UNIVERSITY OF CALIFORNIA
RIVERSIDE

Examining Evidence for Phenotypic and Genetic Convergence in the Guppy (*Poecilia reticulata*)

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Evolution, Ecology, and Organismal Biology

by

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June 2018

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Acknowledgements

I would first like to thank my co-advisors Cheryl Hayashi and David Reznick. I am so lucky to have you both as advisors, as your constant help and support have made this dissertation possible. Thank you to John Gatesy and Jason Stajich, who formed the rest of my dissertation committee, for your expertise and advice.

For their critiques of my dissertation proposal, I would like to thank my Oral Qualifying committee: John Gatesy, Howard Judelson, Joel Sachs, Jason Stajich, and Brad White. I was extremely fortunate to have a committee whose combined research interests complimented my own so well. You advice, especially related to my hypotheses and assumptions, allowed me to pursue a much more scientific dissertation.

I am extremely grateful to many faculty and staff at UCR. Biology Department members: Jon Allen, Sophia Jurisch, Beverly McNeil, Jessica Pacheco, Trish Steen, and the Vivarium staff. Melissa Gomez, Kathy Redd, Amy Ricks, and Dawn Loyola of the CNAS Graduate Student Affairs Center provided frequent guidance and exhibited extreme dedication to their graduate students. The Student Health Services and CAPS members were always friendly, caring, and very helpful in keeping me well during my time at UCR.

My dissertation was made possible from the advice and prior research of many: John Endler, Bonnie Frasier, Swanne Gordon, Kim Hughes, and Axel Künstner. I am especially thankful to all members of the Weigel lab that created a publicly available guppy genome.

Many fellow graduate students and Post-Doctoral Associates helped me during my time at UCR. The members of the Hayashi lab immensely expanded my knowledge of RNA-seq and taught me a new protocol for RNA extractions: Crystal Chaw, Matthew Collin, Sandra Correa, and Jim Starrett. Reznick lab members provided thoughtful discussions during lab meetings and frequent feedback on my writing and experiments: Jeff Arendt, Kristin Edwards, Andrew Furness, Joshua Goldberg, Daniel Goldberg, Samantha Levell, Keenan Morrison, and Robert Prather. The lab manager for the Reznick lab, Yuridia Reynoso, was a constant source of support for any questions related to guppies, lab procedures, and grant funding. The hours you spent organizing the care for the guppies can never be repaid.

It took a small army of undergraduates to feed and maintain the fish used in this dissertation. Your commitment to work that is often very tedious was much appreciated. I thank Jasmine Hinh and Sarah Ruckman, both very talented undergraduate researchers, who provided a significant amount of help with Chapter 3 and Chapter 4, respectively. That work would not have been possible without their hours writing and executing computer codes. Diana Tran undertook the tireless task of analyzing guppy photographs for an earlier version of Chapter 3 in addition to being a valuable vivarium helper.

The text of this dissertation, in part or in full, is a reprint of the material as it appears in *Evolution and Development* (Chapter 1: in revision), *PeerJ* (Chapter 2: in revision), *Ecology and Evolution* (Chapter 3: in revision), and *Molecular Ecology* (Chapter 4: in prep). The co-authors Cheryl Hayashi and David Reznick listed in those publications directed and supervised the research, which forms the basis for this

dissertation. Jeff Arendt (Chapter 1), Jasmine Hinh (Chapter 3), and Sarah Ruckman (Chapter 4) also contributed to the publications by participating in data analysis and manuscript preparation/editing.

Dedication

I wish to express my gratitude to my family for their love and support during my time at UCR. My friends Megan and Aaron deserve special mention. You gave me much needed laughter, good times, and delicious meals, even if I had to remain awake past my bedtime to experience it. Lastly, thank you to Justin. You may not have always understood why I was so excited to download millions of base pairs of sequencing data, but you were there to share in my successes, listen to my problems, and provide a constant reminder of how proud you were of me. I also appreciate you pretending that my degree makes me the smart one when we both know which one of us kills the other at Jeopardy.

ABSTRACT OF THE DISSERTATION

Examining Evidence for Phenotypic and Genetic Convergence in the Guppy (*Poecilia reticulata*)

by

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Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, June 2018
Dr. Cheryl Hayashi, Co-Chairperson
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Convergent evolution of a trait can occur at interspecific or intraspecific levels. Traits that are convergent within species are predicted to have a similar genetic basis due to recently shared ancestry. However, it is unclear how likely it is to find similar genetics during cases of rapid evolution or evolution on historic timescales. My dissertation examines evidence for phenotypic and genetic convergence in guppies, which are found in recently-introduced and historically-existing sites that differ in predation regime. I use ornamental tail coloration as it varies highly among developmental stages within an individual, males and females, individuals within a site, and between sites.

In Chapter one, I demonstrate that color develops in a consistent order among males from different predation regimes and I propose that early maturing males are the ideal stage for future experiments. In Chapter two, I determine that black melanin-related genes are likely candidates for genetic differences among fish varying in the presence of ornamental coloration. In Chapter three, I find evidence that phenotypic color pattern

differences during rapid evolution follow a mixture of unique and similar trajectories based on current selective environment experienced. The color gene differences exhibit partial molecular convergence, indicating that guppies maintain variation within and between sites. In Chapter four, I discover phenotypic color pattern similarity among independently derived drainages and predator communities with substantial genetic divergence. I find a lack of molecular convergence in the identity of differentially expressed color genes among geographic locations. Taken together, these results suggest that the scale at which convergence is examined is very important. Sites may exhibit an overall signal for convergence, but may also follow slightly different trajectories upon closer examination. Convergent evolution of a trait should not be considered as being entirely present or not present, but rather a mixture of contingent and similar evolutionary processes acting at the level considered.

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List of Abbreviations

CPM: counts per million mapped reads
DE: differential expression or differentially expressed
DEG: differentially expressed gene
HP: high predation
LP: low predation
SNP: single nucleotide polymorphism

Introduction

Convergent evolution refers to the independent evolution of similar phenotypes in different lineages (Darwin 1859). Nonconvergent or divergent evolution refers to the evolution of different traits in lineages and is thought to have four common causes (Kaeuffer et al. 2012):

1. *Ecological*: different lineages might actually experience slightly different selective pressures beyond the main one used to categorize the environment (Leal et al. 2002, Siwertsson et al. 2013).
2. *Genetic*: different genetics among lineages can generate different phenotypes (Blount et al. 2008).
3. *Functional*: performance may be the same among different phenotypes from different lineages, generating convergence in function, but not phenotype (McGee and Wainwright 2013).
4. *Sexual*: sexual selection may be different among lineages and can oppose what one would predict based on natural selection alone (Alexander and Breden 2004).

Examples of convergence abound (Dawkins 1986, Colosimo et al. 2005, Gross et al. 2009, Nosil et al. 2009, Colombo et al. 2013, Westram et al. 2014, Baumgarten et al. 2015), but a lack of convergence can also inform researchers about the evolutionary process. For example, it can provide information about the effects of contingency on the outcome of evolution (Travisano et al. 1995, Blount et al. 2008). Additionally, knowing whether phenotypes and their underlying genetics have evolved similarly or dissimilarly

can help determine the repeatability of evolution (Conte et al. 2012). The proposed studies will test the conditions under which color pattern evolution is similar and the prevalence of convergence or nonconvergence/divergence.

Study System

The guppy (*Poecilia reticulata*) is a small live-bearing fish found in the rivers of Trinidad. Guppies have colonized three main independent drainages containing numerous rivers across northern Trinidad. Across drainages, fish have been diverging from one another for 0.1–1.2 million years (Fajen and Breden 1992). Within drainages, upstream guppies in different rivers have been evolving independently from one another. Some gene flow can occur between different rivers, especially between downstream sites, but most rivers remain genetically divergent from one another (Willing et al. 2010). Guppies from downstream communities co-occur with many predators, hence are referred to as high predation (HP) communities. Guppies from headwater streams and above barrier waterfalls live with few other species and experience reduced predation, hence are referred to as low predation (LP) communities. HP guppies differ from LP guppies in a diversity of attributes, including life-history, morphological, and behavioral traits (Reznick and Endler 1982, Endler 1995, Reznick and Bryga 1996). HP guppies have occasionally crossed barrier waterfalls to colonize LP sites (Willing et al. 2010), after which these guppies evolve and acquire LP phenotypes. In addition to colonizing natural streams, HP guppies have been introduced into previously guppy-free LP environments multiple times to examine evolution in action. After introduction, fish have evolved traits

similar to natural LP guppies (Endler 1980a, Reznick et al. 1990, Reznick et al. 1997). The similarities among LP guppies from multiple independent sites are a classic example of convergence, but other studies have found evidence of phenotypic nonconvergence (Kemp et al. 2009, Fitzpatrick et al. 2014).

One key and highly variable morphological trait is ornamental male coloration. Males exhibit a wide variety of genetically determined color patterns (Brooks and Endler 2001) that vary within and between sites (Fig. I.1), while females exhibit no ornamental coloration. However, there can be similarity in the color patterns of males from different sites (Fig. I.2). The size, area, and number of spots on the body are known to be greater in LP sites (Endler 1995). Surprisingly, convergence or nonconvergence in tail coloration has not been examined even though it has long been known to be highly color polymorphic (Winge 1927) and plays a key role in courtship displays (Farr 1980). Tail coloration has many advantages over body coloration for studies of gene expression. For example, tail epidermis is easy to separate from other tissues and displays an even greater range of coloration (from no coloration to being completely colored) than the body. Color differences in the tail are most likely due to regional changes in gene expression, as guppies with no tail coloration still have body coloration. Therefore, I adopt a whole transcriptome approach.

There are over 100 color genes annotated in the guppy genome (Table I.1). The main color pathways produce black, orange/yellow, or iridescent (structural) coloration. Orange/yellow color in guppies includes pteridines, which tend to be red and are synthesized, and carotenoids, which tend to be yellow and are acquired through the diet

(Grether et al. 2001). Carotenoids are located in the same color containing cells as pteridines (Kottler et al. 2014), so genes involved in the development of those cells are relevant to carotenoids.

Guppies are an ideal system to test for molecular convergence or nonconvergence. Guppies have traditionally been used to study phenotypic evolution (Endler 1995), with recent research focused on identifying the number, name, and function of genes (Kottler et al. 2013, Kottler et al. 2015, Sandkam et al. 2015), especially in the context of convergent evolution (Fraser et al. 2015). Although genes underlying convergence have been identified in other systems (Miller et al. 2007, Hohenlohe et al. 2010, Reed et al. 2011, Colombo et al. 2013), guppies are among the few species (Vignieri et al. 2010, Baumgarten et al. 2015) where the evolutionary significance of the trait (i.e. polymorphic tail coloring) has been clearly demonstrated (Olendorf et al. 2006, Hughes et al. 2013). This is in contrast to other well-characterized systems, where the adaptive advantage of the trait is unclear (Sucena et al. 2003, Prud'Homme et al. 2006, Le Rouzic et al. 2011, Greenwood et al. 2012).

Dissertation Chapters

In Chapter one, I examine the phenotypic and genetic trajectory of coloration in male guppies. The aims of the chapter are to compare phenotypic trajectories between males from different predation regimes to ensure that they are identical and then examine the genetic association between coloration and its underlying gene expression. I also identify the ideal developmental stage to sample male tails. Results indicate that high and

low predation males have conserved sequences of color development, making it possible for future chapters to accurately compare males across predation regimes as long as they are in the same stage. The results also show that only the appearance of black coloration is concurrent with the expression of the underlying synthesis genes producing it. Males in the early stages of color development were chosen as the optimal stage to sample for future work.

In Chapter two, I establish sex-biased differences in color gene expression. The goal of the chapter is to determine whether males with ornamental coloration express color genes at different levels than females with no ornamental coloration. The results show that males consistently express color genes at higher levels than females, especially in melanin-related genes. The genes identified in this chapter are subsequently used as candidates for being importantly involved in color variation.

In Chapter three, I determine if there is tail color convergence between males from a high predation location and males that were introduced into four previously guppy-free low predation locations. I also examine gene expression patterns and the fixation of single nucleotide polymorphisms (SNPs) among all locations. The low predation Introduction males evolved to have similar color patterns to one another, but patterns within these locations also exhibit unique phenotypic trajectories. There are no significantly differentially expressed genes between the high predation location and any Introduction location, but there are fixed color gene SNPs with partial genetic convergence in the Introduction locations.

In Chapter four, I take a directed approach to examining evidence for convergent evolution. I sample individuals at multiple, genetically diverged locations to identify color patterns similarities and differences. I then select individuals with similar color patterns and ask whether the associated genetics are the same among locations. Across drainages, there is much more pattern similarity than expected by chance, but this signal differs at individual locations. There is at least one color gene more highly differentially expressed by colored compared to uncolored individuals in three of the five locations tested, but no overlap in the identity of these genes among locations.

These dissertation chapters integrate the identification of genes of interest with an analysis of the genetics associated with color pattern evolution. Fundamentally, this research examines the predictability of evolution (Conte et al. 2012, Bailey et al. 2015) and concludes that evolution may be predictable on higher levels of biological organization, but is frequently unpredictable upon closer examination. Therefore, both similar and unique processes are important in the study of trait evolution.

Figures

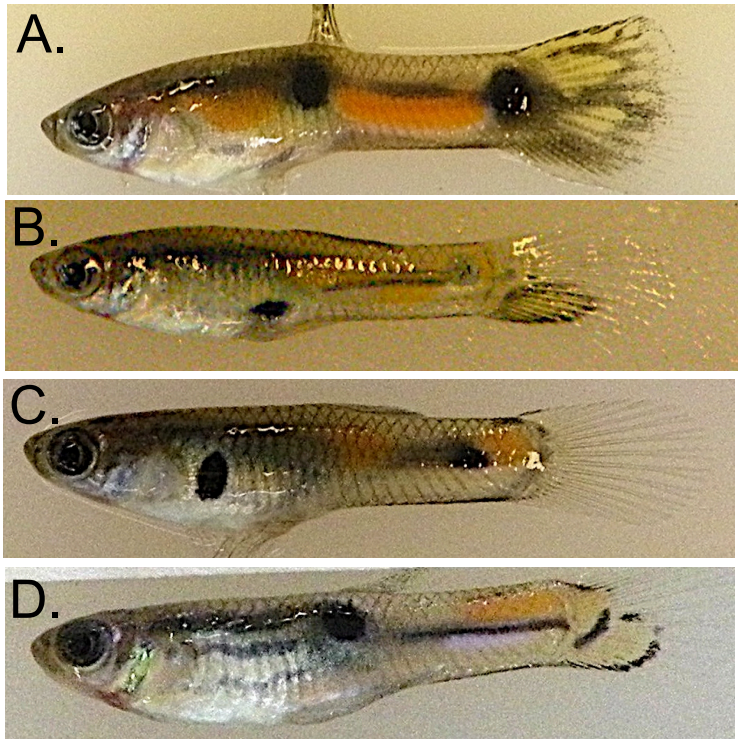

Figure I.1. Male guppy tail color variation. Tail color in guppies can vary between (A,B) and within (C,D) sites.

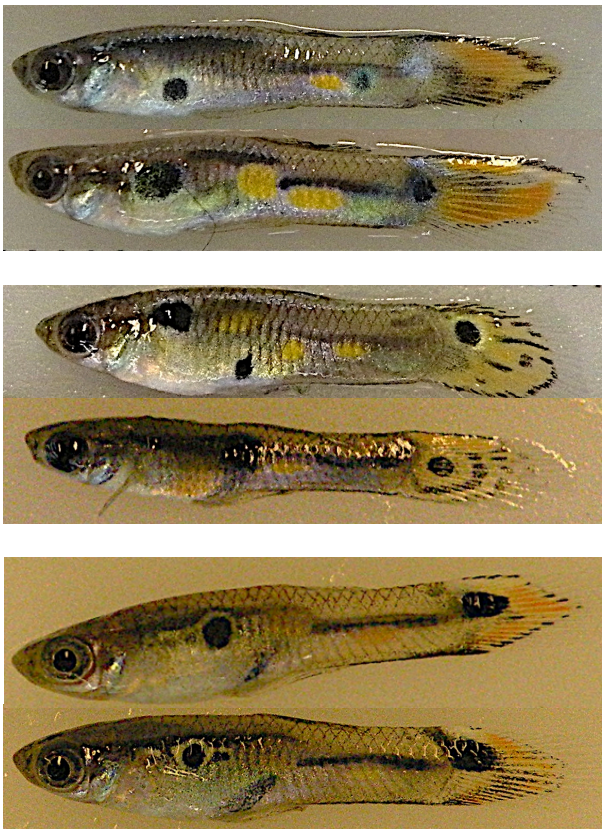

Figure I.2. Male guppy color pattern similarity across sites. Three different color patterns are shown. Each color pattern is represented by photos of fish from different sites.

Table I.1. Color genes annotated in guppies that were of special interest for all dissertation chapters. Reference lists where the identity of the color gene was described.

