Investigating the developmental and gene regulatory basis of color diversification in cichlid fish

A framework for evolutionary developmental studies in the Midas cichlid species complex (*Amphilophus spp.*)

Dissertation submitted for the degree of Doctor of Natural Sciences

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Konstanz, 2017

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Date of the Oral Examination: 22 December, 2017

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Summary

With their explosive speciation and astounding levels of diversity, cichlid fishes are an excellent model system for the study of the relationship between genotype and phenotype. In particular, the recent radiations of the Midas cichlids represent an opportunity to examine the molecular basis of rapid adaptive diversification and phenotypic change. In this thesis, I propose a framework in which the Midas cichlids can be used as a model for future research using an evo-devo approach. In its chapters, the suitability of this group for evo-devo studies is assessed, and methods to facilitate future research are outlined. Finally, an empirical study into the regulatory basis of morphological change is conducted, identifying target genes for future research.

Chapter I defines a developmental staging system for the Midas cichlid species complex (*Amphilophus spp.*). The first seven days following fertilization are described in detail, providing an important framework for future studies in cichlid evolution and development. The morphogenesis of key innovations found in the Midas cichlid, including adhesive glands and chromatophore development, were noted in detail. Six main developmental periods are defined - the zygote, cleavage, blastula, gastrula, segmentation, and post-hatching periods. Using these ontogenetic landmarks, development in the Midas cichlid was compared to that of other teleosts such as zebrafish and medaka. Additionally, we tested for the effects of temperature on the rate of development. Compared to the zebrafish, early development in Midas cichlids proceeds slowly, and is highly sensitive to changes in temperature. Overall, this chapter provides an outline of standardized developmental landmarks, facilitating both comparative studies of ontogeny and functional experiments using transgenesis and genome editing techniques.

For the first time in a Neotropical cichlid species, a method for producing transgenic Midas cichlids is outlined in **Chapter II**. Protocols used in zebrafish and other model teleosts were adapted for use in the Midas cichlid – a crucial step in establishing this species complex as a model for evolutionary developmental studies. Using optimized microinjection protocols and *Tol2* transposon-mediated transgenesis, this chapter describes the generation of a stable transgenic line of Midas cichlids. F1 fish with ubiquitously-expressed fluorescence were produced by inserting the *ubi::eGFP* plasmid construct, combining the *ubiquitin* promoter region with the gene for enhanced GFP (*eGFP*), into the Midas genome.

In **Chapter III**, gene regulation is characterized among African cichlid species. Using RNA sequencing, genome-wide expression patterns were examined in three unpaired fin types (anal, dorsal and caudal fins) in five African cichlid species. The species examined in this study (*Oreochromis niloticus*, *Neolamprologus brichardi*, *Astatotilapia burtoni*, *Metriaclima zebra* and *Pundamilia nyererei*) represent both species within the Haplochromine lineage, which is known as a textbook example of an adaptive radiation, as well as more distantly-related, non-radiating species. Within this context, we describe general patterns of cis-regulatory evolution and identify target genes associated with color patterning and fin morphology which can be examined in future research using techniques developed in the previous chapters.

In summary, this thesis establishes a framework in which evo-devo studies can be carried out in cichlid fishes. Future research using an evolutionary developmental approach will contribute to a greater understanding of the link between genotype and phenotype and the role of cis-regulatory changes in the generation of adaptive diversity.

Zusammenfassung

Mit ihrer explosionsartigen Artbildung und verblüffenden Vielfalt auf vielerlei Ebenen sind Buntbarsche ein exzellentes Modelsystem, um das Zusammenspiel zwischen Genotyp und Phänotyp zu erforschen. Besonders die kürzliche adaptive Radiation Zentralamerikanischer Buntbarsche der Gattung Amphilophus (*Amphilophus spp.*) bietet uns nun die Möglichkeit die molekulare Basis schneller adaptiver Diversifikation und phänotypischen Wandels zu untersuchen. In dieser Dissertation lege ich die Grundlagen für die Etablierung von *Amphilophus* Arten als Modellorganismus zukünftiger wissenschaftlicher Arbeiten im Bereich der evolutionären Entwicklungsbiologie. In den vorderen Kapiteln werde ich die Eignung dieser Artgruppe für die evolutionäre Entwicklungsbiologie beurteilen und Methoden für deren zukünftige Erforschung erläutern. Zum Schluss stelle ich eine empirische Studie vor, welche die regulatorische Basis morphologischen Wandels untersucht und nenne Zielgene für weitere Studien in dieser Richtung.

Kapitel I, stellt ein System zur Bestimmung der zeitlichen Entwicklung von Arten des Amphilophus Komplexes vor. Die Entwicklung in den ersten sieben Tagen nach der Befruchtung wird im Detail beschrieben und liefert somit eine wichtige Grundlage für weitere Studien der Evolution und Entwicklungsbiologie von zentralamerikanischen Buntbarschen. Die Morphogenese von Schlüsselinnovationen innerhalb der *Amphilophus* Arten wird genau beschrieben, einschließlich der Entwicklung von Klebdrüsen und Chromatophoren. Sechs große entwicklungsbiologische Zeitabschnitte werden definiert – die Zygote, die Furchung, die Blastula, die Gastrula, die Segmentierung, sowie Zeitabschnitte nach dem Schlüpfen. Mithilfe dieser ontogenetischen Merkmale haben wir die Entwicklung der *Amphilophus* Arten mit anderen Modellorganismen innerhalb der Knochenfischen, wie dem Zebrabärbling oder dem Reiskärpfling („Medaka") verglichen. Zusätzlich, haben wir die Auswirkung von Temperaturveränderung auf die Entwicklung getestet. Im Vergleich zum Zebrabärbling schreitet die frühe Entwicklung in *Amphilophus* Arten nur langsam voran und ist sehr empfindlich gegenüber Temperaturveränderungen. Insgesamt liefert dieses Kapitel eine Zusammenfassung standardisierter entwicklungsbiologischer Orientierungspunkte, welche sowohl

vergleichende Studien der Ontogenese als auch funktionelle Experimente (z.B. Transgenese und Genom-Editierung) zukünftig erleichtern werden.

In **Kapitel II** wird zum ersten Mal eine Methode zur Erstellung transgener Buntbarsche, anhand neotropischen Buntbarsche, beschrieben. Für diesen Zweck haben wir bereits existierende Protokolle, die bei Zebrabärblingen und anderen Modellorganismen verwendet werden, an unseren Modellorganismus angepasst. Das war ein wesentlicher Schritt, um *Amphilophus* Arten zukünftig als Modellorganismus für die evolutionäre Entwicklungsbiologie zu etablieren. Indem wir verbesserte Protokolle für Mikroinjektionen und die *Tol2* transposonvermittelte Transgenese verwendeten, konnten wir in diesem Kapitel die Herstellung einer stabilen transgenetischen Linie von *Amphilopus* Arten erreichen. F1 Nachkommen mit allgegenwertigen exprimierter Fluoreszenz wurden herangezogen, indem wir das *ubi::eGFP* Plasmidenkonstrukt, gekoppelt mit einer ubiquitin Promoterregion mit dem Gen für verstärkte GFP Expression in das Genom injizierten.

In **Kaptiel III** wird Genregulierung in afrikanischen Buntbarschen charakterisiert. Mit Hilfe von RNA Sequenzierung wurden genomweite Expressionsmuster in den drei unpaarigen Flossentypen (Anal-, Rücken- und Schwanzflosse) in fünf afrikanischen Buntbarscharten untersucht. Die in dieser Studie verwendeten Arten (*Oreochromis niloticus*, *Neolamprologus brichardi*, *Astatotilapia burtoni*, *Metriaclima zebra* and *Pundamilia nyererei*) repräsentieren sowohl die Haplochrominen, welche ein Lehrbuchbeispiel für adaptive Radiationen sind, als auch eine weniger nah verwandte Art aus einer weniger artenreichen Linie. In diesem Kontext, beschreiben wir allgemeine Muster der cis-regulierten Evolution und identifizieren Zielgene, die mit Farbmustern und Flossenmorphologie assoziiert sind. Dafür benutzen wir die in den vorangegangenen Kapiteln beschriebenen neuen Techniken.

Zusammenfassend, legt diese Dissertation die Grundlage für weitere Studien im Bereich der evolutionären Entwicklungsbiologie bei Buntbarschen. Zukünftige Forschung mit einen evolutionär-entwicklungsbiologischen Ansatz wird sowohl unser Verständnis der Prozesse, die den Genotyp und Phänotyp verknüpfen, erweitern sowie die Rolle von cis-regulativen Veränderungen, die adaptiver Diversifizierung zugrundeliegenden, beleuchten.

General Introduction

Evolution and evo-devo

The astounding diversity of life on Earth and how it came to be has always been a fascinating subject. Even in antiquity, scholars such as Aristotle and Pliny the Elder attempted to categorize and describe the vast array of biological diversity (Elder 1991; Aristotle et al. 2002). Hundreds of years later, Linnaeus published *Systema Naturae*, formalizing the classification system for plants and animals which is still used to this day (Linnaeus 1758). However, it was not until 1859, when Charles Darwin proposed his revolutionary theory of evolution through natural selection, that biologists truly began to explore the mechanisms through which this diversity arose (Darwin 1859). It is now well known that phenotypes develop as a result of the genes being expressed, the environment affecting the organism, and the interaction of these two forces. Yet even today, understanding the precise manner in which genetic changes translate into the diverse phenotypes seen in nature remains one of the most pressing questions in evolutionary biology.

The Modern Synthesis was developed in the early 20th century, incorporating the fields of genetics, systematics, morphology and paleontology into a comprehensive theoretical framework for evolutionary theory (Huxley 1942). One limitation of the Modern Synthesis was that it overlooked the role of development in explaining phenotypic change during the course of evolution. This shortcoming was later rectified in the 1980s, with the integration of the field of evolutionary developmental biology, or evo-devo (Müller 2007). Evo-devo research seeks to understand the causal mechanisms underlying phenotypic diversity. In this thesis, I establish a framework in which evo-devo studies can be carried out in cichlid fish.

The origins of diversity

The idea that changes in gene regulation can play an essential role, perhaps an even greater role than protein-coding changes, in diversification was first proposed in 1975, when King and Wilson noted that coding regions in humans and chimps are nearly identical (King & Wilson 1975). This notion was later expanded upon by François Jacob, who described evolution as working like a “tinkerer,” adjusting existing mechanisms leading to phenotypic change, rather than “engineering” new ones (Jacob 1977). With recent advances in sequencing technology, an increasing number of whole-genome sequences have been assembled and published – with each new genome, our understanding of the connection between genotype and phenotype becomes clearer, and it is now accepted that regulatory evolution is an important mechanism in the evolution of complexity and diversity (Levine & Tjian 2003). Despite this progress, the exact manner in which regulatory and protein-coding mutations interact is still a topic of debate (Carroll 2008; Craig 2009; Hoekstra & Coyne 2007). Those who advocate for a primary role of cis-regulatory changes postulate that low rates of protein coding mutations in closely-related yet phenotypically divergent species, in conjunction with the reduced risk of mutations in regulatory regions being deleterious, make these types of changes contribute more to evolution than structural mutations (Carroll 2008). It has even been shown in some cases that changes in gene regulation play a major role in the early stages of reproductive isolation and speciation (Haerty & Singh 2006).

One of the main goals of evolutionary developmental biology is to understand how differences in gene regulation can ultimately lead to quantifiable phenotypic changes. Seemingly-small changes in the location (heterotopy), level (heterometry) and timing (heterochrony) of gene expression are thought to drive phenotypic diversification throughout development. These gene expression changes are controlled by an estimated one million regulatory elements (de Laat & Duboule 2013). Due to this extreme complexity, it has been difficult for researchers to study the nature of genes and regulatory elements in an evolutionary context until recently. However, with technological advances such as RNA sequencing and ChIP-seq, it has become feasible to identify the mechanisms underlying phenotypic diversity. By applying these genomic methods, in conjunction with recently-developed genome engineering techniques such as CRISPR-Cas9, it is becoming increasingly possible to study the ways in which evolution tinkers to generate the astounding diversity found on the planet.

Cichlids as a model for adaptive diversification

Cichlid fishes are one of the most species-rich vertebrate families on the planet; they are renowned among biologists for their remarkable diversity, rapid rates of speciation and parallel evolution of adaptive traits (Salzburger & Meyer, 2004; Seehausen, 2006; Stiassny & Meyer, 1999). African cichlids in particular are known for their extreme diversity and explosive speciation. It has been estimated that

Lake Tanganyika, one of the older East African Rift Lakes, formed around ten million years ago (Cohen et al. 1993). Lakes Malawi and Victoria formed even more recently – estimates suggest that Lake Victoria, which contains over 500 species of cichlids, was completely dry roughly 12,000 years ago (Johnson et al. 1996). Overall, almost 2000 species of cichlids have evolved in the African Lakes; as a result of this accelerated rate of speciation, they have become known as a textbook case of an adaptive radiation (Kocher 2004; Seehausen 2006; Turner et al. 2001). Interestingly, many of the adaptive traits found within these radiations evolved convergently – similar stripe patterns, body shapes and craniofacial morphologies can be found within each lake. These convergent evolution patterns are also reflected in the Central American Midas cichlids, which represent a smaller and younger radiation where one can more easily tackle questions of adaptive radiation and diversification using an evo-devo approach.

To study the evolution of the Midas cichlid species flock, one must first understand the complex geological history of the landscape in which it occurred. Central America, including Nicaragua, is located along the "Ring of Fire," an area of increased geological activity situated around the edge of the Pacific Ocean. The geography of western Nicaragua was formed by uplift in response to the subduction of two tectonic plates, and is characterized by its high level of volcanism (Kutterolf et al. 2007). Nicaragua is home to the two largest lakes in Central America, Lake Managua and Lake Nicaragua, in addition to several smaller crater lakes that arose more recently due to volcanic activity. The large lakes – shallow, turbid and ancient – house ancestral populations of *Amphilophus*. The crater lakes, which range from 25,000 to as young as under 1000 years old, were independently colonized by the ancestral source populations. These isolated crater lakes represent independent natural experiments; in each newly-colonized lake, founder populations adapted and diversified rapidly (in less than a few hundred generations), often diverging into distinct species despite the lack of geographic barriers. This makes the Midas cichlids one of few empirical cases of sympatric speciation in the wild (Barluenga & Meyer 2004; Barluenga et al. 2006). As such, the crater lakes are excellent examples of parallel evolution, adaptive radiations and sympatric speciation.

Midas cichlids as an evo-devo model system

One of the central aims of this dissertation is to contribute to the establishment of the Midas cichlid species complex (*Amphilophus spp*. (Günter 1864a)) as a model for both evolutionary and developmental studies – an evo-devo model system. Indeed, they possess many traits that facilitate their utility as an evo-devo model species: continuous and adaptive patterns of phenotypic variation, the ability to breed and raise a sufficient number of animals in the lab, the possibility to perform crosses between populations or species, and access to high-quality genomic information (Parsons & Albertson 2013). The ecology and evolution of adaptive phenotypic diversity is well-described in Midas cichlids (Elmer, Kusche, et al. 2010; Muschick et al. 2011a). It is possible to easily and efficiently produce both pure species and hybrids in the lab via natural or *in vitro* fertilization – many population crosses have already been performed to carry out QTL and linkage mapping studies (Franchini et al., 2014; Henning et al., 2010; Recknagel, Elmer, & Meyer, 2013). Finally, genomic resources have become increasingly available in recent years (Brawand et al. 2014; Elmer et al. 2014). Therefore, the Midas cichlids have the potential to serve as an excellent model for studies of evolution and development, integrating the two fields to uncover the genetic and developmental basis of complex adaptive traits. The first chapter of this thesis describes early development in the Midas cichlids, and serves as a foundation for future evo-devo studies in *Amphilophus*. In this chapter, we define a developmental staging system for the Midas cichlid, similar to those found in model teleosts such as zebrafish and medaka.

Transgenesis in the Midas cichlid

Genetic engineering methods such as transgenesis have proven to be particularly valuable in evo-devo research. While recombinant DNA technology was first developed in the early 1970s, it was not until 1988 until the first transgenic zebrafish was produced. Since then, countless transgenesis experiments have been performed in model teleost species. In addition, increasingly efficient methods, such as *Tol2* transposon-mediated transgenesis, have been developed (Kawakami 2007; Suster et al. 2009). In recent years, transgenesis has been applied in an increasing number of non-model teleost species (Juntti et al. 2013; Yamamoto et al. 2011; Hosemann et al. 2004; Valenzano et al. 2011). A key step in laying the foundation for evo-devo studies in the Midas cichlid is to establish an efficient and reliable method for genomic engineering in this taxon. The genetic architecture underlying adaptive change in Midas cichlids has been studied using genome scans and quantitative trait loci (QTL) mapping studies (Kautt, Elmer, & Meyer, 2012). However, functional analysis is necessary to fully bridge the gap between genotype and phenotype, and fully understand how genetic changes translate to phenotypic variation. In the second chapter of my thesis, I adapt methods for *Tol2* transposon-mediated transgenesis to produce the first stable line of transgenic *Amphilophus spp*, thereby significantly expanding the molecular toolkit available to investigate the genetic and developmental basis of adaptive phenotypic change. By developing a method to test candidate genes and regulatory elements *in vivo*, this chapter provides an essential framework for uncovering the mechanisms that lead to diversity.

The regulatory basis of adaptive evolution in cichlids

Regulatory evolution has been repeatedly suggested to be a main driver of diversification among cichlid species. It has been suggested that an enrichment in novel miRNAs may have had an impact on the explosive rates of speciation found within the Haplochromine cichlid radiation (Brawand et al. 2014). Some of the key axes along which cichlid diversification occurs is in coloration and in body patterning (Kocher 2004). The final chapter of this thesis focuses on understanding how changes in genome-wide differences in gene regulation may contribute to adaptive phenotypic change. Using an RNA-sequencing approach to characterize gene expression differences among three unpaired fin types from five species of African cichlids, I uncover general patterns of transcriptome diversity in both adaptively radiating and non-radiating lineages. In addition, species- and fin-specific differentially expressed genes are identified, revealing the molecular functions and pathways associated with phenotypic divergence. Finally, I identify several candidate genes that may potentially be involved in the development of color patterns and fin morphologies found in these astoundingly diverse fishes. The candidate genes revealed in this study will be tested *in vivo* in the Midas cichlids, using the methods designed in previous chapters. Therefore, this thesis represents a comprehensive work, providing a framework for future studies of evolution and development in Midas cichlids, facilitating the study of fundamental questions in evolutionary biology and illumination of the molecular underpinnings of adaptive phenotypic diversity.

I. Chapter I

Embryonic and larval development of the Midas cichlid: a model teleost to investigate adaptive novelties and species differences from an evo-devo perspective

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Published in BMC DEVELOPMENTAL BIOLOGY, 2015

Abstract

Background

Central American crater lake cichlid fish of the Midas species complex (*Amphilophus spp.*) are a model system for sympatric speciation and fast ecological diversification and specialization. Midas cichlids have been intensively analyzed from an ecological and morphological perspective. Transcriptomic and genomic data sets have recently been collected, and a high-quality draft genome is now available. Many ecologically-relevant species-specific traits such as pigmentation and cranial morphology arise during ontogeny. Detailed descriptions of the early development of Neotropical cichlid fish, and the Midas cichlid in particular, are needed to investigate the ontogeny of species differences and adaptations.

Results

We describe the embryonic and larval development of the crater lake cichlid, *Amphilophus xiloaensis*, until seven days after fertilization. Similar to previous studies on the teleost development, we describe six periods of embryogenesis - the zygote, cleavage, blastula, gastrula, segmentation, and post-hatching period. Furthermore, we define homologous stages to model teleosts such as medaka, zebrafish as well as other cichlid species, and highlight key morphological differences found in Midas cichlid embryos. The latter includes the presence of adhesive glands and different early melanophore patterns, as well as differences in developmental timing compared to other teleosts.

Conclusions

The developmental staging of the Midas cichlid will aid researchers in the comparative investigation of teleost ontogeny. It will facilitate comparative developmental biology studies of Neotropical and African cichlid fish in particular. In the past, the species flocks of the African Great Lakes have received the most attention from researchers, but some of the lineages of the 300-400 species of Central American lakes are a fascinating model system for adaptive radiation and rapid phenotypic evolution. The availability of genetic resources, their status as a model system for evolutionary research, and the possibility to perform functional experiments including transgenesis makes the Midas cichlid complex a very attractive model for evolutionary-developmental research.

Introduction

Cichlids are famous for their astonishing rate of phenotypic diversification and speciation. With over 2000 described species, cichlid fish form one of the most diverse and species-rich groups of animals (Salzburger & Meyer 2004). Lake cichlids in Africa and in the Neotropics of South and Central America provide well-known examples of adaptive radiations (Barluenga et al. 2006; Stiassny & Meyer 1999; Meyer et al. 1990). In particular, the radiations of cichlids in Nicaraguan crater lakes (Figure I.1, Table I.1) provide a good opportunity to study the early stages of speciation and diversification. This is because members of the Midas cichlid species complex have diverged repeatedly in several crater lakes, both sympatrically and allopatrically, often within a few thousand years (Barluenga et al., 2006; Elmer et al., 2014; Elmer, Lehtonen, Fan, & Meyer, 2013; Kautt, Elmer, & Meyer, 2012). Little is known so far about the molecular and developmental mechanisms that drive the observed phenotypic diversity between recently diverged species. The Midas cichlid complex underwent a rapid diversification within a very short time (between 2000 and 25,000 years) and, interestingly, repeatedly evolved several adaptive traits (hypertrophied lips, elongate body shapes, dentitional innovations) in parallel in multiple crater lakes (Figure I.1, Table I.1). Therefore, Midas cichlids are an excellent and unique model system for the comparative study of the phenotype-genotype relationship.

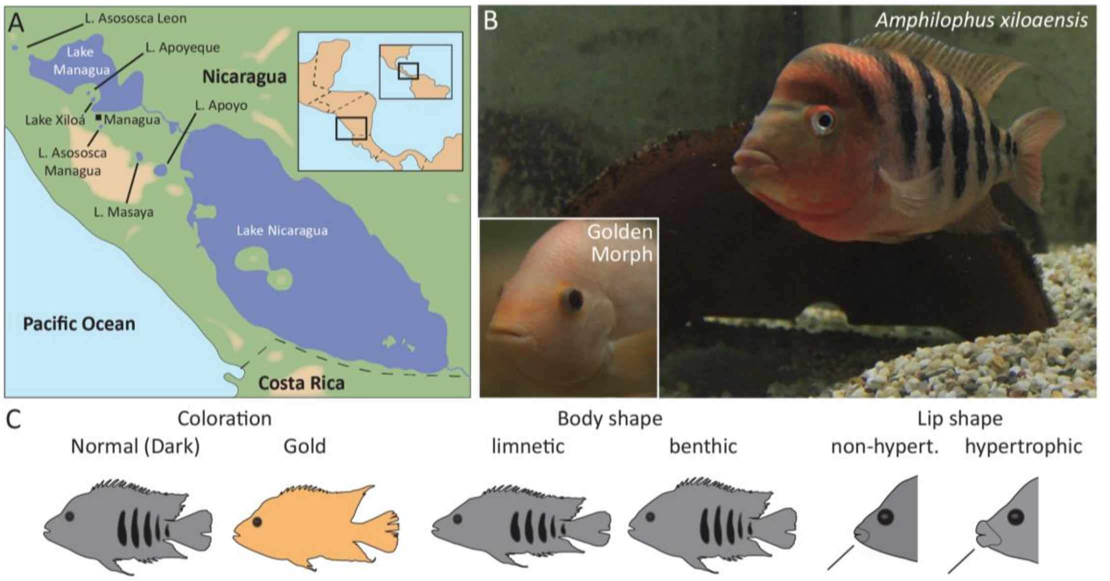

Figure I.1. Range and prominent phenotypic differences of members of the Midas cichlid species complex.
(A) Map of the Pacific coast of Nicaragua in Central America. Besides the large Nicaraguan lakes (Managua and Nicaragua), multiple crater lakes (Asososca Leon, Apoyeque, Xiloá, Asosoca Managua, Masaya and Apoyo) have appeared in the course of the last 25,000 years. These crater lakes have been colonized by Midas cichlids from the large lakes, resulting in new species. (B) Midas cichlids from Lake Xiloá, *Amphilophus xiloaensis*, the focal species of this study. (C) Three selected traits that are interesting from an evolutionary-developmental angle. In the large lakes and in many crater lakes, cichlid species and morphs show differences in coloration, body shape and lip shape.

The Midas cichlid species complex currently includes 13 described species (Table I.1). Two ancestral "source" species occur in the big lakes, Lake Managua and Lake Nicaragua - *Amphilophus labiatus* (Günter 1864a) and *A. citrinellus* (Günter 1864b). These two source species colonized the much younger crater lakes to give rise to several endemic species. Since the late 1970s, many endemic crater lake species have been described. Six species, *A. zaliosus*, *A. astorquii*, *A. chancho*, *A. flaveolus*, *A. globosus* and *A. supercilius* were described from Lake Apoyo (Barlow & Munsey 1976; Geiger, McCrary & Stauffer 2010; Stauffer et al. 2008), along with four in Lake Xiloá (*A. amarillo*, *A. sagittae*, *A. xiloaensis* and *Amphilophus viridis*) (Stauffer & McKaye 2002; Recknagel, Kusche, et al. 2013) and one in Lake Asososca Managua, *A. tolteca* (Recknagel, Kusche, et al. 2013). Despite these numerous discoveries, more Midas cichlids surely await scientific description (Elmer, Lehtonen, et al. 2010; Geiger, McCrary & Schliewen 2010).

The species we are focusing on in this study, *Amphilophus xiloaensis*, was first described in 2002 (Stauffer & McKaye 2002) and is endemic to Lake Xiloá (Figure

Species	Described by	Endemic to	Coloration	Body shape	Lip shape	Genome
A. citrinellus	Günther, 1864	-	normal or gold	benthic	non-h.# or hypertrophic*	high quality (unpublished)
A. labiatus	Günther, 1864	-	normal or gold	limnetic	hypertrophic	low coverage†
A. zaliosus	Barlow & Munsey 1976	L. Apoyo	normal	limnetic	non-hypertrophic	low coverage†
A. astorquii	Staufer et al., 2008	L. Apoyo	normal	benthic	non-hypertrophic	low coverage†
A. chancho	Staufer et al., 2008	L. Apoyo	normal	benthic	non-hypertrophic	low coverage†
A. flaveolus	Staufer et al., 2008	L. Apoyo	normal	benthic	non-hypertrophic	low coverage†
A. globosus	Geiger et al., 2010	L. Apoyo	normal	benthic	non-hypertrophic	low coverage†
A. supercilius	Geiger et al., 2010	L. Apoyo	normal	benthic	non-hypertrophic	low coverage†
A. amarillo	Staufer et al., 2002	L. Xiloá	normal (gold rare or absent)	benthic	non-hypertrophic	low coverage†
A. sagittae	Staufer et al., 2002	L. Xiloá	normal or gold	limnetic	non-hypertrophic	low coverage†
A. xiloaensis	Staufer et al., 2002	L. Xiloá	normal or gold	benthic	non-hypertrophic	low coverage†
A. viridis	Recknagel et al. 2013	L. Xiloá	normal	benthic	non-hypertrophic	low coverage†
A. tolteca	Recknagel et al. 2013	L. As. Managua	normal or gold	benthic or limnetic	non-hypertrophic	-

Table I.1. Summary of discovered Midas cichlid species.
Selection of their observed phenotypic variations for coloration, body and lip shape and genome status. (# Lake Managua and Nicaragua, * crater lakes, † Elmer et al., 2014b).

I.1b). This crater lake is estimated to be approximately 6100 years old (Elmer et al. 2013; Kutterolf et al. 2007). Of the Nicaraguan crater lakes, Lake Xiloá has the highest fish species richness (Elmer, Fan, et al. 2010), including four described cichlid species with an exceptionally high haplotype diversity relative to the lake’s age (Elmer, Kusche, et al. 2010).

Many studies have assessed the early ontogeny of fishes in classic model organisms such as zebrafish, *Danio rerio* (Kimmel et al. 1995); medaka, *Oryzias latipes* (Iwamatsu 2004); stickleback, *Gasterosteus aculeatus* (Swarup 1958) and rainbow trout, *Oncorhynchus mykiss* (Ballard 1973). However, there are only a few studies on cichlid fishes, most of which describe the development of African cichlids (Fujimura & Okada 2007; Balon 1977; Holden & Bruton 1994). Studies of Neotropical cichlids also exist (Meijide & Guerrero 2000; Balon 1960a; Balon 1960b; Jones 1972; Mattos 2014), but most of them are not very detailed. Additionally, because ontogeny can differ strongly amongst species, there is a general necessity for independent developmental studies (Kunz 2004).

Midas cichlids are a famous example of parallel evolution and rapid diversification. This makes them interesting, not only from an evolutionary and ecological standpoint, but also from a developmental, “evo-devo” perspective. A detailed description of the embryonic development of the Midas cichlid is still lacking. The present study will allow researchers to tackle evolutionary questions from a developmental, evo-devo perspective and form a foundation for future studies examining the genetic and developmental factors leading to phenotypic diversification among extremely young species of a particularly species-rich lineage of cichlid fish.

Methods

Maintenance of adult fish

Midas cichlids of *Amphilophus xiloaensis* (wild caught from crater Lake Xiloá, Nicaragua in 2010) were kept under constant conditions (28 ± 1°C, 12 h dark/light cycle, pH 8.5±0.5) in 480L (113,5 (length) x 50 (height) x 85 cm (depth)) or 550 L (110 x 50 x 100 cm) tanks. Two to five pairs are usually kept per tank to minimize aggressive behavior while maximizing reproductive success. Gravel was used as a substrate for the tanks. Each tank was equipped with clay flower pots split into halves as spawning substrate (Figure I.1B). Cichlids are able to use the pots to hide, reducing stress and the frequency of attacks between fish. Pairs usually occupy one of the pots as their territory. If eggs are not removed by stripping, the female deposits her eggs on the inside of the pots, where the male fertilizes them. Specimens analyzed in this story were obtained both by regular spawning (eggs can be easily removed from a pots) and stripping combined with in vitro fertilization.

Stripping of eggs and fertilization

To obtain eggs, it is crucial that the fish are stripped at the right time. As soon as couples pair up and begin to defend their territory, the female must be checked daily for eggs. A few days before spawning, both the male and female become more aggressive. The female’s genital papilla starts to swell, protrude and turn reddish in color. Females showing these signs were removed from the water with a net and the eggs were stripped by applying light pressure to the abdominal region anterior of the genital papilla, followed by a slight squeezing movement towards the genital pore. Eggs should come out easily; if not, the female is not yet ready to spawn. If only a few eggs come out, it is likely that the eggs are not yet mature. Eggs were stripped directly into a petri dish (diameter 90mm) filled with tank water. Between 400 and 1100 eggs can be obtained using this method (average ~700). Females spawn regularly (every four to six weeks) and throughout the year.

