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Consequences of mito-nuclear interactions in life history traits of zebrafish

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Prologue

The present dissertation was carried out in collaboration of two laboratories (the Laboratory of Population Genetic and Evolution and the Laboratory of Marine and Fish Biology) of the Biology Department, University of Crete. At this point, I would like to thank some people who contributed to its completion.

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Abstract

Mitochondria’s most important function is the oxidative phosphorylation (OXPHOS). They also participate in other functions such as cell death, signalling, synthesis of amino acid, haem, nucleotides and lipids, ion homeostasis, cell motility and proliferation. Hence, mutations in mtDNA have been associated with severe human disorders. These diseases are quite common and can be severe, eventually leading to death. At the moment, mitochondrial diseases cannot be cured. They only can be prevented with mitochondrial replacement therapy (MRT). However, regarding MRT, questions have been raised about the potentially harmful genetic effects of combining the recipient's nuclear genome with the donor's mitochondrial genome. These questions need more research with an experimental system model that will be closer to mammals, but will be much easier to handle. Previous researches have mainly been done in invertebrates such as Drosophila or vertebrates such as mice. Zebrafish (*Danio rerio*) is a particularly suitable candidate for such an experimental system model because of its easy maintenance and continuous, and almost infinite, supply of eggs. This thesis contains the first steps in using zebrafish as an experimental model for studying mito-nuclear interactions. We examined which strains and species are suitable for the future study of mito-nuclear interactions and heteroplasmy in chimeric fish through DNA sequencing. Genetic distances showed that the genetically more distant strains are AB and SJD (0,75%) and the genetically closest species are *D. rerio*'s AB and *D. nigrofasciatus* (6,43%). Therefore, after selection through genetic distances, the experiments

for measurement of life history traits such as critical swimming speed, heart rate, total length-at-hatch, mortality, hatching rate and growth rate were performed through a series of replicated trials with *D. rerio's* AB and SJD strains and their hybrid. Through these experiments we characterized the paternal strains and their hybrids for "default" history traits. The species *D. nigrofasciatus* was not well studied, mostly due to difficulties in its reproduction, which could be solved in the future by *in vitro* fertilization.

Keywords: life history traits, mito-nuclear interactions, zebrafish, *D. rerio*, *D. nigrofasciatus*, PCR.

Περίληψη

Τα μιτοχόνδρια πραγματοποιούν πολλές κρίσιμες κυτταρικές λειτουργίες όπως ο κυτταρικός θάνατος, η σηματοδότηση αρκετές μεταβολικές διεργασίες όπως η παραγωγή ATP, η σύνθεση αμινοξέων, νουκλεοτιδίων και λιπιδίων, η ομοιόσταση ιόντων, η κινητικότητα και ο πολλαπλασιασμός των κυττάρων. Ως εκ τούτου, οι μεταλλάξεις στο mtDNA έχουν συσχετιστεί με σοβαρές ανθρώπινες διαταραχές. Οι θεραπείες μιτοχονδριακής αντικατάστασης είναι από τις πιο υποσχόμενες θεραπείες για την πρόληψη των μιτοχονδριακών ασθενειών. Ωστόσο, οι ανησυχίες σχετικά με τις τεχνικές μιτοχονδριακής αντικατάστασης είναι θεμελιώδη θεωρητικά ερωτήματα σχετικά με τη γενετική και την εξέλιξη του mtDNA. Αυτή η έρευνα χρειάζεται ένα πειραματικό μοντέλο συστήματος που θα μοιάζει με θηλαστικά, αλλά θα ήταν πολύ πιο εύκολο να το χειριστούμε. Οι προηγούμενες έρευνες κυρίως έχουν πραγματοποιηθεί μόνο σε ασπόνδυλα όπως η δροσόφυλλα ή σπονδυλωτά όπως τα ποντίκια. Το zebrafish (*Danio rerio*) είναι ένας πολύ καλός υποψήφιος για ένα τέτοιο μοντέλο πειραματικού συστήματος λόγω της εύκολης συντήρησης και της συνεχούς, και σχεδόν άπειρης, παροχής αυγών. ανίχνευση των συνεπειών

που έχει η συνεργασία του μιτοχονδρίου με τον πυρήνα στην αρμοστικότητα των ατόμων zebrafish, όπως η μέτρηση σε διάφορα χαρακτηριστικά της ζωής τους (life history traits). Το πρώτο βήμα είναι ο καθορισμός γενετικών αποστάσεων των έξι διαφορετικών στελεχών του zebrafish και τριών γειτονικών ειδών του γένους *Danio* που έδειξε ότι τα στελέχη με τις περισσότερες γενετικές διάφορες είναι το AB και το SJD και όσον αφορά το είδος το *Danio nigrofasciatus* έχει την πλησιέστερη γενετική απόσταση με το *D. rerio*. Επίσης, δημιουργήθηκαν οι ειδικοί primers για την επόμενη μελέτη των μιτο-πυρηνικών αλληλεπιδράσεων. Επομένως, η μέτρηση σε life history traits όπως στην κρίσιμη ταχύτητα κολύμβησης, στο καρδιακό ρυθμό, στο μήκος σώματος ανά εκκόλαψη, στο ειδικό ρυθμό ανάπτυξης, στη θνησιμότητα και στο ποσοστό των εκκολαπτόμενων αυγών πραγματοποιήθηκε μέσω σειράς επαναλήψεων πειραμάτων με τα στελέχη του zebrafish AB και SJD και το υβρίδιο τους.

Οι εργαστηριακές αναλύσεις πραγματοποιήθηκαν στο Εργαστήριο Πληθυσμιακής Γενετικής και Εξέλιξης του Τμήματος Βιολογίας του Πανεπιστημίου Κρήτης. Λήφθηκαν όλα τα απαραίτητα μέτρα για την εξασφάλιση βέλτιστων συνθηκών αποστείρωσης, καθώς και για την αποφυγή μόλυνσης.

Τα κύρια στάδια της μοριακής ανάλυσης ήταν τα ακόλουθα:

α) εξαγωγές DNA,

β) Αλυσιδωτή Αντίδραση Πολυμεράσης (PCR),

γ) προσδιορισμός αλληλουχίας νουκλεοτιδίων προϊόντων PCR σε αυτοματοποιημένη συσκευή προσδιορισμού αλληλουχίας,

δ) δημιουργία εκκινητών ειδικών για το στέλεχος/είδος.

Τα κύρια μέρη της φαινοτυπικής ανάλυσης ήταν τα ακόλουθα:

α) πείραμα μέτρησης της κολυμβητικής ικανότητας στα ψάρια στο στάδιο της μεταμόρφωσης μέσω μέτρησης της σχετικής κρίσιμης κολυμβητικής ταχύτητας σε ειδική κολυμβητική συσκευή,

β) πείραμα μέτρησης του καρδιακού ρυθμού μέσω των video στα αυγά, 48 ώρες μετά την γονιμοποίηση,

γ) πείραμα μέτρησης του μήκους στην εκκόλαψη μέσω φωτογραφιών των λάρβων που εκκολάπτονταν κάθε 30 λεπτά, 48 ώρες μετά την γονιμοποίηση,

δ) μέτρηση των ρυθμών θνησιμότητας, εκκόλαψης και αύξησης μέσω των παραπάνω πειραμάτων.

Τα αποτελέσματα των μετρήσεων του καρδιακού ρυθμού μας έδειξαν ότι το στέλεχος AB είχε σημαντικά υψηλότερο καρδιακό ρυθμό από δύο άλλα στελέχη. Γενικά, δεν υπάρχουν τόσες πολλές μελέτες σχετικά με τη σύγκριση του καρδιακού ρυθμού εμβρύων διαφορετικών στελεχών/ειδών του zebrafish στα 48 hpf. Η συντριπτική πλειονότητά τους συνήθως χρησιμοποιούν το στέλεχος AB ως ομάδα ελέγχου για τη μελέτη των επιπτώσεων διαφόρων περιβαλλοντικών παραγόντων. Στη μελέτη μας χαρακτηρίσαμε τον «default» καρδιακό ρυθμό για μελλοντικές μελέτες και στόχος μας θα είναι να μάθουμε εάν ο υψηλότερος καρδιακός ρυθμός του στελέχους AB οφείλεται σε μιτοχονδριακό πολυμορφισμό μεταξύ των στελεχών.

Το μήκος στην εκκόλαψη ήταν διαφορετικό για κάθε στέλεχος και το στέλεχος AB είχε μεγαλύτερο μήκος στην εκκόλαψη ενώ το στέλεχος SJD είχε μικρότερο μήκος στην εκκόλαψη και το υβριδικό στέλεχος ήταν ενδιάμεσο μεταξύ τους. Στο μέλλον θα ήταν χρήσιμο να επαληθεύσουμε αυτά τα πειράματα για τα έμβρυα που εκκολάπτονται με 72 hpf καθώς και να ελεγχθεί εάν υπάρχει διαφορά στο μήκος στην εκκόλαψη μεταξύ εμβρύων με τη πρώιμη εκκόλαψη (48-72 hpf) και αργότερη εκκόλαψη (72-96 hpf) σε σχέση με το στέλεχος. Επίσης, είναι ενδιαφέρον ότι το υβριδικό στέλεχος είχε ενδιάμεσο μήκος στην εκκόλαψη μεταξύ δύο

μητρικών στελεχών και όχι το ίδιο με το στέλεχος AB. Θα ήταν ενδιαφέρον να ελέγξουμε εάν αυτό μπορεί να οφείλεται στο mtDNA που κληρονομήθηκε από το στέλεχος SJD ή σε οποιουσδήποτε πολύπλοκους περιορισμούς στους οποίους συμμετέχουν και τα δύο γονικά DNA.

Δεν μπορούμε να ερμηνεύσουμε πλήρως τα αποτελέσματα των μετρήσεων του ρυθμού ανάπτυξης λόγω της σημαντικής επίδρασης της αλληλεπίδρασης μεταξύ της επανάληψης και του στελέχους ως παράγοντες, επομένως, δεν μπορούμε να γενικεύσουμε τη σημασία του στελέχους και για τις δύο επαναλήψεις μαζί. Για να αποφευχθεί αυτό στις μελλοντικές μελέτες, η πειραματική διάταξη πρέπει να σχεδιαστεί μόνο για αυτό το πείραμα ώστε να συγκρίνεται ο ρυθμός ανάπτυξης των ψαριών χωρίς να χρησιμοποιείται μεγάλο μέρος τους για το πείραμα κολυμβητικής ικανότητας.

Στη μελέτη μας τα παρουσιαζόμενα αποτελέσματα της κολυμβητικής δοκιμασίας έδειξαν σημαντική διαφορά μεταξύ του RUcrit του στελέχους SJD και του στελέχους AB. Ωστόσο, δεν υπάρχουν σημαντικές διαφορές στο μήκος των ψαριών που συμμετείχαν στο τεστ κολύμβησης και δεν υπάρχει συσχέτιση μεταξύ του RUcrit και του μήκους των ψαριών. Υπάρχει πιθανότητα ότι ο μιτοχονδριακός πολυμορφισμός είναι ο λόγος που το SJD είχε χαμηλότερη κολυμβητική ταχύτητα από ότι το AB επειδή σε μια άλλη μελέτη έχουν παρατηρήσει τα ίδια αποτελέσματα για το στέλεχος WIK που είναι συγγενικό με το SJD. Το επόμενο βήμα στις μελέτες μπορεί να είναι η έρευνα για το εάν η λιγότερη κολυμβητική ικανότητα των στελεχών SJD και WIK εξαρτάται από τη μιτοχονδριακή ποικιλομορφία ή από φαινοτυπικούς παράγοντες

Ως αποτελέσματα αυτής της μελέτης, επίσης προέκυψε ότι ο ρυθμός θνησιμότητας και ο ρυθμός εκκόλαψης δεν διέφεραν σε σχέση με το στέλεχος.

Αυτές οι μετρήσεις ήταν τυποποιημένες και λάβαμε τα "default" life history traits αυτών των στελεχών. Το είδος *D. nigrofasciatus* δεν έχει μελετηθεί καλά και υπάρχουν προβλήματα

στην αναπαραγωγή του, τα οποία προσπαθούμε να ξεπεράσουμε χρησιμοποιώντας διαφορετικές μεθόδους, συμπεριλαμβανομένης της *in vitro* γονιμοποίησης.