Gene ID	**Reference**
abhd11	Braasch et al. 2009
adamts20	Braasch et al. 2009
ap3b1	Braasch et al. 2009
ap3d1	Braasch et al. 2009
apc	Braasch et al. 2009
asip	Braasch et al. 2009
atoh7	Braasch et al. 2009
atox1	Braasch et al. 2009
atp6ap1	Braasch et al. 2009
atp6ap2	Braasch et al. 2009
atp6v0d1	Braasch et al. 2009
atp6v1e1	Braasch et al. 2009
atp6v1h	Braasch et al. 2009
atp7a	Braasch et al. 2009
atrn	Braasch et al. 2009
bloc1s3	Braasch et al. 2009
creb1	Braasch et al. 2009
csf1r	Braasch et al. 2009
dct	Braasch et al. 2009
drd2	Braasch et al. 2009
dtnbp1	Braasch et al. 2009
ebna1bp2	Braasch et al. 2009
ece1	Braasch et al. 2009
eda	Braasch et al. 2009
edn3	Braasch et al. 2009
egfr	Braasch et al. 2009
erbb3	Braasch et al. 2009
fgfr2	Braasch et al. 2009
fig4	Braasch et al. 2009
foxd3	Braasch et al. 2009
frem2	Braasch et al. 2009
fzd4	Braasch et al. 2009
gch1	Braasch et al. 2009
gchfr	Braasch et al. 2009
gja5	Braasch et al. 2009

gpc3	Braasch et al. 2009
gpnmb	Braasch et al. 2009
gpr143	Braasch et al. 2009
gpr161	Braasch et al. 2009
hps1	Braasch et al. 2009
hps3	Braasch et al. 2009
hps4	Braasch et al. 2009
hps5	Braasch et al. 2009
hps6	Braasch et al. 2009
ikbkg	Braasch et al. 2009
itgb1	Braasch et al. 2009
kcnj13	Braasch et al. 2009
kit	Braasch et al. 2009
kitlg	Braasch et al. 2009
lef1	Braasch et al. 2009
lyst	Braasch et al. 2009
mbtps1	Braasch et al. 2009
mc1r	Braasch et al. 2009
mcoln3	Braasch et al. 2009
mgrn1	Braasch et al. 2009
mitf	Braasch et al. 2009
myc	Braasch et al. 2009
mycbp2	Braasch et al. 2009
myo7a	Braasch et al. 2009
nsf	Braasch et al. 2009
oca2	Braasch et al. 2009
pabpc1	Braasch et al. 2009
paics	Braasch et al. 2009
pax3	Braasch et al. 2009
pax7	Braasch et al. 2009
pcbd1	Braasch et al. 2009
pcbd2	Braasch et al. 2009
pmel	Braasch et al. 2009
pts	Braasch et al. 2009
qdpr	Braasch et al. 2009
rab27a	Braasch et al. 2009
rab32	Braasch et al. 2009
rab38	Braasch et al. 2009

rabggta	Braasch et al. 2009
rpl24	Braasch et al. 2009
rps20	Braasch et al. 2009
scarb2	Braasch et al. 2009
skiv2l2	Braasch et al. 2009
slc24a4	Braasch et al. 2009
slc24a5	Braasch et al. 2009
slc45a2	Braasch et al. 2009
snai2	Braasch et al. 2009
sox10	Braasch et al. 2009
spr	Braasch et al. 2009
tfap2a	Braasch et al. 2009
tpcn2	Braasch et al. 2009
trim33	Braasch et al. 2009
txndc5	Braasch et al. 2009
tyr	Braasch et al. 2009
vps11	Braasch et al. 2009
vps18	Braasch et al. 2009
vps33a	Braasch et al. 2009
vps39	Braasch et al. 2009
wnt1	Braasch et al. 2009
wnt3a	Braasch et al. 2009
xdh	Braasch et al. 2009
zic2	Braasch et al. 2009
bcmo1	Walsh et al. 2012
scarb1	Walsh et al. 2012
stard3	Walsh et al. 2012
stard5	Walsh et al. 2012
star	Walsh et al. 2012
mlana	Sharma et al. 2014
cxcl12	Svetic et al. 2007
igsf11	Eom et al. 2012
mmp2	Ellis & Crawford 2006

Chapter 1

The Developmental and Genetic Trajectory of Coloration in the Guppy

Abstract

Examining the association between trait variation and development is crucial for understanding the evolution of phenotypic differences. Male guppy ornamental caudal fin coloration is one trait that shows a striking degree of variation within and between guppy populations. Males initially have no caudal fin coloration, then gradually develop it as they reach sexual maturity. For males, there is a trade-off between female preference for caudal fin coloration and increased visibility to predators. This trade-off may reach unique endpoints in males from different predation regimes. Caudal fin coloration includes black melanin, orange/yellow pteridines or carotenoids, and shimmering iridescence. This study examined the phenotypic trajectory and genetics associated with color development. We found that black coloration always developed first, followed by orange/yellow, then iridescence. The ordering and timing of color appearance was the same regardless of predation regime. The increased expression of melanin synthesis genes correlated well with the visual appearance of black coloration, but there was no correlation between carotenoids or pteridine synthesis gene expression and the appearance of orange/yellow. The lack of orange/yellow coloration in earlier male caudal fin developmental stages may be due to reduced expression of genes underlying the development of orange/yellow xanthophores.

Introduction

Selection acts on heritable variation in the phenotype, but phenotypic variation is necessarily due to changes in the underlying developmental process (Atchley and Hall 1991). For example, regional and developmental alterations to the expression level of the *Agouti* gene caused subspecies of *Peromyscus polionotus* beach mice to shift the location of pigment production and mainland mice to have partially colored hair (Manceau et al. 2011). Thus examining how trait variation develops is crucial for understanding the evolution of phenotypic variation (White 2001). Of particular interest is the striking degree of morphological variation seen within and among species. Examples include trichomes in *Drosophila* species (Stern 1998, Sucena and Stern 2000), reduction in armor plates and the pelvic girdle in sticklebacks (Colosimo et al. 2005, Chan et al. 2010), limb length in *Anolis* lizards (Losos et al. 1997), and beak size in Darwin's finches (Lamichhaney et al. 2016). With these and other examples, it was crucial to determine the point when developmental trajectories start to diverge to fully make a connection between genotype and phenotype. In addition, it is important to determine developmental changes in gene expression because the appearance of a trait need not indicate when the genes producing that trait are active (Rodriguez-Trelles et al. 2005).

Color pattern is a morphological character that often changes throughout ontogeny, such as cryptic coloration in fawns that is lost in adult deer (Hiller et al. 2008) or showy plumage in songbirds that is only displayed when males reach sexual maturity (McGraw et al. 2005). Coloration is a complex trait formed by multiple component color types that interact to determine the final color pattern. In vertebrates, color-containing

cells called chromatophores develop from the neural crest (Sauka-Spengler and Bronner-Fraser 2006). Neural crest cells are multipotent, migratory cells that lead to a diversity of cell lineages such as craniofacial cartilage and bone or peripheral neurons and glia (Parichy 2006). In vertebrates, the precursor cells destined to contain color differentiate into one of several types of chromatophores. Chromatophores include dark melanophores, orange or yellow xanthophores, and silver iridophores (Braasch et al. 2009). Xanthophores can develop from neural crest cells or from progenitors derived from the neural crest, whereas melanophores and iridophores develop from migratory cell progenitors (Nüsslein-Volhard and Singh 2017). Variation in color appearance might, therefore, reflect variation in development of the chromatophores or variation in the production of pigment.

Trinidadian guppies (*Poecilia reticulata*) are an ideal system for examining color development over time because males exhibit a wide variety of genetically determined ornamental color patterns (Brooks and Endler 2001) that represent a trade-off between mating success and predation risk. Sexual selection favors brightly colored males while natural selection favors less colorful males (Endler 1983). The interplay between these two factors can cause coloration to vary among predator communities (Endler 1980). Independent of predator community, male color varies within and between sites, while females exhibit no ornamental coloration. Both genetic and environmental effects influence male coloration at a site. For example, male coloration is genetically correlated with female preference for the trait (Houde and Endler 1990) and faithfully passed from father to son in certain sites (Haskins et al. 1961a). Site-specific factors such as nutrient

availability (Endler 1993) or light environment (Grether et al. 1999, Grether et al. 2001) can also cause differential evolution of coloration. Color varies within the lifetime of a male, as males initially have no ornamental coloration and then gradually develop it as they mature. In particular, the development of caudal fin coloration is important as the caudal fin is highly color polymorphic (Winge 1927) and plays a key role in courtship displays (Farr 1980). The evolutionary significance of caudal fin coloration has been previously demonstrated (Olendorf et al. 2006, Hughes et al. 2013) as caudal fin coloration has an important role in negative frequency dependent selection. The more rare of two color types (either colored or uncolored caudal fins) has higher short-term survival (Olendorf et al. 2006) and reproductive success (Hughes et al. 2013) than the more common type. The result is that both the presence or absence of coloration, and likely its diversity, persists in natural guppy populations.

There are major color cell types found in interconnected genetic networks with >100 total known genes involved in producing black, orange/yellow, and iridescent coloration. Black and orange/yellow colors are pigment-based, while iridescent coloration is structural and composed of reflective guanine platelets (Braasch et al. 2008). These known genes have various functions such as the development of chromatophores, regulation of coloration, or pigment synthesis (Braasch et al. 2009). Males with no caudal fin coloration still have body coloration once sexually mature. Therefore, color differences in the caudal fin may be due to regional changes in gene expression and factors acting either cell autonomously or cell nonautonomously. For example, factors could act autonomously within a precursor color cell to promote its development (Parichy

and Tumer 2003) or could interact nonautonomously with other cells in the extracellular environment (Parichy 2006). In guppies, calendar age is an unreliable indicator of sexual maturation as development varies widely even among fish from the same litter (Reznick 1990). Fortunately, development of the gonopodium is highly stereotyped (Reznick 1990) and can be used as an index of maturation. In juvenile guppies, the male and female anal fins are identical. When male guppies start to mature three fin rays begin to elongate, transforming the anal fin into an intromittent organ, the gonopodium, used to transfer sperm during internal fertilization.

The first goal of this study is to examine the phenotypic development of coloration over time between individuals differing in predation regime. The second goal is to determine the gene expression patterns associated with the development of coloration in individuals from the same predation regime. We study gene expression to identify candidate genes so further investigations can take a targeted approach to examining the causal genetic changes. The phenotypic and gene expression studies were undertaken in fish exposed to common laboratory conditions to determine the role of genetics in coloration. Color is known to differ among predation regimes (Endler 1980a). Fish from high predation sites also mature at an earlier age so the correlation between maturity level and color presence could vary among predation regimes. However, we anticipate that the developmental timing of each color will be conserved between predation regimes. We also hypothesize that the development of each specific color happens in identical sequence among individuals, with minimal overlap of color appearance. Relative amount of color is known to differ among predation regimes

(Endler 1980). Fish from high predation sites also mature at an earlier age so the correlation between maturity level and color presence could vary between predation regimes. However, the process of maturation, once begun, is highly stereotyped and similar regardless of predation regime (see details below). As such, we predict that color development will be similar between predation regimes both in terms of relative timing and the sequence of color production. We expect genes involved in cell development, such as transcription factors, will be expressed earliest followed by genes directly involved in the synthesis of each color. This is because the specialized color containing cells should be present prior to the synthesis of the color within that cell. Furthermore, we hypothesize that the visual appearance of a specific color will be synchronous with the expression of the genes producing that color.

Materials and Methods

Time Series Analysis of Color Development

The anal fin of male guppies undergoes a complex metamorphosis that takes approximately 21 days (Reznick 1990). The metamorphosis is well correlated with the development of the gonadotrophic zone of the brain and maturation of the testes (Turner 1941, Kallman and Schreibman 1973, Schreibman and Kallman 1977). During metamorphosis, the number of segments on the third ray of the anal fin increases from about 10 to 32, so segment number is a numerical index of development. Mature males tend to have segment numbers ≥ 27. As the segments form, the anal fin passes through morphologically distinct points that can also be used as a qualitative index of

development. Male coloration begins to develop at the same time, making anal fin metamorphosis an objective external indicator of the maturation process that can be aligned with color development (Reznick 1990).

Guppies were collected from a high predation (HP) and a low predation (LP) site in the Guanapo River in the Northern Range Mountains of Trinidad. Fish were bred for two generations in the laboratory at University of California, Riverside (UCR). Fish were reared on a 12:12 hour day:night lighting schedule with overhead fluorescent lights. All fish were fed *ad libitum* on identical diets of wet fish food (Community Crave, Xtreme Aquatic Foods, FL) in the mornings and brine shrimp in the afternoons. Any manipulations of the fish were performed in the morning hours to control for differences in light environment on coloration. During Summer 2015, immature males with no color present were taken from the lines and housed in individual tanks. Approximately 2-3 times per week, males were anaesthetized in MS-222 and examined under an Olympus SZ Stereo Microscope (Tokyo, Japan) under 4× magnification. The number of segments on the anal fin was taken as an average of three separate counts and the presence of black, orange/yellow, and iridescent coloration on the caudal fin was recorded. Once all colors were present, males were put back into their original tanks. Data were collected for 18 HP males from April-August 2015 and 23 LP males from June-September 2015.

We used a mixed effect model to test whether the number of anal fin segments present when a color first appeared depended on predation regime and the type of color. Since HP males mature earlier than LP males and the predators of HP males impact color evolution, it is possible that color development may exhibit heterochrony between HP

and LP males (i.e. whole pathways moved in time; Gould 2002). However, as noted in the Introduction, we do not expect there to be a differences between predation regimes when development is scaled to the anal fin. For analyses, data were simplified to only include the number of anal fin segments when each of the three color categories was first observed. Number of anal fin segments was log-transformed to meet assumptions of normality. Since there were slight differences in the time at which measurements were taken for HP and LP fish, the day when each male had ≥ 20 segments was included as a covariate. Data were tested in R version 3.1.3 (R Core Development Team) with lmer in lme4 (Bates et al. 2015). Log segment number was the dependent variable with predation regime, color, and day an individual had ≥ 20 segments as fixed effects and fish ID as the random effect. No interaction was included between predation regime and color since preliminary analyses indicated it was not a significant term. Bootstrapped 95% confidence intervals were calculated using bootMer in lme4 as were parametric bootstrapped p-values. For simplicity, figures show effect estimates and confidence intervals with the log transformation reversed.

Genetic Sampling and RNA Extractions

Male and female guppies were collected from the same HP site described above and crosses were performed to generate two different iso-male lines. We used the HP location fish for this analysis because HP fish produce the large broods required for pooled sampling. Caudal fin color elements are inherited as a single trait that segregates with the sex-determining gene (Haskins et al. 1961a) so all sons develop the same color

pattern and can be combined when tissue from a single individual is limited. Fish were bred in the laboratory at UCR for two generations to reduce environmental effects. Male caudal fins were sampled at three different stages of color development that also correlated with the development of the male's anal fin (Fig. 1.1). As the anal fin develops into the gonopodium, it passes through distinct points that were used to standardize sampling. Stage 1 males had no caudal fin coloration present as rays 3-5 of their anal fins began elongating (immature). Stage 2 males had early developing caudal fin coloration with further elongation of rays 3-5 into a butter knife shape or with early formation of the tip of the gonopodium (maturing). Stage 3 males had late developing caudal fin coloration with the tip of the gonopodium fully formed (mature). Males were anaesthetized in MS-222 and 10 caudal fins from sibling males at the same developmental stage were removed, combined into a single sample, and frozen in liquid nitrogen. Prior research indicated that tissue from 10 caudal fins was needed for sufficient RNA yield. This was done for each stage and line to yield two biological replicates per stage-each composed of a pool of 10 individuals-for a total of six samples. After caudal fin removal, males were immediately sacrificed in a lethal dose of MS-222 with no recovery in between. Samples were stored at -80°C until RNA extraction.

Caudal fins were homogenized in Trizol (Invitrogen, Carlsbad, CA) using a Tissue Tearor (BioSpec Products, Bartlesville, OK) and total RNA was purified using a Qiagen RNeasy Mini Kit (Valencia, CA). RNA was treated with TURBO DNase (Ambion, Carlsbad, CA) and quantified with a Qubit 2.0 Fluorometer (Invitrogen). RNA

quality was assessed with an Agilent Bioanalyzer (Santa Clara, CA). All samples had RNA Integrity values ≥ 8.8.

Illumina Sequencing

Libraries were prepared by the University of California, San Diego Institute for Genomic Medicine using the Illumina TruSeq kit v2 (San Diego, CA). Procedures followed the manufacturer's recommendations, with each sample receiving a unique barcode. Samples were pooled into equimolar amounts and sequenced on two lanes of an Illumina HiSeq2500 at UCR using 100 bp single-end sequencing. Each sample yielded between 19.3-52.7 million raw reads (Table 1.1). Raw sequence reads have been deposited in NCBI's Sequence Read Archive (Accession: SRP108073).

Quality Control, Alignment, Read Counting and Differential Expression

Raw RNA-seq reads were cleaned using the fastq_quality_filter tool of the FASTX-Toolkit (Hannon Lab, Cold Spring Harbor Laboratory, NY). Reads were required to have a Phred +33 quality score of at least 20 in 100% of bases. Retained reads had residual adapter sequences trimmed using Trimmomatic 0.20 (Bolger et al. 2014). The first 12 bps of each read were then removed after not passing several criteria in the report taken from the FastQC tool (Babraham Institute, Cambridge, UK). All reads were required to have a minimum length of 50 bps or were discarded. This left 15.3-41.0 million cleaned reads per sample (Table 1.1).

Reads passing all quality control filters were aligned to the annotated guppy genome (Kunstner et al. 2016); NCBI accession GCF_000633615.1) using TopHat2 (Trapnell et al. 2009). Default options were used except the number of threads was four and the minimum intron size was 50 bps. The BAM files output by TopHat2 were sorted by sequence name and converted to SAM files. Read counts for each gene were generated using ht-seqcount in the union mode (Anders et al. 2014).

Analysis of differential expression was performed using DESeq2 (Anders and Huber 2010) in R. Samples were grouped according to stage so that the two different lines were considered biological replicates. Although fish chosen for each line had slight variations to caudal fin coloration, adult males of both lines had all color types present. Grouping the fish this way allowed for color-specific developmental genes to be detected, rather than genes specific to individual pattern elements (i.e. spot versus stripe). For a gene to be considered for differential expression analysis, it was required to have at least one count per million mapped reads for at least two samples. Contrasts were generated between stages 1-2, 1-3, and 2-3 and differential expression was tested using a false-discovery rate cutoff of 0.05 (Benjamini and Hochberg 1995).

Color Gene Expression Analyses

Genes known to be involved in coloration were extracted from the list of differentially expressed genes (Svetic et al. 2007, Braasch et al. 2009, Eom et al. 2012, Walsh et al. 2012, Ellis and Crawford 2016). Attention was paid to genes that were involved in production of black melanin or orange/yellow pteridine pigments. These

genes were listed by Braasch et al. (2009) under the headings of "pteridine synthesis" or "components of melanosomes". Genes involved in carotenoid coloration were also extracted. Although Walsh et al. (2012) described 11 carotenoid genes, only six of those were located in the guppy genome. Individual gene expression levels in figures were calculated from counts per million mapped reads (CPM). To test whether developmental genes were expressed at earlier stages than pigment synthesis genes, genes were categorized by their function (development: xanthophore development + melanophore development or synthesis: pteridine synthesis + components of melanosomes; (Braasch et al. 2009) and the stage at which expression measured as CPM was largest. This analysis was repeated with just melanophore development and components of melanosome genes alone. Iridescence was not included because genes associated with the production of iridescence have not been described. For both tests, a Fisher's Exact Test was performed in JMP Pro (version 12, SAS Institute, Irvine, CA).

Results

Time Series Analysis of Color Development

Black coloration always developed first when males had about 11 segments on their anal fin (Fig. 1.2). This was followed by orange/yellow coloration at about 20 segments then iridescent coloration at about 28 segments (Fig. 1.2). Fish developed orange/yellow and iridescent coloration at significantly later segment numbers than black coloration (Table 1.2). Although Guanapo HP fish developed coloration slightly earlier than Guanapo LP fish (Fig. 1.2), the differences between predation regimes were not

significant (Table 1.2). Therefore, we performed RNA-seq analyses on fish from a single predation regime (HP).

