayashi, C., Hilbrant, M., Hughes, D.S.T., Janssen, R., Lee, S. L., Maeso, I., Murali, S.C., Muzny, D.M., Da Fonseca, R.N., Paese, C.L.B., Qu, J., Ronshaugen, M., Schomburg, C., Schönauer, A., Stollewerk, A., Torres-Oliva, M., Turetzek, N., Vanthournout, B., Werren, J.H., Wolff, C., Worley, K.C., Bucher, G., Gibbs, R.A., Coddington, J., Oda, H., Stanke, M., Ayoub, N.A., Prpic, N-M., Flot, J-F., Posnien, N., Richards, S., & McGregor, A.P. 2017. The house spider genome reveals an ancient whole-genome duplication during arachnid evolution. *BMC Biology.* 15. <https://doi.org/10.1186/s12915-017-0399-x>.

Scott, M.A., Dhillon, R.S., Schulte, P.M., Richards, J.G., 2014. Physiology and performance of wild and domestic strains of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in response to environmental challenges. *Can. J. Fish. Aquat.* 72, 125-134. <https://doi.org/10.1139/cjfas-2013-0450>.

Simonot, D.L., Farrell, A.P., 2009. Coronary vascular volume remodelling in rainbow trout *Oncorhynchus mykiss*. *J. Fish. Biol.* 75, 1762-1772.

<https://doi.org/10.1111/j.1095-8649.2009.02427.x>.

- Snyder, G.K., Sheafor, B.A., 1999. Red blood cells: Centerpiece in the evolution of the vertebrate circulatory system. *Am. Zoo.* 39, 189-198.
<https://doi.org/10.1093/icb/39.2.189>.
- Soltis, D.E., Albert, V.A., Leebens-Mack, J., Bell, C.D., Paterson, A.H., Zheng, C., Sankoff, D., Depamphilis, C.W., Wall, P.K., Soltis, P.S. Polyploidy and angiosperm diversification. *Am. J. Bot.* 96, 336-348.
<https://doi.org/10.3732/ajb.0800079>.
- Swarup, H. 1959. Effect of triploidy on the body size, general organization, and cellular structure in *Gasterosteus aculea*. *Journal of Genetics.* 56, 143-155.
- Verhille, C., Antilla, K., Farrell, A.P., 2013. A heart to heart on temperature: Impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comp. Biochem. Physiol. A. Mol. Integ. Physiol.* 164, 653-657. <http://dx.doi.org/10.1016/j.cbpa.2013.01.011>.
- Wattus S.J., Zon, L.I., 2018. Stem cell safe harbor: the hematopoietic stem cell niche in zebrafish. *Blood advances.* 2, 3063-3069.
<https://dx.doi.org/10.1182%2Fbloodadvances.2018021725>.
- Witeska, M., 2013. Erythrocytes in teleost fishes: a review. *Zoo. Ecol.* 23, 275-281.
<http://dx.doi.org/10.1080/21658005.2013.846963>.
- Zhang, N., Bao, Y., Xie, Z., Huang, X., Sun, Y., Feng, G., Zeng, H., Ren, J., Li, Y., Xiong, J., Chen, W., Yan, C., & Tang, M. (2019). Efficient characterization of tetraploid watermelon. *Plants.* 8. <https://doi.org/10.3390/plants8100419>.

2.0 Chapter 2:

Early, non-lethal ploidy and genome size quantification using confocal microscopy in zebrafish embryos

Christopher D. Small¹, James P. Davis¹, Bryan D. Crawford¹, Tillmann J. Benfey¹

¹Department of Biology, University of New Brunswick, Fredericton, NB, Canada, E3B 5A3

Correspondence

Tillmann Benfey, Department of Biology, University of New Brunswick, Fredericton, NB, Canada, E3B 5A3.

Email: Benfey@unb.ca

Abstract

Ploidy transitions through whole genome duplication have shaped evolution by generating redundant copies of genes and thereby facilitating their sub- and neo-functionalization to express novel traits. Polyploidy was historically thought to be most relevant to evolution within plants, but the age of genomics has uncovered ploidy transitions within all taxa of eukaryotes examined aside from birds and mammals. The nuclear:cytoplasmic (n:c) ratio is maintained in polyploid vertebrates resulting in larger cells, but body size is maintained by a concomitant reduction in cell number. Ploidy can be manipulated easily in most teleosts, and the zebrafish, already well established as a model system for biomedical research, is therefore an excellent system in which to study the effects of increased cell size and reduced cell numbers in polyploids on development and physiology. Here I describe a novel technique to assess genome size and determine ploidy non-lethally at 48 hours post-fertilization in transgenic zebrafish modified to

express fluorescent histones and show that this technique can be used to separate diploids and triploids from a mixed-ploidy pool of embryos for subsequent experimentation.

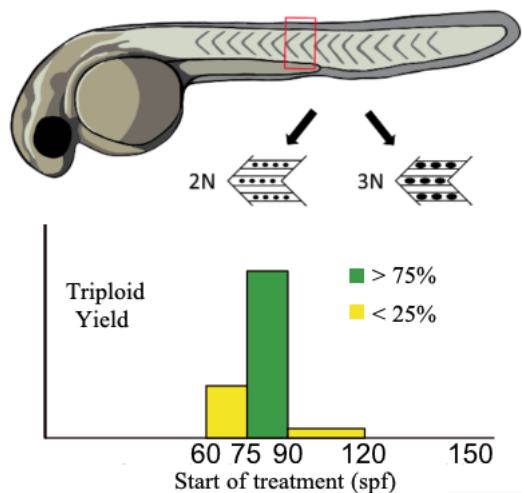
Keywords: Triploid, Polyploid, Whole Genome Duplication, Cell size, Genome size, Zebrafish.

Research highlights:

Triploid zebrafish can be produced and identified non-lethally at 48 hours post-fertilization using myofiber nucleus volume analysis.

Triploid zebrafish are a valuable tool for studying the effects of ploidy and cell size changes on development and physiology.

Graphical abstract:



2.1 Introduction

Variation in genome size among species is extreme, estimated at ~80 000 fold among eukaryotes (Gregory & Hebert, 1999) and ~350 fold among vertebrates (Cavalier-Smith, 1978; Gregory, 2001). Investigations into the evolution of genome size have revealed a pattern: the amount of DNA in the nucleus correlates with the volume of the nucleus and the cell itself (Gregory, 2001; Levy & Heald, 2010), a pattern that holds true across tissues and taxa for most eukaryotic cells (Cavalier-Smith, 1978; Gregory, 2002; Gillooly, Hein, & Damiani, 2015). Understanding the mechanism(s) linking nucleus volume and cell volume to the amount of nuclear DNA is a long-standing goal in fields of biology as diverse as cell biology (Turner, Ewald, & Skotheim, 2012; Ginzberg, Kafri, & Kirschner, 2015; Neurohr et al., 2019), development (Levy & Heald, 2010; Roth & Walkowiak, 2015; Miller, Brownlee, & Heald, 2020), physiology (Benfey, 1999; Saranyan, Ross, & Benfey, 2017; Christensen et al., 2019), and evolutionary biology (Otto, 2007; Smith & Gregory, 2009; Elliott & Gregory, 2015; Mueller, 2015).

Ploidy transitions are the most drastic and sudden changes to genome size observed in evolution, yet these dramatic changes in the amount of DNA per cell occasionally persist while the ancestral lineage goes extinct (Braasch & Postlethwaite, 2012; Van de Peer et al., 2009). Early studies comparing diploid, triploid, and tetraploid salamanders (Fankhauser, 1939, 1945) and diploid and triploid fish (Swarup, 1959) showed that animals at higher ploidy levels have larger cells, but body size is unaffected by ploidy due to a concomitant reduction in cell number. This pattern linking DNA content, cell size and cell number is consistent across vertebrates, as documented in fish (Benfey, 1999), amphibians (Roth & Walkowiak, 2015; Miller et al., 2020), reptiles (Tiersch & Figiel, 1991), birds (Abdel-Hameed & Neat, 1971; De Boer et al., 1984;

Gregory, 2018), and mammals (Henery & Kaufman, 1992; Henery, Bard, & Kaufman, 1992). This stands in stark contrast to the pattern in plants and some invertebrates, in which polyploid individuals are larger than diploids because cell numbers are not reduced to balance increased cell size (Nell, 2002; Lozano et al., 2006; Aversano et al., 2012). We still lack a complete understanding of the mechanisms underlying this compensatory relationship between cell volume and cell number in vertebrates that maintains body size independent of ploidy. Given the importance of allometric relationships between body size and major determinants of ecological niche placement, such as metabolism (Kleiber and Rogers, 1961; West et al., 1997; Anderson-Teixeira et al., 2009), thermal tolerance (Leiva, Calosi, & Verberk, 2019), prey preference and hunting behaviour (Kalinkat et al., 2011; Dunic & Baum, 2017), and the evidence for at least 10 ploidy transitions in teleosts alone (Braasch & Postlethwait, 2012), it is tempting to speculate that cell number compensation evolved to maintain optimum body size in the face of genome size variation and ploidy transitions.

Dr. Susumo Ohno suggested in his book *Evolution by Gene Duplication* (Ohno, 1970) that gene/genome duplications could be evolutionarily advantageous due to increased redundancy and relaxed selection on highly conserved genes, thereby reducing negative selection on mutations and increasing the potential for functionally advantageous alleles to evolve. Recent studies have supported this hypothesis by linking the divergence of ohnologues (i.e., paralogous genes arising from a Whole Genome Duplication) to the evolution of novel traits such as migrating between freshwater and marine environments in salmonids mediated via branchial Na^+/K^+ -ATPase α -isoform switching (Richards et al., 2003; Bystriansky et al., 2006, 2007) and metabolizing lactic acid into ethanol via pyruvate decarboxylase during prolonged hypoxia/anoxia in

goldfish (*Carrassius auratus*) and crucian carp (*C. carrassius*) (Fagernes et al., 2015). The development of techniques to manipulate ploidy in fish (Piferrer et al., 2009) and amphibians (Fankhauser, 1945) enables us to study these questions in controlled laboratory environments and ask: what are the negative consequences that arise when genome copy number and cell volume increase? Or in other words, what are the trade-offs for the advantages gained from genome duplication that explain the relative scarcity of polyploidy in vertebrates?

The ability to manipulate ploidy has been useful for both aquaculture and agriculture as triploids cannot complete meiosis making farmed triploid animals reproductively sterile (Benfey, 1999; Sattler, Carvalho, & Clarindo, 2016; Benfey, 2016; Zhang et al., 2019). Since the pioneering works of early developmental biologists on polyploids (Griffiths, 1941; Fankhauser, 1945; Swarup, 1959), research on the physiological consequences of polyploidy in vertebrates has largely focused on testing the feasibility of using triploid fish in aquaculture to prevent gene flow between farmed escapees and wild fish. Such studies have shown that triploid fish are less able to tolerate sub-optimal conditions that impose physiological challenges (e.g., Ojolick et al., 1995; Hyndman et al., 2003; Verhille, Antilla, & Farrell, 2013), which has prevented the wide-scale adoption of this technology. However, there has been a resurgence of interest in assessing triploid salmonid fishes for use in aquaculture due to improvements in our understanding of their thermal (Verhille et al., 2013; Sambraus et al., 2018; Riseth et al., 2020) and hypoxia tolerance (Scott et al., 2014; Hansen et al., 2015; Sambraus et al., 2017; Benfey & Devlin, 2018), and dietary requirements (Sambraus et al., 2020). Despite these advances in understanding the biology of triploid salmonids, they are slow-growing, cold-water fish with a long generation time (~3 years) making them a

rather impractical model organism. Taking advantage of a well-established model teleost like zebrafish (*Danio rerio*), with their rapid growth and short generation time, would complement triploid research in common aquaculture species as it has for other branches of aquaculture science both currently (Ulloa, Medrano, & Feijoo, 2014; Ribas & Piferrer, 2014) and in the future (Wargelius et al., 2016).

Triploid zebrafish have been produced by inhibiting extrusion of the second polar body via temperature treatment shortly after fertilization (Kavumpurath & Pandian, 1990; Baars et al., 2016; Van de Pol et al., 2020). Given that hydrostatic pressure is widely used to produce triploids of other species of fish (Piferrer et al., 2009) and has been used in zebrafish to produce gynogenetic homozygous diploids after fertilization with irradiated sperm (Streisinger et al., 1981), I reasoned that this technique would produce triploid zebrafish if eggs were fertilized with intact sperm. The rapid development of the zebrafish at 28.5°C (Kimmel et al., 1995) presents a challenge as the temporal window of opportunity to produce triploids is very short (between 1-3 minutes after fertilization depending on the type of treatment).

Current techniques to determine ploidy level in zebrafish are lethal until fish are adult-sized (approximately 3 months old) and, therefore, large enough that blood can be sampled for erythrocyte size measurement (Kavumpurath & Pandian, 1990). While flow cytometric measurement of cellular DNA content in cell suspensions made from embryos is a useful strategy to ensure that triploidy induction is successful at the population level (Delomas & Dabrowski, 2018; Van de Pol et al., 2020), this also requires destructive sampling and introduces the potential confound of competition among individuals of different ploidy within a tank (O’Keefe & Benfey, 1997; Taylor et al., 2014). Furthermore, many of the powerful molecular techniques available in

zebrafish such as qPCR and RNAseq require embryo homogenization, making it challenging to validate ploidy in the embryos. The development of a non-lethal technique to determine ploidy level in zebrafish embryos would help address these challenges to ensure that the zebrafish is being used to its full potential as a model for triploid research.

The transgenic zebrafish Tg(h2a.f/z:H2Af/z::eGFP) was used as it expresses green fluorescent protein fused to the H2A.f/z histone subunit under the regulatory control of the endogenous h2a.f/z promoter thereby making the chromatin inside the nucleus of every cell relatively easy to image *in vivo* (Pauls et al., 2001). At 48 hours post-fertilization (hpf), the nuclei of polynucleated myofibers in the developing myotomes are roughly uniform in shape, size, and orientation, making them ideal candidates for early and non-lethal ploidy determination. I applied hydrostatic pressure to *in vitro* fertilized zygotes at various times spanning the undefined critical window for triploid induction and found that triploid yield is maximized when pressure treatment of zygotes begins between 75 and 90 seconds post-fertilization (spf). Although diploid and triploid embryos are typically morphologically indistinguishable, there is a tendency for triploid embryos to have minor cardiac edemas and some irregular tissues, and mortality rate is significantly higher in triploids during embryonic development. To confirm that zebrafish exhibit some of the same physiological patterns observed in triploid salmonids (Benfey & Sutterlin, 1984; Benfey & Bennett, 2009), I present the results from a simple experiment measuring heart rate of 56 hpf diploid and triploid embryos at 27°C and 32°C to demonstrate that viable triploid embryos are comparable physiologically and are a valuable tool for investigations of polyploid biology.

2.2 Methods

2.2.1. Animal husbandry

Adult zebrafish were reared in flow-through dechlorinated municipal water in the University of New Brunswick Zebrafish Facility in standard 25 x 11 x 15 cm tanks (Pentair Aquatic Ecosystems, Apopka, FL, USA) at 28°C on a 14:10 h light:dark photoperiod. They were fed zebrafish pellets (Gemma 500 Micro, Skretting, Vancouver, BC, Canada) twice per day supplemented with live *Artemia* once per day. Embryos from these adults were reared in glass Petri dishes in Embryo Rearing Medium (ERM: 13 mM NaCl, 0.5 mM KCl, 0.02 mM Na₂HPO₄, 0.04 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃, pH 7.4) with 0.01% methylene blue at 28.5°C until 120 hpf when they were transferred to 1 L larval tanks (6 x 3 x 4 cm). Larvae were fed a series of increasingly larger pellets (Gemma Micro 75-300, Skretting) using automated feeders set to feed 4 times per day. The transgenic lines used in this study were Tg(h2a.f/z:H2A:f/z::eGFP) (Pauls et al., 2001) and Tg(myf7:mCherry) (Palencia-Desai et al., 2011). All procedures involving animals were approved by the UNB Animal Care Committee, according to the guidelines of the Canadian Council of Animal Care.

2.2.2 *In vitro* fertilization and triploid induction

2.2.2.1 Gamete collection

Adult zebrafish were allowed to spawn naturally, either in 1 L breeding tanks or in their home tanks (using a tray of marbles as spawning substrate) to ensure that the fish were healthy and spawning. These fish were then given 10-14 days to recover and gametes were collected for *in vitro* fertilization (IVF). Healthy and robust adults were sorted by sex (Westerfield, 2000) into separate tanks the evening before IVF. Ten minutes after the lights came on the next morning, males were anaesthetized in tris-buffered MS-222

(168 $\mu\text{g/mL}$) (Westerfield, 2000) and sperm was collected manually by applying gentle pressure to the body wall in a slow wave from anterior to posterior. Sperm was collected in a 9 μL capillary tube (Sigma-Aldrich, St. Louis, MO, USA) attached to a mouth aspirator (Sigma-Aldrich) for suction. Sperm samples that were not white and opaque were discarded. Samples were expelled into 60 μL of ice-cold Hank's Full Strength solution (0.37 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4 , 0.44 mM KH_2PO_4 , 1.3 mM CaCl_2 , 1.0 mM MgSO_4 , 4.2 mM NaHCO_3) (Westerfield, 2000) in 0.5 mL microfuge tubes and stored on ice to maintain viability, with sperm pooled from multiple males until a cloudy consistency was achieved. Eggs were collected into a Petri dish by applying gentle pressure ventrally to the belly of anaesthetized (as above) gravid females and using a plastic micropipette tip to handle the eggs. Egg batches containing eggs that were not predominantly yellow and round were discarded. Any sperm or egg samples that were exposed to water after extraction or were not fertilized within 5 minutes of extraction were discarded. Adults were quickly moved to a well-aerated recovery tank after gamete collection.

2.2.2.2 Fertilization and triploid induction

Fertilization was achieved by pipetting 20 μL of sperm solution into the egg mass followed immediately by 60 μL of ERM to activate sperm motility. At 30 seconds post fertilization (spf), an additional 1 mL of ERM was added to facilitate the transfer of embryos in the next step. Fifteen seconds before pressure treatment, 50-70% of the embryos were transferred using a transfer pipette into a custom-designed capsule made by cutting off the end of a 1 mL pipette tip and capping both ends of the remaining cylinder with 40 μm nylon mesh using small elastics or custom-fitted plastic rings. Following this, the capsule was placed into a 75 mL French press chamber (model FA-

073; SLM Aminco, Urbana, IL, USA) that had been completely filled with water from the adult home tank. Excess water was displaced by leaving the pressure-relief valve open while pushing in the piston to ensure that no air was trapped within the chamber. The pressure-relief valve was then closed, and the chamber placed in a 12-ton manually operated Carver lab press (model 3851-0; Cole-Palmer, Montréal, QC, Canada). At 75 spf, pressure was rapidly (less than 5 seconds) increased to 58.6 MPa and held for 5 minutes. Forty mL of ERM was added to the Petri dish with the untreated controls during this interval, to avoid desiccation and to prevent embryos from sticking together. After pressure treatment, embryos were transferred into a Petri dish with ~40 mL of ERM and any clumps of embryos were dissociated with gentle swirling using a pipette tip. Embryos were incubated at 28.5°C for 1 hour before sorting on a dissecting microscope (Leica Microsystems model MZ205A; Wetzlar, Germany) to remove unfertilized eggs and quantify fertilization rate. Embryos were raised in these conditions (<50 embryos per Petri dish) until 120 hours post fertilization (hpf) and inspected every 12 hours to quantify and remove deformed or dead embryos. Rearing solutions were refreshed every 24 hours.

2.2.3 Determining ploidy with Tg(h2a.f/z:H2A.f/z::eGFP) embryos

2.2.3.1 Confocal microscopy

Presumptive diploids (control) and triploids (pressure-treated) were produced from Tg(h2a.f/z:H2A.f/z::eGFP) adults, as described above. At 48 hpf, individual embryos were anaesthetized in 168 µg/mL tris-buffered MS-222 and mounted (in anaesthetic) on a slide using vacuum grease footpads to hold the cover slip in place. Confocal images were collected using a scanning confocal microscope (Leica model SP2 with 63x 1.2 NA lens or model SP8 with 25x 0.9 NA lens) to focus on the elliptical, anterior-posterior

oriented, fluorescent nuclei in the developing myotomes. Stacks were collected mediolaterally, starting at the notochord and moving out to the epidermis, dorsal to the posterior margin of the yolk extension. Step sizes were either 1 μm (Leica SP2) or 0.5 μm (Leica SP8). After imaging, the slide cover was removed using forceps under a dissecting microscope and the embryo was transferred into a recovery dish with fresh ERM using a wide-bore glass pipette and monitored until recovery. Individual embryos were transferred into separate 2 mL microfuge tubes filled with ERM (after recovery from anesthesia) and incubated at 28.5°C until ploidy was determined from the confocal data. Rearing solution in the microfuge tubes was changed every 12 hours.

2.2.3.2 Image processing

Confocal stacks were first processed in FIJI (Schindelin et al., 2012) for embryo orientation, Region of Interest selection, and histogram normalization. I applied a 3D Gaussian blur (1.0 sigma value in X, Y, and Z) to eliminate artifactual holes and hot spots in the images of nuclei, facilitating accurate selection of nuclei by thresholding and extraction of individual nuclei using Fluorender (Wan et al., 2012, 2017). FIJI-processed confocal stacks were opened in Fluorender, in which 30-50 nuclei that were well-imaged (i.e., not cut-off at the edge of a stack and not overlapping another object) were manually selected and separated from any poorly-resolved or incomplete nuclei (purple and green nuclei, respectively, in Figure 1) using the paintbrush tool. Stacks of the extracted nuclei were returned to FIJI for volume measurement using the 3D Object Counter (Bolte & Cordelières, 2006). This function outputs an array of volume measurements (number of voxels in object multiplied by voxel size) and an object map linking each volume measurement to a specific nucleus in the stack. Histograms for each embryo (bin size 10 μm^3) were generated using ggplot2 (Wickham, 2016) in R (R Core

Team, 2019) and ploidy assigned using the modal volume in these histograms, on the basis that nuclear volume is significantly larger in triploids than in diploids (Benfey, 1999).

2.2.3.3 Validation of ploidy assignment

Thirty-two control diploid embryos and 32 putative triploid embryos (based on confocal analysis of Tg(h2a.f/z:H2A.f/z::eGFP) embryos) were raised to adult-size for independent ploidy confirmation based on erythrocyte size. Blood was collected from these fish at 4 months of age using a modified version of the technique described in Zang et al., 2013. Hypodermic needles (28.5 gauge) were heparinized (18 I.U./ml heparin, 0.9% (w/v) NaCl) by drawing the solution up and down, then allowing 2 hours to air dry. Fish were anaesthetized in tris-buffered MS-222 (168 µg/mL) and 2-4 µL of blood was collected from the caudal vasculature through the body wall just ventral to the lateral line. Blood was stored on ice in heparinized (18 I.U./ml heparin, 0.9% (w/v) NaCl) 0.5 mL microfuge tubes until blood smears could be made. Erythrocytes were imaged live using DIC microscopy on a Leica DM RXA25 microscope with a 63X lens and a Leica DC500 camera, and the major axis was measured using the line tool in FIJI. The effect of ploidy on erythrocyte size was analyzed using a paired Student's t-test and plotted using R.

2.2.4 Optimizing triploid induction using hydrostatic pressure

The triploid induction protocol described in section 2.2.2.2 was optimized by varying the start time of the hydrostatic pressure treatment (60, 75, 90, 120, and 150 spf) and comparing triploid induction success using confocal analysis of Tg(h2a.f/z:H2A.f/z::eGFP) embryos as described in sections 2.2.3.1 and 2.2.3.2. A sham-treatment at 75 spf was included to control for the effect of handling on the

pressure-treated groups, with sham-treated zygotes treated identically except that the pressure was not increased in the pressure chamber. Each start time was tested with 5 different batches of eggs. For each treatment, any IVF with less than 50% fertilization rate in the diploid control was discarded and not counted in this cumulative total. Fertilization was quantified by comparing the number of fertilized and unfertilized eggs at 1-3 hours after IVF (unfertilized eggs were subsequently removed). Mortality proportions were determined by counting dead embryos at 48 hpf. Triploid induction success was compared by analyzing 10 embryos per treatment and comparing the number of triploids detected. Triploid yield was calculated for each sample by multiplying triploid induction, survival, and fertilization rates. The effects of pressure treatment timing on fertilization rate, survival rate, ploidy induction rate, and triploid yield were each analyzed by one-way ANOVA followed by Tukey's Honest Significant Differences method for post-hoc analysis in R where significant ($p < 0.05$) differences in the ANOVA were detected.

2.2.5 Survival rate in diploid and triploid zebrafish embryos

Diploid and triploid embryos were produced as described in section 2.2.2 and sorted at the 8-cell stage into 15 4-well Petri dishes (1 embryo per well, 30 embryos per ploidy) and reared as described in section 2.2.1. This experiment was performed three times on separate days and survival curves represent the average of the three experiments ($n = 3$). Dead/deformed embryos were counted and removed every 3 hours for the first 24 hours, then every 12 hours until 120 hpf. Ploidy was determined at 48 hpf as described in section 2.2.3.1 and 2.2.3.2. Ploidy could not be determined in mortalities that occurred before 48 hpf, but in all cases triploid induction rate was above 95% in the surviving embryos. Kaplan-Meier survival curves were generated and compared using the log-rank

test in R using the following packages: ggplot2 (Wickham, 2016), survival (Therneau & Grambsch, 2000), and survminer (Kassambara & Kosinski, 2017). Bright field micrographs of embryos were captured using a Leica MZ205A dissecting microscope with a DFC360 FX greyscale low-light camera attachment.

2.2.6 Measuring heart rate in diploid and triploid zebrafish embryos

Diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos were produced as described in section 2.2.2 and reared in Petri dishes at 28.5°C until 40 hpf. Ten viable triploids were identified using confocal microscopy at 48 hpf as described in sections 2.2.3.1 and 2.2.3.2. At 56 hpf, heart rate was measured by recording the heartbeat of the 10 confirmed triploids and 10 diploid controls at 27°C and again after a 1-hour incubation at 32°C using a dissecting microscope (Boreal, St. Catharines, ON, Canada) with a camera attachment (model MU-900; AmScope, Irvine, CA, USA) for at least 30 seconds up to a maximum of 60 seconds. Experimental temperatures were achieved using water baths; Petri dishes with embryos were placed onto platforms in the water bath so they were partially submerged. Temperature in Petri dishes was measured using a Traceable Total-Range Digital Thermometer (VWR, Radnor, PA, USA). Videos were analyzed using VLC media player (www.videolan.org) and the effects of temperature and ploidy on heart rate were analyzed by repeated measures ANOVA using R with a Tukey's Post-hoc analysis.

2.3 Results

2.3.1 Nucleus volume can be measured non-lethally in zebrafish embryos at 48 hours post fertilization

I imaged the trunk musculature of putative diploid and triploid

Tg(h2a.f/z:H2A.f/z::eGFP) embryos dorsal to the posterior tip of the yolk extension

(Figure 2.1A). Compared to controls (putative diploids), embryos derived from pressure-treated zygotes (putative triploids) had larger nuclei in the developing myotomes (Figure 2.1B,C, respectively). Importing confocal volumes into Fluorender made it possible to select and isolate specific nuclei in their entirety (no partial nuclei) and without any overlapping objects obscuring them from analysis (Figure 2.1B,C; selected nuclei are purple, excluded nuclei are green). Plotting the distribution of nuclear volumes from individual embryos as histograms revealed a shift to the right in putative triploids (Figure 2.1D,E).

2.3.2 Embryos with larger nuclei that develop from pressure-treated zygotes are *bona fide* triploids

Treating several hundred zygotes with hydrostatic pressure at 75 spf reliably produced populations of embryos with a high proportion of putative triploids (Figure 2.2A). This pattern was consistent across families with nucleus volumes clustering around $200\ \mu\text{m}^3$ and just above $300\ \mu\text{m}^3$ in putative diploid and triploid populations, respectively (Figure 2.2B). Subsequent measurement of erythrocyte size in adults from these same populations confirmed that they were of the predicted ploidy (Figure 2.2C-F).

2.3.3 Optimal triploid yield is achieved by pressure-treating zygotes at 75 or 90 spf

Pressure treatment at 60 spf significantly reduced both fertilization success and survival compared to later treatments (Figure 2.3A,B, respectively, $p < 0.001$, $n = 5$). Triploids were reliably produced with treatments at 60, 75 and 90 spf, but triploid induction success was much lower at 120 spf and no triploids were detected in embryos treated or at 150 spf (Figure 2.3C). Triploid yields from treatments at 60 and 120 spf were significantly lower than those from 75 or 90 spf (Figure 2.3D, $p < 0.001$, $n = 5$) due to poor fertilization and survival rates, and to low triploid induction rate, respectively. No

significant differences were detected between the 75 and 90 spf treatments for any of these variables. No triploids were detected in the sham-treatment group.

2.3.4 Survival rate during early development is reduced in triploid embryos

Most triploid embryos were morphologically indistinguishable from sibling diploids (Figure 2.4A,B); however, deformities such as pericardial edema were observed (Figure 2.4C). More subtle phenotypic aberrations, such as incomplete or malformed tail fin folds were more common (Figure 2.4D), but it was unclear if these minor deformities affect survival. Triploid embryos had a significantly lower survival rate between the 4-8 cell stage and first feed at 120 hpf (Figure 2.4E, $p < 0.01$). Most of the mortalities of triploid embryos occurred between 7 and 13 hpf, i.e. during epiboly, gastrulation and early somitogenesis; these embryos were often very asymmetrical, with the cell mass spreading unevenly over the yolk.

2.3.5 Embryonic heart rate is not affected by ploidy

The beating heart could be visualized in diploid and triploid embryos at 56 hpf (Figures 2.5A-D). No differences were observed in the development or morphology of the heart: at 48-54 hpf, diploid and triploid hearts had a distinct atrium and ventricle, strongly expressed the myosin light chain 7 (*myl7*) transgene (Figures 2.5C,D), and had a rhythmic heartbeat. Heart rate was significantly increased at high temperature ($p < 0.001$) but was not affected by ploidy ($p = 0.523$) and there was no interaction between temperature and ploidy ($p = 0.564$) (Figure 2.5E). Nuclear volume analysis at 48 hpf as described in section 2.2.2.2 and 2.2.2.3 confirmed that all embryos in the triploid group were indeed triploid (Figure 2.5F).

2.4 Discussion

As evidence mounts for Ohno's assertion that gene/genome duplication is an important driver of the evolution of novel function (Ohno, 1970; Richards et al., 2003; Braasch & Postlethwait, 2012; Fagernes et al., 2015), it is clear that scientists must also understand the negative effects on fitness that arise from changes in ploidy and cell volume to understand the role of polyploidy in evolutionary processes. While ploidy transitions persist more frequently in plants than in vertebrates (Mable, 2004; Schoenfelder & Fox, 2015; Mueller, 2015), they have occurred in nearly all major taxa in the animal kingdom (De Boer et al., 1984; Otto & Whitton, 2000; Gregory & Mable, 2005; Mable et al., 2011; Kenny et al., 2016; Schwager et al., 2017), with mammals being a curious exception (Svartman, Stone, & Stanyon, 2005). Research on triploid Atlantic salmon has revealed subtle physiological differences between ploidies, suggesting that tetraploid individuals after a WGD would likely experience selective pressures differently, for instance with respect to competition for resources (Taylor et al., 2014), temperature tolerances (Riseth et al., 2020), or nutritional requirements (Sambraus et al., 2020). Taking advantage of the molecular tools developed for zebrafish to ask questions pertaining to the fitness of polyploid animals and developmental constraints imposed by changes in cell size and cell number in tissue primordia will help us to understand the evolutionary trade-offs that take place when ploidy or genome size changes. Furthermore, a deeper understanding of the physiological differences between diploid and triploid fish will enable fish farmers optimize rearing protocols for triploids.

Ploidy can now be determined non-lethally by measuring nuclear volume in the developing myotome using confocal microscopy in *Tg(h2a.f/z:H2A.f/z::eGFP)* zebrafish embryos followed by image processing and analysis using freely available, open-source

software, thereby making it possible to detect and isolate triploid embryos from a mixed pool of individuals of unknown ploidy without the need to sacrifice them. The utility of myofiber nuclear volume analysis for identifying triploids is clear, but this technique has produced some curious observations that are difficult to explain. Diploid embryos consistently have one tight peak of nuclear volumes and a second, smaller peak of nuclei that are approximately double the size (perhaps pre-mitotic cells in the process of DNA replication as suggested in Van de Pol et al., 2020). While triploid embryos do produce a peak nuclear volume approximately 50% larger than that of diploids (as expected), the values are not normally distributed, extending into and below the modal volume of diploid embryos. This may reflect some degree of aneuploidy; indeed, recent work has demonstrated a high frequency of chromosomal aberrations in pressure-induced triploid Atlantic salmon especially in cases when the rate of triploid induction is low (Glover et al., 2020).

Considering the narrow induction window and lower triploid induction rate in zebrafish compared to Atlantic salmon (Benfey, 2016), it is possible that aneuploidy could be even more prevalent in pressure-shocked zebrafish embryos. Classic ploidy validation techniques such as erythrocyte size measurement or flow cytometric measurement of nuclear DNA content are not well suited for ruling out this hypothesis without further experimentation. That said, we favour an alternative hypothesis. The Tg(h2a.f/z:H2A.f/z::eGFP) transgenic has fluorescently labelled histones, so our measurements are likely susceptible to differences in heterochromatinization and DNA packing. This technique does not measure the actual mass of DNA present in each nucleus or even the volume of the nucleus as defined by its membrane, but rather it measures the amount of space occupied by the cluster of chromosomes contained within

the nucleus. Nuclei with more tightly packed DNA (i.e., more heterochromatin) would likely appear smaller using this technique than would a nucleus with an equal amount of DNA but with more euchromatin. Gene expression studies have found differences between diploids and triploids (Ching et al., 2010; Christensen et al., 2019; Van de Pol et al., 2020), but I am unaware of studies comparing the general amount and rate of transcription between ploidies. If there are differences in the ratio of heterochromatin to euchromatin in some myofiber nuclei, this could explain the broader range of nuclear volumes observed in triploids. In a parallel study, I have demonstrated that myofibers typically have 3 nuclei in triploids and 4 nuclei in diploids (Chapter 3), so triploid myofibers have 9 copies of the genome per cell whereas diploid myofibers have only 8. Perhaps increased heterochromatin is a compensatory response to increased ploidy that maintains gene expression in cells with more copies of the genome. The zebrafish system is well-suited to experiments to assess this hypothesis.

Using this technique for early ploidy determination, we have optimized the timing of hydrostatic pressure treatment to maximize triploid yield and have also identified a developmental threshold after which triploids can no longer be produced. Optimal triploid yields are achieved by pressure-treating zygotes between 75 and 90 spf. Treating at 60 spf reduces both fertilization and survival rates. The former is likely simply because embryos must be removed from the fertilization solution after only 45 seconds, giving eggs less time to be fertilized. I cannot explain the latter observation, but this pattern of high mortality when pressure-treating too early is consistent with other fish species such as salmonids (Benfey & Sutterlin, 1984; unpublished observations). Reduced triploid induction rates with pressure treatments beginning later than 90 spf is intriguing considering that Kavumpurath and Pandian (1990) determined the optimal

timing of heat treatment for triploid induction to be 150 spf and that Van de Pol and colleagues (2020) successfully produced triploids using cold treatment at 180 spf. Hydrostatic pressure treatment is preferred to thermal treatments for commercial-scale triploid induction because, unlike changes in temperature, all eggs are immediately subjected to the same treatment and optimal treatments are not dependent on pre-treatment incubation temperature (Benfey, 2016). It is generally assumed that there are no differences among triploids produced by the various induction techniques (temperature, pressure, chemical, etc.) but these observations suggest that this assumption should be re-examined. Little is known about the mechanism of second polar body retention after hydrostatic pressure treatment that results in triploidy aside from an electron microscopy study showing microtubule depolymerization in an *Actinosphaerium* protist subjected to similar hydrostatic pressure treatments (Tilney et al., 1966). The involvement of microtubules in this mechanism is attractive due to the observation that microtubule toxins such as colchicine successfully induce ploidy transitions in plants (Corneillie et al., 2019) and in fish (Smith & Lemoine, 1979), but actin is also clearly involved as well since cytochalasin B has been shown to induce ploidy transitions in molluscs (Melo et al., 2015) and in fish (albeit with low survival rates) (Bolla & Refstie, 1985). This is consistent with the idea that microtubules are necessary for karyokinesis, and actin-based contractile rings are necessary for cytokinesis. Now that we can quickly and easily measure ploidy in zebrafish embryos, we have an efficient system to compare the mechanisms of second polar body retention and ploidy manipulation affected by the various ploidy manipulation techniques.

Viable triploid zebrafish embryos are morphologically indistinguishable by eye from diploid controls, but I observed some common deformities in pressure-treated

triploids such as pericardial edema and malformed epithelial layers. I examined the developmental effects of triploidy on tissue morphogenesis in a parallel study (Chapter 3) so here I focus on the survival of macroscopically normal triploid embryos. These had significantly lower survival rates compared to untreated diploid controls. Delomas & Debrowski (2018) reported reduced survival in heat-induced triploid zebrafish over a longer time span (2-4 cell stage until 70 days), with most mortalities in the first 5 days after fertilization. Mortality rates were similar between their heat-treated triploids and our pressure-treated triploids until 48 hpf, after which mortality rate improved greatly in pressure-treated embryos (our study) but not in heat-treated embryos (Delomas & Debrowski, 2018). Survival in cold-treated triploid embryos was 77% but was only recorded until 24 hpf (Van de Pol et al., 2020) confirming that mortality is increased in the first 24 hours regardless of the technique used to manipulate ploidy. I have shown that that if a pressure-treated triploid embryo survives beyond 24 hpf, they are likely to be viable, but I am unable to discern between the true effects of increasing ploidy and the effects of the physical treatments on embryo survival from our study. This novel technique to measure ploidy non-lethally creates the opportunity to compare survival between triploids and “failed triploids”, i.e., diploids that were exposed to the pressure treatment but not induced to become triploid, and thereby distinguish between these ploidy and physical treatment effects.

Fertilization rate was not reported in the previously mentioned studies presumably because with their protocols, triploid embryos remain in the fertilization solution long enough to achieve saturation whereas fertilization is a limiting factor when embryos are treated at 60 spf (our study). Deciding which triploid induction technique is best will require a comparison between heat-, cold- and pressure-treatments measuring

fertilization, survival, and triploid induction rates simultaneously; using this early, non-lethal technique to determine ploidy makes this more feasible. The convenience of the temperature treatments is relevant as temperature treatments do not require specialized equipment like a hydrostatic pressure chamber and are more practically forgiving since they allow more time after fertilization before treatment. Similar to Van de Pol and colleagues (2020), high mortality rates using heat-treatments to induce triploidy (our unpublished observations) led us to use an alternative ploidy manipulation technique. I chose to use hydrostatic pressure as is it the most common protocol used in aquaculture, but if cold-treated triploids have comparable survival rates after 24 hpf as well, this could be the ideal protocol for triploid induction in zebrafish.

A simple experiment was designed to strengthen our argument for triploid zebrafish as a model for studying the physiology of triploid fish by demonstrating that the previously documented absence of an effect of triploidy on heart rate in larval brook charr (*Salvelinus fontinalis*) (Benfey & Bennett, 2009) holds true for zebrafish. This demonstrates the feasibility of using confocal microscopy to identify triploid individuals from a pool of zebrafish embryos of mixed ploidy for use in subsequent experiments. While we should not assume that triploid zebrafish will be comparable to other species in all aspects of physiology, I have shown that at least some aspects of their physiology are comparable. Taking into account the comparable gene expression patterns between diploid and triploid zebrafish reported by Van de Pol et al. (2020) and the lack of a ploidy effect on the link between temperature and heart rate reported here, I am confident that triploid zebrafish will contribute to our growing understanding of the biological effects of whole genome duplication and resultant changes in cell size and number.

2.5 Conclusion

A notable difference between plants and animals is that polyploid animals compensate for larger cells by reducing cell number to maintain body size, whereas plants do not. Sudden and drastic changes in body size would likely result in major shifts in the ecological niche that an organism is best suited for. For example, body size correlates with prey type and nutritional requirements such that in coral reef fish, prey type and feeding frequency change as gape sizes increases and the fish can eat larger prey (Dunic & Baum, 2017). If animals were larger after a genome duplication, it could have cascading effects on food webs as a predator could suddenly have access to entirely new species of prey. There is no clear consensus for the pattern employed by invertebrates in response to changes in ploidy and cell size but recent discoveries of WGDs in spiders (Schwager et al., 2017) and horseshoe crabs (Kenny et al., 2016) emphasize that the developmental and morphological consequences of WGDs are relevant for invertebrates as well. As we discover more genome duplications events, we are learning about the genetic consequences of polyploidy in animals (Postlethwait, 2007; Otto, 2007; Braasch & Postlethwait, 2012; Fagernes et al., 2015; Mueller, 2015; Conant, 2020) but we are lagging behind in understanding how these changes affect development and physiology. Perhaps the evolution of compensatory mechanism(s) that maintain allometric relationships independently of cell size changes were driven by changes in genome size. Triploid zebrafish will be a valuable tool for these types of studies now that we can reliably produce and identify them early and non-lethally.