1.Introduction

1.1. Mitochondrial diseases and mitochondrial replacement therapy

The mitochondria are the only organelles in animal cells that have their own DNA. A cell can have thousands of mitochondria, each of which can contain two to ten copies of mtDNA (per cell, there can be 100 to 10,000 separate copies of mtDNA) (Dunham-Snary & Ballinger, 2015). Their most important function is the oxidative phosphorylation (OXPHOS). Cell death, signaling, synthesis of amino acid, haem, nucleotides and lipids, ion homeostasis, cell motility and proliferation are also all carried out by mitochondria. Mito-nuclear interactions must be coordinated well for eukaryotic cells to function properly (Mossm an et al., 2019). Mutations in mtDNA (alone or in combination with nDNA that encodes mitochondrial proteins) have been linked to severe human disorders (myopathies, neurodegenerative diseases, diabetes, cancer, infertility PNT, and so on), multisystem complications, and death during childhood or adolescence, and sometimes, adult onset can cause premature death, for which there are currently no known cure (Dunham-Snary & Ballinger, 2015; Wolf et al., 2015). Because mitochondrial biogenesis is jointly encoded by two different genomes, the genetics of mitochondrial disease and mitochondrial dysfunction are complicated. The nuclear genome (nDNA) encodes over 1000 gene products that operate in the mitochondria, while the mitochondrial genome (mtDNA) contains 13 protein-coding genes, 22 transfer RNAs, and two ribosomal RNAs that are expressed within the organelle (Mossman et al., 2019). As a result, precise and synchronised coordination between the two genomes is essential for appropriate assembly and operation of the electron transport chain components and mitochondrial functions (Anderson et al., 2021). Namely, for eukaryotic cells to function properly, mito-nuclear interactions must be coordinated incredibly well (Pesole et al., 2012). Disruptions to this pathway, whether caused by mutations in the mitochondrial or nuclear sides,

can cause a wide spectrum of changes in life history traits of organism, and in severe cases, mitochondrial disease (Anderson et al., 2021). These diseases are quite common (1 in 5000 newborns) and can be severe, eventually leading to death.

At the moment, mitochondrial diseases cannot be cured (Gorman et al., 2016; Farnezi et al., 2020). They only can be prevented with mitochondrial replacement therapy. This includes transplanting the nuclear genome of an oocyte (or early embryo) from a mother with diseased mitochondria to an enucleated oocyte from an unaffected donor (Herbert & Turnbull, 2018; Mossman et al., 2019). Mitochondrial replacement can be performed before or after fertilization. The fact that mammalian eggs stall in metaphase of meiosis II in preparation for fertilization provides an opportunity for transplantation before fertilization. Polarized light birefringence can be used to see the meiosis II spindle, allowing it to be transplanted along with its accompanying chromosomes. Following fertilization, the haploid maternal and paternal genomes are packaged individually in pronuclei, which may be easily seen and transplanted using light microscopy. Distinguishing the male and female pronucleus in human zygotes is difficult, hence pronuclear transfer entails transplanting both the maternal and paternal pronuclei (Herbert & Turnbull, 2018).

Mitochondrial replacement can radically affect the expression profiles of nuclear genes and affect a range of critical aspects such as individual development, cognitive behavior, and vital health markers, according to studies on model species ranging from mice to fruit flies (Reinhardt et al., 2013; Anderson et al., 2021). Questions have been raised about the potentially harmful genetic effects of combining the recipient's nuclear genome with the donor's mitochondrial genome. Mito-nuclear communication and interaction is a standard part of proper cell function, and in normal sex, half of the mother's nuclear genome will be inherited with mitochondria, with which it is relatively compatible because these nuclear and mitochondria

genomes have co-evolved. However, mitochondrial replacement can disrupt this form of natural selection and adaptation, which could have unintended consequences for cellular bioenergetics and physiology (Dunham-Snary & Ballinger, 2015; but see Eyre-Walker, 2017). One of the most pressing question concerning mitochondria replacement is whether the experimental treatment of the oocyte affects the embryo's development and fitness (Tachibana et al., 2009; Hyslop et al., 2016) as well as whether it creates harmful incompatibilities due to mito-nuclear interactions when the donor's mtDNA must collaborate with the recipient's foreign nuclear background (Gemmell & Wolff, 2015; Wallace, 2016; Yu-Wai-Man, 2016). Another concern regards the small amount of mutated mtDNA that can be transferred to the oocyte along with the nucleus and produce embryos with two types of mtDNA (heteroplasmy) the mutated and the wild-type ones. Because of non-Mendelian genetics of mtDNA the mutated type can spread and out-compete the wild type, jeopardizing mitochondrial replacement therapy's success (Kang et al., 2016; Sato & Sato, 2017).

Despite that the first babies have been born with mitochondrial replacement technique (Zhang et al., 2017; Pompei & Pompei, 2019) the questions above have not been studied in detail. Addressing these questions needs large number of experiments and increased sample sizes, which cannot be performed in humans, mainly because of ethical reasons. Therefore, an experimental system for performing these experiments would be particularly useful. Such a system could be the zebrafish as it will be described below (paragraph 1.2).

In mito-nuclear interactions nuclear genes evolve continuously to alleviate dysfunctions caused by mutations in mitochondrial genes which accumulate at much higher rate and reside more frequently in a genome with lower recombination rate (Hill et al., 2019). Barreto et al. (2018) pointed out that mitochondrial proteins encoded by nDNA are evolving rapidly in related populations of intertidal copepod *Tigriopus californicus* and this might represent adaptive

changes within each population as a result of fast and constantly changing mtDNA. Rand & Mossman (2020) clarify in their work that mitochondria is playing a role of one of the links between phenotype and genotype through environmental and mito-nuclear interactions which govern ongoing mito-nuclear evolution. However, given the ubiquity of co-functioning and co-evolution of mitochondrial and nuclear genes, our knowledge about an influence of mito-nuclear interactions on phenotype of metazoan (and in natural populations too) is still incomplete and needed further research (Hill et al., 2019).

1.2. The zebrafish as a "model" organism in the study for mitochondrial replacement therapy

The zebrafish *Danio rerio* (Hamilton, 1822) is a tropical freshwater species of the Cyprinidae family. The natural habitats of the species are in rivers and lakes of the tropical areas of South-East Asia, Pakistan, India, Bangladesh, Nepal and Myanmar (Figure 1).

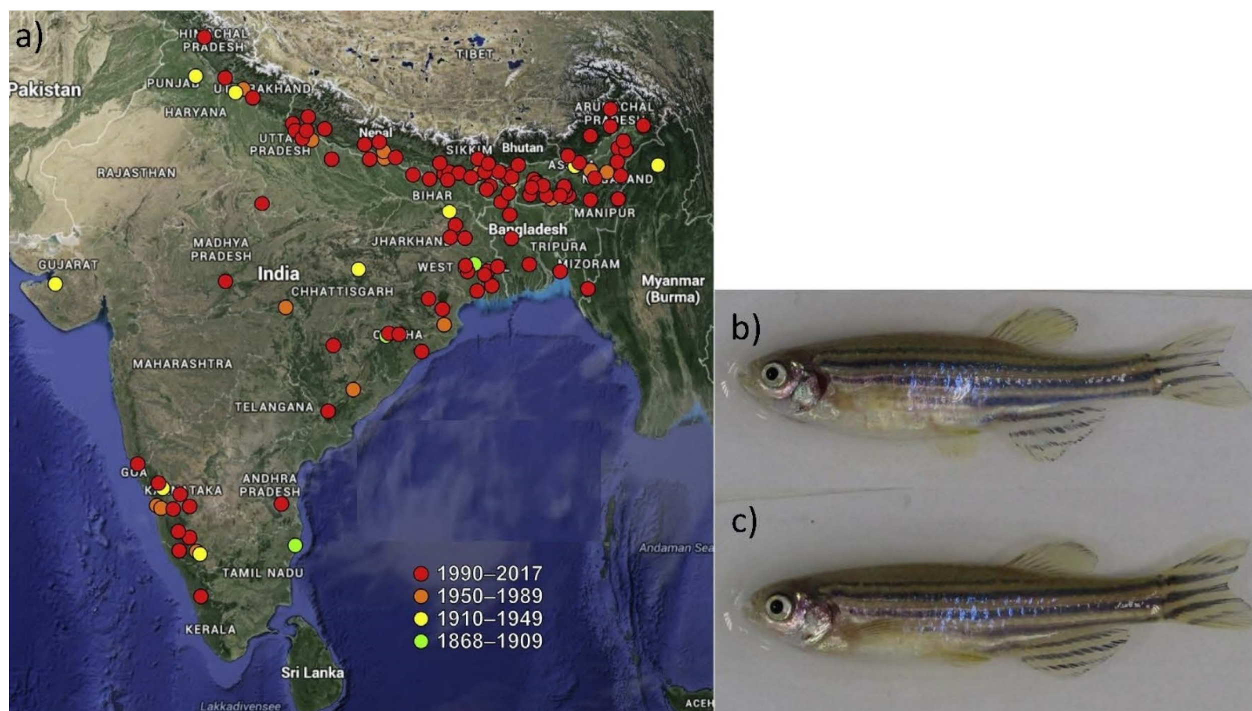

Figure 1. a) Historical and recent sites of zebrafish's geographical distribution. Image by Parichy & Postlethwait, 2020. b) Adult female zebrafish, and c) adult male zebrafish, image from the current study.

Zebrafish in the wild are found in small flocks (5-20 individuals) and feed on zooplankton, insects and phytoplankton. At the same time, it is a widespread species with the temperature in its living environments ranging from 6 °C in winter to 38 °C in summer, at a slightly alkaline pH (~8.0) (Lawrence, 2007; Spence et al., 2008).

The species is oviparous, with asynchronous maturation of the gonads and without parental care of the offspring. As it adapts to the monsoon climate, reproduction is synchronised with the arrival of the rains. Reproduction highly depends on food availability, the levels of which positively depend on increasing rainfall (Spence et al., 2008), and is also regulated by the photoperiod. Zebrafish spawn in the early morning hours both in the laboratory (Selman et al., 1993) and in the wild (Spence & Smith, 2006). The eggs of this species are benthic and, depending on the conditions (temperature, water chemistry), they hatch in 4-7 days in free-swimming larvae (Lawrence, 2007). At 28 °C hatching takes place 48-72 hours after fertilization. At the same temperature, the larval stage is completed in about 20 days after fertilization. Under laboratory conditions, the species can spawn continuously when subjected to 14:10 L:D photoperiod conditions (light:dark). Generation time ranges from 3 to 4 months and lifespan from 5 to 7 years (Westerfield, 2007).

The zebrafish mtDNA is a typical animal mitochondrial genome in terms of size and gene content. It is a circular, double-stranded, 16596-nucleotide genome that encodes seven NADH dehydrogenase subunits (Complex I), one cytochrome c reductase subunit (Complex III), three cytochrome c oxidase subunits (Complex IV), and two ATP synthase subunits (Complex V). Furthermore, transcription of this genome generates components of the genome's own

translational apparatus, namely 22 tRNAs and 2 rRNAs, highlighting its semi-autonomous nature because these processes rely on the nuclear genome as well (Artuso et al., 2012).

Zebrafish has been one of the best study models in many fields of biology in recent years and it is a particularly suitable candidate for the study of mitochondrial replacement for several reasons:

- it is a vertebrate and as vertebrate has the same major organs and tissues as humans; over 70% of human genes have a true ortholog in the zebrafish genome (Beffagna, 2019);
- it has already been used as a model organism in genetics, developmental and molecular biology, and there are numerous molecular tools designed for zebrafish available for use;
- it is an oviparous species with external fertilisation, easy to be maintained and handled in the lab, which has a continuous, and almost infinite, supply of eggs (Uusi-Heikkiï et al., 2010; Lawrence et al., 2012). Also, its life cycle contains only 60 days (Spence et al., 2008) or even shorter. All this makes zebrafish a model organism for the study of mitochondrial diseases (Steele et al., 2014);
- the ethical restrictions for the use of zebrafish in the laboratory are considerably less strict than those for mammals.

1.3. Life history traits

An organism's life history includes its pattern of survival and reproduction, as well as the qualities that directly affect survival and the timing or amount of reproduction. In organisms with complex life cycles, rates of survival and reproduction can be assessed across age classes or at different stages. Growth rate, age and size at sexual maturity, the temporal pattern or schedule of reproduction, the number, size, and sex ratio of offspring, the distribution of intrinsic or extrinsic

mortality rates (e.g., patterns of senescence), and patterns of dormancy and dispersal are examples of life history traits (Fox & Messina, 2013).

For our study, the following life history traits of zebrafish were chosen: swimming capacity, heart rate, total length at hatching, growth rate, mortality and hatching rate, which are important indicators of morphological differences between strains and species.

Fish swimming performance can be divided into three categories: continuous swimming, prolonged swimming, and burst swimming. Continuous swimming is achieved at low speeds with the participation of primarily red muscle fibers that are supplied with aerobic metabolism energy, while burst swimming includes white muscle fibers that use anaerobic metabolism energy (Beamish, 1978). In the case of prolonged swimming, red muscle fibers are initially employed, while at speeds greater than 80% of critical swimming speed (U_{crit}), pink and white muscle fibers are also recruited (Beamish, 1978; Hammer, 1995; Reidy et al., 2000).

