Pigment cell organization and genetic analysis of color pattern formation in the guppy (*Poecilia reticulata*)

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Summary

The evolution of vertebrate pigment patterns has attracted much attention for decades. The color patterns of animals serve various functions, including camouflage, mate choice, and photoprotection, and they often evolve quickly. To understand their evolution, the complex genetic networks regulating the differentiation, migration, proliferation, and survival of the neural crest-derived pigment cells need to be studied.

In contrast to mammals and birds, fish have several types of pigment cells that interact with each other during color pattern development. The pigment pattern of male guppies (*Poecilia reticulata*) is exceptionally variable and subject to strong natural and sexual selection. Previous studies suggested that a plethora of color genes located on the autosomes and the sex chromosomes of the guppy direct color pattern formation in this small livebearer.

In this thesis, I present evidence that the ornaments of male guppies consist of three types of pigment cells, black melanophores, orange xanthophores, and blue reflective iridophores and that iridophores are present in each of the male color ornaments. Further, I show that early Kita-dependent and late Kita-independent melanophores are essential for the formation of the guppy female and male net-like reticulate pattern. I also found that most xanthophores of the guppy require the ancient paralog of Kita, Colony-stimulating factor 1 receptor a (Csf1ra), for their development. Analysis of the phenotypes of *kita* and *csf1ra* mutant males demonstrated that both gene products are required for male pigment pattern formation. In the case of Csf1ra, I suggest that this is mediated by interactions between melanophores and xanthophores. Identification of *blond* as the guppy ortholog of *adenylate cyclase 5* (*adcy5*) and analysis of the effects of the *adcy5* loss-of-function mutation revealed that all melanophores of the guppy require Adcy5 for their differentiation. The mutation in *adcy5* also strongly affects most of the male-specific orange ornaments, whose formation probably depends on cues given by the melanophores to the xanthophores.

Taken together, my work revealed that Kita-, Csf1ra-, and Adcy5-signaling are fundamentally important for guppy pigment pattern formation.

The identification of the function of these autosomal pigmentation genes represents an important step towards understanding the genetics and evolution of guppy coloration, especially as modifications in the Kita and Csf1ra pathways have been shown to be involved in the evolution of the color patterns of other vertebrates.

Zusammenfassung

Die Evolution der Farbmuster von Wirbeltieren hat während der letzten Jahrzehnte viel Aufmerksamkeit bekommen. Die Farbmuster von Tieren evolvieren oft schnell und erfüllen viele Funktionen, unter anderem dienen sie der Tarnung, Partnerwahl und Photoprotektion. Um ihre Evolution zu verstehen, müssen die komplexen genetischen Netzwerke, die die Differenzierung, Migration, Proliferation und das Überleben der von der Neuralleiste abstammenden Pigmentzellen regulieren, untersucht werden.

Im Gegensatz zu Säugetieren und Vögeln haben Fische mehrere verschiedene Arten von Pigmentzellen, die während der Entwicklung der Farbmuster miteinander interagieren. Das Farbmuster von männlichen Guppys (*Poecilia reticulata*) ist außergewöhnlich variabel und ist starker natürlicher und sexueller Selektion unterworfen. Frühere Studien weisen darauf hin, dass eine Vielzahl von Farbgenen auf den Autosomen und den Geschlechtschromosomen des Guppys die Entwicklung des Farbmusters in diesem kleinen lebendgebärenden Fisch steuern.

In der vorliegenden Arbeit zeige ich, dass die Ornamente von männlichen Guppys aus drei verschiedenen Arten von Pigmentzellen bestehen, nämlich aus schwarzen Melanophoren, orangefarbenen Xanthophoren und blau reflektierenden Iridophoren, und dass Iridophoren in allen männlichen Ornamenten vorhanden sind. Außerdem weise ich nach, dass frühe Kita-abhängige und späte Kita-unabhängige Melanophoren für die Entwicklung des weiblichen und männlichen Netzmusters essenziell sind. Zusätzlich habe ich entdeckt, dass die meisten Xanthophoren des Guppys das sehr alte Paralog von Kita, Colony-stimulating factor 1 receptor a (Csf1ra), für ihre Entwicklung benötigen. Die Untersuchung der Phänotypen von *kita*- und *csf1ra*-mutanten Männchen zeigte, dass die Produkte von beiden Genen für die Entwicklung des männlichen Farbmusters benötigt werden. Im Fall von Csf1ra halte ich es für sehr wahrscheinlich, dass diese Abhängigkeit indirekt durch Interaktionen zwischen Melanophoren und Xanthophoren zustande kommt. Durch die Identifizierung von *blond*, welches das Guppy Ortholog von *adenylate cyclase 5* (*adcy5*) ist, und die Untersuchung der Auswirkungen des Funktionsverlusts dieses Gens stellte sich heraus, dass alle Melanophoren des Guppys Adcy5 für ihre Differenzierung benötigen. Die Mutation in *adcy5*

beeinflusst auch die meisten der männlich-spezifischen orangefarbenen Ornamente, für deren Entwicklung vermutlich Signale benötigt werden, die von den Melanophoren an die Xanthophoren gegeben werden.