Color Gene Expression Analyses

Of the 105 genes with at least one function in coloration annotated in the guppy genome, 100 met the expression cut-off of at least one count per million in at least two samples. Stage 2 males expressed 96 color genes at detectable levels (> 1 CPM in both replicates), while stage 3 and stage 1 males expressed 95 and 94 color genes, respectively.

Most of the DE color genes were between stages 1 and 3, while stages 2 and 3 were more similar in their gene expression levels. The two genes more highly DE in stage 1 compared to stages 2 and 3 had functions in pteridine synthesis (Table 1.3). The genes more highly expressed in stages 2 and 3 compared to stage 1 were frequently related to melanin or involved in the development of chromatophores (Table 1.3). None of the carotenoid genes were significantly differentially expressed between any of the stage comparisons.

All nine genes (three DE, six non-DE) involved in melanin synthesis steadily increased their expression levels between stages 1-3 (Fig. 1.3A). The average fold expression change in counts per million mapped reads (CPM) between stages 1-3 of the three DE melanin synthesis genes was 21.30. Despite not showing up as DE, the other melanin synthesis genes had an average fold change of 2.57 in stage 3 compared to stage 1. The 10 genes involved in pteridine synthesis (two DE, eight non-DE) had more

variable expression levels among stages (Fig. 1.3B). The non-DE pteridine synthesis genes only had expression levels that were 1.89 times larger in stage 3 compared to stage 1. The two DE pteridine synthesis genes were more highly expressed in stage 1 so had an average fold expression change from stages 1-3 of 0.69. All six of the carotenoid genes were non-DE and had expression levels that were 1.81 times larger in stage 3 compared to stage 1 (not shown).

Since carotenoid and pteridine synthesis genes had expression patterns that were not concurrent with the visual production of the coloration, eight genes involved in the development of the orange/yellow xanthophores were examined. Three genes were DE and had expression levels that were significantly higher in stage 3 compared to stage 1 (Fig. 1.4).

Synthesis genes involved in producing melanin or pteridines were not expressed at significantly later stages than developmental genes involved in making melanophores and xanthophores (Fisher's Exact $p = 0.13$). However, when only considering melanin-related genes, there were significant differences between gene function and stage of highest gene expression (Fisher's Exact $p = 0.0024$). All the melanin synthesis genes were more highly expressed at stage 3, while melanophore development genes had approximately equal numbers of genes with highest expression across stages (stage 1=6 genes, stage 2=10 genes, stage 3=8 genes).

Discussion

Time Series Analysis of Color Development

Among fish, the time course of color development has been most often studied in the zebrafish *Danio rerio* and other *Danio* species (McClure 1999, Parichy 2006, Patterson et al. 2014). Zebrafish have body and caudal fin coloration, but guppy caudal fin coloration most closely parallels zebrafish body coloration. Melanophores and xanthophores in zebrafish caudal fins develop at the same time from a common precursor cell (Tu and Johnson 2010), but black coloration in guppies appeared first. During zebrafish embryogenesis, body melanophores are known to differentiate first at about 24 hours postfertilization (hpf), followed by xanthophores at 42 hpf and then iridophores a few hours later (Kimmel et al. 1995, Ziegler 2003). Other *Danio* species have distinct body color patterns between larval and adult stages, but the transition between stages is marked first by a movement of melanophores followed by variation among species in whether xanthophores or iridophores are next to move (McClure 1999). The order of events in the development of ornamental guppy caudal fin coloration agrees with zebrafish body coloration since black consistently developed prior to orange/yellow and then iridescence (Fig. 1.2). This indicates that the order of color development has been conserved for over 200 million years (Hedges et al. 2015).

The timing and order of color development is also conserved between guppies from high and low predation regimes. From a molecular genetics viewpoint, movement of whole pathways forward and backward in time (termed heterochrony; Gould 2002) can require alterations to the identity of transcription factors that bind or a change in the

expression timing of regulatory molecules (Yanai et al. 2011, Pham et al. 2017). One study in frogs found that heterochronic changes were less likely than changes to gene expression level (termed heterometry; Arthur 2000), especially among developmental genes (Yanai et al. 2011). Differences between predation regimes in color amount were not quantified here since the males used in the phenotypic component needed to have all color types present. However, guppies from different predation regimes can have varying amounts of color presence (Endler 1980). Since there is no developmental timing variation, any color differences between predation regimes could be due to the differences in gene expression levels. In addition, there could be differences in the ultrastructure of the spots between predation regimes, although divergent strains from different predation regimes were previously found to have similar organization of their color cells (Kottler et al. 2014).

Color Gene Expression Analyses

Black melanin coloration

Differences in melanin synthesis gene expression correlated well with the appearance of that coloration. In zebrafish bodies, adult melanophores develop from precursor cells and not from early larval melanophores (Parichy and Spiewak 2015). New development of melanophores would require increased expression of developmental genes and gene expression should be concurrent with the appearance of black in zebrafish adults. The expression patterns for melanin genes that we found for guppies matches this expectation.

Other fish studies have found that melanin-related genes were more highly expressed in color morphs of Midas cichlid with more black pigmentation (Henning et al. 2013) or in dark versus light skin bars of freshwater stickleback (Greenwood et al. 2012), but with no overlap in the specific genes that are differentially expressed. The differentially expressed genes we describe here also represent a different subset of melanin-related genes associated with melanin expression. One exception is the gene *igsf11*, which has been shown to play a role in melanophore stripe formation of adult zebrafish bodies and pigment patterning of adult African cichlid caudal fins (Eom et al. 2012, Ahi and Sefc 2017). This gene was more highly expressed in stage 3 compared to stage 1 male guppies and provides a link to melanin pattern formation across distantly related species (Hedges et al. 2015). Black coloration in guppies confirms the expected association seen between phenotypic trait variation and differential gene expression (Yamamoto et al. 2004, Gompel et al. 2005, Passador-Gurgel et al. 2007, Steiner et al. 2007, Ayroles et al. 2009, Whitehead et al. 2012). The genes we found associated with black melanin coloration are important candidates for future studies aimed at determining the mechanism by which color develops.

Orange/yellow pteridine or carotenoid coloration

Our hypothesis that carotenoid and pteridine synthesis gene expression would correlate with the visual appearance of orange/yellow coloration was not supported. Pteridine synthesis and carotenoid gene expression was more constant across developmental stages than melanin gene expression. In zebrafish, adult xanthophores

derive directly from early larval xanthophores, which lose pigment and then produce it again (Parichy and Spiewak 2015). If this developmental process happened in guppies, we would expect increased expression of synthesis genes, but not developmental genes, when orange/yellow was visually apparent. We saw the opposite pattern since there was a correlation between orange/yellow presence and xanthophore development gene expression. This finding indicates that color development needs to be studied for the fish species of interest, as it may not be identical to the zebrafish model. The three development genes significantly downregulated in stage 1 compared to stage 3 were *csf1r* (*colony-stimulating factor 1 receptor*), *sox10* (*SRY-related HMG-box 10*), and *pax3* (*paired box 3*). Guppies mutant in *csf1r* have almost no xanthophores, no orange skin pigmentation, and disrupted black ornamentation due to alterations in xanthophore-melanophore interactions (Kottler et al. 2013). *Pax3* mutants in zebrafish have a reduction in xanthophores, indicating the gene is involved in early xanthophore specification (Minchin and Hughes 2008). *Sox10* is involved in neural crest cell differentiation and mutants have defects in their peripheral nervous system as well as pigmentation (Dutton et al. 2001). Based on these other studies, we propose that the lack of orange/yellow pigmentation in early stages is associated with lowered expression of those three genes with important functions in xanthophore development. However, some of the xanthophore development genes have multiple functions. We cannot exclude the possibility that their expression levels might be caused by an unrelated factor that is correlated with the appearance of orange/yellow.

Future Directions

By taking previously known color genes and associating them with guppy pattern development, this experiment facilitates further study of the genetics and evolution of guppy coloration. The genes identified here are candidates for involvement in other aspects of coloration. For example, additional experiments are underway to examine sex-biased differences in color gene expression as well as convergence of coloration and associated gene expression in different watersheds.

In future studies, the addition of more biological replicates per stage is likely to identify further DE genes. Our expression analysis program is known to maintain the false positive rate even with two biological replicates (Robles et al. 2012), but it is possible that truly differentially expressed genes were overlooked due to the reduction of statistical power. In addition, the genes of interest identified here can be validated with another technique such as qRT-PCR. Lastly, new color genes are frequently discovered in model fish species, such that future studies can add new genes to the list of candidate color genes.

This experiment also establishes the optimal sampling strategy. Males at stage 2 expressed slightly more color genes than stage 1 or 3 males. Stage 2 males also have anal fins that are still developing, making it possible to more easily standardize the sampling. The anal fins of stage 3 males represent the endpoint of anal fin metamorphosis so it is not possible to distinguish between a male that has just matured and one that matured weeks ago. Future studies examining genes associated with coloration will therefore focus on sampling males at stage 2.

Conclusion

Our findings support the hypothesis that appearance of color in male guppy caudal fins happens in identical sequence between HP and LP fish. In addition, there was minimal overlap in the appearance of each of the three main color types tested (black, orange/yellow, iridescence). Our hypothesis that developmental genes would be expressed earlier than genes involved in the production of melanin was only partially supported. All melanin synthesis genes were more highly expressed in stage 3, but developmental genes were not all more highly expressed in stage 1. Different developmental genes reached their peak expression at approximately equal numbers across the three stages. Lastly, we found that with black coloration, the visual appearance of the color correlated with the expression of the melanin synthesis genes. This was not true with orange/yellow coloration and pteridine synthesis or carotenoid gene expression. We instead propose that the lack of orange/yellow coloration in stage 1 may be due to downregulation of genes needed for development of the xanthophores that eventually contain the pteridines and carotenoids. This study confirms that trait appearance does not necessarily indicate that the genes producing the trait are highly expressed compared to other developmental time points. Gene expression is expected to precede the phenotype in certain cases such as limb development (Tabin 1992), but may be expressed outside of the expectation for other traits. This occurred in the present study and in other examples of coloration where a phenotype is superimposed on an already existing structure (Aymone et al. 2013, Martin et al. 2014, Kronforst and Papa 2015).

Figures

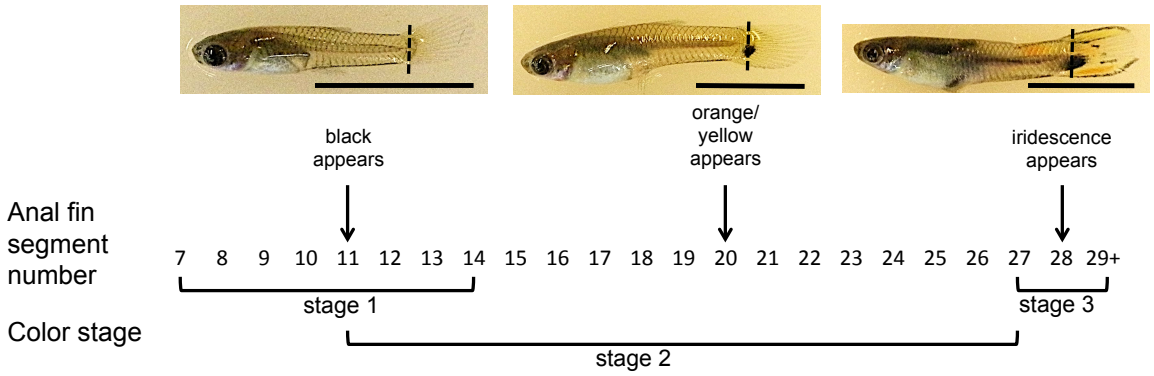

Figure 1.1. Stages sampled for genetic analyses of color gene expression. Anal fin segment number is used as an index of maturity. Color stage refers to the groupings used in genetic samplings. Photographs above text demonstrate a representative male sampled for each of the three stages. Although black may appear around 11 segments, stage 1 fish chosen for genetic sampling did not have any visible color present. Solid black scale bars indicate 1 cm in length. Dashed black lines indicate where the caudal fin would have been dissected.

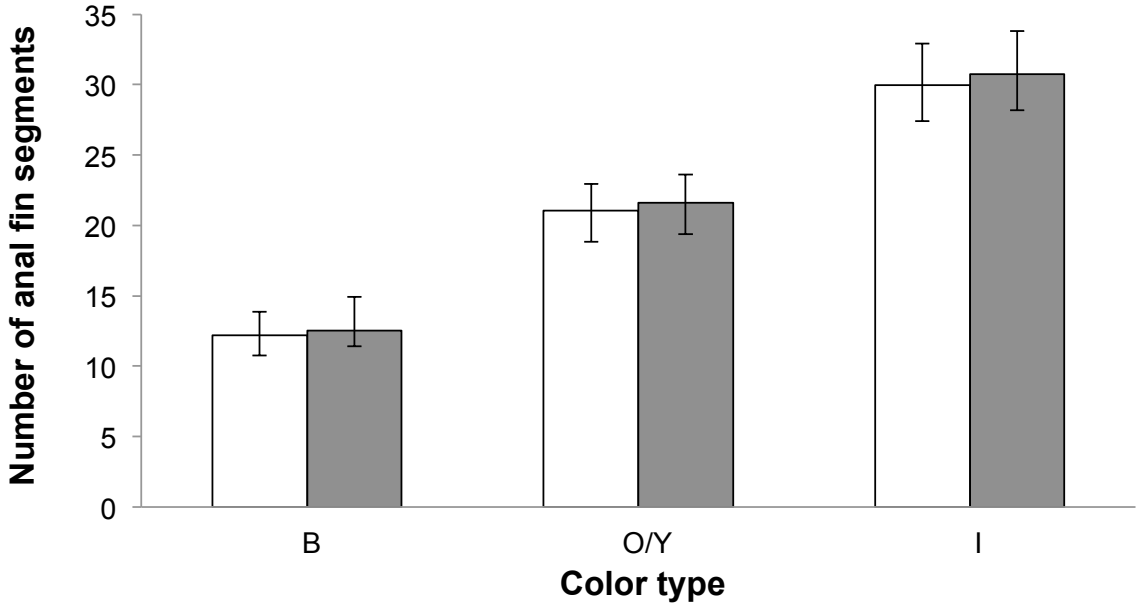

Figure 1.2. Number of segments present on the male anal fin when each color type developed. Segments are shown for black (B), orange/yellow (O/Y), and iridescent (I) coloration in males from Guanapo HP (white) and Guanapo LP (gray). Bars represent 95% confidence intervals.

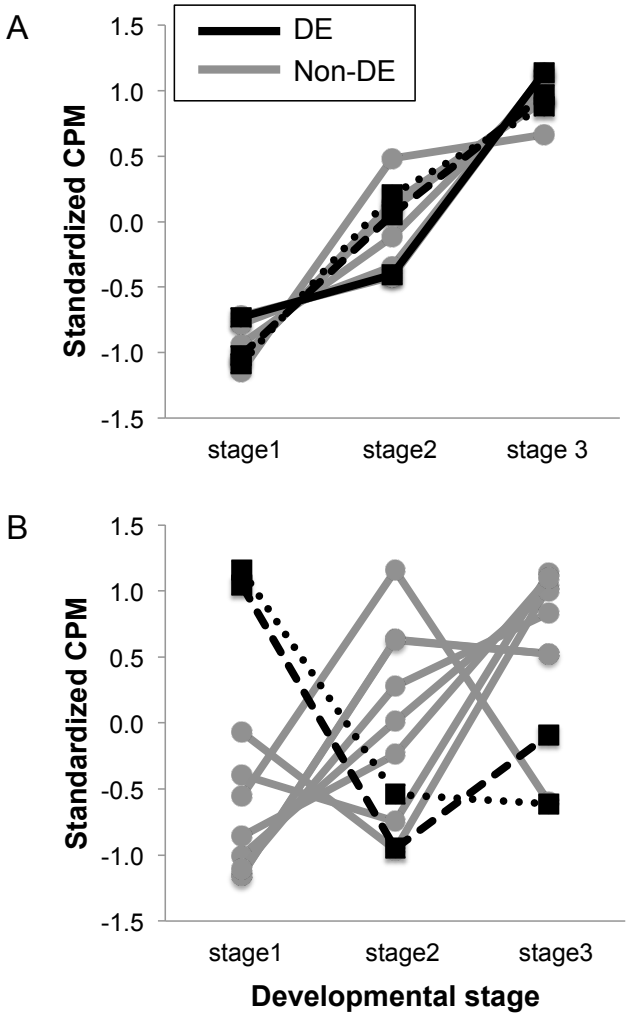

Figure 1.3. Standardized counts per million mapped reads (CPM) of pigment production genes across developmental stages. A. Melanin production genes. B. Pteridine synthesis genes. Genes in gray were not differentially expressed (DE) in the expression analysis, while genes in black were DE in the analysis. Standardization of expression levels was performed so all genes could be graphed on the same axis and was calculated as: stage_expression_level - mean_expression_among_stages / standard_deviation_among_stages.

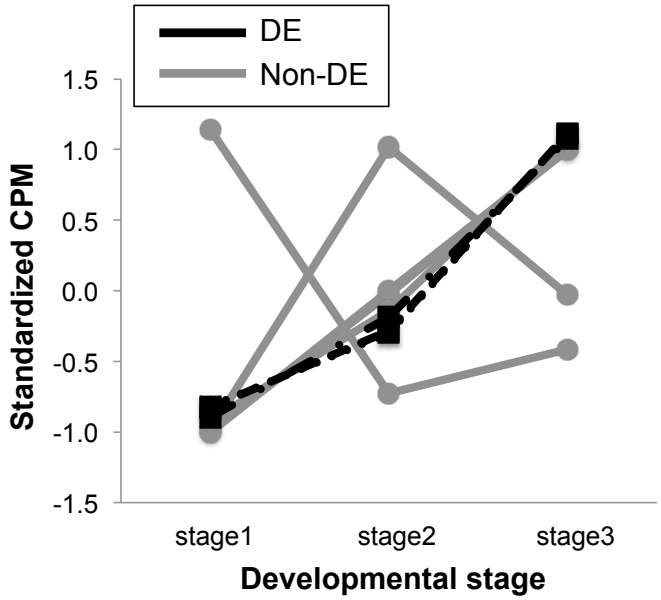

Figure 1.4. Standardized counts per million mapped reads (CPM) of xanthophore development genes across developmental stages. Genes in gray were not differentially expressed (DE) in the expression analysis, while genes in black were DE in the analysis. Standardization of expression levels was calculated as in Figure 1.3.

Tables

Table 1.1. Number of RNA sequencing reads obtained for each sample. Cleaned reads represent the number remaining after quality control filtering. Reads were then mapped to the annotated guppy genome.