2.6 Acknowledgements

The authors would like to thank Dr. Jim Kieffer (University of New Brunswick – Saint John) for his helpful comments regarding the manuscript and Dr. Ian Scott (SickKids

Hospital – Toronto) for generously donating the Tg(h2a.f/z:H2A.f/z::eGFP) transgenic line. Thanks are owed to Robyn O’Keefe, Robyn Shortt, and Krista Latimer for excellent fish care, Stephen Cogswell and Susan Belfry from UNB Microscopy and Microanalysis for technical assistance, and Charlotte Bartlett for artwork. This research was funded by NSERC Discovery Grants (BDC and TJB), an NSERC Engage Grant (TJB) in partnership with Canada Cryogenetics Services, and an NBIF Research Assistantship Initiative grant (TJB).

2.7 References

- Abdel-Hameed, M. F., & Neat, H. J. (1971). A study on erythrocyte cell volume and number in normal adult and triploid intersex chickens. *Poultry Science*. 51, 1376-1382. <https://doi.org/10.3382/ps.0511376>.
- Anderson-Teixeira, K. S., Savage, V. M., Allen, A. P., & Gillooly, J. F. (2009). Allometry and metabolic scaling in ecology. In: *Encyclopedia of Life Sciences (ELS)*. John Wiley & Sons, Ltd: Chichester. <https://doi.org/10.1002/9780470015902.a0021222>.
- Aversano, R. E., Ercolano, M. R., Caruso, I., Fasano, C., Rosellini, D., & Carputo, D. (2012). Molecular tools for exploring polyploid genomes in plants. *International Journal of Molecular Sciences*. 13, 10316-10335. <https://doi.org/10.3390/ijms130810316>.
- Baars, D. L., Takle, K. A., Heier, J., & Pelegri, F. (2016). Ploidy manipulation of zebrafish embryos with heat shock 2 treatment. *Journal of Visualized Experiments*. 118, e54492. <https://doi.org/10.3791/54492>.

- Benfey, T. J., & Sutterlin, A. M. (1984). Triploidy induced by heat shock and hydrostatic pressure in landlocked Atlantic salmon (*Salmo salar*). *Aquaculture*. 36, 359-367. [https://doi.org/10.1016/0044-8486\(84\)90328-4](https://doi.org/10.1016/0044-8486(84)90328-4).
- Benfey, T. J. (1999). The physiology and behavior of triploid fishes. *Reviews in Fisheries Science*. 7, 39-67. <http://dx.doi.org/10.1080/10641269991319162>.
- Benfey, T. J., & Bennett, L. E. (2009). Effect of temperature on heart rate in diploid and triploid brook charr, *Salvelinus fontinalis*, embryos and larvae. *Comparative Biochemistry and Physiology A - Molecular & Integrative Physiology*. 152, 203-206. <https://doi.org/10.1016/j.cbpa.2008.09.025>.
- Benfey, T. J. (2016). Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (*Salmo salar*) as a case study. *Reviews in Aquaculture*. 8, 262-282. <https://doi.org/10.1111/raq.12092>.
- Benfey, T. J., & Devlin, R. H. (2018). Ploidy has minimal effect on hypoxia tolerance at high temperature in rainbow trout (*Oncorhynchus mykiss*). *Physiological and Biochemical Zoology*. 91, 1091-1101. <https://doi.org/10.1086/700218>.
- Bolla, S., & Refstie, T. (1985). Effect of cytochalasin B on eggs of atlantic salmon and rainbow trout. *Acta zoologica*. 66, 181-188. <https://doi.org/10.1111/j.1463-6395.1985.tb00837.x>.
- Bolte, S., & Cordelières, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy*. 224, 213-232. <https://doi.org/10.1111/j.1365-2818.2006.01706.x>.
- Braasch, I. P., & Postlethwait, J. H. (2012). Polyploidy in fish and the teleost genome duplication. In: P. A. Soltis & D. E. Soltis (Eds.), *Polyploidy and Genome*

Evolution (1st ed., p. 341-386). Berlin: Springer. https://doi.org/10.1007/978-3-642-31442-1_17.

Bystrianski, J. S., Richards, J. G., Schulte, P. M., & Ballantyne, J. S. (2006). Reciprocal expression of gill Na⁺/K⁺-ATPase α -subunit isoforms α 1a and α 1b during sea water acclimation of three salmonid fishes that vary in their salinity tolerance. *The Journal of Experimental Biology*. 209, 1848-1858.

<https://doi.org/10.1242/jeb.02188>.

Bystrianski, J. S., Frick, N. T., Richards, J. G., Schulte, P. M., & Ballantyne, J. S. (2007). Failure to up-regulate gill Na⁺,K⁺-ATPase α -subunit isoform α 1b may limit seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*). *Comparative Biochemistry and Physiology, Part A*. 148, 332-338.

<https://doi.org/10.1016/j.cbpa.2007.05.007>.

Cavalier-Smith, T. (1978). Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. *Journal of Cell Science*. 34, 247-278.

Ching, B., Jamieson, S., Heath, J. W., Heath, D. D., & Hubberstey, A. (2010). Transcriptional differences between triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*) during live *Vibrio anguillarum* challenge. *Heredity*. 104, 224-234. <https://doi.org/10.1038/hdy.2009.108>.

Christensen, K. A., Sakhrani, D., Rondeau, E. B., Richards, J. G., Koop, B. F., & Devlin, R. H. (2019). Effect of triploidy on liver gene expression in coho salmon (*Oncorhynchus kisutch*) under different metabolic states. *BMC Genomics*. 20. <https://doi.org/10.1186/s12864-019-5655-8>.

- Conant, G. C. (2020). The lasting after-effects of an ancient polyploidy on the genomes of teleosts. *PLoS ONE*. 15(4), e0231356.
<https://doi.org/10.1371/journal.pone.0231356>.
- Corneillie, S., De Storme, N., Van Acker, R., Fangel, J. U., De Bruyne, M., De Rycke, R., Geelan, D., Willats, W. G. T., Vanholme, B., & Boerjan, W. (2019). Polyploidy affects plant growth and alters cell wall composition. *Plant Physiology*. 179, 74-87. <https://doi.org/10.1104/pp.18.00967>.
- De Boer, L. E. M., De Groen, T. A. G., Frankenhuys, M. T., Zonneveld, A. J., Sallevelt, J., & Belterman, R. H. R. (1984). Triploidy in *Gallus domesticus* embryos, hatchlings, and adult intersex chickens. *Genetica*. 65, 83-87.
<https://doi.org/10.1007/BF00056766>.
- Delomas, T. A., & Dabrowski, K. (2018). Why are triploid zebrafish all male? *Molecular Reproduction and Development*. 85(7), 612-621.
<https://doi.org/10.1002/mrd.22998>.
- Dunic, J. C., & Baum, J. K. (2017). Size structuring and allometric scaling relationships in coral reef fishes. *Journal of Animal Ecology*. 86, 577-589.
<https://doi.org/10.1111/1365-2656.12637>.
- Elliott, T. A., & Gregory, T. R. (2015). What's in a genome? The C-value enigma and the evolution of eukaryotic genome content. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 370
<http://dx.doi.org/10.1098/rstb.2014.0331>.
- Fagernes, C. E., Stensløkken, K-O., Røhr, Ä. K., Berenbrink, M., Ellefsen, S., & Nilsson, G. E. (2015). Extreme anoxia tolerance in crucian carp and goldfish through neofunctionalization of duplicated genes creating a new ethanol-

- producing pyruvate decarboxylase pathway. *Scientific Reports*. 7.
<https://doi.org/10.1038/s41598-017-07385-4>.
- Fankhauser, G. (1939). Polyploidy in the salamander, *Eurycea bislineata*. *The Journal of Heredity*. 30, 379-388.
- Fankhauser, G. (1945). The effects of changes in chromosome number on amphibian development. *The Quarterly Review of Biology*. 20, 20-78.
<https://doi.org/10.1086/394703>.
- Gillooly, J. F., Hein, A., & Damiani, R. (2015). Nuclear DNA content varies with cell size across human cell types. *Cold Spring Harbor Perspectives in Biology*. 7.
<https://doi.org/10.1101/cshperspect.a019091>.
- Ginzberg, M. K., Kafri, R., & Kirschner, M. (2015). On being the right (cell) size. *Science*. 348, 1245075. <https://doi.org/10.1126/science.1245075>.
- Glover, K. A., Harvey, A. C., Hansen, T. J., Fjellidal, P. G., Besnier, F. N., Bos, J. B., Ayllon, F., Taggart, J. B., Solberg, M. F. (2020). Chromosome aberrations in pressure-induced triploid Atlantic salmon. *BMC Genetics*. 21.
<https://doi.org/10.1186/s12863-020-00864-0>.
- Gregory, T. R., & Hebert, P. D. N. (1999). The modulation of DNA content: Proximate causes and ultimate consequences. *Genome Research*. 9, 317-324.
<https://doi.org/10.1101/gr.9.4.317>.
- Gregory, T. R. (2001). Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biological Reviews*. 76, 65-101.
<https://doi.org/10.1017/S1464793100005595>.
- Gregory, T. R. (2002). Genome size and developmental complexity. *Genetica*. 115, 131-146. <https://doi.org/10.1023/A:1016032400147>.

- Gregory, T. R., & Mable, B. K. (2005). Polyploidy in animals. In: T. R. Gregory (Eds.). *The Evolution of the Genome* (1st ed., p. 427-517). <https://doi.org/10.1016/B978-012301463-4/50010-3>.
- Gregory, T. R. (2018). Genome size and brain cell density in birds. *Canadian Journal of Zoology*. 96(4), 379-382. <https://doi.org/10.1139/cjz-2016-0306>.
- Griffiths, R. (1941). Triploidy (and haploidy) in the newt, *Triturus viridescens*, induced by refrigeration of fertilized eggs. *Genetics*. 26, 69-88.
- Hansen, T. J., Olsen, R. E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T., & Fjelldal, P. G. (2015). Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*. 435, 354-360.
<http://dx.doi.org/10.1016/j.aquaculture.2014.10.017>.
- Henery, C. C., Bard, J. B. L., & Kaufman, M. H. (1992). Tetraploidy in mice, embryonic cell number, and the grain of the developmental map. *Developmental Biology*. 152, 233-241. [https://doi.org/10.1016/0012-1606\(92\)90131-Y](https://doi.org/10.1016/0012-1606(92)90131-Y).
- Henery, C. C., & Kaufman M. H. (1992). Relationship between cell size and nuclear volume in nucleated red blood cells of developmentally matched diploid and tetraploid mouse embryos. *The Journal of Experimental Zoology*. 261, 472-478. <https://doi.org/10.1002/jez.1402610414>.
- Hyndman, C. K., Kieffer, J. D., Benfey, T. J. (2003). Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture*. 221, 629-643. [https://doi.org/10.1016/S0044-8486\(03\)00119-4](https://doi.org/10.1016/S0044-8486(03)00119-4).

- Kalinkat, G., Rall, B. C., Vucic-Pestic, O., & Brose, U. (2011). The allometry of prey preferences. *PLoS ONE*. 6, e25937.
<https://doi.org/10.1371/journal.pone.0025937>.
- Kassambara, A., & Kosinski, M. Survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.4.4. URL: <https://CRAN.R-project.org/package=survminer> (31 December 2019).
- Kavumpurath, S., & Pandian, T. J. (1990). Induction of triploidy in the zebrafish, *Brachydanio rerio* (Hamilton). *Aquaculture Research*. 21, 299-306.
<https://doi.org/10.1111/j.1365-2109.1990.tb00468.x>.
- Kenny, N. C., Chan, K. W., Nong, W., Qu, Z., Maeso, I., Yip, H. Y., Chan, T. F., Kwan, H. S., Holland, P. W. H., Chu, K. H., Hui, J. H. L. (2016). Ancestral whole-genome duplication in the marine chelicerate horseshoe crabs. *Heredity*. 116, 190-199. <https://doi.org/10.1038/hdy.2015.89>.
- Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics*. 203, 253-310. <https://doi.org/10.1002/aja.1002030302>.
- Kleiber, M., & Rogers, T. A. (1961). Energy Metabolism. *Annual Reviews in Physiology*. 23, 5-36. <https://doi.org/10.1146/annurev.ph.23.030161.000311>.
- Leiva, F. P., Calosi, P., & Verberk, W. C. E. P. (2019). Scaling of thermal tolerance with body mass and genome size in ectotherms: A comparison between water- and air-breathers. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 374, 20190035. <http://dx.doi.org/10.1098/rstb.2019.0035>.
- Levy, D. L., & Heald, R. (2010). Nuclear size is regulated by importin α and Ntf2 in *Xenopus*. *Cell*. 143, 288-298. <https://doi.org/10.1016/j.cell.2010.09.012>.

- Lozano, E., Sáez, A. G., Flemming, A. J., Cunha, A., & Leroi, A. M. (2006). Regulation of growth by ploidy in *Caenorhabditis elegans*. *Current Biology*. 16, 493-498.
<https://doi.org/10.1016/j.cub.2006.01.048>.
- Mable, B. K. (2004). 'Why polyploidy is rarer in animals than in plants': myths and mechanisms. *Biological Journal of the Linnean Society*. 82, 453-466.
<https://doi.org/10.1111/j.1095-8312.2004.00332.x>.
- Mable, B. K., Alexandrou, M. A., & Taylor, M. I. (2011). Genome duplication in amphibians and fish: An extended synthesis. *Journal of Zoology*. 284, 151-182.
<https://doi.org/10.1111/j.1469-7998.2011.00829.x>.
- Melo, E. M. C., Gomes, C. H. A. d. M., da Silva, F. C., Sühnel, S., & de Melo, C. M. R. G. (2015). Chemical and physical methods of triploidy induction in *Crassostrea gigas* (Thunberg, 1793). *Boletim do Instituto de Pesca*. 41, 889-898.
- Miller, K. E., Brownlee, C., & Heald, R. (2020). The power of amphibians to elucidate mechanisms of size control and scaling. *Experimental Cell Research*. 392, 112036. <https://doi.org/10.1016/j.yexcr.2020.112036>.
- Mueller, R. L. (2015). Genome biology and the evolution of cell-size diversity. *Cold Spring Harbor Perspectives in Biology*. 7, a019125.
<https://doi.org/10.1101/cshperspect.a019125>
- Nell, J. A. (2002). Farming triploid oysters. *Aquaculture*. 210, 69-88.
[https://doi.org/10.1016/S0044-8486\(01\)00861-4](https://doi.org/10.1016/S0044-8486(01)00861-4).
- Neurohr, G. E., Terry, R. L., Lengefeld, J., Bonner, M., Brittingham, G. P., Moretto, F., Miettinen, T. P., Vaites, L. P., Soares, L. M., Pauloa, J. A., Harper, J. W., Buratowski, S., Manalis, S., van Werven, F. J., Holt, L. J., & Amon, A. (2019).

- Excessive cell growth causes cytoplasm dilution and contributes to senescence. *Cell*. 176, 1083-1097. <https://doi.org/10.1016/j.cell.2019.01.018>.
- Ohno, S. (1970). *Evolution by gene duplication*. Berlin: Springer Berlin Heidelberg. <https://doi.org/10.1007/978-3-642-86659-3>.
- Ojolick, E. J., Cusack, R., Benfey, T. J., & Kerr, S. R. (1995). Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture*. 131, 177-187. [https://doi.org/10.1016/0044-8486\(94\)00338-O](https://doi.org/10.1016/0044-8486(94)00338-O).
- O'Keefe, R. A., & Benfey, T. J. (1997). The feeding response of diploid and triploid Atlantic salmon and brook trout. *Journal of Fish Biology*. 51, 989-997. <https://doi.org/10.1111/j.1095-8649.1997.tb01537.x>.
- Otto, S. P., & Whitton, J. (2000). Polyploidy incidence and evolution. *Annual Review in Genetics*. 34, 401-437. <https://doi.org/10.1146/annurev.genet.34.1.401>.
- Otto, S. (2007). The evolutionary consequences of polyploidy. *Cell*. 131, 452-462. <https://doi.org/10.1016/j.cell.2007.10.022>.
- Palencia-Desai, S., Kohli, V., Kang, J., Chi, N. C., Black, B. L., & Sumanas, S. (2011). Vascular endothelial and endocardial progenitors differentiate as cardiomyocytes in the absence of Etsrp/Etv2 function. *Development*. 138, 4721-4732. <https://doi.org/10.1242/dev.064998>.
- Pauls, S., Geldmacher-Voss, B., Campos-Ortega, J. A. (2001). A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for *in vivo* studies of embryonic development. *Developmental Genes and Evolution*. 211, 603-610. <http://dx.doi.org/10.1007/s00427-001-0196-x>.

- Piferrer, F., Beaumont, A., Flaguère, J.-C., Flajšhans, M., Haffray, P., & Colombo, L. (2009). Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture*. 293, 125-156. <http://dx.doi.org/10.1016/j.aquaculture.2009.04.036>.
- Postlethwait, J. H. (2007). The zebrafish genome in context: ohnologs gone missing. *Journal of Experimental Zoology*. 308B, 563-577. <https://doi.org/10.1002/jez.b.21137>.
- R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. URL: <https://www.R-project.org/>.
- Ribas, L., & Piferrer, F. (2013). The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture*. 6. <https://doi.org/10.1111/raq.1204>.
- Richards, J. G., Semple, J. W., Bystriansky, J. S., & Schulte, P. M. (2003). Na⁺/K⁺-ATPase α -isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *Journal of Experimental Biology*. 206, 4475-4486. <https://doi.org/10.1242/jeb.00701>.
- Riseth, E. N., Fraser, T. W. K., Sambraus, F., Stien, L. H., & Hvas, M. (2020). Is it advantageous for Atlantic salmon to be triploid at lower temperatures? *Journal of Thermal Biology*. 89, 102548. <https://doi.org/10.1016/j.jtherbio.2020.102548>.
- Roth, G., & Walkowiak, W. (2015). The influence of genome and cell size on brain morphology in amphibians. *Cold Spring Harbor Perspectives in Biology*. 7, a019075. <https://doi.org/10.1101/cshperspect.a019075>.

- Samraus, F., Olsen, R. E., Remen, M., Hansen, T. J., Torgersen, T., & Fjelldal, P. G. (2017). Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture*. 473, 1-12. <https://doi.org/10.1016/j.aquaculture.2017.01.024>.
- Samraus, F., Remen, M., Olsen, R. E., Hansen, T. J., Waagbø, R., Torgersen, T., Lock, E. J., Imsland, A., Fraser, T. W. K., & Fjelldal, P. G. (2018). Changes in water temperature and oxygen: the effect of triploidy on performance and metabolism in large farmed Atlantic salmon. *Aquaculture Environment Interactions*. 10, 157-172. <https://doi.org/10.3354/aei00260>.
- Samraus, F., Hansen, T. J., Daae, B. S., Thorsen, A., Sandvik, R., Stien, L. H., Fraser, T. W. K., & Fjelldal, P. G. (2020). Triploid Atlantic salmon *Salmo salar* have a higher dietary phosphorus requirement for bone mineralization during early development. *Journal of Fish Biology*. 97, 137-147. <https://doi.org/10.1111/jfb.14338>.
- Saranyan, P. V., Ross, N. W., & Benfey, T. J. (2017). Erythrocyte heat shock protein responses to chronic (*in vivo*) and acute (*in vitro*) temperature challenge in diploid and triploid salmonids. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*. 206, 95-104. <https://doi.org/10.1016/j.cbpa.2017.01.007>.
- Sattler, M. C., Carvalho, C. R., & Clarindo, W. R. (2016). The polyploidy and its key role in plant breeding. *Planta*. 243, 281-296. <https://doi.org/10.1007/s00425-015-2450-x>.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J-Y., White, D. J.,

- Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*.
<https://doi.org/10.1038/nmeth.2019>.
- Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. 9, 671-675.
<https://doi.org/10.1038/nmeth.2089>.
- Schoenfelder, K. P., & Fox, D. T. (2015). The expanding implications of polyploidy. *Journal of Cell Biology*. 209, 485-491. <https://doi.org/10.1083/jcb.201502016>.
- Schwager, E. E., Sharma, P. P., Clarke, T., Leite, D. L., Wierschin, T., Pechmann, M., Akiyama-Oda, Y., Esposito, L., Bechsgaard, J., Bilde, T., Buffry, A. D., Chao, H., Dinh, H., Doddapaneni, H., Dugan, S., Eibner, C., Extavour, C. G., Funch, P., Garb, J., Gonzalez, L. B., Gonzalez, V. L., Griffiths-Jones, S., Han, Y., Hayashi, C., Hilbrant, M., Hughes, D. S. T., Janssen, R., Lee, S. L., Maeso, I., Murali, S. C., Muzny, D. M., Da Fonseca, R. N., Paese, C. L. B., Qu, J., Ronshaugen, M., Schomburg, C., Schönauer, A., Stollewerk, A., Torres-Oliva, M., Turetzek, N., Vanthournout, B., Werren, J. H., Wolff, C., Worley, K. C., Bucher, G., Gibbs, R. A., Coddington, J., Oda, H., Stanke, M., Ayoub, N. A., Prpic, N-M., Flot, J-F., Posnien, N., Richards, S., & McGregor, A. P. (2017). The house spider genome reveals an ancient whole-genome duplication during arachnid evolution. 15. <https://doi.org/10.1186/s12915-017-0399-x>.
- Scott, M. A., Dhillon, R. S., Schulte, P. M., & Richards, J. G. (2014). Physiology and performance of wild and domestic strains of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in response to environmental challenges. *Canadian*

- Journal of Fisheries and Aquatic Sciences*. 72, 125-134.
<https://doi.org/10.1139/cjfas-2013-0450>.
- Smith, E. M., & Gregory, T. R. (2009). Patterns of genome size diversity in the ray-finned fishes. *Hydrobiologia*. 625. <https://doi.org/10.1007/s10750-009-9724-x>.
- Smith, L. T., & Lemoine, H. L. (1979). Colchicine-induced polyploidy in Brook Trout. *Progressive Fish-Culturist*. 41, 86-88. [https://doi.org/10.1577/1548-8659\(1979\)41\[86:CPIBT\]2.0.CO;2](https://doi.org/10.1577/1548-8659(1979)41[86:CPIBT]2.0.CO;2).
- Streisinger, G., Walker, C., Dower, N., Knauber, D., & Singer, F. (1981). Production of clones of homozygous diploid zebra fish. *Nature*. 291, 293-296.
<https://doi.org/10.1038/291293a0>.
- Svartman, M., Stone, G., Stanyon, R. (2005). Molecular cytogenetics discards polyploidy in mammals. *Genomics*. 85, 425-430.
<https://doi.org/10.1016/j.ygeno.2004.12.004>.
- Swarup, H. (1959). Effect of triploidy on the body size, general organization, and cellular structure in *Gasterosteus aculea*. *Journal of Genetics*. 56, 143-155.
- Taylor, J. F., Bozzolla, P., Frenzl, B., Matthew, C., Hunter, D., & Migaud, H. (2014). Triploid Atlantic salmon growth is negatively affected by communal ploidy rearing during seawater grow-out in tanks. *Aquaculture*. 432, 163-174.
<http://dx.doi.org/10.1016/j.aquaculture.2014.05.014>.
- Therneau, T. M., & Grambsch, P. M. (2000). *Modeling survival data: Extending the Cox model*. Cham: Springer. New York: Springer-Verlag New York.
<https://doi.org/10.1007/978-1-4757-3294-8>.
- Tiersch, T. R., & Figiel, C. R. (1991). A triploid snake. *Copeia*. 3, 838-841.

- Tilney, L. G., Hiramoto, Y., & Marsland, D. (1966). Studies on the microtubules in heliozoa III. A pressure analysis of the role of these structures in the formation and maintenance of the axopodia of *Actinosphaerium nucleofilum* (Barrett). *The Journal of Cell Biology*. 29, 77-95. <https://doi.org/10.1083/jcb.29.1.77>.
- Turner, J. J., Ewald, J. C., & Skotheim, J. M. (2012). Cell size control in yeast. *Current Biology*. 22, R350-R359. <https://doi.org/10.1016/j.cub.2012.02.041>.
- Van de Peer, Y., Maere, S., & Meyer, A. (2009). The evolutionary significance of ancient genome duplications. *Nature Reviews Genetics*. 10, 725-732. <https://doi.org/10.1038/nrg2600>.
- Van de Pol, I. L. E., Flik, G., & Verberk, W. C. E. P. (2020). Triploidy in zebrafish larvae: Effects on gene expression, cell size and cell number, growth, development and swimming performance. *PLoS ONE*. 15, e0229468. <https://doi.org/10.1371/journal.pone.0229468>.
- Verhille, C., Antilla, K., & Farrell, A. P. (2013). A heart to heart on temperature: Impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*. 164, 653-657. <http://dx.doi.org/10.1016/j.cbpa.2013.01.011>.
- Ulloa, P. E., Medrano, J. F., & Feijoo, C. G. (2014). Zebrafish as animal models for aquaculture nutrition research. *Frontiers in Genetics*. 5. <https://doi.org/10.3389/fgene.2014.00313>.
- Wan, Y., Otsuna, H., Chien, C-B., & Hansen, C. (2012). FluoRender: An application of 2D image space methods for 3D and 4D confocal microscopy data visualization

- in neurobiology research. *IEEE Pacific Visualization Symposium*. 201-208.
<https://doi.org/10.1109/PacificVis.2012.6183592>.
- Wan, Y., Otsuna, H., Holman, H. A., Bagley, B., Ito, M., Lewis, A. K., Colasanto, M., Kardon, G., Ito, K., & Hansen, C. (2017). FluoRender: Joint freehand segmentation and visualization for many-channel fluorescence data analysis. *BMC Bioinformatics*. 18. <https://doi.org/10.1186/s12859-017-1694-9>.
- Wargelius, A., Leininger, S., Skaftnesmo, K. O., Kleppe, L., Andersson, E., Taranger, G. L., Schulz, R. W., & Edvardsen, R. B. (2016). Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Scientific Reports*. 6, 21284. <https://doi.org/10.1038/srep21284>.
- West, G. B., Brown, J. H., & Enquist, B. J. (1977). A general model for the origin of allometric scaling laws in biology. *Science*. 276, 121-126.
<https://doi.org/10.1126/science.276.5309.122>.
- Westerfield, M. (2000). *The Zebrafish Book. A guide for the laboratory use of zebrafish (Danio rerio)*. Eugene: University of Oregon Press.
- Wickham, H. (2016). *ggplot2*. Cham: Springer. <https://doi.org/10.1007/978-3-319-24277-4>.
- Zang, L., Shimada, Y., Nishimura, Y., Tanaka, T., & Nishimura, N. (2013). A novel, reliable method for repeated blood collection from aquarium fish. *Zebrafish*. 10, 425-432. <https://doi.org/10.1089/zeb.2012.0862>.
- Zhang, N., Bao, Y., Xie, Z., Huang, X., Sun, Y., Feng, G., Zeng, H., Ren, J., Li, Y., Xiong, J., Chen, W., Yan, C., & Tang, M. (2019). Efficient characterization of tetraploid watermelon. *Plants*. 8. <https://doi.org/10.3390/plants8100419>.

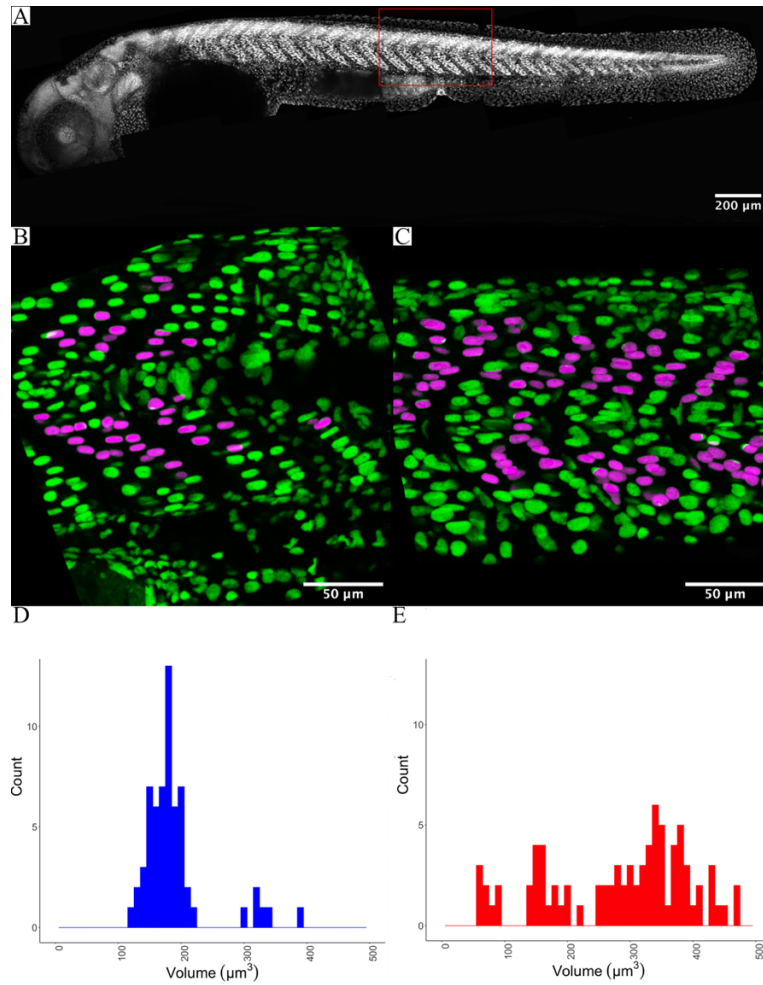


Figure 2.1 Large numbers (100+) of myofiber nuclei can be imaged simultaneously using confocal microscopy in Tg(h2a.f/z:H2A.f/z::eGFP) zebrafish embryos (A, red box). Compared to embryos from the control group (B; presumptive diploid), putative triploids (C; pressure treated as zygote) have visibly larger nuclei. Intact nuclei can be selected and extracted using FluoRender (selected nuclei are purple). Nuclear volume distributions measured from these selected nuclei show an increase in size going from presumptive diploid to triploid embryos (D and E, respectively).

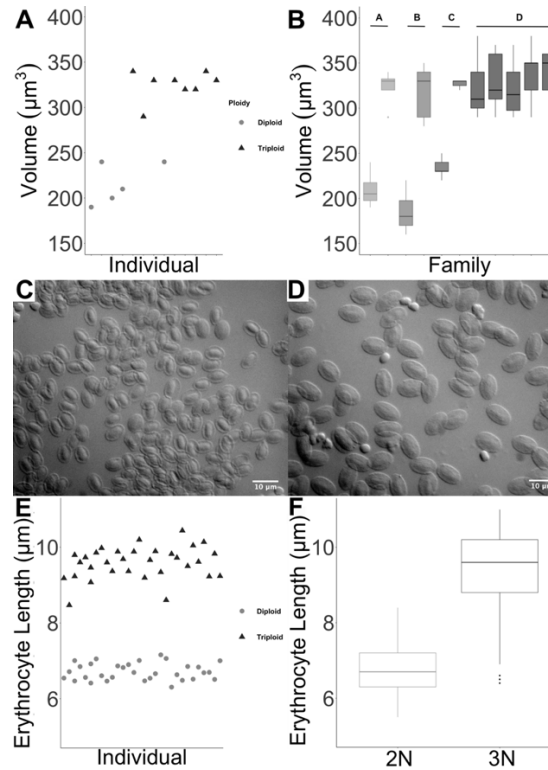


Figure 2.2 Hydrostatic pressure reliably produces embryos with larger nuclei and that are *bona fide* triploids. **A)** Within-family variation of modal nucleus volume reveals two non-overlapping clusters separating putative triploids (fish 5-7 and 9-13) from controls (fish 1-4) and an embryo from the pressure treatment group that did not become triploid (fish 8). **B)** Among-family variation in modal nucleus volume indicates that this pattern of larger nuclei in putative triploid embryos is repeatable and consistent across families (A-C denote families of sibling diploids and triploids; D denotes 5 distinct families of triploid embryos). **C & D)** Diploid erythrocytes from adult zebrafish (C) are visibly smaller than triploid erythrocytes (D). **E & F)** Erythrocyte major axis analysis reveals two non-overlapping clusters separating triploids from diploids (E); all 32 Tg(h2a.f/z:H2A.f/z::eGFP) embryos identified as putative triploids using confocal microscopy had larger erythrocytes as adults (F, n = 32 fish/ploidy, $p < 0.001$).

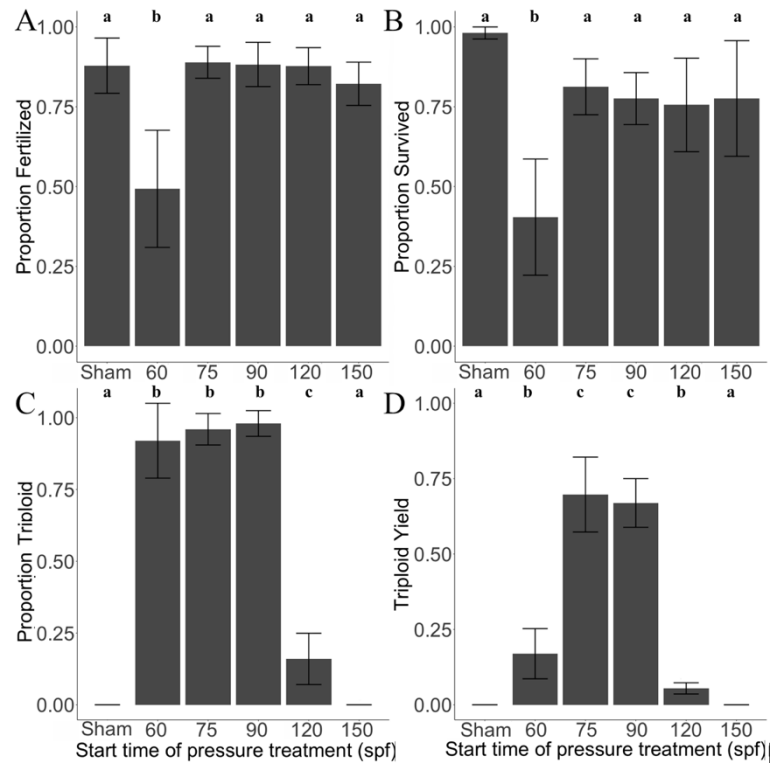


Figure 2.3 Start time of hydrostatic pressure treatment affects fertilization rate (**A**), survival rate to 48 hpf (**B**), triploid induction rate (**C**, 10 embryos per IVF), and triploid yield (**D**) ($n = 5$ IVFs for each start time, $p < 0.001$ for all experiments). $N = 10$ embryos per IVF for (**C**). Letters denote significant differences between groups, error bars represent standard deviation.

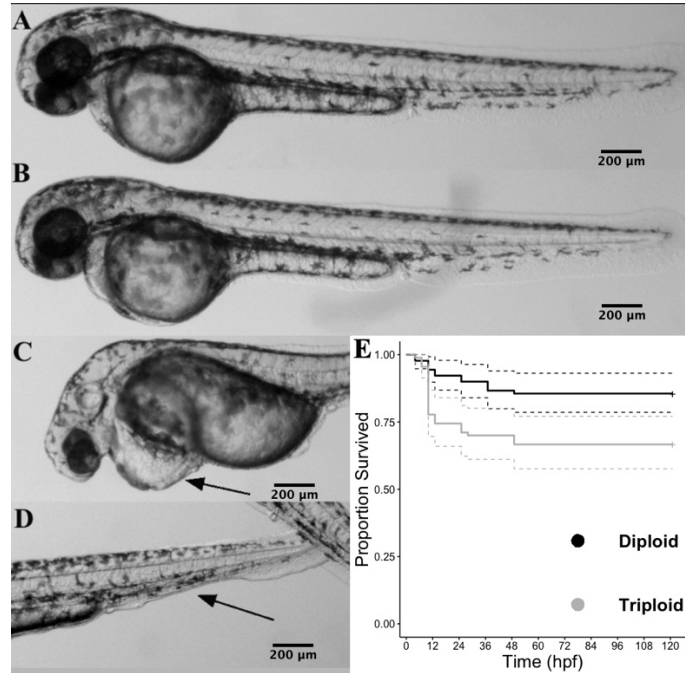


Figure 2.4 Survival until 120 hpf is significantly affected by ploidy. **A & B)** Most triploid embryos were morphologically indistinguishable from sibling diploids (48 hpf; A is diploid and B is triploid). **C & D)** Triploid embryos that survived until 24 hpf occasionally had severe deformities such as pericardial edema (C, arrow) and a malformed yolk sac, but more subtle deformities such as malformed fin folds as seen in the tail fin epithelium were more common (D, arrow). **E)** Kaplan-Meier survival curve with 95% confidence intervals (dotted lines) for diploid (black) and triploid (grey) embryos. Survival analysis indicated that ploidy significantly affected survival between the 4-cell stage (time = 0.5-1 hpf) and first feed (time = 120 hpf) ($n = 3$, 30 embryos/ploidy/replicate, $p < 0.01$).

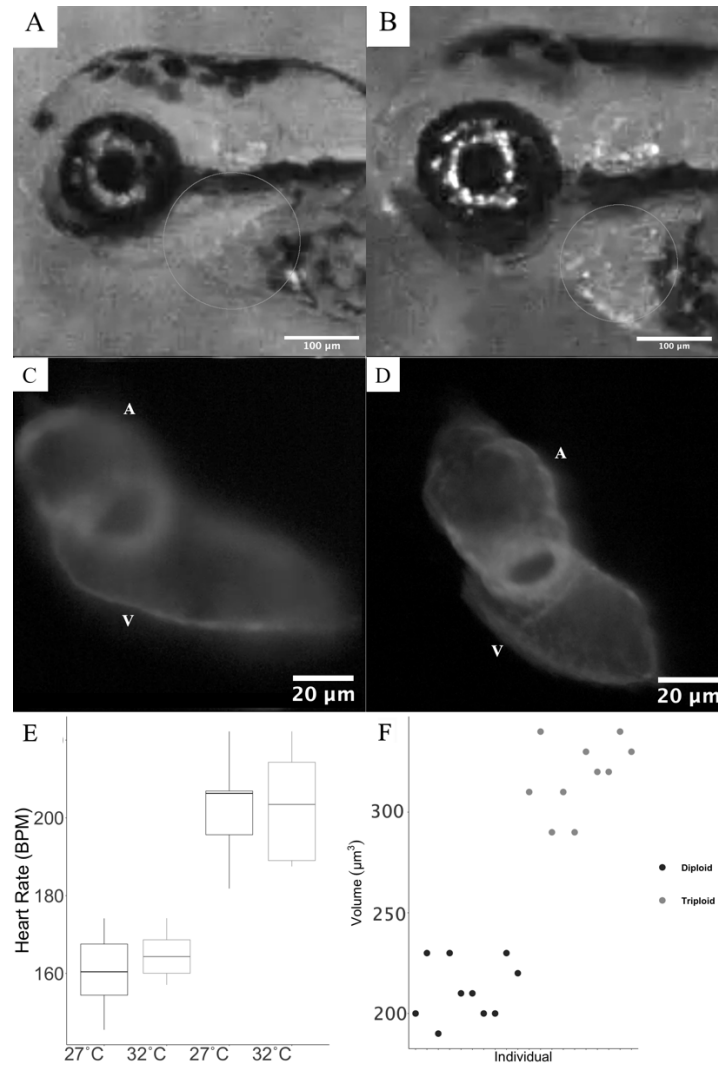


Figure 2.5 Heart rate is affected by temperature but not ploidy at 56 hpf. Diploid (A & C) and triploid (B & D) were not morphologically distinguishable in overall morphology (A & B; the heart is located within the white circle) or cardiac morphology (C & D). **A-D**) Single frames from videos of Tg(myl7:mCherry, h2a.f/z:H2A.f/z::eGFP) zebrafish embryo (A and V indicate the atrium and ventricle, respectively). **E**) Heart rate was not affected by ploidy at 27°C or at 32°C ($p = 0.523$, $n=10$ embryos/ploidy; diploids in black and triploids in grey) but was affected by temperature ($p < 0.001$). **F**) Histogram of modal nuclear volumes from confocal analysis used to identify diploid and triploid individuals for subsequent heart rate analysis.

3.0 Chapter 3:

Tissue-specific patterns of compensation for increased cell size maintains organ size and body size in triploid zebrafish.