Zusammenfassend lässt sich sagen, dass meine Arbeit gezeigt hat, dass die Kita-, Csf1ra-, und Adcy5-Signalwege fundamental für die Entwicklung des Farbmusters des Guppys sind. Die Charakterisierung der Funktionen dieser autosomalen Farbgene ist ein wichtiger erster Schritt in Richtung eines verbesserten Verständnisses der Genetik und Evolution des Farbmusters des Guppys, vor allem, da bereits bewiesen wurde, dass Modifikationen in den Kita- und Csf1ra-Signalwegen zu der Evolution der Farbmuster von anderen Wirbeltieren beitragen.

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Publications

Accepted publications

KOTTLER, V. A., A. KÜNSTNER, I. KOCH, M. FLÖTENMEYER, T. LANGENECKER, M. HOFFMANN, E. SHARMA, D. WEIGEL and C. DREYER, 2015 *Adenylate cyclase 5* is required for melanophore and male pattern development in the guppy (*Poecilia reticulata*). Pigment Cell & Melanoma Research [Epub ahead of print].
doi: 10.1111/pcmr.12386

KOTTLER, V. A., I. KOCH, M. FLÖTENMEYER, H. HASHIMOTO, D. WEIGEL and C. DREYER, 2014 Multiple pigment cell types contribute to the black, blue, and orange ornaments of male guppies (*Poecilia reticulata*). PLoS ONE **9:** e85647.
doi: 10.1371/journal.pone.0085647

KOTTLER, V. A., A. FADEEV, D. WEIGEL and C. DREYER, 2013 Pigment pattern formation in the guppy, *Poecilia reticulata*, involves the Kita and Csf1ra receptor tyrosine kinases. Genetics **194:** 631-646.
doi: 10.1534/genetics.113.151738

Additional accepted publications not part of this thesis

KOTTLER, V. A., A. KÜNSTNER and M. SCHARTL, 2015 Pheomelanin in fish? Pigment Cell & Melanoma Research **28:** 355-356.
doi: 10.1111/pcmr.12359

SHARMA, E., A. KÜNSTNER, B. A. FRASER, G. ZIPPRICH, **V. A. KOTTLER**, S. R. HENZ, D. WEIGEL and C. DREYER, 2014 Transcriptome assemblies for studying sex-biased gene expression in the guppy, *Poecilia reticulata*. BMC Genomics **15:** 400.
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1 Introduction

1.1 Vertebrate pigmentation and pigment cell types

The astonishing complexity of vertebrate pigmentation has fascinated animal breeders and scientists for a long time. In *The Origin of Species*, crosses between domestic pigeons differing in coloration, feather shape, and skeletal features led Charles Darwin to the conclusion that all pigeon breeds are derived from a single species, the rock pigeon (*Columba livia*) (DARWIN 1859). In the 17th century, spontaneous pigmentation mutants of mouse (*Mus musculus*) were propagated in China and Japan, while the breeding of ornamental carps (*Cyprinus carpio*) commonly known as "koi" or "nishikigoi" was initiated in the Nīgata prefecture of Japan in the 19th century (BALON 1995; MORSE 1978). Recently, the genetic basis of naturally occurring color varieties of *Peromyscus* mice, cats, wolves (*Canis lupus*), birds, and sticklebacks (*Gasterosteus aculeatus*) has attracted much attention from researchers (ANDERSON *et al.* 2009; HOEKSTRA *et al.* 2006; KAELIN *et al.* 2012; LINNEN *et al.* 2013; MANCEAU *et al.* 2011; MILLER *et al.* 2007; MUNDY *et al.* 2004; THERON *et al.* 2001; XU *et al.* 2013).