Since there are no clear external signs to indicate the maturity of the males, we usually obtained sperm from one to three males. Stripping was performed using the same method as for females. We found no way to confirm that sperm was obtained, but in most cases (five out of six clutches collected) eggs were fertilized; the combination of survival rate and fertilization rate was estimated to be between 30 and 90% at three days post-fertilization (dpf). All experiments were in accordance with the animal research facility of the University of Konstanz, Germany and have been granted by the Regierungspräsidium Freiburg, Germany.

Raising conditions

After fertilization, eggs were kept for five minutes in the petri dish, which is sufficient for a successful fertilization. As a next step, eggs were transferred into a new dish containing clean, autoclaved tank water. The eggs were distributed into multiple petri dishes (50 eggs per plate) and kept in a 28°C incubator (HIR10M Grant, Boekel) or in 25°C or 31°C water baths (1003, GFL), without agitation or aeration. The embryos were moved into fresh petri dishes with new autoclaved tank water every 24 hours.

Visualization of xanthophores

To visualize xanthophores in developing Midas cichlids a modified version of the method described for zebrafish and African cichlids (Odenthal et al. 1996; Salzburger et al. 2007) was used. Embryos were mounted in 3% methylcellulose (1000 µl) mixed with ammonium hydroxide solution (20 µl), and β-mercaptoethanol (1 µl). The pH was checked to be above pH9 using pH indicator strips (Macherey-Nagel). Auto-fluorescence could only be detected with the addition of ammonium hydroxide and β-mercaptoethanol and using an UV-filter. Without the solutions no auto-fluorescence could be detected. Auto-fluorescence in the GFP channel seemed to be unspecific and was detected with and without the addition of the chemicals. We cannot exclude that the chromatophores we visualized by UV light do not also include or even are solely leucophores that are similar in their developmental specification and differentiation (Kimura et al. 2014). Since the cells are colorless and their maturation might be induced later we had to rely on the available protocols (Odenthal et al. 1996) for xanthophore detection.

Image acquisition

Photographs were taken on a stereomicroscope (Leica MZ10 F with Leica DMC2900 Camera) using the Leica Application Suite software 4.5.0. Note that, due the use of a non-achromatic lens, some of the images taken at high magnifications contain chromatic aberrations, causing the edges of objects to appear yellow or violet in color. To improve the depth of field, we used the "Multifocus Montage" module/plugin of the Leica Application Suite software. Eight photographs at different focus levels were matched and combined, retaining the best-focused parts of each photograph and resulting in a single sharp image. Images of UV epiluminescence were taken with a Zeiss AxioCam Mrc digital camera using a Zeiss SteREO Lumar V.12 Stereomicroscope with UV filter. Photographs of adult fish were taken with a Canon EOS 7D SLR with a 17-40mm lens.

Results

Description of the early development of the Midas cichlid

The early development of the Midas cichlid *Amphilophus xiloaensis* during the first seven days following fertilization was documented at 28°C. We illustrate and discuss the main features of 32 developmental stages in the first seven days of development and compare them to previous descriptions of teleost development. As a reference, we mainly used the well-documented development staging of the zebrafish (Kimmel et al. 1995), the medaka (Iwamatsu 2004) and two of the most complete and detailed descriptions of cichlid development - the Nile tilapia *Oreochromis niloticus* (Fujimura & Okada 2007) and the south American cichlid *Cichlasoma dimerus* (Meijide & Guerrero 2000). Lastly, we discuss differences in the rate of early development, which is comparatively slow in Midas cichlids.

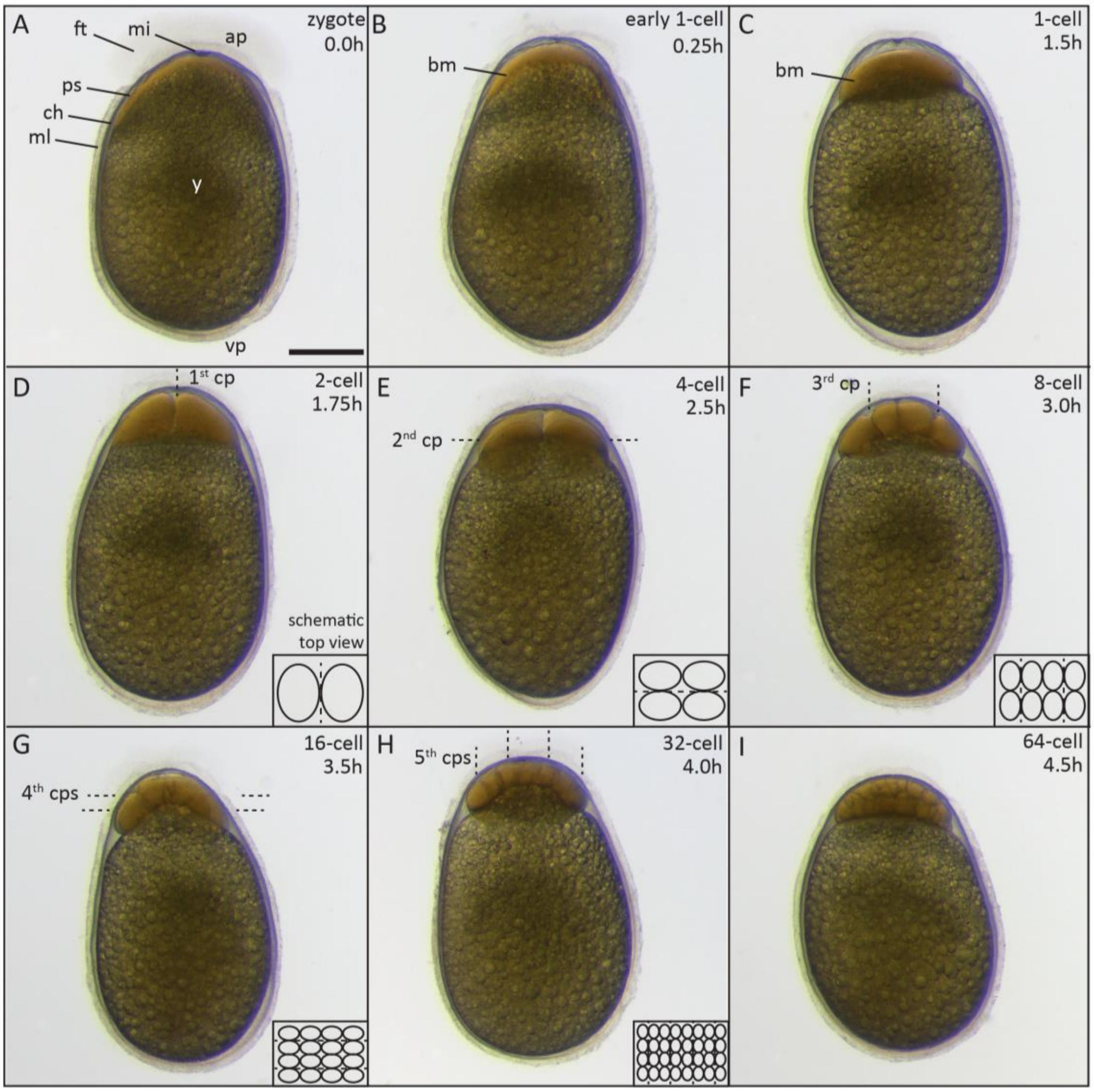

Figure I.2. Embryos during cleavage and blastula stages.
(A) Zygote stage (0hpf); (B) Early 1-cell stage (0.25hpf); (C) Late 1-cell stage (1.5hpf); (D) 2-cell stage (1.75hpf); (E) 4-cell stage (2.5hpf); (F) 8-cell stage (3hpf); (G) 16-cell stage 3.5hpf); (H) 32-cell stage (4hpf); (I) 64-cell stage (4.5hpf). Schemes illustrate the position of cells and cleavage planes from a top-down view (D-H). Abbr.: ap, animal pole; bm, blastomeres; ch, chorion; cp(s), cleavage plane(s); ft, filament tuft; mi, micropyle; ml, mucous layer; ps, perivitelline space; vp, vegetal pole; y, yolk. Scale bar = 500 µm.

Zygote period (0-1.75 hpf at 28°C)

Unfertilized or newly-fertilized eggs of *A. xiloaensis* have an ovoid shape, with the longitudinal axis longer (2.14 ± 0.09 mm) than the transverse axis (1.42 ± 0.07 mm) and the animal pole narrower than the vegetal pole (Figure I.2A). The egg is surrounded by the chorion, a translucent envelope that sticks closely to the egg (Figure I.2A, I.3A). This persists throughout later developmental stages, when there is almost no perivitelline space between the chorion and the vitellus (egg yolk). The vitellus is composed of large dark-yellow yolk globules/platelets of varying sizes

(0.01-0.09 mm), giving it a grainy appearance, as reported previously for the Midas cichlid and closely-related Neotropical cichlids (Kunz 2004; Oldfield 2011; Chellappa et al. 2005) (Figure I.2A, I.3A). The micropyle, the pore in the membrane that guides sperm to the oocyte (Amanze & Iyengar 1990), has a funnel or cone-shaped configuration. It is surrounded by a tuft of filament that can be best observed with dark field illumination (Figure I.3A), and can only be seen until the first four to six cell divisions (Figure I.2A-I). After spawning (both natural and by stripping) the eggs are held together and stuck to the substrate, or under laboratory conditions to the petri dish, by a mucous secretion. (Figure I.3B, C) In contrast to in zebrafish (Kimmel et al. 1995), the chorion does not swell and lift away from the fertilized egg during the zygote period, which lasts until the first cleavage occurs around 1.75 hpf (28°C).

One-cell stage (0hpf)

Fertilization induces cytoplasmic movements at the animal pole, where the blastodisc increases in volume and replaces the yolk. (Figure I.2B, C). The blastodisc gradually segregates from the yolk and forms a more prominent, clearly-defined cell at 1.5 hours, and the perivitelline space becomes visible. The cytoplasm is uniform, but darker than in other teleosts (Kimmel et al. 1995; Meijide & Guerrero 2000) (Figure I.2).

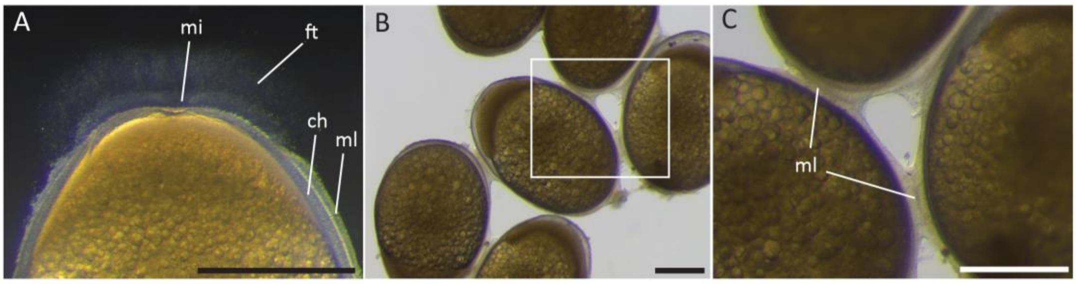

Figure I.3. Micropylar region and mucous layer.
(A) At the one-cell stage, the micropylar region is surrounded by the filament tuft. (B, C) The mucous layer adheres the eggs to substrate and/or to one another at low (B) and high magnification (C). Abbr.: ch, chorion; mi, micropyle; ft, filament tuft; ml, mucous layer. Scale bar = 500 μm.

Cleavage period (1.75-5hpf)

After 1.75 hours, cleavages occur every 35 minutes (at 28°C). The cleavage mode is meroblastic discoidal, as in other teleosts. The six synchronously-occurring divisions of this period result in stereotyped arrays of blastomeres, as reported previously (Kimmel et al. 1995; Iwamatsu 2004; Meijide & Guerrero 2000) (Figure

I.2). The egg is telolecithal and the meroblastic (incomplete) divisions keep a connection between yolk and blastodisc during the cleavage period.

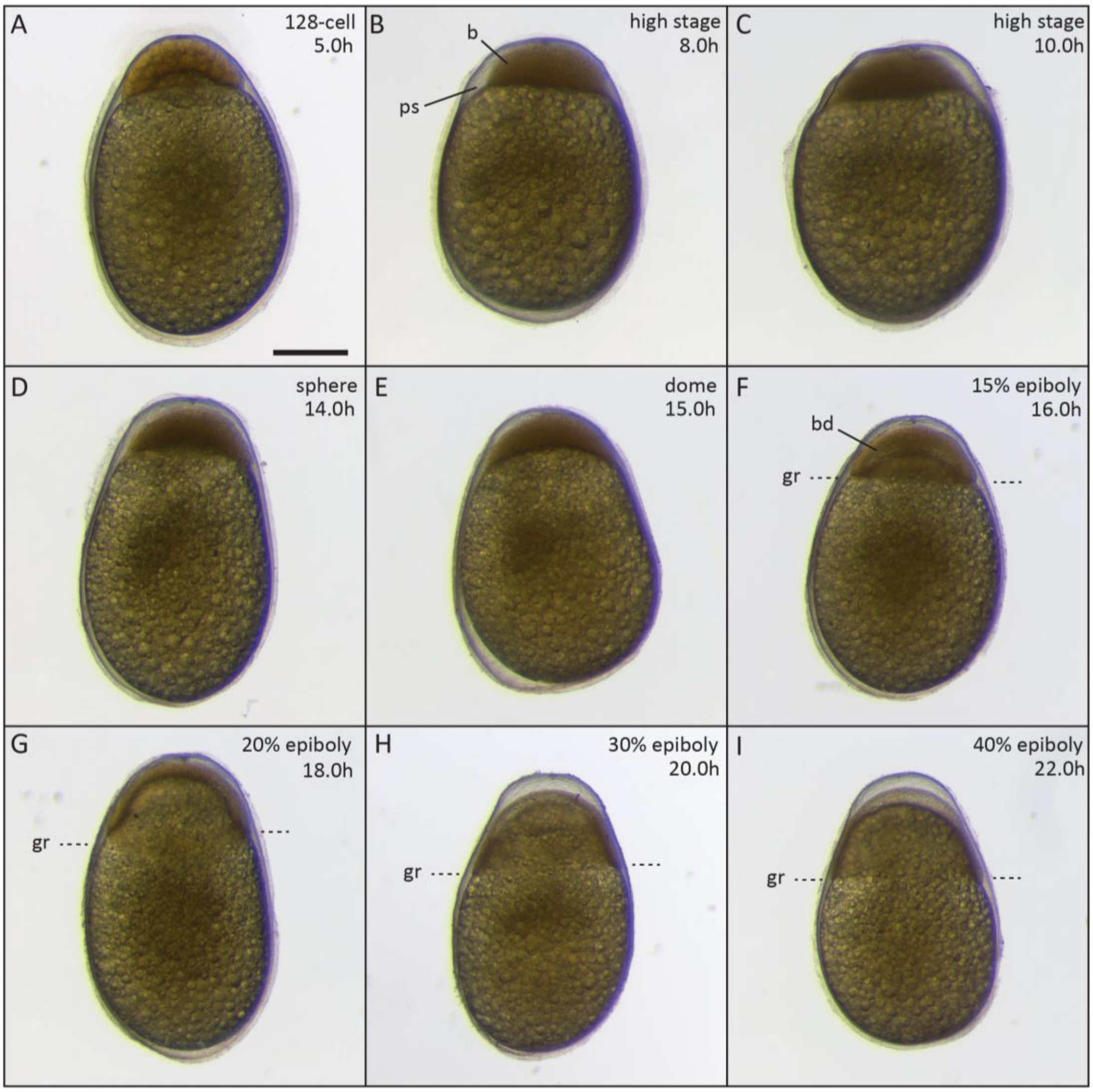

Figure I.4. Embryos during late blastula and early gastrulation phases.
(A) 128-cell stage (5 h); (B) early high stage (8 h); (C) late high stage (10 h); (D) sphere stage (14 h); (E) dome stage (15 h); (F) 15% epiboly (16 h); (G) 20% epiboly (18 h). (H) 30% epiboly (20 h). The position of the germ ring (gr in F-H) is indicated by the dashed lines. Abbreviations: b, blastodisc; bd, blastoderm; es, embryonic shield, ez, evacuation zone, gr, germ ring; ps, perivitelline space. Scale bar = 500 µm.

Two-cell stage (1.75hpf)

The first cleavage furrow is vertically oriented (meridional), dividing the blastodisc into two cells (blastomeres) of equal size. Both cells stay connected to the underlying yolk (meroblastic cleavage) (Figure I.2D).

Four-cell stage (2.5hpf)

In the second division, the cleavage plane is oriented in a right angle to the first cleavage plane, resulting in four blastomeres arranged in a 2 x 2 array if viewed from the animal pole (Figure I.2E).

Eight-cell stage (3hpf)

The third set of cleavages occurs in two planes parallel to the first cleavage plane, dividing the four blastomeres into eight blastomeres. They are arranged in a 2 x 4 array. Viewed laterally, only four cells are visible (Figure I.2F).

16-cell stage (3.5hpf)

The fourth cleavage plane also occurs on two planes, this time parallel to the second cleavage plane. The two rows of four blastomeres are divided into four rows of four blastomeres (4 x 4 array) (Figure I.2G).

32-cell stage (4hpf)

The fifth set of cleavages generates a 4 x 8 array of cells, although the pattern is less stereotypic than in previous stages. All cells are still in contact with the yolk. Often, the blastodisc curves around the yolk, shaping the underlying yolk in a dome-like structure (Figure I.2H).

64-cell stage (4.5hpf)

During the sixth set of divisions, cells start to be cleaved completely from the others, forming a second layer of cells on top of those that are still connected to the yolk (marginal cells). Unlike in previous stages, there are no regularly-patterned cleavage planes or stereotypical cell arrangements. (Figure I.2I).

Blastula period (5-24hpf)

The blastula period extends from the 128-cell stage until gastrulation. Cleavages occur with increasing irregularity. The blastodisc gets a more uniform appearance, and starts to thin and spread around the yolk (epiboly). At 50% epiboly, when half of the yolk is covered by the blastodisc, gastrulation begins (Figure I.4).

Morula stage (5hpf)

Cleavages continue to occur. As in the 64-cell stage divisions, no clear cleavage planes can be identified. The seventh, eighth and ninth cleavages result in 128, 256 and 512 blastomeres. Consequently, cells gradually become smaller, without a clear increase in the size of the blastodisc (Figure I.4A).

High stage (8 / 10hpf)

The blastodisc is a thick, ball-like structure on top of the yolk, the hallmark of this stage compared to later stages (Figure I.4B, C).

Sphere stage (14hpf)

After the high stage, the blastodisc gradually flattens, resulting in a spherical shape (Figure I-4D).

Dome stage (15hpf)

The flattening of the blastodisc continues, starting to cover the top of the yolk, which bulges towards the animal in a dome-like shape, as described for zebrafish by Kimmel et al. (Kimmel et al. 1995) (Figure I.4E).

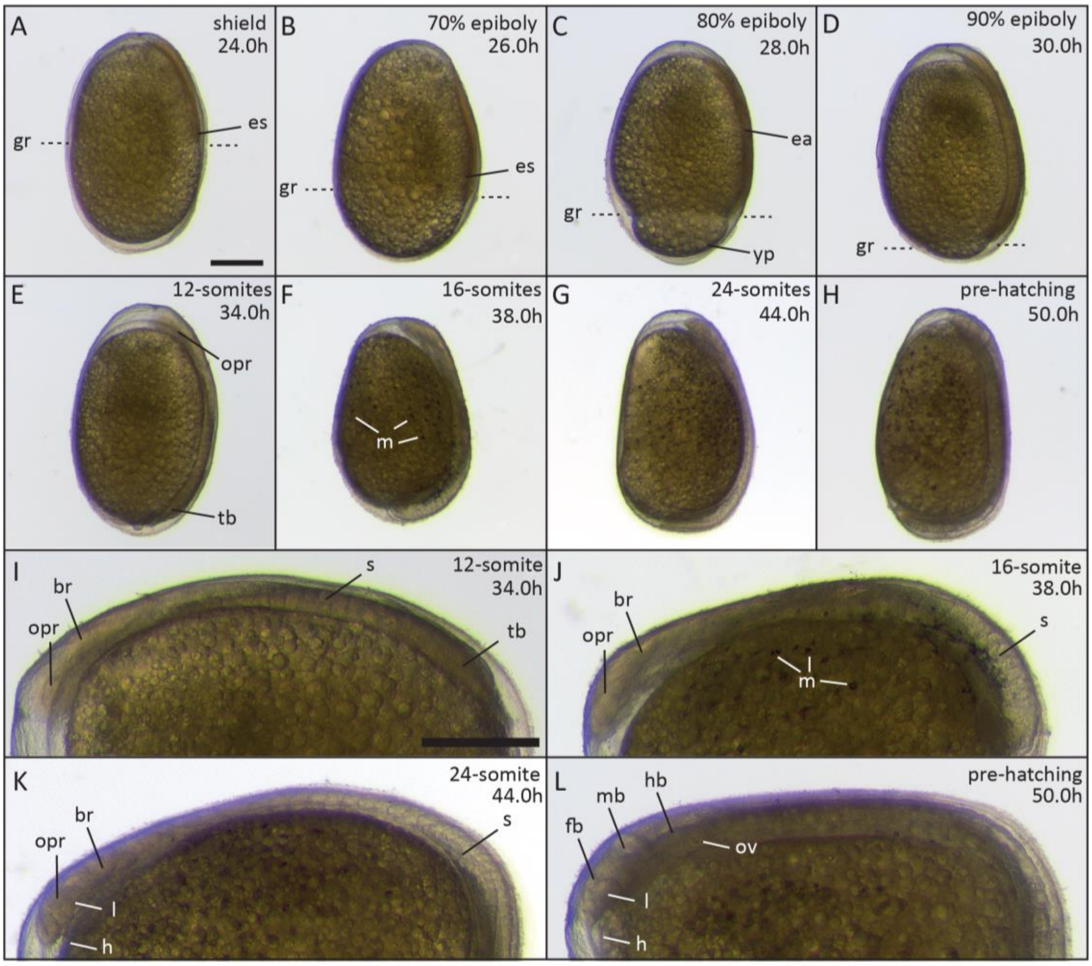

Figure I.5. Embryos during gastrulation and segmentation stages.
(A) Shield stage (24hpf); (B) 70% Epiboly (26hpf); (C) 80% Epiboly (28hpf); (D) 90% Epiboly (30hpf); (E, I) 8 somites (34hpf); (F, J) 12 somites (38hpf); (G, K) 20 somites (44hpf); (H, L) Pre-hatching stage (50hpf). The position of the germ ring (gr in A-D) is indicated by the dashed lines. Abbr.: br, brain; ea, embryonic axis; fb, forebrain; gr, germ ring; h, heart; hb, hindbrain; l, lens; ym, yolk melanophores; mb, midbrain; opr, optic primordium; ov, otic vesicle; s, somites; tb, tailbud; yp, yolk plug; Scale bar = 500µm.

Early Epiboly stages (15% - 16hpf / 20% - 18hpf)

The blastodisc, which gradually transforms into a uniformly thick layer, starts to cover the yolk and is now called the blastoderm. This stage can be measured by percentage of epiboly. We defined two stages of early epiboly depending on how far the blastoderm margin (the germ ring) has spread over the yolk: 15% and 20%, measured by the ratio between the distances between the animal pole and blastoderm margin, and between the animal and vegetal pole. (Figure I.4F-G).

Gastrula period (24-34hpf)

When 30% epiboly is reached, cells start to accumulate at one position in the blastoderm margin, which is now defined as the dorsal side. Gastrulation starts at this position by involution of cells, eventually giving rise to the three germ layers. Epiboly continues until the blastoderm completely covers the yolk. In contrast to zebrafish segmentation, the next period of development, starts before 100% epiboly is reached (Figure I.4H, I.5A-C).

30% Epiboly – Shield Stage (20hpf)

When epiboly has progressed to 30% of the yolk, a thickening appears at one position of the blastoderm margin (now defined as the dorsal side). This thickening is referred to as the “shield” (Kimmel et al. 1995) and is the result of cellular movements. Gastrulation and cell involution take place in this part of the blastoderm (Figure I.4H). In zebrafish, this stage occurs later, at 50% epiboly. Due to epiboly and convergence towards the embryonic shield, the blastoderm becomes thin at the animal pole. Due to the reduced number of cells, it is referred to as the evacuation zone (Kimmel et al. 1995) (Figure I.4H).

Late Epiboly Stages (50% - 24hpf / 70% - 26hpf / 80% - 28hpf)

At 50% epiboly the dorsal side of the blastoderm thickens further and the future embryonic axis becomes visible, with the anterior end in direction of the animal pole (Figure I.5A-C). After 70% of the yolk is covered, the speed of epiboly continues at a constant rate of about 5% per hour (three times slower than in zebrafish (Kimmel et al. 1995)). Later stages of epiboly are characterized by the presence of the yolk plug, the section of yolk at the vegetal pole that has not yet been encompassed by the blastoderm (Figure I.5C).

Segmentation period (30-66hpf)

During the segmentation period, the embryo forms and extends further, even before epiboly finishes completely. Structures including the somites, tail, eye and auditory vesicle begin to take shape. Additionally, the brain starts to grow in size. Pigmentation appears first on the yolk sac and later on the body axis. (Figure I.5D-H).

6-somite stage / 90% epiboly (30hpf)

Somitogenesis starts before the end of epiboly. At 90% epiboly, eight somites have formed and the tail bud appears at the posterior end of the body axis. At the anterior end of the embryo, the brain primordium without visible morphological subdivisions and the optic primordia that evaginates from the future diencephalon part of the brain primordium are visible. The otic (acoustic) vesicle is forming in the posterior head region. (Figure I.5D).

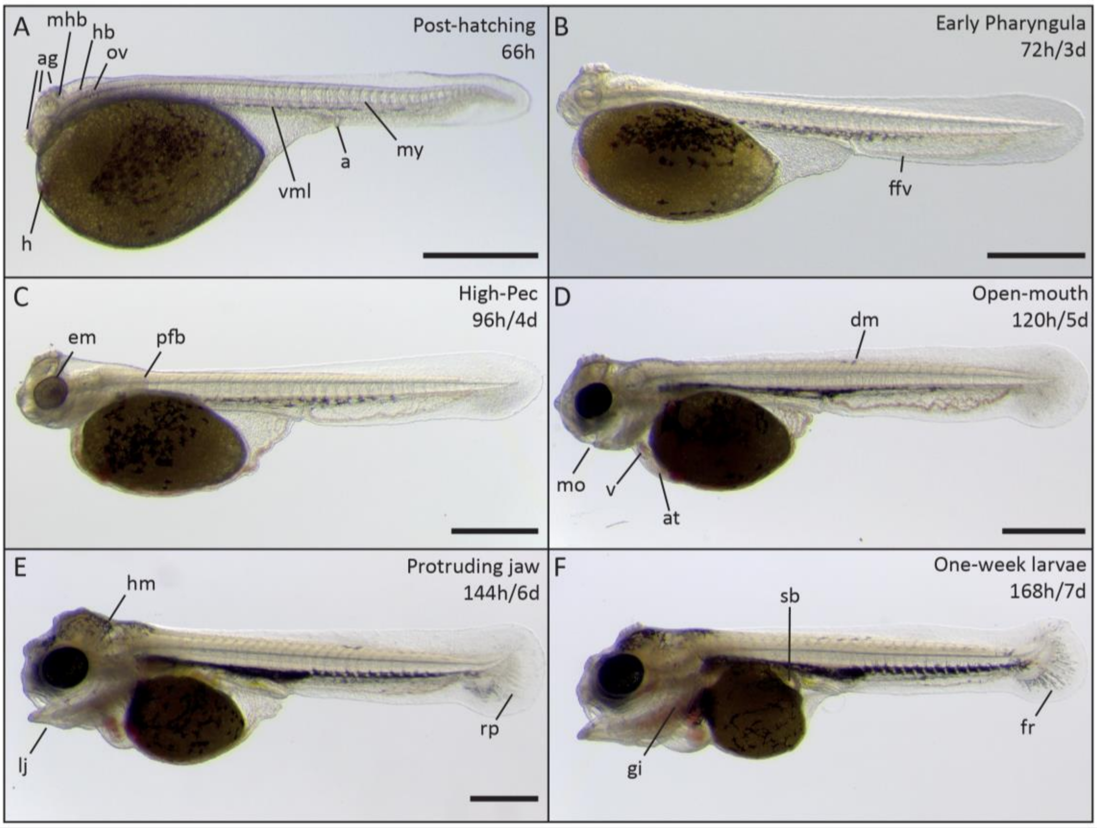

Figure I.6. Larvae in early post-hatching stages (66-168hpf).
(A) Post-hatching stage (66hpf); (B) Early pharyngula period (72hpf); (C) High-pec stage (96hpf); (D) Open-mouth stage (120hpf); (E) Protruding-mouth stage (144hpf); (F) Free swimming larvae (168hpf); The position of the germ ring (gr in A-D) is indicated by the dashed line. Abbr.: a, anus; ag, adhesive glands; at, atrium; dm, dorsal melanophores; em, eye melanophores; ffv, fin fold veins; fr, fin rays; gi, gills; h, heart; hb, hindbrain; hm, head melanophores; lj, lower jaw; m, melanophore; mhb, midbrain-hindbrain boundary; mo, mouth opening; my, myomeres; ov, otic vesicle; pfb, pectoral fin bud; rp, rays primordia; sb, swim bladder; v, ventricle; vml, ventral (melanophore) stripe. Scale bars = 1 mm.

12-somite stage (34hpf)

At the 12-somite stage, epiboly is finished and the entire yolk is covered by blastoderm. The tail bud and optic primordia become more prominent. (Figure I.5E, I).

16-somite stage (38hpf)

At the 16-somite stage, the first melanophores appear on top of the yolk sack as well as in the posterior part of the embryo. The tail extends further and starts to curl inside the chorion. The pericardial sac forms between the anteriormost region of the yolk and the head region, slightly lifting the head from the yolk (Figure I.5F, J).