Christopher D. Small¹, Tillmann J. Benfey¹, Bryan D. Crawford¹

¹Department of Biology, University of New Brunswick, Fredericton, NB, Canada, E3B 5A3

Correspondence

Tillmann Benfey, Department of Biology, University of New Brunswick, Fredericton, NB, Canada, E3B 5A3.

Email: benfey@unb.ca

Abstract:

Whole genome duplications (WGDs) and ploidy transitions have occurred in nearly every major taxon of eukaryotes (except for mammals), but they are far more common in plants than in animals. Increased DNA content in polyploids generates organisms with larger cells and, in plants, polyploids are larger than diploids as cell number does not change. Conversely, vertebrate body size does not correlate to cell size and ploidy as they compensate for increased cell size to maintain body size. This was thought to be explained by a simple reduction in cell number that matches the increase in cell size to maintain body size as ploidy increases, but here I show that the compensatory mechanism that maintains body size in triploid zebrafish is tissue-specific: A) blood cells respond in the classical pattern with a reduced number of larger erythrocytes in circulation, B) muscle, a tissue comprised of polynucleated muscle fibers, compensates by reducing the number of larger nuclei in each myofiber such that myofiber and

myotome size is unaffected by ploidy, and C) vascular tissue compensates by thickening blood vessel walls at the expense of lumen space. Understanding the physiological implications of ploidy on tissue function requires a detailed description of the specific morphological compensation for each tissue in order to truly understand how ploidy changes affect development and physiology.

3.1 Introduction

Morphogenesis and growth interact during development to produce tissues, organs, and bodies that are sized appropriately for the environment using mechanisms such as developmental,¹⁻⁴ or phenotypic-plasticity.⁵⁻⁹ These processes are regulated by the genome and therefore have been optimized by selection for any ecological niches occupied by that species. The relationships between organ/body size and function are typically non-linear as surface area does not scale linearly with volume and organ function is often limited by functional surface area.¹⁰⁻¹² An example of this is Kleiber's Law: metabolic rate scales proportionately to approximately 3/4 of an animal's mass.¹³ Huxley and Tessier¹⁴ defined these relationships as allometric, though they were primarily focused on the relationship between organ size and overall body size. The study of allometry has expanded beyond physical traits to include physiological (e.g., metabolism^{13,15}) and ecological traits (e.g., habitat and prey preference.^{16,17}). Our mechanistic understanding of the interactions among body size, physiological tolerances, and ecological niches are incomplete, but patterns have emerged as ecological principles such as Bergmann's Rule (colder climates favour larger bodies)^{18,19} and Allen's Rule (colder climates favour rounder bodies and short limbs).^{20,21} Evolutionary changes in body size and morphology must manifest as changes in the number, size, and/or spatial arrangement of cells (and the extracellular matrix in which they reside) as the animal

develops²²⁻²⁵. The evolutionary selective pressures on morphology determining which genomes survive must therefore be selecting genomes that vary in ways that alter cell proliferation, apoptosis, matrix remodeling, cell volume, and the number of cells allocated to specific structures. This linkage between evolutionary and developmental biology has become a popular field of investigation during the past few decades, as our understanding of the genetic, molecular, and cellular underpinnings of morphogenesis grows.²²⁻²⁵

In eukaryotes, nuclear and cell volume (i.e., the N:C ratio) are linked to the amount of DNA in the cell.^{12,26-29} Genome size varies 80000-fold across eukaryotes³⁰ and 350-fold across vertebrates^{26,27}; however, the N:C ratio is remarkably consistent across taxa and tissues.¹² Variation in genome size can be partially explained by numerous Whole Genome Duplications (WGDs).³¹⁻³³ These ploidy transitions have persisted more often in plants than in vertebrates.^{31,33-35} leading early researchers to conclude that polyploidy played a negligible role in the evolution of animals.³⁶

Ohno (1970) proposed that two WGD events occurred at the base of the vertebrate lineage; these ploidy transitions could be advantageous because of increased redundancy that relaxes selection on highly conserved genes.³⁷ Advances in genomics have provided clear evidence not only for two WGDs at the root of the vertebrates,³⁸ but also for others that have occurred since including more than 10 in teleosts alone.^{32,39} For example, phylogenomic analyses of ohnologues (paralogous genes having arisen through WGDs) have uncovered extensive divergence since these WGDs⁴⁰⁻⁴² including functional diversification of hemoglobins,⁴³ matrix metalloproteinases,^{44,45} sox genes,⁴⁶ and circadian rhythm genes.⁴⁷ Since the discovery of these WGDs, research has focused mostly on the genetic consequences of ploidy transitions.^{31,32,40,48-50} Determining the

evolutionary fate of ohnologues that arose from WGDs has convincingly refuted the idea that polyploidy has not been important in animal evolution, but these are not the only consequences that could influence how ploidy transitions persist in evolution.

Maintaining the N:C ratio requires sudden changes in DNA content from WGD to be associated with significant changes in cell volume. This highlights a curious difference between plants and vertebrates in how they respond to ploidy transitions: vertebrates compensate for larger cells by reducing cell number to maintain organ and body size,⁵¹⁻⁵³ whereas plants do not compensate for larger cells and polyploid plants are therefore larger than diploids.^{54,55} A developmental mechanism that decouples cell volume from organ size and body size could improve tolerance to WGDs by maintaining the allometric relationships shaped by evolution for an organism in its ecological niche.¹⁶ Differences among tissues and organs with respect to how they compensate for ploidy changes or in how these changes affect function may shed light on such mechanisms.

In most teleost species, ploidy can be easily manipulated if *in vitro* fertilization is possible.⁵⁶ Treating zygotes with a sudden temperature shift or with hydrostatic pressure after DNA replication but before cytokinesis will induce a WGD (a diploid to tetraploid transition).⁵⁶⁻⁶¹ Alternatively, a diploid-triploid transition can be induced by treating embryos before the extrusion of the second polar body.^{52,62-64} Triploids have been studied more extensively than tetraploids as they are reproductively sterile making them useful for aquaculture – triploidy prevents gene flow between farmed and wild fish in the event of escapes and prevents females from allocating resources to gamete production,⁶⁵ but triploid fish seem less tolerant of stressful conditions and succumb more readily to physiological challenges.^{66,67} Tetraploid fish are fertile, but they are

typically even less robust than triploids.⁵⁷⁻⁶¹ The observation that ploidy transitions affect physiological tolerances and increase mortality rate when reared in conditions that are optimized for diploid performance is intriguing. If polyploids underperform diploids in conditions that are finely tuned to maximize diploid performance, are there conditions that would favour polyploids over diploids? Even if performance was equal between ploidies, polyploidy could be favoured under those conditions over time due to the presence of ohnologues that can provide adaptive benefits as they evolve novel functions. WGD generates an immediate reproductive barrier between populations that occupy the same ecological niches (because hybrids between diploids and tetraploids would likely be sterile triploids), therefore diploids and tetraploids would be in direct competition for the same resources. Predicting conditions favourable for polyploids requires a detailed understanding of the consequences of WGDs and associated changes in cell volume/cell number on the physiology of the various cell types, tissues and organs, and on the organism.

WGDs can be induced in zebrafish, but tetraploid zebrafish die within 10 days of fertilization when reared in standard zebrafish conditions optimized for diploids⁵⁹⁻⁶¹. Triploid zebrafish can be reliably produced⁶²⁻⁶⁴ and ploidy can be measured non-lethally using confocal microscopy to measure nucleus volume in the developing myofibers (Chapter 2).⁶⁸ The optical clarity of zebrafish embryos and the availability of molecular tools and transgenic lines to label specific tissues make the triploid zebrafish an ideal model to study how different cell and tissue types compensate for changes in ploidy, cell volume, and cell number. Here, I use live imaging of transgenic zebrafish embryos (bright-field, DIC, and confocal microscopy) and immunohistochemistry to compare

three very different tissue types – blood, muscle, and vasculature – to demonstrate tissue-specific differences in their compensatory response to polyploidy.

Blood is a fluid tissue comprised of large numbers of individual mono-nucleated cells that lack cell-cell and cell-matrix adhesions.^{69,70} Erythrocytes respond to ploidy changes in the expected manner – erythrocytes are larger, but there are fewer of them in circulation in triploid compared to diploid fish.⁵² The skeletal musculature of the trunk in fish is a series of chevron-shaped myotomes made of stacked bundles of contractile, multinucleate myofibers aligned in parallel along the anterior-posterior axis of the embryo.^{71,72} In addition to undergoing fusion between myoblasts to generate multinucleate myofibers,⁷² these cells maintain extensive cell-matrix adhesions at all stages of their differentiation,^{45,73} but it is not yet clear how myotome development is affected by ploidy changes *in vivo*. Vascular tissue is an endothelial network of various-sized tubes that must spread fractally throughout the embryo to circulate blood throughout the body. These cells maintain both cell-cell and cell-matrix adhesions and are patterned by extrinsic signaling molecules presented by the extracellular matrix of the tissue through which they grow but we do not know how these processes are affected by ploidy transitions.

Using triploid zebrafish, I demonstrate that ploidy transitions and the concomitant changes in cell volume and cell number have diverse effects on different tissues in the teleost embryo. Hypotheses regarding developmental mechanisms that allow vertebrates to compensate morphologically for sudden and dramatic changes in cell volume and number, and how they affect the evolution of WGDs and polyploidy, need to account for any physiological implications that arise due to changes in the cellular composition of the various tissues and organs in the body. Ohnologues do not

have time to accumulate mutations and diverge if the cell volume/cell number changes reduce fitness so dramatically that polyploids die before reproducing and passing on their genes. There is a balance between the consequences of cell volume/cell number changes that affect selection on individual organisms and the advantages associated with extra gene copies in polyploids over evolutionary time. Understanding the evolution of polyploids requires an understanding of these developmental constraints relating to cell size and the numbers of cells in organ primordia, as well as the evolutionary genetic consequences.

3.2 Results:

3.2.1 Early cleavage rate and blastomere volume are not significantly different in diploid and triploid zebrafish

Diploid and triploid zebrafish embryos were morphologically indistinguishable between the 4-cell and 512-cell stages (Fig. 3.1A-F) and did not differ in the number of cell divisions during the first 230 minutes of development (to the 512-cell stage) (Fig. 3.1G, $n = 8$ embryos per ploidy, $p = 0.232$). At the earliest stages (4-8 cell), triploid embryos were consistently one cell cycle behind diploid embryos at any moment in time, but there was no observable difference at later stages between 128 cells until 512 cells, after which staging embryos precisely becomes more challenging. At the 16-cell stage, blastomere width and height (Fig. 3.1B and E, red and green lines, respectively) were not significantly affected by ploidy (Fig. 3.1H; $p = 0.506$ and 0.910 , respectively).

3.2.2 Triploid embryos have fewer, larger erythrocytes in circulation

At 48 hpf, diploid and triploid zebrafish embryos had a beating heart and robust circulation of erythrocytes in large blood vessels like the dorsal aorta (Fig. 3.2A and B) as well as in the intersomitic vessels (ISVs) (Fig. 3.2C and D). Triploid erythrocytes

were larger (major axis) than diploid erythrocytes in the dorsal aorta where they are unconstrained by lumen diameter ($n = 8$ embryos per ploidy, 30 cells per embryo, $p < 0.001$, Fig. 3.2A, B and E) and in smaller vessels such as the ISVs that force erythrocytes to stretch and deform during erythrocyte transit ($n = 8$ embryos, $n = 5-8$ cells per embryos, $p < 0.001$, Fig. 3.2C, D and F). Triploid erythrocytes stretched to a greater degree while traversing an ISV compared to diploid erythrocytes ($n = 8$ embryos per ploidy, $p < 0.001$, Fig. 3.2G). Triploid embryos had fewer circulating erythrocytes than diploid embryos ($n = 8$ embryos per ploidy, $p < 0.001$, Fig. 3.2H).

3.2.3 Myofiber length is constrained by myotome size and N:C ratio is maintained by reducing myonuclear count in triploids

Despite having larger nuclei in the developing myofibers at 48 hpf (Chapter 2),⁶⁸ triploid myotomes were no larger than diploid myotomes with average length (anterioposterior) of $82.6 \mu\text{m} \pm 5.3$ and $81.3 \mu\text{m} \pm 8.9$ and average height (dorsoventral) of $199.2 \mu\text{m} \pm 18.0$ and $191.9 \mu\text{m} \pm 25.2$ in diploids and triploids respectively ($n = 5$ embryos per ploidy, 3 myotomes per embryo, $p = 0.175$ and 0.988 for myotome length and height, Fig. 3.3A and B). This is consistent at finer resolutions: sarcomere length was always $2 \mu\text{m}$ ($n = 8$, $p = 1$, Fig. 3.3C and D) and myofiber length (2N: $84.7 \mu\text{m} \pm 5.1$, 3N: 80.2 ± 6.2) and width (2N: $7.1 \mu\text{m} \pm 1.5$, 3N: $7.3 \mu\text{m} \pm 2.4$) were all unaffected by ploidy ($n = 3$ embryos per ploidy, 10 myofibers per embryo, $p = 0.360$ and 0.618 respectively, Fig. 3.4A and B). Furthermore, the number of myofibers per myotome in triploids was not significantly reduced compared to diploids ($n = 3$ embryos per ploidy, $p = 0.065$, Fig. 3.4C-E) and the somites from which they develop are established at the same rate (Fig. 3.3E). Diploid myofibers typically had 4 nuclei compared to 3 nuclei in triploids (Fig. 3.5, $n = 10$ fibers per embryo, 8 embryos per ploidy, $p < 0.001$).

3.2.4 Triploid blood vessels have thicker vessel walls and lumen diameter is not increased

At 48 hpf, diploid and triploid embryos have three large vessels that transport blood along the anterior-posterior axis of the embryo connected by a series of smaller vessels between each pair of somites.⁷⁴ Despite the change in nucleus volume, there were no qualitative differences between ploidies in the patterned network of blood vessels, and no significant differences in the measured distance between the dorsal aorta and the dorsal longitudinal anastomotic vessel ($120.7 \mu\text{m} \pm 8.1$ in diploids and $116.8 \mu\text{m} \pm 11.3$ in triploids) or the distance between ISVs ($70.1 \mu\text{m} \pm 4.6$ in diploids and $72.7 \mu\text{m} \pm 6.3$ in triploids) ($n = 5$ embryos per ploidy, $p = 0.751$ and $p = 0.476$ respectively, Fig. 3.6A and B). Resliced confocal projections showing the vascular network in cross-section reveals that the distance ISVs extend out laterally from the midline was also not affected by ploidy ($36.4 \mu\text{m} \pm 4.5$ in diploids and $33.8 \mu\text{m} \pm 5.1$, $n = 5$ embryos per ploidy, $p = 0.087$, Fig. 3.6C and D). Diploid and triploid ISVs were consistently made of 3 vascular endothelial cells ($n = 5$ embryos per ploidy, Fig. 3.7). The dorsal-most nucleus was located in the mediolateral section of the vessel just where ISV connects to the dorsal longitudinal anastomotic vessel before the vessel turns and grows ventrally (Fig 3.7C and E, magenta arrows). The medial and ventral (blue and yellow arrows respectively) were found in the dorsoventral running section of the vessel before it connects to the posterior cardinal vein (Fig. 3.7D, F and G). Renders from FluoRender^{75,76} of the ISVs (Fig. 3.8A, B, E, and F) and the dorsal aorta (Fig. 3.8 C, D, G, and H) show that the blood vessel walls were thicker in triploids than in diploids ($n = 5$ embryos per ploidy, $p < 0.001$ in both cases; Fig. 3.8) and that this expansion of the vessel walls comes at the expense of lumen space rather than expanding out into neighbouring tissues.

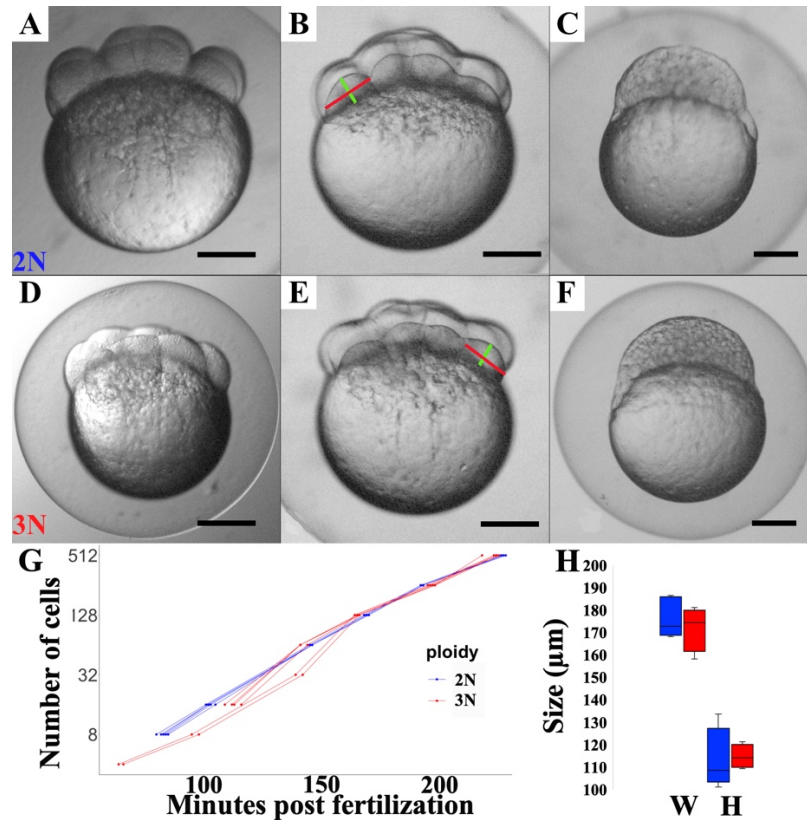


Figure 3.1. Cell cycle and cell volume are not affected by ploidy in pre-gastrula zebrafish embryos. Diploids (A-C) and triploids (D-F) are morphologically indistinguishable at the 8-cell (A and D), 16-cell (B and E) and 512-cell (C and F) stages. **A-F)** Bright-field micrographs of pre-gastrula stage diploid and triploid zebrafish embryos (scale bar = 50 μm). **G)** Cleavage rate from the 4-cell stage until the 512-cell stage is not significantly affected by ploidy (2 blastomeres per embryo, 8 embryos per ploidy, $p = 0.232$). **H)** Cell size is not significantly affected by ploidy in diploid (blue) and triploid (red) blastomeres. ‘W’ is cell width and ‘H’ is cell height ($n = 8/\text{ploidy}$, $p = 0.506$ and 0.910 respectively).

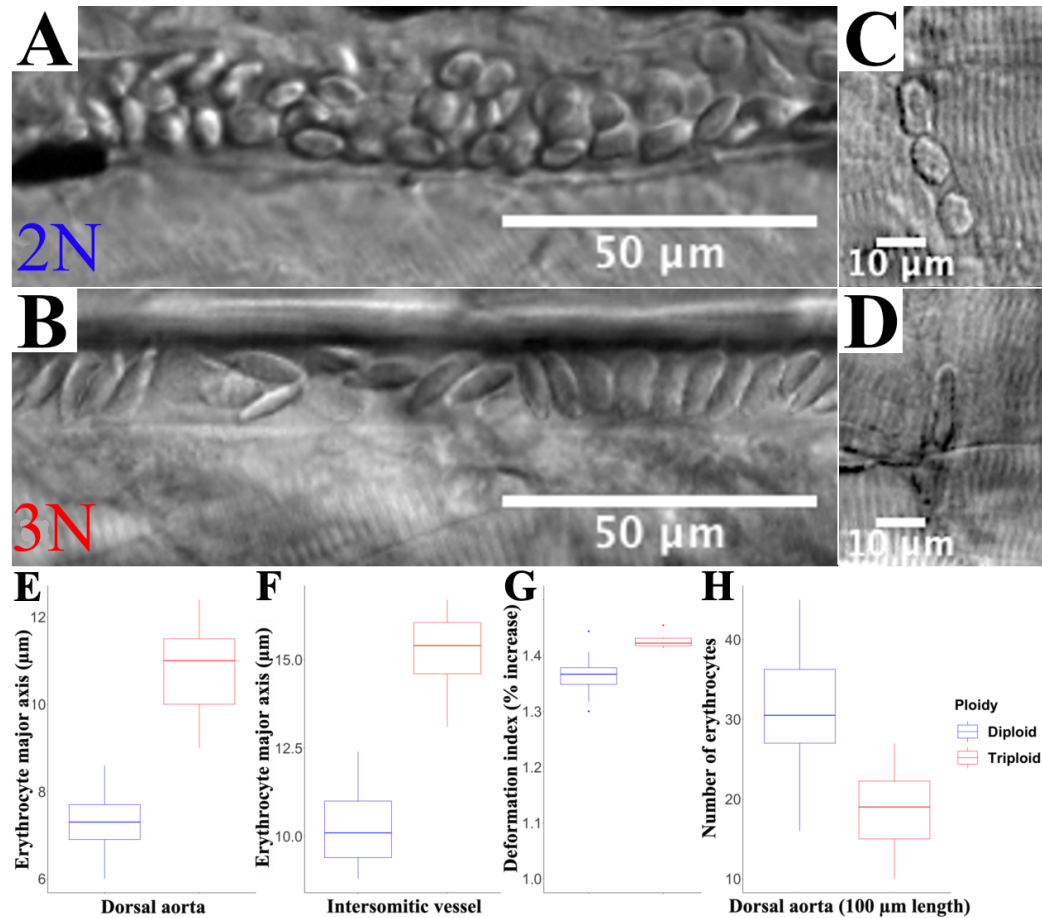


Figure 3.2. Erythrocytes are larger, but fewer in number in triploid zebrafish embryos. **A+B)** DIC micrographs of embryonic erythrocytes in the dorsal aorta at 48 hpf in a diploid (A) and a triploid (B) embryo. **C+D)** DIC micrographs of embryonic erythrocytes traversing an ISV at 48 hpf in a diploid (C) and triploid (D). **E)** Triploid erythrocytes are larger than diploid erythrocytes in the dorsal aorta (n=8 embryos per ploidy [30 cells per embryo], $p < 0.001$) and **F)** in the ISVs (n=8 embryos per ploidy (5-8 cells per embryo), $p < 0.001$). **G)** Triploid erythrocytes deform relatively more than diploid cells when traversing ISVs (8 embryos per ploidy, $p < 0.01$). **H)** Triploid embryos have fewer circulating erythrocytes (n=8 embryos per ploidy, $p < 0.001$).

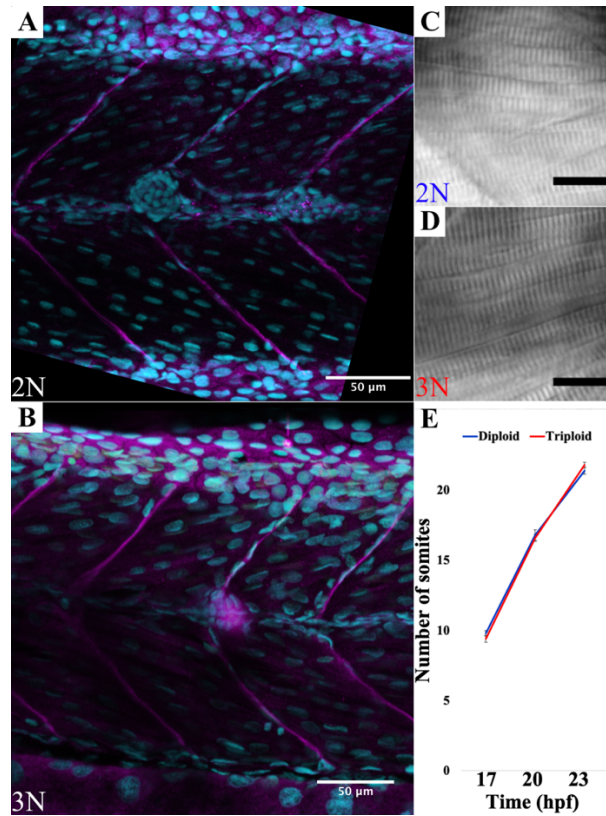


Figure 3.3. Ploidy does not affect myotome size, sarcomere size, or the rate of somitogenesis in zebrafish embryos. **A+B)** Confocal micrographs of anti-laminin immunostains (magenta channel) co-stained with Draq5 (cyan channel) of diploid (A) and triploid (B) myotomes. Myotome size was not significantly different along the anterioposterior axis or the dorsoventral axis ($n = 5$ embryos per ploidy, $p = 0.175$, and $p = 0.988$ respectively). **C+D)** DIC micrographs of diploid and triploid myofibers. Sarcomere size was not significantly affected by ploidy ($n = 8$ embryos per ploidy, $p = 1$). **E)** The rate of somitogenesis is not affected by ploidy ($n = 8$ embryos per ploidy, $p = 0.22$).

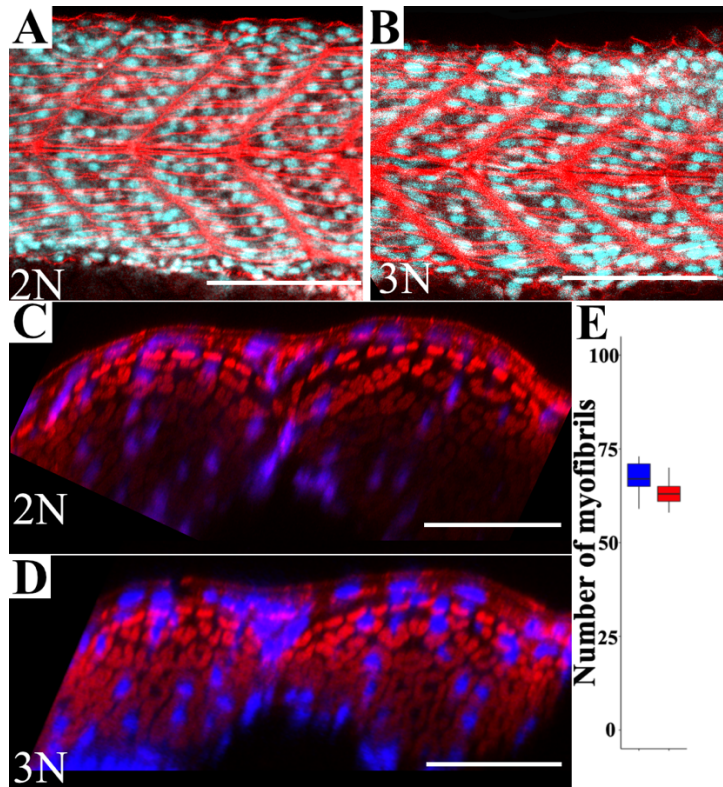


Figure 3.4. The size and number of myofibers in each myotome are not affected by ploidy. **A+B)** Confocal micrographs of diploid (A) and triploid (B) myotomes with actin immunostained with anti-phalloiden 568 (red channel) and DNA stained with DAPI (cyan channel). Scale bars are 100 μ m. **C+D)** Single-frame images from confocal stacks from A+B resliced along the AP axis. The number of myofibers in a diploid myotome (C) is not significantly different than the number in triploid myotomes (D) ($n = 3$ embryos per ploidy, $p = 0.065$) (E; diploid in blue, triploid in red).

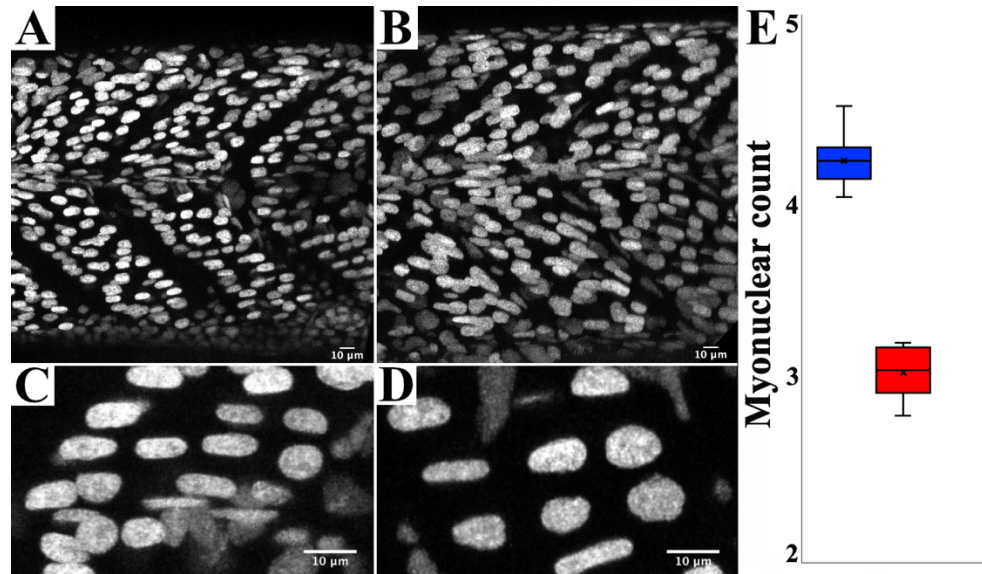


Figure 3.5. The number of nuclei per myofiber is significantly reduced in triploid embryos. **A+B)** Confocal projections of myotomes in diploid (A) and triploid (B) Tg(h2a.f/z:H2A.f/z::eGFP) embryos at 48 hpf. **C+D)** Higher magnification, single planes of polynucleated myofibers from A+B. **E)** Diploid myofibers have significantly more nuclei than triploid myofibers (blue and red, respectively; n = 10 fibers per embryo, 8 embryos per ploidy, $p < 0.001$).

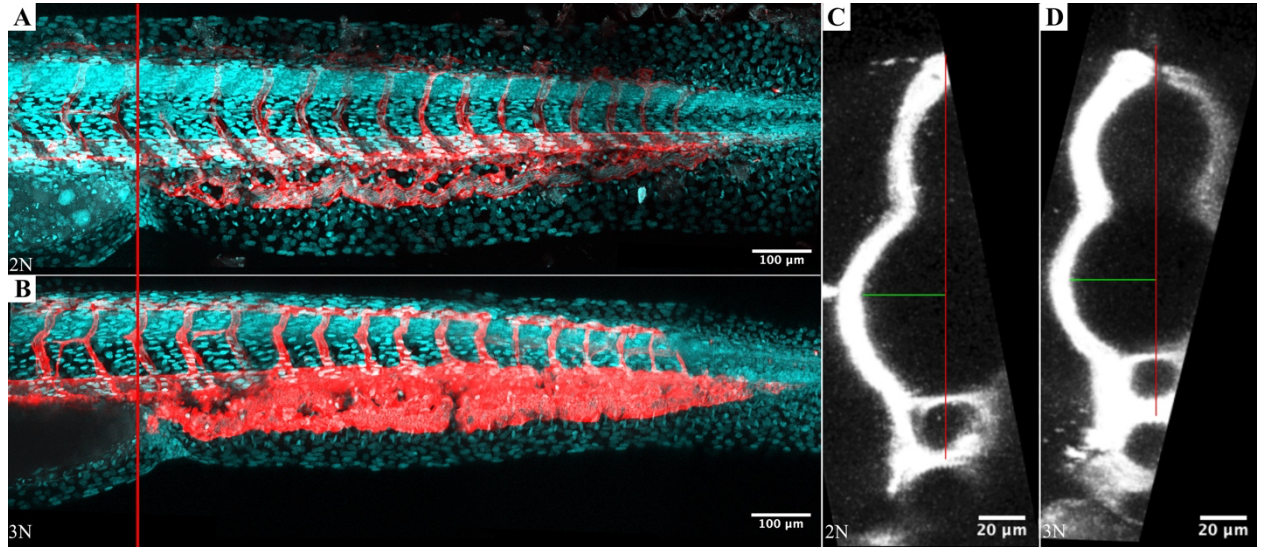


Figure 3.6. Vascular patterning is not significantly affected by ploidy. **A+B)** Composite confocal micrographs of the developing vascular system of diploid (A) and triploid (B) Tg(h2a.f/z:H2A.f/z::eGFP; Flk1:mCherry) embryos. ISV length and the distance between each ISV along the AP axis are not significantly affected by (n = 5 embryos per ploidy, $p = 0.751$ and $p = 0.476$, respectively). Red line indicates the plane projected in C+D. **C+D)** Confocal projections of resliced stacks from A and B. The distance between the midline of the dorsal aorta (red line) and the lateral-most point in the ISV (green line) was not significantly affected by ploidy (n = 5 embryos per ploidy, $p = 0.087$).

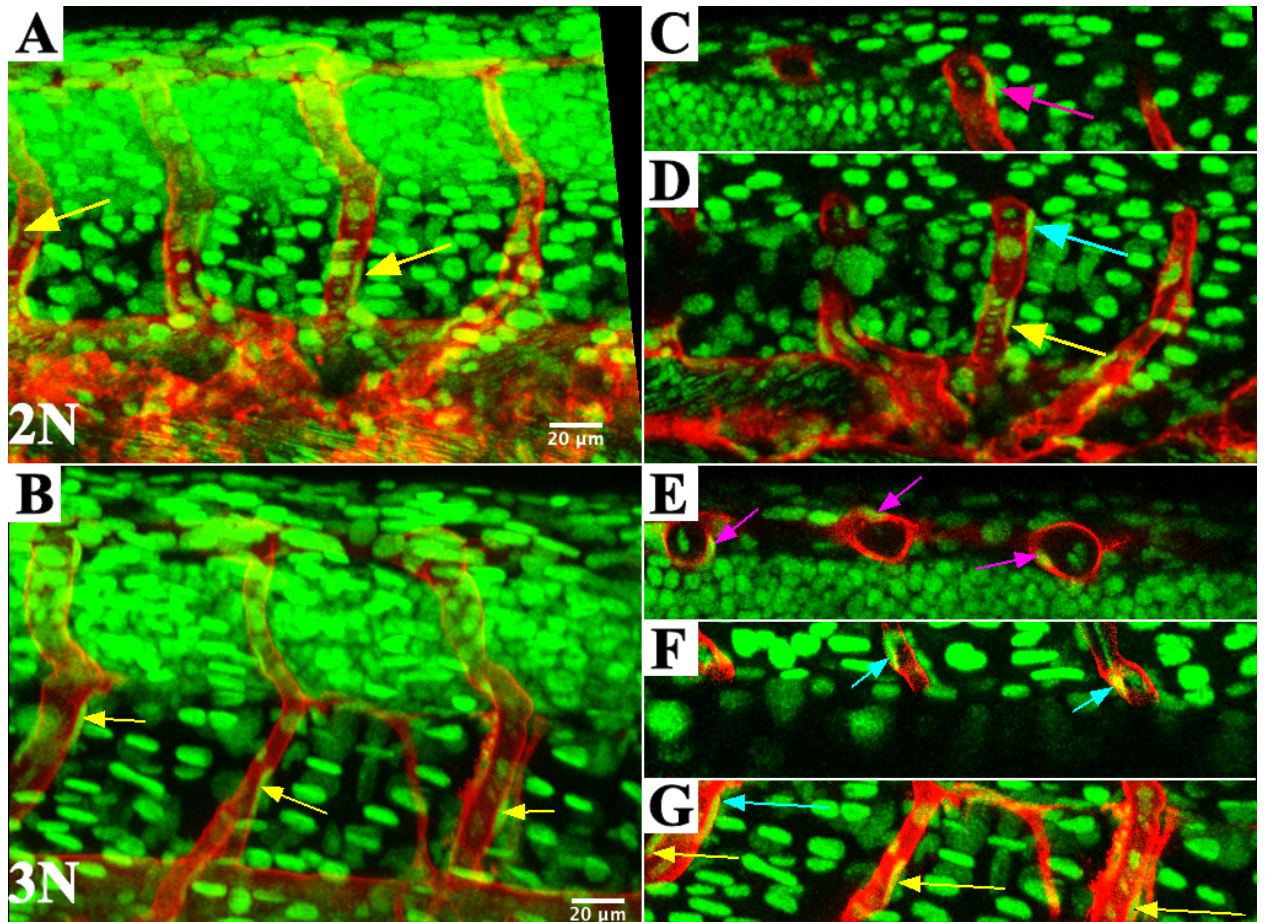


Figure 3.7. Triploid ISVs are not comprised of fewer cells than diploid ISVs in 48 hpf zebrafish embryos. **A+B)** Composite confocal micrographs of diploid (A) and triploid (B) Tg(h2a.f/z:H2A.f/z::eGFP; Flk1:mCherry,) embryos, showing endothelial cell nuclei (yellow arrows). **C+D)** Cropped, single plane micrographs at Z-planes selected to highlight endothelial cell nuclei in the dorsal (C, magenta arrow), medial (D, blue arrow), and ventral (D, yellow arrow) planes in a diploid embryo. **E-G)** Cropped, single plane micrographs at Z-planes selected to highlight endothelial cell nuclei in the dorsal (E, magenta arrow), medial (F, blue arrow), and ventral (G, yellow arrow) in a triploid embryo. There is no effect of ploidy on the number of cells in the ISVs at 48 hpf ($n = 3$ embryos per ploidy).

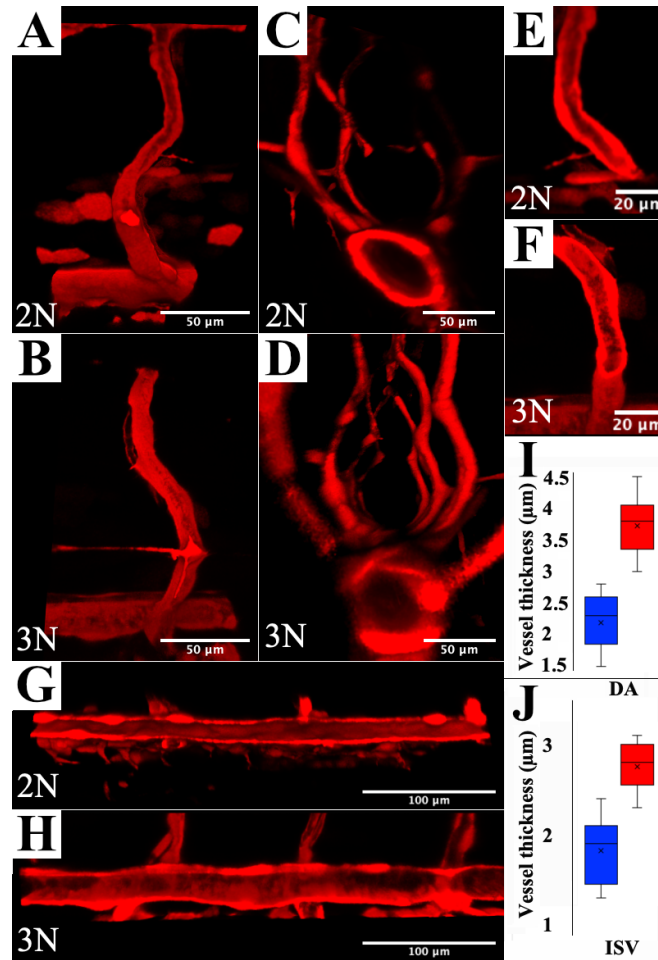


Figure 3.8. Vascular endothelial cells are thicker in triploid zebrafish. **A+B)** Lateral view of rendered confocal projections of an ISV and a section of the dorsal aorta in a diploid (A) and triploid (B) *Tg(h2a.f/z:H2A.f/z:eGFP; Flk1:mCherry)* embryo (red channel only). **C+D)** Rendered confocal projections from A and B rotated 90° to see the dorsal aorta in cross section in a diploid (C) and triploid (D) embryo. **E+F)** Optical longitudinal section at the midline of an ISV from the renders in A (E, diploid) and B (F, triploid). **G+H)** Optical longitudinal section at the midline of the dorsal aorta from the render in A (G, diploid) and B (H, triploid). **I)** Blood vessel walls of the dorsal aorta (from G and H) are an ISV (from E and F) are thicker in triploid than in diploid embryos (red and blue, respectively; $n = 5$ embryos per ploidy, $p < 0.001$ in both cases).

3.3 Discussion

As we discover more examples of genetic innovations and increasing morphological diversity after ploidy transitions, we reinforce Ohno's statement that "natural selection merely modifies, while redundancy creates".³⁷ Tracing the fates of ohnologous gene pairs using genomics has revealed that sub-functionalization and neo-functionalization are far more common than previously thought,^{40,48,49} leading some to wonder if there are patterns that influence which genes are retained as ohnologous pairs and which lose one member as a pseudogene.^{40,50} Focusing only on the genetic implications of polyploidy is not sufficient for truly understanding the roles that ploidy transitions have played in evolution if we consider the physiological differences between fish of different ploidies that would affect survival. Most studies that have documented physiological differences between ploidies have focused on triploids,^{52,77-80} but given that tetraploids are even less robust than triploids⁵⁶⁻⁶¹ it is likely that these physiological differences would affect their survival after a ploidy transition. WGDs and ploidy transitions are also associated with sudden changes in cell volume and we do not fully understand the physiological implications of these effects. In vertebrates, these increases in cell size are compensated for by reducing cell number in the organism^{12,26-29}. This compensation maintains the overall body size of the animal, making it more likely that it will be able to occupy the same ecological niche as its diploid ancestor/new competitor, but the developmental mechanism(s) that maintain allometric relationships between tissues and organs, and other developmental constraints such as limitations on the minimum number of cells in a primordium, are poorly understood.