Line	Replicate	Stage	Raw reads	Cleaned reads	Mapped reads (%)
GH4	1	1	27,996,643	21,639,979	19,885,247 (91.9)
GH5	2	1	52,744,726	41,043,905	37,508,187 (91.4)
GH4	1	2	19,318,402	15,314,393	13,943,457 (91.0)
GH5	2	2	33,937,307	26,476,819	24,218,195 (91.5)
GH4	1	3	48,974,057	38,325,918	34,946,982 (91.2)
GH5	2	3	30,714,140	24,039,884	21,945,597 (91.3)

Table 1.2. Fixed effects parameter estimates for linear mixed effect model fitted to log-transformed number of segments present on the anal fin.

Fixed effect	**Factor**	**Estimate**	**Standard error**	***t* value**	**p-value**
Intercept		1.086	0.0229	47.51	< 0.001
Predation	HP	0	0	.	.
	LP	0.012	0.0310	0.38	N.S.
Color	Black	0	0	.	.
	Orange/yellow	0.237	0.0196	12.12	< 0.001
	Iridescent	0.391	0.0196	19.96	< 0.001
Day 20 segments		-0.001	0.0004	-2.34	0.026

Table 1.3. Color genes differentially expressed in each stage comparison tested. Gene functions are modified from Braasch et al. (2009) or Eom et al. (2012).

Stages tested	**Gene name**	**Gene function**	**Log_2 fold change**	**Stage higher expressed**	**Corrected p-value**
1 / 2	*qdpr*	Pteridine synthesis	1.296	1	0.013
1 / 2	*gja5*	Uncategorized	1.427	2	0.028
1 / 2	*gpnmb*	Melanin synthesis	1.914	2	0.024
1 / 2	*sox10*	Chromatophore dev.	1.260	2	0.022
1 / 3	*pcdb1*	Pteridine synthesis	1.087	1	0.039
1 / 3	*csf1r*	Xanthophore dev.	1.517	3	0.002
1 / 3	*gja5*	Uncategorized	2.163	3	<0.001
1 / 3	*gpnmb*	Melanin synthesis	3.832	3	<0.001
1 / 3	*igsf11*	Melanin patterning	2.062	3	<0.001
1 / 3	*lyst*	Melanosome construct.	1.377	3	0.042
1 / 3	*mc1r*	Melanin regulation	2.975	3	<0.001
1 / 3	*mitf*	Melanophore dev.	1.566	3	0.005
1 / 3	*pax3*	Melano/Xantho dev.	1.391	3	0.024
1 / 3	*rab38*	Melanin synthesis	1.297	3	0.049
1 / 3	*slc24a4*	Melanin synthesis	1.720	3	0.016
1 / 3	*sox10*	Chromatophore dev.	2.431	3	<0.001
2 / 3	*mc1r*	Melanin regulation	1.839	3	0.007

Chapter 2

Sex-Biased Expression Between Guppies Varying in the Presence of Ornamental Coloration

Abstract

Sex-biased gene expression provides a mechanism to resolve sexual conflict across a genome largely shared by both sexes. Trinidadian guppies are ideal to examine questions of sex-bias as they exhibit sexual dimorphism in ornamental coloration with male only expression. Here we use RNA-sequencing to quantify whole transcriptome gene expression differences, with a focus on differential expression of color genes between the sexes. We determine whether males express gene positively correlated with coloration at higher levels than females. We find that all the differentially expressed color genes were more highly expressed by males. Males also expressed all known black melanin synthesis genes at higher levels than females, regardless of whether the gene was significantly differentially expressed in the analysis. This correlates with the visual color differences between sexes at the stage sampled, as all males had ornamental black coloration apparent. We propose that selection is resolving the sexual conflict over ornamental coloration by favoring male-biased expression.

Introduction

When a trait is beneficial to one sex and detrimental to the other, intralocus sexual conflict results and one or both sexes cannot reach their fitness optimum (Lande 1980). The evolution of sexual dimorphism is one way of resolving the conflict (Pennell and Morrow 2013). However, the capacity for sexual dimorphism is limited since males and females share nearly all of their genome. Different mechanisms can evolve to reduce the negative fitness consequences to one sex during cases of sexual conflict. Genes coding for or regulating the trait could be genetically linked to a non-recombining region of the heterogametic sex chromosome (Kirkpatrick and Hall 2004, Basolo 2006). Alternatively, sex-biased expression can occur, where the gene(s) for the trait are expressed exclusively or more highly by one sex (Ellegren and Parsch 2007). The second mechanism is the focus of this study because it is thought to account for a majority of sexual dimorphism (Ellegren and Parsch 2007, Mank 2009). For example, a study on birds found that sexual selection had a greater impact on gene expression evolution than sequence evolution (Harrison et al. 2015). Similarly, there has been a link between sexual dimorphism and sex-biased expression in dominant and subordinate wild turkeys (Pointer et al. 2013). An analysis of the evolution of complex sexually dimorphic traits requires correlating the identity and functions of sex-biased genes with sex-specific variation.

The Trinidadian guppy (*Poecilia reticulata*) provides an ideal system to examine sex-biased expression on an evolutionarily relevant trait (Sharma et al. 2014). Guppies have an XY sex determination system with males as the heterogametic sex (Winge 1922). The Y chromosome is believed to be relatively young in guppies, as it is cytologically

indistinguishable from the X chromosome (Winge 1927, Wright et al. 2017). Linkage analyses indicate that the Y chromosome has a freely recombining region and two male-specific non-recombining regions, one of which contains the sex-determining locus (Tripathi et al. 2009).

Both male and female guppies have a reticulate black camouflage and light yellow color to their bodies, but only males possess ornamental coloration (Fig. 2.1). The ornament gradually develops on the body and caudal fin as the male matures (Fig. 2.1). Ornamental coloration in guppies represents a trade-off between sexual and natural selection (Endler 1980). Sexual selection favors bright coloration, while natural selection by diurnal, visually oriented fish predators favors duller coloration. Early studies determined that ornamental color pattern elements have a variety of linkage patterns (Winge 1927, Haskins and Haskins 1951). Many of these were Y-linked, although Y linkage can vary among geographic sites. For example, high predation fish have more Y-linked body coloration, while low predation fish have more autosomal and X linkage (Haskins et al. 1961b, Gordon et al. 2011). These results could be due to selection on standing genetic variation for non-Y-linked color or increased recombination rates between X and Y chromosomes in low predation fish (Gordon et al. 2011).

To separate expression of sex-specific ornamental versus non-sex-specific coloration, tissue from the caudal fin is used here. The caudal fin is the only skin location where there are reliable sex-specific differences and a lack of camouflage coloration that would otherwise prevent the detection of sex-specific ornamental coloration. Caudal fin coloration is used in courtship displays (Farr 1980) and is subject to both natural and

sexual selection (Olendorf et al. 2006, Hughes et al. 2013). Although body coloration extends slightly into the base of the caudal fin of both sexes, females largely have clear caudal fins.

The goal of this study was to characterize sex-biased expression in guppy caudal fin color genes. Ornamental caudal fin coloration in the males includes black melanin, orange/yellow pteridines or carotenoids, and shimmering iridescence. We hypothesize that any sex-biased genes that are positively correlated with coloration will have higher expression in males than females. Since the fish were sampled at a stage when melanin coloration was beginning to appear, we also hypothesize that males will express melanin synthesis genes at consistently higher levels than females.

Materials and Methods

Sampling and RNA Extractions

Guppies were collected from a high predation (HP) site in the Guanapo River in the Northern Range Mountains of Trinidad. Crosses were performed to generate two different iso-male lines. Fish were bred for two generations in the laboratory at University of California, Riverside (UCR; IACUC AUP approval: A-20140003). Male caudal fins were sampled at a time when their anal fins were morphing into the gonopodium, which also correlated with early development of caudal fin coloration. Female fins were also collected, although females lack an independent marker of maturation. Therefore, females were taken from the same litter as the males so were the same age and genetic background. Fish were anaesthetized in MS-222 and 10 caudal fins

from sibling males or females were removed, combined into a single sample per sex, and frozen in liquid nitrogen. This was done for each line for a total of four samples and two biological replicates per sex. Although it is preferable to include more replicates, funding and vivarium space were limited. The differential expression analysis program used (see below) can accommodate two biological replicates and maintain the false positive rate below threshold values (Robles et al. 2012). Therefore, it is more likely that significantly differentially expressed (DE) genes would be overlooked due to reduced power compared to mistakenly calling a truly non-DE gene as DE. After caudal fin collection, fish were sacrificed in a lethal dose of MS-222 with no recovery in between. Samples were stored at -80°C until RNA extraction. The two male samples are the same as obtained during Chapter 1.

Caudal fins were homogenized in Trizol (Invitrogen, Carlsbad, CA) using a Tissue Tearor (BioSpec Products, Bartlesville, OK). Total RNA was purified using a Qiagen RNeasy Mini Kit (Valencia, CA) and then treated with TURBO DNase (Ambion, Carlsbad, CA). RNA was quantified with a Qubit 2.0 Fluorometer (Invitrogen) and integrity was measured with an Agilent Bioanalyzer (Santa Clara, CA). All samples had RNA Integrity values ≥ 7.7.

Illumina Sequencing

RNA-seq libraries were prepared by the University of California, San Diego Institute for Genomic Medicine using the Illumina TruSeq unstranded RNA Library Preparation kit v2 (San Diego, CA). The manufacturer's recommendations were followed

and each sample received a unique barcode. Samples were pooled into equimolar amounts and sequenced on an Illumina HiSeq2500 at UCR using 100 bp single-end sequencing. Each sample yielded between 8.5-33.9 million reads (Table 2.1). Sequence reads have been deposited in NCBI's Sequence Read Archive (Accession: SRP111128).

Quality Control, Alignment, Read Counting and Differential Expression

RNA-seq reads were cleaned using the fastq_quality_filter tool of the FASTX-Toolkit (Hannon Lab, Cold Spring Harbor Laboratory, NY). Reads were required to have a Phred +33 quality score of at least 20 in 100% of bases. Residual adapter sequences were then trimmed using Trimmomatic 0.20 (Bolger et al. 2014). The first 12 bps of each read were removed after not getting passing scores in the FASTQC report (Babraham Institute, Cambridge, UK). Reads with a minimum length less than 50 bps were discarded. This left 6.4-26.5 million reads per sample (Table 2.1).

Cleaned reads were aligned to the annotated guppy genome (Kunstner et al. 2016; NCBI accession GCF_000633615.1) using TopHat2 (Trapnell et al. 2009). Default options were used except the number of threads was four and the minimum intron size was 50 bps. The output BAM files from TopHat2 were sorted by sequence name and converted to SAM files. Read counts for each gene were obtained using ht-seqcount in the union mode (Anders et al. 2014).

Differential expression analysis was performed using DESeq2 (Anders and Huber 2010) in R version 3.1. Although there was variation in the total number of reads mapped per sample, DESeq2 is able to handle varying read counts (Anders and Huber 2010).

Briefly, DESeq2 calculates a size factor, based on sampling depth, for each library and then scales gene counts by this coverage value. It does this prior to performing differential expression estimates. Samples were grouped according to sex so that the two different lines were considered biological replicates. Genes considered for differential expression were required to have at least one count per million mapped reads (CPM) for at least two samples. A contrast was generated between males and females and differential expression was tested using a false-discovery rate cutoff of 0.05 (Benjamini and Hochberg 1995). There was no threshold in $\log_2$ fold changes to call a gene significant, although all DE genes had $\log_2$ fold-change values ≥ 1.1, corresponding to over a two-fold change in expression level.

Genes known to be involved in coloration were the focus and were obtained from multiple citations (Svetic et al. 2007, Braasch et al. 2009, Eom et al. 2012, Walsh et al. 2012, Sharma et al. 2014, Ellis and Crawford 2016). Special attention was also paid to certain melanin, pteridine, and carotenoid genes listed by Braasch et al. (2009) and Walsh et al. (2012). Although three of the carotenoid genes have functions that may preclude expression in the caudal fin (*bcmo1*, *scarb1*, and *scarb2*), they were retained in the analysis. Individual gene expression levels were calculated from counts per million mapped reads.

We performed a Fisher's Exact Test to examine whether the chromosomal distribution of DE color genes was significantly different from the chromosomal distribution of non-DE color genes. For this test, DE genes were scored as being on the X chromosome or not on the X chromosome and then this scoring was repeated for non-DE

color genes. Four genes, all of which were non-DE, were not included in the analysis as their chromosomal positions were unknown.

Results

Males and females had similar percentages of reads mapping to the guppy genome (Table 2.1). The guppy genome contains 26,071 loci and 18,568 met the cut-off imposed for differential expression estimates of at least one count per million in at least two samples. Of the 106 genes with at least one function in coloration annotated in the guppy genome, 102 met the cut-off imposed as above.

Ten of the color genes were differentially expressed (DE) between sexes, with all ten more highly expressed in males (Fig. 2.2, Table 2.2). More than half of these DE color genes are involved in the eventual formation of black melanin pigmentation (Table 2.3) and this correlates with the visual presence of black coloration at the stage sampled. The other four genes had more general, unknown, or pteridine synthesis functions.

There are nine genes exclusively involved in melanin synthesis in the guppy genome. Three of them were DE in the analysis, but all of them consistently had higher expression in males (Fig. 2.3A), which also correlates with the presence of black in male caudal fins only. There was less of a clear trend to whether males or females more highly expressed orange/yellow pteridine synthesis genes, but only one gene was DE (Fig. 2.3B). Orange/yellow carotenoid genes usually had higher expression in males, but none were DE (Fig. 2.3C).

There were no significant differences in the distribution of DE color genes on and off the X chromosome compared to the distribution of non-DE color genes on and off the X chromosome (Fisher's Exact P = 0.1052; DE on X chromosome=2, non-DE on X chromosome=4, DE off X chromosome=8, non-DE off X chromosome=88 genes).

Discussion

Almost all of the color genes tested had annotations in the guppy genome. Since the genome was assembled from a female guppy, these color genes must be X- or autosomal-linked and are therefore candidates for being under sexual conflict. This study has found that the evolution of sex-biased expression in sexually dimorphic guppies is at least one mechanism that can resolve this conflict (Ellegren and Parsch 2007).

Our hypothesis that males with ornamental pigmentation would have increased expression of color genes was supported. Specifically, males had significantly more highly expressed color genes when compared to conspecific females. We predict that these differences in color gene expression promote the formation of male ornamental color patterns and are advantageous during courtship of females. However, there may still be trade-offs between predation risk and sexual selection (Endler 1980a).

There were 10 DE genes (*gja5*, *igsf11, mitf*, *mlana*, *pax7*, *rab38*, *slc45a2*, *sox10*, *tyr*, *xdh*) between males and females, with all 10 more highly expressed by males. Although the *gja5* gene is uncategorized by Braasch et al. (2009), another study found that zebrafish mutants had a reduction of melanophores (Watanabe et al. 2006). *Igsf11* is involved in adult melanin pattern formation and zebrafish mutant in this gene have

defects in the survival and migration of melanophores (Eom et al. 2012). *Mitf* is involved in melanophore development and positively regulates *mlana* (Du et al. 2003), which can both regulate a melanin synthesis gene. *Pax7* is expressed in early xanthophore cells and mutants have reduced yellow pigmentation (Minchin and Hughes 2008). *Rab38* targets a melanin synthesis enzyme to the melanophores (Montoliu et al. 2011). *Slc45a2* and *tyr* have functions in producing melanin and mice mutant in these genes have reduced or absent melanin pigmentation (Montoliu et al. 2011). *Sox10* is involved in differentiation from the neural crest cell and mutants have pigmentation defects in addition to defects in the peripheral nervous system (Dutton et al. 2001). *Xdh* plays an ultimate role in the synthesis of pteridines, specifically yellow sepiapterins (Braasch et al. 2007).

Most of these sex-biased DE genes have general functions in the regulation of melanophores, xanthophores, or neural crest cells. These genes may have more effects than the genes whose only function is to synthesize melanin or pteridines, although future studies could confirm the degree of pleiotropy. Since all females still have melanin camouflage body coloration, regional and tissue-specific differences in gene regulation are clearly important in generating the complex trait that is guppy ornamental coloration. We expect trait variation among color polymorphic males is at least partially explained by regulatory changes in transcription regulators and developmental genes.

Expression differences between guppy males and females in our study were especially apparent in black melanin related genes. More than half of the differentially expressed genes (6/10) had functions related to melanin and all melanin synthesis genes, regardless of differential expression status, were higher expressed in males. The agrees

with our hypothesis that melanin genes would exhibit more sex-bias than pteridines or carotenoids because we sampled the fish at a stage when the melanin was visually appearing and melanin synthesis gene expression is known to increase (Chapter 1).

There was no trend for the differentially expressed color genes to be more or less often located on the X chromosome. Theory predicts that when a gene is beneficial in males and detrimental in females, there will be an excess of X-linkage if the sexually antagonistic mutation is recessive and a deficit of X-linkage if the mutation is dominant (Ellegren and Parsch 2007). We have yet to identify any causal mutations, but empirical studies of global gene expression indicate that the autosomes contain most of the sex-biased genes (reviewed in Mank 2009). Although we found no difference in our study, there were also very few DE genes between males and females that could be examined.

Sex-biased gene expression is widely distributed and has been found in a variety of species varying from highly sexually dimorphic birds to lowly sexually dimorphic alga (Pointer et al. 2013, Lipinska et al. 2015). Sex-biased expression of color genes was recently discovered in damselflies, although females of this species are the ones that exhibit color polymorphism (Chauhan et al. 2016). Previous studies of guppy sex-biased expression that included caudal peduncle tissue (located on the body) found 33 color genes with differential expression, 29 of which were male-biased (Sharma et al. 2014). Two of their most male-biased color genes (*tyr*, *xdh*) were also found to be DE in this study. In guppies, the sexual conflict over ornamental coloration is at least partly resolved by male-biased expression of color genes.