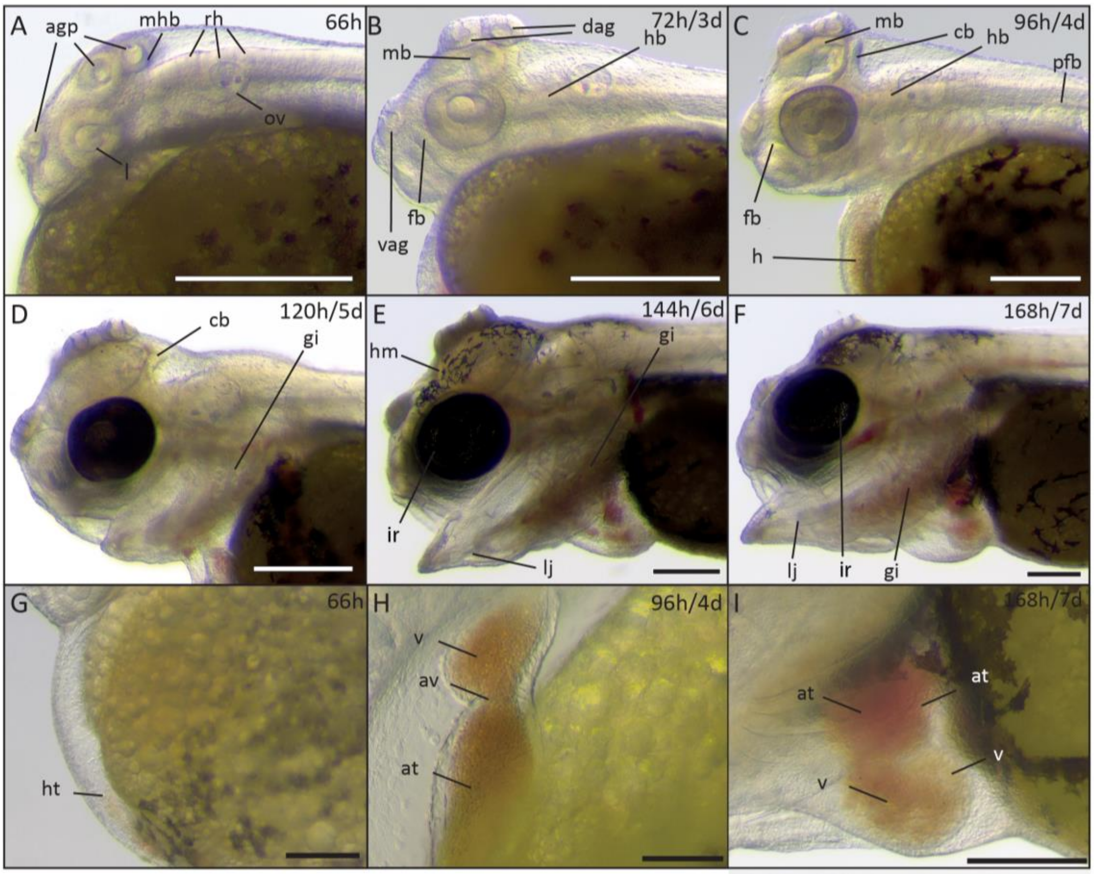

Figure I.7. Head and heart development in post-hatching stages (66-168 hpf).
(A) Post-hatching stage (66hpf); (B) Early pharyngula period (72hpf); (C) High-pec stage (96hpf); (D) Protruding-mouth stage (120hpf); (E) Open-mouth stage (144hpf); (F) Free swimming larvae (168hpf). (G-I) The developing heart at 66hpf (G), 96hpf (H) and 168hpf (I). Abbr.: agp, adhesive gland primordium; at, atrium; av, atrio-ventricular valve; cb, cerebellum; dag, dorsal adhesive gland; fb, forebrain; gi, gills; h, heart; ht, heart tube; ir, iridophores; l, lens; lj, lower jaw; hm, head melanophores; mb, midbrain; mhb, midbrain-hindbrain boundary; ov, otic vesicle; pfb, pectoral fin bud; rh, rhombomeres; v, ventricle; vag, ventral adhesive gland. A-F, I: Scale bar = 500 µm; G: Scale bar = 200 µm; H: Scale bar = 100 µm.

24-somite stage (44hpf)

At 24-somite stage, the lens primordium can easily be seen. Myotomal contractions start to occur (Figure I.5G, K).

Pre-hatching stage (50hpf)

The eye and lens have expanded in size and the head thickens due to brain growth. At this point, the three brain vesicles forebrain, midbrain, and hindbrain have become structurally differentiated and can easily be distinguished under high magnification. Muscle contractions become more frequent. Embryos hatch between 50 and 60hpf. Because hatching is variable, it is not particularly useful as a staging index. The tail is still curled and the head is bent around the yolk. The elongated tube-shaped heart, that cannot be morphologically divided into atrium and ventricle yet, starts to beat at this stage (Figure I.5H, L).

Post-Hatching period (66-168hpf)

After the embryos (now called larvae, or fry) have hatched paired fins and craniofacial skeleton start to develop rapidly in the four days after hatching (Figure I.6-8). The rudiments of all organs are present and their morphogenesis (organogenesis) continues until the end of the first week of development.

Post-hatching stage (66hpf)

Melanophores start to form ventrally in what is called the “ventral stripe” in zebrafish (Haffter et al. 1996). Unlike in zebrafish, no melanophores are formed in the dorsal and lateral stripes during the early stages of development (Figure I.6A, I.7A, I.8A). In contrast to zebrafish but similar to medaka, stickleback and other cichlids (Swarup 1958; Fujimura & Okada 2007; Meijide & Guerrero 2000; Furutani-Seiki & Wittbrodt 2004), the yolk has no posterior extension. In zebrafish, a small projection of the yolk extends posteriorly up towards the anal region (Kimmel et al. 1995). Still, the yolk sac is not completely round, and forms a cone-like tip at the posterior end. Further posterior to the end of the yolk sac, the digestive tract and anus can be seen. Shortly after hatching, the adhesive gland apparatus forms. The apparatus consists of two pairs of glands in the dorsal head region above the midbrain (dorsal glands) and one pair of glands anterior to the eye (ventral gland) (Figure I.7A-F). They are used by the larvae to attach to the substrate and to each other before they reach the free-swimming stage, as previously described in cichlids (Meijide & Guerrero 2000;

Groppelli et al. 2003) and the cave fish *Astyanax mexicanus* (Pottin et al. 2010). Under the described laboratory conditions in petri dishes, larvae mainly stick to particles such as remnants of the chorion that remain in the dish after hatching. Thereby, groups of larvae may all connect to a single particle and group together. The first red blood cells start to move through the circulatory system that allows the visualization of the developing heart that still has a tube shaped form (Figure I.7G).

Early Pharyngula period (72hpf)

The brain vesicles increase in size resulting in a further thickening of the head region (Figure I.6B, I.7B) and the isthmus, the connection between mid- and hindbrain, becomes more prominent (Figure I.7B). The head starts to lift from the yolk. Also, vascularization starts along the ventro-caudal part of the medial fin fold (caudal aorta and caudal vein) (Figure I.6B). The tail has straightened and the surface of the fin fold has increased, especially ventrally (Figure I.6B). The adhesive glands are becoming more prominent at this stage (Figure I.7B).

High-pec stage (96hpf)

Melanophores start to form in the eye (Figures I.6C, I.7C), but only a few can be seen along the ventral zone of the body axis (Figure I.8D). The pectoral fin can be seen as an elongated blade-shaped tissue projecting dorsally from the yolk. Head and body axes now have nearly the same orientation and the head lifts up from the yolk (Figure I.6C). The adhesive glands have started to decrease in size and brain ventricles, midbrain and isthmus can be seen more clearly (Figure I.7C). The heart can now be morphologically separated into ventricular and atrial chamber, separated by the atrio-ventricular valve. The cardiac looping is already in process that moves the atrium to a more dorsal position and transiently generating a S-shaped structure, comparable to heart development in zebrafish (Stainier 2001; Keßler et al. 2012) (Figure I.7H).

Open mouth-stage (120hpf)

A few melanophores start to form dorsally, and eye melanophores have increased in density, causing the eye to become opaque (Figure I-6D). Silvery reflective iridophores can be detected in the eye and become more prominent after the fifth day of development (Figure I.7E, F). The mouth opening and gills become visible (Figure I.7D). Also, the chambers of the heart have become fully differentiated

(Figure I.6D) and the caudal fin starts to form, acquiring a more rounded shape (Figure I.8B).

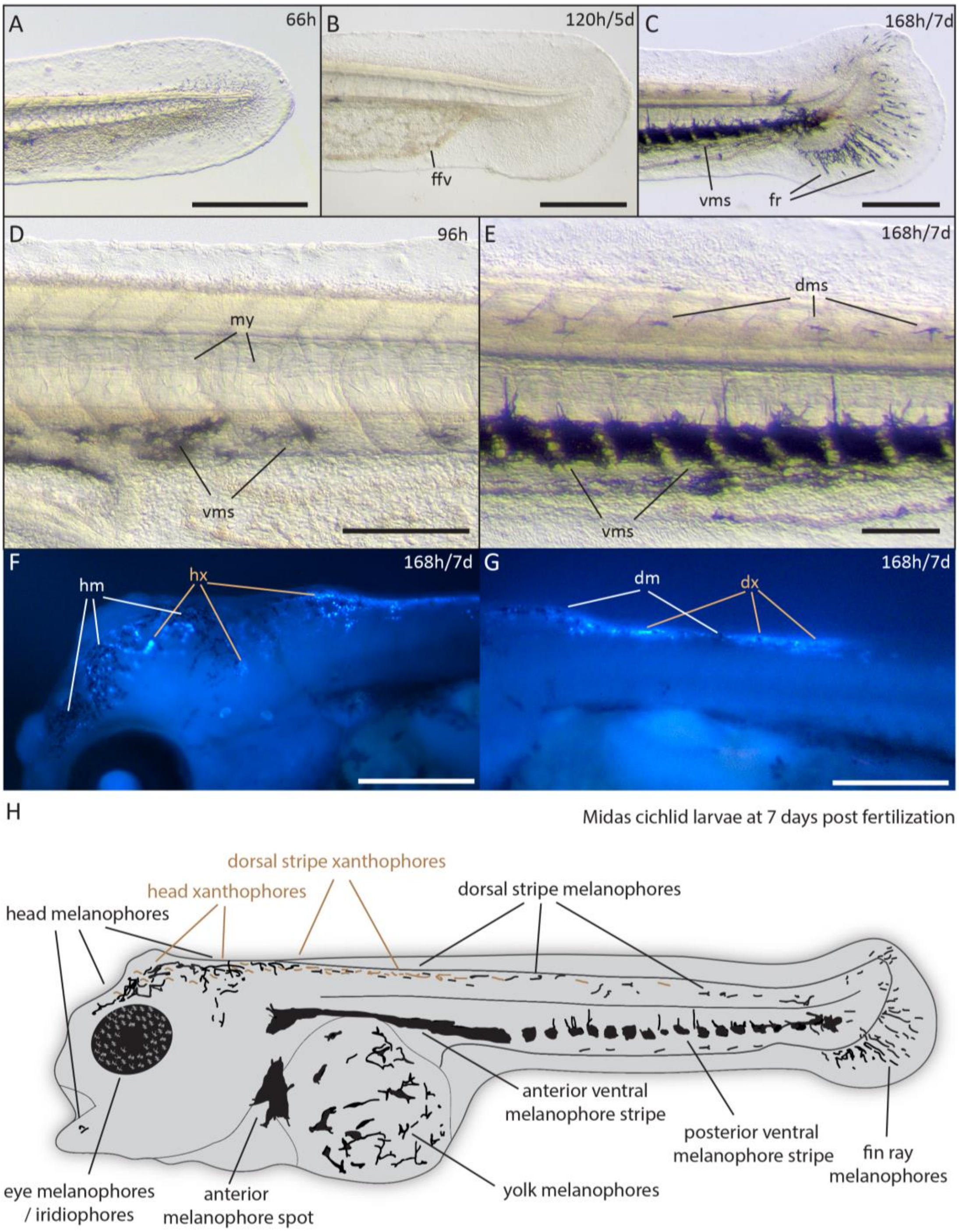

Figure I.8. Detail of tail, melanophore and xanthophore development in post-hatching stages (68-168 hpf).
(A-C) Caudal fin development at post-hatching stage (66hpf, A), protruding-mouth stage (120hpf, B) and free swimming larvae stage (168hpf, C). (D, E) Formation and dendrite extension of melanophores at high-pec stage (96hpf, D) free swimming larvae stage (168hpf, E). (F, G) Xanthophores on head (F) and in the dorsal stripe above the yolk (G) visualized under UV light. (H) Scheme summarizing the chromatophore distribution at 168hpf/7dpf. Abbr.: dm, dorsal melanophores; dx, dorsal xanthophores; dms, dorsal melanophore stripe; hm, head melanophores; hx, head xanthophores; ffv, fin fold veins; fr, fin rays; my, myomeres; vms, ventral (melanophore) stripe. A-C, F, G. Scale bars: 500 µm. D-E. Scale bars: 250 µm.

Protruding jaw-stage (144hpf)

Melanophores form ventrally and appear for the first time in the head region. The ventral melanophores condense in the posterior part giving them a segmented appearance that correlates with myomere position; while anteriorly and also dorsally to the heart they are a coherent mass of cells (Figure I.6E). The lower jaw extends anteriorly, stretching the head in a more anterior direction. (Figure I.7E). The caudal fin starts to develop fin rays and the rays are readily populated by melanophores that most likely migrated from the ventral stripe (Figure I.6E). In contrast to earlier stages, the strong vascularization in the ventral medial fin fold becomes less evident (Figure I.6E).

One-week larvae (168hpf)

The larva further increases in size, and the gills can be seen more clearly than in previous stages. The jaw becomes thicker and more strongly vascularized, and the larva is able to open and close its mouth freely (Figure I.6F, I.7F). The melanophores increase in number, and they aggregate more clearly (Figure I.8C, E, H). Some of them project dendrites dorsally into the space between two myomeres. Xanthophores can be detected now both at head and in the dorsal stripe in close proximity to melanophores (Figure I.8F, G). They appear colorless both in reflected and under transmitted light and could only be detected using UV-light (see Methods). Silvery reflective iridophores are less prominent then in zebrafish, medaka and tilapia (Kimmel et al. 1995; Iwamatsu 2004; Fujimura & Okada 2007) and could only be detected in the eye (Figure I.7E,F; Figure I.8H). The caudal fin rays have become thicker, and elongated melanophores are arranged around them (Figure I.8C). The heart is now fully developed and the heart can be divided in right and left atrium as well as the two ventricles that are located ventrally. The ventricular walls thickened, indicated by the reduced visibility of red blood cells (Figure I.7I). The swim bladder develops on the ventral side of the body, dorsal to the posterior end of the yolk plug. (Figure I.6F). Between days seven and eight, the swim bladder inflates and the larva begins to swim upright.

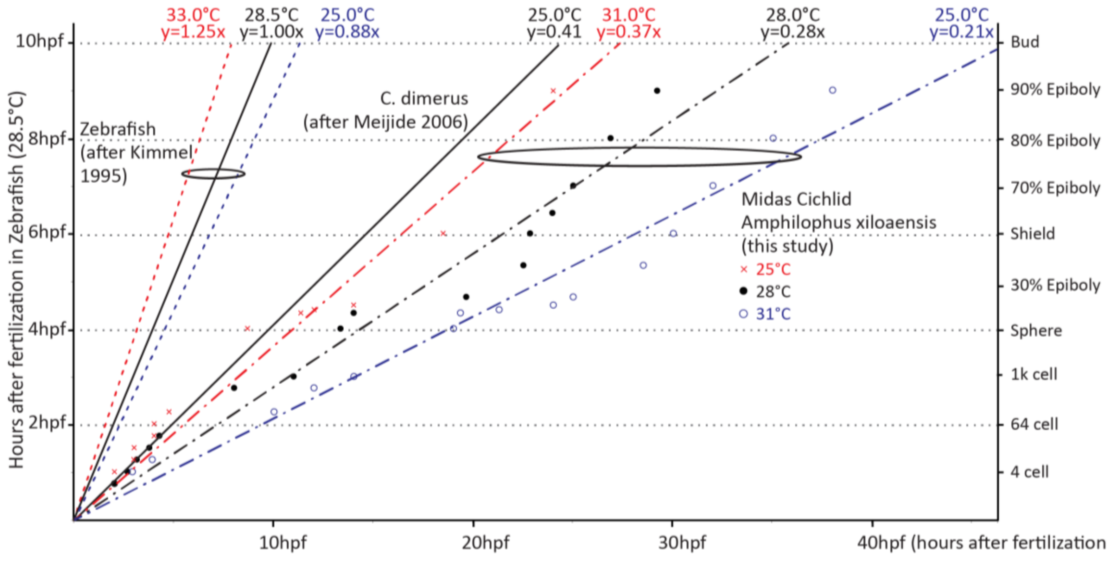

Figure I.9. The effects of temperature on developmental time.
Developmental rates in *D. rerio* (zebrafish) at 25, 28.5 and 33°C (from Kimmel et al. 1995), *C. dimerus* (South-American substrate-brooding cichlid) at 25°C (from Meijide & Guerrero, 2006) and the Midas cichlids at 25, 28 and 33°C (this study), standardized to zebrafish development at 28.5°C (Kimmel et al. 1995).

Midas cichlid development is greatly influenced by temperature

The early development of the Midas cichlid is slower than that of genetic model teleosts such as medaka and zebrafish. However, it is comparable to the African Nile tilapia (Figure I.9). We compared the homologous developmental stages to the South American cichlid *Cichlasoma dimerus* and the zebrafish *Danio rerio.* We show that, when raised at the same temperature (25°C), the developmental rate between fertilization and 100% epiboly is approximately two times slower than in *C. dimerus* and over four times slower than in zebrafish (Figure I.9, Figure I.10B). In particular, the rate of epiboly seems to be decelerated compared to zebrafish. The influence of temperature on developmental rate is far greater than in zebrafish, where there is only a 1.42-fold difference between embryos developing at 25°C and 31°C (Kimmel et al. 1995). In Midas cichlids, the difference is 1.76-fold. Despite this, later developmental stages seem to be less affected by temperature, with only minor differences in hatching time, development of pectoral fins and mouth opening between *C. dimerus* and *A. xiloaensis* (Figure I.10A, B).

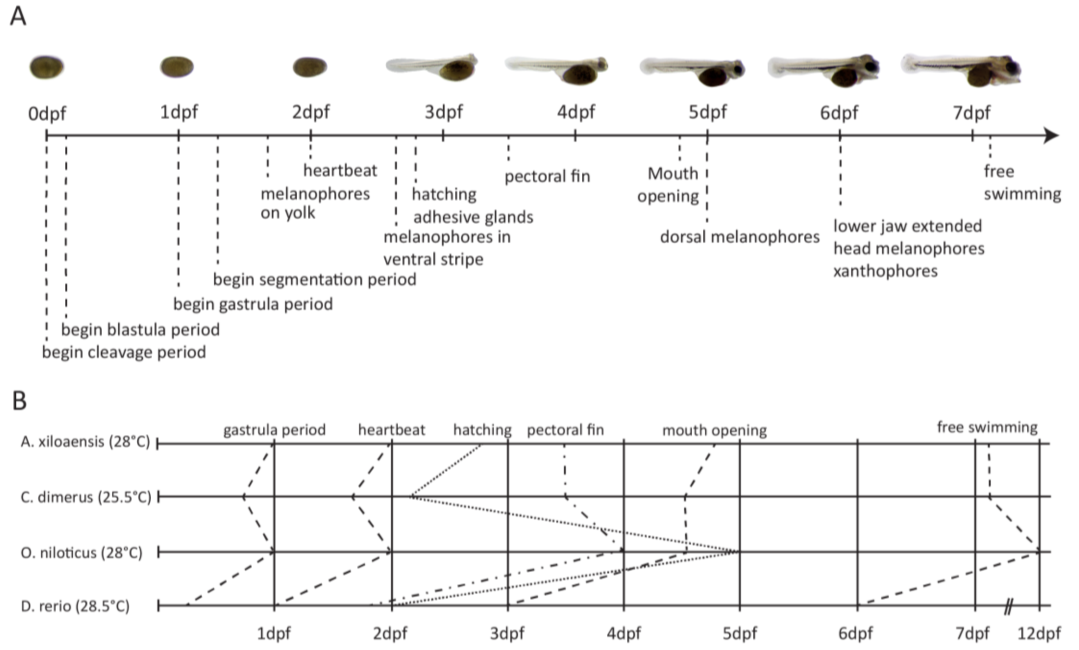

Figure I.10. Summary of Midas cichlid development and comparison to the development of other teleosts.
(A) Summary of the most important steps of the first week of Midas cichlid development at 28°C. (B) Comparison between this study and three further studies on teleosts including *C. dimerus*, a South American cichlid, *O. niloticus*, an African cichlid and *D. rerio*, the zebrafish.

Discussion

We describe the embryonic and larval development of *Amphilophus xiloaensis* as a representative of the Midas cichlid species complex. Midas cichlids are an excellent example of rapid adaptation (Muschick et al. 2011b; Fan et al. 2012) and parallel evolution (Elmer, Kusche, et al. 2010; Manousaki et al. 2013) They allow to integrate studies of genomics, adaptive radiation and phenotypic divergence into the field of “evo-devo”. The rate of divergence in many traits, including coloration and pigmentation, morphology of body shapes, lips, jaws and teeth as well as neural systems such as vision is much higher in cichlid fishes than in most other vertebrate groups (Elmer, Kusche, et al. 2010; Barluenga et al. 2006; Barluenga & Meyer 2010). In Nicaraguan crater lakes, speciation and phenotypic diversification took place over a very short time and endemic species have been described from crater lakes that are less than 2000 years old (Recknagel, Elmer, et al. 2013). In less than 25,000 years, at least eleven species have evolved, carrying various traits that are divergent from the ancestral population (Henning & Meyer 2014; Elmer, Fan, et al. 2010; Barluenga et al. 2006). A few mutations of major effect expected to play important roles in driving phenotypic richness and ecological diversity as studies from other cichlids (Salzburger et al. 2007; Schulte et al. 2014; Henning et al. 2014; Seehausen et al. 2008;

Brawand et al. 2014; Powder et al. 2014; Albertson et al. 2014; Roberts et al. 2009; Roberts et al. 2011) or sticklebacks (Chan et al. 2010; Colosimo 2005; Cleves et al. 2014) suggest. The mutation rate of Midas cichlids has been estimated to be between 6.6×10^{-8} and 7.1×10^{-8} mutations, which is comparable to the vertebrate average (Recknagel, Elmer, et al. 2013; Barluenga & Meyer 2004). Still, genetic differences between different Midas cichlid species are small due to their long generation times (conservative estimate one year (Barluenga & Meyer 2004)) and recent time of divergence (2,000 -25,000 years (Elmer, Lehtonen, et al. 2010)).

Differences in developmental pathways are often involved in the basis of ecologically relevant phenotypic differences (Braasch et al. 2015) such as those observed in the Midas species complex, including body shape and craniofacial shape (Franchini et al. 2014; Klingenberg et al. 2003; Recknagel et al. 2014), lip shape (Manousaki et al. 2013; Machado-Schiaffino et al. 2014; Barlow 1976), coloration (Barlow 1976; Torres Dowdall et al. 2014; Henning et al. 2013; Elmer et al. 2009) and pharyngeal jaw morphology (Barluenga et al. 2006) (Figure I.1C, Table I.1). Although most of these phenotypes arise later during ontogeny, differences might be already detectable on a subtle morphological and gene expression level – especially for craniofacial phenotypes associated with benthic-limnetic differences as recently shown in other species (e.g. craniofacial skeleton of Malawi cichlids (Powder et al. 2014) or the Arctic charrs (Ahi et al. 2014). To examine if indeed inter-species differences for some of these morphologies and gene expression differences are already present at early stages of Midas cichlid development, a standardization of embryonic timing and a comprehensive - and comparative - staging system are necessary as a baseline for hypothesis driven research in this field. This staging forms the basis for future comparative developmental work on different species of Midas cichlids and closely related Neotropical cichlids (Geiger, McCrary & Schliewen 2010). Since development is greatly influenced by temperature, easily recognizable landmarks, along with a standardized temperature-time protocol must be defined. This will ease the collection of comparable stages for molecular biological experiments, such as *in situ* hybridization or RNA extraction.

Availability of transcriptomic and genomic data sets, including a high-quality draft genome of *A. citrinellus* and low-coverage genomic information of eleven Midas cichlid complex species (Elmer et al. 2014) are available (Table I.1).

In Midas cichlids, embryonic traits such as the prominent adhesive glands and the early melanocyte and xanthophore patterns differ from other model teleosts such as medaka and zebrafish. Adhesive glands have recently been studied in the cavefish *Astyanax mexicanus* (divergence time approximately 265 million years (Hedges et al. 2006)) and described in other cichlids (Meijide & Guerrero 2000; Groppelli et al. 2003), but neither medaka nor zebrafish possess adhesive organs.

The embryonic melanophore patterns we observe are also very different from model teleosts such as Medaka and zebrafish (Kimmel et al. 1995; Iwamatsu 2004; Furutani-Seiki & Wittbrodt 2004). The prominent dorsal and lateral stripes of melanophores is almost completely absent in Midas cichlid embryos, suggesting different migration of neural crest cells, which are thought to generate all but the yolk melanocytes in teleosts (Kelsh et al. 1996). It has been proposed that some melanophores migrate from the yolk to populate the embryo, especially in the ventral zone; however, there has been some controversy surrounding this claim (Jones 1972). Further histological analysis using neural crest markers could solve this controversy and clarify the genetic cause of the different melanophore patterns observed in Midas cichlids.

The embryos of substrate brooders such as the Midas cichlid are also easier to use for genetic manipulations such as transgenesis than the massive, yolky eggs of mouth-brooding cichlids from the African Great Lakes, although those were the first species where transgenesis has been successfully performed (Fujimura & Kocher 2011; Juntti et al. 2013). Functional assays like those performed in zebrafish can also be carried out in Midas cichlids (Kratochwil et al., unpublished results). Large clutch sizes and the slow development before the one-cell stage allow for the injection of considerable amounts of eggs. This method will allow researchers to transiently map the influence of gene overexpression or the activity of regulatory elements. Additionally, genetic manipulations by morpholinos or CRISPR-Cas that both have been shown to work in the Nile Tilapia (Li et al. 2014; Pabic et al. 2010) might be applicable in the Midas cichlid as well. One limitation is the longer generation time, which is about nine to twelve months under laboratory conditions. Despite this limitation, it may still be possible to generate stable transgenes or knockouts, which would be relevant for experiments in Neotropical, substrate-brooding cichlids. Furthermore, the Midas cichlid could also serve as an excellent outgroup for the

African cichlid species flocks, allowing for the functional screen or assay of genes or cis-regulatory elements (Kratochwil & Meyer 2015; Kratochwil & Meyer 2015b). As shown here, Midas cichlids can be easily maintained, bred, stripped and raised in large numbers under laboratory conditions.

Conclusions

It is still barely understood which genes and mutations underlie the parallel evolution of traits and the quickly-evolving species richness exhibited by Midas cichlids. This study adds valuable information about the course of early development to help to tackle questions about the molecular basis of phenotypic novelties from an evolutionary-developmental, evo-devo angle. The staging system in a representative Midas cichlid species will set the foundation for future experiments and ease interspecies comparisons. It will help to reproducibly select standardized developmental stages during development to analyze gene function and differences in gene expression and patterns. Midas cichlids have embryonic traits (adhesive glands, melanophore patterns) that differ from the classical developmental model teleosts, medaka and zebrafish. It will be interesting to analyze the genetic cause of these differences. We also propose the Midas cichlid as a new model organism for evolutionary developmental research. In addition to the availability of genetic resources and the possibility to perform functional experiments, Midas cichlids and their adaptively relevant phenotypic diversity are well-described from an ecological and evolutionary standpoint. These advantages, taken together, make this system very attractive for evolutionary-developmental questions.

II. Chapter II

Tol2 transposon-mediated transgenesis in the Midas cichlid (*Amphilophus citrinellus*) — towards understanding gene function and regulatory evolution in an ecological model system for rapid phenotypic diversification

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Published in BMC DEVELOPMENTAL BIOLOGY, 2017

Abstract

Background

The Midas cichlid species complex (*Amphilophus spp.*) is widely known among evolutionary biologists as a model system for sympatric speciation and adaptive phenotypic divergence within extremely short periods of time (a few hundred generations). The repeated parallel evolution of adaptive phenotypes in this radiation, combined with their near genetic identity, makes them an excellent model for studying phenotypic diversification. While many ecological and evolutionary studies have been performed on Midas cichlids, the molecular basis of specific phenotypes, particularly adaptations, and their underlying coding and cis-regulatory changes have not yet been studied thoroughly.

Results

For the first time in any New World cichlid, we use Tol2 transposon-mediated transgenesis in the Midas cichlid (*Amphilophus citrinellus*). By adapting existing microinjection protocols, we established an effective protocol for transgenesis in Midas cichlids. Embryos were injected with a *Tol2* plasmid construct that drives enhanced green fluorescent protein (*eGFP*) expression under the control of the ubiquitin promoter. The transgene was successfully integrated into the germline, driving strong ubiquitous expression of *eGFP* in the first transgenic Midas cichlid line.

Additionally, we show transient expression of two further transgenic constructs, *ubiquitin::tdTomato* and *mitfa::eGFP*. Transgenesis in Midas cichlids will facilitate further investigation of the genetic basis of species-specific traits, many of which are adaptations.

Conclusion

Transgenesis is a versatile tool not only for studying regulatory elements such as promoters and enhancers, but also for testing gene function through overexpression of allelic gene variants. As such, it is an important first step in establishing the Midas cichlid as a powerful model for studying adaptive coding and non-coding changes in an ecological and evolutionary context.

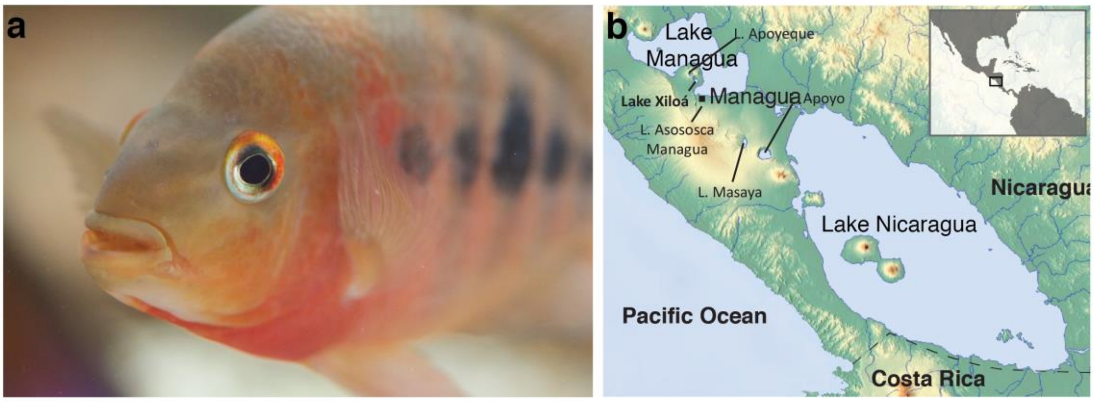

Figure II.1. The study system.
(a-b) Within the last 25,000 years Midas cichlids (*Amphiliphus spp.*; here Amphilophus amarillo from Lake Xiloa (a)) from Nicaragua (b) colonized several small crater lakes from the large lakes L. Nicaragua and L. Managua. Within the crater lakes Midas cichlids underwent rapid and parallel adaptive evolution and formed several new species.