Within the animal kingdom, some of the primary functions of pigmentation are protection against harmful ultraviolet radiation, thermoregulation, camouflage, and intra- and interspecific signaling, e.g., attraction of mates, warning of potential predators, and mimicry (PROTAS and PATEL 2008). The color patterns of vertebrates are generated by pigment cells (chromatophores) that contain either light-absorbing pigments or reflective structures. Only one type of pigment cell, the melanophore (frequently called melanocyte), is present in the skin of mammals and birds (FUJII 2000; HOEKSTRA 2006; MILLS and PATTERSON 2009). Within mammalian and bird melanophores, either black to brown eumelanin or light brown to yellow pheomelanin is synthesized from tyrosine (HOEKSTRA 2006; ITO and WAKAMATSU 2003; JACKSON 1997; MARKS and SEABRA 2001). The synthesis and storage of the melanins takes place in special lysosome-related organelles, the melanosomes, which are subsequently transferred to neighboring keratinocytes in the skin (MARKS and SEABRA 2001; SEIBERG 2001). Keratinocytes in the hair and feather follicles then deposit the pigments into the developing hair and feathers (SLOMINSKI *et al.* 2005; YU *et al.* 2004). The switch between eu- and pheomelanin production and the resulting differences in the spatial distributions of the pigment types generate the diverse fur and plumage color patterns of mammals and birds (HOEKSTRA 2006; ITO and WAKAMATSU 2003; JACKSON 1997; MILLS and PATTERSON 2009; MUNDY 2005). Reddish carotenoids derived from the food and structural colors additionally contribute to the brilliant colors of many bird species (BADYAEV and HILL 2000; LANDEEN and BADYAEV 2012; MILLS and PATTERSON 2009; YU *et al.* 2004).

In contrast to mammals and birds, several pigment cell types have been reported in other vertebrates. At least five different types contribute to the vivid color patterns of fish: melanophores, yellow to red xanthophores (sometimes called erythrophores), bluish to whitish iridescent iridophores, white leucophores, and blue cyanophores (FUJII 2000; GODA and FUJII 1995; HIRATA *et al.* 2003; NAGAO *et al.* 2014; WUCHERER and MICHIELS 2012). The melanosomes of fish exclusively contain eumelanin; pheomelanin has not been found in fish skin so far (ADACHI *et al.* 2005; ADACHI *et al.* 2010; BRAASCH *et al.* 2009; HOEKSTRA 2006; ITO and WAKAMATSU 2003; MILLS and PATTERSON 2009). Xanthophores contain pteridines, which are synthesized de novo from guanosine triphosphate and are stored within xanthosomes (HIRATA *et al.* 2003; HIRATA *et al.* 2005; MATSUMOTO 1965; OBIKA 1993). Additionally, like birds, fish can make use of carotenoids that are contained in certain types of food (GRETHER *et al.* 2001; OBIKA 1993). Iridophores are highly reflective cells, as their iridosomes embody stacked guanine crystals that can appear bluish to whitish, which is caused by thin film interference and refraction of incoming light (FUJII 2000; GUNDERSEN and RIVERA 1982; TAKEUCHI 1976). The nature of the only other type of pigment cell that can appear white, the leucophore, is still somewhat elusive, but leucophores have been described as white cells that most likely contain uric acid crystals within their roundish leucosomes (HAMA 1975; MENTER *et al.* 1979; NAGAO *et al.* 2014; TAKEUCHI 1976). Pigmentation mutants of medaka (*Oryzias latipes*) suggest that leucophores are closely related to xanthophores (KELSH *et al.* 2004; KIMURA *et al.* 2014; NAGAO *et al.* 2014). Blue cyanophores have been identified in the skin of two species of the family Callionymidae (*Synchiropus splendidus* and *S. picturatus*) and are filled with cyanosomes containing a blue pigment (GODA and FUJII 1995). Different combinations of pigment cell types and variation in their shapes, numbers, and distributions in the skin generate the diverse color patterns of fish, which can vary greatly, even between closely related species (morphological color change) (FUJII 2000; PARICHY 2006; SANTOS *et al.* 2014; SEEHAUSEN *et al.* 2008; SINGH and NÜSSLEIN-VOLHARD 2015). Additionally, like many amphibians and "reptiles," fish can adapt rapidly to environmental cues by aggregating or dispersing their pigment-containing organelles within the chromatophores, e.g., to adapt to the coloration of the background or for intraspecific communication, which is under hormonal and neural control (physiological color change) (FUJII 2000).

All pigment cells except for the ones of the retinal pigment epithelium are derived from the neural crest (BAGNARA *et al.* 1979; KELSH *et al.* 2009; SAUKA-SPENGLER and BRONNER-FRASER 2008). The neural crest is a transient, multipotent cell population unique to vertebrates, whose formation is initiated at the neural plate border during gastrulation (SAUKA-SPENGLER and BRONNER-FRASER 2008). After specification, neural crest cells undergo epithelial to mesenchymal transition and migrate to various regions within the body, where they give rise to many cell types, e.g., chromatophores, bone and cartilage cells of the craniofacial skeleton, and neurons and Schwann cells of the peripheral nervous system (SAUKA-SPENGLER and BRONNER-FRASER 2008). The chromatophore precursors, the chromatoblasts, either migrate along the dorsolateral pathway, between the somites and the non-neural ectoderm, or along the ventral (medial) pathway, between the somites and the neural tube, to their point of destination. The melanoblasts of mouse and chicken (*Gallus gallus*) migrate along the dorsolateral pathway, while the melanoblasts of zebrafish (*Danio rerio*) utilize both pathways (KELSH *et al.* 2009). Zebrafish xanthoblasts, however, use only the dorsolateral pathway, whereas iridoblasts are exclusively found along the ventral pathway (KELSH *et al.* 2009). Lately, it has been shown that a substantial proportion of the skin melanophores of mouse is derived from multipotent "Schwann cell precursors" that move along nerves on the ventral pathway (ADAMEYKO *et al.*

2009). Consistent with these findings, recent studies demonstrated that the melano- and iridoblasts contributing to the adult zebrafish pigment pattern are associated with dorsal root ganglia and migrate to the skin along nerves (BUDI *et al.* 2011; DOOLEY *et al.* 2013; SINGH *et al.* 2014).