Figures

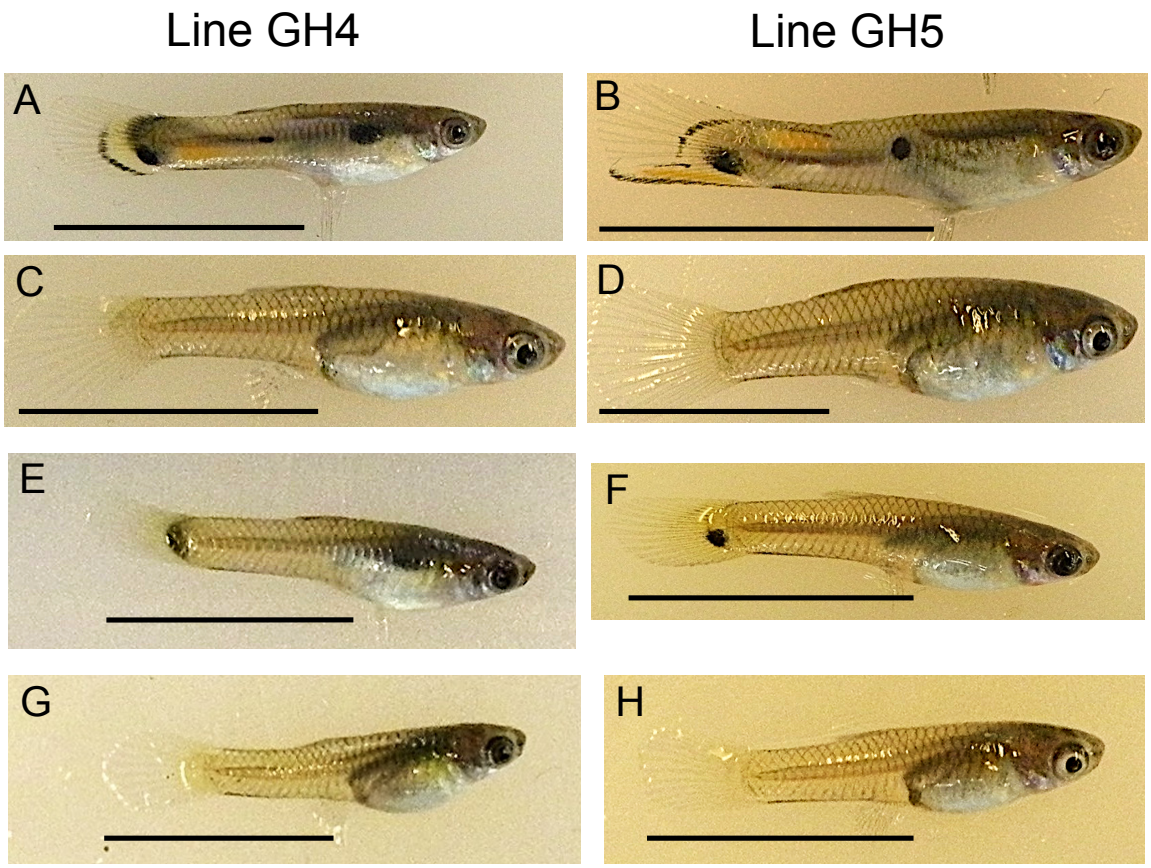

Figure 2.1. Guppies from the two lines sampled. Adult males have ornamental coloration (A, B), while females (C, D) do not. Maturing caudal fins were sampled from males (E, F) and females (G, H) for RNA-seq analyses. Scale bar 1 cm.

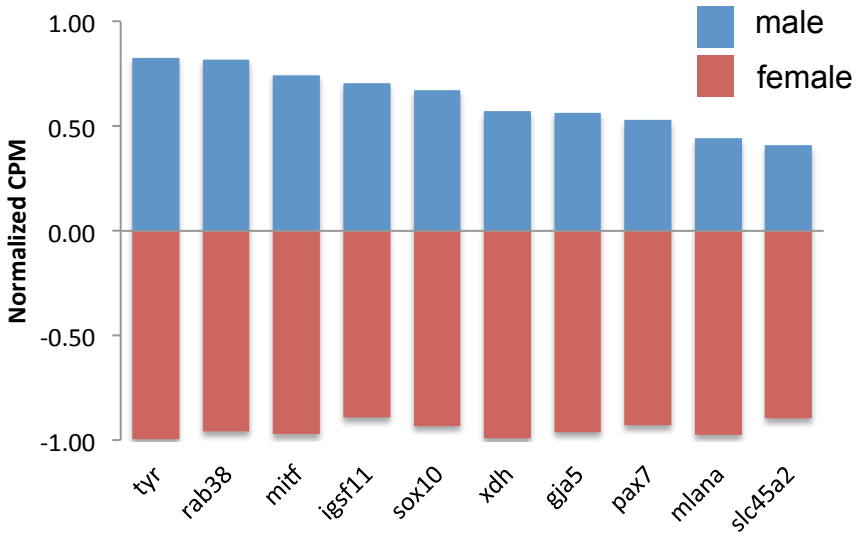

Figure 2.2. Expression of differentially expressed color genes by male (blue) and female (red) guppies. Normalization of counts per million mapped reads (CPM) was performed so all genes could be graphed on the same axis. To do so, feature scaling was applied to each sample and then the scaled values were averaged within sex to yield a single male and single female normalized value bounded by -1 and +1. Feature scaling for each gene was calculated as: x + ((sampleCPM - minimumCPM)*(y - x) / (maximumCPM - minimumCPM)), where x = -1 and y = +1. If the biological replicates within sexes tended to agree, then one sex would have a positive value and the other sex would have a negative value.

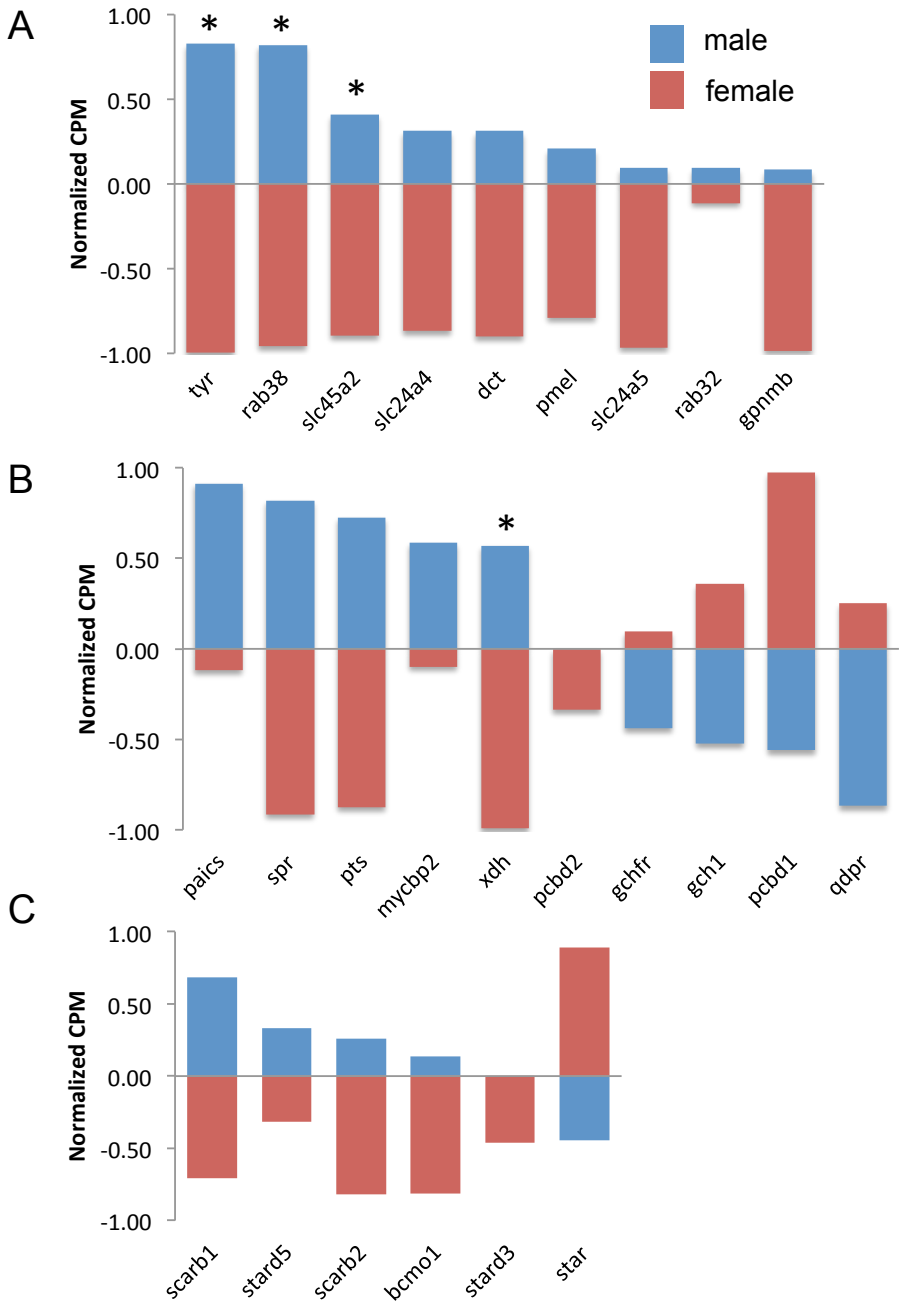

Figure 2.3. Expression of melanin synthesis (A), pteridine synthesis (B) and carotenoid (C) genes by male (blue) and female (red) guppies. Normalization and feature scaling was performed as in Figure 2. Asterisks above bars indicate DE genes between males and females.

Tables

Table 2.1. Number of RNA sequencing reads obtained for each sample. Reads were mapped to the annotated guppy genome (NCBI GCF_000633615.1).

Line	Replicate	Sex	Raw reads	Cleaned reads	Mapped reads (%)
GH4	1	M	8,527,408	6,397,511	5,835,774 (91.2)
GH5	2	M	33,937,307	26,510,922	24,218,195 (91.5)
GH4	1	F	10,227,445	7,896,411	7,174,677 (90.9)
GH5	2	F	29,135,360	18,989,337	18,986,132 (92.3)

Table 2.2. Number of more highly differentially expressed (DE) genes in genome and involved in coloration for each sex. A gene was counted as DE it if was more highly expressed by a given sex with FDR-corrected p-value ≤ 0.05.

Number of genes	**Males**	**Females**
DE in genome	123	1
DE and involved in color	10	0

Table 2.3. Identification, function, and final color type expressed for the color genes more highly differentially expressed by males.

Gene name	**Function**	**Final color type expressed**
gja5	Uncategorized function	Unknown
igsf11	Melanin pattern formation	Melanin
mitf	Melanophore development	Melanin
mlana	Melanogenesis regulation	Melanin
pax7	Xanthophore development	Pteridines or carotenoids
rab38	Melanosome components	Melanin
slc45a2	Melanosome components	Melanin
sox10	Chromatophore development	Any
tyr	Melanosome components	Melanin
xdh	Pteridine synthesis	Pteridines

Chapter 3

Convergent Evolution of Coloration in Experimental Introductions of the Guppy

Abstract

Despite the multitude of examples of evolution in action, relatively fewer studies have taken a replicated approach to understand the repeatability of evolution. Here we examine convergent evolution of adaptive coloration in experimental introductions of guppies from a high predation (HP) environment into four low predation (LP) environments. LP introductions were replicated across two years and in two different forest canopy cover types. We take a complementary approach by examining both phenotypes and genetics. For phenotypes, we categorize the whole color pattern on the tail fin of male guppies and analyze evolution using a correspondence analysis. We find that coloration in the introduction sites diverged from the founding Guanapo HP site. Sites group together based on canopy cover, indicating convergence in response to light environment. However, the axis that explains the most variation indicates a lack of convergence. Therefore, evolution may proceed along similar phenotypic trajectories, but still maintain unique variation within sites. For the genetics underlying the phenotypes, we examine expression levels and the fixation of single nucleotide polymorphisms (SNPs) in genes associated with coloration. Although we find no differentially expressed color genes between Guanapo HP and any of the introduction sites, four different color genes have fixed SNPs in at least two introduction sites. The finding of incomplete SNP fixation across all four introduction sites supports the phenotypic result that there is variation in the genetics associated with color evolution. In addition, incomplete SNP fixation indicates that substantial genetic variation is maintained within and between guppy sites despite rapid evolution.

Introduction

Examples of microevolution in populations have demonstrated that evolution can proceed much more rapidly than previously believed (Darwin 1859, Hendry and Kinnison 1999). Species introductions provide one way to examine evolution in action as populations experience new environments, resources, competitors, or predators (Reznick and Ghalambor 2001). Examples include *Drosophila* (fruit fly) body size clines when adapting to a new climate, *Geospiza* (finch) morphological evolution as food resources change, and *Poecilia* (guppy) life history evolution in response to altered predator community (Reznick and Bryga 1987, Reznick et al. 1990, Grant and Grant 1995, Huey et al. 2000).

When introductions are replicated, they can provide information about the repeatability of rapid evolution. The populations in question can respond to the selection pressures by evolving convergently along similar trajectories. Alternatively, different populations may adapt to the same selection by evolving along different trajectories (historical contingency) or may instead respond via phenotypic plasticity. Convergent evolution has been proposed as evidence that the options for adaptive evolution are limited (Achaz et al. 2014). Historical contingency occurs when random events unique to each population in question cause them to evolve along different trajectories (Gould 2002, Blount et al. 2008). Plasticity could cause divergence among populations if differences are induced as a response to unique features of the environment (Oke et al. 2016).

Trinidadian guppies provide the opportunity to evaluate whether populations in replicated introductions evolve convergently. Guppies from downstream communities co-occur with many predators, hence are referred to as high predation (HP) communities. Guppies from headwater streams above barrier waterfalls live with few other species and experience reduced predation, hence are referred to as low predation (LP) communities. HP guppies differ from LP guppies in a diversity of attributes, including life-history, morphology, and behavior (Reznick and Endler 1982, Endler 1995, Reznick and Bryga 1996). HP guppies have occasionally crossed barrier waterfalls to colonize LP sites (Willing et al. 2010), after which these guppies evolve and acquire LP phenotypes. HP guppies have been introduced into previously guppy-free LP environments multiple times to examine evolution in action. After introduction, fish have evolved traits similar to natural LP guppies (Endler 1980a, Reznick et al. 1990, Reznick et al. 1997).

Guppy male ornamental coloration is thought to evolve under convergent evolution between HP and LP populations. Although ornamental coloration is a complex multicomponent trait, LP males are known to have greater spot size, area, and number than HP males (Endler 1995). This is caused by a trade-off between natural and sexual selection: natural selection favors less colorful males while sexual selection in the form of female choice favors more colorful males (Endler 1983).

Researchers have conducted several introductions in the Caroni drainage system to examine color evolution. Kemp (2009) re-examined the original Aripo introduction by Endler (1980) as well as the El Cedro introduction to determine if body color evolution was predictable. They found that body color did increase in both introductions, but along

different routes. Orange/yellow, black, and iridescence increased in Aripo, but only iridescence increased in El Cedro and orange and black actually declined. More recently, introductions in 2008 and 2009 have been made from Guanapo HP into the LP Upper Lalaja, Lower Lalaja, Taylor, and Caigual Rivers. Gordon et al.'s work examined color evolution in two of those introductions. They found that black coloration decreased in Upper and Lower Lalaja compared to Guanapo HP, while orange/yellow coloration increased (Gordon et al. 2015). Common garden experiments indicated that black changes were most likely due to plasticity and orange/yellow changes were the result of genetic effects. Kemp et al. (in review) recently determined that Upper Lalaja, Lower Lalaja, Taylor, and Caigual males had significantly more iridescent body coloration than Guanapo HP males several years after introduction. These four 2008 and 2009 introductions along with Guanapo HP are the focus here.

The goals of this study are two-fold. First, we examine phenotypic convergence in tail coloration between the four experimental LP introductions in comparison to the HP ancestor from which they originated. We choose the tail as it is highly color polymorphic (Winge 1927) and plays a key role in courtship displays (Farr 1980), despite rarely being the exclusive subject of color studies in guppies. We hypothesize that the introduction sites will diverge from the HP site based on canopy cover and year of introduction. Second, we examine tail color gene expression and allelic differences between contemporary males from the four experimental introductions and the HP site. We hypothesize that there would be either an increased expression level of color genes or

identical color genes with fixation of a single nucleotide polymorphism (SNP) in all the introduction sites compared to the HP site.

Methods

Study System

In 2008, juvenile guppies were removed from Guanapo HP and reared to maturity in the laboratory before being placed into mating groups. Mating groups consisted of a single tank with up to five males and five females. Additional mating groups were placed in separate tanks for a total of 38 fish of each sex. All fish were then introduced into previously guppy free LP reaches of the Upper and Lower Lalaja tributaries. The canopy cover was regularly thinned in Upper Lalaja to mimic that experienced by HP guppies, but left intact in Lower Lalaja. Males from a cross were introduced into a separate site from the females they mated with in the laboratory. This ensured that starting genetic diversity was similar because all males were represented in both sites, either as stored sperm in introduced females or by being themselves introduced. In 2009, a similar experiment was performed in the Taylor (thinned canopy; 52 males and 52 females) and Caigual (intact canopy; 64 males and 64 females) tributaries. All introduced fish were individually marked and photographed. A monthly mark-recapture census was initiated immediately after introduction. Virtually all guppies at a site were collected and photographed plus new recruits were individually marked, measured, and photographed, then all fish were released at the site of capture. After a male reached maturity (at least 14 mm in length), a photograph was taken in which the medial fins were spread open.

Phenotypic Analysis of Color Convergence

Photographs were taken of wild-caught adult males from the ancestral HP site located in the Guanapo river (n=28 photographs) in May 2008 and from the four introduced, experimental sites of guppies four years after introduction (Upper Lalaja: n=25, Lower Lalaja: n=28, Taylor: n=24, and Caigual: n=24 photographs). All photographs analyzed have been uploaded to FigShare (DOI: 10.6084/m9.figshare.6130352). Potential brothers, identified based on having near identical body and tail coloration, were first filtered out such that males with identical tail color patterns within sites would not be full siblings (Fig. 3.1). Males were visually placed into categories according to whether the tail coloration was minimal (<25% colored) or moderate (>25% colored). The tail was partitioned into three sections: proximal near the caudal peduncle, dorsal, and ventral. The distal end was not included as a partition because any distal coloration, if present, was an extension of color from another section. Sections were scored for the presence of three color patterns: flags, swords, and highlights (Fig. 3.2). Flags consist of black spots on a field of orange/yellow. Swords are very thin stripes of black or black plus orange/yellow on the dorsal and/or ventral margins. Highlights are thick stripes of orange/yellow color bounded by black on at least two sides. If color was present and did not conform to one of the three patterns (for example, a single spot), it was scored as having black only, orange/yellow only, or black plus orange/yellow (Fig. 3.2). The same researcher performed two independent categorizations for each male photograph on different days to ensure consistency. Any

photographs with color score disagreements were checked a third time to correct any data entry mistakes.

To test for color pattern convergence, a correspondence analysis was performed using R package FactoMineR (Le et al. 2008) on a count table of the number of individuals having 35 unique pattern types for each of the five sites. The first three axes accounted for 84.6% of the variation and were chosen for further analyses. R package factoextra was used to construct symmetric biplots plotting all five sites from the correspondence analysis.