Using triploid zebrafish embryos, I have uncovered several alternative strategies that different tissues and cell types utilize to compensate for increased nuclear volume as

ploidy increases and I discuss how these different strategies could have different implications that are specific to each tissue. The classical “fewer, but larger cells” explanation for body size compensation in polyploids^{51,52} is not true for all tissues. It is important to know how these compensations affect each organ’s or tissue’s function in order to understand how and when fertile polyploids will survive to pass on their genes.

3.3.1 Early development

I have shown that triploid embryos are morphologically indistinguishable from sibling diploids during cleavage. Interestingly, the duration of the cell cycle is not affected by ploidy suggesting that DNA replication is not rate-limiting during early cleavage. In most cases, the length of the cell cycle is inversely proportional to ploidy,^{30,64,81} so I predicted that developmental rate would be slower in cleavage-stage triploids. However, despite triploid cells having 50% more DNA to replicate during each cell cycle, triploid embryos progressed from the 4- or 8-cell stage until the 512-cell stage no slower than sibling diploids. The early cleavage stage is unique in that blastomeres are dividing rapidly by skipping the G1 and G2 phases of the cell cycle.⁸² By switching to M phase and dividing immediately after DNA replication, the number of cells can increase rapidly but blastomeres get smaller with every division. These results suggest that at these early stages, the capacity for DNA replication is not limiting the cell cycle in cleavage stage zebrafish embryos.

The observation that blastomere size is not affected by ploidy at these earliest embryonic stages implies that the N:C ratio is not an inherent property of all zebrafish cells; rather, it becomes locked in at some later point in development as seen in urchin and frogs.⁸³ This suggests a possible mechanism explaining the difference in cell size between diploid and triploids. The N:C ratio is set at the mid-blastula transition (MBT)

when the rapid cell cycling of blastomeres stops and cells no longer get smaller with each cell division.⁸⁴ In *Xenopus*, there is a sudden drop in the cytoplasmic concentration of maternally-provided Importin α at the MBT^{84,85} and a concurrent activation of zygotic genome transcription.⁸⁶ Importin α regulates the transport of cytoplasmic proteins (including maternally-provided nuclear structure proteins such as LaminB) into the nucleus⁸⁴ acting as a cell surface-area-to-volume sensor by becoming progressively more sequestered to the plasma membrane as the ratio of membrane:cytoplasm increases with each cell division.⁸⁵ Eventually, most of the maternally-provided pool of Importin α is bound in the plasma membranes and the import of cytoplasmic proteins into the nucleus slows, triggering the MBT and the end of reductive blastomere division. A triploid embryo (with larger nuclei) would consume more of the maternally-provided nuclear structure proteins with each cell division. Perhaps triploid animals have larger cells because the supply of nuclear structure proteins dilutes more rapidly and becomes limiting one cell cycle earlier, thereby triggering the MBT before the cells can undergo one more round of reductive cell division.

The establishment of the N:C ratio and zygotic genome activation both occur at the MBT, but it is becoming clear that these processes are linked and not merely a temporal coincidence. Zygotic genome activation (ZGA) occurs in haploid *Drosophila* one cell cycle later than in diploids, as would be predicted by this model^{87,88} and manipulating the N:C ratio by injecting exogenous DNA triggers ZGA early.⁸⁹ *Xenopus* embryos in which cell size has been reduced experimentally by manually removing cytoplasm have precocious zygotic genome activation,^{90,91} presumably due to the sudden drop in cytoplasm:membrane ratio that causes Importin α to become limiting sooner.

Since blastomeres are skipping the G1 and G2 phases, they are getting smaller with each division so by undergoing the mid-blastula transition after fewer cell cycles, the N:C ratio is maintained by altering how many reductive cleavage divisions occur before the zygotic genome activates. Testing this model would require the ability to modulate the amount of LaminB in the cytoplasm perhaps by microinjecting *in vitro* translated proteins into the cytoplasm of triploid zebrafish zygotes or 2-cell embryos to see if cell size increase can be decoupled from ploidy increase.

3.3.2 Blood

The blood is the most-studied tissue in the biology of polyploid vertebrates because triploid fish are particularly susceptible to physiological stressors that affect aerobic metabolism such as hypoxia, temperature, and exercise.^{77-80,92} Also, erythrocyte analyses are the classic diagnostic tools for measuring ploidy either by comparing length of the major axis using microscopy⁹³ or by measuring DNA content using flow cytometry.⁹⁴ Most of these studies focus on adult blood since samples can be collected easily from larger fish, but here I show that embryonic erythrocytes conform to the same “fewer but larger” pattern of compensation to polyploidy that is known to occur in adult blood. Embryonic erythrocytes differentiate directly from the mesoderm without a stem cell phase,^{95,96} making them distinct from adult erythrocytes that differentiate from hematopoietic stem cells, but it seems they conform to the same pattern of compensation.

Blood is unique in being a viscous liquid rather than the typical solid, static tissues that make up most of the organism. The lack of cohesion means that blood cells are not constrained by neighbouring tissues and can therefore compensate for increased ploidy by getting larger in all dimensions. The physiological consequences of increased

cell volume on erythrocyte function are not well-understood, but this strategy of compensation maintains hematocrit and hemoglobin so that these hematological variables generally are not affected by ploidy.⁵²

A long-standing mystery in triploid hematology has been the preponderance of erythrocytes with bisected nuclei when examined on blood smear.⁵² The circulating blood is a population of mostly mature cells with a short half-life, and most studies of triploid hematology focus only on these cells. If bisected nuclei are indicative of damage, these erythrocytes will be eventually be removed by macrophages and replaced by new erythrocytes that differentiate from a pool of hematopoietic stem cells that colonize the anterior lobe of the kidney. To our knowledge, the only studies comparing the number of stem cells for any tissue between animals of different ploidy level were cell culture studies comparing the differentiation rate and number of myoblasts in diploid and triploid trout and salmon muscle showing that triploids had slower/reduced differentiation rates and fewer myoblasts.^{97,98} If these patterns hold true in the blood as well, this could limit the ability of fish to replace erythrocytes that accumulate damage while squeezing through narrow capillary vessels. The zebrafish is an ideal model system for this line of investigation as there are already hundreds of hematopoiesis-relevant mutants and transgenics available due to decades of research on development of the hematopoietic system and its relation to cancers of the blood.^{69,70,99}

3.3.3 Muscle

Studies focused on understanding the effects of ploidy changes on skeletal muscle development and physiology^{97,98,100-102} are relatively scarce compared to the hematological studies. Myofibers are multinucleated cells that form by membrane fusion of myoblast precursor cells,⁷² and then cells elongate along the anterior-posterior axis

until they span the distance between somite boundaries.^{71,73} Previously, I have shown that these myofiber nuclei are larger in triploid embryos than in diploids and I can use these nuclei to identify triploid individuals from a pool of mixed ploidy embryos (Chapter 2).⁶⁸ Somite morphology and the rate of somitogenesis are not affected by ploidy, likely because somite boundaries are spaced equally in diploid and triploid embryos and the length of the myofibers that span the somite are not affected by ploidy. I predicted that these cylindrical myofibers would have a larger cross-sectional diameter in order to maintain the N:C ratio, but did not detect a difference in the width of individual myofibers in cross-section or in the total number of myofibers in an optical cross-section of a myotome, suggesting that this tissue responds to ploidy change differently than in blood. Close examination of diploid and triploid myofibers revealed the mechanism: myofibers are constrained in size along the A-P dimension by the somite boundaries, but because they are multinucleated cells, they can reduce the amount of DNA in any given myofiber by reducing the number of nuclei per myofiber. Diploid myofibers typically have four nuclei per fibre whereas most triploid myofibers have only three. Presumably, this is a result of the fusing together of fewer myoblast precursor cells, which suggests an interesting N:C sensitive mechanism regulating myoblast fusion. Despite this compensation in the number of nuclei, it is still curious that triploid myofibers do not appear to be larger than diploid myofibers. A diploid myofiber with four nuclei would have eight copies of the genome per cell whereas a triploid with three nuclei per fibre would have nine copies, so there could still be a subtle effect of ploidy on the cross-sectional area or on the number of myofibers. Indeed, the difference in the number of myofibers between diploid and triploid in this study approaches significance. The poor signal-to-noise ratio in the deep tissues near the midline of immunostained

embryos limited the analysis of myofiber cross-sectional area to measurements of myofiber width in 2-dimensional confocal projections rather than 3-dimensional analysis of resliced confocal projections. Labelling cell membranes by injecting mRNA encoding for a membrane-targeting fluorescent protein¹⁰³ in Tg(h2a.f/z:H2A.f/z::eGFP) embryos would allow for more precise measurements of cross sectional area to detect for subtle effects on the size and number of myofibers in a diploid (with 2 copies of the haploid genome in ~4 nuclei) compared to triploid myofibers (with 3 copies of the haploid genome in ~3 nuclei).

An intriguing observation from this study is that sarcomere length is consistent between ploidies. Scaling of subcellular structures to cell size has been demonstrated for other organelles,^{84,85,105,106} so it is not surprising that sarcomere size is maintained when myofiber size is not affected by ploidy. Perhaps this unique strategy utilized by myofibers ensures that the force-generating contractile apparatus in the skeletal muscle remains unchanged in the face of ploidy and cell size changes. Sudden changes in the force-generating ability of the sarcomeres could affect even the most basic functions like swimming and ventricular contractions. Preventing these changes increases the likelihood of a polyploid surviving and reproducing in the niche once occupied by its diploid ancestors.

3.3.4 Blood Vessels

The lack of apparent differences in the patterning of the vascular network between diploids and triploids in 48 hpf old embryos suggests that this repulsion-based mechanism, which is directed by Semaphorin-Plexin signalling,^{107,108} is sufficiently robust to tolerate changes in cell size without affecting the pattern produced. I predicted that triploids would reduce the number of vascular endothelial cells for any given length

of blood vessel, but rejected this hypothesis by counting three flattened nuclei embedded in vessel walls (rather than inside the lumen) in both diploid and triploid ISVs. Vascular endothelial cells are thicker in triploids embryos compared to those in diploids thereby maintaining the N:C ratio. Strikingly, rather than increasing the diameter of the vessel wall, the cells expand into the empty space of the lumen decreasing lumen diameter. This contradicts my initial prediction that lumen diameter would increase in order to accommodate the increased size of the erythrocytes that circulate within these vessels. Recent work using zebrafish to test models of microcapillary flow emphasized the importance of pressure-feedbacks driven by vessel occlusion to evenly distribute erythrocytes throughout the vascular network,¹⁰⁹ but it is unclear how a sudden change in the cell size without a concomitant increase in vessel diameter affects erythrocyte flow patterns. The observation that the larger triploid erythrocytes must traverse a network of vessels that are smaller in diameter than those travelled by the smaller diploid erythrocytes could contribute to the well-documented limitations of triploid fish to cope with oxygen stress, whether it be due to high temperatures, hypoxia, or exercise.

When the animal becomes reliant on the efficient delivery of blood to all of its tissues for aerobic metabolism, perhaps the network of vessels compensates since it is so plastic. There are no studies comparing oxygen diffusion distance or distance between vessels in capillary beds between adult animals of different ploidies but it could be that animals will compensate for ploidy changes by modifying capillarization of tissues. It is also possible that the altered thickness of the vascular endothelial cells affects the rate of oxygen transported from the blood into the tissues, so there could well be angiogenic differences that emerge as the fish grows and the vascular system is shaped by local oxygen demands in the adjacent tissues. At this early stage, lumen diameter is largely

regulated by the size of the vascular endothelial cells that form the tube and by internal pressure from blood flow. Later, from 4-6 days post-fertilization onwards, pericytes and vascular smooth muscle cells adhere to the abluminal surface of blood vessels, conferring the ability to dynamically regulate vessel lumen diameter.¹⁰⁸ It would be interesting to study to number and function of these cells on vessels that have thicker endothelial cells to see if lumen diameter and vasocontractility are affected by ploidy.

3.3.5 Conclusions and future directions

Morphology and physiology are inextricably linked.^{5,10,110} Vertebrates have evolved developmental mechanisms that compensate for the cell size increases that accompany WGDs and ploidy transitions so that organ and body size are maintained in polyploids. Does changing the cellular density of an organ or tissue affect its function if organ size is maintained? Individual triploid erythrocytes contain more hemoglobin than diploid erythrocytes, but the reduction in the number of erythrocytes compensates so that the concentration of hemoglobin per unit blood volume typically is consistent in diploids and triploids.⁵² Does packaging hemoglobin into fewer, larger units affect the ability of an organism to efficiently transport oxygen from its respiratory surfaces to its tissues? Functional studies of respiratory surfaces suggest that thicker epithelial layers significantly affect the rates of oxygen diffusion into the blood,^{10,110} but little is known about how blood vessel thickness affects physiology and oxygen transport. Erythrocytes with bisected nuclei are frequently observed in triploid blood smears,⁵² perhaps due to squeezing through blood vessels that have not increased in diameter to accommodate larger erythrocytes. Packing more hemoglobin into each erythrocyte means that a triploid loses a larger proportion of the total amount of circulating hemoglobin whenever a cell is damaged than would a diploid, thereby amplifying the consequences of

erythrocyte damage. In mammalian erythrocytes, cell size is inversely proportional to respiration rate due to a reduced surface to volume ratio^{111,112} suggesting that respiration rate would be reduced in a triploid erythrocyte despite having more hemoglobin. There must be a metabolic cost associated with replacing erythrocytes at a faster rate and with having more damaged erythrocytes in circulation, but little is known about erythrocyte turnover in fish. Simonot and Farrell^{113,114} noted that triploid rainbow trout (*Oncorhynchus mykiss*) were not able to recover from phenylhydrazine-induced anaemia, whereas diploids had, suggesting that triploids had a reduced capacity for erythropoiesis and further implicating erythrocyte turnover as a limiting factor for triploid physiology.

This study focuses on triploid zebrafish, but most evolutionarily-relevant ploidy transitions would be diploid to tetraploid transitions. It is possible to produce tetraploid zebrafish, but they are usually very deformed and do not survive to adulthood⁵⁹ (our unpublished observations). This is curious as tetraploids are viable in other species of teleost.^{57-59,60} McMillen and colleagues¹¹⁶ argue that the zebrafish somite is an extremely reduced tissue consisting of two border cells that abut the anterior and posterior myotendinous junction and two interior cells that have a different fate because they lack this attachment to the extracellular matrix at the myotendinous junction. It seems likely that triploid somites would be even further reduced to having only three cells in total, but still able to define two distinct cell types (border and interior) and form a functional myotome. However, a tetraploid somite would be reduced even further to two cells, both of which would therefore become border cells and thus making it impossible to form a functional myotome. The reason why tetraploid zebrafish are not viable may be due to their size: an animal with so few cells dedicated to each developmental primordium may

not be able to establish all necessary cellular identities from which to build essential structures when these rudiments are now comprised of only half the number of cells. A larger fish can afford to reduce the number of cells by half because there is still a sufficient number of precursor cells remaining to differentiate into the various cell types needed for a tissue or organ to function, but a myotome that is only two cells across can only produce border cells.

This study has revealed that the mechanism of compensation to maintain body size in the face of cell size changes is not conserved across tissues. Blood compensates for ploidy transitions by reducing the number of cells thereby maintaining the concentration of hemoglobin in the tissue. Muscle compensates by reducing the number of myoblast fusions to produce myotomes, myofibers, and sarcomeres whose size is not affected by ploidy. The vascular system compensates by thickening vessel walls and maintaining the dimensions of the network. The zebrafish is well-suited to testing the functional consequences of these changes on whole-animal physiology that could shed light on the conditions that would minimize the negative consequence of WGD in evolution and on conditions that will ameliorate the performance of triploids in aquaculture.

3.4 Experimental Procedures

3.4.1 Animal husbandry

Adult zebrafish were reared in flow-through dechlorinated municipal water in the University of New Brunswick Zebrafish Facility in standard 25 x 11 x 15 cm tanks (Pentair Aquatic Ecosystems, Apopka, FL) at 28°C on a 14:10 h light:dark photoperiod. They were fed adult zebrafish pellets (Gemma Micro, Skretting) twice per day supplemented with live *Artemia* once per day. Embryos were reared in glass Petri dishes

in Embryo Rearing Medium (ERM: 13 mM NaCl, 0.5 mM KCl, 0.02 mM Na₂HPO₄, 0.04 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃, pH 7.4) with 0.01% methylene blue at 28.5°C until 120 hpf when they were transferred to 1L larval tanks. Larvae were fed a series of increasingly larger pellets (Gemma Micro 75-300, Skretting) using automated feeders set to feed 4 times per day. Transgenic lines used in this study were Tg(h2a.f/z:H2A.f/z::eGFP),¹¹⁷ Tg(Flk1:eGFP),¹¹⁸ and crosses between Tg(h2a.f/z:H2A.f/z::eGFP) and Tg(Flk1:mCherry).¹¹⁹

3.4.2 Triploid production and identification

Triploids and diploid controls were produced and identified using the protocol described in Chapter 2. Briefly, triploidy was induced by applying hydrostatic pressure (58.6 MPa for 5 minutes) to *in vitro* fertilized zygotes 70-80 seconds after fertilization. Unless otherwise noted below, triploids were always identified using confocal microscopy to measure nucleus volume in the myofibers at 48 hpf.⁶⁸ This was done *in vivo* for Tg(h2a.f/z:H2A.f/z::eGFP) embryos, whereas Tg(Flk1:eGFP) and Tg(Flk1:mCherry) embryos were fixed and stained in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) with 20 µM of Draq5 (Thermo Scientific, Waltham, MA, USA) in PBS with 0.1% Triton-X100 (PBSTx) overnight at 4°C and then washed thrice in PBSTx prior to confocal microscopy.

3.4.3 Blastomeres

3.4.3.1 Cleavage rate

Symmetrically dividing diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos (8 per ploidy) were sorted individually into four-chambered Petri dishes at the 4- to 8-cell stage and held at 28°C in an incubator until the 512-cell stage. Bright-field images were taken periodically (Leica MZ205A dissecting microscope), with the embryos quickly returned

to the incubator after imaging to maintain temperature. Several pressure-derived embryos were asymmetrical at the 8-cell stage and therefore not likely to be viable; these were replaced with embryos from the same pressure treatment and kept in the same incubator. Hence, some of the triploid embryos were first imaged at the 16-cell stage. Embryos were staged as described in Kimmel et al.¹²⁰ and images were processed, annotated, and assembled in FIJI.¹²¹ The rate of cell division (number of cell divisions / elapsed time [minutes]) was compared between diploids and triploids using a two-sample Student's t-test in R.¹²²

3.4.3.2 Blastomere volume

Blastomere volume was approximated by measuring the width and height of blastomeres from these same images using the line tool in FIJI (8 embryos per ploidy at the 16-cell stage). The two lateral-most blastomeres in each embryo were measured and averaged (these cells were selected because their edges were easier to resolve clearly) and the effect of ploidy on blastomere dimensions was analyzed using separate two-sample Student's t-tests for width and height.

3.4.4 Erythrocytes

Diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos were raised at 28°C in Petri dishes (<50 embryos per dish) until 48 hpf, at which time they were anaesthetized with tris-buffered MS-222 (0.168 mg/ml), mounted onto a slide in anaesthetic using a coverslip with vacuum grease footpads, and imaged on a Leica SP8 confocal microscope. DIC XYT stacks of blood flow in the dorsal aorta and in the ISVs were collected. Individual erythrocytes could not be resolved in most of the XY frames (Figure S1), but occasionally a frame would capture cells between heart beats and then they could be resolved. Erythrocyte number was estimated from these images by

counting the cells along a 100 μm line drawn parallel to the blood vessel. DIC XYT stacks of the ISVs were collected until at least 5 cells had been imaged. Erythrocyte major axis lengths in the dorsal aorta and ISV were measured using the line tool in FIJI (dorsal aorta (DA): 30 erythrocytes per embryos, ISV: 5-8 erythrocytes per embryo, 8 embryos per ploidy). Erythrocyte deformation was calculated by dividing mean erythrocyte length in an ISV by mean erythrocyte length in the dorsal aorta for each embryo. Erythrocyte length was averaged for each embryo and the effect of ploidy on mean erythrocyte length (DA and ISV), erythrocyte deformation, and erythrocyte number were analyzed by independent two-sample Student's t-tests in R. Ploidy of each embryo was confirmed by myofiber nucleus volume analysis after blood flow images were collected.

3.4.5 Muscle

3.4.5.1 Myotome morphology

Diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos were fixed in Dent's fixative (80% methanol, 20% DMSO) overnight at 4°C and then blocked for 8 h in 5% BSA in PBSTx (blocking buffer) at 4°C. They were then incubated in primary antibody (rabbit anti-laminin at 1:1000 in blocking buffer, catalogue #PA1-16730; ThermoFisher Scientific, Waltham, MA, USA) overnight at 4°C, washed thrice in PBSTx, incubated in secondary antibody (goat anti-Rabbit Alexa-488 at 1:2000 in blocking buffer; Invitrogen, Carlsbad CA, USA) with 20 μL Draq5 (Thermo Scientific, Waltham, MA, USA) overnight at 4°C, and again washed thrice in PBSTx. Myotome dimensions (anterior-posterior = length, dorsal-ventral = height) were measured in three adjacent myotomes beginning with the one immediately dorsal to the tip of the yolk extension. Embryos treated identically except for no primary antibody incubation exhibited very

weak and ubiquitous autofluorescence. Confocal stacks were collected using a Leica SP8 confocal microscope, fitted with a 25× 0.9 NA lens. Images and plate assembly, Z-projection, and annotation were done in FIJI. The effects of ploidy on myotome height and length were assessed using two-sample Student's t-tests in R (n = 5 embryos per ploidy).

3.4.5.2 Rate of somitogenesis

At 12 hpf, 8 diploid and 8 triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos were manually dechorionated using #5 watchmaker forceps, pooled in a Petri dish, and blindly sorted into individual wells of 4-welled Petri dishes. Somites were counted on a Leica MZ205A dissecting microscope at 17, 20 and 23 hpf, with the embryos kept separated until ploidy confirmation at 48 hpf. Rate of somitogenesis was calculated as the number of new somites formed during the 6-hour experiment (final somite number – initial somite number) and the effect of ploidy on this value determined by two-sample Student's t-test in R (n = 8 embryos per ploidy).

3.4.5.3 Myofiber morphology

Diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) overnight at 4°C, blocked for 8 h in 5% BSA in PBSTx (blocking buffer) at 4°C, and then incubated in Alexa Phalloidin 568 (1:20 in blocking buffer; Molecular Probes Eugene, OR, USA) with DAPI (1:10000 in blocking buffer; Thermo Scientific, Waltham, MA, USA) overnight at 4°C. Embryos were washed thrice in PBSTx after each step. Embryos treated identically without the stains exhibited very weak and ubiquitous autofluorescence and a barely visible GFP signal in cell nuclei. Confocal stacks were collected using a Leica SP8 confocal microscope fitted with a 25× 0.9 NA lens. Images

were assembled, Z-projected and made into annotated composites using FIJI. A myotome dorsal to the tip of the yolk sac extension was isolated using the Crop tool. Myofiber length and width were measured by drawing lines bisecting the nuclei at the centre of the fibre (Figure S2 red line) and perpendicular to the widest part of the fibre (Figure S2 blue line), respectively, with 10 fibers measured in each embryo. Myofibers were counted using the same confocal stacks resliced along the anterior/posterior axis in FIJI with 0.568 μm stepsize. In these resliced stacks, a myofiber appears as bundles of red actin that surround blue nuclei. These myofibers were counted manually from the resliced stacks in one half of the embryo (the lateral side of the embryo nearest to the objective from notochord to the ectoderm). The effects of ploidy on myofiber length, width and number ($n=3$ embryos per ploidy, 10 fibers per embryo) were determined using two-sample Student's t-tests in R.

3.4.5.4 Sarcomere length

DIC micrographs of sarcomeres from the embryos in section 4.4.2 were collected (Leica SP8 confocal microscope, fitted with a 25×0.9 NA lens) at 48hpf during ploidy determination. Sarcomere length was measured in the myotome immediately dorsal to the posterior tip of the yolk extension using the line tool in FIJI. Mean sarcomere lengths (5 measurements from 8 myofibers per embryo) between diploids and triploids were compared using a two-sample Student's t-test.

3.4.5.5 Myonuclear count

Myonuclear counts were performed in diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) embryos by panning through confocal stacks to find a series of nuclei that could unequivocally be assigned to a single myofiber and manually counting them. Ten fibres were counted per embryo. The effect of ploidy on the mean number of nuclei per

myofiber (n = 8 embryos per ploidy) was determined using a two-sample Student's t-test in R.

3.4.6 Vasculature

3.4.6.1 Vascular patterning

Overlapping confocal stacks were collected from the yolk extension to the vascular plexus of diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP; Flk1:mCherry) embryos using a Leica SP8 confocal microscope fitted with a 25× 0.9 NA lens. Images were assembled, Z-projected and made into annotated composites using FIJI. Inter-ISV space (mean of 3 adjacent ISV pairs dorsal to yolk sack extension) and ISV length (measured as the distance between the dorsal aorta (DA) and the dorsal longitudinal anastomotic vessel (DLAV, mean value of 3 measurements made at the centre of each ISV pair)) was measured using the line tool in FIJI. The lateral growth of the ISV was measured by reslicing the confocal stacks from anterior to posterior (0.568 µm stepsize) and measuring the distance between the midline of the dorsal aorta and the outermost part of the ISV (always below the lateral line) (Figure 6 C+D, green line). The effects of ploidy on inter-ISV distance, DA-DLAV distance and ISV lateral growth (n = 5 embryos per ploidy, p = 0.087) were assessed using two-sample Student's t-tests in R.

3.4.6.2 ISV cellularity

From the confocal stacks in 4.5.1, the number of nuclei in each ISV was determined by manually scanning through stacks and counting the flattened, GFP-positive nuclei embedded in the mCherry-positive blood vessels for 3 ISVs per embryo (Fig. 7A+B; 5 embryos per ploidy). ISVs are 3-dimensional structures and do not lie in a single plane *in vivo*, making it impossible to show a single image with all nuclei visible in a single blood vessel without nuclei from the surrounding myotome tissue impeding the view.

Individual planes from the stack were selected to highlight the dorsal (Fig. 7C+E, diploid and triploid respectively), medial (Fig. 7D+F, diploid and triploid respectively), and ventral (Fig. 7D+G, diploid and triploid respectively) ISV nuclei. Images were cropped, stitched and annotated using FIJI.

3.4.6.3 Vessel wall thickness

The red channel from the confocal stacks in 4.5.1 was imported into Fluorender^{75,76} to facilitate 3D rotation and optical slicing so I could measure thickness of the ISV (Figure 8 A+B and E+F) and dorsal aorta (Figure 8 C+D and G+H) vessel walls. Using the orthoslice functions in Fluorender, I navigated to the widest section of the blood vessel (excluding any nuclei) and measured the thickness of the vessel wall at three places along the vessel wall (averaged for each embryo). For the ISVs, I measured three ISVs per embryo and averaged them. Mean vessel thickness was compared between diploids and triploids by two-sample Student's t-test ($n = 5$ embryos, $p < 0.001$ in both cases).

3.5 References:

1. Burggren WW, Reyna KS. Developmental trajectories, critical windows and phenotypic alteration during cardio-respiratory development. *Resp. Physiol. Neurobi.* 2011;178:13-21.
2. Christou M, Iliopoulou M, Witten PE, Koumoundouros G. Segmentation pattern of zebrafish caudal fin is affected by developmental temperature and defined by multiple fusions between segments. *J Exp Zool Part B.* 2018;330:330-340.
3. Mendez-Sanchez JF, Burggren WW. Hypoxia-induced developmental plasticity of larval growth, gill and labyrinth organ morphometrics in two anabantoid fish: The facultative air-breather Siamese fighting fish (*Betta splendens*) and the obligate air-breather the blue gourami (*Trichopodus trichopterus*). *J. Morphol.* 2019;280:193-204.
4. Lafeunte E, Beldade P. Genomics of developmental plasticity in animals. *Front Genet.* 2019;10:720.

5. Matey V, Richards JG, Wang Y, Wood CM, Rogers J, Davies R, Murray BW, Chen X-Q, Du J, Brauner CJ. The effect of hypoxia on gill morphology and ionoregulatory status in the Lake Qinghai scaleless carp, *Gymnocypris przewalski*. *J Exp Biol*. 2008;211:1063-1074.
6. Keen AN, Klaiman JM, Shields HA, Gillis TE. Temperature-induced cardiac remodelling in fish. *J Exp Biol*. 2017;220:147-160.
7. Pan Y, Ern R, Morrison PR, Brauner C, Esbaugh AJ. Acclimation to prolonged hypoxia alters hemoglobin isoform expression and increases hemoglobin oxygen affinity and aerobic performance in a marine fish. *Sci. Rep*. 2017;7:7834.
8. Gilmour KM, Perry SF. Conflict and compromise: Using reversible remodeling to manage competing physiological demands at the fish gill. *Physiol*. 2018;33:412-422.
9. Williams KJ, Cassidy AA, Verhille CE, Lamarre S, MacCormack TJ. Diel cycling hypoxia enhances hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*): evidence of physiological and metabolic plasticity. *J Exp Biol*. 2019;222:jeb206045.
10. Hughes GM. Scaling of respiratory areas in relation to oxygen consumption of vertebrates. *Experientia*. 1984;40:519-524.
11. Meredith TL, Kajiura SM. Olfactory morphology and physiology of elasmobranchs. *J Exp Biol*. 2010;213:3449-3456.
12. Gillooly JF, Hein A, Damiani R. Nuclear DNA content varies with cell size across human cell types. *Cold Spring Harb. Perspect. Biol*. 2015;7:a019091.
13. Kleiber M, Rogers TA. Energy metabolism. *Annu. Rev. Physiol*. 1961;23:5-36.
14. Huxley J, Tessier G. Terminology of relative growth. *Nature*. 1936;137:780-781.
15. Miettinen TP, Björkland M. Mitochondrial function and cell size: An allometric relationship. *Trends Cell Biol*. 2017;27:393-402.

16. Anderson-Teixeira K, Savage VM, Allen AP, Gillooly JF. Allometry and metabolic scaling in ecology. In Janssen R. *Encyclopedia of Life Science*. West Sussex: John Wiley & Sons; 2009:1-10.
17. Kalinkat G, Rall B, Vucic-Pestic O, Brose U. The allometry of prey preferences. *PLoS one*. 2011;6:e25937.
18. Blackburn TM, Gaston KJ, Loder N. Geographic gradients in body size: a clarification of Bergmann's rule. *Divers Distrib*. 1999;5:165-174.
19. James G. Bergmann's Rules and Allen's Rules. In Trevathan, W. *The International Encyclopedia of Biological Anthropology*. Wiley-Blackwell;2018:1-3.
20. Tilkens MJ, Wall-Scheffler C, Weaver TD, Steudel-Numbers K. The effects of body proportions on thermoregulation: an experimental assessment of Allen's rule. *J. Hum. Evol*. 2007;53:286-291.
21. Alho JS, Herczeg G, Laugen AT, Räsänen K, Laurilas A, Merilä J. Allen's rule revisited: quantitative genetics of extremity length in the common frog along a latitudinal gradient. *J. Evol. Biol*. 2010;24:59-70.
22. Day SJ, Lawrence PA. Measuring dimensions: the regulation of size and shape. *Development*. 2000;127:2977-2987.
23. Müller G. Why an extended evolutionary synthesis is necessary. *Interface Focus*. 2017;7: 20170015.
24. Smith SJ, Rebeiz M, Davidson L. From pattern to process: studies at the interface of gene regulatory networks, morphogenesis, and evolution. *Curr. Opin. Genet. Dev*. 2018;51:103-110.
25. Amano T. Gene regulatory landscape of the sonic hedgehog locus in embryonic development. *Dev Growth Diff*. 2020;62:334-342.
26. Cavalier-Smith T. Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. *J. Cell. Sci*. 1978;34:247-278.

27. Gregory TR. The bigger the C-value, the larger the cell: Genome size and red blood cell size in vertebrates. *Blood Cells Mol. Dis.* 2001;27:830-843.
28. Gregory TR. Genome size and developmental complexity. *Genetica.* 2002;115:131-146.
29. Levy D, Heald R. Nuclear size is regulated by importin α and ntf2 in *Xenopus*. *Cell.* 2010;143:288-298.
30. Gregory TR, Hebert PDN. The modulation of DNA content: Proximate causes and ultimate consequences. *Genome Res.* 1999;9:317-324.
31. Otto S. The evolutionary consequences of polyploidy. *Cell.* 2007;131:452-462.
32. Braasch I, Postlethwait, J. Polyploidy in fish and the teleost genome duplication. In Soltis P, Soltis DE. *Polyploidy and Genome Evolution*. Berlin Heidelberg: Springer-Verlag; 2012:341-383.
33. Mueller R. Genome biology and the evolution of cell-size diversity. *Cold Spring Harb. Perspect. Biol.* 2015;7:a019125.
34. Masterson J. Stomatal size in fossil plants: Evidence for polyploidy in majority of angiosperms. *Science.* 1994;264:421-424.
35. Moghe G, Shiu S. The causes and molecular consequences of polyploidy in flowering plants. *Ann. N. Y. Acad. Sci.* 2014;1320:16-34.
36. Muller HJ. Why polyploidy is rarer in animals than in plants. *Am Nat.* 1925;59:346-353.
37. Ohno S. *Evolution by gene duplication*. Berlin Heidelberg: Springer-Verlag; 1970.
38. Dehal P, Boore J. Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol.* 2005;3:e314.

39. Hoegg S, Brinkmann H, Taylor JS, Meyer A. Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J. Mol. Evol.* 2004;59:190-203.
40. Postlethwait J. The zebrafish genome in context: Ohnologs gone missing. *J Exp Zool Part B.* 2007;308:563-577.
41. Pasquier J, Braasch I, Batzel P, Cabau C, Monfort J, Nguyen T, Jouanno E, Berthelot C, Klopp C, Journot L, Postlethwait J, Guiguen Y, Bobe J. Evolution of gene expression after whole-genome duplication: New insights from the spotted gar genome. *J Exp Zool Part B.* 2017;328:709-721.
42. Sullivan C, Lage C, Yoder J, Postlethwait J, Kim. Evolutionary divergence of the vertebrate TNFAIP8 gene family: Applying the spotted gar orthology bridge to understand ohnolog loss in teleosts. *PLoS ONE.* 2017;12:e0179517.
43. Opazo JC, Butts GT, Nery MF, Storz JF, Hoffmann FG. Whole-genome duplication and the functional diversification of teleost fish hemoglobins. *Mol Biol Evo.* 2013;30:140-153.
44. Wyatt RA, Keow JY, Harris ND, Haché CA, Li DH, Crawford BD. The zebrafish embryo: A powerful model system for investigating matrix remodeling. *Zebrafish.* 2009;6:347-354.
45. Matchett EF, Wang S, Crawford BD. Paralogues of mmp11 and timp4 interact during the development of the myotendinous junction in the zebrafish embryo. *J. Dev. Biol.* 2019;7:22.
46. Voldoire E, Brunet F, Naville M, Volff JN, Galiana D. Expansion by whole genome duplication and evolution of the sox gene family in teleost fish. *PLoS ONE.* 2017;12:e0180936.
47. Toloza-Villalobos J, Arroyo JI, Opazo JC. The circadian clock of teleost fish: A comparative analysis reveals distinct fates for duplicated genes. *J. Mol. Evol.* 2014;80:57-64.

48. Braasch I, Gehrke AR, Smith JJ, Kawasaki K, Manousaki T, Pasquier J, Amores A, Desvignes T, Batzell P, Catchen J, Berlin AM, Campbell MS, Barrell D, Martin KJ, Mulley JF, Ravi V, Lee AP, Nakamura T, Chalopin D, Fan S, Weisel D, Cañestro C, Sydes J, Beaudry FEG, Yi Sun, Hertel J, Beam MJ, Fasold M, Ishiyama M, Johnson J, Kehr S, Lara M, Letaw JH, Litman GW, Litman RT, Mikami M, Ota Y, Saha NR, Williams L, Stadler PF, Wang H, Taylor JS, Fontenot Q, Ferrara A, Searle SMJ, Aken B, Yandell M, Schneider I, Yoder JA, Volff JN, Meyer A, Amemiya CT, Venkatesh B, Holland PWH, Guiguen Y, Bobe J, Shubin NH, Di Palma F, Alföldi J, Lindblad-Toh K, Postlethwait JH. The spotted gar genome illuminates vertebrate evolution and facilitates human-teleost comparisons. *Nat. Genet.* 2016;48:427-437.
49. Lien S, Koop BF, Sandve SR, Miller JR, Kent MP, Nome T, Hvidsten TR, Leong JS, Minkley DR, Zimin A, Grammes F, Grove H, Gjvusland A, Walenz B, Hermansen RA, von Schalburg K, Rondeau EB, Di Genova A, Samy JKA, Vik JO, Vigeland MD, Caler L, Grimholt U, Jentoft S, Våge DI, de Jong P, Moen T, Baranski M, Palti Y, Smith DR, Yorke JA, Nederbragt AJ, Tooming-Klunderud A, Jakobsen KS, Jiang X, Fan Dingding, Hu Y, Liberles DA, Vidal R, Iturra P, Jones SJM, Jonassen I, Maass A, Omholt SW, Davidson WS. The Atlantic salmon genome provides insights into rediploidization. *Nature.* 2016;533:200-205.
50. Conant K. The lasting after-effects of an ancient polyploidy on the genomes of teleosts. *PLoS ONE.* 2020;15:e0231356.
51. Fankhauser G. Polyploidy in the salamander *Eurycea bislineata*. *J. Hered.* 1939;30:379-388.
52. Benfey TJ. The physiology and behavior of triploid fishes. *Rev Fish Sci.* 1999;7:39-67.
53. Miller KE, Brownlee C, Heald R. The power of amphibians to elucidate mechanisms of size control and scaling. *Exp. Cell Res.* 2020;392:112036.
54. Song C, Liu SJ, Xiao J, He WG, Zhou Y, Q QB, Zhang C, Liu Y. Polyploid organisms. *Sci. China Life Sci.* 2012;55:301-311.
55. Aversano R, Ercolano MR, Caruso I, Fasano C, Rosellini D, Carputo D. Molecular tools for exploring polyploid genomes in plants. *Int. J. Mol. Sci.* 2012;13:10316-10335.
56. Piferrer F, Beaumont, A, Falguière J-C, Flajšhans M, Haffray P, Colombo L. Polyploid fish and shellfish: Production, biology and applications to aquaculture

- for performance improvement and genetic containment. *Aquaculture*. 2009;293:125-156.
57. Myers JM, Hershberger WK, Iwamoto, RN. The induction of tetraploidy in salmonids. *J World Aquacult Soc*. 1986;17:1-7.
 58. Chourrout D, Chevassus B, Krieg F, Happe A, Burger G, Renard P. Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females - Potential of tetraploid fish. *Theor Appl Genet*. 1986;72:193-206.
 59. Heier J, Takle KA, Hasley AO, Pelegri F. Ploidy manipulation and induction of alternate cleavage patterns through inhibition of centrosome duplication in the early zebrafish embryo. *Dev Dynam*. 2015;244:1300-1312.
 60. Weber GM, Hostuttler MA, Semmens KJ, Beers BA. Induction and viability of tetraploids in brook trout (*Salvelinus fontinalis*). *Can J Fish Aquat Sci*. 2015;72:1443-1449.
 61. Baars DB, Takle KA, Heier J, Pelegri F. Ploidy manipulation of zebrafish embryos with heat shock 2 treatment. *J. Vis. Exp*. 2016;118:e54492.
 62. Kavumpurath S, Pandian TJ. Induction of triploidy in the zebrafish, *Brachydanio rerio* (Hamilton). *Aquac. Res*. 1990;21:299-306.
 63. Delomas TA, Dabrowski K. Why are triploid zebrafish all male? *Mol Reprod Dev*. 2018;85:612-621.
 64. van de Pol ILE, Flik G, Verberk WCEP. Triploidy in zebrafish larvae: Effects on gene expression, cell size and cell number, growth, development and swimming performance. *PLoS ONE*. 2020;15:e0229468.
 65. Benfey TJ. Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (*Salmo salar*) as a case study. *Rev Aquac*. 2015;8:264-282.
 66. Maxime V. The physiology of triploid fish: current knowledge and comparisons with diploid fish. *Fish Fish*. 2008;9:67-78.