The most established way of calculating the aerobic swimming ability of fish is by measuring U_{crit}. According to Brett (1964), U_{crit} is the maximum prolonged swimming speed achieved at the point of fatigue, at which the maximum prolonged oxygen uptake takes place. U_{crit} is used as an important physiological parameter for calculating maximum aerobic exercise in fish (Brett, 1964; Hammer, 1995; Farrell, 2007). At the same time, the ecological importance of this speed for fish is crucial for migration (Plaut, 2001), the avoidance of prey (Fuiman et al., 2006), but also for the search for food (Reidy et al., 2000). Therefore, U_{crit} is an important determinant of fish fitness and is often used as a means of calculating it (Hammer, 1995; Reidy et al., 2000). Finally, U_{crit} is widely used to assess the impact of different environmental factors on fish aerobic exercise (Hicken et al., 2011; Scott & Johnston, 2012; Dimitriadi et al., 2018; Kourkouta et al., 2021; Sergi et al., 2022).

One of the first organ systems to function in the developing fish is the cardiovascular system emerging by around 24 hours post fertilisation (hpf) in zebrafish (Stainier & Fishman, 1994; Bagatto, 2005). Heart of zebrafish plays a crucial role in fish survival and, also, zebrafish can efficiently regenerate its heart (Kikuchi, 2014; Beffagna, 2019)). Hence, it is a renowned model for many heart diseases studies and heart rate measurement is one of crucial life history traits of zebrafish. Size of zebrafish is a major indicator of its developmental progress and cannot be substituted by just age in hours or days post fertilization (Higgs et al., 2002; Parichy et al., 2009; Christou et al., 2018). Hence, length at hatching is an important trait which shows whether there is any difference in embryo development from the start of fish life. Specific growth rate is often used in various dietary and environmental studies (Dabrowski & Miller, 2018; Bai et al., 2019; Meirelles et al., 2021; Printzi et al., 2021; Sergi et al., 2022) along with mortality.

1.4. The purpose of this research

This thesis regards the first steps in using zebrafish as an experimental model for studying mito-nuclear interactions. We examine which strains and species are suitable for the future study of mitochondrial interactions and heteroplasmy in chimeric fish through molecular methods. We also characterize the paternal strains and their hybrids for "default" life history traits.

2. Materials and methods

2.1. Molecular analyses

The laboratory analyses were performed in the Laboratory of Population Genetics and Evolution of Biology Department of the University of Crete. All necessary measures were taken to ensure optimal sterilisation conditions, as well as to avoid contamination.

The main stages of the laboratory analysis were as follows:

a) DNA extractions,

b) Polymerase Chain Reaction (PCR),

c) nucleotide sequencing of PCR products in an automated sequencing device,

d) strain/species-specific primer creation.

2.1.1. DNA extractions

The samples from the 6 strains - AB, Wageningen, Casper, SJD, TL, WIK and 3 species (*Danio albolineatus*, *Danio aesculapii*, *Danio nigrofasciatus*) (Table 2.1) were taken for genetic analysis purposes. Every sample was dissolved in the lab mortar with liquid nitrogen, after that, extraction solution was added (400µl of lysis buffer, 10 µl of proteinase k and 30 µl of 10% SDS for every sample) and put in the water bath with t°=55°C for 3-4 hours. Then 280 µl of saturated solution NaCl (6M) was added, and the mix was put in the vortex for 15 minutes and in the centrifuge (1300 turns) for 30 minutes. The supernatant was transferred to another eppendorf, 500 µl of chloroform was added and eppendorf was put in the centrifuge (1300 turns) for 5 minutes. After that supernatant suspension was transferred again, 600 µl of cold isopropanol was added and this solution was put in the fridge for 30 minutes for DNA to be formed. After that, the solution was centrifuged for 15 minutes for DNA to form a pellet, propanol was removed and

a little bit of 70% ethanol was added. After complete drying, 50 μl of pure water was added to the pellet.

Table 2.1. Genus Danio species and strains (Froese & Pauly, n.d.)

name	source	species	characteristic phenotypes
AB	Marine and Fish Biology of the University of Crete, Greece	*Danio rerio*	uniform, pigmented, horizontal, blue stripes on the side of the body
Wag (sub-population of AB that were bred in the source lab)	Marine and Fish Biology of the University of Crete, Greece	*Danio rerio*	uniform, pigmented, horizontal, blue stripes on the side of the body
Casper	Marine and Fish Biology of the University of Crete, Greece	*Danio rerio*	translucent skin and scales
SJD	European Zebrafish Resource Center of Institute of Biological and Chemical Systems - Biological Information Processing, Germany	*Danio rerio*	uniform, pigmented, horizontal, blue stripes on the side of the body
Tübingen long fin (TL)	European Zebrafish Resource Center of Institute of Biological and Chemical Systems - Biological Information Processing, Germany	*Danio rerio*	spotted, long-finned
Wild Indian Karyotype (WIK)	European Zebrafish Resource Center of Institute of Biological and Chemical Systems - Biological Information Processing, Germany	*Danio rerio*	uniform, pigmented, horizontal, blue stripes on the side of the body
D. aesculapii	Max Planck Institute for Biology in Tübingen, Germany	*D. aesculapii*	6-7 brown vertical bars anteriorly on side, two horizontal rows of small brown spots posteriorly
D. albolineatus	Max Planck Institute for Biology in Tübingen, Germany	*D. albolineatus*	body pink with two light yellow-white stripes from below dorsal origin to caudal base
D. nigrofasciatus	Max Planck Institute for Biology in Tübingen, Germany	*D. nigrofasciatus*	striped, spotted, a fine net of dorsal scales lined in soft gray

2.1.2. Polymerase Chain Reaction (PCR)

Multiplication of selected genetic loci is the next step for genetic distance analysis. In the present work, two mitochondrial genetic loci were selected:

- the COXI (cytochrome c oxidase subunit I) gene, which is one of the most popular markers used for molecular systematics predicted to enable heme binding activity and metal ion binding activity and to contribute to cytochrome-c oxidase activity (Madden, 2002),

- the 16s RNA gene, which is a ribosomal RNA encoded by the mitochondrial genome and is necessary for the translation of messenger RNAs into mitochondrial proteins (Yang et al., 2014).

The polymerase chain reaction (PCR) technique was used to amplify the markers, each reaction containing 1 μl of genomic DNA and 19 μl of Master Mix, containing 2.5 units of Taq polymerase, 1X Taq buffer, 1.5 mM MgCl2, 0.2 μM dNTPs, 0.2 μM from each primer (2 primers per reaction) and ultra-pure water until complete volume.

The primer pairs, as well as information on their sequence and expected product size, are listed in Table 2.2, While the PCR conditions used for gene amplification are listed in Table 2.3.

Table 2.2. The genetic loci studied in the present work, the names and sequences of the primers used, the size of their products.

genetic loci	primers	sequence (5'-3')	size of DNA part
16sRNA	16SAR	CGCCTGTTTATCAAAAACAT	614
	16SBR	CCGGTYTGAACTCAGATCAYGT	
COX1	LCO1490	GGTCAACAAATCATAAAGATATTGG	708
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	

Table 2.3. Conditions of PCR reactions for the two primer pairs used in the present study. Each cycle was repeated 35 times.

genetic loci	primers	conditions		
		stage	t (°C)	time (sec)
16sRNA	16SAR	Denaturation	94	30
		Annealing	42	30
	16SBR	Extension	72	60
COX1	LCO1490	Denaturation	95	30
		Annealing	48	30
	HCO2198	Extension	72	60

2.1.3. Electrophoresis of PCR products in agarose gel

To test the PCR results, the samples were electrophoresed in 1% agarose gel containing 5 μl of ethidium bromide. 1 μl of DNA, 2 μl of agarose gel loading buffer 6X and 8 μl of ultra-pure water were placed in each well of the gel. The samples were electrophoresed for approximately 35 mins at 120 volts. The gel was then placed in a special chamber, exposed to UV radiation, and photographed. DNA becomes visible as fluorescent bands within the gel as ethidium bromide binds to DNA and fluoresces when exposed to UV radiation.

2.1.3. PCR product sequencing

Sequencing was performed by the sequencing company CeMIA (Larissa) on an automated sequencing device (ABI3730XL DNA Analyzer by Applied Biosystems), using PCR forward and reverse primers for all genes. This device recognises the labelled nucleotides of the synthesised sequence upon electrophoresis of the product on a polyacrylamide gel. In total, all individuals were successfully sequenced for COX1 and 16s RNA genes.

2.1.4. Sequence correction and alignment

After DNA sequencing, the quality of the sequences was evaluated, as well as their homology to other known sequences using the heuristic BLAST algorithm in the NCBI database (Madden, 2002). The sequences were then checked and corrected "by eye" in MEGA X (v. 10.2.4) (Kumar et al., 2018). Some bases were corrected and the ends of the sequences were removed where the "reading" by the sequencer was of low quality.

The sequences were then aligned using the MAFFT algorithm (Kuraku et al., 2013; Katoh et al., 2019) through the MAFFT online program (v.7). The alignment is intended to determine the homologous positions of the sequences, since based on this the evolutionary relations of the sequences are calculated. After a comparison of all the sequences with the corresponding sequences in BLAST, almost 100% similarity was observed. BLAST has a partial mitochondrial genome for every strain and species that were used in analysis and because of the said similarity, the decision was made to use this partial mitochondrial genome from BLAST for comparison of genetic distances between strains and species.

2.1.5. Strain/species-specific primer creation

Primer designing: Sequences for primers for AB, SJD, and *D. nigrofasciatus* were chosen after carefully and manually comparing each gene of mtDNA of these strains/species. After that, those sequences were sent to Eurofins Scientific for primers creation.

Primers of high quality should generally have the following properties:

- Length of 18-24 bases
- 40-60% G/C content
- Start and end with 1-2 G/C pairs

- Melting temperature (Tm) of 50-60°C
- Primer pairs should have a Tm within 5°C of each other
- Primer pairs should not have complementary regions

2.2. Experimental design and phenotypic analyses

2.2.1. Fish maintenance

Male and female zebrafish were obtained from the laboratory of Marine and Fish Biology of the University of Crete and from European Zebrafish Resource Center of Institute of Biological and Chemical Systems - Biological Information Processing in Germany. *D. aesculapii*, *D. albolineatus* and *D. nigrofasciatus* were kindly provided by the Max Planck Institute for Biology in Tübingen, Germany. Fish were raised for 6 months and maintained at 28 °C and a 14/10 light/dark cycle (Westerfield, 2007). All fish were housed in 8-litre tanks at a density about 6 fish/L or less in a recirculating freshwater system (Zebtec, Tecniplast Group). Adult fish were fed twice daily to apparent satiation with a commercial zebrafish feed (Sparos Lda., Portugal) and bloodworms. All strains/species of zebrafish were raised from embryos under identical conditions.

2.2.2. Swimming performance

Fifteen pairs of fish (five for each group respectively: AB, SJD and their hybrid: SJD females X AB males) were chosen in triplicate for spawning. Fertilised eggs were collected 6-8 hours after spawning to enable their easy discrimination from the unfertilised eggs. The same number of eggs (about 40 eggs) was taken from every pair in the group that spawned.

Egg incubation and larval rearing were performed in 100 L aquarium with three (4.5 L each) incubators (100 eggs/incubator initial density) connected to closed recirculation systems, at

pH 7.0–7.5, 500–700 μS/cm conductivity, 85–95% oxygen saturation, 14/10 h light/dark photoperiod. Larvae were fed on newly hatched *Artemia* nauplii five times per day. After 5-6 mm TL, commercial diet was provided to the experimental populations (Sparos Lda., Portugal), and provision of *Artemia* nauplii was gradually terminated (Printzi et al., 2021). Swimming performance was estimated in when fish reached 10-12 mm total length (TL). An experimental protocol was conducted with three replicates, where zebrafish larvae were raised and treated likewise until adulthood.

According to Koumoundouros et al. (2009), swimming performance was assessed by estimating the sustained critical swimming speed U_{crit} with a custom-designed swimming apparatus (Figure 2.1., with a swim tunnel of 70 cm length, 10 cm depth and 5 cm width). Different flow regimes were obtained using external magnetic pumps and adjustable valves. An electromagnetic flow-meter sensor (Valeport, model 801) was used to calibrate water speed in the tunnel. A bunch of plastic straws helped in maintaining laminar flow through the swimming channel and in preventing fish from forward escape. Temperature was maintained constant at 28 °C and oxygen saturation at >95%.