Genetic Analysis of Color Convergence

Genetic Sampling and RNA Extractions

Twenty guppies were collected from Guanapo HP and the four introduction sites in 2013. Fish were bred in the laboratory at the University of California-Riverside (UCR) for two generations and maintained in a stock tank. The UCR Institutional Animal Care and Use Committee (IACUC) approved all procedures involving the fish. Male tails were sampled at a stage in the development of the anal fin when color was just beginning to develop. Fish were anaesthetized in MS-222 and 10 tails largely represented by unrelated males were combined into a single sample per site. Some of the stock males within a sample may have been brothers based on color pattern similarity, but no more than two brothers were allowed per family in a sample. This was repeated for another biological replicate taken from the stock tanks and at all five sites/stock tanks for a total of 10 samples. Fish were then immediately sacrificed in a lethal dose of MS-222 and were not

allowed to awake from anesthesia in between. Samples were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA was extracted by homogenizing caudal fins in Trizol (Invitrogen, Carlsbad, CA), purifying with a Qiagen RNeasy Mini Kit (Valencia, CA), and treating with TURBO DNase (Ambion, Carlsbad, CA). RNA quality was gauged with an Agilent Bioanalyzer (Santa Clara, CA). All samples had RNA Integrity values ≥ 8.1.

Illumina Sequencing and Quality Control

The University of California-San Diego Institute for Genomic Medicine constructed libraries using the Illumina TruSeq kit v2 (San Diego, CA), following the manufacturer's recommendations and with each sample receiving a unique barcode. Samples were pooled into equimolar amounts and sequenced on two lanes of an Illumina HiSeq2500 at UCR (1 x 100bps). The reads were cleaned using the fastq_quality_filter tool of the FASTX toolkit (Hannon Lab, Cold Spring Harbor Laboratory, NY). Reads needed to have a Phred +33 quality score of at least 20 in 100% of bases to pass quality controls. Any retained reads then had residual adapter sequences trimmed using Trimmomatic 0.20 (Bolger et al. 2014). Each sample yielded 18.8-40.8 million reads (Table 3.1). Raw sequence reads have been deposited in NCBI's Sequence Read Archive (Accession: SRP114275). After cleaning, there were 15.9-32.6 million reads per sample (Table 3.1).

Read Alignment, Read Counting, and Differential Expression

Reads passing all quality control filters were aligned in TopHat2 to the reference guppy genome (NCBI accession GCF_000633615.1) using default options, except the number of threads was four and the minimum intron size was 50 bps. Read counts for each gene were obtained using htseq-count in union mode (Anders et al. 2014). Differential expression (DE) analyses were performed using DESeq2 (Anders and Huber 2010). A gene was only analyzed if it had at least one count per million mapped reads for at least two samples. Contrasts were generated between Guanapo HP and each of the four introduction sites and differential expression tests utilized a false-discovery rate cutoff of 0.05. Genes known to be involved in coloration were extracted from the list of differentially expressed genes.

SNP Calling and Base Pair Fixations

GATK was used to call sequence variants from the RNA-seq data, according to the best practices guide (DePristo et al. 2011). Picard (Broad Institute) was first run to add read group information and coordinate sort the BAM file output by TopHat2. Duplicate reads were marked and the BAM file was reordered to match the reference guppy genome file.

GATK recommends performing base quality score recalibration of sample data against a known database of single nucleotide polymorphism (SNPs), which had to be generated for each sample prior to calling final SNPs. To do so, SplitNCigarReads, RealignerTargetCreater and IndelRealigner were run for each sample in GATK, followed

by the HaplotypeCaller to generate base calls for the full transcriptome. The SelectVariants and VariantFiltration tools generated high quality SNP calls with Fisher Strand values > 30.0 and Quality by Depth values < 2.0. Base quality score recalibration using the high quality SNPs was performed and then HaplotypeCaller was run on just the genome regions where color genes were located. Variant selection and filtering generated the final SNP calls for each sample. Lastly, CombineVariants was used on both biological replicates at a site to isolate the common SNPs between the two samples. Final outputs consisted of a list of color gene sequence variants between samples at each site and the reference guppy genome.

The interest was in sequence variants between Guanapo HP and each introduction site, so manual inspection in Excel was used to determine such SNPs. It was assumed that adaptation from standing variation would be more likely over short time scales (Elmer and Meyer 2011), so the inspection procedure required Guanapo HP to have segregating variation at a SNP site and the introduction site fixed for either base pair. Since samples represented a pool of 10 males with up to 20 unique alleles, segregating variation was defined as having allele frequencies > 0.2 and < 0.8 for the minor and major alleles, respectively. Fixation for a SNP in an introduction site was defined as having allele frequencies ≤ 0.2 or ≥ 0.8 for the minor and major alleles, respectively. A SNP was only called if the depth of coverage (DP) over both biological replicates was > 20. Identical SNPs were examined across intact or thinned canopy sites, 2008 or 2009 introduction sites, as well as across all sites.

Results

Phenotypic Analysis of Color Convergence

The variation in tail color patterns among the four LP introduction sites compared to Guanapo HP was due to a mixture of non-similar and similar trajectories (Fig. 3.3). The first dimension, accounting for over 36% of the variation, separated both the 2009 introductions (Taylor and Caigual) from all other sites (Fig. 3.3A). The second dimension separated Guanapo HP from all of the introduction sites, with no trend to the separation within introductions (Fig. 3.3A or 3.3B). The third dimension separated introduction sites according to whether their canopies were thinned (Upper Lalaja and Taylor) or intact (Lower Lalaja and Caigual) (Fig. 3.3B).

Genetic Analysis of Color Convergence

Gene expression

There are 26,071 genes in the guppy genome and 19,897 met the cut-off imposed for DE estimates of at least one count per million (CPM) in at least two samples. Of the 106 genes with at least one function in coloration located in the guppy genome, 100 met the cut-off for DE tests. There were very few DE genes (range: 77-207) between Guanapo HP and any of the individual introduction sites across the transcriptome. None of the DE genes had known functions in coloration.

SNP variation

At least one SNP in a color gene was located at sites introduced in the same year or sites with the same canopy cover (Table 3.2). There were no SNPs fixed in common at all four introduction sites. However, Taylor, Caigual, and Upper Lalaja all shared the same fixed SNP in the color gene *atp6v0d1* (Table 3.2).

Discussion

Organism responses to environmental change can occur along similar or different trajectories for both phenotypes and genotypes. Identical phenotypic trajectories would be an example of convergent evolution, while varied trajectories indicate factors such as historical contingency (Gould 2002, Achaz et al. 2014), phenotypic plasticity (Oke et al. 2016), or genetic drift (Bock et al. 2015). Phenotypic convergence with identical genetic trajectories could indicate that the phenotypes are constrained to evolve along certain directions (Schluter 1996) while a lack of genetic convergence could indicate that the same phenotype can be produced using many-to-one mapping (Rosenblum et al. 2010).

The identification of convergence can depend on the scale being studied and this is true for both phenotypes and genetics (general versus specific phenotypic traits or genomic regions versus base pair substitutions). In *Drosophila*, overall wing size clines were replicated across three continents, yet the exact wing features that evolved were dissimilar (Gilchrist et al. 2004). Similarly, White Sands lizards evolved reduced pigmentation via mutations in the gene *Mc1r*, yet the molecular mechanisms were different across the species tested (Rosenblum et al. 2010). The present study has found

that the biological level under examination is important when deciding to label a trait as convergent since phenotypes were more similar than their underlying genetics.

Phenotypic Analysis of Color Convergence

Trait evolution after movement to a new environment can occur via selection or founder effects/drift, among other factors. Although guppy introductions were initiated with <100 individuals, a recent study found that the effective population sizes of the introduction sites were large and most likely not under the influence of drift (Kemp et al. in review). In this study, we believe that congruent phenotypic changes among identical predator communities and canopy treatments are largely the result of selection as opposed to drift.

Most of the color pattern variation explained in the correspondence analysis largely implied a lack of convergence (Fig. 3.3A). However, there was a partial signal for the timing of introduction to be important as both the 2009 introductions (Taylor and Caigual) grouped together. For these two sites, the direction of color pattern evolution could have been biased by the starting genetic variation present. The evolution of orange pigmentation is known to evolve in parallel with female preference for orange, although the outcome of this evolution differs among guppy sites (Houde and Endler 1990). The breeding design homogenized the starting genetic variation in males within each year of introduction, so sites introduced in the same year would be expected to have similar genetic variation for male orange ornamentation and female preference for that color. It is unclear why the 2008 introductions would not have grouped together if this were the

case, although Upper Lalaja had the fewest number of color patterns present (data not shown).

The second largest percentage of variation indicated that all introduction sites had divergent color patterns from Guanapo HP, but there was no grouping trend among the introduction sites (Fig. 3.3). Color patterns in the introduction sites were different from their HP ancestor, but they changed along unique trajectories. The divergence from Guanapo HP may be due to the selective effects of a release from predation pressure. In LP sites, sexual selection via female choice is expected to predominate (Endler 1980b) and could cause introduction sites to diverge from their HP ancestor. The unique trajectories among introduction sites could be due to historical contingency. Alternately, a lack of convergence could have occurred if there was a differential loss of color patterns among introduction sites as a result of genetic drift (Bock et al. 2015). Differences in the magnitude or direction of phenotypic differences among sites have also been found in classic systems of convergence such as stickleback, White Sands lizards, and lake whitefish (Rosenblum and Harmon 2010, Kaeuffer et al. 2012, Siwertsson et al. 2013).

The third largest percentage of variation implied convergent evolution as that dimension separated sites according to canopy cover (Fig. 3.3B). This change to color pattern is significant, as it happened regardless of any biases to the starting genetic variation present at introduction. Light environment must play a more important role in the direction of evolution. Another recent study determined that canopy cover impacted guppy body coloration, with greater divergence between Guanapo HP and the thinned

canopy sites (Kemp et al. in review). Canopy cover could impact the direction of pattern evolution in one of two ways. First, additional light increases food availability via increased primary productivity, which leads to stronger orange/yellow carotenoid colors (Grether et al. 1999, Grether et al. 2001). Second, the light visibility could impact color pattern viewing. Variable light environments can cause differential evolution of sexual signals through sensory drive (Endler and Basolo 1998, Boughman 2002). If female neural capacity was altered under different canopies and this caused a shift in female preferences, certain colors or patterns might be favored. Rivers with thinner canopies are brighter in all wavelengths and are enhanced for shorter (blue-UV) wavelengths in shaded areas, while rivers with thicker canopies are enhanced for middle-to-long wavelengths (Endler 1993). As a result, different color patterns may be favored to increase visibility and color contrast.

Genetic Analysis of Color Convergence

Gene expression

Phenotypic color pattern evolution of the introduction sites was not matched with genetic convergence in color gene expression. The statistical lack of DE color genes between Guanapo HP and the four introduction sites could be caused by a couple of explanations. First, there may not yet be a strong expression component to coloration in these sites. Gordon et al. (2015) determined in a common garden study that alterations in black coloration were a plastic response to the environment. The fish reared in the present study were bred in the lab for two generations to reduce environmental effects so changes

to black coloration may not have been detectable in a genetic analysis. Gordon did find that orange/yellow coloration had a genetic component in the common garden, but such genetic changes were not detected here. Chapter 1 found that genes underlying orange/yellow coloration are most differentially expressed at an earlier developmental stage (when there is no visible color), so it is possible that the fish stage sampled here (developing coloration) was too advanced to detect gene expression differences in that coloration.

Second, the sampling protocol used for genetic analyses may have been inadequate to quantify gene expression changes to coloration. Due to logistical constraints, samples consisted of a pool of tails from 10 males taken in a large stock tank. It is difficult to obtain a sample of 10 that represents an entire site when sites may have 1-2 orders of magnitude more fish censused in any given month. The biological replicates at a site were not always similar based on MDS plots (not shown) created during expression analysis, which also supports the idea that the sampling protocol had inadequate density.

Third, there were very few differentially expressed genes identified between HP and LP fish across the whole transcriptome. This would indicate that genetic differences between HP and LP male tails are minimal or could be reflected in changes other than gene expression, such as SNPs.

SNP variation

There was incomplete sharing of fixed SNPs located within color genes. These results should be interpreted with some caution since only 20 individuals per site were genotyped. Common SNPs were found at sites sharing the same year of introduction and canopy cover. In addition, Upper Lalaja, Caigual, and Taylor shared an identical SNP in the gene *atp6v0d1*, which is a vacuolar (v)-ATPase. Although v-ATPases have several effects, zebrafish mutant in this gene have pigmentation defects, specifically abnormal levels and/or distributions of black melanin pigmentation (Nuckels et al. 2009). According to the guppy genome annotation file, the site in question is located within an untranslated exon of the gene. Future investigation could focus on functional consequences, if any, of this SNP and whether these findings implicate partial genetic convergence.

Figures

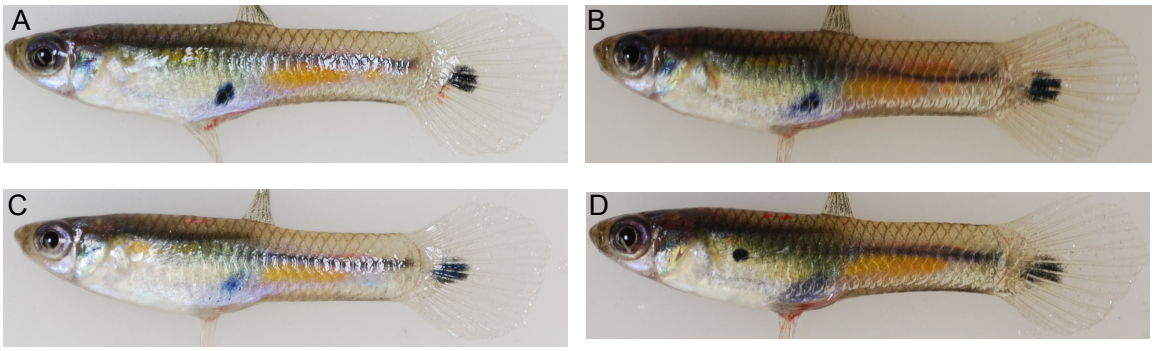

Figure 3.1. Photographs of fish described as related and unrelated. Photos A-C are males that were considered brothers based on nearly identical body and tail color elements. The male in photo D is considered unrelated to males A-C since the main black body spot was in a unique location. Brothers were filtered out such that only males A and D would have been included in the analysis. All males are from Taylor and have a black spot on the front margin of the tail.

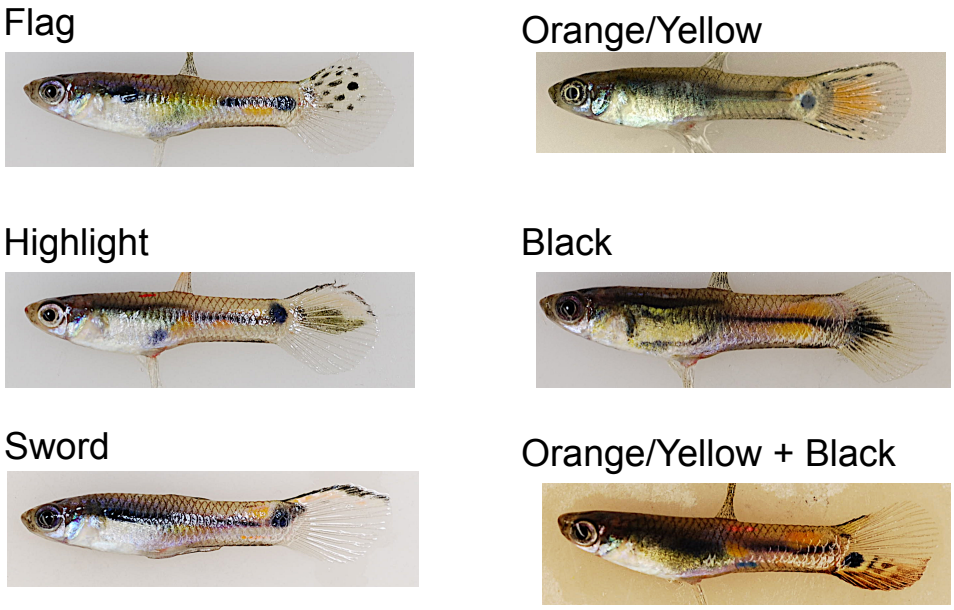

Figure 3.2. Example photographs of the color pattern categories that were scored. Note the orange/yellow fish photograph also has upper and lower swords, as that color element was never present by itself. Other pattern elements could be found individually or in combination with additional elements.

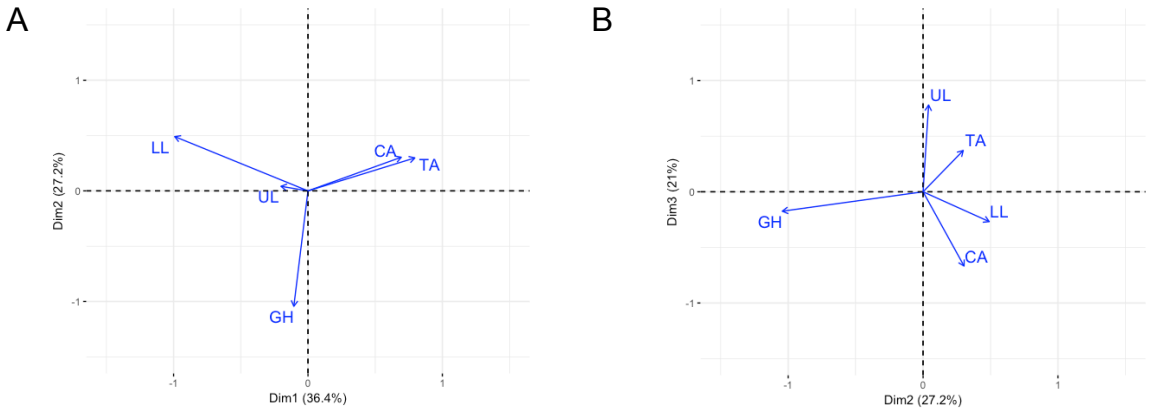

Figure 3.3. Correspondence analysis plots of principal coordinates for Guanapo HP (GH) and each of the four LP introduction sites (Upper Lalaja: UL, Lower Lalaja: LL, Taylor: TA, and Caigual: CA). A. Dimension 1 plotted against Dimension 2. B. Dimension 2 plotted against Dimension 3. UL and LL were founded in 2008, while CA and TA were founded in 2009. UL and TA have thinned canopies, while LL and CA have intact canopies. Acute angles between any of the sites indicate greater similarity.

Tables

Table 3.1. Number of RNA sequencing reads obtained for each sample. Cleaned reads represent the number remaining after quality control. Reads were then mapped to the annotated guppy genome.