Background

Cichlid fishes are a textbook example for phenotypic diversity and rapid rates of speciation (Kocher 2004). They are one of the most diverse groups of vertebrates with over 2000 described species (Henning & Meyer 2014). Biologists have long been fascinated by these teleosts and numerous studies have been conducted on aspects of cichlid biology such as their strikingly diverse color patterns (Seehausen & Van Alphen 1999; Roberts et al. 2009), morphologies (Barluenga et al. 2006; Kusche et al. 2014) and behaviors (York et al. 2015; Lee et al. 2012). Newly-available genomic resources in combination with Quantitative Trait Loci (QTL) and molecular studies allow cichlid scientists to study the exact genetic mechanistic underpinnings of ecologically relevant traits (Kratochwil & Meyer 2015; Brawand et al. 2014). Hence, techniques from 'model teleosts,' such as medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Kawakami 2007) have to be adapted to functionally validate and analyze genotype-phenotype relationships in these new species. Molecular tools, in particular transgenesis, are effective for testing gene function and activity of *cis*-regulatory elements. In recent years, transgenesis technology has increasingly been applied to non-model organisms, especially driven by the use of the *Tol2* transposon-mediated insertion technology that strongly increases the insertion efficiency of recombinant DNA (Kawakami 2007). This expands this powerful toolset to organisms of evolutionary and ecological interest including sticklebacks (Chan et al. 2010), African cichlids (Juntti et al. 2013) and killifish (Valenzano et al. 2011). Here, we have successfully adapted and optimized *Tol2*-mediated transgenesis for the first time in a cichlid from the New World, the Midas cichlid species complex, *Amphilophus spp*.

One of the central aims of evolutionary biology is to understand how genetic changes contribute and translate to adaptive phenotypic changes. The Nicaraguan Midas cichlids (*Amphilophus spp.,* Figure II.1a) are an excellent model system for studying phenotypic diversification and how this might ultimately result in the formation of novel, distinct species. In Nicaragua, several isolated crater lakes have been colonized from the two great lakes, Lake Managua and Lake Nicaragua (Figure II.1b and Elmer, Kusche, et al. 2010). The age of these crater lakes spans from 25000 years (Lake Apoyo), to less than 1000 years (Lake Asososca Managua). These smaller crater lakes have been repeatedly colonized by Midas cichlids (Kautt, Machado-Schiaffino, & Meyer, 2016; Kautt, Machado-Schiaffino, Torres-Dowdall, & Meyer, 2016). Colonization events not only triggered diversification and adaptation to the specific Crater Lake environment (Barluenga & Meyer 2004; Torres-Dowdall et al. 2017), but also gave rise to several novel species that formed both in allopatry and sympatry (Barluenga et al. 2006). Each lake can be seen as a small adaptive radiation, within which species and individuals show a wide variety of morphological characteristics (Recknagel et al. 2014). Several traits have been found to differ between source and crater lakes, as well as between the newly-formed species within the crater lakes (Elmer & Meyer 2011). These include, but are not limited to, variation in body size and shape (i.e. limnetic and benthic ecomorphs) (Barluenga et al. 2006; Meyer 1990; P Franchini et al. 2014; Meyer 1990b), pharyngeal jaws (Barluenga et al. 2006), hypertrophied lips (Machado-Schiaffino et al. 2014), coloration (Henning et al. 2013), and visual sensitivity (Julián Torres-Dowdall et al. 2017). Midas cichlids present an excellent opportunity to determine the genetic architecture of traits using genome scans and QTL mapping studies (Kratochwil & Meyer 2015; Franchini et al. 2014). However, bridging the gap between genotype and phenotype, and understanding how genetic changes translate to phenotypic variation, critically depends on complementary functional approaches (Kratochwil & Meyer 2015). Here, tools such as transgenesis are necessary to facilitate the discovery of the exact genetic changes and mechanisms that underlie phenotypic diversification.

Transgenesis is defined as the process of introducing new genetic information into a living organism. The development of recombinant DNA technology in the early 1970s (Cohen et al. 1973) paved the way for transgenesis to become a widely-used technique in experimental biology. The first transgenic zebrafish was produced in 1988 (Stuart et al. 1988). Since then, more efficient methods of producing transgenic zebrafish have been developed using transposon-mediated insertion. The now-common *Tol2* transposable element was originally isolated from medaka, and *Tol2* transposon-mediated transgenesis (Kawakami 2007), our method of choice, represented a significant improvement in the efficacy of transgenesis compared to previous approaches. Although the use of transgenesis in zebrafish and medaka is widespread, its use in other teleosts has been fairly limited until recently. Within the last several years, transgenesis has been successfully used in non-model organisms such as the Nile Tilapia (*Oreochromis niloticus*) (Fujimura & Kocher 2011), the haplochromine cichlid *Astatotilapia burtoni* (Juntti et al. 2013), the African turquoise killifish (*Nothobranchius furzeri*) (Valenzano et al. 2011) and the three-spined stickleback (*Gasterosteus aculeatus*) (Chan et al. 2010). Our study adds the Midas cichlid to this growing list of non-model teleost species.

In this study, we show that the *Tol2* system of transgenesis can be successfully applied to the Midas cichlid (Figure II.2). We established a stable line of Midas cichlids carrying a ubiquitously expressed enhanced Green Fluorescent Protein (*eGFP*) construct (*ubi::eGFP*). For this study, we used a construct that combines the *ubiquitin (ubi)* promoter region, expressed in all eukaryotic cells, and the gene coding for *eGFP*. This construct was chosen for testing because the fluorescent reporter can be expressed in all cell types, facilitating the quantification of the presence and intensity of transgene expression in treated embryos. The transgene was successfully integrated into the germline, confirming that transgenesis, an important and versatile tool, can be used in Midas cichlids. To further demonstrate the wide applicability of this technology in Midas cichlids, we provide transient expression data for two additional constructs: 1) *ubi::tdtomato*, a construct with the red fluorescent protein *tdTomato* (Williams et al. 2010) under the control of the same *ubiquitin* promoter and 2) *mitfa::eGFP* that drives pigment-cell specific GFP expression under the control of the promoter of the melanoblast/melanophore marker *microphthalmia-associated transcription factor* (*mitfa*) (Curran et al. 2009; Lister et al. 1999).

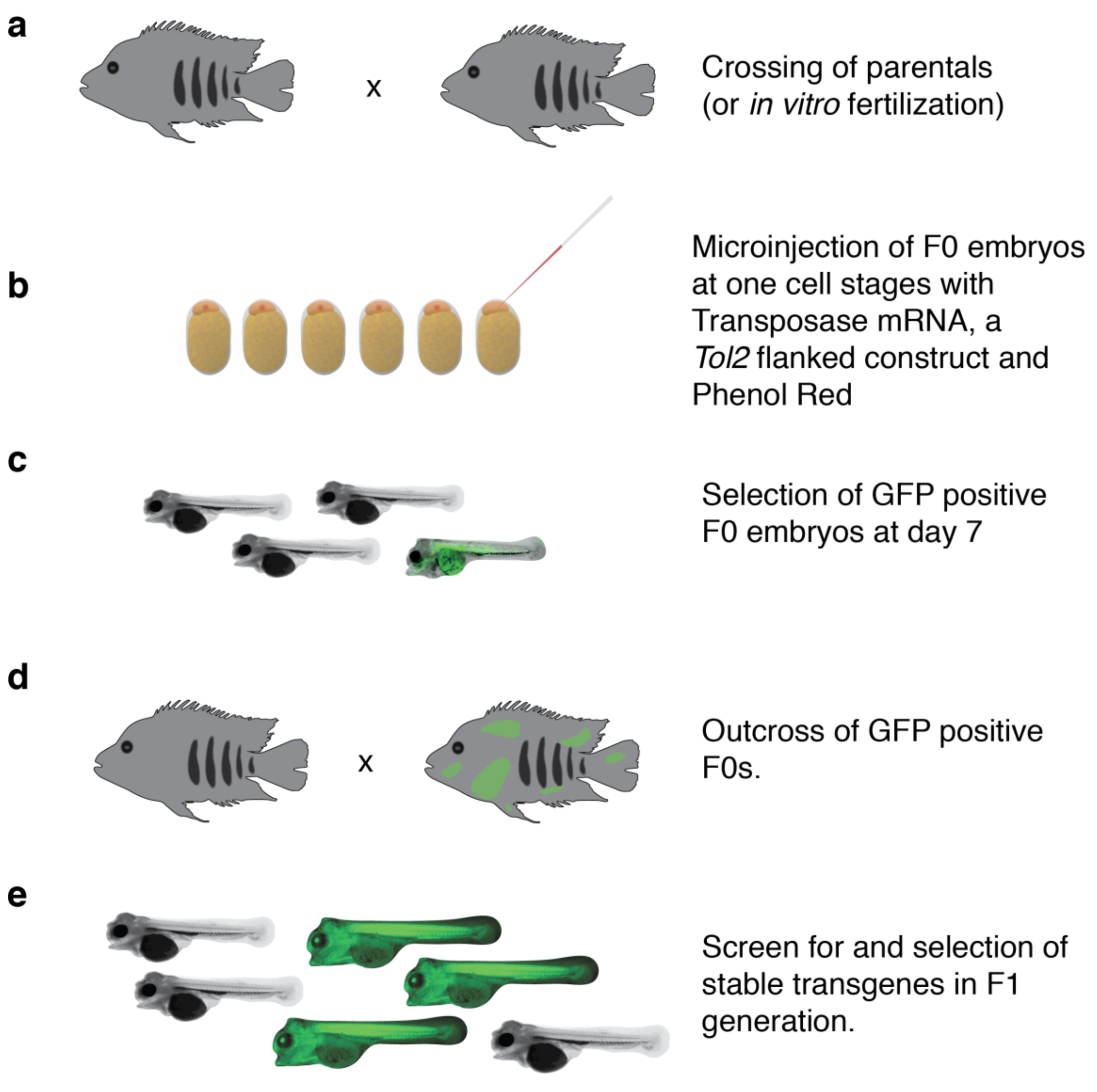

Figure II.2. Experimental overview.
(a) Midas cichlids are crossed. After successful fertilization eggs are immediately collected. Alternatively, eggs can be fertilized in vitro. (b) Embryos at on-cell stage are injected with a mix of Transposase mRNA, phenol red and a Tol2 flanked DNA-construct. (c) Positive embryos show a mosaic pattern of GFP fluorescence. They are screened and selected seven days after fertilization. GFP positive larvae are raised. (d-e) To obtain F1 transgenic embryos, GFP positive individuals are crossed after 9-12 months (d) to obtain stable transgenic Midas cichlid lines (e).

Methods

Fish husbandry and egg collection

Adult Midas cichlids (*Amphilophus citrinellus*) were maintained in aquarium facilities at the University of Konstanz under constant conditions (28 ± 1°C, 12 h dark/light cycle, pH 7.5 ± 0.5) as previously described (Kratochwil et al. 2015). Gravid females with fully-developed eggs ready for fertilization are identifiable by their characteristic swollen and enlarged genital pore (Figure II.3a). Eggs were stripped and fertilized (Figure II.3a, b) or taken promptly after natural fertilization, as previously described (Kratochwil et al. 2015).

Cloning

Transgenes were generated using the construct *pT2A_Ubiquitin-eGFP-pA_pA2* (Figure II.3e). Using site-specific recombination-based cloning (multisite Gateway technology) we combined the promoter region of ubiquitin (*p5E_ubi*, Addgene ID 27320; (Mosimann et al. 2011)) with the *Tol2*-Kit constructs *383_pME-EGFP*, *302_p3E-polyA* and *394_pDestTol2pA2* (Kwan et al. 2007) as well as *pME-tdTomato* (Williams et al. 2010). To generate the *p5e-mitfa* vector, a 1.1kB fragment including 53 bp of 5'UTR and 1054 bp upstream of the 5'UTR were amplified from *A. citrinellus* genomic DNA using the primer pair 5′ – gat cgc tcg agC ATC TTT GTT CCT TAT CC and 5′ – gat cga cta gtT CCC TTT ATC TTG TTA GC (hybridization sequence in uppercase, leader sequence and restriction site in lowercase). The fragment was cloned into the multiple cloning site of p5e-MCS using the restriction enzymes XhoI and SpeI. *pT2A_mitfa-eGFP-pA_pA2* and *pT2A_ubiquitin-tdTomato-pA_pA2* were generated using site-specific recombination-based cloning as previously described (Kwan et al. 2007).

Microinjection

After fertilization, eggs were transferred into 2% agarose plates molded with custom-designed injection trenches (Figure II.3c, d). Using forceps, eggs were inserted into the trenches, oriented in an upright position with the animal pole on top. Injections were performed using glass capillaries (Hilgenberg, length 100mm, outside diameter: 1.0mm; inside diameter 0.58 mm) pulled on a Sutter P-97 Flaming/Brown Micropipette Puller. A solution composed of the plasmid construct (12.5 ng/µl), transposase (12.5 ng/µl), RNAse-free water and phenol red (1%) for visualization was co-injected into the embryos. An air pressure-driven microinjector (Narishige IM-300) was used for injections. Injection volume was adjusted to fill approximately 5% of the egg volume. The solution was injected directly into the developing one-cell stage embryo to maximize successful incorporation into the genome. Because early embryonic development in Midas cichlids proceeds relatively slowly compared to other teleost species (Kratochwil et al. 2015), it is possible to inject 500-1000 eggs before the first cell division takes place, 90 to 100 min after fertilization.

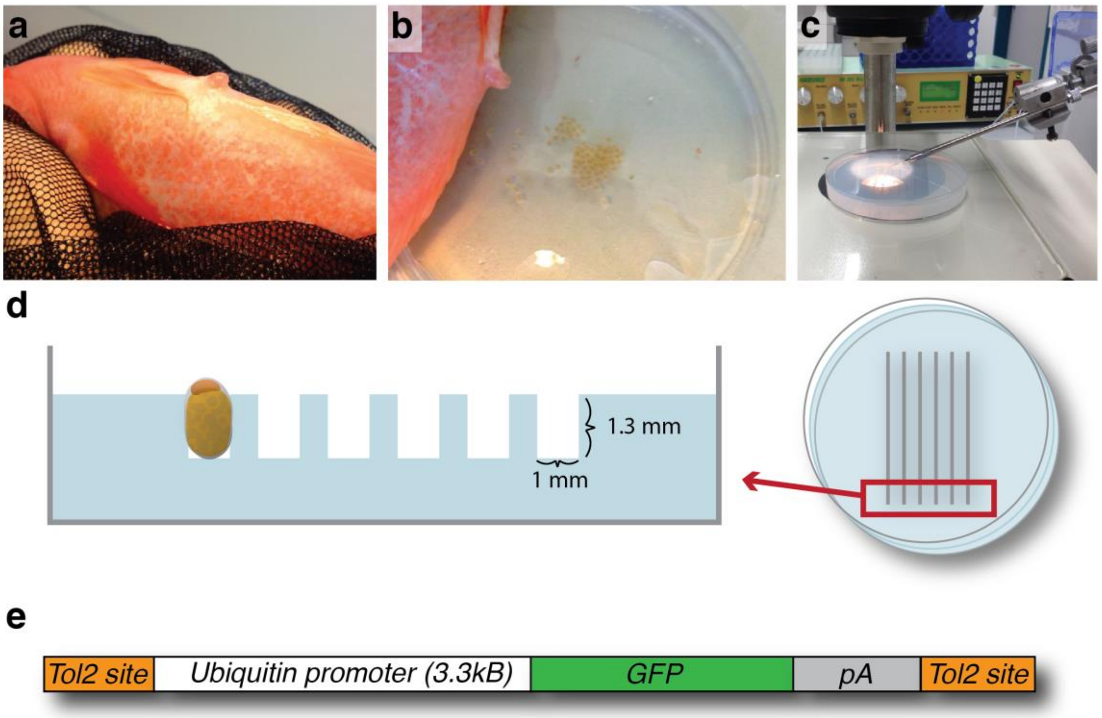

Figure II.3. Egg stripping and microinjection.
(a) Female Midas cichlid (here a golden morph of *Amphilophus xiloaensis*) with enlarged genital pore. (b) For *in vitro* fertilization, eggs are stripped from female fish into petri dishes. To fertilize the eggs, one or more males are stripped. Alternatively, eggs can be taken immediately after 'natural' fertilization). (c) The Microinjection setup that is used for injecting the Midas cichlid embryos. (d) Orientation of eggs in custom-molded agarose injection plates. The eggs must be oriented in an upright position to allow injection precisely into or just below the cell. (e) Scheme of the construct used for the generation of the *ubi::eGFP* line.

Maintenance of larvae, image acquisition and establishment of stable transgenic lines

After injection, eggs were transferred to new plates, with roughly 50 embryos per dish to avoid overcrowding, with fresh autoclaved water from the aquarium facility, and kept in a 28°C incubator (HIR10M Grant, Boekel) without agitation or aeration. Embryos and larvae were previously tested in conditions with and without agitation or aeration, and these two procedures were found to have no effect on survival (Kratochwil et al. 2015). Every 24 hours, surviving embryos were transferred to a new petri dish with clean, autoclaved tank water. At seven days post-fertilization, larvae were selected to be raised to maturity. Here, only the larvae showing strong eGFP fluorescence were kept and raised.

To prepare the embryos and larvae for photography, fish were first anesthetized with 0.04% tricaine (MS-222). They were then positioned on a slide using 3% methycellulose. Color photographs were taken with a stereomicroscope (Leica MZ10 F with Leica DMC2900 Camera) using the Leica Application Suite software 4.5.0. To improve the depth of field, we used the "Multifocus Montage" module/plugin of the Leica Application Suite software as previously described (Kratochwil et al. 2015). Fluorescent images were taken using the same microscope and software, with a Leica Camera (DFC3000G) and a GFP filter.

After screening for fluorescence, F0 larvae displaying widespread expression of the *ubiquitin-eGFP* transgene were raised to maturity under standard aquarium conditions. After reaching sexual maturity, Passive Integrated Transponder (PIT) tags were implanted inter-muscularly into the dorsal side of the body. Tagged males were then stripped to fertilize wild-type eggs in vitro. The fertilized eggs, referred to as the F1 generation, were screened for survival and fluorescence as described above. Of the five breeding pairs analyzed, two produced clutches with fluorescent offspring.

Sectioning and Microscopy

Larval and juvenile fish were sectioned and photographed under a fluorescence microscope. Larvae and juveniles were anaesthetized in tricaine methanosulfonate (MS-222) and fixed for two hours in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C. After fixation, the specimens were rinsed with PBS and transferred into 30% sucrose in PBS at 4°C until the specimens sank. The samples were then embedded at 37°C in pre-heated 11.5% gelatin / 30% sucrose in PBS for 30 minutes and allowed to harden at room temperature. Gel blocks were trimmed to leave ~5mm gel on each side of the sample, then slowly lowered into 2-Methylbutane chilled by dry ice until the block froze through, and kept at -80°C. Sections were cut at 20 µm using a cryostat microtome (HM 500 OM, Microm) at -20°C and mounted on Superfrost™ Plus Microscope Slides (Menzel-Gläser) at room temperature. The slides were air-dried at room temperature for 30 minutes then rinsed three times with PBS for ten-minute intervals. The sections were counterstained with 2 µg/ml 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Sigma) in PBS in dark conditions at room temperature for 20 minutes and rinsed three times with PBS for ten-minute periods. Slides were mounted in Mowiol mounting medium.

Results

Microinjection and screening process

One of the most common techniques to manipulate the genome of teleosts is through transgenesis, the integration of foreign DNA-constructs into the genome (Kratochwil & Meyer 2015). To generate stable transgenic lines, recombinant DNA has to be integrated into the germline (germline transgenesis). In teleosts, transgenesis of somatic and germ cells can be obtained most effectively by the microinjection of recombinant DNA into one-cell stage embryos. The integration can be significantly increased by co-injection of a *Tol2* insertion site-flanked DNA construct and transposase-encoding mRNA that is readily translated and triggers DNA insertion in a cut-and-paste manner. As a first step, we sought to optimize microinjection conditions in Midas cichlids using a construct expressing a fluorescent reporter. The construct selected for use in this study was comprised of the zebrafish *ubiquitin* promoter region (Mosimann et al. 2011) and the *eGFP* reporter gene flanked by *Tol2* insertion sites (Figure II.3e). In zebrafish, the *ubiquitin* promoter drives

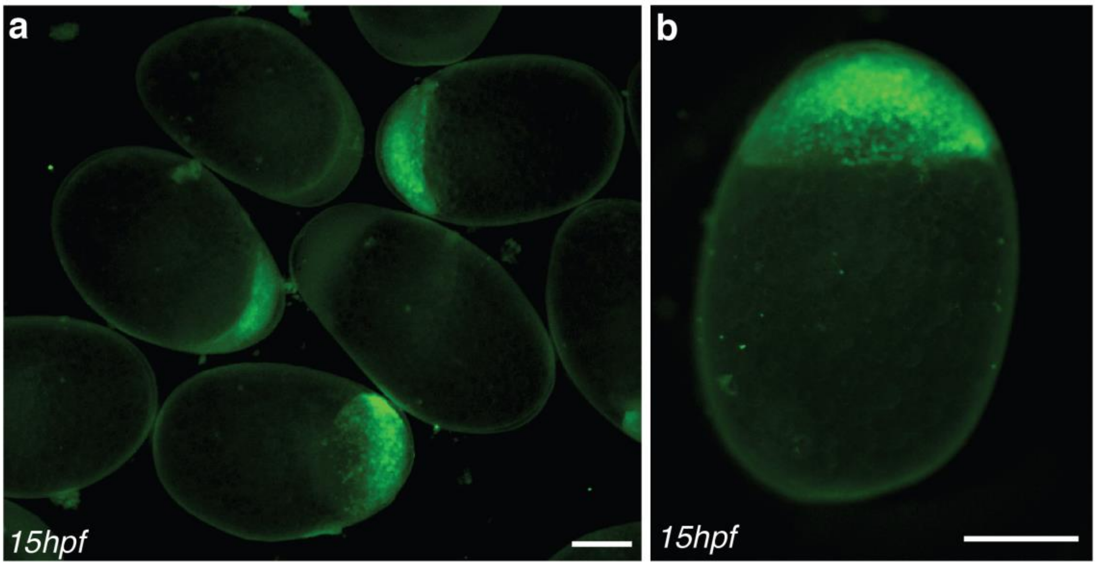

Figure II.4. Onset of GFP fluorescence.
(a-b) 15 hours after fertilization (dome stage) GFP fluorescence can be readily seen and used for selecting positive embryos. Scale bars = 500 µm.

strong and ubiquitous expression during all developmental stages and in all organs. Hence, it is ideal for assessing the applicability and efficacy of transgenesis.

In contrast to the small, round eggs of zebrafish, Midas cichlids eggs are almost two times larger and have an ovoid shape that complicates precise injections. In an effort to optimize injection conditions, we produced agarose trays allowing for the alignment and fixation of embryos in an upright position with the animal pole on the top (Figure 3d). Microinjection of a mixture of transposase mRNA, DNA, RNAse free water and Phenol red was performed directly into the cell or in the yolk slightly underneath the cell. Injections were carried out until the first cell division occurred, approx. 100 min after fertilization. Strong transient fluorescence can be readily seen at 15 h after fertilization (Figure II.4a, b). This stage corresponds to the dome stage in zebrafish at around 4 hours post fertilization (Kratochwil et al. 2015; Kimmel et al. 1995). At 7 days post fertilization (dpf), strong fluorescence can be observed in several cell types and tissues, particularly mesodermal and epidermal derivatives (Figure II.5 a-c).

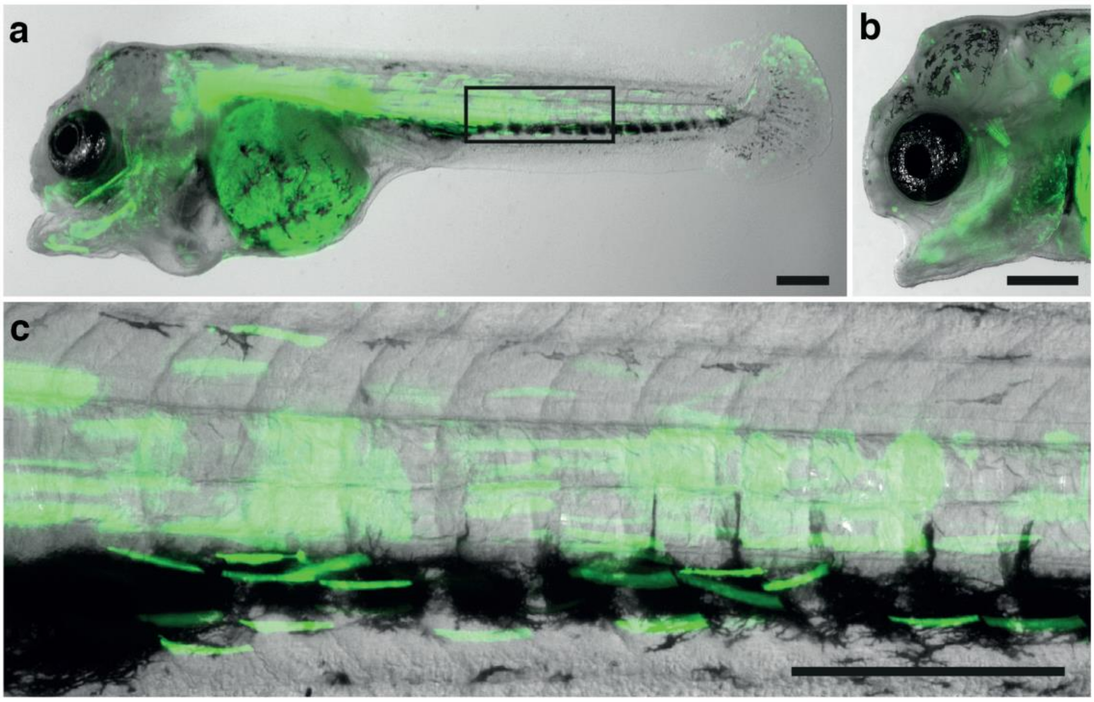

Figure II.5. Transient expression of the *ubi::eGFP* transgene.
(a-c) At seven days after fertilization, fluorescence can be seen in a mosaic pattern across all tissues including trunk musculature, head bones and muscles, fin folds, epidermis and heart. Images are composites of brightfield and GFP-filter photographs. Scale bars = 500 µm.

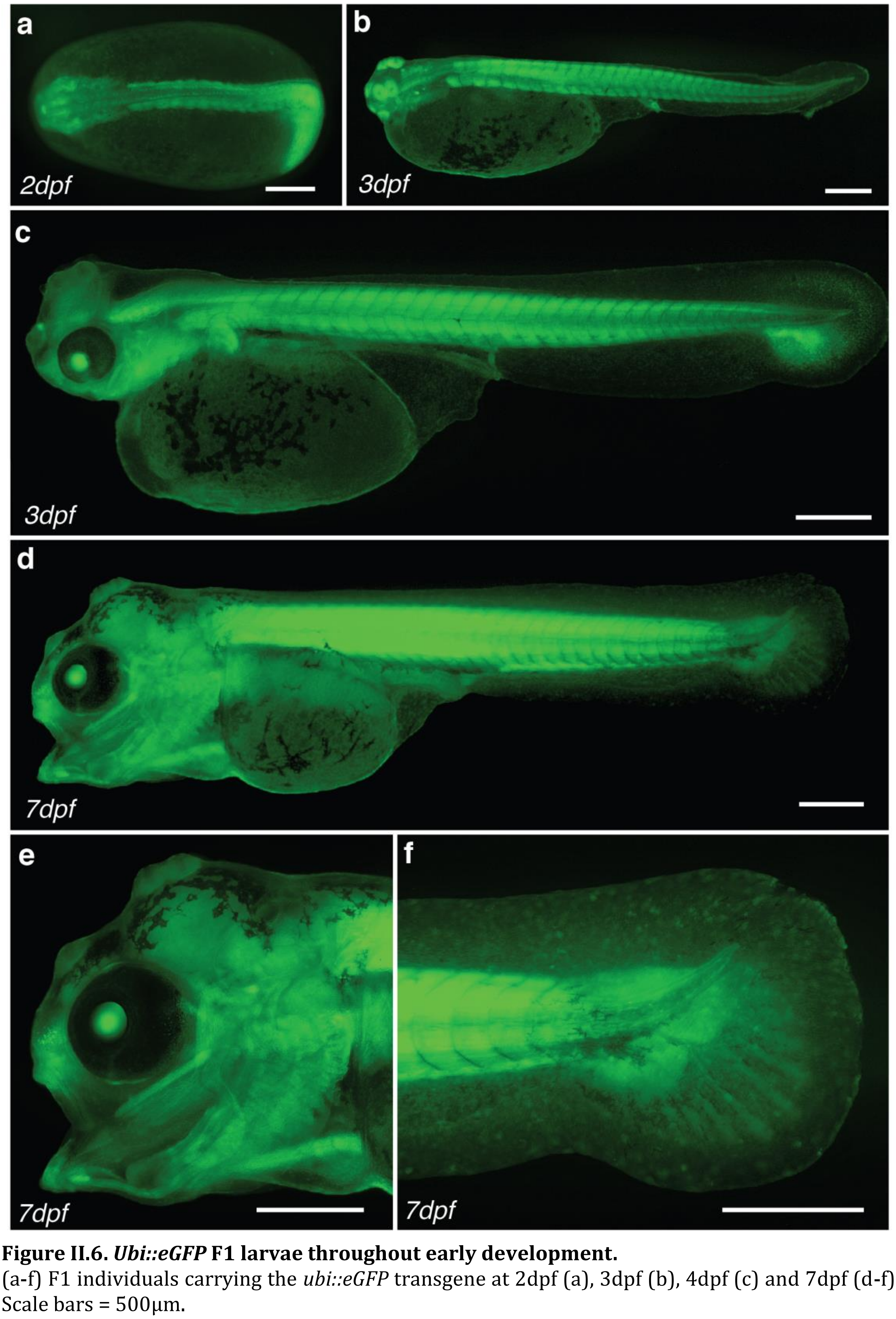

Figure II.6. *Ubi::eGFP* F1 larvae throughout early development.
(a-f) F1 individuals carrying the *ubi::eGFP* transgene at 2dpf (a), 3dpf (b), 4dpf (c) and 7dpf (d-f). Scale bars = 500μm.

Generation and analysis of a stable *ubi-eGFP* transgenic Midas line

Fluorescent individuals were selected and raised in aquaria. Eggs of five independent mating pairs were obtained after one year and screened for fluorescence. Out of five pairs, two produced clutches with embryos ubiquitously expressing eGFP. Around half of the F1 generation fathered by these males were positive for eGFP fluorescence, indicating that the parental males are hemizygotic carriers of the transgenic allele. We documented eGFP fluorescence during the first seven days of development (Figure II.6). The eGFP expression pattern was ubiquitous with particular strong expression in somites (Figure II.6a, b). The expression pattern resembled the transiently expressing embryos. Next, we sectioned 7dpf embryos to show the distribution of eGFP. Notably, coronal sections revealed that the eGFP signal is ubiquitous but not homogenous, with some tissues showing stronger signal then others. Also here, trunk and head muscles show a strong eGFP signal both in whole embryos (Figure II.6 c-f) and in sections (Figure II.7). In adult fish, a strong eGFP signal can be detected in all analyzed organs including brain, eye, liver, heart and fin tissue (Figure II.8). Overall, eGFP fluorescence was strong across all developmental stages and analyzed tissues.