67. Fraser TWK, Fjellidal PG, Hansen T, Mayer I. Welfare considerations of triploid fish. *Rev Fish Sci.* 2012;20:192-211.
68. Small C, Davis J, Crawford BD, Benfey TJ. Early, non-lethal ploidy and genome size quantification using confocal microscopy in zebrafish embryos. In prep.
69. Davidson AJ, Zon LI. The ‘definitive’ (and ‘primitive’) guide to zebrafish hematopoiesis. *Oncogene.* 2004;23:7233-7246.
70. Carradice D, Lieschke GJ. Zebrafish in hematology: Sushi or science? *Blood.* 2008;111:3331-3342.
71. Keenan SR, Currie PD. The developmental phases of zebrafish myogenesis. *J. Dev. Biol.* 2019;7:12.
72. Shi J, Pengpeng B, Jimin P, Hui L, Grishin NV, Bassel-Duby R, Chen EH, Olson EN. Requirement of the fusogenic micropeptide myomixer for muscle formation in zebrafish. *PNAS USA.* 2017;114:11950-11955.
73. Goody MF, Sher RB, Henry CA. Hanging on for the ride: Adhesion to the extracellular matrix mediates cellular responses in skeletal muscle morphogenesis and disease. *Dev Biol.* 2015;401:75-91.
74. Childs S, Chen J-N, Garrity DM, Fishman MC. Patterning of angiogenesis in the zebrafish embryo. *Development.* 2002;129:973-982.
75. Wan Y, Otsuna H, Chien C-B, Hansen C. FluoRender: An application of 2D image space methods for 3D and 4D confocal microscopy data visualization in neurobiology research. *IEEE Pac Vis Symp.* 2012:201-208.
76. Wan Y, Otsuna H, Holman HA, Bagley B, Masayoshi I, Lewis AK, Colasanto M, Kardon G, Ito K, Hansen C. FluoRender: joint freehand segmentation and visualization for many-channel fluorescence data analysis. *BMC Bioinformatics.* 2017;18:280.
77. Ojolick EJ, Cusack R, Benfey TJ, Kerr SR. Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture.* 1995;131:177-187.

78. Verhille C, Anttila K, Farrell AP. A heart to heart on temperature: Impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comp. Biochem. Physiol. A*. 2013;164:653-657.
79. Sambraus F, Olsen RE, Remen M, Hansen TJ, Torgersen T, Fjellidal PG. Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture*. 2017;473:1-12.
80. Riseth EN, Fraser TWK, Sambraus F, Stien LH, Hvas M. Is it advantageous for Atlantic salmon to be triploid at lower temperatures? *J. Therm. Biol.* 2020;89:102548.
81. Wyngaard GA, Rasch EM, Manning NM, Gasser K, Domangue R. The relationship between genome size, development rate, and body size in copepods. *Hydrobiologia*. 2005;532:123-137.
82. Zamir E, Kam Z, Yarden A. Transcription-dependent induction of G1 phase during the zebra fish midblastula transition. *Mol. Cell. Biol.* 1997;17:529-536.
83. Mukherjee RN, Sallé J, Dmitrieff S, Nelson KM, Oakey J, Minc N, Levy DL. The perinuclear ER scales nuclear size independently of cell size in early embryos. *Dev. Cell*. 2020;54:395-409.
84. Levy DL, Heald R. Nuclear size is regulated by importin α and ntf2 in *Xenopus*. *Cell*. 2010;143:288-298.
85. Brownlee C, Heald R. Importin α partitioning to the plasma membrane regulates intracellular scaling. *Cell*. 2019;176:805-815.
86. Lee MT, Bonneau AR, Giraldez AJ. Zygotic genome activation during the maternal-to-zygotic transition. *Annu. Rev. Cell. Dev. Biol.* 2014;30:581-613.
87. Syed S, Wilky H, Raimundo J, Lim B, Amodeo A. The nuclear to cytoplasmic ratio directly regulates zygotic transcription in *Drosophila* through multiple modalities. *bioRxiv*.
88. Chari S, Wilky H, Govindan J, Amodeo AA. Histone concentration regulates the cell cycle and transcription in early development. *Development*. 2019;146:dev177402.

89. Amodeo AA, Jukam D, Straight AF, Skotheim JM. Histone titration against the genome sets the DNA-to-cytoplasm threshold for the *Xenopus* midblastula transition. *PNAS*. 2015;112:E1086-E1095.
90. Chen H, Einstein LC, Little SC, Good, MC. Spatiotemporal patterning of zygotic genome activation in a model vertebrate embryo. *Dev Cell*. 2019;49:852-866.
91. Blythe SA, Wieschaus EF. Establishment and maintenance of heritable chromatin structure during early *Drosophila* embryogenesis. *eLife*. 2016;5:e20148.
92. Hansen TJ, Olsen RE, Stien L, Oppedal F, Torgersen T, Breck O, Remen M, Vågseth T, Fjellidal PG. Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*. 2015;435:354-360.
93. Benfey TJ, Sutterlin AM. The haematology of triploid landlocked Atlantic salmon, *Salmo salar* L. *J. Fish. Biol.* 1984;42:333-338.
94. Lamatsch DK, Steinlein C, Schmid M, Scharl M. Noninvasive determination of genome size and ploidy level in fishes by flow cytometry: Detection of triploid *Poecilia formosa*. *Cytometry*. 2000;39:91-95.
95. Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development*. 2007;134:4147-4156.
96. Bertrand JY, Cisson J, Stachura D. Notch signaling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. *Blood*. 2010;115:2777-2783.
97. Greenlee AR, Kersten CA, Cloud JG. Effects of triploidy on rainbow trout myogenesis *in vitro*. *J. Fish Biol.* 1995;46:381-388.
98. Johnston IA, Strugnell G, McCracken ML, Johnstone R. Muscle growth and development in normal sex ratio and all female diploid and triploid Atlantic salmon. *J. Exp. Biol.* 1999;202:1991-2016.
99. Choudhuri A, Fast EM, Zon ZI. Using zebrafish to study pathways that regulate hematopoietic stem cell self-renewal and migration. *Stem Cell Rep*. 2017;8:1465-1471.

100. Suresh V, Sheehan RJ. Biochemical and morphological correlates of growth in diploid and triploid rainbow trout. *J. Fish. Biol.* 2005;52:588-599.
101. Nathanailides C, Klaoudatos D, Perdikaris C, Klaoudatos S, Kolygas M, Athanassopoulou. Metabolic differentiation of diploid and triploid European sea bass juveniles. *Int Aquat Res.* 2019;11:199-206.
102. Peng L-M, Zheng Y, You F, Wu Z-H, Tan X, Jiao S, Zhang P-J. Comparison of growth characteristics between skeletal muscle satellite cell lines from diploid and triploid olive flounder *Paralichthys olivaceus*. *PeerJ.* 2016;4:e1519.
103. Jackson HE, Ingham PW. Control of muscle fibre-type diversity during embryonic development: The zebrafish paradigm. *Mech. Dev.* 2013;130:447-457.
104. Williams MLK, Sawada A, Budine T, Yin C, Gontarz P, Solnica-Krezel L. Gon4l regulates notochord boundary formation and cell polarity underlying axis extension by repressing adhesion genes. *Nat. Comm.* 2018;9:1319.
105. Hara Y, Merten C. Dynein-based accumulation of membranes regulates nuclear expansion in *Xenopus laevis* egg extracts. *Dev. Cell.* 2015;33:562-575.
106. Chen H, Good MC. Size regulation: Big insights from little cells. *Dev. Cell.* 2016;37:392-394.
107. Torres-Vázquez J, Gitler AD, Fraser SD, Berk JD, Pham VN, Fishman MC, Childs S, Epstein JA, Weinstein BM. Semaphorin-Plexin signaling guides patterning of the developing vasculature. *Dev. Cell.* 2004;7:117-123.
108. Bahrami N, Childs SJ. Development of vascular regulation in the zebrafish embryo. *Development.* 2020;147:dev183061.
109. Chang S-S, Tu S, Baek KI, Pietersen A, Liu Y-H, Savage VM, Hwang S-PL, Hsiai TK, Roper M. Optimal occlusion uniformly partitions red blood cells fluxes within a microvascular network. *PLoS Comput Biol.* 2017;13:e1005892.
110. Gillooly JF, Gomez JP, Mavrodiev EV, Rong Y, McLamore ES. Body mass scaling of passive oxygen diffusion in endotherms and ectotherms. *PNAS.* 2016;113:5340-5345.
111. Yamaguchi K, Jürgens KD, Bartels H, Piiper J. Oxygen transfer properties and dimensions of red blood cells in high-altitude camelids, dromedary camel and goat. *J Comp. Physiol B.* 1987;157:1-9.

112. Chakraborty S, Balakotoah V, Bidani A. Diffusing capacity reexamined: relative roles of diffusion and chemical reaction in red cell uptake of O₂, CO, CO₂, and NO. *J. Appl. Physiol.* 2004;97:2284-2302.
113. Simonot DL, Farrell AP. Cardiac remodelling in rainbow trout *Oncorhynchus mykiss Walbaum* in response to phenylhydrazine-induced anaemia. *J. Exp. Biol.* 2007;210:2574-2584.
114. Simonot DL, Farrell AP. Coronary vascular volume remodelling in rainbow trout *Oncorhynchus mykiss*. *J. Fish. Biol.* 2009;75:1762-1772.
115. Wang Y, Zhang M, Qin Q, Peng Y, Huang X, Wang C, Coa L, Li W, Tao M, Zhang C, Liu S. Transcriptome profile analysis on ovarian tissues of autotetraploid fish and diploid red crucian carp. *Front. Genet.* 2019;10:208.
116. McMillen P, Chatti V, Jülich D, Holley SA. A sawtooth pattern of cadherin2 stability mechanically regulates somite morphogenesis. *Curr. Biol.* 2016;26:542-549.
117. Pauls S, Geldmacher-Voss B, Campos-Ortega JA. A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for *in vivo* studies of embryonic development. *Dev. Genes. Evol.* 2001;211:603-610.
118. Choi J, Dong L, Ahn J, Doa D, Hammerschmidt M, Chen J-N. FoxH1 negatively modulates *flkl* gene expression and vascular formation in zebrafish. *Dev. Biol.* 2007;304:735-744.
119. Wang Y, Kaiser MS, Larson JD, Nasevicius A, Clark KJ, Wadman SA, Roberg-Perez SE, Ekker SC, Hackett PB, McGrail M, Essner JJ. Moesin1 and Ve-cadherin are required in endothelial cells during *in vivo* tubulogenesis. *Dev.* 2010;137:3119-3128.
120. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev. Dyn.* 1995;203:253-310.
121. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White, DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: An open-source platform for biological-image analysis. *Nat Meth.* 2012;9:676-682.
122. R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. URL: <https://www.R-project.org/>.

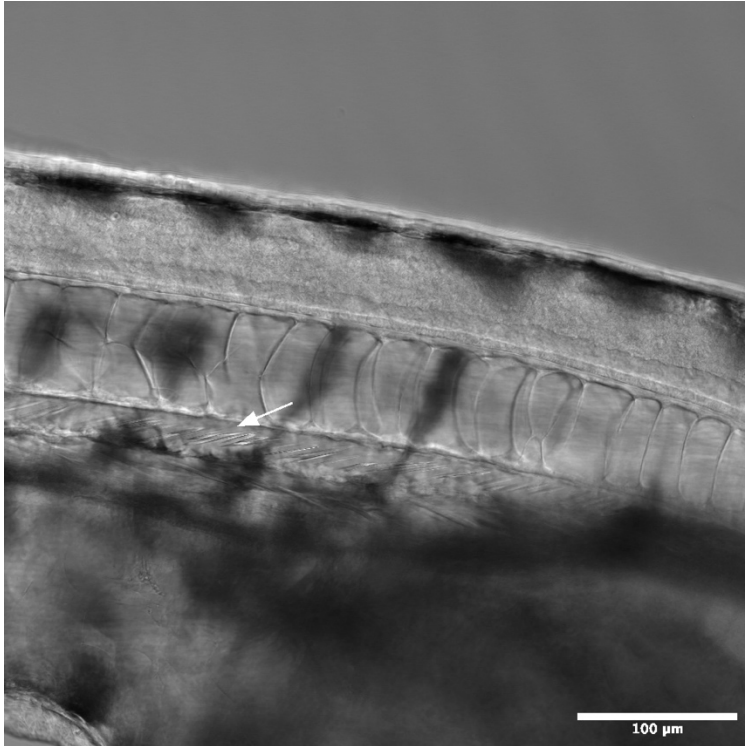


Figure 3.S1. DIC micrograph of streaking blood cells in dorsal aorta (white arrow).

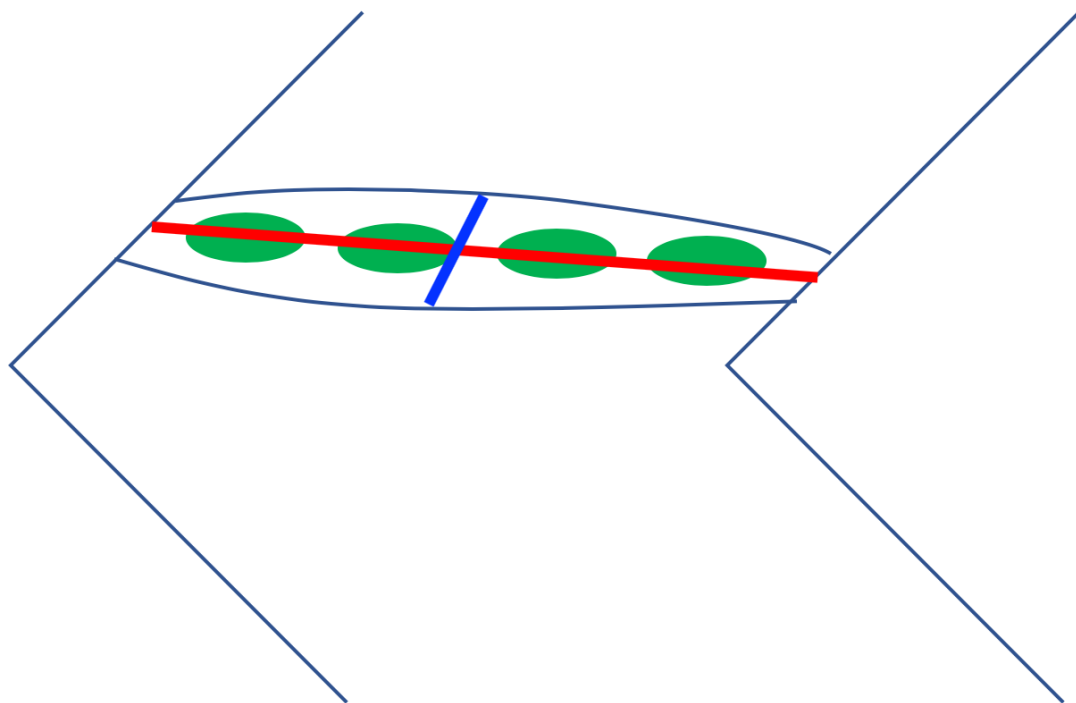


Figure 3.S2. Cartoon model of a multi-nucleated myofiber spanning a myotome.

Chevron-shaped lines represent the myotendinous junction. Red line denotes myofiber length, blue line denotes myofiber width, green ovals denote the nuclei.

4.0 Chapter 4:

Triploids have fewer hematopoietic stem cells and slower steady-state rate of erythropoiesis

Christopher D. Small, Krista MacRae, Bryan D. Crawford, and Tillmann. J. Benfey.

Abstract

The blood of triploid fish is comprised of fewer, larger cells such that hemoglobin concentration is not affected by ploidy, but it is not yet clear if erythrocyte turnover is affected by ploidy. By adapting standard hematological assays for use in small fish, I have shown that this “fewer, larger cells” pattern holds true in triploid zebrafish making them a viable model for studying triploid hematology. Using myotome myofiber nuclear volume analysis to determine ploidy *in vivo* combined with transgenic fish with tissue-specific fluorescent reporters, I have demonstrated that triploids have fewer hematopoietic stem cells and a slower rate of steady-state erythropoiesis. Curiously, triploids were able to recover from experimentally-induced anemia within the same time frame as diploids but it is still not clear if triploids take longer to recover as the time frame of the experiment was too long to capture the rate of recovery.

4.1 Introduction

Ploidy transitions have been relatively rare in the evolution of animals compared to their frequency in plants (Müller, 1925; Masterson, 1994; Braasch and Postlethwait, 2012; Mueller, 2015). Ohno’s (1970) prediction that there were two rounds of genome duplication at the base of the vertebrate lineage (referred to as the 2-R hypothesis) has since been supported through the advent of genomic technologies (Ohno, 1970; Dehal

and Boore, 2005; Braasch and Postlethwait, 2012). Most subsequent ploidy transitions (often referred to as Whole Genome Duplications – abbreviated WGDs) among vertebrates have occurred in teleost and amphibian lineages (Mable, 2004; Mable et al., 2011; Braasch and Postlethwait, 2012; Mueller, 2015). Research on WGDs has traditionally focused on underlying genetic mechanisms and consequences to understand what happens to duplicated genes in subsequent generations (Postlethwait, 2007; Braasch and Postlethwait, 2012; Lien et al., 2016; Braasch et al., 2016; Conant, 2020). These studies have emphatically revealed the role genome duplication has played in evolution by relaxing selection on conserved genes, thereby allowing one copy to mutate and potentially evolve novel functions (Richards et al., 2003; Bystriancki et al., 2006 and 2007; Fagernes et al., 2015). Increasing evolvability could certainly be advantageous *per se*, but little is known about the negative consequences of increased ploidy that have limited its occurrence in evolutionary history.

The correlation between DNA content and cell size is consistent across tissues and taxa (Gregory, 2001; Gregory and Mable, 2005; Gillooly et al., 2015), but the organismal-level response to increased cell size varies among plants, invertebrates, and vertebrates. In plants and many invertebrates, body size is positively correlated with ploidy as the number of cells in tissues and organs is independent of ploidy (Aversano et al., 2012; Nell, 2002; Clarke et al., 2018). In contrast, vertebrates (and some invertebrates) respond to increased ploidy by reducing cell number such that polyploid organisms are no larger than conspecific diploids (Fankhauser, 1939; Benfey, 1999; Piferrer et al., 2009; Miller et al., 2020). Although the physiological implications of building an organism from fewer, larger subunits are not well-studied from an evolutionary perspective, there has been decades of research striving to tease apart the

subtle physiological differences between diploid and experimentally-produced triploid fish (especially salmonids) for the purpose of optimizing triploid performance in aquaculture. By extrapolating what we learn from these more common diploid/triploid comparisons, we can improve our understanding of ploidy transitions in the evolution of vertebrates.

Interest in triploid physiology revolves around the observation that triploid fish are reproductively sterile (Benfey, 1999; Piferrer et al., 2009; Benfey, 2016). If farmed fish escape their physical confinement, this added layer of genetic confinement prevents gene flow from farmed to wild populations and the establishment of invasive species. Moreover, the ovary fails to develop in triploid females so there is no sexual maturation and, therefore, maturation-induced deterioration of flesh quality is prevented (Poontawee et al., 2007; Piferrer et al., 2009; Benfey, 2016). However, triploids are not widely used in aquaculture as they tend to underperform compared to diploids (Maxime, 2008; Fraser et al., 2012; Benfey, 2016). Taking into account the number of ploidy transitions that have persisted in evolution along with the fact that captive rearing conditions are typically optimized for diploids has led some to surmise that maybe polyploids are not well-suited for environments that favour diploids (Riseth et al., 2020). A mechanistic understanding of the trade-offs between the advantages of increased gene copy number at higher ploidy levels and the physiological consequences of having fewer, larger cells is necessary to predict which conditions favour WGDs in evolution perhaps by minimizing the disadvantages associated with polyploidy that have discouraged their adoption by fish farmers.

The physiological differences between diploids and triploids typically relate to their ability to meet oxygen demands. Triploids succumb sooner and more frequently,

and experience more negative sub-lethal effects, than diploids when challenged at high temperature and low oxygen (Ojolick et al., 1995; Verhille et al., 2013; Hansen et al., 2015; Scott et al., 2014; Sambraus et al., 2017 and 2018) or when exercised (Hyndman et al., 2003; Bernier et al., 2004) so research has focused on hematology, aerobic metabolism, and the cardiorespiratory system. While I have recently shown that different tissues have distinct compensatory strategies in response to ploidy changes (Chapter 3), blood is the classic example of a tissue with a “fewer but larger cells” pattern typically ascribed to triploid tissues. Hematocrit and hemoglobin concentration remain constant as ploidy increases due to the reduction in cell number matched by an increase in the amount of hemoglobin per cell (Benfey, 1999; Benfey and Sutterlin, 1984). Although there have been many studies comparing hematology between diploids and triploids, most have focused on the biochemical and metabolic properties of whole blood or mature erythrocytes without considering how erythropoiesis, erythrocyte senescence, and erythrocyte turnover are affected by ploidy. In this chapter, I investigate the effects of ploidy on erythrocyte age demographics in an effort to elucidate the cellular mechanisms underlying physiological differences between diploid and triploid fish.

Vertebrate erythrocytes are continuously produced from a population of self-renewing hematopoietic stem cells (HSCs) that reside in a highly conserved hematopoietic niche (though the anatomical location of this niche varies among taxa) (Martinez-Agosto et al., 2007; Wattrus and Zon, 2018; Kapp et al., 2018; Mahony and Bertrand, 2019). Mammalian erythrocytes are enucleated during differentiation and so they are unable to respond transcriptionally when perturbed (Nikinmaa, 1990). As such, mammalian erythrocytes are short-lived with an average half-life of ~30 days in mice and ~120 days in humans (Franco, 2012; Franco et al., 2013; Higgins, 2015). In order to

replenish and maintain this tissue, mammals must produce billions of new erythrocytes every day and so have large numbers of HSCs that colonize the bone marrow throughout the body (Franco et al., 2013; Higgins, 2015; Kapp et al., 2018; Mahony and Bertrand, 2019). In contrast, teleost erythrocytes remain nucleated and transcriptionally active for most of their lifespan (Nikinmaa, 1990; Götting and Nikinmaa, 2017), but there are very few studies that provide empirical measurements of steady-state erythropoiesis, erythrocyte lifespan, and erythrocyte turnover in fish (Witeska, 2013). Teleosts have relatively few HSCs, and they reside only in the anterior kidney. Absolute numbers defining erythrocyte half-life for any species of teleost are scarce and often conflicting (Weinberg et al., 1976; Avery et al., 1992; Fischer et al., 1998; Morera and MacKenzie, 2011), but the discovery that fixed-angle centrifugation can be used to sort cells into distinct age cohorts provides a means to test for differences between young and old cells even without precise estimates for their chronological age (Lund et al., 2000; Lewis et al., 2012; Götting and Nikinmaa, 2017).

Lund and colleagues (2000) demonstrated in rainbow trout (*Oncorhynchus mykiss*) erythrocytes that while DNA content and total protein concentration do not decline as cells age, there is 90% less total RNA in the oldest age cohort compared to the youngest. Similarly, the rate of total protein synthesis in the oldest cohort is 80% lower than that of the youngest cohort and there is a dramatic reduction in the cellular heat shock response in older erythrocytes at both the mRNA level and the protein level for various genes in this pathway (Lund et al., 2000). The reduction in transcription from young to old cells is also accompanied by a reduction in the number of mitochondria (Moyes et al., 2002) and reduced aerobic metabolism (Phillips et al., 2000; Moyes et al., 2002). Using RNAseq, Götting and Nikinmaa (2017) recently showed that this markedly

reduced transcription appears to be a programmed shut-down rather than a random process since many key housekeeping genes are still expressed in older cells, thus demonstrating that erythrocyte senescence and turnover is a highly regulated process. Taken together, these data suggest that there is a non-negligible population of senescent erythrocytes in circulation that function solely as carriers of hemoglobin that can no longer respond transcriptionally to environmental perturbations. In line with this idea, erythrocyte age demographics are shifted towards a younger cohort following thermal stress in rainbow trout, with a massive increase in the number of young erythrocytes without an increase in hematocrit or hemoglobin (Lewis et al., 2012).

The simplest explanation for these observations is that older erythrocytes are unable to mount a heat shock response in reaction to thermal stress and are rapidly cleared from circulation to be replaced by newer erythrocytes generated by a burst of erythropoiesis. Indeed, transcriptome analysis of rainbow trout blood following heat stress reveals that hematopoietic genes are significantly upregulated 24 hours after stress (Lewis et al., 2010). It follows, then, that a fish with a greater proportion of these aged, transcriptionally-inert erythrocytes in circulation would be less able to respond to physiological stressors and return itself to homeostasis. Intriguingly, Saranyan et al. (2017) recently demonstrated that triploid brook charr (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) consistently had lower blood titres of HSP70, HSP90, HSF1, and ubiquitin (free and total), indicative of reduced capacity to mount a heat shock response and of impaired protein turnover. These data are consistent with a model in which triploids have a preponderance of aged, senescent erythrocytes and therefore are limited in their ability to cope with aerobic stressors. In agreement with these data, triploids were independently shown to have lower basal titres of bilirubin in circulation

indicative of reduced erythrocyte turnover (Hansen et al., 2015), further supporting this idea regarding reduced capacity for erythropoiesis in triploids.

Until recently, little was known about erythropoiesis in non-mammalian vertebrates, making it difficult to pursue these questions mechanistically (Nikinmaa, 1990; Murad et al., 1990). With its hundreds of mutants and transgenics specific to the hematopoietic system, the zebrafish (*Danio rerio*) now provides a powerful system in which to study vertebrate erythropoiesis (Carradice et al., 2008; Wattus and Zon, 2018). The “Odyssey of the Hematopoietic Stem Cell” (Burns and Zon, 2006) has been characterized in exquisite detail from specification, when HSCs first intravasate through the ventral wall of the dorsal aorta at 26 hours post-fertilization (hpf) into circulation, until they extravasate into the caudal hematopoietic tissue at 36 hpf (Murayama et al., 2006; Kissa et al., 2008). While in this transient hematopoietic niche, HSCs differentiate, proliferate, and mature before re-entering circulation to colonize the definitive hematopoietic organs (anterior kidney for erythropoiesis) (Murayama et al., 2006; North et al., 2007). By taking advantage of transgenic tools built to visualize and dissect hematopoiesis in zebrafish, such as transgenics with HSC-specific fluorescent reporters (Tg(c-myb:eGFP; flk1:mCherry)) and erythrocyte-specific fluorescent reporters (Tg(gata1a:dsRed)), we can design experiments to test hypotheses pertaining to erythropoiesis and erythrocyte turnover in triploids.

Triploidy can be induced in zebrafish by preventing the extrusion of the second polar body using an abrupt shift in temperature (Kavumpurath and Pandian, 1990; Van de Pol et al., 2020) or by rapidly increasing hydrostatic pressure (Chapter 2). Ploidy level can be determined in embryos using myofiber nuclear volume analysis either *in vivo* using Tg(h2a.f/z:H2A.f/z::eGFP) embryos with a fluorescent histone subunit or in

fixed embryos stained with fluorescent markers that bind to DNA, such as DAPI or Draq5. In adults, ploidy can be determined by measuring erythrocyte length from a blood smear (Chapter 2).

The aim of this study was to compare the erythropoietic system between diploid and triploid zebrafish by quantifying the number of hematopoietic stem cells and comparing the relative proportion of young, newly differentiated erythrocytes in the blood. Due to the small size of the zebrafish, I first designed pilot studies to confirm that: A) I could make accurate hematological measurements from very small volumes of blood ($<10\ \mu\text{L}$), B) it is feasible to repeatedly (two weeks apart) sample small volumes of blood from adult zebrafish, and C) triploid zebrafish exhibit the pattern of hematological differences between ploidies in other species. I then quantified the number of definitive HSCs in diploid and triploids using both an assay relying on a single genetic marker (Tg(c-myb:eGFP)) (Dimri et al., 2014) and another, more specific assay that uses two genetic markers to label only the definitive HSCs (Esain et al., 2016). Using another transgenic that produces the fluorescent protein dsRed only during erythrocyte differentiation (Long et al., 1997; Di Maria et al., 1999; Travers et al., 2003; Lenard et al., 2016), I then designed and validated an assay to characterize erythrocyte age demographics and the relative rate of erythropoiesis in adult Tg(gata1a:dsRed) zebrafish in order to compare steady-state and stress-induced erythropoiesis in diploids and triploids.

4.2 Methods

4.2.1 Animal care

Juvenile brook charr were haphazardly selected from a population of fish bred and reared at the University of New Brunswick Brook Charr Facility. Fish were fed once

daily to apparent satiation (Floating Trout Feed #5, Corey Aquafeeds, Fredericton, NB, Canada). Adult zebrafish were reared in flow-through dechlorinated municipal water in the University of New Brunswick Zebrafish Facility in standard 25 x 11 x 15 cm tanks (Pentair Aquatic Ecosystems, Apopka, FL) at 28°C on a 14:10 h light:dark photoperiod. They were fed adult zebrafish pellets (Gemma 500 Micro, Skretting, Vancouver, BC, Canada) twice per day supplemented with live *Artemia* once per day. Embryos were reared in glass Petri dishes in Embryo Rearing Medium (ERM: 13 mM NaCl, 0.5 mM KCl, 0.02 mM Na₂HPO₄, 0.04 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃, pH 7.4) with 0.01% methylene blue at 28.5°C until 120 hpf when they were transferred to 1L larval tanks. Larvae were fed a series of increasingly larger pellets (Gemma Micro 75-300, Skretting,) using automated feeders set to feed 4 times per day. The transgenic lines used in this study were Tg(h2a.f/z:H2A:f/z::eGFP) (Pauls et al., 2001), Tg(c-myb:eGFP) (North et al., 2007) in a *mifta*^{-/-}, *royr*^{-/-} background (Travers et al., 2003), Tg(c-myb:eGFP; flk1:mCherry) (Wang et al., 2010) in a *mifta*^{-/-}, *royr*^{-/-} background, and Tg(gata1a:dsRed) (Travers et al., 2003). All procedures involving animals were approved by the UNB Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care.

4.2.2 Hematology

Small volume hematocrit experiment

Blood from three juvenile brook charr anaesthetized in 1% tertiary amyl alcohol (Sigma-Aldrich) was collected into heparinized Vacutainer tubes (Thomas Scientific, Swedesboro, New Jersey, USA) and kept on ice for no longer than 1 hour before analysis. A micropipette was used to transfer specific volumes (0.5, 1, 2, 5 and 10 µL) of this blood onto a piece of waxed paper and each entire drop was then drawn up into a

separate hematocrit tube (Sigma-Aldrich) and centrifuged for 10 minutes (IEC International Micro-capillary centrifuge Model MB; Fisher Scientific, Waltham, MA, USA), after which they were laid flat and imaged using a Leica MZ205A dissecting microscope with a DFC360 FX greyscale low-light camera attachment. Hematocrits were calculated in triplicate from each image using the line tool in FIJI (Schindelin et al., 2012).

Blood was collected from eight adult Tg(gata1a:dsRed) zebrafish using a modified protocol from Zang et al. (2013). Hypodermic needles (28.5 gauge) attached to 1 mL hypodermic syringes were pre-heparinized by drawing a heparin-saline solution (18 I.U. / mL in 0.9% (w/v) NaCl) up and down and given at least two hours to air dry. Fish were anaesthetized in tris-buffered MS-222 (168 μ g / mL) (Westerfield, 2000) and 10-15 μ L of blood was collected from the caudal vasculature through the body wall, ventral to the lateral line. The blood was then expelled from the syringe (after first removing the needle) into 0.5 mL microfuge tubes. Any blood that could not be expelled from the syringe after removing the needle was collected using a 20 μ L pipette and added to the microfuge tube. Blood was kept on ice for up to 15 minutes before analysis. Specific volumes of blood (0.5, 1, 2 and 5 μ L) were then collected in 9 μ L microhematocrit tubes (Sigma) by pipetting the desired volumes of blood onto waxed paper and drawing it up using capillary action. Hematocrit was measured using a microhematocrit centrifuge (StatSpin CritSpin, Beckman-Coulter, Brea, California, USA). Due to the small volume of blood obtained from zebrafish, hematocrit was only measured once at each volume for each fish. All blood samples collected from adult zebrafish hereafter used this protocol except the sampling volume was adjusted for each experiment.

Repeated blood sampling in zebrafish

Blood samples (6-8 μ L) were collected and hematocrit measured from 20 adult Tg(gata1a:dsRed) zebrafish as described above. Fish were promptly returned to their home tank to recover, but one fish did not recover from the procedure. Fourteen days after the initial sampling, blood was collected from these same 19 fish and hematocrit was again measured. There were no mortalities after this second sampling. The effect of repeated sampling on hematocrit was analyzed using a paired Student's t-test (R Core Team, 2019).

Diploid/Triploid comparison

Diploid and triploid Tg(h2a.f/z:H2A.f/z::eGFP) zebrafish were produced by *in vitro* fertilization followed by a hydrostatic pressure treatment (58.6 MPa for 5 minutes) starting between 70 and 85 seconds post-fertilization in half of the zygotes to induce triploidy (Chapter 2). Triploid embryos were identified using myofiber nuclear volume analysis as described in a previous study (Chapter 2) and 30 individuals of each ploidy reared to adulthood in separate tanks. At 6 months old, 10 μ L of blood was collected (as described above) from the surviving 27 diploid and 22 triploid adults. Blood smears were made from 0.5 μ L of blood and imaged using DIC microscopy (Leica DM RXA25 microscope with a 63X lens with a Leica DC500 camera attachment) within 1 hour of sampling. Erythrocyte length (major axis) was measured (30 cells per fish) using the line tool in FIJI. Cell counts were made in triplicate using a Neubauer hemocytometer after diluting blood 1:200 into Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl₂, 5.0 mM HEPES, pH 7.2; Westerfield, 2000). Hematocrit was measured in triplicate using the zebrafish hematocrit protocol described above from 1 μ L of blood per tube.

The effect of ploidy on erythrocyte length, cell count and hematocrit were each analyzed by unpaired Student's t-tests using R.

4.2.3 Counting hematopoietic stem cells in 36 hpf embryos

Single transgene label

HSCs were first quantified in the caudal hematopoietic tissue at 36 hpf using a single transgenic label (Tg(c-myb:eGFP)) in a *mitfa*^{-/-}, *royr*^{-/-} background, relying on the well-described anatomical position of HSCs in the caudal hematopoietic tissue as the more dorsal of two strips of cells that express *c-myb* at this time point, the other (more ventral) being precursor primordial germ cells (Dimri et al., 2014; Esain et al., 2016). Diploids and triploids were produced as above except that ploidy was determined post-hoc (as described below) due to the lack of the Tg(h2a.f/z:H2A.f/z::eGFP) transgene. The microscopy was performed while blind to presumptive ploidy to avoid any bias. Embryos were anaesthetized in MS-222 (168 µg / mL) at 36 hpf, mounted onto a slide, and caudal hematopoietic tissue imaged using a Leica SP2 confocal microscope fitted with a 20x 0.5 NA lens. Embryos were imaged as rapidly as possible, alternating between controls (n=8) and putative triploids (n=8) for an elapsed time of one hour to minimize the effects of HSC proliferation and developmental rate on HSC number. One putative triploid was determined to be a diploid and was therefore excluded from further analysis. Z-projections of the confocal stacks were produced using FIJI and HSCs (GFP-positive cells in the dorsal strip) were counted manually. The effect of ploidy on this number was analyzed using an unpaired Student's t-test.

Double transgene label

In a subsequent experiment, specificity of HSC identification was improved by using the double transgenic label Tg(c-myb:eGFP; flk1:mCherry) in a *mitfa*^{-/-}, *royr*^{-/-}

background. Similar to the single transgene protocol, three untreated (diploid) control and three putative triploid embryos were anaesthetized and imaged (blind to ploidy) at 36 hpf using a Leica SP8 confocal microscope fitted with a 25×0.9 NA water immersion lens. Confocal stacks from the green channel (*c-myb*) and the red channel (*flkl*) were overlaid in FIJI so that *bona fide* HSCs (that express both *c-myb* and *flkl*) appear as yellow. HSCs were counted by manually scrolling through the stacks and the effect of ploidy on the number of HSCs was compared using an unpaired Student's t-test. To ensure that only cells located in the caudal hematopoietic tissue were counted, the overlaid confocal stacks were resliced in FIJI from left to right and cells that did not reside within the vasculature of the caudal hematopoietic tissues were not counted.

Ploidy validation without the Tg(h2a.f/z:H2A.f/z::eGFP) transgene

Diploid and triploid zebrafish were produced using the early pressure protocol described above (Chapter 2) except that myofiber nuclear analysis to determine ploidy was conducted post-hoc in fixed embryos stained using the nucleic acid stain Draq5 (Sigma). Briefly, embryos were carefully removed from the slide after imaging using watchmaker forceps, sorted into individually labelled microfuge tubes, and fixed overnight at 4°C in Dent's fixative (80% methanol, 20% dimethyl sulfoxide) to abolish most of the fluorescence from the transgenic fluorescent proteins. Embryos were then washed thrice with PBS with 0.1% Triton-X100 (PBSTx) and stained overnight at 4°C with 20 μ M Draq5 in PBSTx. Stained embryos were washed thrice in PBSTx, mounted and imaged again using a Leica SP2 confocal microscope fitted with a 63x 0.7 NA lens, and myofiber nuclear volume analysis was performed in FIJI as described in Chapter 2.

4.2.4 Measuring erythropoiesis using Tg(gata1a:dsRed) zebrafish

Bleeding-induced erythropoietic recovery pilot study

Thirty adult, homozygous Tg(*gata1a:dsRed*) zebrafish were haphazardly sorted into three groups of 10 fish: initial control (C1), final control (C2) and bled treatment (T). Groups C1 and T were anaesthetized in MS-222 (168 µg / mL) and ~10-15 µL of blood collected using the protocol described in section 4.2.2, with no mortalities. Three blood smears were generated from each blood sample and erythrocytes were imaged precisely 10 minutes after blood smears were produced, providing only enough time for cells to settle. DIC and epifluorescence micrographs were captured sequentially using a Leica DM RXA25 microscope with a 63× 0.7 NA lens using a Leica DC500 camera attachment. Images for each fish were sorted into individual folders and assigned a random number as a folder name by an independent observer (www.random.org) so the data could be analyzed while blind to treatment. One hundred erythrocytes were selected and marked on the DIC image to blind the observer to the fluorescent signal while identifying cells for fluorescence intensity measurements. Care was taken to select cells that were not damaged or overlapping any other cells. Using a custom macro in FIJI that measures mean fluorescence intensity in a circle (radius of 5 pixels) centred on a mouse-click, I measured cytoplasmic fluorescence intensity for each of the 100 erythrocytes marked on the DIC micrograph. Fluorescence intensity was measured 3 times for each erythrocyte (in non-overlapping regions of the cytoplasm) and then averaged. Cells were then grouped by mean fluorescence intensity into two bins using a threshold of 150 A.U. to calculate how many erythrocytes express *gata1a* (highly fluorescent, newly differentiated erythrocytes) relative to weakly- or non-fluorescent erythrocytes. Cell count and hematocrit were determined for each blood sample as described in section 4.2.2, using 1 µL per microhematocrit tube. After a two-week recovery period, this protocol was repeated using the second control group (C2; naïve fish) and the bled

treatment group. Differences in number of highly fluorescent erythrocytes, hematocrit and cell count between groups C1, C2, T (initial) and T (final) were analyzed using one-way ANOVAs followed by Tukey's Honest Significant Differences post-hoc analyses to identify differences among groups.

Diploid/Triploid comparison

Diploid and triploid Tg(gata1a:dsRed) embryos were generated from adults that were homozygous for the transgene as described in section 4.2.2. Thirty diploid and 30 putative triploid embryos were raised in separate tanks (as in section 4.2.1). At 6 months, 6-8 μL of blood was collected from each fish using the protocol from section 4.2.2 to produce blood smears (in triplicate; 0.5 μL per slide) and to measure hematocrit (in triplicate; 1 μL per microhematocrit tube). Blood smears were analyzed using the fluorescence intensity protocol described in section 4.2.4, and hematocrit and circulating cell count was measured as in section 4.2.2. Ploidy was determined by measuring erythrocyte length using the DIC micrographs of the blood smears confirming that all fish in the experiment were of the expected ploidy. This process was repeated after a two-week recovery period. The effect of ploidy on the number of strongly fluorescent cells, cell count and hematocrit were analyzed using two-way ANOVAs with ploidy and sampling time as factors.