Sample	Site	Rep	Raw reads	Cleaned reads	Mapped reads (%)
1	Guanapo HP	1	26,870,174	22,586,691	20,766,878 (92.0)
2	Guanapo HP	2	39,086,645	32,622,487	30,141,929 (92.4)
3	Upper Lalaja	1	21,814,280	18,212,053	16,555,056 (90.9)
4	Upper Lalaja	2	18,881,969	15,857,847	14,703,350 (92.7)
5	Lower Lalaja	1	31,063,239	26,112,166	24,316,619 (93.1)
6	Lower Lalaja	2	23,971,652	20,331,650	18,682,796 (91.9)
7	Taylor	1	32,035,905	25,681,736	23,205,491 (90.4)
8	Taylor	2	25,701,105	20,676,206	18,621,562 (90.1)
9	Caigual	1	24,075,451	19,278,165	17,624,322 (91.4)
10	Caigual	2	40,809,576	32,639,238	29,653,795 (90.9)

Table 3.2. Summary of SNPs in common among Upper Lalaja (UL), Lower Lalaja (LL), Taylor (TA), and Caigual (CA). Intact or thinned refer to canopy cover. The years 2008 and 2009 refer to when introductions were initiated. Ref is the base pair found in the guppy reference genome and Alt is the variant located by GATK. Asterisks next to the Ref or Alt base pair denote which one was fixed in the introduction sites compared to Guanapo HP, which had segregating variation.

Site type	**Gene**	**Function**	**Ref**	**Alt**
Intact (LL, CA)	*rab27a*	Melanosome transport	A*	T
Thinned (UL, TA)	*atp6v0d1*	Systemic effects	G*	C
2008 (UL, LL)	*ece1*	Melanophore development	G*	A
	ece1	Melanophore development	G*	T
	mycbp2	Pteridine synthesis	A	G*
2009 (CA, TA)	*atp6v0d1*	Systemic effects	G*	C
UL, TA, CA	*atp6v0d1*	Systemic effects	G*	C

Chapter 4

Convergent and Divergent Evolution of Guppy Coloration Among Genetically Dissimilar Locations

Abstract

Phenotypic convergence describes the independent evolution of similar traits, while molecular convergence refers to similarity of the underlying genetic changes. An examination of convergence and divergence allows for an understanding of how constraint and diversification influence species evolution. Guppies are ideal for research on phenotypic and molecular convergence or divergence, as they are located across distantly related drainages in rivers representing unique and common selective environments. This study examined ornamental male caudal fin color patterns and the genes associated with coloration at five guppy locations. Caudal fin coloration is a trait used in courtship displays and is highly variable within and among locations. We find that color patterns were more similar between than within drainages. Two locations had significantly more individuals with similar color patterns to other locations than expected by chance. Correspondence analysis of color patterns only partially grouped locations by drainage. Gene expression analyses determined divergence in the identity of differentially expressed color genes among the different locations, except when color types were contrasted across multiple locations. There was some similarity among locations in the identity of color genes containing fixed single nucleotide polymorphisms (SNPs), but most maintained unique SNPs. We propose that guppy coloration exhibits convergent phenotypic evolution with mostly divergent genetics.

Introduction

Examining the convergent evolution of traits allows for an understanding of the roles of constraint and diversification in biology (Agrawal 2017). Phenotypic convergence can occur on multiple levels of biological organization, such as among distant lineages, closely related species, or within species. Respective examples include morphological similarities between marsupials and placental mammals (Dawkins 1986), thick lips for foraging in cichlid fish (Colombo et al. 2013, Baumgarten et al. 2015), and armor plate losses in freshwater stickleback (Colosimo et al. 2005). The cause of similar phenotypes between lineages varies and can due to selective factors, biases, or genetic drift (Losos 2011, Washburn et al. 2016). Phenotypic convergence can happen with or without molecular convergence, which occurs when the genetic changes underlying convergence are similar (Lenser and Theiben 2013).

The term molecular convergence can be used when there are different mutations within the same gene (Protas et al. 2006, Rosenblum and Harmon 2010) or identical mutations within the same gene (Colosimo et al. 2005). It can occur due to mechanisms such as reversion to an ancestral state, sorting of ancestral polymorphism, or introgression via hybridization (Martin and Orgogozo 2013). The chance of finding molecular convergence is more likely as the age between taxa is reduced (Conte et al. 2012). A particular gene can also have properties that favor it for molecular convergence such as a large mutational target size, an important position within a regulatory network, or few pleiotropic effects (Gompel and Prud'homme 2009).

Guppies are an ideal system for examining questions of phenotypic and molecular convergence. Trinidadian guppies originated from mainland South American and have colonized rivers within three main independent drainages in Trinidad (Northern, Caroni, and Oropouche). Colonization of drainages most likely occurred via a two arcs hypothesis (Boos 1984). The first arc from South America populated Western parts of Trinidad including the Northern and Caroni drainages, while the second arc populated parts of Eastern Trinidad that include the Oropouche drainage (Boos 1984). Rivers within the Northern drainage have been separated from the Caroni drainage by 150,000-200,000 years, while those two drainages have been separated from the Oropouche drainage by 500,000-600,000 years (Fajen and Breden 1992). Some gene flow can occur between different rivers within drainages, especially downstream sites, but most rivers remain genetically divergent from one another (Willing et al. 2010).

Guppies in downstream communities co-occur with many predators and are referred to as high predation (HP) communities. Guppies from headwater streams above barrier waterfalls live with few other species and experience reduced predation, hence are referred to as low predation (LP) communities. The similarities among multiple independent locations of LP guppies in life history, morphological, and behavioral traits are a classic example of convergence (Endler 1980b, Reznick et al. 1990, Reznick et al. 1997). However, selective pressures may act similarly on guppy traits and cause convergence irrespective of predation regime (Olendorf et al. 2006).

Male ornamental coloration is one trait that may exhibit convergence regardless of predation regime. Male coloration is genetically determined (Brooks and Endler 2001)

and highly variable within and between sites. Tail coloration in particular displays a large range of coloration compared to the body, as it varies from no coloration to being completely colored. There are over >100 total known color genes with a variety of color-related functions (Svetic et al. 2007, Braasch et al. 2009, Eom et al. 2012, Walsh et al. 2012, Sharma et al. 2014, Ellis and Crawford 2016). The main color pathways in guppies produce black and orange/yellow pigment or structural iridescent coloration. Orange/yellow includes synthesized pteridines and dietary carotenoids (Grether et al. 2001), while black is synthesized melanin.

The goals of this study are two-fold. First, we examine phenotypic color pattern in males from 1-2 locations within each of the three main guppy drainages in Trinidad. We hypothesize that there will be color pattern similarity among independent locations, representing a possible case of convergent evolution. Second, we take males representing extreme phenotypes (colored versus uncolored) within each of the same sites and ask whether the differentiating genes between color morphs are the same between sites. We hypothesize that there will be complete molecular convergence between locations.

Methods

Phenotypic Analysis of Color Pattern Similarity

Photographic Techniques and Pattern Classification

During Summer 2017, photographs were taken in the field of mature males from five geographic locations located in the Northern (Yarra HP River), Caroni (Caroni HP River, Maracas HP River), or Oropouche (Quare LP River, La Seiva HP River)

drainages. These drainages are genetically divergent (Willing et al. 2010). Each geographic location was visited between 10 am to 2 pm and photographs of males were taken with a Nikon Coolpix P80 camera (Tokyo, Japan) using the macro setting and no flash. To standardize across variable field conditions, each male was placed facing left on top of a white tray with a ruler containing color markings.

Photographs were analyzed as described in Chapter 3. Two independent categorizations were performed for each male photograph on different days to ensure consistency. Any photographs with color score disagreements were checked a third time to correct any data entry mistakes and determine if consistent score designations could be determined. Agreements could not be made in 10 cases so these photographs were removed from further analyses, which left 213 photographs.

Statistical analyses

A Pearson's chi-square test was used to test whether similar patterns were more likely to be found within drainages or between drainages. The 26 patterns that were found in multiple geographic locations were scored as "between" or "within". Anytime a pattern was found in a mix of drainage types it was scored as "between". This was tested against the null of a 0.50 expectation of being located within or between drainages.

Fisher's exact tests were performed on each geographic location separately to examine frequency differences between unique or similar patterns. A 2x2 table was made and counts were tallied as to the number of times a pattern was shared with any other geographic location (similar) or only found at the geographic location of interest (unique)

for each of two frequency types: singleton (only one individual at that geographic location had that pattern) or repeated (at least two individuals at the geographic location had the same pattern).

We performed tests for each geographic location and all geographic locations combined to determine if individuals fell into shared patterns more often than expected by chance. For all geographic locations combined, data were considered on the drainage level such that a pattern was only labeled similar if it was shared across different drainages. An observed value was calculated as: number of individuals found in shared categories / total number of individuals sampled at a geographic location. This was compared against a null distribution, which was generated and tested using the dplyr and stats packages in R (version 3.4.1). Sampling with replacement was performed assuming a population size equal to the number of individuals sampled at a geographic location and allowing for equal probability of a pattern found at that geographic location to be sampled. At each of 10,000 sampling iterations, the percentage of individuals falling into shared categories was calculated. A histogram corresponding to the null distribution was plotted and p-values were calculated by comparing the number of times an expected value exceeded the observed similarity value.

Lastly, we performed a correspondence analysis to test for color pattern similarity among geographic locations. We used R package FactoMineR (Le et al. 2008) on a count table of the number of individuals having 58 unique color pattern types for each of the five geographic locations. After viewing scree plots using R package factoextra, the first two axes were chosen for further analyses as they accounted for 62.36% of the variation.

Factoextra was also used to construct symmetric biplots from the correspondence analysis.

Genetic Analysis of Coloration

Genetic Sampling and RNA Extractions

Guppies were collected from the same five geographic locations as above during 2015. Fish were bred in the laboratory at UCR for two generations. A single female was crossed to either a colored or uncolored caudal fin male. There were two colored and two uncolored caudal fin crosses representing two biological replicated per caudal fin color type. To control for any female contribution to coloration, all the females were sisters from the same line and were unrelated to any male line. Male caudal fins were sampled at Stage 2 in the development of the anal fin (see Chapter 1). Fish were anaesthetized in MS-222 and up to 10 caudal fins from males were combined into a single sample per cross. There were a total of 20 samples. Fish were then immediately sacrificed in a lethal dose of MS-222 with no recovery in between. Samples were stored at -80°C until RNA extraction. RNA was extracted and quantified with integrity measured as in Chapter 1. All samples had RNA Integrity values ≥ 7.0.

Illumina Sequencing and Quality Control

Libraries were prepared, sequenced, and cleaned as in Chapter 1 with some modifications. Sequencing was performed on either a HiSeq2500 in Rapid Run Mode or a NextSeq500 in Mid Output Mode. For both instruments, 150 bp single-end sequencing

was performed. Each sample yielded 18.2-96.3 million reads (Table 4.1). Raw sequence reads have been deposited in NCBI's Sequence Read Archive (Accession: SRP126780). Due to quality issues, the first 12 bps and the last 50 bps were removed from each read. Reads from samples sequenced on the HiSeq2500 were required to have Phred+33 quality scores of at least 20 in 100% of bases. Reads from samples sequenced in the NextSeq500 were required to have Phred+33 quality scores of at least 20 in 85% of bases to prevent the FASTX Toolkit (Hannon Lab, Cold Spring Harbor Laboratory, NY) from removing almost all the reads. After cleaning, there were 14.3-66.1 million reads per sample (Table 4.1).

Read Alignment, Read Counting, and Differential Expression

Reads passing all quality control filters were aligned and counted with differential expression (DE) analyses performed as in Chapter 1 except contrasts in DESeq2 were generated between colored and uncolored caudal fin males at a single geographic location. The data were also analyzed in DESeq2 using a design matrix to test for the overall effect of color type on differential expression while controlling for differences among geographic locations. The second test only included La Seiva, Caroni, and Maracas due to issues with the presence of some coloration in uncolored caudal fins from Quare and Yarra (see Discussion for more information). Genes known to be involved in coloration were extracted from the list of differentially expressed genes.

Results

Phenotypic Analysis of Color Pattern Similarity

Pattern similarity was significantly more likely to be found between than within drainages ($\chi^2 = 12.462$, d.f. = 1, $p = 0.00042$). There was a significant effect of pattern type (similar or unique) and occurrence (singleton or repeated) for Maracas (Fisher's exact $p = 0.0371$) and La Seiva (Fisher's exact $p = 0.0231$), suggesting that unique patterns were more likely to be singletons and/or similar patterns were more likely to be found repeatedly within these two geographic locations. Quare (Fisher's exact $p = 0.0573$) had the same trend as Maracas and La Seiva, but there was no effect for Caroni or Yarra.

Comparing the observed percentage of individuals falling into shared categories among drainages revealed a much larger proportion of similar individuals than expected by chance under a null distribution (Fig. 4.1; $p < 0.0001$). When tested on separate locations, Maracas ($p = 0.007$) and La Seiva ($p = 0.021$) had more similar individuals than expected by chance. Quare bordered on having the same trend ($p = 0.061$), but Caroni and Yarra did not differ from the expectation ($p = 0.132$ and $p = 0.100$, respectively).

For the correspondence analysis, the variation in caudal fin color patterns among the five locations indicated that Caroni, Maracas, and Yarra grouped together (Fig. 4.2). The first dimension separated Quare, an Oropouche drainage location, from all other locations. The second dimension separated La Seiva, the other Oropouche drainage location, from all other locations.

Genetic Analysis of Coloration

Differential Expression

Of the 106 genes with at least one function in coloration annotated in the guppy genome, 103 met the cut-off for DE estimates of at least one count per million (CPM) in at least two samples. At least one color gene was differentially expressed (DE) between colored and uncolored males in the individual comparisons for Caroni, Maracas, and La Seiva, but not Quare and Yarra (Table 4.2). Of these DE color genes, all were more highly expressed by colored individuals, but none were DE in more than one location. When looking at overall effects of color type on gene expression and controlling for location differences among Caroni, Maracas, and La Seiva, six genes were DE, all of which were more highly expressed in colored types (Fig. 4.3).

Discussion

The evolution of shared phenotypes in different lineages has been associated with both similar and dissimilar genetics (Elmer and Meyer 2011). The exact genetic mechanisms vary, as both coding sequence and gene expression have been importantly associated with phenotypic change (Hoekstra and Coyne 2007, Stern and Orgogozo 2008). The present study joins other research reporting both shared and unique features of variation (Kaeuffer et al. 2012, Ravinet et al. 2013, Siwertsson et al. 2013, Collar et al. 2014). However, our study has discovered shared patterns of variation that surpass selective boundaries typically considered as being heavily associated with trait similarity.

Traditional classifications of habitat type may not fully explain the selective pressures experienced by isolated groups within species (Kaeuffer et al. 2012).

Phenotypic Analysis of Color Pattern Similarity

The correspondence analysis separated La Seiva and Quare, both in the Oropouche drainage, from all other locations. Based on a molecular phylogeny, some consider Oropouche guppies to be a different species from guppies in the Caroni and Northern drainages (Schories et al. 2009). Another study found genetic admixture between Yarra HP and the Caroni drainage (Willing et al. 2010), which could explain the lack of separation between these locations.

In the random sampling tests, La Seiva and Maracas had significantly more pattern similarity than expected under a null distribution. The observed pattern similarity for Caroni and Yarra were not different from the expectation, which could be caused by features of the data or their biology. We frequently only found one male that had a shared pattern for Caroni and Yarra, which could cause insignificance as compared to having many individuals with a fewer number of similar patterns. Divergent processes acting on individuals at these locations could be impacting the color patterns present as well. Caroni is located most downstream so could potentially be receiving an influx of fish from other locations during flooding and the Yarra location has very intense predation. Quare LP bordered on having significant pattern similarity to the HP locations, which indicates that male selection pressures may be similar even across divergent predation regimes.

Multiple selective pressures could be causing the color pattern similarity or dissimilarity. If male guppies with certain patterns are more visible to all predators, such as by predators forming search images (Olendorf et al. 2006), it could drive the evolution of color patterns not preferred by predators. Alternatively, aspects of female choice are variable among locations and can cause the correlated evolution of dissimilar male ornaments (Endler and Houde 1995). In addition, these locations differ based on many abiotic factors such as canopy cover, which can cause morphological variation among locations (Endler 1993).

Among the Caroni and Oropouche drainages, we determined that there were many more individuals falling into shared patterns than predicted by chance. In addition, similar patterns were more frequently found between drainages than within drainages. Of the patterns that were shared across locations, multiple individuals at a location were usually sampled with that pattern. The patterns unique to a geographic location were rare, as they were typically only found in one individual. We located multiple unrelated individuals exhibiting the same pattern within a location, indicating that we were successful in sampling deeply enough to obtain sufficient replication. Taken together, we believe our findings implicate a role for convergent evolution.

The finding of possible convergence is impressive since our locations represent a variety of histories and selective environments (Langerhans and DeWitt 2004). We have sampled LP (Quare) and HP (all other locations) predation regimes and have three drainages (Northern, Caroni, and Oropouche) thought to represent two invasion events from mainland South America (Boos 1984). Lastly, for the HP locations, the predators

are derived from different sources. Caroni, Maracas, and La Seiva have characin and cichlid predators derived from mainland South America sources, whereas the Yarra location has gobies and mullets derived from marine sources (Reznick and Bryga 1996).

Genetic Analysis of Coloration

Differential Expression

All the locations had differential expression (DE) of at least one color gene, except Quare and Yarra, which could be due to a sampling issue. It was noted during caudal fin dissection that at least one of the two uncolored biological replicates at these locations had a small amount of black at the base of the caudal fin. This spot was not present in the fathers to these crosses, so the female line could have impacted caudal fin coloration in unexpected ways. It was not possible to sample this spot since it was so close to the body, but the genetics could have been altered compared to a son with a completely clear caudal fin.

All DE genes at the other locations where more highly expressed by colored caudal fin males. Most of the DE genes had functions related to black melanin, which was expected given that the males were sampled at a stage when the melanin was just developing in colored caudal fins (Chapter 1). None of the DE color genes in an individual location were identical to any other location. This indicates that the genetics associated with uncolored or colored caudal fins are divergent at unrelated locations and there are multiple genetic paths associated with similar phenotypes.

The four DE genes at Caroni have all been found in one or two previous studies of caudal fin coloration between males and females or between developmental stages (Chapters 1 and 2). The gene *gja5*, also called Connexin 40 in mammals, has now been associated with color variation in the present study and both the prior guppy caudal fin color studies. This gene is uncategorized by Braasch et al. (2009), but another study found that zebrafish mutant in this gene had reduced melanophores and spots in place of stripes (Watanabe et al. 2006). Future studies should investigate the functional consequences of expression variation at this gene.