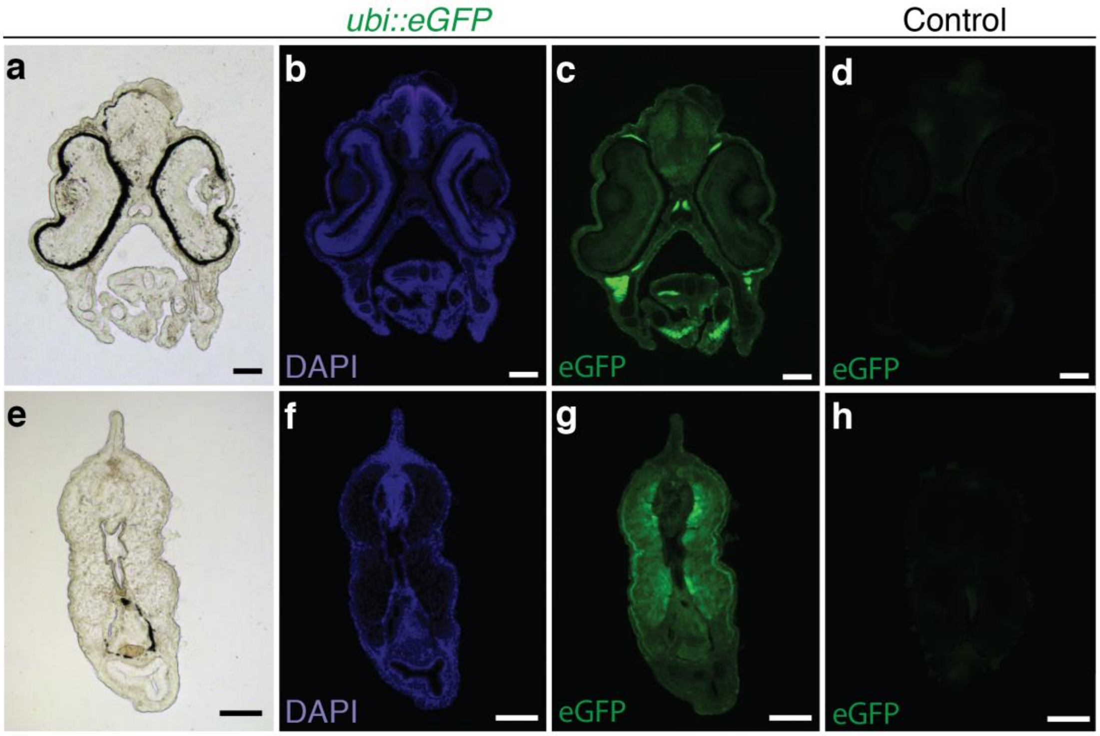

Figure II.7. Coronal sections of ubi::eGFP and wild type larvae at 7dpf.
(a-h) All larvae were stained with DAPI (b, f) and photographed under the same conditions. While F1 ubi::eGFP larvae (a, e) show bright fluorescence under GFP filtered light (c, g), wild types show minimal autofluorescence (d, h). Scale bars = 100µm.

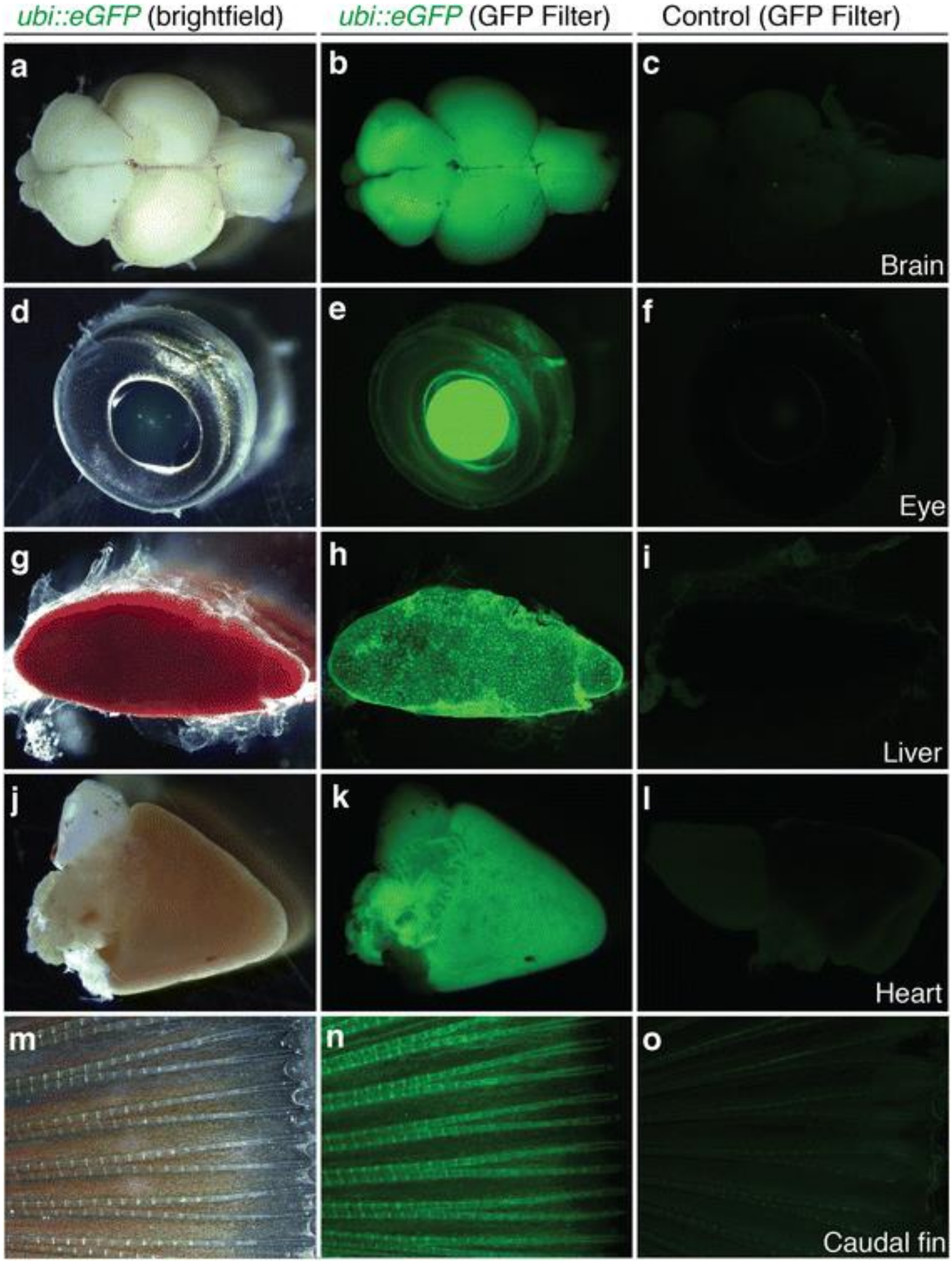

Figure II.8 *ubi::eGFP* transgene expression in F1 organs.
(a-o) F1 individual shows bright fluorescence throughout the body including brain (a, b), eye (d, e), liver (g, h), heart (j, k) and fins (m, n) when viewed under fluorescent light with a GFP filter (b, e, h, k, n). Organs of non-transgenic fish show minimal levels of autofluorescence in every organ examined (c, f, i, l, o)

Transient expression patterns of two additional transgenic constructs: *ubiquitin::tdTomato* and *mitfa::eGFP*

To demonstrate that the transgenesis approach is widely applicable in Midas cichlids, we generated two additional constructs: *ubiquitin::tdTomato*, which uses a different (red fluorescent) reporter, and *mitfa::eGFP*, that labels pigment cells under the control of a 1.1 kb promoter element of the *microphthalmia-associated transcription factor* (*mitfa*). For *ubiquitin::tdTomato* (Fig. II.9a), strong transient fluorescence is displayed in the embryos, with an expression pattern resembling that of the *ubiquitin::eGFP* construct (Fig. II.9b-c). To test a more cell-specific promoter, we used the promoter sequence 1.1kB upstream of the *A. citrinellus mitfa* coding sequence (Fig. II.10a) to create *mitfa::eGFP* (Fig. II.10b). A similar construct using the proximal promoter sequence of zebrafish *mitfa* has previously been shown to drive melanoblast-specific expression in zebrafish embryos (Curran et al. 2009). Indeed, GFP fluorescence could be detected in non-pigmented dendritic cells on the head and trunk (Fig. II.10c-d) suggesting that the construct is able to drive expression specifically in melanoblasts (i.e. melanophore precursors).

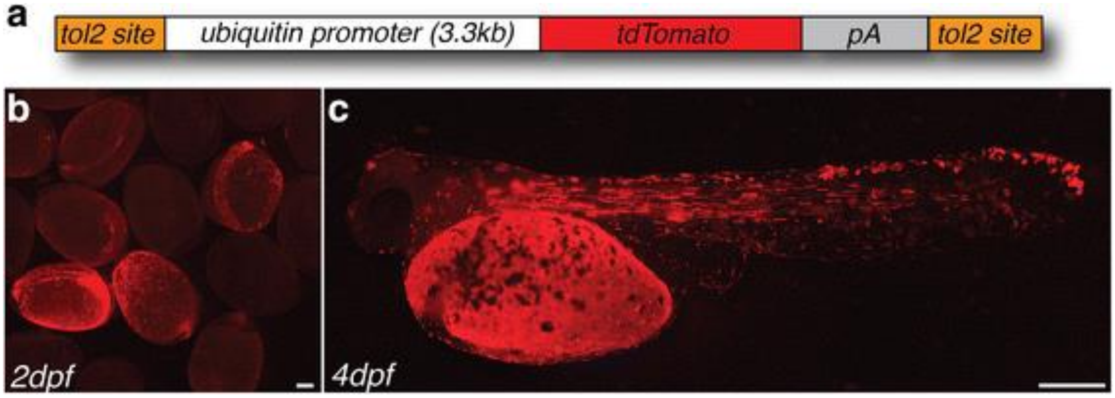

Figure II.9. Transient expression of *ubi::tdTomato*.
(a-c) Similar to ubi::eGFP, embryos injected with *ubi::tdTomato* (a) show bright fluorescence in a mosaic pattern across all tissues (2dpf, b; 4dpf, c). Scale bar = 500 μm.

Discussion

In this study, we adapt existing protocols to perform transgenesis in the Midas cichlid (*Amphilophus citrinellus*). Using the *Tol2* transposon system, we produced the first transgenic Midas cichlid. As such, this work represents the first step towards testing genes and regulatory elements underlying adaptive traits in this adaptively-radiating species complex. Several important life history traits make transgenesis in this species group particularly feasible and convenient. First, unlike many of the African cichlid species, Midas cichlids are substrate-brooding fish. This facilitates the fertilization of eggs in vitro, granting more flexibility in planning experiments. Each clutch may contain over one thousand eggs, allowing for a large sample size and robust statistical analysis in any transgenic study on this species.

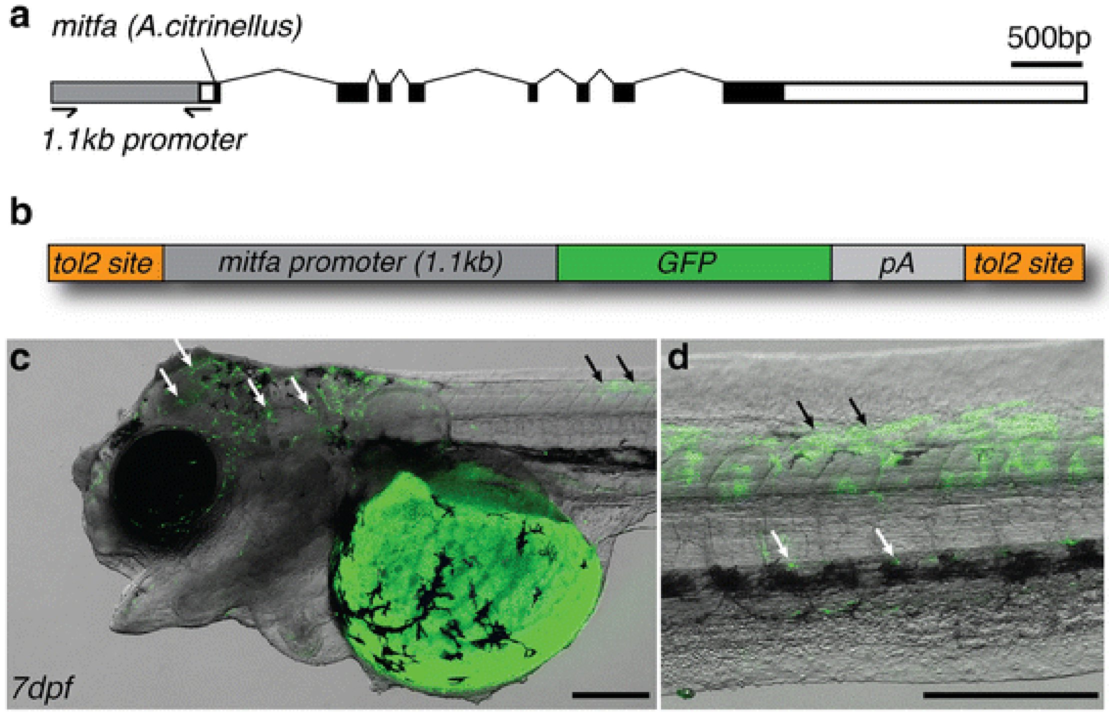

Figure II.10. Transient expression of *mitfa::eGFP*
(a-b) To test a cell-type specific promoter, we cloned a 1.1kB promoter fragment of *A. citrinellus mitfa* (a), a melanoblast marker, upstream of eGFP (b). c At 7dpf, GFP was expressed in dendritic cells on the head (white arrows) and trunk region (black arrows). d On the trunk, cells expressing GFP were mainly located dorsally (black arrows), similar to expression patterns seen in zebrafish [33]. A few cells could be found more ventrally, entangled with melanophores in the ventral melanophore stripe (white arrows). Scale bar = 500 µm.

Applications of transgenesis

Transgenesis enables the insertion of novel genetic information into the target genome. Therefore it is particularly well-suited for two applications: 1) Reporter assays for testing the activity and expression pattern of cis-regulatory elements such as promoters and enhancers and 2) overexpression experiments to analyze gene function. This methodology would therefore allow to test regulatory element candidates obtained from QTL studies or association studies (Chan et al. 2010; O'Brown et al. 2015), as well as through methods such as ChIP-seq and ATAC-seq that allow for genome-wide identification of active regulatory elements (Kratochwil & Meyer 2015; Kratochwil & Meyer 2015b). Gene function can also be assessed using overexpression, in which the expression of a gene of interest is increased by integrating another copy of the gene. This gene can be under the control of a ubiquitous promoter, or can be further specified in space and time using tissue-specific promoters. Overexpression can be effectively used to mimic regulatory changes that might ultimately explain phenotypic differences. On the other hand, phenotypes that result from gene loss or hypomorphic mutations affecting gene function can be rescued by the overexpression of the respective gene (Kratochwil & Meyer 2015). Cell-type specific constructs such as *mitfa::eGFP* will be especially valuable resources to improve the understanding of pigmentation phenotypes in cichlid fishes, a family known for its rich diversity of hues and color patterns.

Advantages and pitfalls of performing transgenesis in Midas cichlids

Several factors determine the suitability of a teleost species for transgenesis studies. Critical factors are 1) frequent breeding under lab conditions, 2) the possibility of raising larvae under lab conditions, 3) possibility to obtain one to two-cell stage embryos, 4) large clutch sizes, 5) regular breeding times, 6) a penetrable chorion that permits microinjection and 7) short generation times to obtain F1 individuals. For many ecological model systems, one or more of these factors hampers efficient transgenesis. In sticklebacks, an excellent system for analyzing gene function and regulatory divergence, transgenesis is particularly complicated by seasonal breeding behavior and small clutch sizes (O'Brown et al. 2015). Likewise, African cichlids are an excellent model system for understanding phenotypic diversification, but suffer from drawbacks regarding transgenesis. In the case of the African cichlids, the combination of small clutch sizes, mouth-brooding and the difficulty of timing fertilization make the application of transgenesis at large scales prohibitively challenging. Midas cichlids exhibit several traits that make transgenesis a suitable tool for this model system. A few days before fertilization, Midas cichlids form monogamous pairs (Barlow 1986). At the time the genital papillae swells, fertilization can be predicted to occur within the next 24 hours. Consequently, eggs can be collected directly after natural fertilization, or artificially fertilized as previously described (Kratochwil et al. 2015). The clutches are large (up to 1500 or more eggs) and develop relatively slowly. Larvae are robust and can be easily raised in tap water under lab conditions (Kratochwil et al. 2015). One of the major drawbacks of Midas cichlids is their long generation time, which can range from nine to twelve months.

While the aforementioned advantages ease transient analysis, long generation times make it time- and space consuming to obtain stable transgenes.

From Midas genotypes to Midas phenotypes

Midas cichlids are an excellent example of rapid phenotypic changes. This includes adaptive variation in body shapes (i.e. limnetic and benthic forms) (Barluenga et al. 2006; Franchini et al. 2014), hypertrophied lips (Machado-Schiaffino et al. 2014), teeth and pharyngeal jaws (Barluenga et al. 2006), the gold/dark polymorphism of Midas cichlids (Henning et al. 2013), and visual sensitivity (Torres-Dowdall et al. 2017). However, to further understand which genetic elements contribute to phenotypic variation, it is essential to pinpoint and validate their functional relevance. Testing of regulatory elements using GFP transgenesis assays and overexpression of target genes (Kratochwil & Rijli 2014) via transgenesis are important tools that will bring researchers closer to understanding the relationship between genotype and phenotype.

Conclusion

Transgenesis is a key technology for understanding the genetic and molecular basis of adaptive traits. For the first time, we used *Tol2*-mediated transgenesis in the Midas cichlid, a model system for fast and repeated parallel evolution of adaptive phenotypes. This technological advancement opens up new possibilities for studying the genotypic and molecular basis of adaptive traits in Midas cichlids, and provides a workflow for other substrate brooding cichlids and teleosts. We anticipate that the use of transgenesis in Midas cichlid will contribute novel insights into the genetic underpinnings of early stages of diversification.

III. Chapter III

Investigating the transcriptomics of fin diversity in African cichlids

Maggie M. Sefton, Claudius F. Kratochwil, and Axel Meyer

Abstract

Cis-regulatory evolution is an important driving force of morphological diversification. Small-scale differences in morphology between closely-related species can be tested, to examine how these phenotypic changes might be driven by gene regulatory divergence. Here, a large-scale transcriptomic approach is applied including five species of East African cichlids to investigate patterns of regulatory evolution across their adaptive radiations. We focus on unpaired fins (anal, caudal, and dorsal fin) that underwent morphological diversification during cichlid evolution. Our analysis reveals general patterns of gene expression and fin- and species-associated differentially expressed genes. Furthermore, it reveals possible candidate genes for specific coloration patterns and fin morphologies. In summary, we present novel data giving insights on how cis-regulatory changes may contribute to diversification in African cichlids.

Introduction

The diversity of living organisms is one of the most fundamental and fascinating topics in biology. Across the tree of life, there exists an astounding array of diversity in form and function. One of the central goals of evolutionary biology is to reveal the processes and mechanisms underlying this variation in phenotypes and the origin of species. To investigate how gene regulatory changes can contribute to adaptive evolution on a microevolutionary scale, this study applies an RNA sequencing approach to three fin types from five species of African cichlid fish. Using the cichlids as a model for rapid phenotypic diversification, we aim to answer to what extent changes in coloration and fin morphology can be explained by differential gene regulation.

Untangling the relationship between genotype, phenotype and the environment is one of main goals of evolutionary biology. Since it was first recognized in the 1970s that regulatory change can have a profound impact on phenotype (King & Wilson 1975), many studies have been dedicated to uncovering the relative importance of protein coding mutations and mutations affecting gene expression (Hoekstra & Coyne 2007; Carroll 2008; Craig 2009). With recent advances in sequencing technology, an increasing number of whole-genome sequences have been assembled and published – with each new genome, our understanding of the connection between genotype and phenotype becomes increasingly clear, and it is now accepted that regulatory evolution is an important mechanism in the evolution of complexity and diversity (Levine & Tjian 2003). Those who advocate for a primary role of cis-regulatory change in microevolutionary processes argue for low rates of protein coding mutations in closely-related yet phenotypically divergent species, in conjunction with the reduced risk of mutations in regulatory regions being deleterious, as factors that make these types of changes contribute more to evolution than structural mutations (Carroll 2008). These factors allow evolution to work like a "tinkerer," generating morphological diversity through small changes in regulatory regions (Jacob 1977; Kratochwil & Meyer 2015). By comparing gene regulation in closely-related yet phenotypically divergent species groups, such as cichlid fishes, it may be possible to further our understanding of how tinkering with gene expression can result in a wide array of phenotypes.

Cichlid fishes are among the most diverse vertebrate lineages on the planet, with roughly 3000 species described worldwide (Kocher 2004). African cichlids in particular are known for their extreme diversity and rapid rate of speciation. The East African Rift Lakes are estimated to have formed recently, ranging from ten million years ago in Lake Tanganyika (Cohen et al. 1993) to Lake Victoria, which is thought to have been completely dry only 12,000 years ago (Johnson et al. 1996). In this short period of time, nearly 2000 species of cichlids have evolved in the African Lakes, resulting in their notoriety as a textbook case of adaptive radiation (Kocher 2004; Seehausen 2006; Turner et al. 2001). Within these radiations, an impressive amount of morphological, physiological and behavioral diversity can be found, often evolving in parallel in different lakes (Meyer et al. 1990; Rüber & Adams 2001; Salzburger & Meyer 2004). Due to their highly divergent phenotypes and close genetic relatedness, they can be used as a “natural mutagenesis screen,” in which it is possible to examine the role of genes and regulatory elements in specific adaptations (Kratochwil & Meyer 2015; Henning & Meyer 2014). Therefore, they represent an interesting model for studies of phenotypic diversification and the origin of species on a microevolutionary scale.

Changes in gene regulation are hypothesized to be an especially important driver of diversification in cichlids. Recent technological advances have made it possible to obtain large amounts of genomic data from five cichlid species from East Africa, revealing valuable information on the genomic landscapes underlying cichlid diversity. Genome-wide analyses have found that East African cichlid radiations have increased rates of gene duplication, as well as expression changes due to transposable element insertions, compared to the ancestral Nile tilapia and other teleost species (Brawand et al. 2014). In addition to genome-wide expression patterns, previous studies have found evidence that gene expression changes and mutations in non-coding DNA may be associated with specific traits in cichlid fish. For example, a transposable element insertion into the cis-regulatory region of *fhl2b* has been suggested to be linked to the egg dummy phenotype found in the males of many haplochromine species (Santos et al. 2014). Therefore, investigations into the regulatory changes associated with divergent phenotypes have potential to provide further information into the link between genotype and phenotype in this lineage.

In the present study, samples were taken from the following five species of African cichlid fish: *Oreochromis niloticus*, *Astatotilapia burtoni*, *Neolamprologus brichardi*, *Metriaclima zebra*, and *Pundamilia nyererei* (Figure III.1a). These species were chosen because they represent a broad phylogenetic and phenotypic range of African cichlids, as well as the availability of high-quality genomic data (Brawand et al. 2014). The ancestral Nile tilapia, *O. niloticus*, is estimated to have diverged from the haplochromines of Lakes Victoria and Malawi roughly 18 million years ago (Won et al. 2006). *Neolamprologus brichardi*, the fairy cichlid, represents the older yet less species-rich lineages found in Lake Tanganyika (Nishida 1991; Salzburger et al. 2002). It is also distinguished by its pale coloration; therefore, differentially expressed genes in this study may be implicated in the evolution of its colorless phenotype. The three remaining species are representatives of the explosively-speciating haplochromine radiations. The riverine haplochromine species

Astatotilapia burtoni, has been used in a wide array of studies, especially including previous genomic research (Lang et al. 2006; Sanetra et al. 2009). Notably, it has been used as a model to investigate the mechanisms underlying novel traits such as egg dummies on the anal fin (Heule & Salzburger 2011; Theis et al. 2012). Representing the haplochromines from Lake Malawi is *Metriaclima zebra*, known for its distinct blue and black barred pattern. Finally, the colorful *Pundamilia nyererei* was chosen to represent the haplochromines from Lake Victoria, the most species-rich lake. By examining patterns of gene regulation in these species, we aim to uncover whether, as hypothesized, transcriptome evolution is accelerated in rapidly-evolving, phenotypically-diverse lineages such as the haplochromines.

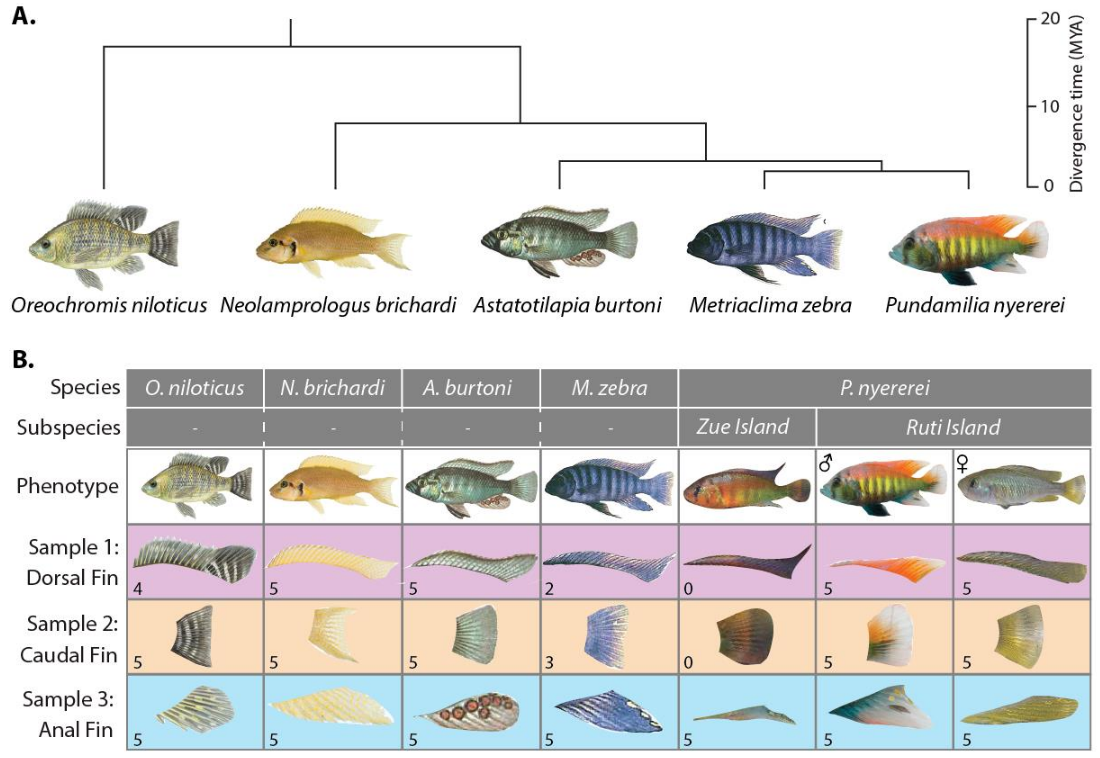

Figure III.1. Overview of study design.
A. Phylogenetic relationships and divergence times of species sampled in the present study; B. Sample size for each species and fin type included in the final analysis.

In addition to analyzing interspecies expression differences, we also characterize gene expression patterns within a single species, *Pundamilia nyererei*. Within this species, samples were taken from two phenotypically divergent populations from Ruti Island and Zue Island (Figure III.1b). In these populations, dominant males exhibit distinct coloration patterns – we hypothesize that differential gene regulation contributes to phenotypic differences within species. In addition, both male and female individuals were sampled from the Ruti Island population of *P. nyererei*. This allows us to investigate the how gene expression is involved in sexual color dimorphism – a trait which is found in haplochromine species, but absent in more basal cichlid lineages. One of the most conspicuous sex-associated traits is the egg spots on the anal fin. These egg spots play a central role in the mating behavior of mouthbrooding cichlid species and are therefore undoubtedly involved in sexual selection. Therefore, we aim to investigate the manner in which gene regulation differences contribute to sex-specific phenotypes.

Beyond examining the species differences in gene regulation among African cichlids, this study also characterizes expression patterns among different fin types. Samples were taken from three different types of unpaired fin from each species – the dorsal, caudal and anal fins. Sampling these fins allows us to look at differences in gene regulation along both an anterior-posterior axis and a dorso-ventral axis across the body. This will provide insights about the evolutionary and developmental basis of different body plans in fish, as well as possibly provide information about differential color patterning which is a characteristic of the African cichlid radiations. In the brightly colored and varied haplochromine species, each of these fin types may display a differently colored or patterned fin. Especially in species with egg spots, comparing gene expression in the anal fin compared to the dorsal may reveal candidate genes that contribute to this evolutionary novelty.

In this study, RNA-sequencing data is collected and analyzed to examine the role of regulatory evolution in African cichlids. Using principal component analysis, expression-based phylogenies, differential expression analysis and characterization of gene ontology terms, we aim to elucidate the transcriptomic mechanisms underlying changes in color and fin morphology in this fascinating group of fish. Using these approaches, general patterns of expression change are described, and several interesting genes for further study are identified.

Methods

Sampling and tissue collection

In total, 96 samples were initially used in this study to examine the transcriptomic basis of diversification in cichlids. Fin clips from unpaired fin types (anal, dorsal and caudal fins) were sampled from several clades of cichlid fish. Because one of the phenotypes we planned to examine in this study is coloration, all samples were taken from male fish unless otherwise specified. This is because, in sexually dimorphic cichlid species, the males tend to be the more colorful sex, while the females generally have more cryptic coloration. The following species were sampled in this experiment: the neotropical *Amphilophus citrinellus* (the Midas cichlid – which was eventually excluded from the study due to low sample quality), *Oreochromis niloticus* (Nile tilapia), *Astatotilapia burtoni*, *Neolamprologus brichardi*, *Metriaclima zebra*, and *Pundamilia nyererei*. Within *P. nyererei*, three subgroups are represented in the samples – phenotypically divergent subspecies from Zue Island and Ruti Island, as well as sexually-dimorphic males and females from Ruti Island. Species and populations used in this study were selected primarily based on availability of genomic resources, to facilitate downstream analysis, and to encompass a wide range of genetic diversity (Figure III.1).

All African fish used in the study were purchased from Cichlidenstadl (cichliden-stadl.de) a German cichlid specialist shop. The fish were sexed, then isolated for 14 days in small tanks to recover from moving-related stress. In species with a vibrantly-colored dominant male, this acclimation period allowed the fish to express their full dominant coloration. Midas cichlids (*A. citrinellus*) were selected from our pre-existing stocks to obtain anal fin samples. To acquire tissue samples, fish were anesthetized on ice, then weighed and photographed. Fins were removed and immediately placed in 3 ml of RNAlater (Sigma-Aldrich), then stored at 4°C until extraction.