4.3 Results

4.3.1 Hematology

Hematocrit measurements from small volumes of brook charr blood (Figure 4.1A) were consistent from 10 μL down to 1 μL , never varying by more than $\pm 2\%$ for any individual fish. At 0.5 μL , mean hematocrit was 10-15% lower than in larger samples and the within-individual replicates varied by more than 10% in two of the three fish assayed

(Figure 4.1B). This same pattern was found for zebrafish, including a cut-off between 0.5 μ L for repeatable measurements (Figure 4.1C). Repeated sampling of small volumes of blood (6-8 μ L) from adult zebrafish was feasible (only one mortality between blood samples) and hematocrit recovered between sampling events ($p = 0.953$; Figure 4.1D). Triploid erythrocytes were significantly larger than diploid erythrocytes ($p < 0.001$, Figure 4.2A-C). Cell count was significantly lower in triploids ($p < 0.001$, Figure 4.2D) but hematocrit was not affected by ploidy ($p = 0.078$, Figure 4.2E).

4.3.2 Hematopoietic stem cells in 36 hpf embryos

The number of HSCs in the dorsal-most strip of GFP⁺ cells in Tg(*c-myb*:eGFP) embryos at 36 hpf was significantly lower in triploids (15 ± 3 , $n = 7$) than in diploids (20 ± 3 HSCs, $n = 8$) ($p < 0.01$, Figure 4.3). The same pattern was observed in the double transgenic with significantly fewer GFP⁺/mCherry⁺ HSCs in the triploids (9 ± 2 , $n = 3$) than the diploids (14 ± 1 , $n = 3$) ($p < 0.05$, Figure 4.4). HSC counts were higher in the less specific, single transgenic assay, but the difference between ploidies was fairly consistent, with approximately 25 and 35 % fewer HSCs in triploids when counted using only *c-myb* expression and more precisely using both *c-myb* and *flkl* expression as markers, respectively.

4.3.3. Measuring rate of erythropoiesis using Tg(*gata1a*:dsRed) zebrafish.

The effect of removing blood from adult Tg(*gata1a*:dsRed) zebrafish on the proportion of highly-fluorescent erythrocytes was significant (Figure 4.5A-C, $n = 10$ fish per group, $p < 0.001$). Tukey's post-hoc analysis revealed that there were no significant differences among the two control groups and the initial treatment group, but the final treatment group of previously bled fish had a significantly greater proportion of highly-fluorescent erythrocytes in circulation. There were no significant differences in hematocrit

(Figure 4.5D, $n = 10$ fish per group, $p = 0.115$) or cell count (Figure 4.5E, $n = 10$ fish per group, $p = 0.6$) among any of the groups.

The proportion of highly-fluorescent erythrocytes in circulation was significantly affected by both ploidy and sampling time ($p < 0.001$ in both cases), but the interaction between ploidy and time was not significant ($p = 0.21$). Triploids had significantly fewer highly-fluorescent erythrocytes at both time points, and the number of these cells was higher at the second sampling than the first in both ploidies (Figure 4.5F). Relative to initial values, the proportions of highly-fluorescent erythrocytes increased by 61% in diploids and 67% in triploids in the two weeks between sampling events. Hematocrit was not affected by ploidy or sampling time (Figure 4.5H; $p = 0.470$ and 0.232 , respectively). Cell count was significantly affected by ploidy ($p < 0.001$) but not sampling time ($p = 0.138$) or the interaction term ($p = 0.160$), with triploid cell counts significantly lower than diploid (Figure 4.5H).

4.4 Discussion

Vertebrate erythrocytes come in a surprisingly diverse variety of shapes and sizes despite sharing the primary function of delivering oxygen to tissues and removing carbon dioxide from them (Nikinmaa, 1990; Snyder and Sheafor, 1999). Mammalian erythrocytes extrude their nucleus and mitochondria during erythropoiesis, and so, despite being much smaller, their hemoglobin concentration is very high relative to other vertebrates (Ji et al., 2011). Avian erythrocytes are similarly small and packed with hemoglobin, but their mitochondria remain intact and active while their nucleus is highly condensed but likely still transcriptionally active (Morera and MacKenzie, 2011; Stier et al., 2013; Clark, 2014). Fish and amphibian erythrocytes are substantially larger and their nucleus occupies a significant amount of the space inside the cell, therefore

limiting the amount of hemoglobin that can be carried by each cell (Snyder and Sheafor, 1999). Despite these fascinating taxonomic patterns across vertebrate phyla, the literature is dominated by studies focused on mammalian or fish erythrocytes so we must make inferences relying mostly on data from these two taxa (Nikinmaa, 1990; Snyder and Sheafor, 1999; Götting and Nikinmaa, 2015).

In mammalian erythrocytes, small size and lack of the nucleus facilitates deformability and perfusion of capillaries with a lumen diameter smaller than that of the cells that traverse them (Chien, 1987; Nader et al., 2019) at the cost of transcriptional regulation of the proteome. This inability to transcribe genes and thereby change the proteins being produced necessitates the rapid turnover of these very short-lived cells (Franco, 2012; Franco et al., 2013; Higgins, 2015). In contrast, fish erythrocytes maintain transcription throughout most of their lifespan allowing them to respond to environmental stressors at the level of gene expression (Lewis et al., 2010 and 2012; Saranyan et al., 2017; Götting and Nikinmaa, 2017), but their rheology is impaired as a result of the retained nucleus and larger size (Nash and Egginton, 1993). Fish typically have fewer circulating erythrocytes and lower blood hemoglobin concentration than mammals, perhaps reflective of their lower metabolic rates or limiting the upper limits of their metabolic rate (Nikinmaa, 1990; Hillman et al., 2013; Van de Pol et al., 2017). We are lacking rigorous, empirical measurements of erythrocyte lifespan and turnover for fish, but we can infer that fish erythrocytes will likely be longer-lived with much slower turnover rates due to their ability to maintain transcription and recycle their proteins to maintain function as they age.

In order to maintain homeostasis, mammals must produce billions of new erythrocytes every day (Franco, 2012; Franco et al., 2013; Higgins, 2015) and this

requires an extensive network of hematopoietic tissues. As such, HSC niches in adult mammals are located throughout the bone marrow so there are large numbers of these cells distributed throughout the body that can produce the enormous numbers of newly differentiated erythrocytes required (Baron et al., 2012; Palis, 2014; Martinez-Agosto et al., 2017, Kapp et al., 2018). There have been fewer studies on the rate of erythropoiesis in adult fish aside from studies aimed at measuring erythropoietic recovery following the induction of anemia using phenylhydrazine (Chudzik and Houston, 1983; Murad et al., 1990; Houston and Murad, 1992; Simonot and Farrell, 2007 and 2009) or bleeding (Weinberg et al., 1976; McLeod et al., 1978). The primary hematopoietic niche in adult fish is localized entirely in the anterior-most section of the kidney (known as the head kidney) (Burns and Zon, 2006, Esain et al., 2016; Kobayashi et al., 2019). Comparing the size of the hematopoietic tissues relative to overall body size in mammals to that in fish suggests that the demand and capacity for erythropoiesis is much lower in fish. If the triploid hematopoietic system conforms to the standard triploid pattern of fewer, larger cells (Benfey, 1999), then we would predict that triploids will have even fewer HSCs than diploids and produce erythrocytes at a slower rate.

In this study, I have shown that it is feasible to use classic hematology techniques to make reliable hematological measurements in adult zebrafish despite their small size, and that hematology of triploid zebrafish conforms to the typical pattern shown for other triploid fishes, i.e., larger, fewer erythrocytes and no difference in hematocrit (Benfey, 1999). Using two transgenic lines to visualize and count HSCs in embryonic zebrafish, I observe that triploid zebrafish consistently have fewer HSCs in the caudal hematopoietic tissue at 36 hpf. Although these assays do not directly compare the number of HSCs in the anterior kidney of adult triploids, several groups have demonstrated that the number

of HSCs in this transient tissue correlates to the number of HSCs in adults when measured using flow cytometry on dissected and dissociated kidneys (Harris et al., 2013; Dimri, et al., 2014; Esain et al., 2016; Choudhuri et al., 2017; Kapp et al., 2018; Mahony and Bertrand, 2019; Kobayashi et al., 2019). It would be interesting to confirm that this correlation holds true for triploids as well, as it is conceivable that the number of HSCs in adults is somewhat plastic and that they expand this HSC pool to a greater degree due to having a smaller pool initially. Without the ability to confirm this empirically in my thesis, I proceed under the assumption that this correlation is true but acknowledge that I have not ruled out this alternative hypothesis.

Expression of *gata1* is a highly conserved feature in the differentiation and maturation of both mammalian and fish erythrocytes but in both cases, *gata1* expression ceases before terminal maturation (Long et al., 1997; De Maria et al., 1999; Yaqoob et al., 2009). Tg(*gata1a:dsRed*) zebrafish produce a red fluorescent protein in their differentiating erythrocytes (Long et al., 1997; Yaqoob et al., 2009) that should no longer be produced upon erythrocyte maturation when *gata1a* expression ceases. This transgenic has been recently used to directly image erythrocyte toxicity (Eum et al., 2016; Lenard et al., 2016) and subsequent regeneration (Lenard et al., 2016) *in vivo* in embryos, but I am not aware of any studies using Tg(*gata1a:dsRed*) zebrafish to study erythropoiesis and erythrocyte demographics in adults. This transgenic line has been used extensively to study erythropoiesis and hemorheology in embryos when all of the circulating erythrocytes are newly differentiated and therefore express high levels of dsRed, making them strongly fluorescent (Yaqoob et al., 2009; Lenard et al., 2016; Eum et al., 2016). I observe that only 10%-20% of circulating erythrocytes are strongly fluorescent in adult Tg(*gata1a:dsRed*) zebrafish consistent with other studies that noted

that approximately 10%-12% of circulating erythrocytes (depending on species) were immature erythrocytes (erythroblasts) based on their morphology (Keen et al., 1989; Quentel and Obach, 1992). The remaining 80%-90% circulating cells are either weakly fluorescent or not fluorescent at all. The weak fluorescence is likely caused by residual amounts of dsRed protein that remains in the cytoplasm after cessation of *gatala* expression that has not yet been turned-over by the proteosome. I therefore grouped these cells with the non-fluorescent cells for analyses. I reasoned that the relative proportion of highly-fluorescent erythrocytes in circulation compared to the number of non-fluorescent cells would be a measure of the rate of erythropoiesis and erythrocyte demographics if hematocrit and circulating cell numbers remain constant, i.e., as the number of highly-fluorescent erythrocytes in circulation increases, the more young, transcriptionally active cells are present. Inducing anemia by bleeding fish is a classic technique to induce erythropoiesis (Weinberg et al., 1976; McLeod et al., 1978), so I stimulated erythropoiesis in this way in adult Tg(*gatala*:dsRed) and observed that the ratio of fluorescent to non-fluorescent cells does indeed increase. Having validated this model for measuring the rate of erythropoiesis, I produced triploid Tg(*gatala*:dsRed) zebrafish and observed that triploids consistently have fewer young, *gatala* expressing erythrocytes in circulation. Surprisingly, the rate of recovery from anemia was comparable between ploidies with a 61% increase in the proportion of *gatala* expressing cells in diploids compared to a 67% increase in triploids. Since hematological values of both diploids and triploids returned to homeostatic levels between sampling events, there could be a difference in the recovery time but I was unable to capture this difference because I sampled after recovery rather than during recovery due to fish welfare concerns. Measuring the rate of erythropoietic recovery would require reducing the

amount of time between samples in order to collect samples before hematocrit and circulating cell counts return to baseline levels. Regardless, the proportion of newly-differentiated, *gata1a* expressing cells was significantly lower in triploids at baseline and after recovery from anemia suggesting that triploids will have proportionally fewer new, transcriptionally-active cells and therefore, by extension, more aging, senescent cells.

The transcriptional response of circulating erythrocytes in teleosts is a widely-studied phenomenon, especially with respect to probing mechanisms of tolerance and acclimation to environmental challenges such as high temperature (Currie et al., 2005; Lewis et al., 2010; Jeffries et al., 2012; Ooman and Hutchings, 2017) and hypoxia (Martos-Sticha et al., 2017; Ooman and Hutchings, 2017; Pan et al., 2017; Pelster and Egg, 2018). Recent work demonstrating that erythrocytes lose the ability to respond transcriptionally as they age (Götting and Nikinmaa, 2017) suggests that we need to consider erythrocyte age demographics along with the standard hematological variables such as hematocrit and hemoglobin. If a significant proportion of the cells in a blood sample are not able to respond to an experimental treatment, then standard gene expression techniques such as qPCR and RNAseq are unable to distinguish between low levels of transcript expressed by all cells in circulation from a large number of transcripts expressed by only a few cells in circulation. Studies such as repeated thermal challenges (Lewis et al., 2012; Corey et al., 2017) or hypoxic challenges (Williams et al., 2019) that demonstrate an increased response or increased tolerance in subsequent exposures of a stressor could be interpreted as an abrupt shift in erythrocyte age demographics following pre-treatment. Having more cells in the young, transcriptionally-active age class means that more of the cells are able to mount a heat

shock response thereby improving tolerance (Lund et al., 2000, Lewis et al., 2012; Phillips et al. 2000).

Triploids have been shown to have higher routine or standard metabolic rates, and hence reduced aerobic scope, when tested at temperatures optimized for diploids, leading many to suggest that triploids have lower thermal optima (Atkins and Benfey, 2008; O'Donnell et al., 2017; Leal et al., 2019; Riseth et al. 2020). Saranyan et al. (2017) posited that the lower basal titres of heat shock proteins at any temperature in triploids suggests that they would have higher standard metabolic rates as they would have to spend more energy refolding damaged proteins. To compound this, data I present here showing that triploids cycle their erythrocytes more slowly suggests that triploids have more old, transcriptionally-inactive erythrocytes, and that the lower titres of heat shock proteins could be a result of the breakdown of the proteosome and protein recycling. This would exacerbate the small metabolic inefficiencies predicted by Saranyan and colleagues (2017) and could explain the higher standard metabolic rate that is thought to be the culprit for their reduced aerobic scope at higher temperatures.

4.5 Conclusion

From an evolutionary perspective, the effects of ploidy on erythrocyte demographics highlights another physiological difference between fish of different ploidy levels that is relevant for understanding the evolutionary consequences of polyploidy. Evolutionary innovations are clearly made possible by genome duplications, and so it is essential to understand the trade-offs. Fish at higher ploidy levels have fewer HSCs and this slows erythrocyte turnover thereby shifting erythrocyte age demographics to an older cohort perhaps causing consequences such as a reduced capacity to mount an appropriate transcriptional response to stressors and higher basal metabolic rates. The

benefits of extra gene copies have been demonstrated, but it is still unclear what sorts of environments would minimize the physiological disadvantages of polyploidy such that evolution has time to innovate. The zebrafish model will be an invaluable asset for elucidating these mechanisms.

Acknowledgements

The authors would like to thank Robyn O'Keefe, Robyn Shortt, and Krista MacRae for excellent fish care, Stephen Cogswell, and Susan Belfry from UNB Microscopy and Microanalysis for technical assistance. This research was funded by NSERC Discovery Grants (BDC and TJB).

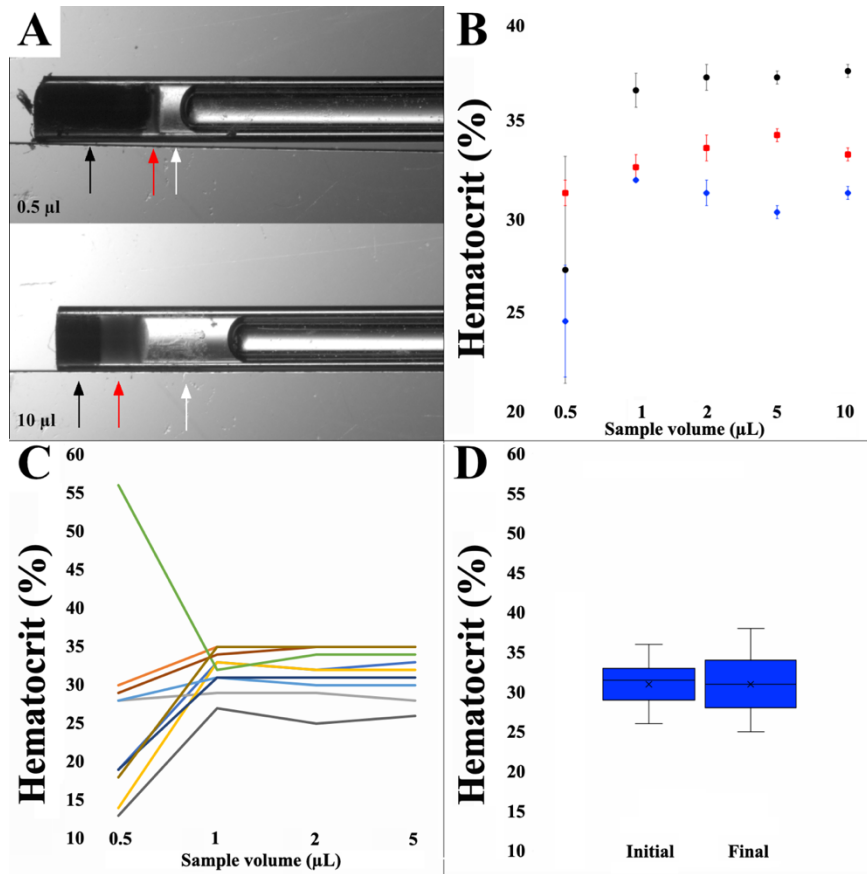


Figure 4.1. Hematocrit can be reliably measured in small volumes ($\geq 1 \mu\text{L}$) of blood (**A**, black arrow is putty, red arrow is erythrocyte fraction, white arrow is plasma; brook charr blood). Samples smaller than $1 \mu\text{L}$ are unreliable in **B**) brook charr blood (mean $\pm$ SEM for 3 replicates per sample; different colours represent individual fish) and **C**) zebrafish blood (different colours represent individual fish; note that lines are not intended to suggest a continuum but are included to demonstrate the threshold below which samples are too small to reliably measure hematocrit). Note that the lines in Figure 4.1C are not intended to suggest a continuum but were included to visually demonstrate the threshold after which samples are too small to reliably measure hematocrit. **D**) Collecting

small volumes of blood (6-8 μL) at two-week intervals did not significantly reduce hematocrit (mean $\pm$ SEM for 20 (initial) and 19 (final) fish; $p = 0.953$).

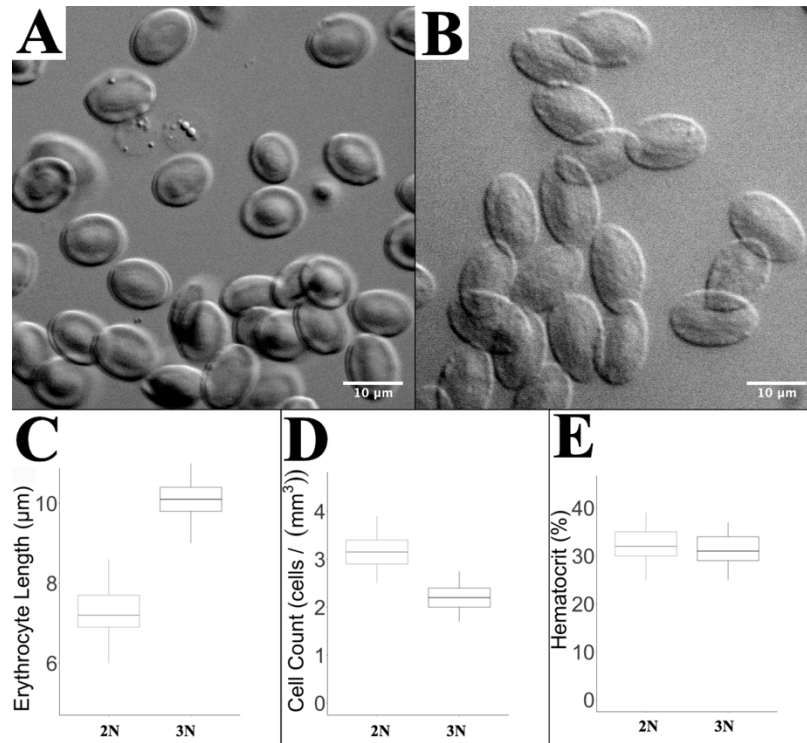


Figure 4.2. Triploid zebrafish have fewer, larger erythrocytes than diploids.

A+B) DIC micrographs of erythrocytes from diploid (A) and triploid (B) adult

zebrafish. **C)** Triploid erythrocytes are larger than diploid erythrocytes

($p < 0.001$). **D)** Cell count is reduced in triploids ($p < 0.001$), but **E)** hematocrit is

unaffected by ploidy ($p = 0.078$). Mean $\pm$ SEM; $n = 27$ diploids and 22 triploids.

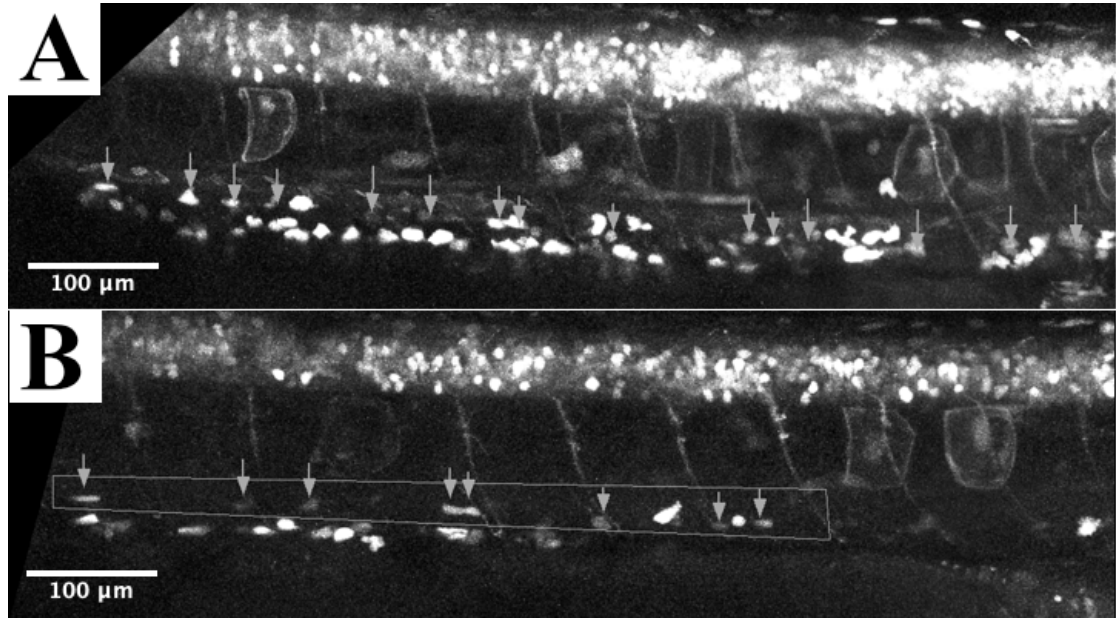


Figure 4.3. Triploids have fewer GFP-positive cells in the caudal hematopoietic tissue of Tg(c-myb:eGFP) zebrafish embryos than diploids at 36 hpf. **A+B)** Stitched Z-projections from confocal stacks of the section of the caudal hematopoietic tissue dorsal to the yolk stack extension of a diploid (A) and a triploid (B). Presumptive hematopoietic stem cells are indicated by the arrows.

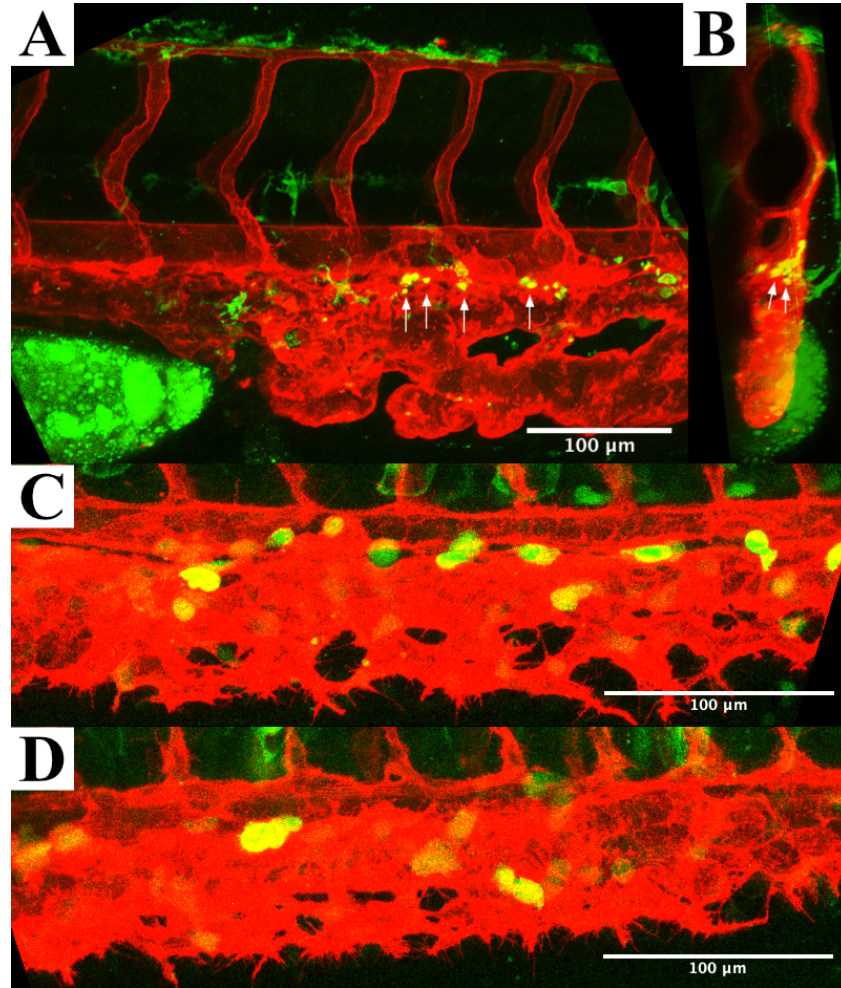


Figure 4.4. Triploid zebrafish embryos have fewer hematopoietic stem cells than diploid embryos at 36 hpf. **A)** Z-projections of an overlay from confocal stacks of Tg(c-myb:eGFP; flk1:mCherry) of a zebrafish embryo. Arrows indicate the double positive GFP⁺ / mCherry⁺ hematopoietic stem cells that appear as yellow in the overlay of the green and red channels. **B)** An optical cross-section made by reslicing the confocal stacks from (A) along the x-axis. Z-projection of the resliced stacks confirm that yellow hematopoietic stem cells (arrows) are restricted to the caudal hematopoietic tissue. **C-D)** The number of hematopoietic stem cells is significantly affected by ploidy. Cropped Z-projections of the

caudal hematopoietic tissue in a diploid (C) and a triploid embryo (D). Arrows indicate hematopoietic stem cells.

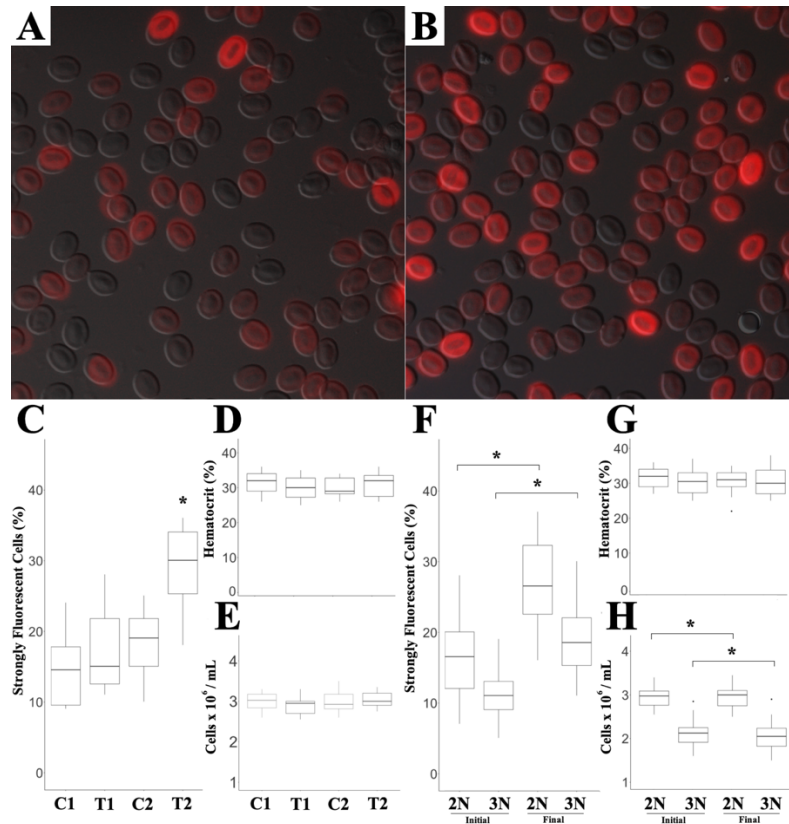


Figure 4.5. Erythropoietic recovery can be measured using Tg(gata1a:dsRed) zebrafish. **A+B)** Epifluorescent micrographs overlaid onto DIC micrographs of blood smears from a control (A) and previously-bled (B) Tg(gata1a:dsRed) zebrafish (both diploid). **C-E)** The number of strongly fluorescent erythrocytes increased significantly in previously bled fish (T2) compared to when blood was collected 14 days earlier (T1) and to naïve, unbled control fish at both times (C1 and C2) (C, 100 cells per fish, $n = 10$ fish per group, $p < 0.001$). There was no effect of treatment on hematocrit (D, $p = 0.115$) or cell count (E, $p = 0.6$). **F-H)** The number of strongly fluorescent erythrocytes was significantly affected by ploidy (2N= diploid and 3N = triploid) and sampling time (F, 100 cells per fish, 30 fish per ploidy, $p < 0.001$ for both factors). Hematocrit (G) was not affected by ploidy ($p = 0.47$) or sampling time ($p = 0.232$). Cell count (H) was

significantly affected by ploidy ($p < 0.001$) but was not sampling time ($p = 0.138$).

Asterisks denote significant differences among groups.

4.6 References:

- Atkins, M.E., Benfey, T.J., 2008. Effect of temperature on routine metabolic rate in triploid salmonids. *Comp. Biochem. Physiol.* 149A, 157–161.
<https://doi.org/10.1016/j.cbpa.2007.11.004>.
- Aversano, R.E., Ercolano, M.R., Caruso, I., Fasano, C., Rosellini, D., Carputo, D, 2012. Molecular tools for exploring polyploid genomes in plants. *Int. J. Mole. Sci.* 13, 10316-10335. <https://doi.org/10.3390/ijms130810316>.
- Avery E.H., Lee B.L., Freedland R.A., Cornelius C.E, 1992. Bile pigments in gallbladder and freshly-secreted hepatic duct bile from fed and fasted rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol.* 101, 857-61.
[https://doi.org/10.1016/0300-9629\(92\)90370-6](https://doi.org/10.1016/0300-9629(92)90370-6).
- Baron, M., Isern, J., Fraser, S.T., 2012. The embryonic origins of erythropoiesis in mammals. *Blood.* 119, 4828-4837. <https://dx.doi.org/10.1182%2Fblood-2012-01-153486>
- Benfey, T.J., 1999. The physiology and behavior of triploid fishes. *Rev. Fish.* 7, 39-67.
<http://dx.doi.org/10.1080/10641269991319162>.
- Benfey T.J., Sutterlin, A.M., 1984. The haematology of triploid landlocked Atlantic salmon, *Salmo salar* L. *J. Fish Biol.* 24, 333-338.
<http://dx.doi.org/10.1111/j.1095-8649.1984.tb04804.x>.
- Benfey, T.J., 2016. Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (*Salmo salar*) as a case study. *Rev. Aquac.* 8, 262-282. <https://doi.org/10.1111/raq.12092>.
- Bernier, N.J., Brauner, C.J., Heath, J.W., Randall, D.J., 2004. Oxygen and carbon dioxide transport during sustained exercise in diploid and triploid chinook

salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat.* 61, 1797-1805.

<http://dx.doi.org/10.1139/f04-110>.

Braasch, I.P., Postlethwait, J.H., 2012. Polyploidy in fish and the teleost genome

duplication. In: Soltis, P.A., Soltis, D.E. (Eds.), *Polyploidy and Genome*

Evolution (1st ed., p. 341-386). Berlin: Springer. https://doi.org/10.1007/978-3-642-31442-1_17.

Braasch, I.P., Gehrke, A.R., Smith, J.J., Kawasaki, K., Manousaki, T., Pasquier, J.,

Amores, A., Desvignes, T., Batzel, P., Catchen, J., Berlin, A.M., Campbell, M.S.,

Barrell, D., Martin, K.J., Mulley, J.F., Ravi, V., Lee, A.P., Nakamura, T.,

Chalopin, D., Fan, S., Weisel, D., Cañestro, C., Sydes, J., Beaudry, F.E.G., Yi,

S., Hertel, J., Beam, M.J., Fasold, M., Ishiyama, M., Johnson, J., Kehr, S., Lara,

M., Letaw, J.H., Litman, G.W., Litman, R.T., Mikami, M., Ota, Y., Saha, N.R.,

Williams, L., Stadler, P.F., Wang, H., Taylor, J.S., Fontenot, Q., Ferrara, A.,

Searle, S.M.J., Aken, B., Yandell, M., Schneider, I., Yoder, J.A., Volff, J.N.,

Meyer, A., Amemiya, C.T., Venkatesh, B., Holland, P.W.H., Guiguen, Y., Bobe,

J., Shubin, N.H., Di Palma, F., Alföldi, J., Lindblad-Toh, K., Postlethwait, J.H

2016. The spotted gar genome illuminates vertebrate evolution and facilitates

human-teleost comparisons. *Nat. Genet.* 48, 427-437.

<https://doi.org/10.1038/ng.3526>.

Burns, C.E., Zon, L.I., 2006. Homing sweet homing: Odyssey of hematopoietic stem

cells. *Immunity.* 25, 859-862. <https://doi.org/10.1016/j.immuni.2006.11.006>.

Bystriancki, J.S., Richards, J.G., Schulte, P.M., Ballantyne, J.S., 2006. Reciprocal

expression of gill Na⁺/K⁺-ATPase α -subunit isoforms α 1a and α 1b during sea

- water acclimation of three salmonid fishes that vary in their salinity tolerance. *J. Exp. Biol.* 209, 1848-1858. <https://doi.org/10.1242/jeb.02188>.
- Bystrianski, J.S., Frick, N.T., Richards, J.G., Schulte, P.M., Ballantyne, J.S., 2007. Failure to up-regulate gill Na⁺,K⁺-ATPase α -subunit isoform α 1b may limit seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*). *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* 148, 332-338. <https://doi.org/10.1016/j.cbpa.2007.05.007>.
- Carradice D., Lieschke G.J., 2008. Zebrafish in hematology: Sushi or science? *Blood.* 111, 3331-3342. <https://doi.org/10.1182/blood-2007-10-052761>.
- Chien, S., 1987. Red cell deformability and its relevance to blood flow. *Annu. Rev. Physiol.* 49, 177-192. <https://doi.org/10.1146/annurev.ph.49.030187.001141>.
- Choudhuri, A., Fast, E.M., Zon, L.I., 2017. Using zebrafish to study pathways that regulate hematopoietic stem cell self-renewal and migration. *Stem Cell Rep.* 8, 1465-1471. <http://dx.doi.org/10.1016/j.stemcr.2017.05.018>.
- Chudzik, J., Houston, H., 1983. Temperature and erythropoiesis in goldfish. *Can. J. Zool.* 61, 1322-1325.
- Clark, P., 2014. Assessment of avian erythrocytes that exhibit variant nuclear morphology. *Comp. Clin. Path.* 24, 485-490. <http://doi.org/10.1007/s00580-014-1926-6>.
- Clarke, E.K., Rivera Gomez, K.A., Mustachi, Z., Murph, M.C., Schvarzstein, M., 2018. Manipulation of ploidy in *Caenorhabditis elegans*. *J. Vis. Exp.* 133, e57296. <https://doi.org/10.3791/57296>.

- Conant, G.C., 2020. The lasting after-effects of an ancient polyploidy on the genomes of teleosts. *PLoS ONE*. 15, e0231356.
<https://doi.org/10.1371/journal.pone.0231356>.
- Corey, E., Linnansaari, T., Cunjak, R.A., Currie, S., 2017. Physiological effects of environmentally relevant, multi-day thermal stress on wild juvenile Atlantic salmon (*Salmo salar*). *Conserv. Physiol.* 5, cox014.
<https://doi.org/10.1093/conphys/cox014>.
- Currie, S., Moyes, C.D., Tufts, B.L., 2005. The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: *in vivo* and *in vitro* comparisons. *Jour. Fish. Biol.* 56, 398-408.
<https://doi.org/10.1111/j.1095-8649.2000.tb02114.x>.
- Dehal P, Boore J., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. *PLoS Biol.* 3, 1700-1708.
<https://doi.org/10.1371/journal.pbio.0030314>.
- De Maria, R., Zeuner, A., Eramo, A., Domenichelli, C., Bonci, D., Grignani, F., Srinivasula, S.M., Alnemri, E.S., Testa, U., Peschle, C., 1999. Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. *Nature*. 401, 489-493. <https://doi.org/10.1038/46809>.
- Dimri, M., Joshi, J., Chakrabarti, R., Sehgal, N., Sureshababu, A., Kumar, I.P., 2014. Todalazine protects zebrafish from lethal effects of ionizing radiation: Role of hematopoietic cell expansion. *Zebrafish*. 12, 33-47.
<https://doi.org/10.1089/zeb.2014.0992>.
- Esain, V., Cortes, M., North, T.E., 2016. Enumerating hematopoietic stem and progenitor cells in zebrafish embryos. In: Kawakami, K., Patton, E.E. Orger, M.

- (Eds). *Zebrafish: Methods and protocols* (2nd edition, 191-206).
<https://doi.org/10.1007/978-1-4939-3771-4>.
- Eum, J., Kwak, J., Kim, H. J., Ki, S., Lee, K., Raslan, A.A., Park, O.K., Chowdhury, M.A.U., Her, S., Kee, Y., Kwon, S-H., Hwang, B.J, 2016. 3D visualization of developmental toxicity of 2,4,6-trinitrotoluene in zebrafish embryogenesis using light-sheet microscopy. *Int. J Mol. Sci.* 17, 1925.
<https://doi.org/10.3390/ijms17111925>.
- Fagernes, C.E., Stensløkken, K-O., Røhr, Ä.K., Berenbrink, M., Ellefsen, S., Nilsson, G.E., 2015. Extreme anoxia tolerance in crucian carp and goldfish through neofunctionalization of duplicated genes creating a new ethanol-producing pyruvate decarboxylase pathway. *Sci. Rep.* 7. <https://doi.org/10.1038/s41598-017-07385-4>.
- Fankhauser, G., 1939. Polyploidy in the salamander, *Eurycea bislineata*. *J. Hered.* 30, 379-388.
- Fischer, U., Ototake, M., Nakanishi, T., (1998). Life span of circulating blood bells in ginbuna crucian carp (*Carassius auratus langsdorfii*). *Fish Shellfish Immunol.* 8, 339-349. <https://doi.org/10.1006/fsim.1998.0144>.
- Franco, R.S., 2012. Measurement of red cell lifespan and aging. *Transfus. Med. Hemother.* 39, 302-307. <https://dx.doi.org/10.1159/000342232>.
- Franco, R.S., Puchulu-Campanella, M.E., Barber, L.A., Palascak, M.B., Joiner, C.H., Low, P.S., Cohen, R.M., 2013. Changes in the properties of normal human red blood cells during *in vivo* aging. *Am. J. Hematol.* 88, 44-51.
<https://doi.org/10.1002/ajh.23344>.