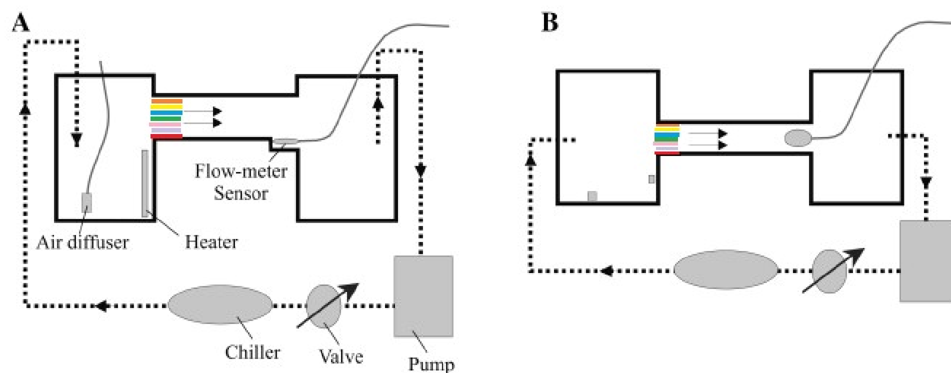

Figure 2.1. Swimming apparatus used in the present study. (A) Lateral view; (B) top view. Arrows indicate the direction of water circulation (Koumoundouros et al., 2009).

Every day eighteen to twenty hours prior to the swimming trials, 4-6 fish from each group and (i.e. 8-12 per group in total) were transferred to three holding aquariums (one for each

group) and deprived of food. For the swimming tests, fish of similar size were placed in the swimming tunnel for 15 min at 2 TLs^{-1} water velocity. Then, water velocity increased every 15 min at a rate of 3 TLs^{-1}. Fatigue was determined when fish left the swimming channel, unable to react to visual or acoustic stimuli from the side or behind (Koumoundouros et al., 2009). Swimming tests were performed at 28.0 ^{o}C and >95% oxygen saturation levels in the water. The experiment was replicate 3 times with identical protocol. TLs^{-1}

The critical swimming speed (U_{crit}) was calculated based on the following formula:

$$U_{crit} = U_i + \left(U_{ii} \times \frac{t_i}{t_{ii}}\right), \text{ (Brett, 2011)}$$

where U_i is the highest swimming velocity (mm s^{-1}) maintained for a full interval of 15 min, U_{ii} is the velocity increment (3 TL s^{-1}), t_i is the time interval that each individual swam at the fatigue velocity, and t_{ii} is the time interval between velocity changes (i.e. 15 min) (Brett, 196). Fatigued fish were anaesthetized (2-phenoxyethanol), photographed under a stereoscope (Olympus SZ61), measured for TL (Image analysis, Lumenera Infinity Analyze Microscopy Software, version 6.5.4, Canada), and fixed in 5% phosphate buffered formalin. Only fish with normal morphology were included in the analysis.

Relative critical swimming speed (RU_{crit}) was calculated as the ratio of U_{crit} to the TL of each specimen ($RU_{crit} = U_{crit}\ TL^{-1}$, Beamish, 1978).

2.2.3. Cardiac rate measurements

Heart rate was estimated on embryos that were collected from the spontaneous spawn of 4 pairs of fish per experimental group (AB, SJD, SJD ♀ x AB ♂) and replicate (three replicates). The breeders were kept under identical conditions (t= 28.0 °C (±0.5 °C), 500–700 μS/cm conductivity, 7.0–7.5 pH, 85–95% oxygen saturation and 14/10 h light/dark photoperiod) in 4 L tanks. After 3-4 days in tank pairs were put in collectors with separators. On the morning of the

next day, separators were taken off at the same time for all pairs. Sixty minutes later, 50-60 eggs were collected from every pair and put in beakers (100 ml), at 28.0 °C. Eight to ten hours post-fertilization (hpf), unfertilized eggs were removed from the beakers. Embryo mortality was recorded at 24 hpf and 48 hpf. Heart rate was measured on videos of embryos at 48 hpf. Video recording was performed under a stereoscope (Olympus SZ61) (Image capture, Lumenera Infinity Analyze Microscopy Software, version 6.5.4, Canada), at 28 ^{o}C. Heartbeats were counted manually on a 14-second video-recording of each embryo. After each recording, water temperature was measured and the embryo was returned to the holding beaker to hatch.

2.2.4. Total length (TL) at hatching

Following the heart rate measurements (48 hpf) all hatched larvae were removed from the beakers. Then, all embryo populations were examined every 30 minutes and newly-hatched larvae were removed, photographed under a stereoscope (Olympus SZ61) (Image analysis, Lumenera Infinity Analyze Microscopy Software, version 6.5.4, Canada) and measured for TL.

2.2.5. Mortality, hatching and growth rate measurements

For mortality and hatching rate measurements, the setup for heart rate and length-at-hatch measurements was used and they were estimated for every replicate.

Mortality rate was estimated by dividing dead eggs removed after 24 hpf and 48 hpf by the total egg number, excluding unfertilised eggs that were removed at 8-10 hpf.

$$mortality\ rate = \frac{dead\ eggs}{(total\ egg\ number - unfertilised\ eggs)} * 100\%$$

Hatching rate was estimated by the use of the following formula:

$$hatching\ rate\ = \frac{total\ egg\ number - (unfertilised\ eggs + dead\ eggs)}{total\ egg\ number} * 100\%$$

For growth rate estimations, all fish that were left at incubators were anaesthetised after the end of swimming performance, stained (Walker & Kimmel, 2009) and total length was measured under a stereoscope (Olympus SZ61) (Image analysis, Lumenera Infinity Analyze Microscopy Software, version 6.5.4, Canada). Only the fish from first and second replicates were used cause there were considerably smaller number of survived fish and consequently smaller number of fish left after swimming challenge.

First replicate: AB (40 individuals) and SJD ♀ x AB ♂ hybrid (43 individuals) were 21 dpf old, SJD (46 individuals) were 27 dpf old cause SJD overall had slower growth and lesser surviving and could not participate in swimming challenge together with AB and SJD ♀ x AB ♂ hybrid. So, we prolonged their Artemia feeding for faster growth.

Second replicate: AB (54 individuals) and hybrid (31 individuals) were 22 dpf old, SJD (9 individuals) were 23 dpf old.

The logarithm of total length of the fish at 4 dpf was subtracted from the logarithm of total length and the number was divided by days after fertilisation (when the fish were anaesthetised) excluding the 4 dpf.

$$growth\ rate = \frac{\ln(TL) - \ln(3.7mm)}{age - 4\ (dpf)}$$

2.3. Statistical analysis

Parametric ANOVA test (Girden, 1992) (or alternatively non-parametric Kruskal-Wallis test (Kruskal & Wallis, 1952) in case of non-fulfilment of ANOVA’s assumptions) was used for statistical analysis for every replicate of each experiments. Two-way ANOVA was used for the pooled dataset with the replicate as the second factor. Also, for unbalanced datasets (the samples

did not have the same size) the ANOVA for unbalanced data (type III) were used. Non-parametric ScheirerRayHare test (Sokal, 1995; Mangiafico, 2016) which is, in some sense, the two-factor version of the Kruskal-Wallis test was also used for the pooled dataset with the replicate as the second factor in cases where the dataset did not fulfil ANOVA's assumptions (like homogeneity of variance) or had small sample size. Also, ANOVA tests were followed by Tukey's HSD test (Tukey John W, 1977) for clarifying information about differences, Dunn test (Dunn, 1961) was used as post-hoc test for the ScheirerRayHare and Kruskal-Wallis tests for the same reason.

3. Results

3.1. Molecular analyses

3.1.1. General information about analysed sequences

We used the sequences of COI and 16sRNA to calculate to characterize genetically six strains of *Danio rerio* (namely, AB, TL, WIK, SJD, Wagenigen and Casper) and three *Danio* species (*D. nigrofasciatus*, *D. albolineatus* and *D. aesculapii*). The sequences that we obtained from these strain and species matched 100% with the respective parts of the whole mtDNA genomes of these strain and species, which were deposited in the GenBank. Therefore, to estimate the genetic distances among these strains and species we used the whole mtDNA sequences of the GenBank with accession numbers KT624627.1 for AB, KT624625.1 for TL, KT624626.1 for WIK, KT624624.1 for SJD, NC_027688.1 for *D. nigrofasciatus,* NC_029771.1 for *D. albolineatus*. For *D. aesculapii* there was not sequences for complete mtDNA genome. That is why only a partial sequence from our study was included and was used for the comparisons.

3.1.2. Genetic distances

The genetic distances were calculated pairwise for all strains and species combinations. The sequences of AB, Wagenigen and Casper were identical hence, only AB, WIK, SJD and TL were included in the following research. Genetic distances showed that the genetically more distant strains are AB and SJD (0,75%) and the genetically closest species are *D. rerio*'s AB and *D. nigrofasciatus* (6,43%) (Table 3.1., Figure 3.1.).

Table 3.1. Estimations of evolutionary divergence in mitochondria between sequences of 4 strains and 2 species. The number of base differences per site from between sequences are shown. This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for

each sequence pair (pairwise deletion option). There was a total of 17626 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 201X)

	strain/species	1	2	3	4	5
1	**AB *(Danio rerio)***					
2	**TL *(Danio rerio)***	0.23%				
3	**WIK *(Danio rerio)***	0.71%	0.67%			
4	**SJD *(Danio rerio)***	0.75%	0.71%	0.04%		
5	***Danio nigrofasciatus***	6.43%	6.47%	6.77%	6.82%	
6	***Danio albolineatus***	25.09%	25.04%	25.08%	25.14%	25.28%

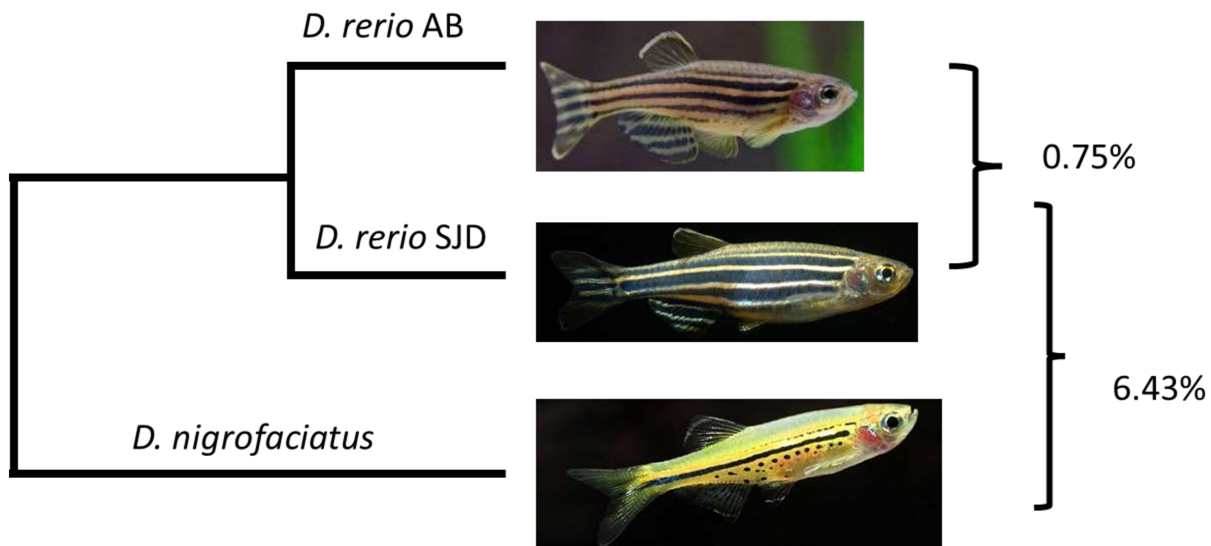

Figure 3.1. Morphological view of strains and species which were selected for further research with designated genetic distances.

3.1.3. Strain/species-specific primer design

We designed strain-specific and species-specific primers. They were 18 to 24 bases long, with 45-54% of G/C content, start and end with 1-2 G/C pairs, melting temperature (Tm) of 52-58°C. Primer pairs have a Tm within max. 2°C of each other, primer pairs have self-annealing and cross-annealing regions max.4 base pairs (Table 3.2.). These properties are falling under the description of a well-working primer, so the primer creation was considered successful after PCR-check (Figure 3.2).