The four remaining DE genes in Maracas or La Seiva (*atp6v1e1*, *egfr*, *mlana*, and *oca2*) have all been implicated in prior studies of coloration. Mutations in *atp6v1e1* have been linked to a reduction of melanophores and little to no xanthophores in zebrafish (Pickart et al. 2004). Alterations to *egfr* impact melanophore development and a duplication of this gene has been associated with melanoma formation in platyfish (Meierjohann and Schartl 2006). *Mlana* is positively regulated by *mitf* and both impact the expression of the gene *pmel*, which has an important role in melanin synthesis (Du et al. 2003). The gene *oca2* has coding sequence deletions in two populations of albino cavefish (Protas et al. 2006) and large expression variation between sexes in another guppy study (Sharma et al. 2014).

When testing for differential expression between colored and uncolored types over the three locations with truly uncolored types (La Seiva, Caroni, and Maracas), there were six DE genes (*cxcl12*, *igsf11*, *mitf*, *mlana*, *oca2*, *pmel*), all of which had higher expression in the colored types. *Cxcl12* is a patterning gene and zebrafish mutant in this

gene have altered migration to neural crest cells impacting the lateral melanin stripe (Svetic et al. 2007). *Igsf11* is also involved in patterning and zebrafish mutants have altered survival and migration of melanophores (Eom et al. 2012). The other four gene functions also impact the eventual formation of melanin as stated above. Three of these six genes (*cxcl12*, *igsf11*, and *pmel*) were not previously identified in the analyses performed on individual locations. This could indicate a weak molecular convergent signal for differential expression between color types among locations.

Conclusions

Guppies at divergent locations sustain a remarkable degree of color pattern variation, even when experiencing female- and/or predator-driven selection. Despite this, there are shared color patterns among individuals from locations that are characterized by different predator communities and colonization events. There was dissimilarity in the genetics associated with extreme phenotypes. Although it is unclear how the gene expression findings would give rise to the phenotypic patterns, the dissimilarity suggests that the locations do not represent complete maintenance of ancestral variation after colonization from the mainland. Taken together, we believe our color pattern findings represent a case of convergent evolution coupled with divergence that maintains distinctive color patterns at a location.

Figures

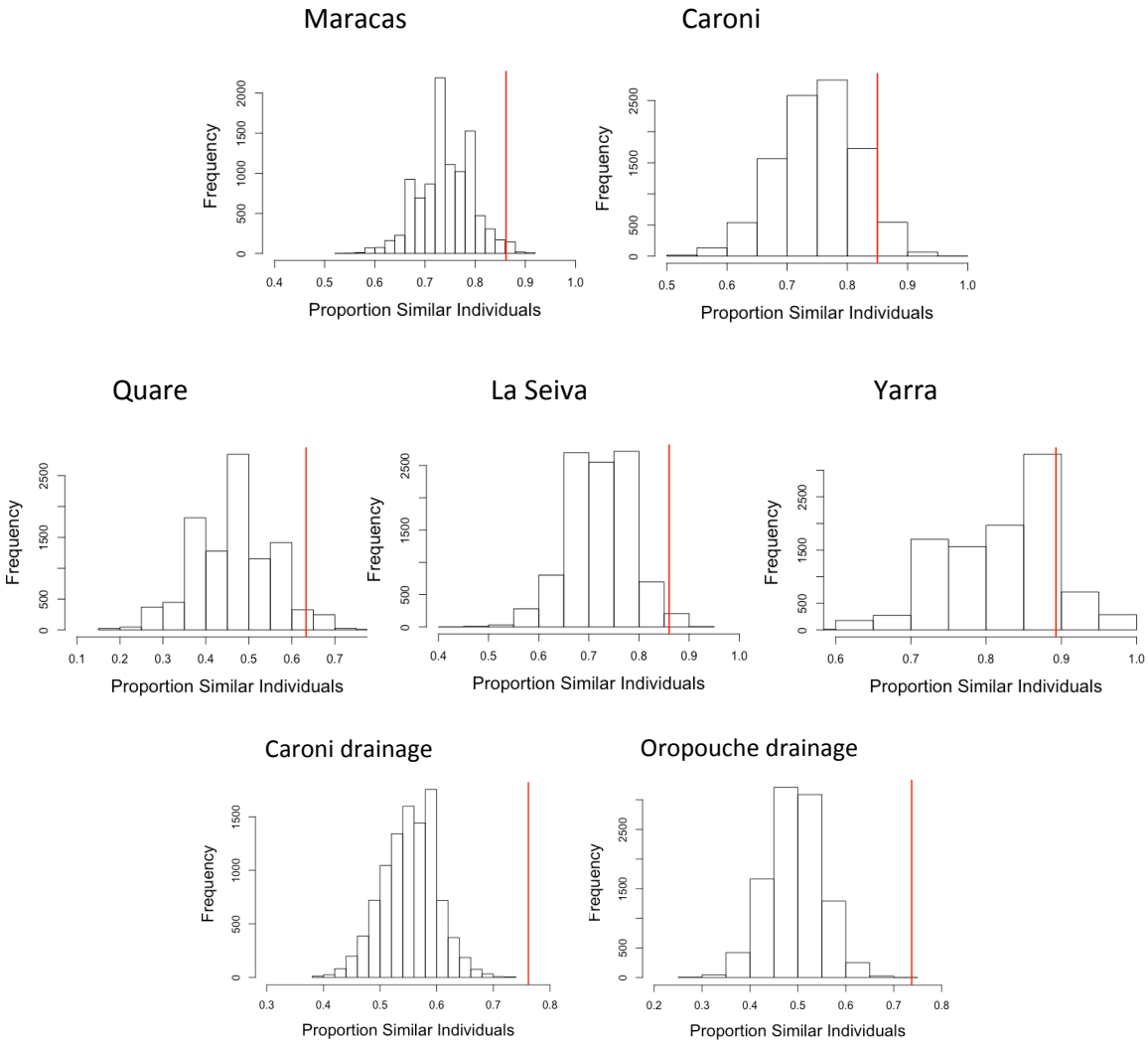

Figure 4.1. Results from random samplings at each location separately and for the drainages. Histograms depict the null distribution for the expected proportion of individuals with shared patterns at a geographic location, while red vertical lines denote the observed proportion of individuals with shared patterns. For the drainage figures, a pattern was only considered shared if it was shared with at least one other drainage. The Northern drainage is not included because the results are identical to the Yarra location.

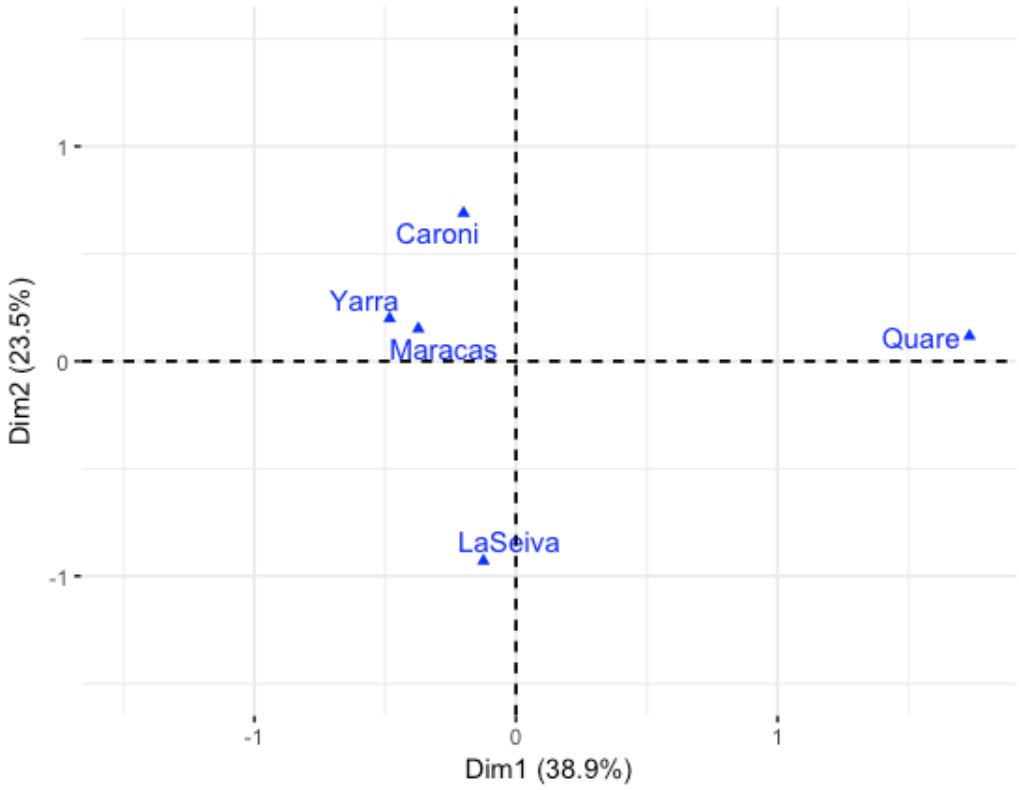

Figure 4.2. Correspondence analysis plot of principal coordinates for each of the five locations. Caroni and Maracas are located in the Caroni drainage, La Seiva and Quare are located in the Oropouche drainage, and Yarra is found in the Northern drainage.

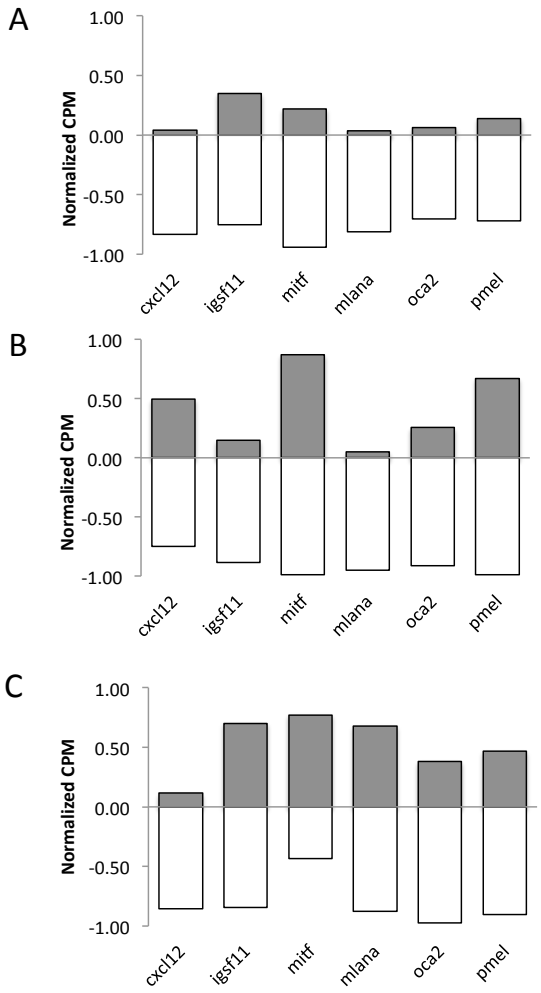

Figure 4.3. Differentially expressed color genes in colored (gray) and uncolored (white) caudal fin guppies. Gene values are shown separately for La Seiva (A), Caroni (B), and Maracas (C). Normalization of counts per million mapped reads (CPM) was performed so all genes could be graphed on the same axis. To do so, feature scaling was applied to each sample and location and then the scaled values were averaged within color types to yield a single colored and single uncolored normalized value bounded by -1 and +1. Feature scaling for each gene at each location was calculated as: x + ((sampleCPM - minimumCPM)*(y - x) / (maximumCPM - minimumCPM)), where x = -1 and y = +1.

Tables

Table 4.1. Number of RNA sequencing reads obtained for each sample. Cleaned reads represent the number remaining after quality control. Reads were then mapped to the annotated guppy genome.

Location	Rep	Color	Raw reads	Cleaned reads	Mapped reads (%)
Caroni HP	1	Uncolored	35,359,803	28,091,772	25,727,606 (91.6%)
Caroni HP	2	Uncolored	40,834,696	32,722,150	29,755,223 (90.9%)
Caroni HP	1	Colored	22,092,554	17,240,068	15,577,288 (90.4%)
Caroni HP	2	Colored	40,467,833	35,903,363	32,897,863 (91.6%)
Maracas HP	1	Uncolored	96,291,475	73,565,767	66,118,131 (89.9%)
Maracas HP	2	Uncolored	35,323,764	31,438,622	28,843,892 (91.7%)
Maracas HP	1	Colored	22,895,043	20,508,593	18,635,920 (90.9%)
Maracas HP	2	Colored	18,234,565	14,278,491	13,065,686 (91.5%)
La Seiva HP	1	Uncolored	26,524,092	20,571,191	18,470,929 (89.8%)
La Seiva HP	2	Uncolored	22,936,953	20,673,091	18,525,098 (89.6%)
La Seiva HP	1	Colored	21,538,754	16,942,646	15,190,818 (89.7%)
La Seiva HP	2	Colored	34,648,449	27,089,906	24,295,160 (89.7%)
Quare LP	1	Uncolored	40,136,593	26,174,399	20,869,195 (79.7%)
Quare LP	2	Uncolored	36,747,405	23,961,694	19,635,482 (81.9%)
Quare LP	1	Colored	33,618,616	21,188,791	17,019,697 (80.3%)
Quare LP	2	Colored	36,573,700	23,797,970	19,467,423 (81.8%)
Yarra HP	1	Uncolored	38,617,090	34,483,806	31,885,265 (92.5%)
Yarra HP	2	Uncolored	23,612,051	18,791,941	17,076,930 (90.9%)

Yarra HP	1	Colored	42,796,155	33,820,062	30,458,628 (90.1%)
Yarra HP	2	Colored	29,512,578	26,407,911	24,188,491 (91.6%)

Table 4.2. Genes more highly differentially expressed by colored individuals in each location. P-values are adjusted to the $\alpha = 0.05$ level using the Benjamini-Hochberg procedure implemented in DESeq2. Gene functions are taken from Braasch et al. (2009) or Du et al. (2003). Melanosomes and melanophores are involved in black melanin, while xanthophores are involved in orange/yellow pteridines and carotenoids. FC: $\log_2$ fold-change between colored and uncolored individuals. Positive values indicate colored individuals had higher expression of the gene.

Location	**Gene**	**FC**	**P-value**	**Gene Function**
Caroni HP	*csf1r*	1.387	0.047	Xanthophore development
Caroni HP	*gja5*	2.616	0.005	Uncategorized
Caroni HP	*gpnmb*	2.428	0.022	Components of melanosomes
Caroni HP	*pax3*	2.370	0.026	Melanophore, xanthophore development
Maracas HP	*atp6v1e1*	0.967	0.046	Xanthophore dev., systemic effects
Maracas HP	*mlana*	1.934	0.013	Melanin regulation
Maracas HP	*oca2*	1.721	0.012	Melanosome construction
La Seiva HP	*egfr*	1.522	0.033	Melanophore development
Quare LP	N/A	.	.	.
Yarra HP	N/A	.	.	.

Concluding Remarks

Arguments for the occurrence of convergent evolution state that shared selective responses and/or genetics cause groups to evolve along similar trajectories when faced with the same environmental pressure. Alternatively, small variations unique to each group being considered may bias the direction of evolution to a different endpoint. Studies reporting both convergence and nonconvergence/divergence in trait evolution are increasing in popularity and this dissertation follows that trend. In this dissertation, I first develop a sampling protocol for the detection of color gene variation and then I examine evidence for convergent evolution of phenotypes and genetics in guppies evolving under cases of rapid and historical evolution.

Chapter one determined that males of both predation regimes follow the same developmental ordering of coloration, making it possible to accurately compare fish independent of predator community. This chapter also determined genes importantly associated with the development of coloration, which facilitates further study of the genetics and evolution of guppy coloration. Specifically, genes involved in synthesizing melanin pigmentation were highly correlated with the visual appearance of black coloration. Finally, this experiment establishes the optimal sampling strategy. Early maturing males expressed slightly more color genes than either of the other two stages sampled. Their anal fins are also still developing, making it possible to more easily standardize the sampling. Future studies examining genes associated with coloration will therefore focus on sampling early maturing males.

Chapter two found that expression of color genes correlated with the presence of

ornamental coloration, especially for black melanin-related genes. Genes involved in the synthesis of melanin were always more highly expressed by males, regardless of differential expression status. These results suggest that genes involved in melanin synthesis are likely candidates for being involved in color differences of fish with variable ornamental coloration levels.

Chapter three examined convergent evolution of phenotypic coloration and its associated genetics in a case of rapid evolution during experimental introductions. Phenotypic coloration in low predation Introductions diverged from their high predation ancestor. However, there was an overall lack of convergence, despite the influence of environmental conditions and year of introduction on color pattern. There was incomplete fixation of base pair substitutions among Introduction site comparisons. Both results indicate that evolution may proceed along similar trajectories, but substantial variation causing unique differences are maintained within and between streams.

Chapter four studied convergent evolution of phenotypes and genetics, but in a case of genetic divergence between locations via historical evolution. Similarity in color pattern was more common between than within drainages, suggesting that convergence may have caused the color evolution rather than immigration between sites. None of the color genes more highly expressed by colored males were identical among sites, indicating heterogeneity in the genetics associated with coloration. These findings corroborate Chapter three results and indicate that even genetically diverged sites exhibit a signal towards convergent evolution despite maintaining unique variations.

Predictions of evolutionary trajectories often prefer to report convergence even

when divergence exists. Instead, a combinatorial approach should be taken, whereby groups should be studied for the presence of convergence and divergence in the trait being considered. The remarkable generation of complex adaptations is not diminished just because groups in question maintain unique variation underneath a convergent signal. Moreover, understanding the biological level at which convergence is examined is necessary when labeling a trait as convergent. Groups may seemingly display convergence for a general trait, but unique features may be present upon close scrutiny of specific traits. Both contingent and similar processes are clearly important when examining the evolution of traits in natural organisms.

Future Directions

By determining candidate genes associated with the convergent evolution of male guppy tail ornamental coloration, this dissertation is an important first step to understand the genetics of adaptation. The same genes with expression changes have now been identified in multiple chapters representing several guppy locations or with other studies of fish coloration. These genes are candidates for being significant in guppy color evolution. It is therefore recommended that future studies examine these candidate genes more closely using targeted DNA sequencing and expression analysis. By expanding the genetic studies to more individuals and locations, it may be possible to determine the mechanism by which male guppies have repeatedly adapted to their environment.

In addition, this dissertation has generated whole transcriptome data between fish differing in developmental stage (Chapter 1), gender (Chapter 2), and predation regime

(Chapters 3 and 4). The sequencing data for all samples is publicly available on NCBI's SRA database. It is highly encouraged that researchers utilize this whole transcriptome data to computationally answer their own evolutionary questions. For example, the SNP list obtained in Chapter 3 can be expanded to determine SNPs across the whole transcriptome that might be associated with the diversity of traits known to differ between HP and LP fish. The guppy system remains an excellent model to ask interesting evolutionary questions and it is my hope that this sequencing data will make further impacts to the field.

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