Additional samples

In addition to the samples collected in the lab, our dataset was supplemented with previously-existing data from NCBI's Sequence Read Archive. Raw reads from previous RNA-seq studies in African cichlids were downloaded and analyzed using similar protocols as our own dataset. The additional samples included in our analysis included skin tissue from *A. burtoni*, *N. brichardi*, *M. zebra*, *P. nyererei* and *O. niloticus* (Brawand et al., 2014), and anal fin samples from both male and female *A. burtoni* (Santos et al., 2014). This data was used for additional quality control and validation, to ensure that the data collected in this study were comparable to previously-sampled specimens of the same tissues and species.

RNA Extraction and Sequencing

Tissue samples were first blotted on filter paper to remove excess RNAlater, then transferred to homogenization tubes containing ceramic beads. Samples were homogenized on a FastPrep-24 homogenizer, then frozen until RNA extraction. To extract total RNA from the homogenized samples, the Qiagen RNeasy Mini Kit was used with a modified protocol, and DNA contamination was removed using a Qiagen DNAse treatment. The concentration of extracted RNA was then quantified on a Qubit fluorometer. Barcoded, paired-end libraries were prepared using the Illumina TruSeq Library Preparation Kit v2 according to the manufacturer's instructions. Prepared libraries were checked for quality on an Agilent 2100 Bioanalyzer. The samples were subsequently sequenced on four lanes of Illumina HiSeq 4000 by BGI.

Quality control

We successfully acquired sequences from all 96 samples. In total, we collected 2,367,541,746 raw paired-end reads, with an average of 24,661,893 reads per sample. Before proceeding with any further analysis, reads were inspected and quality was confirmed using FastQC (Andrews 2010). Next, Trimmomatic was used to remove remaining adapters, trim reads and filter for quality (Bolger et al. 2014). These two preliminary steps are essential to prevent false mapping rates and ensure that downstream analyses are unbiased. Samples with insufficient sequencing depth were excluded from further analysis. To accurately quantify expression levels of lowly-expressed genes, it is important to have a sufficient amount of mapped reads in each sample. Two samples were excluded from the analysis due to low (i.e. fewer than 500,000) read counts. All five Midas cichlid anal fin samples were excluded from the final analysis due to low mapping rate to the draft Midas genome. After all quality control and filtration steps, 89 high-quality samples remained for further analysis.

Read mapping and quantification

For tests of differential expression, trimmed and quality filtered reads from all species were mapped to the *Oreochromis niloticus* reference genome using Tophat2 (Kim et al. 2013). As an important aquaculture species, *O. niloticus* has a high quality and well-annotated genome, making it ideal as a reference for comparative studies. Although genomes are currently available for all the species included in this study, mapping to the *O. niloticus* genome allowed us to obtain comparable data for each species. For each species, several alignment parameters (mismatches, gap length and edit distance) were first tested to maximize the percentage of concordant mapped reads while remaining as conservative as possible. After determining optimal mapping parameters, quality-controlled reads were mapped to the *O. niloticus* genome. Then, accepted hits from the Tophat2 output were processed using HTSeq-count (Anders et al. 2015), to determine the number of reads mapped to each gene in the *O. niloticus* genome. The raw abundance counts were assembled into a count matrix using bash scripts, and proceeded to be analyzed using the DESeq2 package in R (Love et al. 2014) and custom scripts in R and Python.

Analysis of global expression patterns

To ensure sample quality and to visualize the overall expression patterns, Principal Component Analysis (PCA) was performed using the plotpca function in DESeq2. The raw read counts were first transformed using a Variance Stabilizing Transformation (vst) with the DESeq2 built-in vst function. These transformed data were then plotted in a PCA.

As a preliminary quality control step, PCAs were first performed including the additional cichlid samples from the SRA. Skin and brain samples from all species, obtained in previous studies, as well as previously-published anal fin samples from *A. burtoni*, were added to our dataset and plotted as a PCA. This allowed us to determine whether our samples are consistent with data collected in previous studies. These samples clustered by species together with samples from the present study, therefore validating our methods of read mapping and quantification.

Further analysis was performed only on samples taken during the present study. First, all samples were considered in a PCA plot to see whether fin or species level changes had a greater impact on transcriptome divergence. After determining patterns of expression divergence for the complete dataset, various subsets of samples were visualized using PCA, to determine how overall expression patterns differed according to species, lineage or tissue type. To examine how cichlid transcriptomes diverge among different species, each fin from each species studied was plotted separately. Next, in order to visualize how different fin transcriptomes differ within species, species were examined individually and each fin type was plotted in a species-specific PCA.

Genome-wide trends in gene expression were further examined in a phylogenetic tree. First, the Pearson correlation coefficients of the normalized read counts of all samples were calculated and assembled into a distance matrix using R scripts. The phylogenetic tree was generated using functions from the ape package in R, and branch lengths were scaled with a square root function to improve visualization.

Differential expression analysis

Genes differentially expressed in specific fins or lineages were calculated using the normalized count output from DESeq2. A series of t-tests were used to perform all meaningful pairwise comparisons. Then, for each group examined (i.e. each tissue for each species), genes were selected which were significantly differentially expressed in all comparisons involving that group, and no other pairwise comparisons. Significant genes (p-value ≤ 0.05) were then filtered by log2 fold change (with an absolute value greater than or equal to one). These group-exclusive DE genes were then visualized with heatmaps made using the pheatmap package in R (Kolde 2012).

GO term analysis

Functional analysis was conducted using R scripts and tools from Blast2GO (Conesa et al. 2005) and Ensembl (Aken et al. 2016). Gene Ontology terms were used to infer the function of genes that were differentially expressed between species and tissue types (Ashburner et al. 2000; Gene Ontology Consortium 2015). Lists of differentially-expressed genes were produced for each pairwise comparison using DESeq2 and custom R scripts as described above. DE genes were sorted into different classes for GO term analysis – DE specifically in each species, DE specifically in each fin, and DE in all fins or species. The Ensembl IDs of significantly differentially expressed genes were imported into Ensembl’s Biomart online tool to obtain their associated GO ID terms. Text files containing the Ensembl Gene ID and associated GO terms were exported from Biomart, then subsequently loaded into Blast2GO, for further analysis. Functions in Blast2GO were used to complete the annotations and find associated enzyme codes. This data was then used to visualize the distribution of gene ontology terms for biological processes, molecular function and cellular component. This allows us to determine the functions of genes that are differentially expressed in each group and make predictions and inferences about how regulatory changes in specific pathways may effect phenotype and species divergence.

To determine whether specific pathways or processes were enriched in the differentially expressed gene sets, the *Oreochromis niloticus* genome and its annotations was used as a control. For all of the genes expressed in our dataset (i.e. with a row sum greater than or equal to one in our table of normalized gene counts), gene IDs and their associated GO terms were downloaded from Ensembl's Biomart and imported into Blast2GO. The annotations were then augmented using the Annex function, adding a second gene ontology layer to ensure that the annotations were as complete as possible (Myhre et al. 2006). Next, GO-EnzymeCode mapping was performed to match gene ontology terms to enzyme codes. After adding the mapped enzyme codes to the annotation, KEGG Pathway maps were downloaded and loaded into Blast2GO. Finally, combined graphs of the GO terms for biological process, molecular function and cellular component were produced. The relative proportion of GO categories was quantified to represent a cichlid fin under neutral conditions. This set of genes and their functional annotations was used as a reference for comparative studies of different groups of differentially expressed genes. This makes it possible to determine whether specific pathways are over- or underrepresented in the genes that exhibit regulatory divergence between individual lineages, species or tissue types.

After obtaining the complete set of expressed genes and their functional annotations, one-sided Fisher's Exact tests were used to test for gene functions that were overrepresented in the differentially expressed genes. For each species, a list of DE genes was generated and exported as a text file. This was then used as input for Blast2GO's enrichment analysis. The same analysis was performed in all tissues and all species.

Results

Global expression patterns and Principal Component Analysis

To understand how gene expression levels varied among samples, global expression patterns were visualized using Principal Component Analysis (PCA). Using the plotPCA function from the DESeq2 R package, a series of PCA plots were produced to reveal how genome-wide expression clusters among different species and fin types. We found that clustering occurs both by species and by tissue type (Figure III.2). In all species, the dorsal and anal fins cluster together in principal component space, while the caudal fin is more distant and displays more variation. In addition, within a single fin, samples cluster according to evolutionary distance (Figure III.3), with rapidly radiating haplochromine species forming a distinct group, while the more distantly-related *Oreochromis niloticus* clusters separately from the other species.

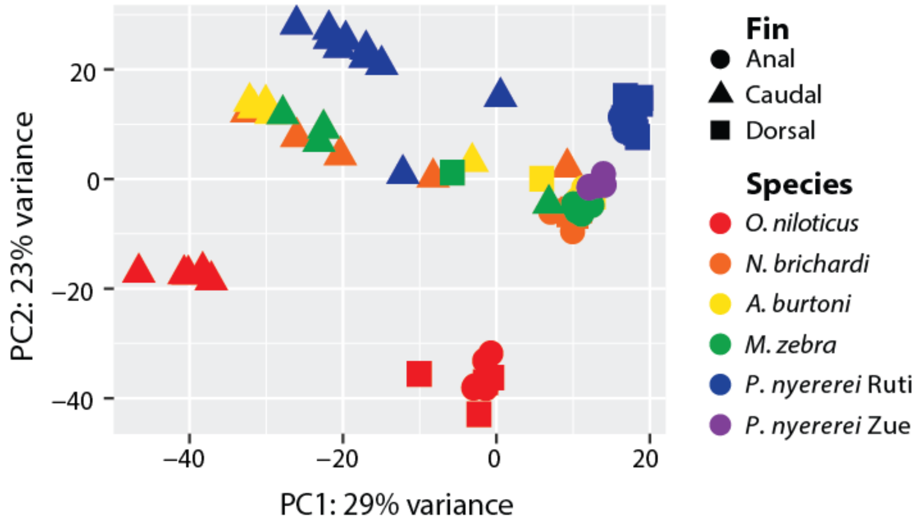

Figure III.2. PCA of gene expression in all samples.

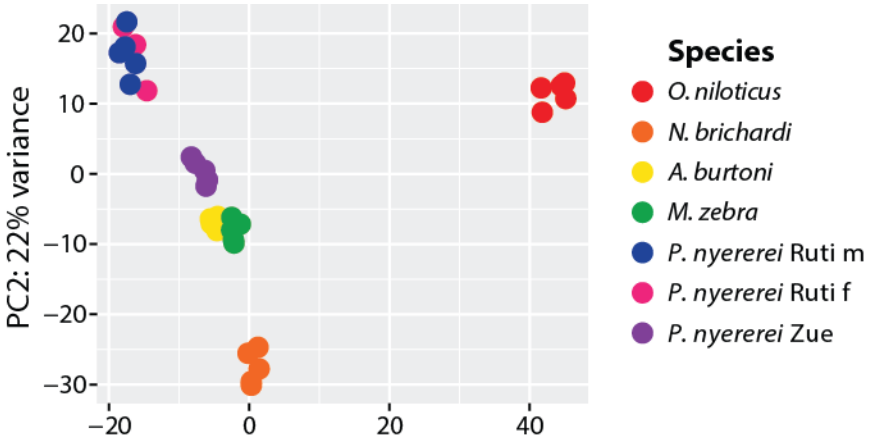

Figure III.3. PCA of gene expression in the anal fins of all sampled groups.

Next, to understand how gene regulation can differ within species, expression patterns within *Pundamilia nyererei* were examined. Males and females from the Ruti Island population, despite their clear differences in color pattern, were indistinguishable from each other in a Principal Component Analysis. In contrast, the anal fins of males from Ruti Island clustered separately from those from Zue Island.

Expression differences between species

In addition to examining transcriptome-wide regulatory differences and fin-specific DE genes, we asked whether specific genes can be associated with a particular species or phenotype. To identify genes that show species-specific regulatory divergence, differential expression analysis was performed. For each species, genes that showed differential expression patterns in all fins compared to the other species were defined as "species-specific" DE genes, which are visualized as heatmaps in Figures III.4-7. The number of species-specific DE genes was variable across species. Several genes of interest, which may be associated with species-specific color phenotypes, were identified. The following species-specific DE genes have previously been implicated in coloration: 1. *atp6v1e1a* in *O. niloticus*; 2. *igfbp7* in *N. brichardi*; 3. *mcoln3a* in *A. burtoni*; and 4. *irf4b* in *P. nyererei*.

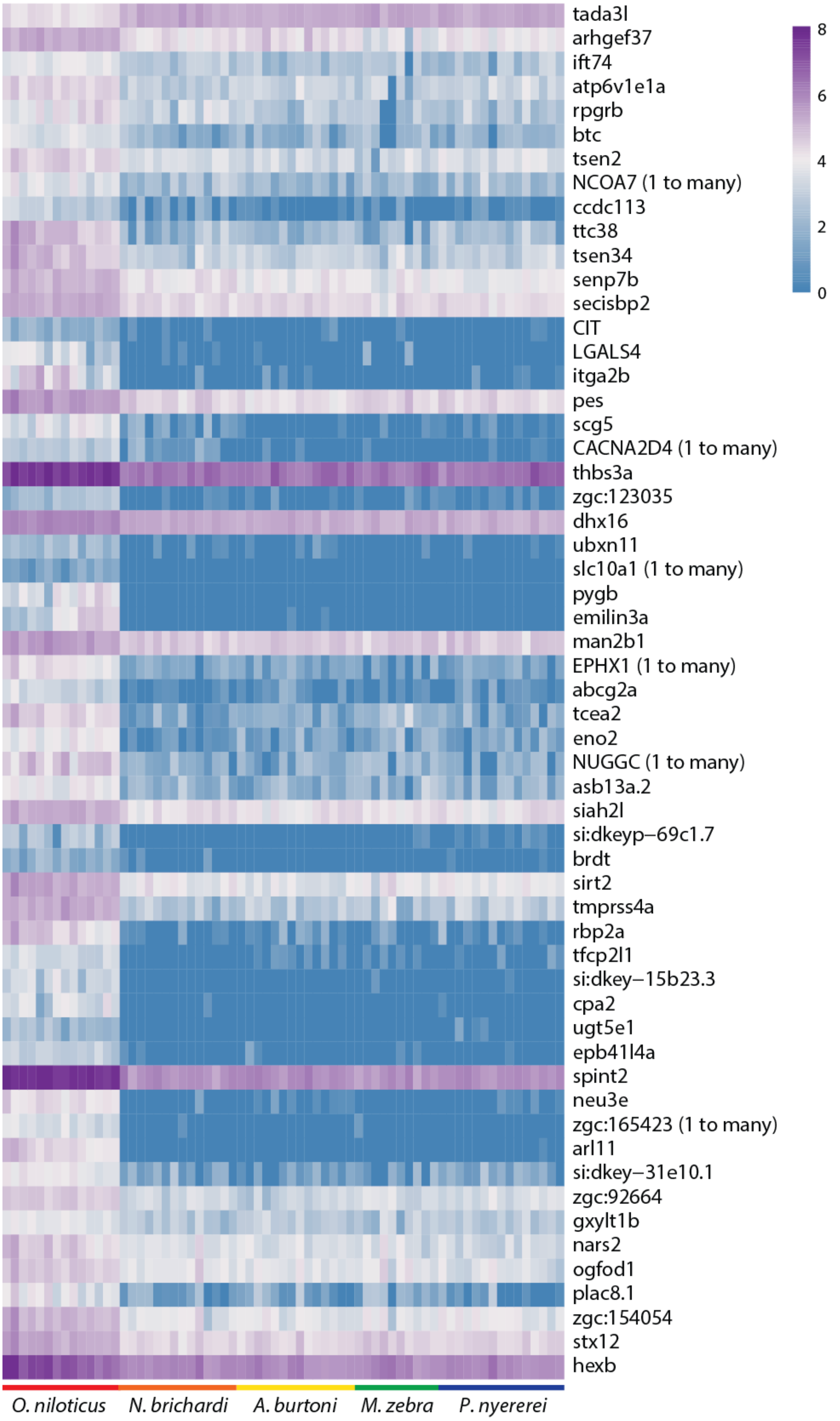

Figure III.4. Heatmap of genes differentially expressed in *O. niloticus*.
Genes displaying significant ($p < 0.05$) up- or down-regulation (|log2 fold change| > 1) relative to all other species in all fin types.

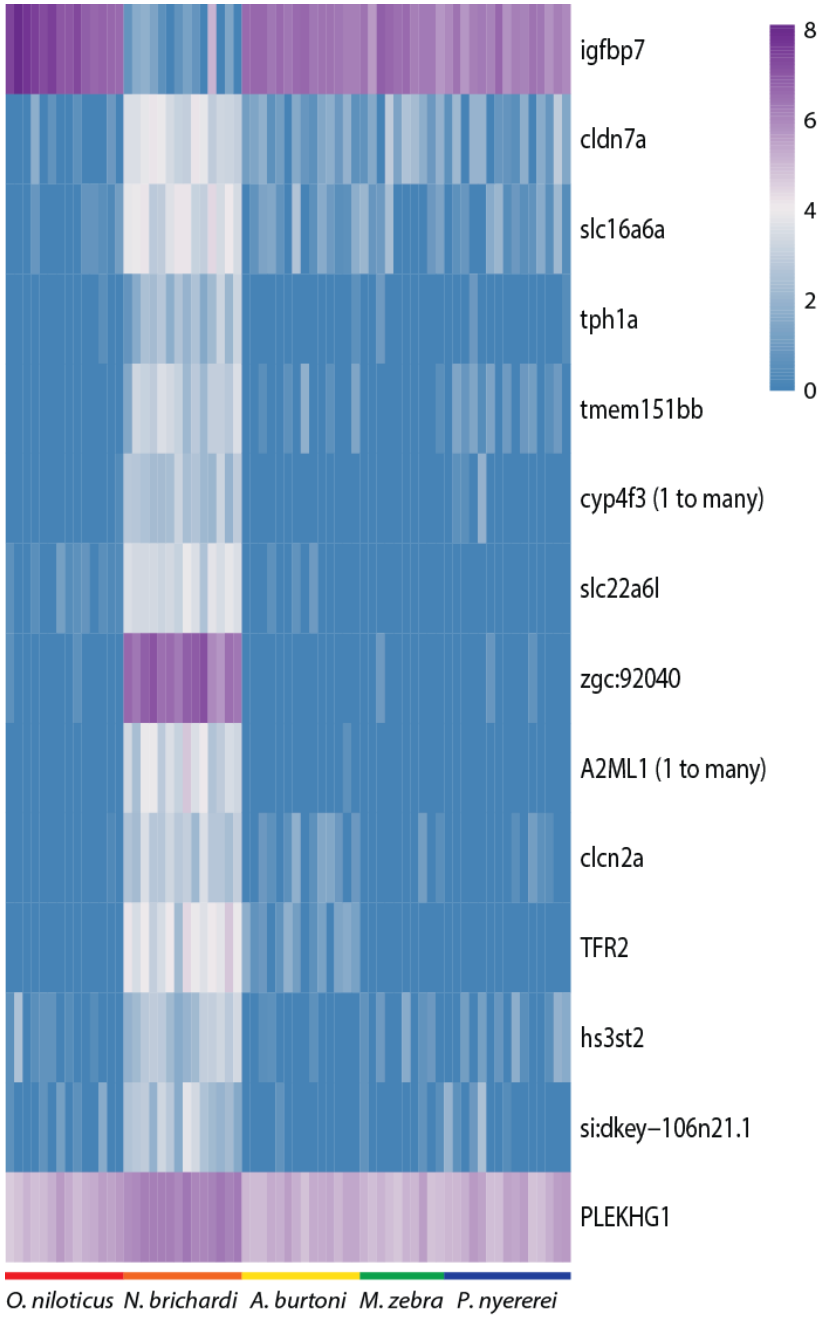

Figure III.5. Heatmap of genes differentially expressed in *N. brichardi*.

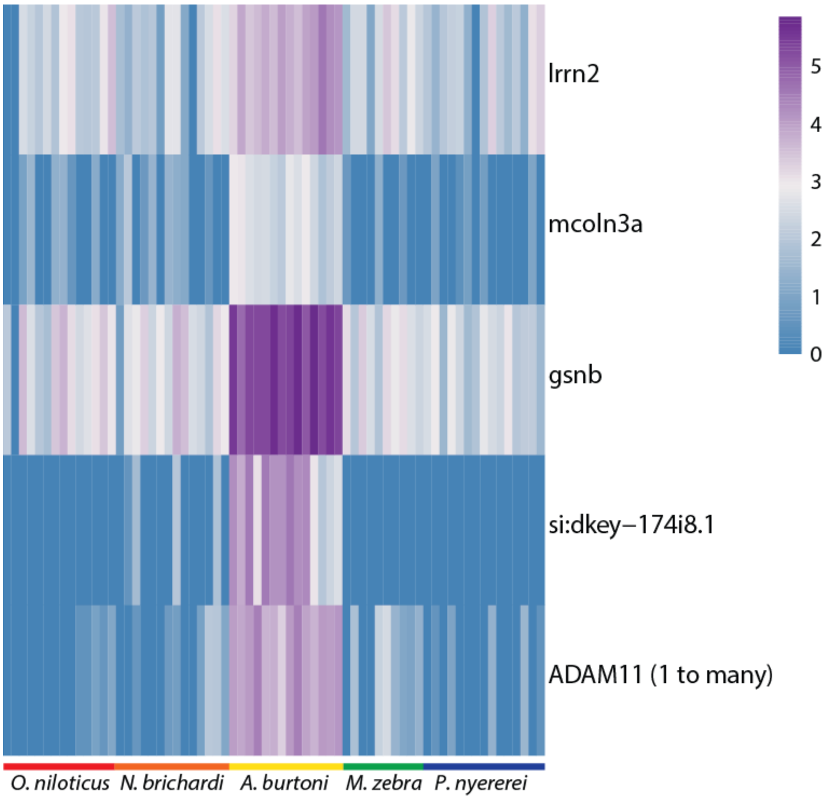

Figure III.6. Heatmap of genes differentially expressed in *A. burtoni.*

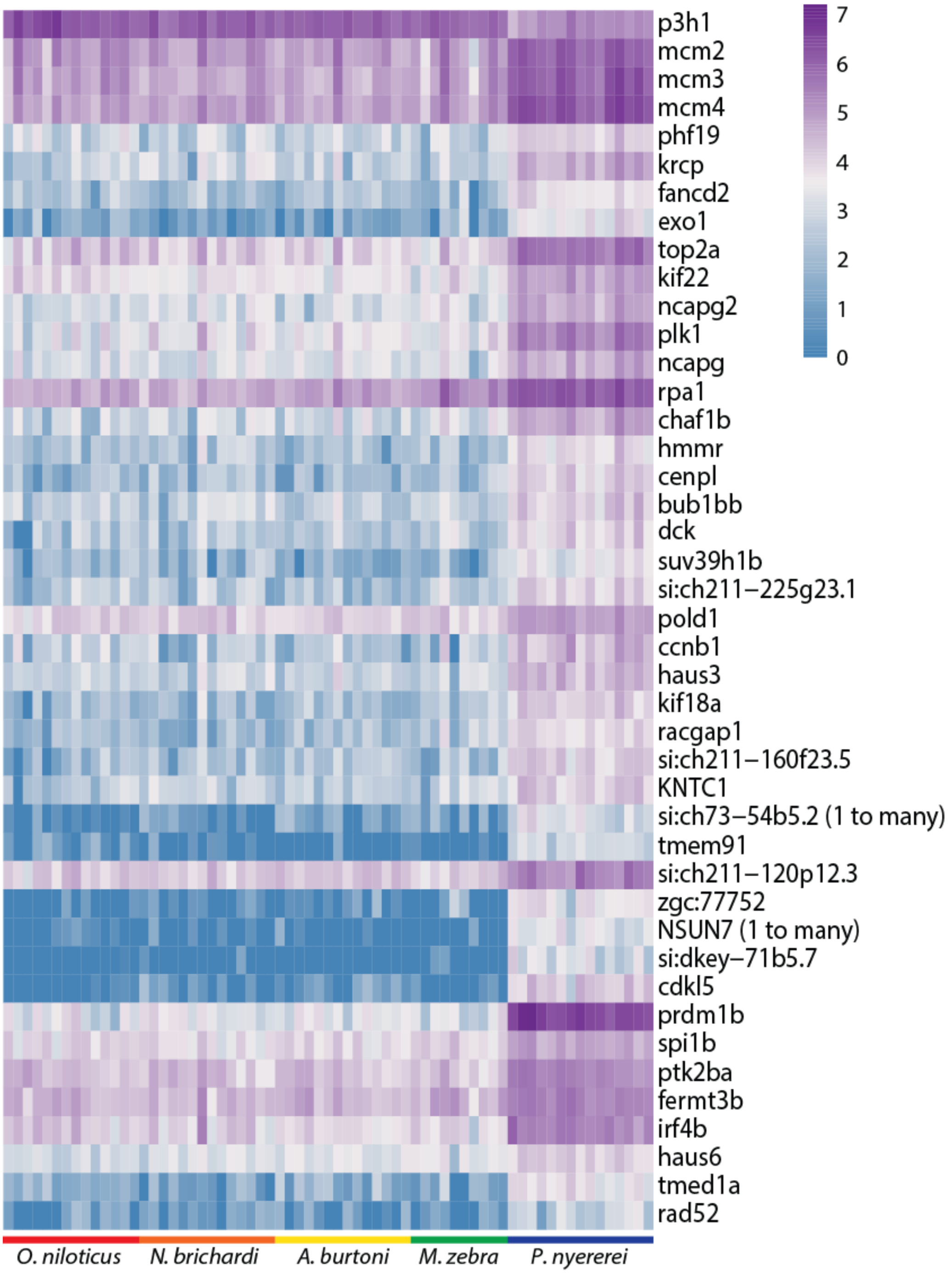

Figure III.7. Heatmap of genes differentially expressed in *P. nyererei*.

Expression differences between fins

To discover whether there are gene expression differences associated with divergent fin color and morphology, differential expression analysis was conducted between the anal, dorsal, and caudal fin in each species. Genes that were differentially expressed in all pairwise comparisons (i.e. dorsal/anal, dorsal/caudal and anal/caudal) in all species were defined as "fin-specific" DE genes (Figure III.8). Although a majority of fin-specific genes were found in the caudal fin, genes potentially associated with fin morphology or color were identified only in the anal fin (*asip1*) and the dorsal fin (*zic1/zic4*).

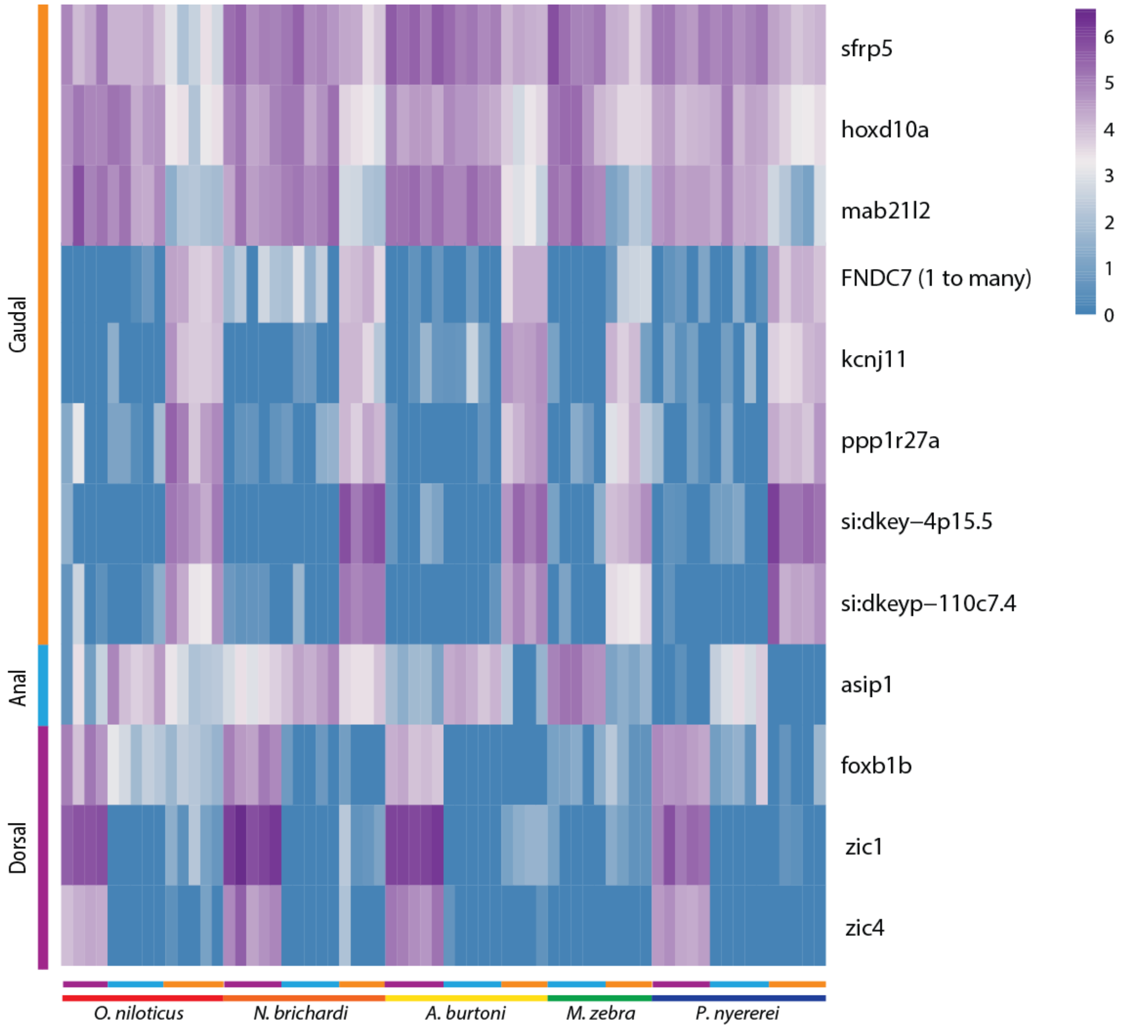

Figure III.8. Heatmap of fin-specific differentially expressed genes.

Expression differences within *Pundamilia nyererei*

The next question addressed in this study was whether particular genes can be associated with population- and sex-specific phenotypic differences within a species. To answer this question, differential expression analysis was carried out within different sexes and populations of *P. nyererei*. No differentially expressed genes were found to be present between males and females of the Ruti Island population in any fin. Between the anal fins of males from the Ruti and Zue Island populations, several differentially expressed genes were identified, but these did not include any previously-described color genes (Figure III.9).

Figure III.9. Heatmap of genes differentially expressed between 2 populations of *P. nyererei*.

Expression differences between haplochromines and non-haplochromines

To answer whether there are specific genes associated with the rapidly-diversifying haplochromine radiations, differential expression analysis was performed between haplochromine and non-haplochromine species. The analysis revealed 25 differentially expressed genes between the two groups, including the potential color gene, *slc2a11a* (Figure III.10).