Table 3.2. Sequences of primer pairs, as well as information on their size, melting temperature and other parameters

species/strain	primer	primer's direction	sequence	length (bp)	GC (%)	Tm (°C)	self-annealing (bp)	cross-annealing (bp)	size of DNA part (bp)	primer's place in mtDNA
AB	AB-F	forward	5'- AAC ACC CTA ACA ACG ACC -3'	18	50	52.2	2	3	607	5667-5684
	AB-R	reverse	5'- CTG AAG ACG CAG GTA AAC C -3'	19	52.6	53.3	3			6291-6309
SJD	SJD-F	forward	5'- TAC CCT AAC AAC AAC ATG ACC G -3	22	45	54.8	4	3	601	5669-5690
	SJD-R	reverse	5'- CTG AAG ACG CAG GCA AAT G -3'	19	52.6	54.5	2			6291-6309
Danio nigrofasciatus	Nigro-F	forward	5'- GAC CTT CTC ACA GGG TTG ATC CTC -3'	24	54.2	58.8	3	4	561	5360-5383
	Nigro-R	reverse	5'- GGA AGT GAA GTT TGG GTT GAT TGC -3'	24	45.8	56.9	2			5944-5967

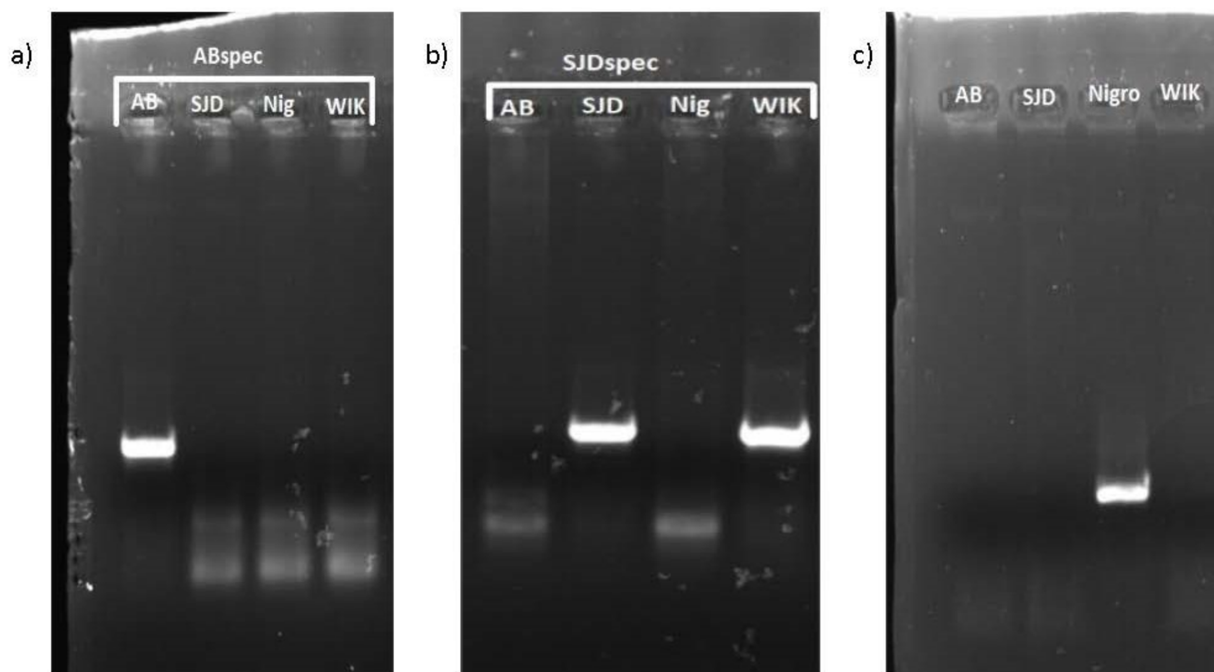

Figure 3.2. Results of primer's check with PCR. a) Specific-AB strain primer. b) Specific SJD strain primer. c) Specific-*D.nigrofasciatus* primer. SJD and WIK are genetically close strains, hence a band is shown for 2 strains.

3.2. Phenotypic analysis

3.2.1. Swimming performance

Separated replicates provided different results such as in first and third replicates there was no significant difference in RU_{crit} between strains ($p>0.05$, one-way ANOVA followed by Tukey's HSD test) but in the second replicate RU_{crit} of fish from SJD strain differs significantly from AB strain ($p=0.007$, one-way ANOVA followed by Tukey's HSD test). This difference in the responses between the different replicates, indicates that the real significant factor might be not the strain. For the pooled dataset, however, two-way ANOVA test presented following results: there was a significant difference in RU_{crit} between first and third replicate ($p=0.004$) and between the strains ($p=0.02$), there was no interaction between two factors ($p>0.05$). The results showed that SJD had lower critical swimming speed than AB strain. Mean and SD for the pooled dataset were: 10.5 ± 0.9 TL s^{-1} for AB, 10.0 ± 0.9 TL s^{-1} for SJD and 10.3 ± 0.9 TL s^{-1} for their hybrid (Figure 3.3).

RU_{crit} (TL s^{-1})

Figure 3.3. Relative critical swimming speed per strain. Average relative critical swimming speed is depicted per strain for each of three replicates and for pooled data. Error bars equal to 1 SD. Numbers inside the bars indicate the sample size. RU_{crit} of SJD is significantly lower than of AB strain in pooled data set and second replicate.

There was no correlation between RU_{crit} and TL of the fish, hence RU_{crit} did not change with the change in TL in any strain (Figure 3.4).

RUcrit (TL s^{-1})

Figure 3.4. Correlation of RU_{crit} and TL. Graphic depicts per strain the relationships between relative critical swimming speed and total length of the fish for pooled data. Numbers in parentheses indicate the sample size.

However, measurement of TL of the fish which performed in swimming test showed that in first and third replicate there is significant difference between the TL of SJD and AB ($p=0.04$, one-way ANOVA test and $p=0.03$, Kruskal-Wallis test respectively) but there is no significant difference in TL in the second replicate ($p>0.05$, one-way ANOVA test). For the pooled dataset, however, non-parametric ScheirerRayHare test followed by Dunn test presented following results: there was a significant difference between the second and other replicates ($p=0.0001$) but not between strains ($p>0.05$), also, there was no interaction between these two factors ($p>0.05$). Mean and SD for the pooled dataset were: 11.1 ± 0.4 mm for AB, 10.7 ± 0.9 mm for SJD and 11.1 ± 0.9 mm for their hybrid (Figure 3.5).

TL (mm)

Figure 3.5. Total length per strain. Average total length is depicted per strain for each of three replicates and for pooled data. Error bars equal to 1 SD. Numbers inside the bars indicate the sample size. There is no significant difference between strains, but there is significant difference between replicates (replicate 2 differed from other replicates) ($p<0.05$, two-way ANOVA followed by Tukey's HSD test).

3.2.2. Heart rate measurements

This part examines differences in the heart rate of different strains through the measurements of heart rate in 48 hpf embryos. It appeared that fish strain showed a significant effect on embryos' heart rate (Figure 3.6). Statistical analysis with non-parametric ScheirerRayHare test followed by post-hoc Dunn test for the pooled dataset showed that embryos of AB strain had significantly higher heart rate than the embryos of the other strains ($p=0$): 218.3 ± 6.9 beats per minute (bmp) versus 198.6 ± 9.3 bmp for SJD and 197.7 ± 8.8 bmp for their hybrids. However, test also showed a significant difference in heart rate between the first and three other replicates ($p=0.00003$). There was no interaction between strains and replicates ($p>0.05$).

Also, the same results were shown for each replicate separately: AB strain had significantly higher heart rate than other strains (p≈0, p≈0, p≈0, p=0.0006 for every replicate respectively, one-way ANOVA for all replicates).

HR (beats/min)

Figure 3.6. Heart rate of 48 hpf embryos of the different strains. Average heart rate in beats per minute is depicted per strain for each of four replicates and for pooled data. Error bars equal to 1 SD. Numbers inside the bars indicate the sample size. AB strain shows much higher heart rate than SJD and their hybrid in all the replicates and in the pooled dataset.

3.2.3. Total length at hatching measurements

This part examines the TL at hatching of different strains through the length measurements of newly hatched larvae every 30 minutes at 48 hpf (Figure 3.7). Results of separated replicates provided different information such as in the first and third replicates TL at hatching differed between all strains significantly (p≈0, Kruskal-Wallis test), in the second replicate TL at hatching of fish from SJD strain differed significantly from hybrid strain (p≈0, Kruskal-Wallis test) but there was no significant difference in TL at hatching between strains in the fourth replicate (p>0.05, Kruskal-Wallis test). However, the results of statistical analysis (ScheirerRayHare followed by Dunn test) performed for pooled dataset showed that there was a

significant difference in TL at hatching between all the strains (p≈0) and between pairs of replicates (p≈0, first and forth replicates differed from second and forth replicates but not from each other). Also, there was no interaction between strains and replicates ($p>0.05$). Mean and SD for the pooled dataset were: 3.6 ± 0.1 mm for AB, 3.4 ± 0.1 mm for SJD x AB and 3.2 ± 0.1 mm for SJD.

TL (mm)

Figure 3.7. Total length at hatching per strain. Average total length at hatching (mm) is depicted per strain for each of four replicates and for pooled dataset. Error bars equal to 1 SD. Numbers inside the bars indicate the sample size. Every strain showed significant differences in their total length-at-hatch comparing with other strains.

3.2.4. Mortality, hatching and growth rates at metamorphosis

Mortality and hatching rates were measured after 72 hpf when more than 50% of embryos have hutched. There were four replicates that were reared at the same conditions (set up for heart rate and TL at hatching measurements).

It seems like average mortality and hatching rates for each strain differed greatly from replicate to replicate (see Figure 3.8, 3.9): pooled mortality rate for AB strain was 69.3%, SJD 75.3%, SJD*AB 61.0% and pooled hatching rate of AB was 19.7%, SJD 18.5% and SJD*AB 33.7%. However, the statistical analysis of each replicate ($p>0.05$, Kruskal-Wallis test) and of

pooled datasets (p>0.05, ScheirerRayHare test followed by post-hoc Dunn test) did not show any significant difference in mortality and hatching rate neither between strains, nor between replicates. There was also no interaction between strains and replicates (p>0.05).

Mortality rate (%)

Figure 3.8. Mortality rate. Average mortality number is depicted per strain for each of four replicates and for pooled dataset. Error bars equal to 1 SD. Numbers inside the bars indicate the number of pairs that spawned. No significant difference is showed in mortality numbers through different replicates and pooled dataset.

Hatching rate (%)

Figure 3.9. Percentage of hatched eggs. Average percentage of hatched eggs is depicted per strain for each of four replicates and for pooled dataset. Error bars equal to 1 SD. Numbers inside the bars indicate the number of pairs that spawned. No significant difference is showed in hatching rate through different replicates and pooled dataset.

Growth rate was measured at the metamorphosis period when the swimming test were performed. The time for performing swimming test were based on fish length but as it was said earlier (chapter 2. Materials and methods) different strains grew with different speed hence all the fish were in their metamorphosis period regarding their length but not in the same age by dpf. There were only two replicates (see why in paragraph 2.2.5) that were reared at the same conditions (set up for swimming performance). Results of separated replicates provided different information such as in the first replicate growth rate did not differ significantly from others ($p>0.05$, Kruskal-Wallis test), in the second replicate growth rate of hybrid strain differed significantly from AB strain ($p<0.05$, Kruskal-Wallis test). The results for pooled dataset showed that there was a significant difference in growth between all the strains ($p = 0.01$) and between replicates ($p \approx 0$) (ScheirerRayHare followed by Dunn test). However, statistical analysis showed that there is an interaction between these two factors. Since the interaction effect is significant ($p=0.02$), the 'strain' effect cannot be generalised for both replicates together. Mean and SD for the pooled dataset were: 0.049 ± 0.01 for AB, 0.042 ± 0.01 days^{-1} for SJD and 0.045 ± 0.01 days^{-1} for hybrid strain.

GR (days^{-1})

Figure 3.8. Specific growth rate. Average specific growth rate is depicted in days^{-1} per strain for each of two replicates and for pooled dataset. Error bars equal to 1 SD. Numbers inside the bars indicate the sample size.

4. Discussion

4.1. Molecular analyses

To make chimeric embryos suitable for study of mito-nuclear interactions and heteroplasmy, it is necessary to choose experimental strains with the farthest genetic distances between them to prevent strains from having the same mitochondria haplotype, considering that in this way no information on mito-nuclear interactions could be obtained. On the other hand, genetic distances between experimental species should be the closest to each other: for species to be able to have fertile offspring and for us to see any mito-nuclear interactions at all.

In our study we found suitable zebrafish strains and Danio species through analysis of genetic distances of 6 strains and 3 species comparing mitochondrial sequences. The results showed that AB and SJD strains are more genetically distant strains from the six that were analysed (0.75% of mitochondrial polymorphism) and *Danio nigrofasciatus* is the closest species to the *Danio rerio* (6.43% of mitochondrial polymorphism).

Flynn et al. (2016) presented unrooted Bayesian phylogenetic tree of 27 zebrafish mitochondrial genomes from six laboratory strains where SJD and WIK strains were more genetically distant strains from AB strain, and TL genetically closer. Guryev et al. (2006) support this in their study: SJD was more distant from AB strain, however WIK and TL were almost identically closer to AB strain (however this study was based on SNP and not on mtDNA). McCluskey & Postlethwait (2015) showed that WIK is the farthest strain from AB (they did not use SJD and TL strains in their analysis and also based it on SNP).

In research of Tang et al. (2010) the result presented *D. nigrofasciatus* as a sister-species for *D. rerio*. *D. albolineatus* was the most distant of our three experimental species. However, in

McCluskey & Postlethwait (2015) the hypothesis that *D. aesculapii* is a *Danio rerio*'s sister-species was supported.