- Fraser, T.W.K., Fjellidal, P.G., Hansen, T., Mayer, I., 2012. Welfare considerations of triploid fish. 20, 192-211. <https://doi.org/10.1080/10641262.2012.704598>.
- Gillooly, J.F., Hein, A., Damiani, R., 2015. Nuclear DNA content varies with cell size across human cell types. *Cold Spring Harb. Perspect. Biol.* 7, a019091. <https://doi.org/10.1101/cshperspect.a019091>.
- Götting, M., Nikinmaa, M.J., 2015. More than hemoglobin – The unexpected diversity of globins in vertebrate red blood cells. *Physiol. Rep.* 3, e12284. <https://dx.doi.org/10.14814%2Fphy2.12284>.
- Götting, M., Nikinmaa, M.J., 2017. Transcriptomic analysis of young and old erythrocytes of fish. *Front. Physiol.* 8, 1046. <https://doi.org/10.3389/fphys.2017.01046>.
- Gregory, T.R., 2001. Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. *Biol. Rev.* 76, 65-101. <https://doi.org/10.1017/S1464793100005595>.
- Gregory, T.R., Mable, B.K., 2005. Polyploidy in animals. In: Gregory, T.R. (Eds.). *The Evolution of the Genome* (1st ed., p. 427-517). <https://doi.org/10.1016/B978-012301463-4/50010-3>.
- Hansen, T.J., Olsen, R.E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T., Fjellidal, P.G., 2015. Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*. 435, 354-360. <http://dx.doi.org/10.1016/j.aquaculture.2014.10.017>.
- Harris, J.M., Esain, V., Frechette, G.M., Harris, L.J., Cox, A.G., Cortes, M., Garnaas, M.K., Carroll, K.J., Cutting, C.C., Khan, T., Elks, P.M., Renshaw, S.A., Dickinson, B.C., Chang, C.J., Murphy, M.P., Paw, B.H., Vander Heiden, M.G.,

- Goessling, W., North, T.E., 2013. Glucose metabolism impacts the spatiotemporal onset and magnitude of HSC induction *in vivo*. *Blood*. 121, 2483-2493. <https://doi.org/10.1182/blood-2012-12-471201>.
- Higgins, J.M., 2015. Red blood cell population dynamics. *Clin Lab Med*. 35, 43-57. <https://dx.doi.org/10.1016%2Fj.cll.2014.10.002>.
- Hillman, S.S., Hancock, T.V., Hedrick, M.S., 2013. A comparative meta-analysis of maximal aerobic metabolism of vertebrates: implications for respiratory and cardiovascular limits to gas exchange. *J. Comp. Physiol. B*. 183, 167-179. <https://doi.org/10.1007/s00360-012-0688-1>.
- Houston, A.H., Murad, A., 1992. Erythro dynamics in goldfish, *Carassius auratus L.*: Temperature effects. *Physiol. Zool*. 65, 55-76.
- Hyndman, C.K., Kieffer, J.D., Benfey, T.J., 2003. Physiology and survival of triploid brook trout following exhaustive exercise in warm water. *Aquaculture*. 221, 629-643. [https://doi.org/10.1016/S0044-8486\(03\)00119-4](https://doi.org/10.1016/S0044-8486(03)00119-4).
- Jeffries, K.M., Hinch, S.G., Sierocinski, T., Clark, T.D., Eliason, E.J., Donaldson, M.R., et al., 2012. Consequences of high temperatures and premature mortality on the transcriptome and blood physiology of wild adult sockeye salmon (*Oncorhynchus nerka*). *Eco. Evol.* 2, 1747-1764. <https://doi.org/10.1002/ece3.274>.
- Ji, P., Murata-Hori, M., Lodish, H.F., 2011. Formation of mammalian erythrocytes: Chromatin condensation and enucleation. *Trends Cell Biol*. 21, 409-415. <https://doi.org/10.1016/j.tcb.2011.04.003>.
- Kapp, F.G., Perlin, J.R., Hagedorn, E.J., Gansner, J.M., Schwarz, D.E., O'Connell, L.A., Johnson, N.S., Amemiya, C., Fisher, D.E., Wölfle, U., Trompouki, E., Niemeyer,

- C.M., Driever, W., Zon, L.I., 2018. Protection from UV light is an evolutionarily conserved feature of the haematopoietic niche. *Nature*. 558, 445–448.
<https://doi.org/10.1038/s41586-018-0213-0>.
- Kavumpurath, S., Pandian, T.J., 1990. Induction of triploidy in the zebrafish, *Brachydanio rerio* (Hamilton). *Aquac. Res.* 21, 299-306.
<https://doi.org/10.1111/j.1365-2109.1990.tb00468.x>.
- Keen, J.E., Calarco Steele, A.M., Houston, A.H., 1989. The Circulating Erythrocytes of Rainbow Trout (*Salmo gairdneri*). *Comp. Biochem. Physiol. A*. 94, 699–711.
[https://doi.org/10.1016/0300-9629\(89\)90620-8](https://doi.org/10.1016/0300-9629(89)90620-8).
- Kissa, K., Murayama, E., Zapata, A., Cortés, A., Perret, E., Machu, C., Herbomel, P., 2008. Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood*. 111, 1147-1156. <https://doi.org/10.1182/blood-2007-07-099499>.
- Kobayashi, I., Kondo, M., Yamamori, S., Kobayashi-Sun, J., Taniguchi, M., Kanemaru, K., Katakura, F., Traver, D., 2019 Enrichment of hematopoietic stem/progenitor cells in the zebrafish kidney. *Sci. Rep.* 9, 14205. <https://doi.org/10.1038/s41598-019-50672-5>.
- Lenard, A., Alghisi, E., Daff, H., Donzelli, M., McGinnis, C., Lengerke, C., 2016. Using zebrafish to model erythroid lineage toxicity and regeneration. *Haematologica*. 101, e164. <https://doi.org/10.3324/haematol.2016.142562>.
- Lewis, J.M., Hori, T.S., Rise, M.L., Walsh, P.J., Currie, S., 2010. Transcriptome responses to heat stress in the nucleated red blood cells of the rainbow trout (*Oncorhynchus mykiss*). *Physiol. Genomics*. 42, 361-373.
<https://doi.org/10.1152/physiolgenomics.00067.2010>.

- Lewis, J.M., Klein, G., Walsh, P.J., Currie, S., 2012. Rainbow trout (*Oncorhynchus mykiss*) shift the age composition of circulating red blood cells towards a younger cohort when exposed to thermal stress. *J. Comp. Physio. B.* 182, 663-671. <https://doi.org/10.1007/s00360-012-0650-2>.
- Lien, S., Koop B.F., Sandve, S.R., Miller, J.R., Kent, M.P., Nome, T., Hvidsten, T.R., Leong, J.S., Minkley, D.R., Zimin, A., Grammes, F., Grove, H., Gjvusland, A., Walenz, B., Hermansen, R.A., von Schalburg, K., Rondeau, E.B., Di Genova, A., Samy, J.K.A., Vik, J.O., Vigeland, M.D., Caler, L., Grimholt, U., Jentoft, S., Våge, D.I., de Jong, P., Moen, T., Baranski, M., Palti, Y., Smith, D.R., Yorke, J.A., Nederbragt, A.J., Tooming-Klunderud, A., Jakobsen, K.S., Jiang, X., Fan, D., Hu, Y., Liberles, D.A., Vidal, R., Iturra, P., Jones, S.J.M., Jonassen, I., Maass, A., Omholt, S.W., Davidson, W.S., 2016. The Atlantic salmon genome provides insights into rediploidization. *Nature.* 533, 200-205. <https://doi.org/10.1038/nature17164>.
- Long, Q., Meng, A., Wang, H., Jessen, J.R., Farrell, M.J., Lin, S., 1997. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development.* 124, 4105-4111.
- Lund, S.G., Phillips, M.C.L., Moyes, C.D., 2000. The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* 203, 2219-2228.
- Mable, B.K., 2004. 'Why polyploidy is rarer in animals than in plants': myths and mechanisms. *Biol. J. Linn. Soc.* 82, 453-466. <https://doi.org/10.1111/j.1095-8312.2004.00332.x>.

- Mable, B.K., Alexandrou, M.A., Taylor, M.I., 2011. Genome duplication in amphibians and fish: An extended synthesis. *J. Zool.* 284, 151-182.
<https://doi.org/10.1111/j.1469-7998.2011.00829.x>.
- Mahony, C.B., Bertrand, J.Y., 2019. How HSCs colonize and expand in the fetal niche of the vertebrate embryo: An evolutionary perspective. *Front. Cell. Dev. Biol.* 7, 34. <https://doi.org/10.3389/fcell.2019.00034>.
- Martinez-Agosto, J.A., Mikkola, H.K.A., Hartenstein, V., Banerjee, U., 2017. The hematopoietic stem cell and its niche: A comparative view. *Genes Dev.* 21, 3044-3060. <http://www.genesdev.org/cgi/doi/10.1101/gad.1602607>.
- Martos-Sitcha, J.A., Bermejo-Nogales, A., Calduch-Giner, J.A., Pérez-Sánchez, J., 2017. Gene expression profiling of whole blood cells supports a more efficient mitochondrial respiration in hypoxia-challenged gilthead sea bream (*Sparus aurata*). *Front. Zool.* 14, 34. <https://doi.org/10.1186/s12983-017-0220-2>.
- Masterson, J., 1994. Stomatal size in fossil plants: Evidence for polyploidy in majority of angiosperms. *Science.* 264, 421-424.
<https://doi.org/10.1126/science.264.5157.421>.
- Maxime, V., 2008. The physiology of triploid fish: Current knowledge and comparisons with diploid fish. *Fish Fish.* 9, 67-78. <https://doi.org/10.1111/j.1467-2979.2007.00269.x>.
- McLeod, T.F., Sigel, M.M., Yunis, A.A., 1978. Regulation of erythropoiesis in the Florida gar, *Lepisosteus platyrhincus*. *Comp. Biochem. Physiol.* 60, 145-150.
[https://doi.org/10.1016/0300-9629\(78\)90220-7](https://doi.org/10.1016/0300-9629(78)90220-7).

- Miller, K.E., Brownlee, C., Heald, R., 2020. The power of amphibians to elucidate mechanisms of size control and scaling. *Exp. Cell Res.* 392, 112036. <https://doi.org/10.1016/j.yexcr.2020.112036>.
- Morera, D., MacKenzie, S.A., 2011. Is there a direct role for erythrocytes in the immune response? *Vet. Res.* 42, 89. <https://doi.org/10.1186/1297-9716-42-89>.
- Moyes, C.D., Sharma, M.L., Lyons, C., Leary, S.C., Leon, M., Petrie, A., Lund, S.G., Tufts, B.L., 2002. Origins and consequences of mitochondrial decline in nucleated erythrocytes. *Biochim. Biophys. Acta.* 1591, 11-20. [https://doi.org/10.1016/s0167-4889\(02\)00224-0](https://doi.org/10.1016/s0167-4889(02)00224-0).
- Mueller, R., 2015. Genome biology and the evolution of cell-size diversity. *Cold Spring Harb. Perspect. Biol.* 7, a019125. <https://doi.org/10.1101/cshperspect.a019125>.
- Muller, H.J., 1925. Why polyploidy is rarer in animals than in plants. *AM NAT.* 59, 346-353.
- Murad, A., Houston, A.H., Samson, L., 1990. Haematological response to reduced oxygen-carrying capacity, increased temperature and hypoxia in goldfish, *Carassius auratus* L. *J. Fish. Biol.* 36, 289-305. <https://doi.org/10.1111/j.1095-8649.1990.tb05610.x>.
- Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H-F., Handin, R.I., Herbomel, P., 2006. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity.* 25, 963-975. <https://doi.org/10.1016/j.immuni.2006.10.015>.
- Nader, E., Skinner, S., Romana, M., Fort, R., Lemonne, N., Guillot, N., Gauthier, A., Antoine-Jonville, S., Renoux, C., Hardy-Dessources, M-D., Stauffer, E., Joly, P., Bertrand, Y., Connes, P., 2019. Blood rheology: Key parameters, impact on

- blood flow, role in Sickle Cell Disease and effects of exercise. *Front. Physiol.* 17, 1329. <https://doi.org/10.3389/fphys.2019.01329>.
- Nash, G.B., Egginton, S., 1993. Comparative rheology of human and trout red blood cells. *J. Exp. Biol.* 174, 109-122.
- Nell, J.A. (2002). Farming triploid oysters. *Aquaculture*. 210, 69-88.
[https://doi.org/10.1016/S0044-8486\(01\)00861-4](https://doi.org/10.1016/S0044-8486(01)00861-4).
- Nikinmaa, M. (1990) Vertebrate red blood cells: Adaptations of function to respiratory requirements. Berlin: Springer Berlin Heidelberg.
<https://doi.org/10.1007/978-3-642-83909-2>.
- North, T.E., Goessling, W., Walkley, C.R., Lengerke, C., Lord, A.M., Weber, G.J., Bowman, T.V., Jang, I-H., Grosser, T., FitzGerald, G.A., Daley, G.Q., Orkin, S.H., Zon, L.I., 2007. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*, 447, 1007-1011. <https://doi.org/10.1038/nature05883>.
- O'Donnell, K.M., MacRae, K.L., Verhille, C.E., Sacobie, C.F.D., Benfey, T.J., 2017. Standard metabolic rate of juvenile triploid brook charr, *Salvelinus fontinalis*. *Aquaculture*. 479, 85-90. <http://dx.doi.org/10.1016/j.aquaculture.2017.05.018>.
- Ohno, S., 1970. *Evolution by gene duplication*. Berlin: Springer Berlin Heidelberg.
<https://doi.org/10.1007/978-3-642-86659-3>.
- Ojolick, E.J., Cusack, R., Benfey, T.J., Kerr, S.R., 1995. Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture*. 131, 177-187.
[https://doi.org/10.1016/0044-8486\(94\)00338-O](https://doi.org/10.1016/0044-8486(94)00338-O).

- Oomen, R.A., Hutchings, J.A., 2017. Transcriptomic responses to environmental change in fishes: Insights from RNA sequencing. *FACETS*. 2, 610–641.
<https://doi.org/10.1139/facets-2017-0015>.
- Palis, J., 2014. Primitive and definitive erythropoiesis in mammals. *Front. in Physiol.* 5, 3. <https://dx.doi.org/10.3389%2Ffphys.2014.00003>.
- Pan, Y.K., Ern, R., Morrison, P.R., Brauner, C.J., Esbaugh, A.J., 2017. Acclimation to prolonged hypoxia alters hemoglobin isoform expression and increases hemoglobin oxygen affinity and aerobic performance in a marine fish. *Sci. Rep.* 7, 7834. <https://dx.doi.org/10.1038%2Fs41598-017-07696-6>.
- Pauls, S., Geldmacher-Voss, B., Campos-Ortega, J.A., 2001. A zebrafish histone variant H2A.F/Z and a transgenic H2A.F/Z:GFP fusion protein for *in vivo* studies of embryonic development. *Dev. Genes Evol.* 211, 603-610.
<http://dx.doi.org/10.1007/s00427-001-0196-x>.
- Pelster, B., Egg, M., 2018. Hypoxia-inducible transcription factors in fish: expression, function and interconnection with the circadian clock. *J. Exp. Biol.* 221, jeb163709. <https://doi.org/10.1242/jeb.163709>.
- Phillips, M.C.L., Moyes, C.D., Tufts, B.L., 2000. The effects of cell ageing on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* 203, 1039-1045.
- Piferrer, F., Beaumont, A., Flaugière, J-C., Flajšhans, M., Haffray, P., Colombo, L., 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture*. 293, 125-156. <http://dx.doi.org/10.1016/j.aquaculture.2009.04.036>.

- Poontawee, K., Werner, C., Müller-Belecke, A., Hörstgen-Schwark, G., Wicke, M., 2007. Flesh qualities and muscle fiber characteristics in triploid and diploid rainbow trout. *J. Appl. Ichthyol.* 23, 273-275. <https://doi.org/10.1111/j.1439-0426.2007.00843.x>.
- Postlethwait, J.H., 2007. The zebrafish genome in context: ohnologs gone missing. *J. Exp. Zool.* 308B, 563-577. <https://doi.org/10.1002/jez.b.21137>.
- Quentel, C., Obach, A., 1992. The cellular composition of the blood and haematopoietic organs of turbot *Scophthalmus maximus* L.. *J. Fish Biol.* 41, 709–716. <https://doi.org/10.1111/j.1095-8649.1992.tb02700.x>.
- R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienne, Austria. URL: <https://www.R-project.org/>.
- Richards, J.G., Semple, J.W., Bystriansky, J.S., Schulte, P.M., 2003. Na⁺/K⁺-ATPase α -isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J. Exp. Biol.* 206, 4475-4486. <https://doi.org/10.1242/jeb.00701>.
- Riseth, E.N., Fraser, T.W.K., Sambraus, F., Stien, L.H., Hvas, M., 2020. Is it advantageous for Atlantic salmon to be triploid at lower temperatures? *J. Therm. Biol.* 89, 102548. <https://doi.org/10.1016/j.jtherbio.2020.102548>.
- Sambraus, F., Olsen, R.E., Remen, M., Hansen, T.J., Torgersen, T., Fjelldal, P.G., 2017. Water temperature and oxygen: The effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture*. 473, 1-12. <https://doi.org/10.1016/j.aquaculture.2017.01.024>.
- Sambraus, F., Remen, M., Olsen, R.E., Hansen, T.J., Waagbø, R., Torgersen, T., Lock, E.J., Imsland, A., Fraser, T.W.K., Fjelldal, P.G., 2018. Changes in water

- temperature and oxygen: the effect of triploidy on performance and metabolism in large farmed Atlantic salmon. *Aquac. Environ. Interact.* 10, 157-172.
<https://doi.org/10.3354/aei00260>.
- Saranyan, P.V., Ross, N.W., Benfey, T.J., 2017. Erythrocyte heat shock protein responses to chronic (*in vivo*) and acute (*in vitro*) temperature challenge in diploid and triploid salmonids. *Com. Biochem. Physiol. A. Mol. Integ. Physiol.* 206, 95-104. <https://doi.org/10.1016/j.cbpa.2017.01.007>.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: An open-source platform for biological-image analysis. *Nature Methods.* 9, 676-682.
<https://doi.org/10.1038/nmeth.2019>.
- Scott, M.A., Dhillon, R.S., Schulte, P.M., Richards, J.G., 2014. Physiology and performance of wild and domestic strains of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in response to environmental challenges. *Can. J. Fish. Aquat.* 72, 125-134. <https://doi.org/10.1139/cjfas-2013-0450>.
- Simonot, D.L., Farrell, A.P., 2007. Cardiac remodelling in rainbow trout *Oncorhynchus mykiss* Walbaum in response to phenylhydrazine-induced anaemia. *J. Exp. Biol.* 210, 2574-2584. <https://doi.org/10.1242/jeb.004028>.
- Simonot, D.L., Farrell, A.P., 2009. Coronary vascular volume remodelling in rainbow trout *Oncorhynchus mykiss*. *J. Fish. Biol.* 75, 1762-1772.
<https://doi.org/10.1111/j.1095-8649.2009.02427.x>.

- Snyder, G.K., Sheafor, B.A., 1999. Red blood cells: Centerpiece in the evolution of the vertebrate circulatory system. *Am. Zoo.* 39, 189-198.
<https://doi.org/10.1093/icb/39.2.189>.
- Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., Gros, F., Royer, C., Massemin, S., Criscuolo, F., 2013. Avian erythrocytes have functional mitochondria opening novel perspectives for birds as animal models in the study of ageing. *Front. Zoo.* 10, 33. <https://doi.org/10.1186/1742-9994-10-33>.
- Traver, D., Paw, B.H., Poss, K.D., Penberthy, W.T., Lin, S., Zon, L.I., 2003. Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat. Immunol.* 4, 1238–1246. <https://doi.org/10.1038/ni1007>.
- Van de Pol, I.L.E., Flik, G., Gorissen, M., 2017. Comparative physiology of energy metabolism: Fishing for endocrine signals in the early vertebrate pool. *Front. Endocrinol.* 8, 36. <https://doi.org/10.3389/fendo.2017.00036>.
- Van de Pol, I.L.E., Flik, G., Verberk, W.C.E.P., 2020. Triploidy in zebrafish larvae: Effects on gene expression, cell size and cell number, growth, development and swimming performance. *PLoS ONE.* 15, e0229468.
<https://doi.org/10.1371/journal.pone.0229468>.
- Verhille, C., Antilla, K., Farrell, A.P., 2013. A heart to heart on temperature: Impaired temperature tolerance of triploid rainbow trout (*Oncorhynchus mykiss*) due to early onset of cardiac arrhythmia. *Comp. Biochem. Physiol. A. Mol. Integ. Physiol.* 164, 653-657. <http://dx.doi.org/10.1016/j.cbpa.2013.01.011>.
- Wang, Y, Kaiser, M.S, Larson, J.D, Nasevicius, A, Clark, K.J, Wadman, S.A, Roberg-Perez, S.E, Ekker, S.C, Hackett, P.B, McGrail M, Essner, J.J., 2010. Moesin1

- and Ve-cadherin are required in endothelial cells during *in vivo* tubulogenesis. *Dev.* 137, 3119-3128. <https://doi.org/10.1242/dev.048785>.
- Wattrus S.J., Zon, L.I., 2018. Stem cell safe harbor: the hematopoietic stem cell niche in zebrafish. *Blood advances*. 2, 3063-3069. <https://dx.doi.org/10.1182%2Fbloodadvances.2018021725>.
- Weinberg, S.R., LoBue, J., Siegel, C.D., Gordon A.S., 1976. Hematopoiesis of the kissing gourami (*Helostoma temmincki*). Effects of starvation, bleeding, and plasma-stimulating factors on its erythropoiesis. *Can. Jour. Zoo.* 54, 1115–1127. <https://doi.org/10.1139/z76-127>.
- Westerfield, M., 2000. The Zebrafish Book. A guide for the laboratory use of zebrafish (*Danio rerio*). Eugene: University of Oregon Press.
- Williams, K., Cassidy, A.A., Verhille, C.E., Lamarre, S.G., MacCormack, T.J., 2019. Diel cycling hypoxia enhances hypoxia tolerance in rainbow trout (*Oncorhynchus mykiss*): Evidence of physiological and metabolic plasticity. *Jour. Exp. Biol.* 222, jeb206045. <https://doi.org/10.1242/jeb.206045>.
- Witeska, M., 2013. Erythrocytes in teleost fishes: a review. *Zoo. Ecol.* 23, 275-281. <http://dx.doi.org/10.1080/21658005.2013.846963>.
- Yaqoob, N., Holotta, M., Prem, C., Kopp, R., Schwerte, T., 2009. Ontogenetic development of erythropoiesis can be studied non-invasively in GATA-1:DsRed transgenic zebrafish. *Comp. Biochem. Physiol. A.* 154, 270-278. <https://doi.org/10.1016/j.cbpa.2009.06.024>.
- Zang, L., Shimada, Y., Nishimura, Y., Tanaka, T., Nishimura, N., 2013. A novel, reliable method for repeated blood collection from aquarium fish. *Zebrafish*. 10, 00. <https://doi.org/10.1089/zeb.2012.0862>.

5.0 Chapter 5:

General Discussion

Whole genome duplications (WGD) produce polyploids and are found on nearly every major branch of the eukaryotic tree of life, but we still know very little about the circumstances under which polyploids survive and out-compete their diploid conspecifics in evolutionary history (Braasch and Postlethwait, 2012; Mueller, 2015). Most research on the evolution of polyploids has focused on genetics and, specifically, on how duplicated genes (referred to as ‘ohnologues’) evolve and diverge after duplication without considering the developmental and physiological consequences that arise from increasing genome size and cell size. Scientists have been studying the physiology of triploid fish for decades in the context of its utility for the aquaculture industry, and have highlighted several physiological limitations associated with polyploidy that would likely affect a polyploid’s ability to thrive and successfully pass on their genes (Benfey, 1999; Piferrer et al., 2009). My intent for this thesis was to blend ideas from both of these fields of research to develop and test hypotheses regarding the developmental and physiological consequences of triploidy that would pertain to the evolutionary fitness of polyploids as well as the performance of triploids in aquaculture.

The zebrafish (*Danio rerio*) has become a popular model vertebrate for developmental biology and is rising in prominence among physiologists as well (Piferrer and Ribas, 2020). In Chapter 2, I developed a novel technique to non-destructively determine ploidy in zebrafish embryos to harness the power and utility of this model system for questions pertaining to the biology of polyploids. Using a transgenic line with fluorescent chromatin, confocal microscopy, and open-source image processing

software, I have optimized a protocol for efficiently producing and identifying triploid zebrafish that can subsequently be used for further experimentation. To demonstrate its utility, I used this technique to unequivocally establish the ploidy of individuals and measured their heart rate at standard (27.5°C) and warm (32°C) temperatures and was able to reproduce a pattern that was observed in a previous study on heart rate in triploid salmonids (Benfey and Bennett, 2009). Now that ploidy can be determined *in vivo*, the zebrafish becomes a valuable resource for studying triploid biology as we can take advantage of the incredible array of tools and techniques that have been established and are continuing to be developed in this model vertebrate.

The developmental consequences of polyploidy piqued the curiosity of early developmental biologists who noted that polyploid animals had larger cells but were no larger than diploid conspecifics (Fankhauser, 1939; Fankhauser, 1945; Swarup, 1959). To compensate for the increase in cell size, polyploids seemed to reduce the number of cells in their tissues such that organ size and body size was largely unaffected by ploidy. This “fewer, larger cells” pattern of compensation was assumed to be true for all tissues, but the technology of the time was limited to bright-field microscopy and so this was only validated for cells in a few easily visualized tissues, such as pigment and blood cells (Fankhauser, 1939; Fankhauser, 1945; Swarup, 1959). In Chapter 3, I took advantage of transgenic zebrafish strains with tissue-specific fluorescent reporter genes stably integrated into their genome and immunohistochemistry with validated antibodies to show that this “fewer, larger cells” pattern is not true for all tissues. The physiological consequences of these tissue-specific compensatory mechanisms that maintain organ size and body size independent of cell size are still not clear, but already we can see the value and utility of the zebrafish system for triploid research. By applying some of the

widely-available tools developed in the zebrafish research community, I was successfully able to refine our view of polyploid development and demonstrate clearly that we should not assume that all tissues in a polyploid animal respond to cell size changes in the same way.

Blood is the classic example of a tissue that conforms to the “fewer, larger cells” pattern of compensation in triploids to the point that erythrocyte size is typically used as a diagnostic measurement to determine ploidy (Benfey, 1999). There have been many hematological comparisons between diploid and triploid blood (e.g., Benfey and Sutterlin, 1984; Saranyan et al., 2017), but these studies have focused on the physiology of mature blood cells and less on the homeostatic mechanisms that maintain hematology in an adult fish. Erythrocyte turnover is a highly-regulated and well-studied phenomenon in mammals (Franco, 2012; Higgins, 2015), but has been largely overlooked in non-mammalian vertebrates as a whole except as a tool for ecologists interesting in determining how energy flows through ecosystems (Vander Zanden et al., 2015). In Chapter 4, I once again made use of the zebrafish system to quantify the number of hematopoietic stem cells in triploid embryos using a highly-specific assay that combines two transgenic strains (Esain et al., 2016). After confirming that triploids have fewer hematopoietic stem cells, I then developed an assay to measure the rate of steady-state erythropoiesis in adult zebrafish using another transgenic strain to show that triploids produce new erythrocytes at a slower rate than diploids.

Having established a technique to measure the rate of erythropoiesis, I then designed an experiment using [10]-gingerol as a feed additive to boost erythropoiesis but this was not successful due to an unexpected effect of the solvent on the hematology of the fish (Appendix 1). The erythropoietic-stimulatory effects of ginger (Ferri-Lagneau et

al., 2012; Haghighi and Rohani, 2013; Kanani et al., 2014; Ferri-Lagneau et al., 2019) should not be overlooked due to the results of this experiment as any positive effects were overshadowed by the negative consequences caused by the solvent. Including ginger compounds in the formulation of the feed pellets increased hematological values in several species of fish, so perhaps this strategy of stimulating erythropoiesis would be viable for increasing the rate of erythrocyte cycling in triploids. This may provide the aquaculture industry with a simple, effective, and inexpensive means of mitigating some of the current drawbacks of farming triploid fish.

Although I have demonstrated that triploids have fewer young, newly differentiated erythrocytes, the conclusion that triploids have more aged, senescent cells is still based on inference rather than anything empirical. I designed a diet-switch experiment using stable isotopes to determine how long it takes for diploids and triploids to turn over their blood (Appendix 2) to address this. I developed a novel technique to infuse standard fish pellets with a ^{13}C -labelled amino acid to produce an isotopically distinct feed that I hoped would allow me to unequivocally demonstrate that the rate of erythrocyte turnover is slower in triploids, and that their blood therefore consists of more older cells. Like the gingerol experiments, this remains a promising approach, and I was clearly able to observe changes in the isotopic composition of the blood over time. Unfortunately, a water failure at the UNB aquatic facility resulted in mass mortality of experimental fish before the experiment reached the half-way point so I was unable to obtain data to determine if erythrocyte turnover is slower in triploids.

Within the last 20 years, several independent studies have demonstrated that erythrocyte aging and senescence is relevant for teleost erythrocytes (Lund et al., 2000; Moyes et al., 2002; Phillips et al., 2000; Götting and Nikinmaa, 2017) as it is in

mammalian erythrocytes (Franco, 2012; Franco et al., 2013; Higgins, 2015). Little is known about the consequences of erythrocyte senescence on physiology in teleosts aside from the fact that extreme thermal challenges produce a marked shift in erythrocyte demographics likely because old erythrocytes are unable to mount a heat shock response, thereby reducing the mean age of remaining erythrocytes (Lund et al., 2000). I have confirmed that triploid zebrafish do indeed have fewer hematopoietic stem cells and that steady-state rate of erythropoiesis is reduced, suggesting that triploid blood likely has proportionally more senescent, sub-optimally functioning erythrocytes in circulation. Despite the expense and duration of the stable isotope diet shift experiment, I would argue that there is value in repeating this for triploid biology and for fish physiology as a whole.

Most hematological studies focus on whole blood rather than on the physiology of individual erythrocytes, but it is becoming clear that not every erythrocyte in circulation is equal (Lund et al., 2000; Moyes et al., 2002; Phillips et al., 2000; Götting and Nikinmaa, 2017). Intriguingly, comparing the heat shock response of triploid blood to diploid blood (Saranyan et al., 2017) produces patterns reminiscent of comparisons between young and old erythrocytes (Lund et al., 2000; Moyes et al., 2002). Erythrocyte demographics is relevant to physiology since older cells have lost their ability to respond to environmental changes, and so the relative proportion of these aged cells must be relevant to an animal's ability to tolerate physiological challenges. The establishment of the zebrafish as a powerful model for triploid development and physiology has improved our ability to address these questions. Understanding the developmental and physiological consequences that arise from building an animal with fewer, larger cells

will help us to understand the trade-offs between the advantages associated with extra gene copies and the disadvantages associated with polyploidy.

References:

- Benfey, T.J., 1999. The physiology and behavior of triploid fishes. *Rev. Fish.* 7, 39-67.
<http://dx.doi.org/10.1080/10641269991319162>.
- Benfey, T.J., & Bennett, L.E., 2009. Effect of temperature on heart rate in diploid and triploid brook charr, *Salvelinus fontinalis*, embryos and larvae. *Comp. Biochem. Physiol. A - Mol. Integr. Physiol.* 152, 203-206.
<https://doi.org/10.1016/j.cbpa.2008.09.025>.
- Benfey T.J., Sutterlin, A.M., 1984. The haematology of triploid landlocked Atlantic salmon, *Salmo salar* L. *J. Fish Biol.* 24, 333-338.
<http://dx.doi.org/10.1111/j.1095-8649.1984.tb04804.x>.
- Braasch, I.P., & Postlethwait, J.H., 2012. Polyploidy in fish and the teleost genome duplication. In: P. A. Soltis & D. E. Soltis (Eds). *Polyploidy and Genome Evolution* (1st ed., p. 341-386). Berlin: Springer. https://doi.org/10.1007/978-3-642-31442-1_17.
- Esain, V., Cortes, M., North, T.E., 2016. Enumerating hematopoietic stem and progenitor cells in zebrafish embryos. In: Kawakami, K., Patton, E.E. Orger, M. (Eds). *Zebrafish: Methods and protocols* (2nd edition, 191-206).
<https://doi.org/10.1007/978-1-4939-3771-4>.
- Fankhauser, G., 1939. Polyploidy in the salamander, *Eurycea bislineata*. *J. Hered.* 30, 379-388.
- Fankhauser, G., 1945. The effects of changes in chromosome number on amphibian development. *Quart. Rev. Biol.* 20, 20-78. <https://doi.org/10.1086/394703>.

- Ferri-Lagneau, K.F., Moshal, K.S., Grimes, M., Zahora, B., Lv, L., Sang, S., Leung, T-C., 2012. Ginger stimulates hematopoiesis via BMP pathway in zebrafish. *PLoS ONE*. 7, e39327. <https://doi.org/10.1371/journal.pone.0039327>.
- Ferri-Lagneau, K.F., Haider, J., Sang, S., Leung, T-C., 2019. Rescue of hematopoietic stem/progenitor cell formation in *plcg1* zebrafish mutant. *Sci. Rep.* 9, 244. <https://doi.org/10.1038/s41598-018-36338-8>.
- Franco, R.S., 2012. Measurement of red cell lifespan and aging. *Transfus. Med. Hemother.* 39, 302-307. <https://dx.doi.org/10.1159%2F000342232>.
- Franco, R.S., Puchulu-Campanella, M.E., Barber, L.A., Palascak, M.B., Joiner, C.H., Low, P.S., Cohen, R.M., 2013. Changes in the properties of normal human red blood cells during *in vivo* aging. *Am. J. Hematol.* 88, 44-51. <https://doi.org/10.1002/ajh.23344>.
- Götting, M., Nikinmaa, M.J., 2017. Transcriptomic analysis of young and old erythrocytes of fish. *Front. Physiol.* 8, 1046. <https://doi.org/10.3389/fphys.2017.01046>.
- Haghighi, M., Rohani, M.S., 2013. The effects of powdered ginger (*Zingiber officinale*) on the haematological and immunological parameters of rainbow trout *Oncorhynchus mykiss*. *J. Med. Plant Herbal Ther. Res.* 1, 8-12.
- Higgins, J.M., 2015. Red blood cell population dynamics. *Clin. Lab. Med.* 35, 43-57. <https://dx.doi.org/10.1016%2Fj.cll.2014.10.002>.
- Kanani, H.G., Nobahar, Z., Kakooli, S., Jafarian, H., 2014. Effect of ginger- and garlic-supplemented diet on growth performance, some hematological parameters and immune responses in juvenile *Huso huso*. *Fish Physiol. Biochem.* 40, 481-490. <https://doi.org/10.1007/s10695-013-9859-6>.

- Lund, S.G., Phillips, M.C.L., Moyes, C.D., 2000. The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* 203, 2219-2228.
- Moyes, C.D., Sharma, M.L., Lyons, C., Leary, S.C., Leon, M., Petrie, A., Lund, S.G., Tufts, B.L., 2002. Origins and consequences of mitochondrial decline in nucleated erythrocytes. *Biochim. Biophys. Acta.* 1591, 11-20.
[https://doi.org/10.1016/s0167-4889\(02\)00224-0](https://doi.org/10.1016/s0167-4889(02)00224-0).
- Mueller, R., 2015. Genome biology and the evolution of cell-size diversity. *Cold Spring Harb. Perspect. Biol.* 7, a019125. <https://doi.org/10.1101/cshperspect.a019125>.
- Phillips, M.C.L., Moyes, C.D., Tufts, B.L., 2000. The effects of cell ageing on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* 203, 1039-1045.
- Piferrer, F., Beaumont, A., Flaugière, J-C., Flajšhans, M., Haffray, P., Colombo, L., 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture.* 293, 125-156. <http://dx.doi.org/10.1016/j.aquaculture.2009.04.036>.
- Piferrer, F., Ribas, L., 2020. The use of the zebrafish as a model in fish aquaculture research. In: Farrell, A.P., Brauner, C., Benfey, T.J. (Eds). *Fish physiology, Vol* 38. <https://doi.org/10.1016/bs.fp.2020.10.003>.
- Saranyan, P.V., Ross, N.W., Benfey, T.J., 2017. Erythrocyte heat shock protein responses to chronic (*in vivo*) and acute (*in vitro*) temperature challenge in diploid and triploid salmonids. *Comp. Biochem. Physiol. A. Mol. Integ. Physiol.* 206, 95-104. <https://doi.org/10.1016/j.cbpa.2017.01.007>.

- Swarup, H., 1959. Effect of triploidy on the body size, general organization, and cellular structure in *Gasterosteus aculea*. *J. Genet.* 56, 143-155.
- Vander Zanden, M.J., Clayton, M.K., Moody, E.K., Solomon, C.T., Weidel, B.C., 2015. Stable isotope turnover and half-life in animal tissues: a literature synthesis. *PLoS ONE*. 10, e0116182. <https://doi.org/10.1371/journal.pone.0116182>.

6.0 Appendix 1:

Stimulating erythropoiesis by using [10]-gingerol as a feed additive in adult zebrafish.

6.1 Introduction

Mammalian erythrocytes are unique among vertebrates in that they are enucleated during differentiation and therefore cannot respond to environmental perturbations at the level of gene expression to replace or make new proteins as needed. Consequently, these erythrocytes are very short-lived, with a half-life of ~30 days in mice and ~120 days in humans, and so mammals must produce millions of new erythrocytes every day to replace cells as they are removed from circulation (Franco, 2012; Franco et al., 2013; Higgins, 2015). Teleost erythrocytes, on the other hand, retain their nuclei and maintain gene expression for part of their lifespan (Götting and Nikinmaa, 2017), leading many to suggest that teleost erythrocytes survive for much longer than mammalian erythrocytes (Morera and MacKenzie, 2011; Witeska, 2013), thereby reducing the need to maintain such high rates of erythropoiesis in fish. In line with this idea, mammals have massively expanded their hematopoietic tissues relative to other vertebrates (Martinez-Agosto et al., 2007; Kapp et al., 2018). Treatment strategies to stimulate erythropoiesis in humans in response to pathological anemias are an ongoing research focus, with one popular strategy being a search for ingredients that stimulate erythropoiesis. Large-scale screens for bio-active molecules have highlighted gingerols as potent immunostimulants and erythropoietic-stimulants (Chen et al., 2013; Ho et al., 2013; Semwal et al., 2015; Smith et al., 2018).

Erythrocyte cycling is not as well studied in fish compared to mammals, but several studies have demonstrated that aged, senescent erythrocytes have greatly reduced transcription (Götting and Nikinmaa, 2017), translation (Lund et al., 2000), mitochondrial activity (Phillips et al., 2000), and aerobic capacity (Moyes et al., 2002). This strongly suggests that the rate of erythrocyte cycling is relevant for a fish's physiology as the proportion of these inefficient, senescent erythrocytes in circulation will significantly impact the aerobic scope of the animal. In fish, ginger-infused feeds increased hematocrit and hemoglobin concentration in rainbow trout (*Oncorhynchus mykiss*) (Haghighi and Rohani, 2013) and beluga sturgeon (*Husa husa*) (Kanani et al., 2014). In zebrafish (*Danio rerio*), incubating embryos in [10]-gingerol was sufficient to expand their hematopoietic tissues, increase the rate of erythropoiesis, and improve recovery after phenylhydrazine-induced anemia (Ferri-Lagneau et al., 2012; Ferri-Lagneau et al., 2019). However, there are no reports of the effects of supplementing feed with [10]-gingerol on erythropoiesis or hematology in adult zebrafish.

In Chapter 3 of this thesis, I demonstrated that hematocrit is unaffected by ploidy in zebrafish, but the blood of triploids is comprised of fewer, larger cells compared to diploids. In Chapter 4, I showed that triploids also have fewer hematopoietic stem cells, suggesting that they could have reduced erythropoietic capacity. I then developed and validated an assay using Tg(gata1a:dsRed) to quantify relative age demographics of erythrocytes based on their fluorescence to show that triploids have proportionally fewer young, newly differentiated erythrocytes, suggesting a slower erythrocyte turnover rate. As outlined in this appendix, I then designed an experiment to make use of the Tg(gata1a:dsRed) erythropoiesis assay to test for erythropoietic-stimulatory effects of a [10]-gingerol infused diet.

6.2 Methods

Fish rearing

90 diploid Tg(gata1a:dsRed) were produced and reared until four months old and then randomly sorted among nine tanks using a random number generator (www.random.org): three control tanks (C1-C3), three vehicle control tanks (V1-V3) and three treatment tanks (T1-T3) with ten fish per tank. At 5.5 months old, fish were anesthetized in tris-buffered MS-222 (168 µg/mL), blotted dry with a Kimwipe, and their body mass measured in order to calculate the amount of feed required such that each treatment group would be fed 0.5% biomass per day (split over two feedings).

Diet preparation

The treatment group diet was produced by dissolving 10 mg of [10]-gingerol (catalogue number: G5798-10mg, Sigma-Aldrich, St. Louis, Missouri, US) into 1 L of methanol and immersing 50 g of adult zebrafish feed pellets (Gemma 500 micro, Skretting, Vancouver, BC, Canada) into this solution in a 2 L glass beaker. The beaker was placed in a fume hood and the methanol allowed to evaporate (with regular mixing) to infuse the [10]-gingerol into the feed pellets. Vehicle control pellets were similarly immersed in methanol (without [10]-gingerol) and the control diet was the untreated pellets. Once completely dry, 0.45 g of each feed type (control, vehicle, and treatment) was measured out into 2 mL microfuge tubes and stored at -20°C until used.