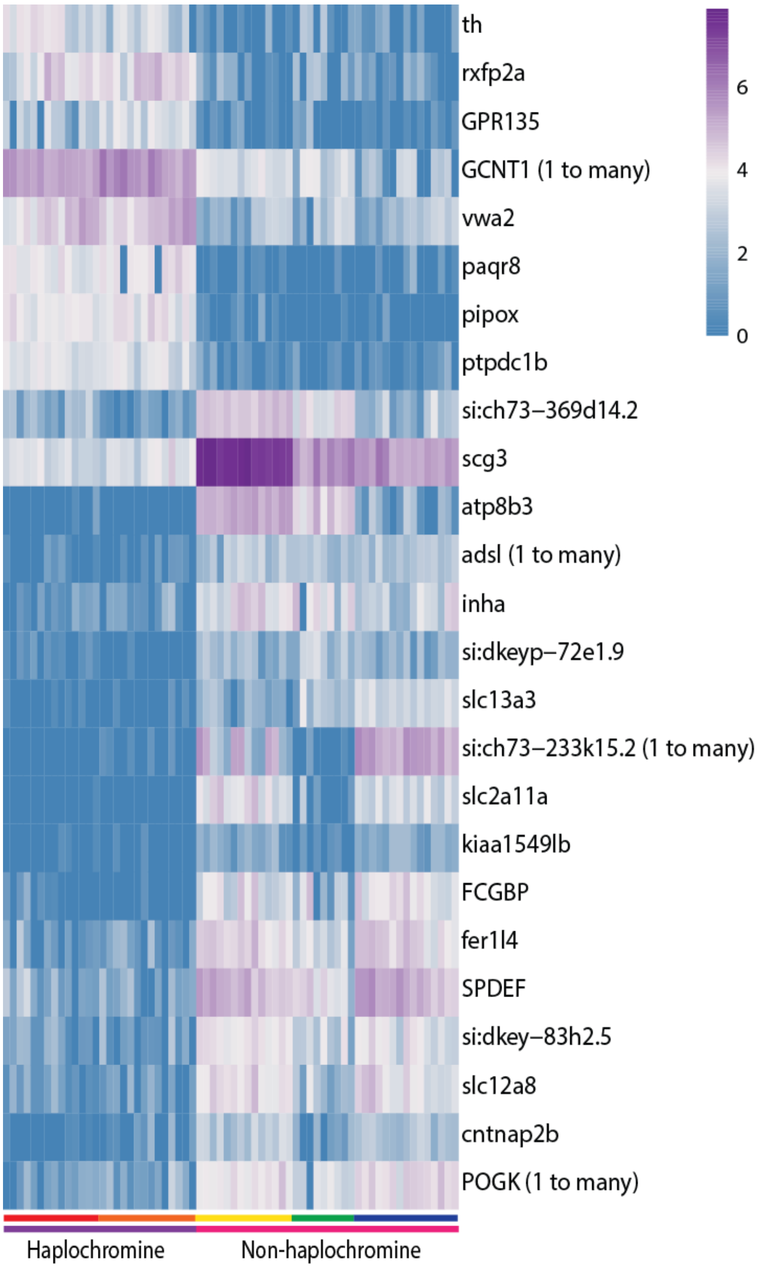

Figure III.10. Heatmap of genes differentially expressed between haplochromine and non-haplochromine cichlids.

Gene Ontology Analysis

After identifying differentially expressed genes in each fin and species, we asked whether any biological processes or functions were over- or underrepresented among these DE genes – to answer this, Gene Ontology (GO term) analysis was carried out. Among the species-specific DE genes, few notable patterns of functional enrichment were observed. In *O. niloticus*, no GO term enrichment was detected. In *N. brichardi*, four GO terms related to transmembrane ion transport were overrepresented. In *A. burtoni*, 42 GO terms were found to be enriched, of which many were also related to transmembrane ion transport. In *M. zebra*, GO terms for phenol-containing compound biosynthetic and metabolic processes were found at a higher proportion in the test set of genes compared to the reference set. *P. nyererei*-specific differentially expressed genes were enriched in 15 gene ontology terms, including several involved in mitosis, nuclear division and other cell cycle processes.

For DE genes associated with different fin types, similar results were obtained. In the anal and dorsal fin genes, no GO term enrichment was detected. In contrast, there were 41 significantly over- or underrepresented gene ontology terms detected in genes differentially expressed in the caudal fin. Overrepresented terms were primarily related to muscle activity and ion transport, while those associated with the nucleus were underrepresented.

Discussion

In this study, a transcriptomics approach was used to investigate gene regulatory patterns in three fins (anal, caudal and dorsal) from five species of African cichlids (*Oreochromis niloticus*, *Neolamprologus brichardi*, *Astatotilapia burtoni*, *Metriaclima zebra*, and *Pundamilia nyererei*) to examine transcriptomic divergence in a model for adaptive radiations. Moreover, population- and sex-specific transcriptomes were also included to help understand within-species changes in regulatory evolution.

General patterns of transcriptome divergence and differential expression

Differences among species

In general, it was found that transcriptome divergence among African cichlid species aligns with evolutionary distance; i.e. more closely-related species cluster together. The ancestral, non-radiating *O. niloticus* diverged from the other species in this study around 18 million years ago (Won et al. 2006), which is reflected in its transcriptional profile – it clusters distantly from the other samples in a PCA. One important caveat to note is the fact that all reads were mapped to the *O. niloticus* genome, which could artificially inflate the number of reads mapped in *niloticus* samples; however, read counts were normalized to mitigate this issue before proceeding with further analysis. The next species to branch off, *N. brichardi*, from the older Lake Tanganyika (Nishida 1991), also forms a distinct cluster. The three closely-related haplochromine species, despite their increased rates of speciation and phenotypic diversification (Kocher 2004), do not show a high level of transcriptome divergence. Therefore, this analysis does not support the hypothesis that fast rates of speciation correlate with transcriptome divergence in this lineage.

Differences among fins

In each species included in this study, the caudal fins were shown to have increased transcriptome divergence compared to the anal and dorsal fins, and the majority of fin-specific differentially expressed genes were found in the caudal fin. The caudal fin is developmentally and morphologically dissimilar from the other two unpaired fins included in this study. Because of this, it is not unexpected that its gene expression levels reflect this difference. The anal and dorsal fins were found to have a reduced number of fin-specific genes; therefore, these genes are more likely to contribute to dorsal-ventral patterning, both in morphology and coloration patterning.

Differences within Pundamilia nyererei

The two phenotypically divergent populations of Pundamilia nyererei males, from Ruti Island and Zue Island, showed an unexpectedly high amount of gene regulatory differentiation in the anal fin. It has previously been shown that sexual selection, driven by female preference for certain color patterns, drives differential male coloration among populations of this species (Selz et al. 2016). Females prefer to mate with males with the color pattern associated with their own population, therefore increasing the amount of reproductive isolation between populations. This study provides further evidence that regulatory changes can contribute to reproductive isolation and population divergence (Haerty & Singh 2006). Because of the close relatedness of these two populations, it is likely that gene expression differences are responsible for much of the diversity of male nuptial coloration patterns found in this species.

In contrast to the high level of divergence found between males from different populations of *P. nyererei*, no differentially expressed genes were detected between males and females from the same population in any of the fins examined in this study. In this species, dominant males display bright nuptial coloration, while females typically show drab, cryptic color patterns. Male coloration in this species is highly plastic – dominant males are brightly colored, but subordinate males are duller and more similar to females in coloration, and an individual's coloration intensity can change rapidly in response to physiological stress or changes in hierarchy. It has been shown in other animals that sex-specific gene expression differences may contribute to sexually dimorphic traits and traits involved in sexual selection, and many phenotypic differences between males and females are have a regulatory basis (Khila et al. 2012; Moczek & Rose 2009). Previous studies have implicated the *csf1ra* and *fhl2b* genes in the development and evolution of the egg spot phenotype (Salzburger et al. 2007; Santos et al. 2014). Because of this, we hypothesized that sex-specific phenotypes in this species could be linked to changes in gene expression, particularly in genes previously linked to egg spots. However, even in the anal fin, no genes were found to show regulatory differences in the individuals sampled in this study. These results indicate that the dimorphic color patterns found between males and females of this species are not affected by local changes in gene expression patterns. This suggests that sexually dimorphic color differences are caused by upstream expression changes, or that they are controlled more by changes in hormone secretion than by gene expression. They also demonstrate a further need for functional research into candidate color genes.

Specific genes of interest

In addition to looking at general patterns of transcriptome divergence, this study identified differentially expressed genes for each species and fin type, to reveal specific genes that may play a role in species and fin diversity. This analysis yielded several interesting candidate genes for future investigation. These genes were previously found in other species to be involved either in morphology, e.g. dorsal-ventral differentiation between the anal and dorsal fins, or in color patterning.

zic1/zic4

In general, very few genes were consistently differentially expressed between the anal and dorsal fin in all species in this study. Of the few genes that did show divergent regulatory patterns between the dorsal and anal fin, the zic family members *zic1* and *zic4* stand out as candidates that are potentially involved in the evolution of morphological differences seen between these two similar fin types (Figure III.11). Previous studies have established that these genes are important in differentiation along a dorsal-ventral axis, and are important for the development of the dorsal region of the body in teleosts (Kawanishi et al. 2013). Therefore, our study provides further evidence that *zic1* and *zic4* are essential to the proper development of the teleost bodyplan. The differential expression of genes such as these in the dorsal and ventral part of the teleost body may contribute to the diversity of dorsal fin shape and structure found among fish.

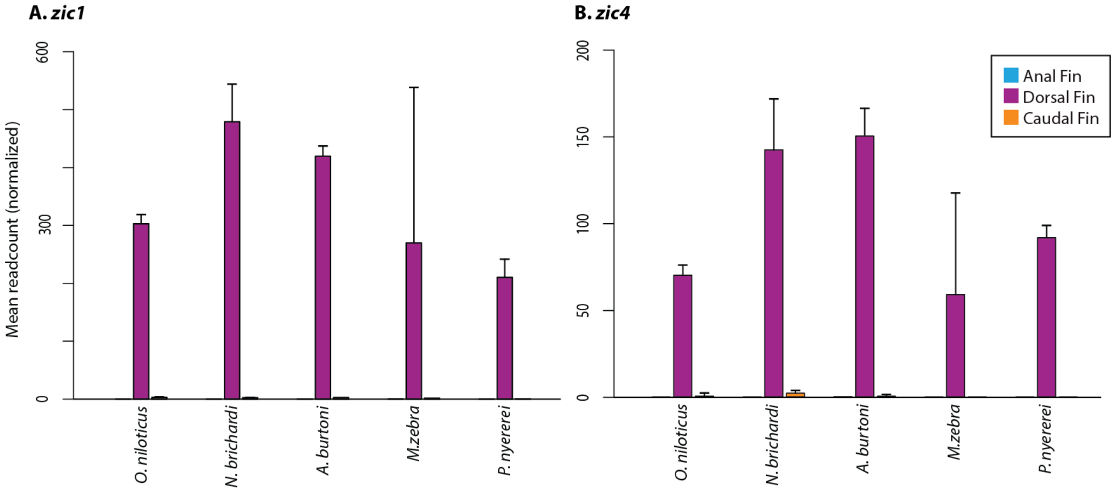

Figure III.11. Expression levels of *zic* genes.
A. *zic1* expression levels; B. *zic4* expression levels.

asip1

Agouti signaling protein 1, or *asip1*, was one of few genes found to be differentially expressed in all species between the anal fin and both other fin types examined. We found elevated expression in the anal fin compared to the dorsal and caudal fin in all species examined, with a particularly strong pattern evident in the haplochromine species (Figure III.12). *Asip1* is a known antagonist of the melanocortin receptor MC1R, and part of the melanin synthesis pathway. Agouti genes display a high level of functional conservation, and have been shown to be involved in patterns of decreased pigmentation in several vertebrate species (Manceau et al. 2011). In the flatfish, this gene was suggested to be related to countershading – a highly conserved coloration pattern found across animals where the ventral part of the body is lighter in color than the dorsal (Guillot et al. 2012). Similar patterns are found across the animal kingdom, with agouti being found to contribute to countershading in mice, chickens, and gar (Manceau et al. 2011; Oribe et al. 2012; Cal et al. 2017). Our results also find that the ventral-most tissue studied had the highest levels of expression, which is consistent with previous research.

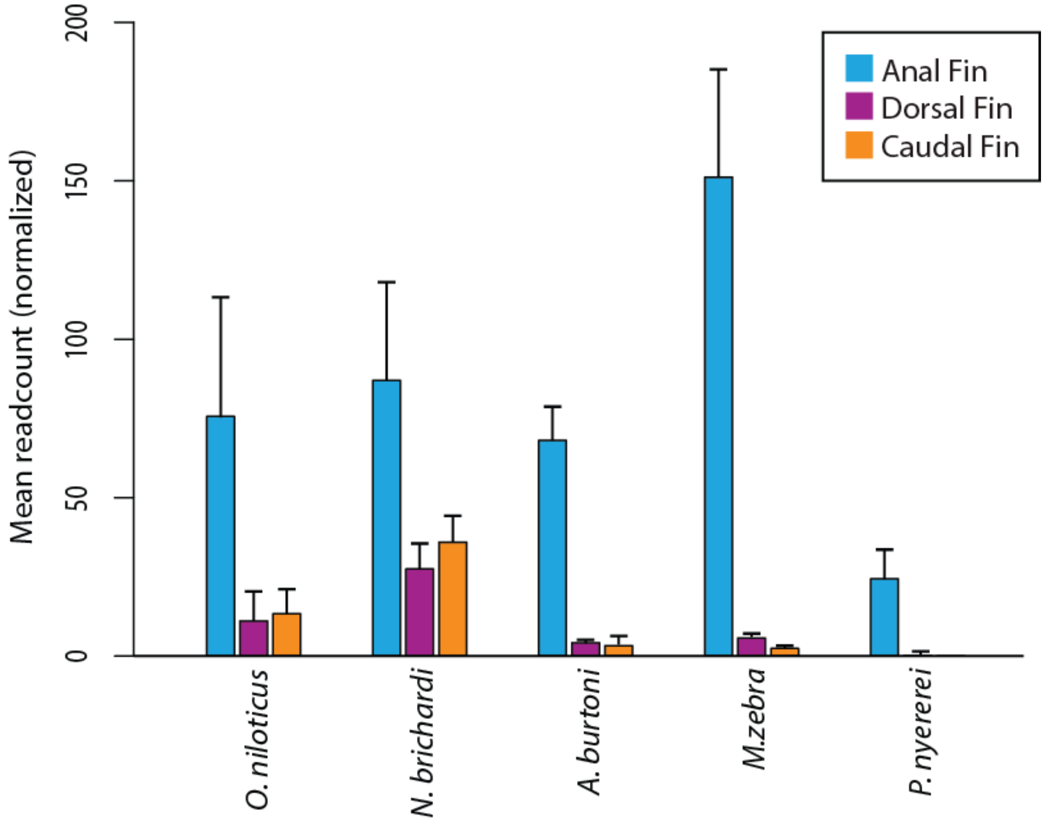

Figure III.12. Expression levels of agouti signaling protein 1, *asip1*.

igfbp7

In the pale-colored *Neolamprologus brichardi*, we found that the *igfbp7* gene was significantly downregulated in all tissues, compared to all other species (Figure III.13). This gene, which codes for insulin-like growth factor binding protein, has been examined in zebrafish and may be related to a decrease in pigmentation across the body. In zebrafish treated with a morpholino to knockout this gene, mutant individuals lacked melanophore pigmentation across the entire body, as well as displaying several other abnormal traits (Hooper et al. 2009). Although this is not confirmed – zebrafish are distantly related to cichlids, and gene knockouts may cause several nonspecific defects – this provides evidence that this gene may be involved in the loss of pigmentation. Combined with the correlative evidence from this study, it is an interesting target for further research into pigment loss. Future studies in cichlids using recently-developed transgenesis techniques (Kratochwil et al, in review) could help reveal whether this gene is responsible for the colorless phenotype seen in *N. brichardi*.

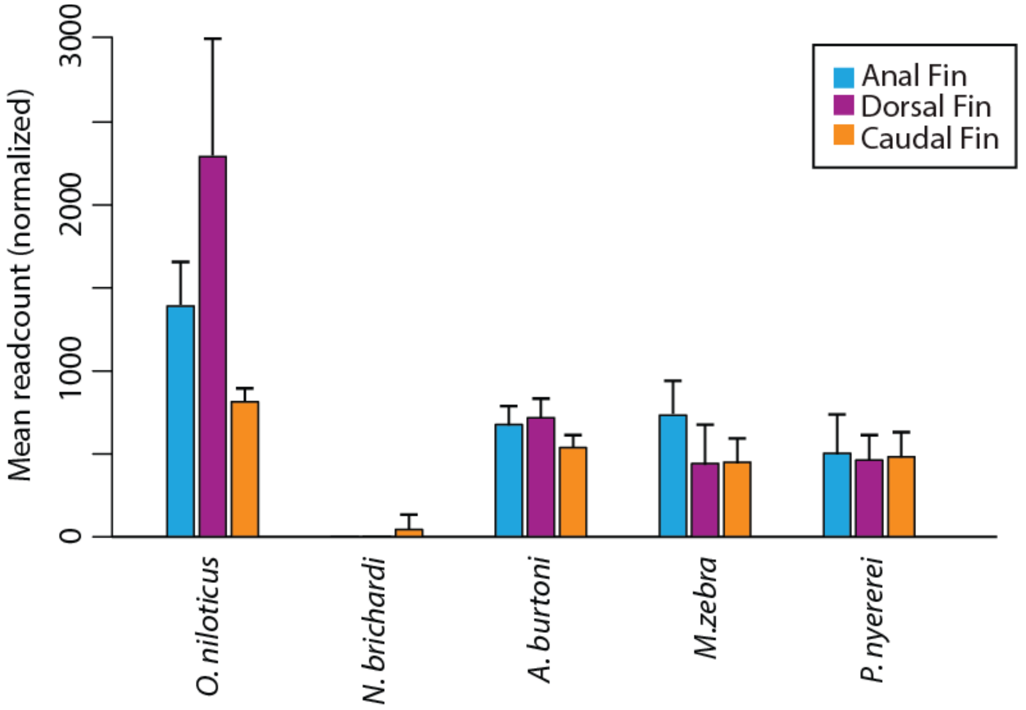

Figure III.13. Expression levels of insulin-like growth factor binding protein, *igfbp7*.

Other species-specific and lineage-specific color genes

In the Nile tilapia, *O. niloticus*, one possible color-associated gene was found: *atp6v1e1a*. This gene codes for the enzyme ATPase, H+ transporting, lysosomal, V1 subunit E1a. In zebrafish, mutations of the subunits of V-ATPase cause variation in pigmentation, suggesting that it plays a fundamental role in the genesis of melanosomes and survival of melanophores (Ramos-Balderas et al. 2013). As such, it may represent a candidate gene for color variation within in *O. niloticus*. A further potential color gene was found to be differentially expressed in *A. burtoni*. *Mucolipin 3a* (*mcoln3a*), was shown previously to be involved in melanophore development in both mammals and teleosts, and arose as a result of the fish-specific genome duplication (FSGD) (Braasch et al. 2009). Among the DE genes specific to *Pundamilia nyererei*, one possible coloration gene was identified, also originating in the FSGD. The *irf4b* gene has been shown to be associated with variation in eye, skin and hair color in humans, but its precise role in pigmentation remains unknown (Braasch et al. 2009).

This analysis yielded one possible candidate for the increased coloration diversity found in the haplochromine cichlids. The *slc2a11a* gene, for solute carrier family 2 (facilitated glucose transporter) member 11a, was upregulated among haplochromine species. This gene's paralog, *slc2a22b*, has been shown to contribute to the differentiation of xanthophores and leucophores in medaka (Kimura et al. 2014). Therefore, its increased presence in haplochromine species may have an implication in their increased color pattern diversity.

Future directions

One potential avenue for further research on the study of gene regulation in African cichlid fins would be to compare the results from the present study to ChIP-seq data. ChIP-seq sequences genomic regions associated with histone modifications to map the cis-regulatory elements such as promoters and enhancers that control gene expression. In conjunction with RNA-sequencing, ChIP-seq has the potential to uncover the regulatory mechanisms underlying phenotypic diversification (Kratochwil & Meyer 2015). Furthermore, future studies could compare the rate of gene expression change to the ratio of synonymous to non-synonymous mutations present in coding regions – the *dN/dS* ratio, which provides information about whether certain genes are being exposed to selection or whether their evolution is mainly stochastic. This would tell us the relative importance of protein coding changes compared to expression changes in specific genes that are implicated in certain traits. For example, if a particular gene shows an increased amount of regulatory divergence among lineages, but a low mutation rate, this suggests that its expression level is more important in the process of phenotypic divergence. It is also possible that genes under positive selection also have increased levels of gene regulatory divergence. These genes that exhibit both differential expression and signs of positive selection would represent ideal candidate genes for further studies.

Another interesting direction for future studies would be to further investigate the genes that show species- or fin-specific expression patterns using an evo-devo approach. Functional analyses of these genes, such as in situ hybridization, transgenesis, gene knockout and overexpression, would give researchers clearer insight into the roles that they play in the process of adaptive radiations. As with all transcriptomic studies, the relationship between genotype and phenotype in this study are correlative – in vivo functional validations of genes is required to prove a causal link between gene expression level and observed phenotype.

Conclusion

Large-scale comparative transcriptomics studies such as this can help to unravel the complex relationship between genotype and phenotype. RNA-seq has become a widely-used method to test between different experimental conditions and controls; it is less frequently applied to examine broader patterns of genome-wide expression evolution across many different tissue types and species. Although transcriptomic analyses can provide only correlative evidence for specific genes associated with phenotypic change, they are useful in identifying signatures of regulatory evolution that may contribute to microevolutionary change. This study and similar research can help us to understand the dynamics of transcriptome evolution during an adaptive radiation. Because adaptive radiations such as those found in the African cichlids represent a rapid burst of speciation, they are an essential resource in the study of evolution and the origin of species.

General Discussion

Main Contributions

This dissertation investigates the regulatory mechanisms underlying the origins of diversity in cichlid fish, as well as providing a framework for evo-devo studies in the Midas cichlids. In addition to contributing new insights into the evolution of cichlid fish, such as uncovering genes associated with morphological changes across an adaptive radiation, the chapters serve as valuable guides and provide experimental methods for future research. The Midas cichlid species complex (*Amphilophus spp.*) is presented as a potential new model for evo-devo research examining the role of cis-regulatory changes in the diversification process – the chapters of this dissertation establish a theoretical and experimental context in which novel and informative experiments can be performed.

Early development in the Midas cichlid species complex

The first chapter provides a developmental staging system for the Midas cichlids, which will serve as a foundation for future experiments and facilitate comparative studies both within the *Amphilophus* species flock and among other cichlid species. Early development in the Midas cichlid is described in detail, and their suitability as a model for evo-devo studies is discussed. The definition of a developmental time is an essential first step in establishing a model organism – similar studies have been published in model teleosts such as zebrafish and medaka, as well as in other cichlid species (Kimmel et al. 1995; Iwamatsu 2004; Fujimura & Okada 2007; Meijide & Guerrero 2000). This study identifies easily recognizable developmental landmarks, enabling the collection of comparable stages for both developmental (e.g. *in situ* hybridization) and molecular biology (e.g. RNA sequencing) experiments. Defining homologous stages between Midas cichlids and other fish species facilitates future comparative developmental studies. The definition of homologous stages also enabled us to quantify the effects of temperature on early development of Midas compared to zebrafish, revealing that temperature changes have a relatively large impact on developmental rate in this species – a quality that has both ecological relevance and allows researchers to manipulate developmental timing. Finally, a method for the reliable visualization of the pale, yellow-colored xanthophores in early embryos was described. Early life patterns of chromatophore development were characterized, providing crucial information for ongoing research on color patterning in cichlids.

Transgenesis in the Midas cichlid species complex

In the second chapter, a microinjection protocol is adapted for use in the Midas cichlids. The *Tol2* transposon-mediated transgenesis method was used to produce transgenic Midas cichlids - the first step towards testing the genes and regulatory elements underlying phenotypic divergence in this adaptively-radiating species complex. Some important factors that facilitate a species' use for transgenesis experiments include the ease of obtaining a large number of early-stage offspring on a regular basis, capacity to maintain large breeding populations in laboratory conditions, traits such as egg size and shape, and short generation times (Kratochwil et al. n.d.). For many ecological model systems, one or more of these factors can hamper the efficient application transgenesis. For example, seasonal breeding and small clutch sizes in the stickleback make transgenic studies logistically difficult on a larger scale, despite being an otherwise excellent system for studying the evolution of gene function and regulatory divergence (O'Brown et al. 2015). Moreover, the African cichlids, with their large-scale adaptive radiations, are an evolutionary and ecological model; however, they are often mouthbrooders with small clutch sizes that can make obtaining large amounts of eggs prohibitively difficult. The *Amphilophus* species flock possesses many traits that are amenable to transgenesis studies; as a result, transgenesis in the Midas cichlid is more feasible than in many other ecological model teleosts. The most significant drawback for transgenesis in Midas cichlids is their long generation times – it can take up to a year for animals to reach full sexual maturity, delaying the time until a stable transgenic F1 line is obtained. Despite this setback, F2 individuals carrying the *eGFP* gene expressed under the *ubiquitin* promoter region were produced in this study. Consequently, this study reports the establishment of the first stable transgenic line of Midas cichlids, and expands the molecular toolkit for future evo-devo studies in this lineage.

Transcriptomics of coloration and fin morphological diversity in African cichlids

In the third chapter, RNA-sequencing was performed on three unpaired fins (anal, caudal and dorsal) from five species of African cichlids (*Oreochromis niloticus*, *Neolamprologus brichardi*, *Astatotilapia burtoni*, *Metriaclima zebra*, and *Pundamilia* *nyererei*) to examine transcriptomic divergence in a model for adaptive radiations. In addition to fin- and species-specific transcriptomes, two aspects within-species divergence were investigated in this study – population-level differences and sex-specific transcriptomes were also scrutinized. In mammals, the rate of transcriptome divergence has been found to vary between lineages (Brawand et al. 2011). Because cis-regulatory changes are thought to play an important role in the adaptive radiations of African cichlids, we hypothesized that transcriptome divergence would be accelerated within the explosively-radiating Haplochromine lineage, relative to non-radiating taxa. Previous studies of African cichlids have identified an increased number of novel miRNAs within haplochromines, providing further evidence for the role of regulatory change within adaptive radiations. However, species-level patterns of transcriptome divergence in African cichlids were described in this study, revealing that transcriptomic differentiation appears to reflect evolutionary distance, as is expected under neutral conditions. At the population level, it was discovered that phenotypically divergent populations of *P. nyererei* exhibited an unexpected degree of transcriptome divergence, hinting at the importance of regulatory evolution in the development of population differences within a species. Also, despite their clear sexual dimorphism, there was no significant difference in gene expression detected between males and females from the same population of *P. nyererei*. Previous studies in mammals have also revealed that different tissue types may exhibit divergent rates of transcriptome evolution (Brawand et al. 2011) – we found that the anal and dorsal fins showed very similar expression patterns, while the caudal fins have divergent regulatory patterns. This may be a reflection of the differences in phenotype found in the anal and dorsal fins compared to the caudal fins, which are very different in shape and tissue composition.

In addition to describing general patterns of gene regulation, this study identified differentially expressed genes in each species and fin type. As is always the case in RNA sequencing experiments, the relationship between these differentially expressed genes and any associated phenotypes is purely correlative. However, some of the genes identified in this study have been associated with similar phenotypes in previous studies. Among these are two members of the zic family, *zic1* and *zic4*, which are known to be associated with dorsal-ventral differentiation in teleosts (Kawanishi et al. 2013). In addition, we see species-specific downregulation of the *igfbp7* gene, previously implicated in pigmentation loss (Hooper et al. 2009), in the colorless *N. brichardi*, making this gene an interesting candidate for future research into pigmentation loss in cichlids. Finally, the *asip1* gene, coding for Agouti signaling protein 1, was found to be significantly upregulated in the anal fin compared to the dorsal and caudal fins in all species examined. Agouti has been previously indicated to be involved in melanin synthesis pathway, and its function is known to be conserved across vertebrates. Notably, it is involved with the development of countershading (decreased pigmentation on the ventral part of the body), in taxa ranging from mice to flatfish (Manceau et al. 2011; Guillot et al. 2012). The increased expression in the ventrally-situated anal fin, therefore, is consistent with previous information about this gene's function and expression – making it an interesting target for future research using the methods developed in the previous chapters.

Future and Outlook

The first chapter of this thesis serves as an essential guide for future studies of cichlid development. For evo-devo studies using methods such as *in situ* hybridization or transgenesis, a developmental staging system describing early life under normalized, controlled conditions is an invaluable resource. Comparative developmental studies examining the ontogeny of coloration patterning in cichlids, using this study as a reference, are already underway. This will undoubtedly provide further information on how and when species-specific phenotypic differences arise.

The second chapter outlines a method in which transgenic Midas cichlids can be produced. Both the methods developed and transgenic animals generated in this study have numerous future experimental uses. With a stable line of fish ubiquitously expressing GFP, it will be possible to perform cell transplantation and lineage-tracing experiments, which have previously been performed in other organisms such as zebrafish and *Xenopus* (Kemp et al. 2009; Gross et al. 2006). One promising application for chimera studies using transgenic Midas cichlids is to investigate the nature of the gold/dark polymorphism, for which the lineage is named. Additionally, transgenesis makes it possible to test the functions of specific regulatory elements using GFP reporter assays, and to overexpress target genes to assess their contribution to phenotypic variation *in vivo*. Finally, the microinjection methods refined for this experiment can be used to perform genome-editing techniques such as CRISPR-Cas9, one of the most powerful available techniques for studying the relationship between genotype and phenotype today.

The third chapter explores the molecular underpinnings of diversity in a rapidly-evolving lineage by characterizing gene expression in three unpaired fins from five species of African cichlids. This study identified many fin- and species-specific differentially expressed genes that may have played a role in phenotypic change among cichlid fish. Of particular interest are genes previously implicated in processes such as coloration and body patterning. Because these phenotypes are both conspicuous and potentially involved in natural and sexual selection, the genes associated with them are excellent targets for follow-up experiments. Using the methods described in the first two chapters, functional analysis can be performed on genes of interest, such as those identified in the third chapter. Consequently, this dissertation can serve as a foundation for future research into the evolution and development of diversity in cichlid fish. In a broader context, this represents a small contribution to the narrowing of the gap between genotype and phenotype – a question that has long perplexed observers of the natural world.

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Acknowledgements

General acknowledgements

First, I would like to thank Prof. Axel Meyer, for providing me with the opportunity to pursue a PhD and conduct research in his lab. Throughout my time in the lab, I have had the chance to learn so much about science; I am glad to have had such an enriching experience.

I am eternally grateful to Dr. Claudius Kratochwil for providing valuable supervision and guidance throughout the last few years, without which I may not have survived my PhD. His dedication and enthusiasm for biology is really inspirational. I'm thankful to be a part of his colorful little research team, along with Yipeng Liang, Sabine Urban, Laura Gießler, Jan Gerwin and all the others.