We expected to find WIK or SJD as the farthest strain for AB, and our expectations were justified but WIK was closer to AB than SJD for 0,05%. That is why we chose SJD for our study. Regarding species we expected to choose one of the two species mentioned in previous researches (*D. aesculapii* or *D.nigrofasciatus)*. But we did not have at least partial mitochondrial genome for *D. aesculapii* and that there was successful attempt in creation of fertile offspring of *D. rerio* and *D. nigrofasciatus* (Endoh et al., 2020). These were the reasons we chose *D.nigrofasciatus* for our research. In future studies it would be interesting to obtain hybrids from *D.rerio* and all three other species of Danio that we analysed in this study.

4.2. Phenotypic analysis

4.2.1. Heart rate, length-at-hatch and growth rate

4.2.1.1. Heart rate

In our study, the embryos of zebrafish were reared, observed, and heart rate was measured at 48 hpf at constant temperature 28 ± 0.5 ^{o}C, and they showed following heart rates: 218.32 ± 6.9 bpm, 198.56 ± 9.3 bpm and 197.66 ± 8.8 bpm for AB, SJD and their hybrid respectively. Also, the results of heart rate measurements showed us that AB strain had significantly higher heart rate than two other strains.

According to Wang et al. (2020), Gazsi et al. (2021), and Scopel et al. (2021) who used AB embryos in their control groups for heart rate measurements, the heart rate of these embryos at 48 hpf should be less than 200 beat per minute (bmp). However, Wang et al. (2020) and Gazsi et al. (2021) studies showed the same heart rate at different temperature: 28.5 ± 0.5 ^{o}C and 25.5 ± 0.5 ^{o}C respectively. Scopel et al. (2021), on the other hand, presented the much lesser heart rate

for their control group - 116 bmp at the same temperature as Wang et al. (2020) – 28.5 ± 0.5^{o}C. According to Barrionuevo & Burggren (1999) heart rate should increase with the increasing temperature. This study measured heart rate at 25, 28 and 31 ^{o}C, and they presented 125 bmp heart rate at 28 ^{o}C. These differences with our study were not necessarily due to the temperature as factor because as we can see heart rate measurements had different results at the same temperature and vice versa, but due to dependance of heart rate on oxygen concentration in water that in its turn depends on egg density in tank and on composition of system water which are unknown for the above studies. Hence, we believe that our results can be valid as well as results from the previous studies.

According to Boonruangkan et al. (2021) zebrafish had the peak heart rate at 48 hpf and then heart rate was abruptly decreasing for yet unknown reason. So, even if there are comparatively more articles with heart rate researches after 48 hpf there is no use for us to reference them for our measurements. For example, Denvir et al. (2008) compared heart rates of AB and Golden strain at 3 days and 5 dpf but no significant difference was found.

In general, there are not so many studies about the comparison of heart rate of embryos of different zebrafish strains/species at 48 hpf. The vast majority of studies usually use AB strain just as a control group for studying effects of various environmental factors. In our study we characterized the "default" heart rate for future studies, and our objective will be to learn whether the higher heart rate of AB strain is due to differences in mtDNA between strains.

4.2.1.2. Total length at hatching

In our study, we found that TL at hatching was different for every strain, and AB strain had greater TL at hatching whereas SJD strain had smaller TL at hatching and the hybrid strain was intermediate between them.

Adams et al. (2020) showed that the water hardness significantly affected the length of larvae measured at 96 hpf and the soft water favoured the growth. To solve this issue in our study we used the same conditions and the water from the same system for parent fish and for every replicate of the experiment. The study of Adams et al. (2020) was designed to compare some life history traits between isogenic homozygous clone and wildtype zebrafish in low pH conditions. They also found significant differences in larvae length between wild type zebrafish and inbred type at 96 hpf when all the fish were hatched. Knowing that fish length increases fast after the hatching event and it is strongly affected by the differences in hatching time between the individuals of the same population, we measured TL at hatching every 30 minutes to obtain newly hatched larvae. Thus, observed differences in our study are not expected to have been affected by this factor. Water temperature has also been proven as critical for the total length at hatching, with elevated temperatures resulting to total length decrease (Polo et al. (1991) for *Sparus aurata*). Also, delay in hatching time was observed with decreasing temperature: at 24 °C hatching occurred later (96 hpf) than at 28°C (48 hpf) (Villamizar et al., 2012). In our study, we ensured the constant optimal temperature for zebrafish eggs at 28 ± 0.5 °C when zebrafish embryo development enhances through continuous temperature measurement on every stage of experiment and for every replicate. According to Uusi-Heikkiï et al. (2010) there was no correlation of the size of the parent fish with either the size of eggs or TL at hatching. Large and small fish offspring had the same TL at hatching but different area-at-hatch and yolk-sac volume. Hence, the size of the parent fish was not taken into account to our research and the TL at hatching was considered as a separate life history trait.

Therefore, as we successfully excluded factors that affect TL at hatching, we can possibly suggest that the difference in TL between strains might be due to “strain” factor as well. Also, it is interesting that the hybrid strain had intermediate TL at hatching between two parental

strains and not the same as AB strain. It would be interesting to check whether this might be due to mtDNA inherited from SJD strain or to any complex limitations in which both of the parental DNA are participating.

Also, it would be useful to verify these results for embryos hatched at 72 hpf as well as to check whether there any difference in TL at hatching between early-hatched (48-72 hpf) and later-hatched (72-96 hpf) embryos with respect to strain. According to Leite-Ferreira et al. (2019) and corresponding behavioral studies later-hatched embryos are less sensible to environmental changes. It would be interesting to learn whether it correlates/depends on their possibly large length at hatching.

4.2.1.3. Growth rate

We cannot fully interpret the results of growth rate measurements due to significant effect of interaction between replicate and strain as factors, hence, we cannot generalize the importance of strain for both replicates together. And separately every replicate showed us different picture might be due to large sample difference in the second replicate (54 individuals for AB, 31 for hybrid and only 9 for SJD). The first replicate had more balanced data and showed us no differences in growth rate between strains.

According to Gonzales & Law (2013) and Abdollahpour et al. (2020) difference in growth rate is related to various feed and feeding regime, and to differences in photoperiod. Experimental fish for our research were grown in the same conditions, hence if there were any differences in growth rate they should be related to the differences between strains. That what we expected to see in our study. It would be useful in the future studies to design setup only for this experiment and compare growth rate of fish without using big part of them for swimming test.

4.2.2. Critical swimming speed

The presented results of swimming test showed significant difference between RU_{crit} of SJD strain and AB strain. However, there is no significant differences in length of the fish that participated in swimming test and no correlation between RU_{crit} and fish length.

Research by Wakamatsu et al. (2019) showed that the swimming capability does not vary between female and male (they used two different protocols and reproduced the results on two successive days) whereas the study of Dimitriadi et al. (2018) supported the hypothesis that there is a strong sexual dimorphism in the RU_{crit} of zebrafish. However, the former study was performed on 6–9-month-old adult fish and the later one on the 4–5-month-old adult fish and that might be the reason for the differences. Our experiment was performed on juvenile fish in metamorphosis stage, so we could not see any sexual dimorphism. Wakamatsu et al. (2019) as well as Plaut (2000) discovered that long-finned fish had lower swimming performance than the fish with regular caudal fin. Our strains had regular fin so the length of the caudal fin did not play role in our swimming test. Also, Wakamatsu et al. (2019) found that WIK strain of zebrafish had significantly lower U_{crit} than AB, TU, TL and PET strains. They suggested that WIK strain originated from a single less capable of swimming pair of wild zebrafish, and that is why it inherited lower swimming capability. To support this hypothesis the swimming test for TU x WIK hybrid was performed, this test showed that the hybrid strain did not have this problem so this trait inherited only in WIK strain and possibly is a recessive-type trait. However, WIK parent in this study was male so we did not really know whether this trait would have been inherited by WIK x TU strain. There is a lack of research on the difference in swimming capability of AB and SJD, hence, we did not know what to expect when we started this study.

As it was discovered in Flynn et al. (2016) SJD and WIK strains' mtDNA form a single cluster in phylogenetic analysis, and this fact suggests that they are more closely related genetically than SJD and AB. Locomotion, such as swimming, strongly depends on metabolic

function, so mutations that impact mitochondrial function are expected to influence locomotor activity (Anderson et al., 2021). Hence, there is a possibility that mitochondrial polymorphism might be the reason SJD (and previously mentioned in Wakamatsu et al., 2019 WIK as a part of a cluster) had lower swimming performance than AB. However, we can only make a hypothesis because swimming capability is also strongly related with heart morphology, skeletal abnormalities on early stages of zebrafish development, body shape, ontogenetic phases and state of the fins and squamation, muscle composition (Plaut, 2000; Koumoundouros et al., 2009; Dimitriadi et al., 2018; Sergi et al., 2022). So, the next step in studies might be research of whether the less swimming capability of SJD and WIK strains depends on mitochondrial diversity or phenotypic factors.

4.2.3. Mortality and hatching rate

As results of this study, it occurred that mortality and hatching rate did not differ with respect of the strain. Cui et al. (2012), Liu et al. (2014), Vankayala et al. (2014), Farhana et al. (2019) and others presented in their studies that hatching rate in zebrafish should be about 80-100%, mortality rate of zebrafish should be no more that 10-15%. Our study showed different values that might be explained by the fact that we only used random 50-60 eggs from each pair, and other studies used mass spawning. Also, Adams et al. (2020) discover that wild type zebrafish had significantly higher survival rate than inbred type. For this experiment also mass spawning were used (in tanks with fish density of 10-20 fish). To exclude the maternal factor in fish survival we used pairs of fish and measures mortality rate for every pair hence it was known for sure that the number of mortality rate that we found was not obtained from eggs of only one female. Also, SJD strain is also an inbred strain so we definitely expected their survival rate to be lower or in our case mortality rate to be higher. Hence, to improve this experiment, it would be

useful to use our set up but to take all the eggs from each pair to get more precise information specifically about mortality and hatching rates.

4.3. *D. nigrofasciatus* and its problems.

According to Endoh et al., 2020 there was a successful attempt in creation of hybrid between the *D. rerio* female and *D. nigrofasciatus* male. Some of these hybrids, unlike hybrids from crossings between *D. rerio* and *D. albolineatus* or *D. aesculapii*, produced aneuploid spermatozoa with varying ploidy levels, ranging from aneuploidy to diploidy with an approximation haploidy mode, with limited fertilization ability. In this study we attempted to obtain hybrids between *D. rerio* and *D. nigrofasciatus* by *in vivo* fertilisation. The attempt was unsuccessful because of the fact that this species is not very well domesticated yet, and because of the absent of knowledge about spawning behaviour of this species. That is why we could not make any successful experiment for heart rate and length-at-hatch measurements with *D. nigrofasciatus*. Their hatching time was about 4 hours earlier than that of zebrafish so there was no chance to make heart rate and length-at-hatch measurements at the same time and the same experimental design as with zebrafish. Nevertheless, we successfully got the second generation of *D. nigrofasciatus* and will continue to extend its population in our laboratory. For now, there are only a few males in every tank and the population of *D. nigrofasciatus* in general is particularly small. As soon as the population of *D. nigrofasciatus* males will be sufficient, the *in vitro* fertilization to obtain hybrids will be available. Recently, this technique was implemented in our study and the first attempts were very promising.

5. Conclusions

This thesis examined and selected the *D. rerio's* strains (AB and SJD) and species (*D. nigrofasciatus*) which are suitable for the study of mitochondrial interactions and heteroplasmy in chimeric zebrafish. For that study, strain/species-specific primers were created and experiments for life history trait measurements of zebrafish were done. For now, the "default" life history traits of strains AB, SJD and their hybrid were standardized. The technique of *in vitro* fertilization was successfully implemented in the study and we intend to start main research as soon as possible.