10-gingerol experiment

At six months old, fish were anesthetized in MS-222 and 6-8 µL of blood was sampled from each fish for a baseline assessment. Blood smears were produced for erythrocyte fluorescence intensity analysis (0.5 µL per slide) and hematocrit and erythrocyte count were measured as described in Chapter 4. Two fish (one from C1 and one from T2) did

not recover. After a two-day recovery period to ensure all fish were swimming strongly, fish were switched to their experimental diets for six weeks, after which blood was sampled again for the same analyses. Three fish died during the experiment (two in T3 and two in V1) and three did not recover from the final sampling (one in each of T1, V3, and C3). Each tank initially had 10 fish, but at the final sampling point this was reduced to 9 in tanks C1 and T2, and 8 in tanks T3 and V1. A one-way ANOVA was performed for each diet, time, and response to rule out any tank effects within treatments. The effects of diet and time point (and any interactions) on the number of strongly fluorescent erythrocytes, hematocrit, and erythrocyte count were each analyzed by repeated measures two-way ANOVA. Tukey's Honest Significant Differences post-hoc analysis was used to detect significant differences within each treatment between the initial and final sampling event.

6.3 Results

Initially, there were no differences among groups in the number of strongly fluorescent cells, hematocrit, or erythrocyte count. There was no effect of tank within treatment, timepoint, or response ($p > 0.05$ in all cases) so tanks were pooled for later analysis.

After 6 weeks on the experimental diets, the effects of diet, time and their interaction on the number of fluorescent *gata1a* expressing cells were all significant ($p < 0.001$). Post-hoc analysis indicated that the number of fluorescent cells was significantly reduced in the vehicle control and the [10-gingerol] diet (both $p < 0.001$), but not in the control diet ($p = 0.773$) (Fig. 6A). The proportion of fluorescent *gata1a* expressing cells in circulation at the end of the experiment was approximately 10% lower in both the 10-gingerol and vehicle control groups than in fish fed untreated pellets. The effects of diet, time and the interaction on hematocrit and on cell count were also significant (both $p <$

0.001) and post-hoc analysis revealed a similar pattern in that hematocrit and cell count were significantly reduced in the vehicle control and in the [10]-gingerol treatment ($p < 0.001$), but not in the control (hematocrit: $p = 0.991$, cell count: $p = 0.999$) (Fig. 1B+C). Final hematocrit and erythrocyte count values were approximately 10% and 30% lower, respectively, in 10-gingerol and vehicle control groups compared to fish fed the untreated pellets.

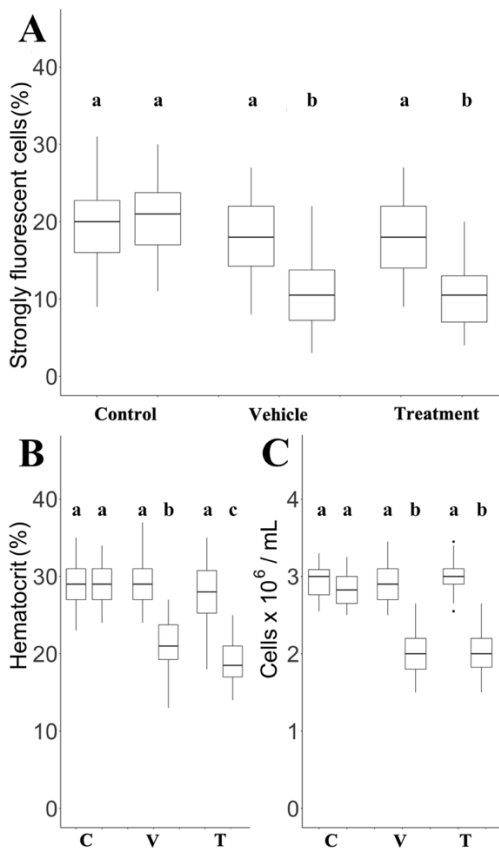


Fig 6.1 Erythropoiesis is impaired in Tg(gata1a:dsRed) zebrafish fed methanol-treated pellets, regardless of addition of [10]-gingerol. Fish fed [10]-gingerol- and methanol-treated feed had significantly reduced proportion of strongly fluorescent erythrocytes (**A**; $p < 0.001$), hematocrit (**B**; $p < 0.001$) and erythrocyte count (**C**; $p < 0.0001$). Asterisks denote significant differences between initial and final values within treatment groups (C = control, V = vehicle, T = treatment).

6.4 Discussion

This experiment was designed to determine the effect of [10]-gingerol on hematopoiesis in adult fish, however, the negative effect of the solvent completely obscures any potential effect this compound may have on hematology. Methanol was selected as the solvent because it was convenient and gingerol had a high solubility, but it appears the solvent may have leached some essential nutrient(s) from the feed and fish health suffered as a consequence. [10]-gingerol was identified as the bioactive molecule in the ginger plant that stimulates erythropoiesis in zebrafish larvae (Ferri-Lagneau et al., 2012; Ferri-Lagneau et al., 2019), but infusing standard zebrafish pellets by evaporating away a methanol solvent had negative effects in this case. Other studies investigating the effect of ginger compounds on fish hematology have had success by including ground ginger in the feed (Haghighi and Rohani, 2013; Kanani et al., 2014). Perhaps including [10]-gingerol or ground ginger into the formulation of the pellets would allow the erythropoietic stimulatory effects seen in embryos to manifest themselves in adult fish. If the rate of steady-state erythropoiesis is slower in triploids, then perhaps including an erythropoietic stimulant in their diet would mitigate some of the physiological vulnerabilities associated with farming triploids in conditions optimized for diploids.

6.5 References:

Chen, H., Soroka, D.N., Haider, J., Ferri-Lagneau, K.F., Leung, T-C., Sang, S., 2013.

[10]-Gingerdiols as the major metabolites of [10]-gingerol in zebrafish embryos and in humans and their hematopoietic effects in zebrafish embryos. *J. Agric. Food Chem.* 61, 5353-5360. <https://doi.org/10.1021/jf401501s>.

- Ferri-Lagneau, K.F., Moshal, K.S., Grimes, M., Zahora, B., Lv, L., Sang, S., Leung, T-C., 2012. Ginger stimulates hematopoiesis via BMP pathway in zebrafish. *PLoS ONE*. 7, e39327. <https://doi.org/10.1371/journal.pone.0039327>.
- Ferri-Lagneau, K.F., Haider, J., Sang, S., Leung, T-C., 2019. Rescue of hematopoietic stem/progenitor cell formation in *plcg1* zebrafish mutant. *Sci. Rep.* 9, 244. <https://doi.org/10.1038/s41598-018-36338-8>.
- Franco, R.S., 2012. Measurement of red cell lifespan and aging. *Transfus. Med. Hemother.* 39, 302-307. <https://dx.doi.org/10.1159%2F000342232>.
- Franco, R.S., Puchulu-Campanella, M.E., Barber, L.A., Palascak, M.B., Joiner, C.H., Low, P.S., Cohen, R.M., 2013. Changes in the properties of normal human red blood cells during *in vivo* aging. *Am. J. Hematol.* 88, 44-51. <https://doi.org/10.1002/ajh.23344>.
- Götting, M., Nikinmaa, M.J., 2017. Transcriptomic analysis of young and old erythrocytes of fish. *Front. Physiol.* 8, 1046. <https://doi.org/10.3389/fphys.2017.01046>.
- Haghighi, M., Rohani, M.S., 2013. The effects of powdered ginger (*Zingiber officinale*) on the haematological and immunological parameters of rainbow trout *Oncorhynchus mykiss*. *J. Med. Plants Res.* 1, 8-12.
- Higgins, J.M., 2015. Red blood cell population dynamics. *Clin Lab Med.* 35, 43-57. <https://dx.doi.org/10.1016%2Fj.cll.2014.10.002>.
- Ho, S-C., Chang, K-S., Lin, C-C., 2013. Anti-neuroinflammatory capacity of fresh ginger is attributed mainly to 10-gingerol. *Food Chem.* 141, 3183-3191. <http://dx.doi.org/10.1016/j.foodchem.2013.06.010>.

- Kanani, H.G., Nobahar, Z., Kakooli, S., Jafarian, H., 2014. Effect of ginger- and garlic-supplemented diet on growth performance, some hematological parameters and immune responses in juvenile *Huso huso*. *Fish Physiol. Biochem.* 40, 481-490. <https://doi.org/10.1007/s10695-013-9859-6>.
- Kapp, F.G., Perlin, J.R., Hagedorn, E.J., Gansner, J.M., Schwarz, D.E., O'Connell, L.A., Johnson, N.S., Amemiya, C., Fisher, D.E., Wölflle, U., Trompouki, E., Niemeyer, C.M., Driever, W., Zon, L.I., 2018. Protection from UV light is an evolutionarily conserved feature of the haematopoietic niche. *Nature.* 558, 445–448. <https://doi.org/10.1038/s41586-018-0213-0>.
- Lund, S.G., Phillips, M.C.L., Moyes, C.D., 2000. The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* 203, 2219-2228.
- Martinez-Agosto, J.A., Mikkola, H.K.A., Hartenstein, V., Banerjee, U., 2007. The hematopoietic stem cell and its niche: A comparative view. *Genes Dev.* 21, 3044-3060. <http://www.genesdev.org/cgi/doi/10.1101/gad.1602607>.
- Morera, D., MacKenzie, S.A., 2011. Is there a direct role for erythrocytes in the immune response? *Vet. Res.* 42, 89. <https://doi.org/10.1186/1297-9716-42-89>.
- Moyes, C.D., Sharma, M.L., Lyons, C., Leary, S.C., Leon, M., Petrie, A., Lund, S.G., Tufts, B.L., 2002. Origins and consequences of mitochondrial decline in nucleated erythrocytes. *Biochim. Biophys. Acta.* 1591, 11-20. [https://doi.org/10.1016/s0167-4889\(02\)00224-0](https://doi.org/10.1016/s0167-4889(02)00224-0).
- Phillips, M.C.L., Moyes, C.D., Tufts, B.L., 2000. The effects of cell ageing on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells. *J. Exp. Biol.* 203, 1039-1045.

- Semwal, R.B., Semwar, D.K., Combrinck, S., Viljoen, A.M., 2015. Gingerols and shogaols: Important nutraceutical principles from ginger. *Phytochemistry*. 117, 554-568. <http://dx.doi.org/10.1016/j.phytochem.2015.07.012>.
- Smith, N.C., Christian, S.L., Taylor, R.G., Santander, J., Rise, M., 2018. Immune modulatory properties of 6-gingerol and resveratrol in Atlantic salmon macrophages. *Mol. Immunol.* 95, 10-19. <https://doi.org/10.1016/j.molimm.2018.01.004>.
- Witeska, M., 2013. Erythrocytes in teleost fishes: a review. *Zoo. Ecol.* 23, 275-281. <http://dx.doi.org/10.1080/21658005.2013.846963>.

7.0 Appendix 2:

Measuring tissue turnover in diploid and triploid brook charr (*Salvelinus fontinalis*) using a diet shift experiment with stable isotopes.

7.1 Introduction

The lifespan of most cells is shorter than the lifespan of the organism itself, so cells must be continuously replaced in order to maintain tissue function. This process of replacing aged or damaged cells with newly differentiated cells is known as tissue turnover (Spalding et al., 2005). Pulse-chase experiments, in which a population of cells is biochemically labelled (the pulse) and then the tissue is sampled periodically until the labelled cells are no longer detectable (the chase) (Franco, 2012), are the gold standard for these analyses when tissues can be non-destructively sampled (i.e., blood). This experimental approach can be technically challenging since it requires reintroducing blood cells into circulation after a sample is collected and labelled *in vitro*. Thus far, the reintroduction of blood cells labelled *ex vivo* has only been used in mice (*Mus musculus*) (Franco et al., 2013) but should work in any species if the challenge of returning blood into the vasculature system can be overcome.

Although not a direct measure of the amount of time that an individual cell survives, another approach to measuring the rate of cell turnover is stable isotope analysis in tissues during a diet-shift experiment (Buchheister and Latour, 2010; Charest, 2016; Busst and Britton, 2017; Winter et al., 2019) which allows determination of the length of time that cells survive within that tissue (Faragher, 2003; Spalding et al., 2005; Bergmann et al., 2015; Karin et al., 2019). Unlike radioisotopes, stable isotopes do not undergo radioactive decay, and their proportions in a given mixture of organic molecules

do not change over time, making them a useful label. Thus, the stable isotope ratios of carbon and nitrogen in an animal's tissues depend on their ratios in its diet and by measuring the relative amounts of these elements in the tissues using mass spectrometry, we can gain insight about what that animal has been eating (Petersen and Fry, 1987; Vander Zanden et al., 2015). This logic is very useful for ecologists to monitor nutrient flux through an ecosystem (Samways et al., 2018; Landsman et al., 2018) and for building food webs (Hayden et al., 2017; Nielsen et al., 2017). Despite the utility of stable isotope analysis in these fields, there are still many unknowns about this technique that need to be examined to improve the inferences that can be drawn from such experiments (Vander Zanden et al., 2015). One critical question that must be addressed is how long it takes for the isotopic signature of any given tissue to shift when the isotopic signature of the diet changes (Post, 2002).

Stable isotope diet-shift studies typically switch between diets with proteins derived from isotopically distinct sources (Charest, 2016; Busst and Britton, 2017; Winter et al., 2019) but that are not necessarily nutritionally identical. The development of a technique to experimentally manipulate the isotopic ratio of a manufactured feed would enhance the utility of these experiments by removing a potential confound. Stable isotope labelling by amino acid in culture (SILAC) is a widely-used technique in quantitative proteomics (Mann et al., 2006; Wang et al., 2018) that has also been used in studies on tissue regeneration in zebrafish (*Danio rerio*) using a manufactured SILAC rodent diet (Westman-Brinkmalm, et al., 2011; Konzer et al., 2013). Here, I present a novel technique to infuse standard fish-feed pellets with a commercially available ^{13}C -labelled amino acid (alanine) to generate an isotopically distinct diet for diet-shift

experiments without having to switch to different pellets that may not be nutritionally identical.

In Chapter 4 of this thesis, I determined that triploids have fewer hematopoietic stem cells and that the rate of steady-state erythropoiesis is slower with proportionally fewer newly differentiated erythrocytes in circulation. Consequently, I predicted that erythrocyte turnover would be slower in triploids compared to diploids due to increased life span of individual erythrocytes. I designed a diet-shift experiment using a diet produced using a modified SILAC protocol to measure the rate at which diploid and triploid brook charr turnover over their blood cells to determine if ploidy affects turnover rate, as has been suggested elsewhere (Simonot and Farrell 2009; Hansen et al., 2015; Chapter 4). The experiment was designed to run for 12 weeks (84 days) based on data from Charest (2016) who measured the turnover rate of blood in diploid brook charr using sulphur isotopes at 14°C and determined that 50% turnover of the blood was achieved at 50 days, but it was nearly 150 days until 90% turnover was achieved. Unfortunately, five weeks (35 days) into the experiment there was a water failure in the UNB aquatic facility that resulted in the death of all the triploids and more than half of the remaining diploids so I was unable to complete the experiment. However, as a proof of principle, this study demonstrated that it is possible to make use of SILAC to produce an isotopically-distinct diet for fish and that animal tissues will shift to match this new diet after a diet-switch.

7.2 Methods:

Fish rearing:

110 diploid and 110 triploid juvenile brook charr from the UNB aquatic facility were anesthetized in 1% tert-amyl alcohol (Sigma-Aldrich, St. Louis, Missouri, USA), size

matched based on mass (mean 125.9 g for diploids and 124.2 g for triploids, ranging from 100 g to 150 g), tagged with coded passive integrated transponders, and sorted into separate tanks (one per ploidy). During the experiment, fish were fed 0.5% of tank biomass per day (divided over two feedings) adjusted each week when fish mass was measured at each sampling event. Temperature during the experiment was measured every day and was always between 16°C and 17°C.

Diet formulation:

500 mg of L-alanine-1-¹³C (Sigma-Aldrich) was dissolved in 7 L of 70% ethanol. Twenty kg of trout feed (Floating Trout Feed #5, Corey Aquafeeds, Fredericton, NB, Canada) were submerged in the L-alanine-1-¹³C solution in flat-bottomed plastic trays and the ethanol was allowed to evaporate in a fume hood until completely dry. The pellets were well-mixed and stored at -20°C until use. To confirm that the infused pellets were isotopically distinct, infused and control pellets (three pellets per sample, three samples per treatment) were oven-dried at 60°C for two days, ground into a homogenous powder, and analyzed by the Stable Isotope in Nature lab at UNB to determine the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios (described in detail below).

Diet-shift experiment:

One week after sorting into experimental tanks but before the diet switch, samples were collected from five fish per ploidy to establish the baseline isotopic signature of blood cells, blood plasma, skin, mucus, and muscle tissue. Fish were haphazardly selected from their home tanks, anaesthetized in 1% tert-amyl alcohol, and measured (fork length and body mass). Blood samples were collected using heparinized vacutainers (Thomas Scientific, Swedesboro, New Jersey, USA) and epidermal mucus samples were collected

using a spatula and stored in a scintillation vial. Fish were then euthanized in 10% tert-amyl alcohol followed by decapitation. Muscle tissue from the lateral body wall ventral to the lateral line was collected using a razor blade. The skin was removed from the muscle tissue and scales were removed by scraping with a razor blade. Muscle and skin samples were also stored in scintillation vials. Muscle, skin, and mucus samples were stored on ice until tissues could be dried in the oven at 60 for two days. Blood was stored on ice until all other samples were collected. Blood samples were centrifuged for 5 min at 10000 rpm in an Eppendorf model 5424 centrifuge to separate cells from plasma, and then a micropipette was used to transfer the plasma to a scintillation vial. Blood and plasma were then dried in the oven at 60 for two days. This same protocol was used to sample 5 fish per ploidy at weekly intervals after the diet switch. Only blood cell samples were analyzed due to cost constraints.

Stable isotope analysis:

Oven-dried tissue samples were ground into a homogenous powder using a mortar and pestle, then 1.000 – 1.200 mg was weighed out into tin capsules. Carbon and nitrogen stable isotope analyses were conducted at the Stable Isotope in Nature Laboratory at UNB on a Costech 4010 (Milan, Italy) elemental analyzer interfaced through a Thermo Finnigan (Bremen, Germany) Conflo III to a Thermo Finnigan Delta XP continuous flow isotope ratio mass spectrometer. Each sample was analyzed in triplicate and data are reported in the standard per mil (‰) δ notation.

7.3 Results:

There was an enrichment of $\delta^{13}\text{C}$ ratio in the spiked pellets (-22.65 ± 0.10) compared to the untreated control pellets (-19.96 ± 1.20), a shift of approximately 2.5-3.0 ‰ but there

was no effect on the $\delta^{15}\text{N}$ ratio (spiked pellet: 3.44 ± 0.16 , control pellet: 3.43 ± 0.09).

At baseline sampling of the fish, the $\delta^{13}\text{C}$ ratio of the blood ($-20.30 \text{ ‰} \pm 0.07$) was enriched by approximately 2.5 ‰ compared to the untreated pellets ($-22.65 \text{ ‰} \pm 0.10$).

After feeding with L-alanine-1- ^{13}C -spiked feed, there was an enrichment of the $\delta^{13}\text{C}$ ratio in the blood for both diploid and triploid fish (Figure 1). A linear trendline produced the best fit for the first five weeks of data in both ploidies but R^2 values were low.

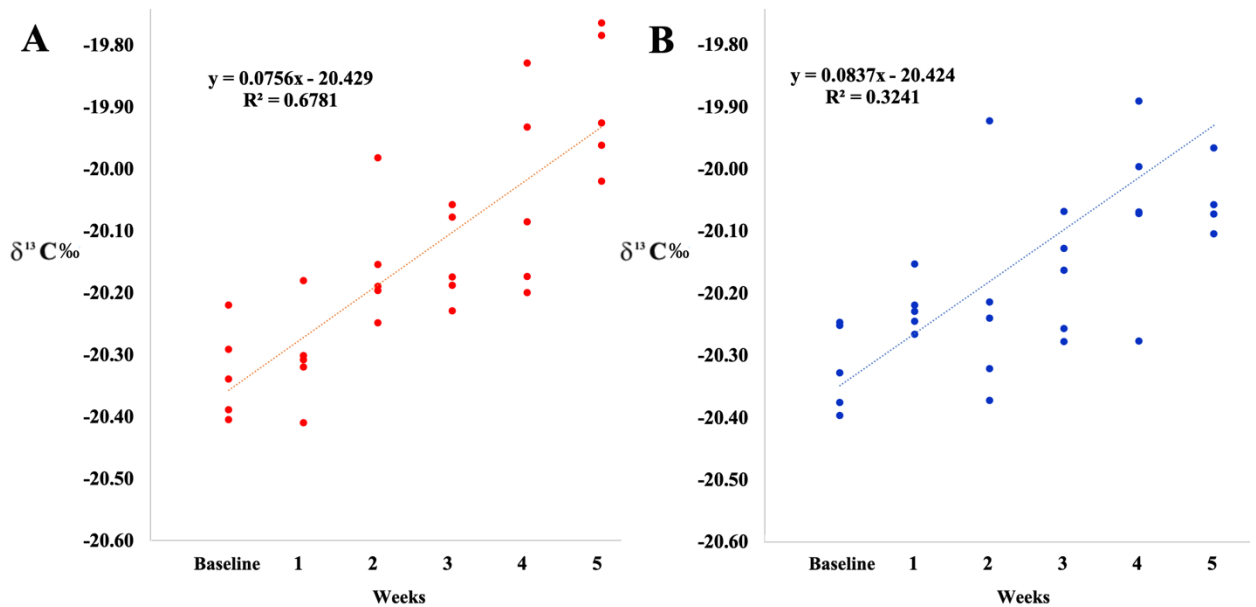


Figure 7.1 $\delta^{13}\text{C}$ increases in blood cells over time after a diet-switching to an isotopically distinct diet in triploid (A, red) and in diploid (B, blue) juvenile brook charr, *Salvelinus fontinalis*.

7.4 Discussion:

This experiment demonstrates that application of a SILAC-based strategy to shift the stable isotope signature of fish pellets for diet-switch experiments is a viable alternative to using distinct feeds derived from different protein sources. The L-alanine-

1- ^{13}C spike resulted in a 2.5-3.0 ‰ $\delta^{13}\text{C}$ enrichment in the feed, and baseline blood values from fish fed this diet were enriched by approximately 3‰ compared to those from fish fed untreated pellets. This shows that the L-alanine-1- ^{13}C spiked feed was sufficiently distinct for use in a diet-shift experiment.

The $\delta^{13}\text{C}$ ratio in the spiked feed was between -19.5 and -20.0 ‰. Given the 2.5‰ enrichment in $\delta^{13}\text{C}$ values between untreated pellets and baseline values from blood samples, I expected the $\delta^{13}\text{C}$ ratio of the blood to eventually equilibrate between -16.5 and -17.0 ‰, a shift of 3‰ compared to the spiked pellets. According to the data from Charest (2016) using sulphur isotopes, tissue equilibration with the diet would take approximately 150 days at 14°C in the blood of adult, diploid brook charr (90% turnover) but this was not feasible for my experiment due to cost and time constraints. Since Charest continued her experiment until tissues had equilibrated, she was able to calculate that 50% turnover of the blood was achieved 49 days after the diet shift. On this basis, my experiment was intended to run for 84 days, which I anticipated would be long enough to reach 50% turnover in triploids even if erythrocyte turnover was slower. I expected $\delta^{13}\text{C}$ ratios to be between -18.0 and -18.5 ‰ by week seven in diploids and I predicated that triploids would take slightly longer to reach these values. Unfortunately, the experiment was terminated after 35 days, before we expected the blood to reach 50% turnover. Although $\delta^{13}\text{C}$ was becoming more enriched in the blood, after 35 days the $\delta^{13}\text{C}$ were only ~0.5‰ more enriched than baseline values. Without any indication that tissues had reached 50% turnover (either by reaching 49 days or by $\delta^{13}\text{C}$ ratios reaching 1.5‰ enrichment), any inferences drawn from these data would be heavily reliant on extrapolation. A linear trendline produced the best-fit for these data based on R^2 , but

these were included only to highlight to positive trend of increased $\delta^{13}\text{C}$ ratio over time rather than for quantitative purposes. I expected that these data would produce a logarithmic curve with rapid change initially (since every cell being removed has no label and every cell being produced is labelled) that slowed over time as the unlabelled cells become rarer (Petersen and Fry, 1987; Vander Zanden et al., 2015; Charest, 2016). Logarithmic curves are frequently produced when biochemists measure enzyme kinetics, but to calculate the maximum rate of reaction (v_{Max}), biochemists use only the initial time points in the reaction when rate of product formation is linear (since every unit of enzyme produces a precise amount of product per unit time). I presume that the linear-fit calculated is an artifact of the fact that we only measured the initial few time points of the shift in $\delta^{13}\text{C}$ ratios of the tissue. Unlike enzyme kinetics assays, there are still too many unknowns regarding the effects of ploidy on the rate of tissue turnover (discussed below) for this initial rate to be useful for comparison.

A critical assumption underlying the logic in this experiment is that the 2‰ - 3‰ enrichment in $\delta^{13}\text{C}$ ratios as carbon moves up a trophic level is not affected by ploidy. There are no data to suggest that this might be affected by ploidy, but that is because it has not yet been tested empirically to rule out this possibility. My experimental design assumed that the scale of the increase in isotope ratios would not be affected by ploidy (i.e., diploids and triploids would both start at X ‰ and equilibrate at $(X + 3)$ ‰), so that I could simply compare how much time had elapsed before diploids and triploids reached a $(X + 1.5)$ ‰ enrichment in the $\delta^{13}\text{C}$ ratio. Repeating Charest's experiments using this modified SILAC protocol for a long-term diet shift experiment until equilibration would certainly be valuable for further study of tissue turnover in triploids

and in fish physiology as a whole. Using a warm-water species such as zebrafish might be a viable alternative strategy to reduce cost since they would both consume far less feed and be expected to turnover their tissues much more rapidly than cold-water species such as brook charr. SILAC experiments in zebrafish for quantitative proteomics are becoming commonplace in research on development (Konzer et al., 2013) and tissue regeneration (Westman-Brinkmalm, et al., 2011), but I am unaware of studies using SILAC diets on adult fish to measure tissue turnover. This novel way to manipulate the isotopic signature of fish pellets provides an alternative way to perform diet-shift experiments that does not require changing to a different feed that may not be nutritionally equivalent.

7.5 References

- Bergmann, O., Zdunek, S., Felker, A., Salehpour, M., Alkass, K., Bernard, S., Sjöström, S.L., Szewczykowska, M., Jackowska, T., dos Remedios, C., Malm, T., Andrã, M., Jashari, R., Nyengaard, J.R., Possnert, G., Jovinge, S., Druid, H., Frisén, J., 2015. Dynamics of cell generation and turnover in the human heart. *Cell*. 161, 1566-1575. <http://dx.doi.org/10.1016/j.cell.2015.05.026>.
- Buchheister, A., Latour, R.J., 2010. Turnover and fractionation of carbon and nitrogen stable isotopes in tissues of a migratory coastal predator, summer flounder (*Paralichthys dentatus*). *Can. J. Fish. Aquat. Sci.* 67, 445-461. <https://doi.org/10.1139/F09-196>.
- Busst, G.M.A., Britton, J.R., 2017. Tissue-specific turnover rates of the nitrogen stable isotope as functions of time and growth in a cyprinid fish. *Hydrobiologia*. 805, 49-60. <https://doi.org/10.1007/s10750-017-3276-2>.

- Charest, M., 2016. Turnover rates and trophic discrimination factors of $\delta^{34}\text{S}$ in brook trout, *Salvelinus fontinalis*. M.Sc. Thesis, Department of Biology, University of New Brunswick (Fredericton).
- Faragher, R.G.A., 2003. The relationship between cell turnover and tissue aging. In: Aspinall, R. (Eds). *Aging of organs and systems*. (1st edition, 1-28).
https://doi.org/10.1007/978-94-017-0673-5_1.
- Franco, R.S., 2012. Measurement of red cell lifespan and aging. *Transfus. Med. Hemother.* 39, 302-307. <https://dx.doi.org/10.1159%2F000342232>.
- Franco, R.S., Puchulu-Campanella, M.E., Barber, L.A., Palascak, M.B., Joiner, C.H., Low, P.S., Cohen, R.M., 2013. Changes in the properties of normal human red blood cells during *in vivo* aging. *Am. J. Hematol.* 88, 44-51.
<https://doi.org/10.1002/ajh.23344>.
- Hansen, T.J., Olsen, R.E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T., Fjellidal, P.G., 2015. Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*. 435, 354-360. <http://dx.doi.org/10.1016/j.aquaculture.2014.10.017>.
- Hayden, B., McWilliam-Hughes, S., Cunjak, R., 2016. Stable isotope analysis reveals limited trophic transfer of allochthonous derived energy in river food webs. *Freshw. Sci.* 35, 544-558.
- Karin, O., Agrawal, A., Porat, Z., Krizhanovsky, V., Alon, U., 2019. Senescent cell turnover slows with age providing an explanation for the Gompertz law. *Nat. Comm.* 10, 5495. <https://doi.org/10.1038/s41467-019-13192-4>.
- Konzer, A., Ruhs, A., Braun, H., Jungblut, B., Braun, T., Krüger, M., 2013. Stable isotope labeling in zebrafish allows *in vivo* monitoring of cardiac morphogenesis.

Mol. Cell. Proteomics. 12, 1502-1512.

<https://dx.doi.org/10.1074%2Fmcp.M111.015594>.

Landsman, S. J., Samways, K. M., Hayden, B., Knysh, K. M., & Heuvel, M. R. van den.

(2018). Assimilation of marine-derived nutrients from anadromous Rainbow Smelt in an eastern North American riverine food web: evidence from stable-isotope and fatty acid analysis. *Freshw. Sci.* 37, 747-759.

<http://doi.org/10.1086/700598>.

Nielsen, J.M., Clare, E.L., Hayden, B., Brett, M.T., Kratina, P., 2017. Diet tracing in ecology: Method comparison and selection. *Methods Ecol. Evol.* 9, 278-291.

<https://doi.org/10.1111/2041-210X.12869>.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. *Annu. Rev. Ecol. Syst.*

18, 293–320. <https://doi.org/10.1146/annurev.es.18.110187.001453>.

Post, D.M., 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecol. Soc. Amer.* 83, 703-718. [https://doi.org/10.1890/0012-9658\(2002\)083\[0703:USITET\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2).

Samways, K., Soto, D.X., Cunjak, R.A., 2018. Aquatic food-web dynamics following incorporation of nutrients derived from Atlantic anadromous fishes. *Jour. Fish Biol.*

92, 399-419. <https://doi.org/10.1111/jfb.13519>.

Simonot, D.L., Farrell, A.P., 2009. Coronary vascular volume remodelling in rainbow trout *Oncorhynchus mykiss*. *J. Fish. Biol.* 75, 1762-1772.

<https://doi.org/10.1111/j.1095-8649.2009.02427.x>.

Spalding, K.L., Bhardwaj, R.D., Buchholz, B.A., Druid, H., Frisé, J., 2005.

Retrospective birth dating of cells in humans. *Cell*. 122, 133-143.

<https://doi.org/10.1016/j.cell.2005.04.028>.

Vander Zanden, M.J., Clayton, M.K., Moody, E.K., Solomon, C.T., Weidel, B.C., 2015.

Stable isotope turnover and half-life in animal tissues: a literature synthesis.

PLoS ONE. 10, e0116182. <https://doi.org/10.1371/journal.pone.0116182>.

Wang, J., Xu, S., Gao, J., Zhang, L., Zhang, Z., Yang, W., Li, Y., Liao, S., Zhou, H.,

Liu, P., Liang, B., 2018. SILAC-based quantitative proteomic analysis of the livers of spontaneous obese and diabetic rhesus monkeys. *Am. J. Physiol. Endocrinol. Metab.* 315, E294-E306.

<https://doi.org/10.1152/ajpendo.00016.2018>.

Westman-Brinkmalm, A., Abramsson, A., Pannee, J., Gang, C., Gustavsson, M.K.,

Otter, M.v., Blennow, K., Brinkmalm, G., Heumann, H., Zetterberg, H., 2011.

SILAC zebrafish for quantitative analysis of protein turnover and tissue regeneration. *J. Proteomics*. 75, 425-434.

<https://doi.org/10.1016/j.jprot.2011.08.008>.

Winter, E.R., Nolan, E.T., Busst, G.M.A., Britton, J.R., 2019. Estimating stable isotope

turnover rates of epidermal mucus and dorsal muscle for an omnivorous fish using a diet-switch experiment. *Hydrobiologia*. 828, 245-258.

<https://doi.org/10.1007/s10750-018-3816-4>.

8.0 Curriculum Vitae:

Christopher D. Small

Current Position:

University of New Brunswick, Fredericton, NB (2014-present)

Ph.D. Candidate co-supervised by Dr. Tillmann Benfey and Dr. Bryan Crawford

Thesis title: Tissue-specific compensatory mechanisms and erythropoiesis in triploid zebrafish.

Education:

Accepted into M.Sc. program at University of New Brunswick, Fredericton (2012-2014)

Projected M.Sc. thesis title: Comparing microvascular blood flow in diploid and triploid zebrafish.

Successfully transferred to Ph.D. program in 2014.

University of New Brunswick, Fredericton NB

B.Sc., Biology. 2012

Publications:

Small, C.D., el-Khoury, M., Deslongchamps, G., Benfey, T.J., Crawford, B.D. Matrix metalloproteinase 13 activity is required for normal and hypoxia-induced precocious hatching in zebrafish embryos. *Journal of Developmental Biology*. **2019**. (Accepted)

Small, C.D. and Crawford B.D. Matrix metalloproteinases in neural development: a phylogenetically diverse perspective. *Neural Regen. Res.* **2016**, 11: 357-362. doi: 10.4103/1673-5374.179030.

Grants and Awards:

2019:

Science Atlantic: Aquaculture and Fisheries

Title: Comparing somite morphology in diploid and triploid zebrafish

Best Graduate Student Talk

Best Overall Communication Award

University of New Brunswick Teaching Award

Teaching Assistant of the Year

Courses:

Animal Physiology, Aquaculture, Human Physiology, Embryology

2018:

Development Day Symposium 2018, Dalhousie University

Title: Comparing tissue structure in diploid and triploid zebrafish

Best Graduate Student Talk

2016:

Science Atlantic: Aquaculture and Fisheries

Title: Testing the water: Adaptive developmental plasticity and its potential for aquaculture

Best Graduate Student Talk

Best Overall Communication Award

2015:

Science Atlantic: Aquaculture and Fisheries

Title: Zebrafish hematology: What can you do with 2 µl of blood?

Best Overall Communication Award

University of New Brunswick Teaching Award

Teaching Assistant of the Year

Courses:

Animal Physiology, Aquaculture, Human Physiology, Embryology

2014:

Aquaculture Association of Canada Scholarship recipient

Research Experience:

2012-Present:

M.Sc. / Ph.D. at University of New Brunswick with Drs. Tillmann Benfey and Bryan Crawford

Thesis title: Examining the role of hematopoietic stem cell number on the rate of erythropoiesis in diploid and triploid fish.

2011/2012:

Undergraduate Independent Study with Dr. Bryan Crawford, UNB.

Thesis title: Collagenase 3 (Mmp13) Accumulation Patterns are Altered in Zebrafish Embryos After Exposure to Environmental Stressors.

Conference Presentations:

2020:

Presentations:

- 1) Title: Tissue-specific compensatory mechanisms in triploid zebrafish

Location: European Zebrafish Meeting, Online conference.

2019:

Presentations:

- 1) Title: Comparing somite morphology in diploid and triploid zebrafish

Location: Science Atlantic, Crandall University, Moncton, NB, Canada.

- 2) Title: The role of ploidy and cell size on vascular morphology in zebrafish

Location: Canadian Society of Zoology, University of Windsor, Windsor, Ont, Canada.

- 3) Title: Matrix metalloproteinase 13 is necessary but not sufficient for hatching in zebrafish embryos

Location: Development Day Symposium, Dalhousie University, Halifax, NS, Canada.

- 4) Title: The role of ploidy and cell size on vascular morphology and microcirculation in diploid and triploid zebrafish embryos

Location: Canadian Society of Ecology and Evolution, University of New Brunswick, Fredericton, NB, Canada.

Posters:

- 1) Title: Body size is independent of cell size in polyploid zebrafish

Location: Society of Developmental Biology, Boston, MA, U.S.A.

2018:

Presentations:

- 1) Title: Comparing tissue structure in diploid and triploid zebrafish

Location: Canadian Society of Zoology, Memorial University, St. John's, Nfld, Canada,
AND Development Day Symposium, Dalhousie University, Halifax, NS, Canada.

- 2) Title: The role of ploidy and cell size on myotome structure in zebrafish

Location: Canadian Society of Developmental Biology, Mont Tremblant, QC, Canada.

- 3) Title: Matrix metalloproteinase 13 regulates precocious hatching in zebrafish embryos

Location: Atlantic Regional Comparative Physiology conference, St. Andrews, NB, Canada.

2017:

Presentations:

- 1) Title: Measuring the Rate of Erythropoiesis in Tg(GATA1a:dsRed) zebrafish

Location: Aquaculture Association of Canada, Halifax, NS, Canada. AND Atlantic Regional Comparative Physiology conference, St. Andrews, NB, Canada.

2016:

Presentations:

- 1) Title: The regulation of MMP13 in early zebrafish development

Location: Development Day Symposium, Dalhousie University, Halifax, NS, Canada.

- 2) Title: Zebrafish as a Model Organism for Triploid Physiology and Development: Baseline Comparisons

Location: Atlantic Regional Comparative Physiology conference, St. Andrews, NB, Canada.

- 3) Title: Testing the Waters: Adaptive Developmental Plasticity and its Potential for Aquaculture

Location: Science Atlantic, Saint Mary's University, Halifax, NS, Canada.

Posters:

- 1) Title: Matrix metalloproteinase 13 proteolytic activity in zebrafish development

Location: Society of Developmental Biology, Boston, MA, U.S.A.

2015:

Presentations:

- 1) Title: Zebrafish Hematology: What can you do with 2 µl of blood?

Location: Science Atlantic, Université de Moncton, Moncton, NB, Canada.

- 2) Title: Idiosyncratic heterogeneity of Mmp13a accumulation during zebrafish development.

Location: North American Zebrafish Researchers, Dalhousie University, Halifax, NS, Canada.

- 3) Title: A non-lethal technique for early identification of triploids in zebrafish embryos.

Location: Atlantic Regional Comparative Physiology conference, St. Andrews, NB, Canada.

2014:

Presentations:

- 1) Title: Blood flow in the microvasculature of diploid and triploid zebrafish

Location: Canadian Society of Zoology, Western University, London, Ont, Canada AND Aquaculture Association of Canada, St. Andrews, NB, Canada.

- 2) Title: Genetics and Transgenesis Made Easy: The Power of the Zebrafish Model

Location: Aquaculture Association of Canada, St. Andrews, NB, Canada.

- 3) Title: Zebrafish Hematology: Overcoming Small Size

Location: Atlantic Regional Comparative Physiology conference, St. Andrews, NB, Canada.

Posters:

- 1) Title: Does capillary lumen size increase to compensate for larger erythrocytes in triploid zebrafish?

Location: Canadian Society of Developmental Biology, Mont Tremblant, QC, Canada

2013:

Presentations:

- 1) Title: The effect of increased cell size on erythrocytes and blood flow in the microvasculature of triploid fish

Location: Canadian Society of Zoology, University of Guelph, Guelph, Ont,
Canada.

- 2) Title: The effect of cell size on blood flow dynamics in the microvasculature of triploid fish

Location: North American Zebrafish Research Conference , University of Maine,
Orono, ME, U.S.A.

- 3) Title: Blood Flow in Triploid Fish

Location: Atlantic Regional Comparative Physiology conference, St. Andrews, NB,
Canada.

2012:

Presentations:

- 1) Title: Using confocal microscopy to measure velocity of individual erythrocytes through a capillary *in vivo*.

Location: Atlantic Regional Comparative Physiology conference, St. Andrews, NB,
Canada.

Posters:

- 1) Title: Collagenase 3 (Mmp13) Accumulation Patterns are Altered in Zebrafish Embryo After Exposure to Environmental Stressors

Location: Canadian Society of Zoology, Mount Allison University, Sackville, NB,
Canada AND The Microscope Society of Canada, Dalhousie
University, Halifax, NS, Canada

Teaching Seminars:

2019:

Title: Insect Development

Location: Guest Lecture in 4th Year Entomology at University of New
Brunswick, Fredericton, NB, Canada

Instructor: Dr. Stephen Heard

2017:

Title: Zygotic Genome Activation in Development

Location: Guest Lecture and Lab Instructor for 3rd Year Embryology laboratory course at University of New Brunswick, Fredericton, NB, Canada
Instructor: Dr. Bryan Crawford

2016:

Title: Capillaries and Microvascular Circulation in Fish Physiology

Location: Guest Lecture for 4th year Fish Physiology at University of New Brunswick – Saint John Campus, Saint John, NB, Canada.
Instructor: Faith Penny, PhD Candidate.