I am also thankful to Prof. Manfred Schartl, for agreeing to referee my thesis and attend my defense. In addition, I would like to thank Prof. Ron Burton for supervising my Masters thesis and inspiring me to pursue a PhD, as well as providing helpful feedback on my PhD work during PAC meetings.

All of my financial support was provided by the Hector Fellow Academy, which also gave me the opportunity to meet great scholars from a number of scientific fields. I'd like to thank all of the IMPRS and especially its coordinator, Mäggi Hieber-Ruiz, for providing support throughout my thesis.

Thanks to the whole Meyer lab for providing support and discussions both scientific and otherwise. Extra thanks to my fellow scholars and friends in the PhD room. I am very grateful to Ralf Schneider for helping me with any and all German language-related issues, of which there were many. Ingrid Bader and Christiane Weber also provided excellent administrative assistance.

Finally, thanks to my parents for supporting me in everything and my brothers for encouraging me, and believing in me even when I didn't believe in myself.

Chapter-specific acknowledgements

Chapter I

The Swiss National Science Foundation (P2BSP3_148629) and the EU FP7 Marie Curie Zukunftskolleg Incoming Fellowship Program, University of Konstanz (grant no. 291784) fund CK. MMS is supported by a Ph.D. fellowship of the Hector Fellow Academy. AM is funded by several grants of the Deutsche Forschungsgemeinschaft (DFG) and advanced grant 297300 “Gen-adapt” by the European Research Council. We appreciate the efforts of two anonymous reviewers for their constructive comments and thank Andreas Kautt, Julián Torres-Dowdall and Joost Woltering for discussion of this work and comments on the manuscript and the staff of the animal care facility of the University of Konstanz.

Chapter II

This work was supported by the Swiss National Science Foundation (P2BSP3_148629), the EU FP7 Marie Curie Zukunftskolleg Incoming Fellowship Program, University of Konstanz (grant no. 291784), the Elite Program for Postdocs of the Baden-Württemberg Foundation and a grant of the German Research Foundation (KR 4670/2-1) to CFK. Funding for MMS was granted by a Ph.D. fellowship of the Hector Foundation. YL is funded by a stipend from the China Scholarship Council (CSC). AM is funded by several grants of the Deutsche Forschungsgemeinschaft (DFG), the University of Konstanz and an advanced grant 297300 “GenAdapt” by the European Research Council. The authors thank Ralf Schneider and Joost Woltering for discussions of this work and comments on the manuscript. Also, we specifically thank the staff of the animal research facility of the University of Konstanz for their excellent care of our fish.

Chapter III

Funding for MMS was provided by the Hector Fellow Academy. The Swiss National Science Foundation (P2BSP3_148629), the EU FP7 Marie Curie Zukunftskolleg Incoming Fellowship Program, University of Konstanz (grant no. 291784), the Elite Program for Postdocs of the Baden-Württemberg Foundation and a grant of the German Research Foundation (KR 4670/2-1) provided funding to CFK. AM is funded by the Deutsche Forschungsgemeinschaft (DFG), the University of

Konstanz and an advanced grant 297300 “GenAdapt” by the European Research Council. The authors thank Daniel Monne-Parera for helping with sample preparation, Paolo Franchini for assistance with the analysis, and Ralf Schneider and Joost Woltering for discussions of the work.

Author contributions

Chapter I

CFK designed the research, established the methods, supervised the experiments, analyzed the data and wrote the manuscript. MMS conducted the experiments, drafted the figures and edited the manuscript. AM designed the research and revised the manuscript.

Chapter II

CFK conceived, designed and supervised the experiments and cloned the constructs. MMS, CFK and YL conducted and optimized the microinjection experiments. MMS, CFK and YL performed histology and analyzed the embryos and sections. MMS wrote the first draft of the manuscript. CFK and AM edited the manuscript. All authors approved the final version.

Chapter III

CFK conceived, designed and supervised the experiments. MMS and CFK prepared the samples; MMS extracted the RNA. MMS analyzed the data, wrote the manuscript and made the figures. CFK revised the manuscript and drafted Figure III.1. AM designed the research.

IV. Supplementary Material

Chapter III. Transcriptomics of fin diversity in African cichlids

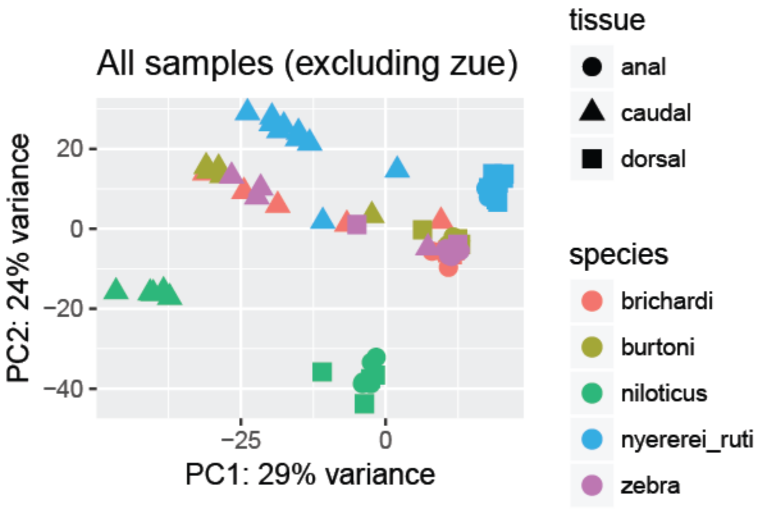

Figure IV.1 PCA of all samples (excluding *P. nyererei* from Zue Island)

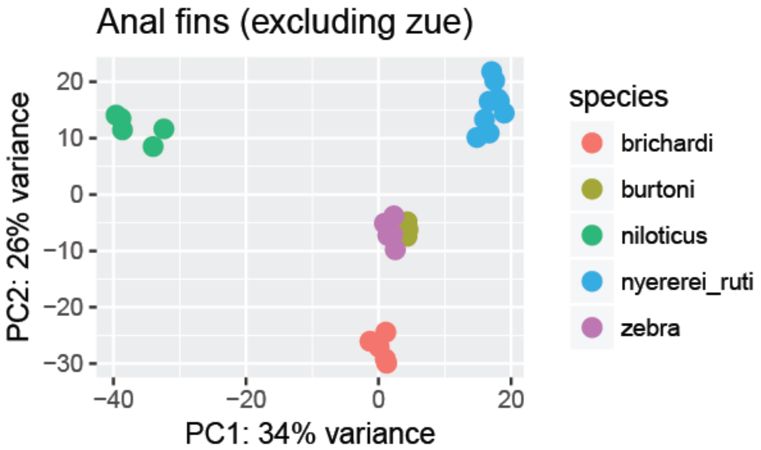

Figure IV.2. PCA of anal fins (excluding *P. nyererei* from Zue Island)

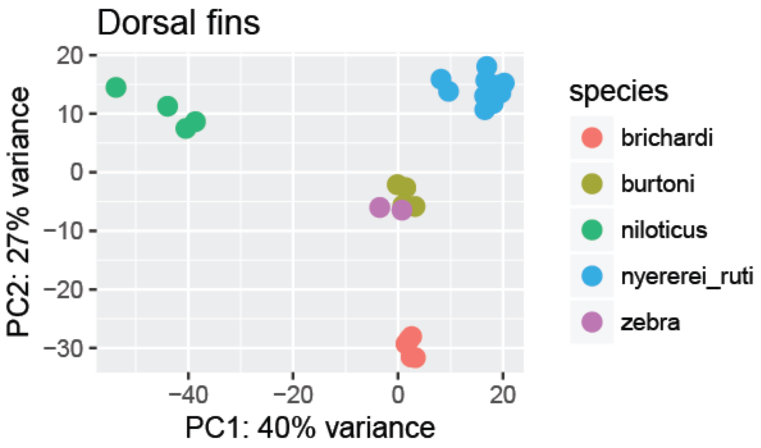

Figure IV.3 PCA of dorsal fins

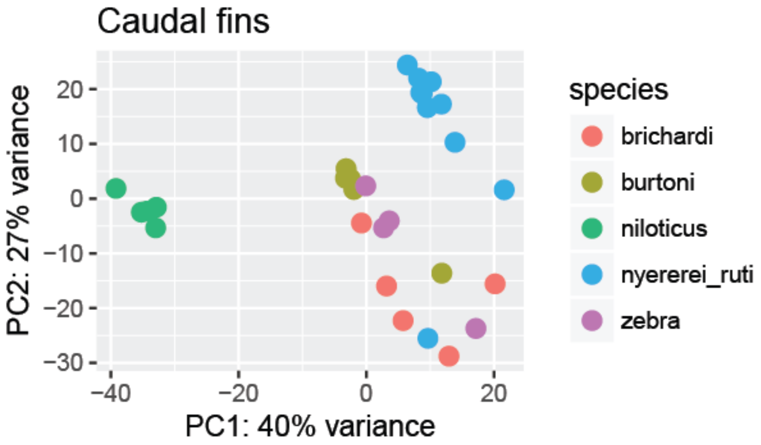

Figure IV.4. PCA of caudal fins

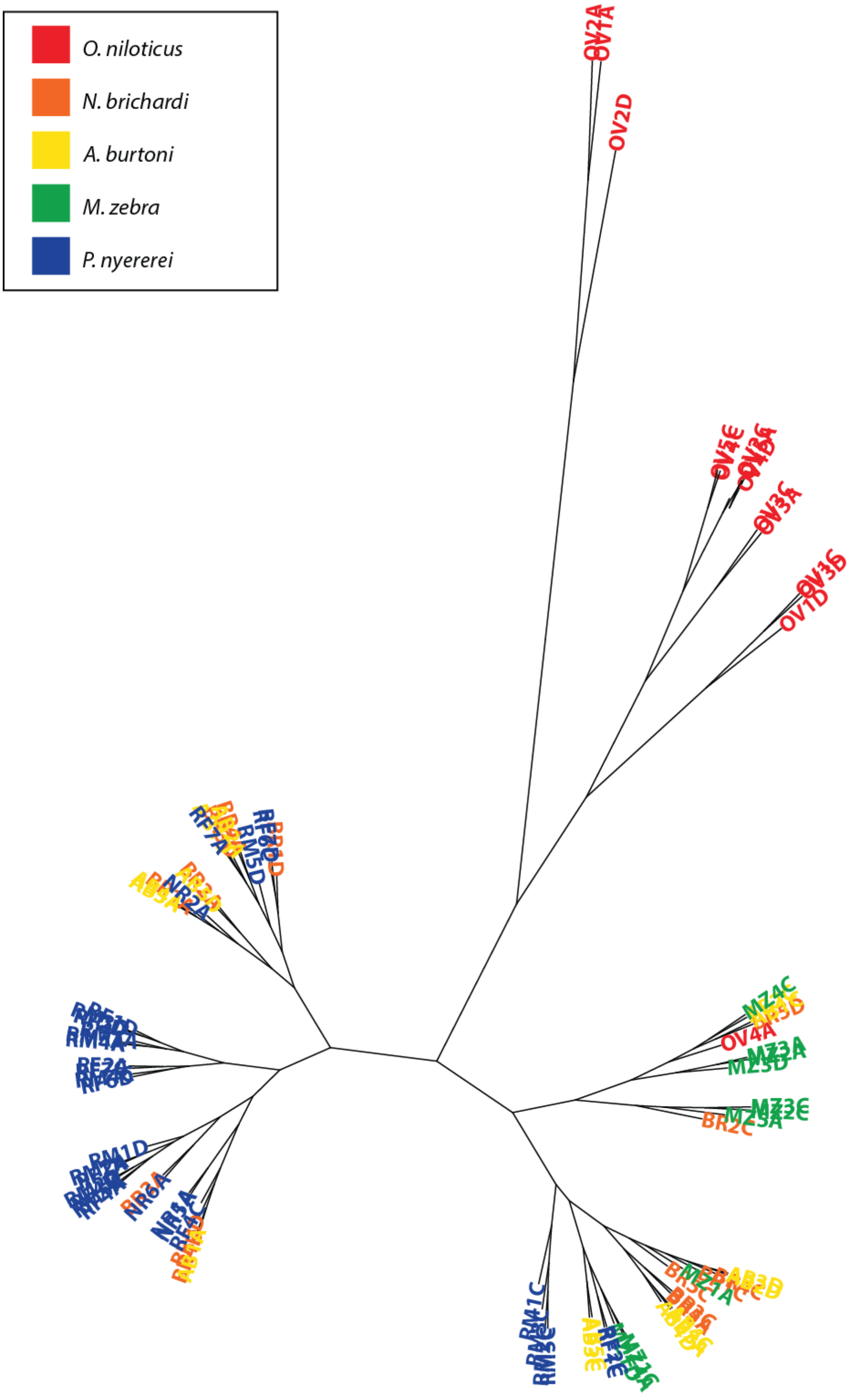

Figure IV.5 Unrooted expression tree of all samples.
The distance matrix is calculated from Pearson correlation coefficients of RLE normalized read counts. Branch lengths are scaled with a square root function for better visualization.

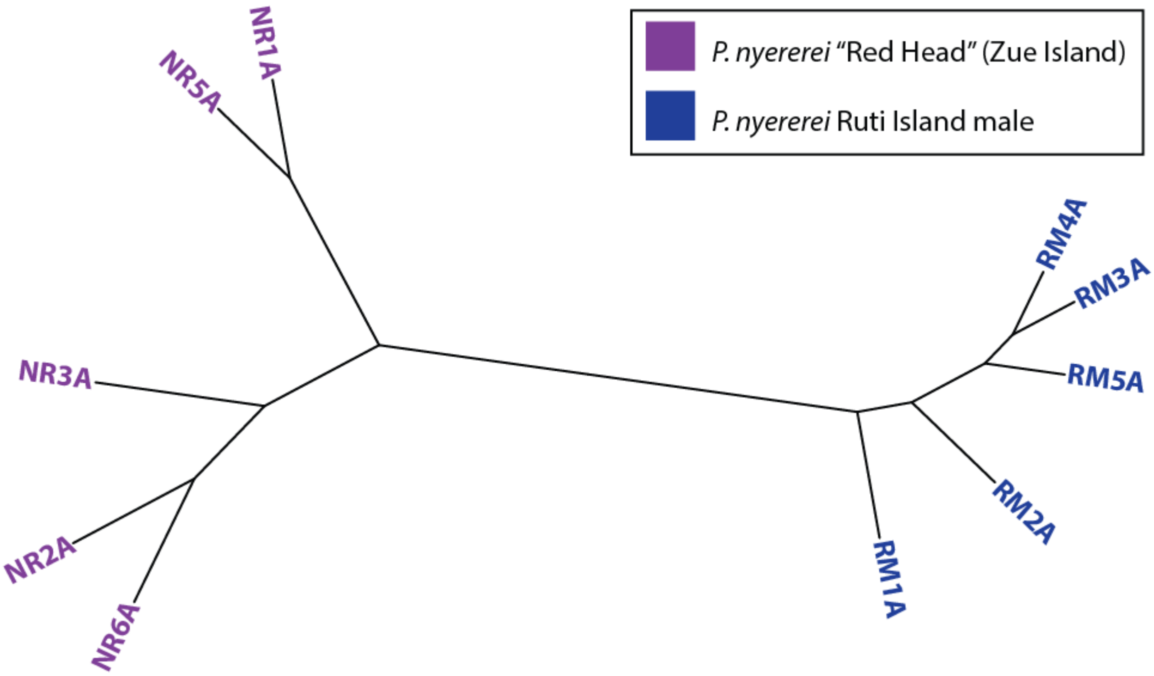

Figure IV.6 Genome-wide population differences in gene expression.
Gene expression tree of anal fin samples from males of two phenotypically-divergent populations of *P. nyererei*. The distance matrix is calculated from Pearson correlation coefficients of RLE normalized read counts. Branch lengths are scaled with a square root function for better visualization.

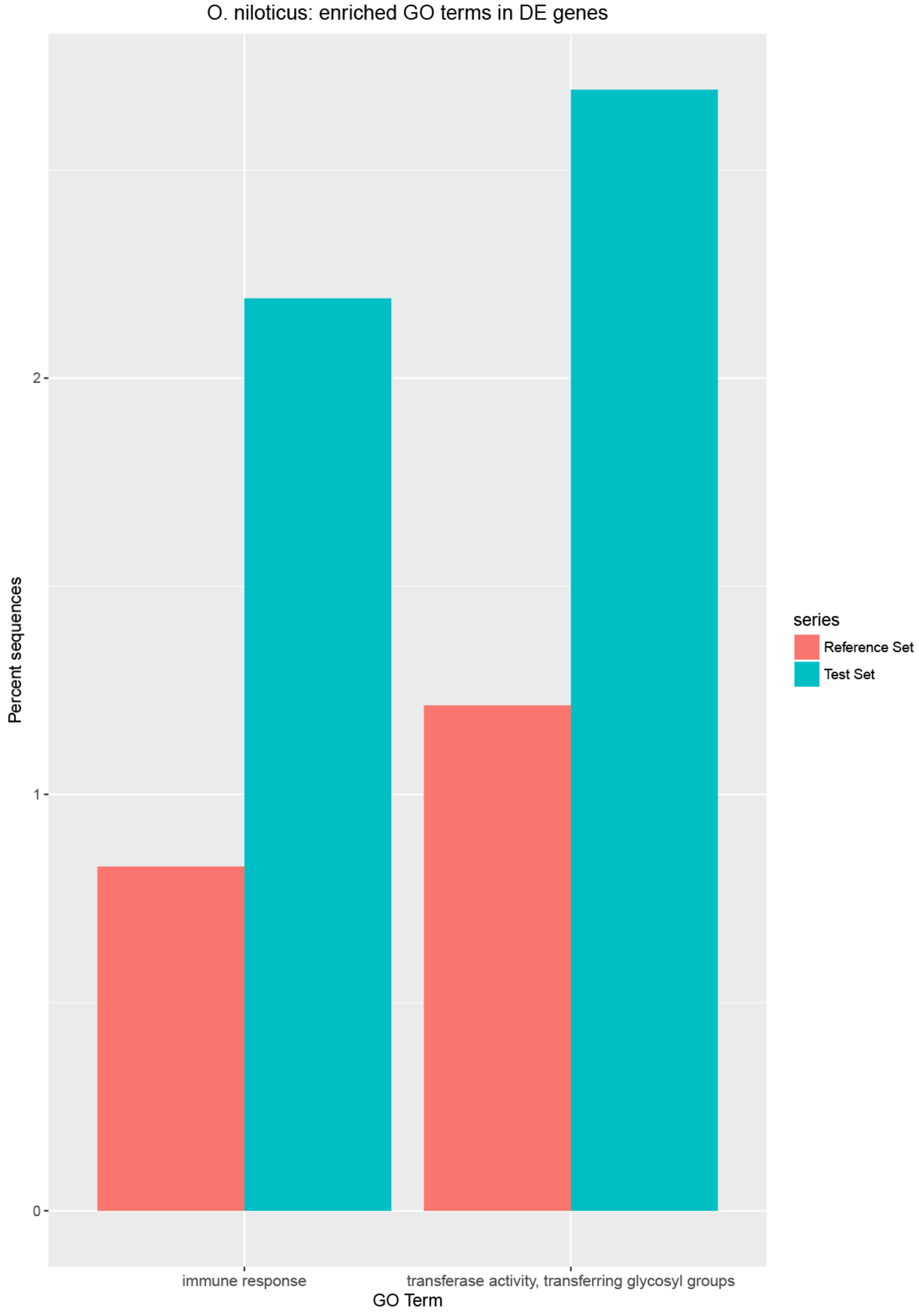

Figure IV.7. GO term enrichment in *O. niloticus*

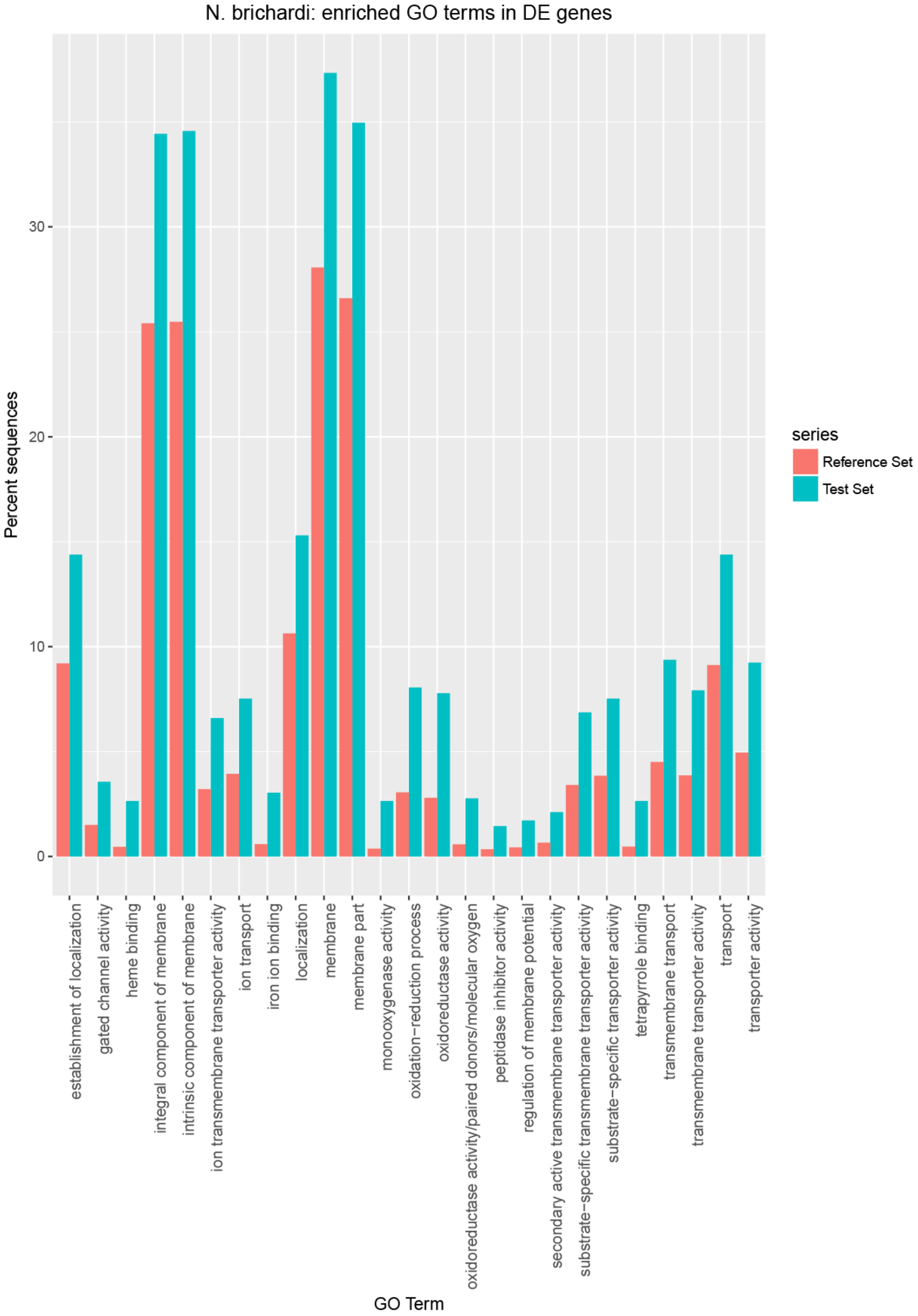

Figure IV.8. GO term enrichment in *N. brichardi*

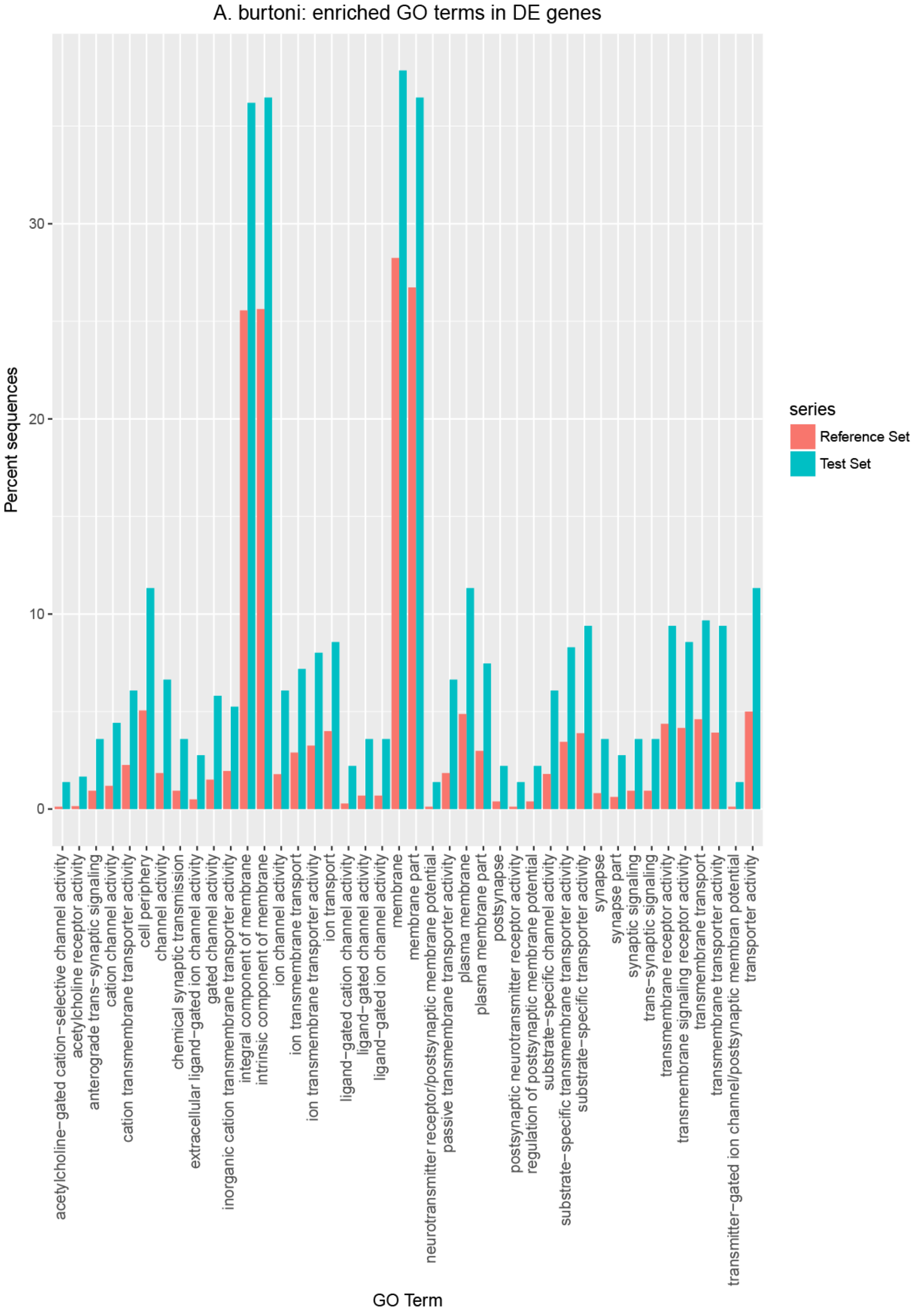

Figure IV.9. GO term enrichment in *A. burtoni*

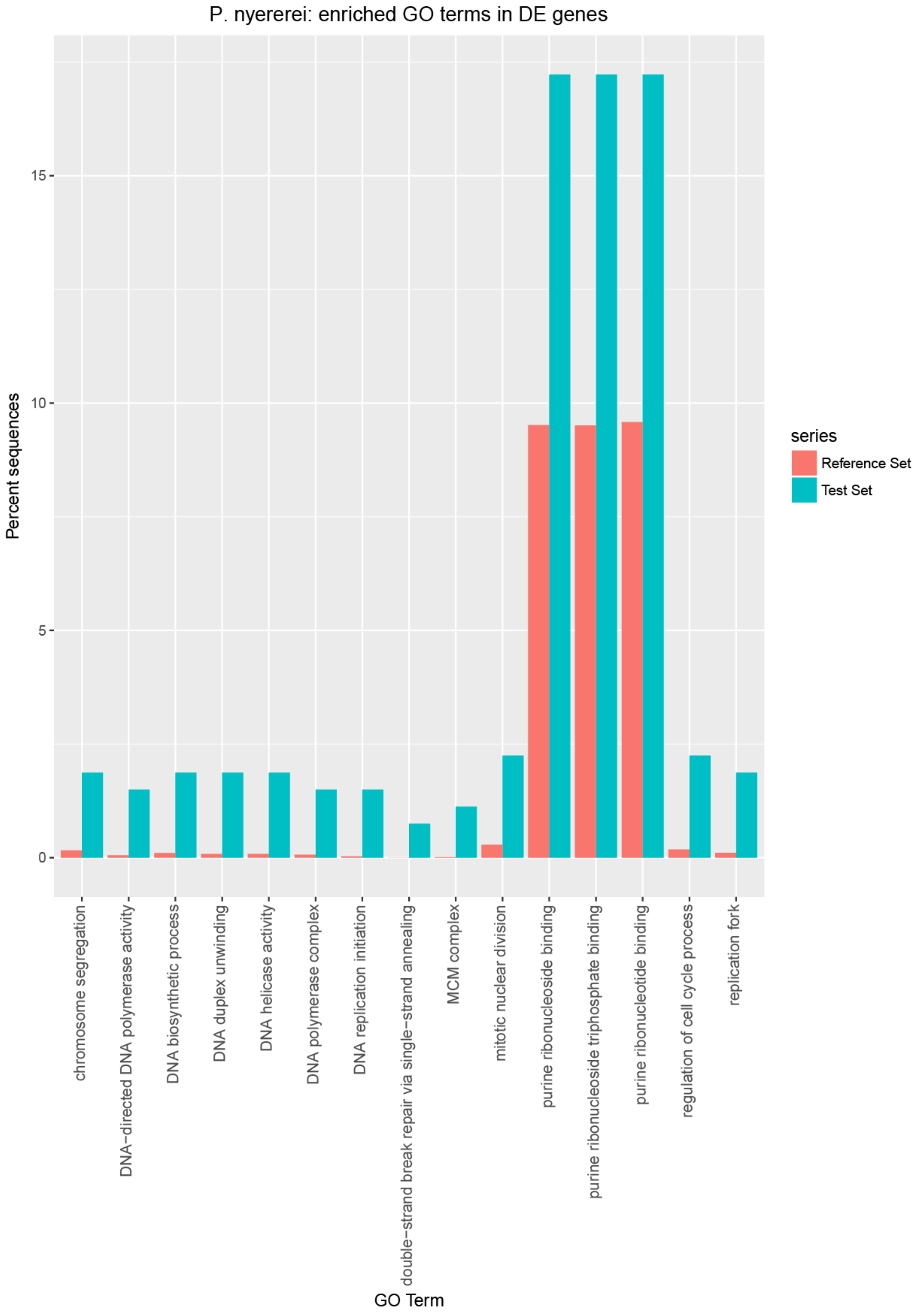

Figure IV.10. GO term enrichment in *P. nyererei*