Bibliography

Abdollahpour, H., Falahatkar, B., & Lawrence, C. (2020). The effect of photoperiod on growth and spawning performance of zebrafish, Danio rerio. *Aquaculture Reports*, *17*, 100295. https://doi.org/10.1016/J.AQREP.2020.100295

Adams, L. G., Gordon, M. S., Buth, D. G., & Hutchings, E. M. (2020). A Comparison of Isogenic Homozygous Clone and Wildtype Zebrafish (Danio rerio): Survival and Developmental Responses to Low pH Conditions. *Https://Home.Liebertpub.Com/Zeb*, *17*(3), 196–210. https://doi.org/10.1089/ZEB.2019.1780

Anderson, L., Camus, M. F., Monteith, K. M., Salminen, T. S., & Vale, P. F. (2021). Mitonuclear interactions affect locomotor activity and sleep in Drosophila melanogaster. *BioRxiv*, 2021.10.30.464953. https://doi.org/10.1101/2021.10.30.464953

Artuso, L., Romano, A., Verri, T., Domenichini, A., Argenton, F., Santorelli, F. M., & Petruzzella, V. (2012). Mitochondrial DNA metabolism in early development of zebrafish (Danio rerio). *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1817*(7), 1002–1011. https://doi.org/10.1016/J.BBABIO.2012.03.019

Bagatto, B. (2005). Ontogeny of cardiovascular control in zebrafish (Danio rerio): Effects of developmental environment. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *141*(4), 391–400. https://doi.org/10.1016/J.CBPB.2005.07.002

Bai, Z., Ren, T., Han, Y., Hu, Y., Schohel, M. R., & Jiang, Z. (2019). Effect of dietary Bio-fermented selenium on growth performance, nonspecific immune enzyme, proximate composition and bioaccumulation of zebrafish (Danio rerio). *Aquaculture Reports*, *13*, 100180. https://doi.org/10.1016/J.AQREP.2019.100180

Barreto, F. S., Watson, E. T., Lima, T. G., Willett, C. S., Edmands, S., Li, W., & Burton, R. S. (2018). Genomic signatures of mitonuclear coevolution across populations of Tigriopus californicus. *Nature Ecology & Evolution 2018 2:8*, *2*(8), 1250–1257. https://doi.org/10.1038/s41559-018-0588-1

Barrionuevo, W. R., & Burggren, W. W. (1999). O2 consumption and heart rate in developing zebrafish (Danio rerio): Influence of temperature and ambient O2. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *276*(2 45-2). https://doi.org/10.1152/AJPREGU.1999.276.2.R505/ASSET/IMAGES/LARGE/AREG70227008X.JPEG

Beamish, F. W. H. (1978). *Swimming capacity. Fish physiology.* . Academic Press.

Beffagna, G. (2019). Zebrafish as a Smart Model to Understand Regeneration After Heart Injury: How Fish Could Help Humans. *Frontiers in Cardiovascular Medicine*, *6*, 107. https://doi.org/10.3389/FCVM.2019.00107

Boonruangkan, J., Farrokhi, H., Rohith, T. M., Kwok, S., Carney, T. J., Su, P.-C., & Kim, Y.-J. (2021). Label-free quantitative measurement of cardiovascular dynamics in a zebrafish embryo using frequency-comb-referenced-quantitative phase imaging. *Https://Doi.Org/10.1117/1.JBO.26.11.116004*, *26*(11), 116004. https://doi.org/10.1117/1.JBO.26.11.116004

Brett, J. R. (2011). The Respiratory Metabolism and Swimming Performance of Young Sockeye Salmon. *Https://Doi.Org/10.1139/F64-103*, *21*(5), 1183–1226. https://doi.org/10.1139/F64-103

Christou, M., Iliopoulou, M., Witten, P. E., & Koumoundouros, G. (2018). Segmentation pattern of zebrafish caudal fin is affected by developmental temperature and defined

by multiple fusions between segments. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *330*(6–7), 330–340. https://doi.org/10.1002/JEZ.B.22825

Cui, J., Zhang, Z., Bai, W., Zhang, L., He, X., Ma, Y., Liu, Y., & Chai, Z. (2012). Effects of rare earth elements La and Yb on the morphological and functional development of zebrafish embryos. *Journal of Environmental Sciences*, *24*(2), 209–213. https://doi.org/10.1016/S1001-0742(11)60755-9

Dabrowski, K., & Miller, M. (2018). Contested Paradigm in Raising Zebrafish (Danio rerio). *Zebrafish*, *15*(3), 295. https://doi.org/10.1089/ZEB.2017.1515

Denvir, M. A., Tucker, C. S., & Mullins, J. J. (2008). Systolic and diastolic ventricular function in zebrafish embryos: Influence of norepenephrine, MS-222 and temperature. *BMC Biotechnology*, *8*(1), 1–8. https://doi.org/10.1186/1472-6750-8-21/FIGURES/3

Dimitriadi, A., Beis, D., Arvanitidis, C., Adriaens, D., & Koumoundouros, G. (2018). Developmental temperature has persistent, sexually dimorphic effects on zebrafish cardiac anatomy. *Scientific Reports 2018 8:1*, *8*(1), 1–10. https://doi.org/10.1038/s41598-018-25991-8

Dunham-Snary, K. J., & Ballinger, S. W. (2015). Mitochondrial-nuclear DNA mismatch matters: Could different nuclear DNA–mitochondrial DNA combinations affect disease severity? *Science (New York, N.Y.)*, *349*(6255), 1449. https://doi.org/10.1126/SCIENCE.AAC5271

Dunn, O. J. (1961). Multiple Comparisons among Means. *Journal of the American Statistical Association*, *56*(293), 52–64. https://doi.org/10.1080/01621459.1961.10482090

Endoh, M., Shima, F., Havelka, M., Asanuma, R., Yamaha, E., Fujimoto, T., & Arai, K. (2020). Hybrid between Danio rerio female and Danio nigrofasciatus male produces aneuploid sperm with limited fertilization capacity. *PLOS ONE*, *15*(5), e0233885. https://doi.org/10.1371/JOURNAL.PONE.0233885

Eyre-Walker, A. (2017). *Mitochondrial Replacement Therapy: Are Mito-nuclear Interactions Likely To Be a Problem?* https://doi.org/10.1534/genetics.116.196436

Farhana, T., Haque, F., Amin, F. B., Zahangir, M. M., & Islam, M. S. (2019). Developmental pliability in zebrafish: An experimental enquiry of acute salinity stress on the early life of zebrafish. *Aquaculture Reports*, *14*, 100189. https://doi.org/10.1016/J.AQREP.2019.100189

Farnezi, H. C. M., Goulart, A. C. X., dos Santos, A., Ramos, M. G., & Penna, M. L. F. (2020). Three-parent babies: Mitochondrial replacement therapies. *JBRA Assisted Reproduction*, *24*(2), 189. https://doi.org/10.5935/1518-0557.20190086

Farrell, A. P. (2007). Cardiorespiratory performance during prolonged swimming tests with salmonids: a perspective on temperature effects and potential analytical pitfalls. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *362*(1487), 2017–2030. https://doi.org/10.1098/RSTB.2007.2111

Flynn, T., Signal, B., Johnson, S. L., & Gemmell, N. J. (2016). Mitochondrial genome diversity among six laboratory zebrafish (Danio rerio) strains. *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis*, *27*(6), 4364–4371. https://doi.org/10.3109/19401736.2015.1089536/SUPPL_FILE/IMDN_A_1089536_SM5222.DOCX

Fox, C. W., & Messina, F. J. (2013). Life History. *Oxford Bibliographies in Ecology*.

Froese, R., & Pauly, D. (n.d.). *FishBase*. Retrieved March 31, 2022, from www.fishbase.org

Fuiman, L. A., Rose, K. A., Cowan, J. H., & Smith, E. P. (2006). Survival skills required for predator evasion by fish larvae and their relation to laboratory measures of performance. *Animal Behaviour*, *71*(6), 1389–1399. https://doi.org/10.1016/J.ANBEHAV.2005.11.013

Gazsi, G., Czimmerer, Z., Ivánovics, B., Berta, I. R., Urbányi, B., Csenki-Bakos, Z., & Ács, A. (2021). Physiological, Developmental, and Biomarker Responses of Zebrafish Embryos to Sub-Lethal Exposure of Bendiocarb. *Water 2021, Vol. 13, Page 204*, *13*(2), 204. https://doi.org/10.3390/W13020204

Gemmell, N., & Wolff, J. N. (2015). Mitochondrial replacement therapy: Cautiously replace the master manipulator. *BioEssays*, *37*(6), 584–585. https://doi.org/10.1002/BIES.201500008

Girden, E. R. (1992). ANOVA : Repeated Measures. *Sage Publications, Inc.*, 1–76.

Gonzales, J. M., & Law, S. H. W. (2013). Feed and Feeding Regime Affect Growth Rate and Gonadosomatic Index of Adult Zebrafish (Danio Rerio). *Zebrafish*, *10*(4), 532. https://doi.org/10.1089/ZEB.2013.0891

Gorman, G. S., Chinnery, P. F., DiMauro, S., Hirano, M., Koga, Y., McFarland, R., Suomalainen, A., Thorburn, D. R., Zeviani, M., & Turnbull, D. M. (2016). Mitochondrial diseases. *Nature Reviews Disease Primers 2016 2:1*, *2*(1), 1–22. https://doi.org/10.1038/nrdp.2016.80

Guryev, V., Koudijs, M. J., Berezikov, E., Johnson, S. L., Plasterk, R. H. A., van Eeden, F. J. M., & Cuppen, E. (2006). Genetic variation in the zebrafish. *Genome Research*, *16*(4), 491. https://doi.org/10.1101/GR.4791006

Hammer, C. (1995). Fatigue and exercise tests with fish. *Comparative Biochemistry and Physiology Part A: Physiology*, *112*(1), 1–20. https://doi.org/10.1016/0300-9629(95)00060-K

Herbert, M., & Turnbull, D. (2018). Progress in mitochondrial replacement therapies. *Nature Reviews Molecular Cell Biology 2018 19:2*, *19*(2), 71–72. https://doi.org/10.1038/nrm.2018.3

Hicken, C. E., Linbo, T. L., Baldwin, D. H., Willis, M. L., Myers, M. S., Holland, L., Larsen, M., Stekoll, M. S., Rice, S. D., Collier, T. K., Scholz, N. L., & Incardona, J. P. (2011). Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(17), 7086–7090. https://doi.org/10.1073/PNAS.1019031108/-/DCSUPPLEMENTAL

Higgs, D. M., Souza, M. J., Wilkins, H. R., Presson, J. C., & Popper, A. N. (2002). Age- and Size-Related Changes in the Inner Ear and Hearing Ability of the Adult Zebrafish (Danio rerio). *JARO: Journal of the Association for Research in Otolaryngology*, *3*(2), 174. https://doi.org/10.1007/S101620020035

Hill, G. E., Havird, J. C., Sloan, D. B., Burton, R. S., Greening, C., & Dowling, D. K. (2019). Assessing the fitness consequences of mitonuclear interactions in natural populations. *Biological Reviews*, *94*(3), 1089–1104. https://doi.org/10.1111/BRV.12493

Hyslop, L. A., Blakeley, P., Craven, L., Richardson, J., Fogarty, N. M. E., Fragouli, E., Lamb, M., Wamaitha, S. E., Prathalingam, N., Zhang, Q., O'Keefe, H., Takeda, Y., Arizzi, L., Alfarawati, S., Tuppen, H. A., Irving, L., Kalleas, D., Choudhary, M., Wells, D., … Herbert, M. (2016). Towards clinical application of pronuclear transfer to

prevent mitochondrial DNA disease. *Nature*, *534*(7607), 383. https://doi.org/10.1038/NATURE18303

Kang, E., Wu, J., Gutierrez, N. M., Koski, A., Tippner-Hedges, R., Agaronyan, K., Platero-Luengo, A., Martinez-Redondo, P., Ma, H., Lee, Y., Hayama, T., van Dyken, C., Wang, X., Luo, S., Ahmed, R., Li, Y., Ji, D., Kayali, R., Cinnioglu, C., … Mitalipov, S. (2016). Mitochondrial replacement in human oocytes carrying pathogenic mitochondrial DNA mutations. *Nature 2016 540:7632*, *540*(7632), 270–275. https://doi.org/10.1038/nature20592

Katoh, K., Rozewicki, J., & Yamada, K. D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160. https://doi.org/10.1093/BIB/BBX108

Kikuchi, K. (2014). Advances in understanding the mechanism of zebrafish heart regeneration. *Stem Cell Research*, *13*(3), 542–555. https://doi.org/10.1016/J.SCR.2014.07.003

Koumoundouros, G., Ashton, C., Xenikoudakis, G., Giopanou, I., Georgakopoulou, E., & Stickland, N. (2009). Ontogenetic differentiation of swimming performance in Gilthead seabream (Sparus aurata, Linnaeus 1758) during metamorphosis. Journal of Experimental Marine Biology and Ecology, 370(1–2), 75–81. https://doi.org/10.1016/J.JEMBE.2008.12.001

Kourkouta, C., Printzi, A., Geladakis, G., Mitrizakis, N., Papandroulakis, N., & Koumoundouros, G. (2021). Long lasting effects of early temperature exposure on the swimming performance and skeleton development of metamorphosing Gilthead seabream (Sparus aurata L.) larvae. *Scientific Reports 2021 11:1*, *11*(1), 1–11. https://doi.org/10.1038/s41598-021-88306-4

Kruskal, W. H., & Wallis, W. A. (1952). Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association*, *47*(260), 583–621. https://doi.org/10.1080/01621459.1952.10483441

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, *35*(6), 1547. https://doi.org/10.1093/MOLBEV/MSY096

Kuraku, S., Zmasek, C. M., Nishimura, O., & Katoh, K. (2013). aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. *Nucleic Acids Research*, *41*(W1), W22–W28. https://doi.org/10.1093/NAR/GKT389

Lawrence, C. (2007). The husbandry of zebrafish (Danio rerio): A review. *Aquaculture*, *269*(1–4), 1–20. https://doi.org/10.1016/J.AQUACULTURE.2007.04.077

Lawrence, C., Adatto, I., Best, J., James, A., & Maloney, K. (2012). Generation time of zebrafish (Danio rerio) and medakas (Oryzias latipes) housed in the same aquaculture facility. *Lab Animal 2012 41:6*, *41*(6), 158–165. https://doi.org/10.1038/laban0612-158

Leite-Ferreira, M. E., Araujo-Silva, H., & Luchiari, A. C. (2019). Individual Differences in Hatching Time Predict Alcohol Response in Zebrafish. *Frontiers in Behavioral Neuroscience*, *13*, 166. https://doi.org/10.3389/FNBEH.2019.00166/BIBTEX

Liu, X. T., Mu, X. Y., Wu, X. L., Meng, L. X., Guan, W. B., Ma, Y. Q., Sun, H., Wang, C. J., & Li, X. F. (2014). Toxicity of Multi-Walled Carbon Nanotubes, Graphene Oxide, and Reduced Graphene Oxide to Zebrafish Embryos. *Biomedical and Environmental Sciences*, *27*(9), 676–683. https://doi.org/10.3967/BES2014.103

Madden, T. (2002). The BLAST Sequence Analysis Tool. In J. McEntyre & J. Ostell (Eds.), *The NCBI Handbook*. Bethesda (MD): National Center for Biotechnology Information (US).

Mangiafico, S. S. (2016). *Summary and Analysis of Extension Program Evaluation in R, version 1.19.10*. Rutgers Cooperative Extension, New Brunswick, NJ. https://rcompanion.org/handbook/F_14.html

McCluskey, B. M., & Postlethwait, J. H. (2015). Phylogeny of Zebrafish, a "Model Species," within Danio, a "Model Genus." *Molecular Biology and Evolution*, *32*(3), 635–652. https://doi.org/10.1093/MOLBEV/MSU325

Meirelles, M. G., Nornberg, B. F., da Silveira, T. L. R., Kütter, M. T., Castro, C. G., Ramirez, J. R. B., Pedrosa, V., Romano, L. A., & Marins, L. F. (2021). Growth Hormone Overexpression Induces Hyperphagia and Intestinal Morphophysiological Adaptations to Improve Nutrient Uptake in Zebrafish. *Frontiers in Physiology*, *12*, 1388. https://doi.org/10.3389/FPHYS.2021.723853/BIBTEX

Mossman, J. A., Biancani, L. M., & Rand, D. M. (2019). Mitochondrial genomic variation drives differential nuclear gene expression in discrete regions of Drosophila gene and protein interaction networks. *BMC Genomics*, *20*(1), 1–23. https://doi.org/10.1186/S12864-019-6061-Y/FIGURES/3

Parichy, D. M., Elizondo, M. R., Mills, M. G., Gordon, T. N., & Engeszer, R. E. (2009). Normal table of postembryonic zebrafish development: Staging by externally visible anatomy of the living fish. *Developmental Dynamics*, *238*(12), 2975–3015. https://doi.org/10.1002/DVDY.22113

Parichy, D. M., & Postlethwait, J. H. (2020). The biotic and abiotic environment of zebrafish. *Behavioral and Neural Genetics of Zebrafish*, 3–16. https://doi.org/10.1016/B978-0-12-817528-6.00001-2

Pesole, G., Allen, J. F., Lane, N., Martin, W., Rand, D. M., Schatz, G., & Saccone, C. (2012). The neglected genome. *EMBO Reports*, *13*(6), 473–474. https://doi.org/10.1038/EMBOR.2012.57

Plaut, I. (2000). Effects of fin size on swimming performance, swimming behaviour and routine activity of zebrafish Danio rerio. *Journal of Experimental Biology*, *203*(4), 813–820. https://doi.org/10.1242/JEB.203.4.813

Plaut, I. (2001). Critical swimming speed: its ecological relevance. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *131*(1), 41–50. https://doi.org/10.1016/S1095-6433(01)00462-7

Polo, A., Yúfera, M., & Pascual, E. (1991). Effects of temperature on egg and larval development of Sparus aurata L. *Aquaculture*, *92*(C), 367–375. https://doi.org/10.1016/0044-8486(91)90042-6

Pompei, M., & Pompei, F. (2019). Overcoming bioethical, legal, and hereditary barriers to mitochondrial replacement therapy in the USA. *Journal of Assisted Reproduction and Genetics*, *36*(3), 383–393. https://doi.org/10.1007/S10815-018-1370-7/FIGURES/1

Printzi, A., Kourkouta, C., Fragkoulis, S., Dimitriadi, A., Geladakis, G., Orfanakis, M., Mazurais, D., Zambonino-Infante, J. L., & Koumoundouros, G. (2021). Balancing between Artemia and microdiet usage for normal skeletal development in zebrafish (Danio rerio). *Journal of Fish Diseases*, *44*(11), 1689–1696. https://doi.org/10.1111/JFD.13487

Rand, D. M., & Mossman, J. A. (2020). Mitonuclear conflict and cooperation govern the integration of genotypes, phenotypes and environments. *Philosophical Transactions of the Royal Society B*, *375*(1790). https://doi.org/10.1098/RSTB.2019.0188

Reidy, S. P., Kerr, S. R., & Nelson, J. A. (2000). Aerobic and anaerobic swimming performance of individual Atlantic cod. *Journal of Experimental Biology*, *203*(2), 347–357. https://doi.org/10.1242/JEB.203.2.347

Reinhardt, K., Dowling, D. K., & Morrow, E. H. (2013). Mitochondrial replacement, evolution, and the clinic. *Science*, *341*(6152), 1345–1346. https://doi.org/10.1126/SCIENCE.1237146/SUPPL_FILE/1237146.REINHARDT.SM.PDF

Sato, K., & Sato, M. (2017). Multiple ways to prevent transmission of paternal mitochondrial DNA for maternal inheritance in animals. *The Journal of Biochemistry*, *162*(4), 247–253. https://doi.org/10.1093/JB/MVX052

Scopel, C. F. v, Sousa, C., Machado, M. R. F., & Santos, W. G. dos. (2021). BPA toxicity during development of zebrafish embryo. *Braz. J. Biol*, *81*(2), 437–447. https://doi.org/10.1590/1519-6984.230562

Scott, G. R., & Johnston, I. A. (2012). Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(35), 14247–14252. https://doi.org/10.1073/PNAS.1205012109/SUPPL_FILE/SD02.XLSX

Selman, K., Wallace, R. A., Sarka, A., & Qi, X. (1993). Stages of oocyte development in the zebrafish, Brachydanio rerio. *Journal of Morphology*, *218*(2), 203–224. https://doi.org/10.1002/JMOR.1052180209

Sergi, E., Orfanakis, M., Dimitriadi, A., Christou, M., Zachopoulou, A., Kourkouta, C., Printzi, A., Zervou, S. K., Makridis, P., Hiskia, A., & Koumoundouros, G. (2022). Sublethal exposure to Microcystis aeruginosa extracts during embryonic development reduces aerobic swimming capacity in juvenile zebrafish. *Aquatic Toxicology*, *243*, 106074. https://doi.org/10.1016/J.AQUATOX.2022.106074

Sokal, R. R. and R. F. J. (1995). *Sokal, R.R. and Rohlf, F.J. (1995) Biometry The Principles and Practice of Statistics in Biological Research. 3rd Edition, W.H. Freeman and Co., New York. - References - Scientific Research Publishing*. W.H. Freeman and Co. https://www.scirp.org/(S(351jmbntvnsjt1aadkposzje))/reference/ReferencesPapers.aspx?ReferenceID=1436974

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews*, *83*(1), 13–34. https://doi.org/10.1111/J.1469-185X.2007.00030.X

Spence, R., & Smith, C. (2006). Mating preference of female zebrafish, Danio rerio, in relation to male dominance. *Behavioral Ecology*, *17*(5), 779–783. https://doi.org/10.1093/BEHECO/ARL016

Stainier, D. Y. R., & Fishman, M. C. (1994). The zebrafish as a model system to study cardiovascular development. *Trends in Cardiovascular Medicine*, *4*(5), 207–212. https://doi.org/10.1016/1050-1738(94)90036-1

Steele, S. L., Prykhozhij, S. v., & Berman, J. N. (2014). Zebrafish as a model system for mitochondrial biology and diseases. *Translational Research*, *163*(2), 79–98. https://doi.org/10.1016/J.TRSL.2013.08.008

Tachibana, M., Sparman, M., Sritanaudomchai, H., Ma, H., Clepper, L., Woodward, J., Li, Y., Ramsey, C., Kolotushkina, O., & Mitalipov, S. (2009). Mitochondrial Gene

Replacement in Primate Offspring and Embryonic Stem Cells. *Nature*, *461*(7262), 367. https://doi.org/10.1038/NATURE08368

Tang, K. L., Agnew, M. K., Hirt, M. V., Sado, T., Schneider, L. M., Freyhof, J., Sulaiman, Z., Swartz, E., Vidthayanon, C., Miya, M., Saitoh, K., Simons, A. M., Wood, R. M., & Mayden, R. L. (2010). Systematics of the subfamily Danioninae (Teleostei: Cypriniformes: Cyprinidae). *Molecular Phylogenetics and Evolution*, *57*(1), 189–214. https://doi.org/10.1016/J.YMPEV.2010.05.021

Tukey John W. (1977). EXPLORATORY DATA ANALYSIS. In *Reading* (Vol. 2). Reading, MA.

Uusi-Heikkiï, S., Wolter, C., Meinelt, T., & Arlinghaus, R. (2010). Size-dependent reproductive success of wild zebrafish Danio rerio in the laboratory. *Journal of Fish Biology*, *77*, 552–569. https://doi.org/10.1111/j.1095-8649.2010.02698.x

Vankayala, R., Chiang, C. S., Chao, J. I., Yuan, C. J., Lin, S. Y., & Hwang, K. C. (2014). A general strategy to achieve ultra-high gene transfection efficiency using lipid-nanoparticle composites. *Biomaterials*, *35*(28), 8261–8272. https://doi.org/10.1016/J.BIOMATERIALS.2014.06.016

Villamizar, N., Ribas, L., Piferrer, F., Vera, L. M., & Sánchez-Vázquez, F. J. (2012). Impact of Daily Thermocycles on Hatching Rhythms, Larval Performance and Sex Differentiation of Zebrafish. *PLOS ONE*, *7*(12), e52153. https://doi.org/10.1371/JOURNAL.PONE.0052153

Wakamatsu, Y., Ogino, K., & Hirata, H. (2019). Swimming capability of zebrafish is governed by water temperature, caudal fin length and genetic background. *Scientific Reports 2019 9:1*, *9*(1), 1–8. https://doi.org/10.1038/s41598-019-52592-w

Walker, M. B., & Kimmel, C. B. (2009). A two-color acid-free cartilage and bone stain for zebrafish larvae. *Http://Dx.Doi.Org/10.1080/10520290701333558*, *82*(1), 23–28. https://doi.org/10.1080/10520290701333558

Wallace, D. C. (2016). Mitochondrial DNA in evolution and disease. *Nature*, *535*(7613), 498. https://doi.org/10.1038/NATURE18902

Wang, D., Zhang, Y., Li, J., Dahlgren, R. A., Wang, X., Huang, H., & Wang, H. (2020). Risk assessment of cardiotoxicity to zebrafish (Danio rerio) by environmental exposure to triclosan and its derivatives. *Environmental Pollution*, *265*, 114995. https://doi.org/10.1016/J.ENVPOL.2020.114995

Westerfield, M. (2007). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). *University of Oregon Press, Eugene*, *5th Edition.* https://zfin.org/ZDB-PUB-101222-52

Wolf, D. P., Mitalipov, N., & Mitalipov, S. (2015). Mitochondrial Replacement Therapy in Reproductive Medicine. *Trends in Molecular Medicine*, *21*(2), 68. https://doi.org/10.1016/J.MOLMED.2014.12.001

Yang, L., Tan, Z., Wang, D., Xue, L., Guan, M. X., Huang, T., & Li, R. (2014). Species identification through mitochondrial rRNA genetic analysis. *Scientific Reports 2014 4:1*, *4*(1), 1–11. https://doi.org/10.1038/srep04089

Yu-Wai-Man, P. (2016). Genetic manipulation for inherited neurodegenerative diseases: myth or reality? *The British Journal of Ophthalmology*, *100*(10), 1322. https://doi.org/10.1136/BJOPHTHALMOL-2015-308329

Zhang, J., Liu, H., Luo, S., Lu, Z., Chávez-Badiola, A., Liu, Z., Yang, M., Merhi, Z., Silber, S. J., Munné, S., Konstandinidis, M., Wells, D., & Huang, T. (2017). Live birth

derived from oocyte spindle transfer to prevent mitochondrial disease. *Reproductive BioMedicine Online*, *34*(4), 361–368. https://doi.org/10.1016/J.RBMO.2017.01.013

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