1.2 *Poecilia reticulata* as a model for pigmentation research

The guppy, *Poecilia reticulata* (Poeciliidae: Cyprinodontiformes), is one of the most popular aquarium fish, due to its robustness, fertility, and diverse coloration. Guppies are small freshwater fish, which are native to northeastern South America, including Venezuela and a few Caribbean islands, most importantly Trinidad (MAGURRAN 2005). They are livebearers with a gestation period of three to four weeks and internal fertilization; the modified anal fin (gonopodium) of the males serves as an intromittent organ (HOUDE 1997). The pigment pattern of male guppies is exceptionally polymorphic, to a degree that each male of a population can be recognized by its unique color pattern consisting of black, yellow, orange, blue, green, and white spots and stripes. Guppy females are camouflaged by an inconspicuous, net-like reticulate pattern and are much larger than males. Previous studies described melanophores, xanthophores/erythrophores, iridophores, and leucophores in guppy skin (FUJII 1966; FUJII and TAGUCHI 1970; GOODRICH *et al.* 1944; GUNDERSEN and RIVERA 1982; TAKEUCHI 1975; TAKEUCHI 1976). The reticulate pattern of the guppy consists of large, so-called "corolla" melanophores, while xanthophores, some corolla melanophores and more superficial "dendritic" melanophores are distributed across the whole body (GOODRICH *et al.* 1944; WINGE and DITLEVSEN 1947). The pigment pattern of newborn guppies resembles the one seen in adult females; in males, the reticulate pattern is partially obscured by the male-specific ornaments, which develop during puberty (HOUDE 1997; MARTYN *et al.* 2006; TRIPATHI *et al.* 2008).

Numerous studies have demonstrated that the color patterns of male guppies are under strong natural and sexual selection. Translocation experiments on Trinidad showed that guppy life-history traits (e.g., age at maturity and brood size), behavior, and male colorfulness co-vary with predation intensity and change rapidly once the predation regime is altered

(Endler 1980; Endler 1995; Godin and McDonough 2003; Magurran 2005). Guppy populations therefore provide the unique opportunity to study complex evolutionary processes in a relatively short amount of time, i.e., within a few years.

Four different families of cone visual pigments (opsins) are present in the retina of the guppy, which are required for the perception of ultraviolet (SWS1, short-wave sensitive 1), bluish (SWS2, short-wave sensitive 2), green (RH2, rhodopsin-like), and orange to red (LWS, long-wave sensitive) light, providing excellent color vision (Archer *et al.* 1987; Hoffmann *et al.* 2007; Trezise and Collin 2005; Weadick and Chang 2007). Guppy females in general prefer conspicuous males with a high percentage of orange coloration, which is an indirect way to estimate the feeding ability (uptake of carotenoids) and general physical fitness (predator avoidance) of a potential mate (Endler 1983; Godin and McDonough 2003; Grether *et al.* 2001; Houde 1987; Kodric-Brown 1985; Magurran 2005). During courtship, male guppies preferentially orient their "more orange side" towards the females and intensify the coloration of their black ornaments, which accentuates their orange and iridescent markings (Brooks 1996; Endler 1983; Gross *et al.* 2007). At least three LWS opsin isoforms differing in their absorption maxima are expressed in guppy eyes, suggesting that the visual system of the guppy is especially tuned towards the perception and discrimination of orange to red hues (Archer *et al.* 1987; Archer and Lythgoe 1990). The LWS opsin isoforms are derived from several LWS opsin loci; sequencing of genomic DNA revealed four LWS genes in the Cumaná guppy from Venezuela and in a close relative of the guppy, the green swordtail (*Xiphophorus helleri*) (Ward *et al.* 2008; Watson *et al.* 2011; Watson *et al.* 2010). Studies have found many polymorphic sites within the LWS opsin genes between and within guppy populations, which might explain some of the variation in female preferences for male color patterns that has been observed among populations (Archer and Lythgoe 1990; Endler and Houde 1995; Hoffmann *et al.* 2007). Iridescent ornaments, the frequency of the overall phenotype of a male, and environmental factors (for instance, water color or density of the canopy affecting carotenoid availability and brightness) influencing male coloration or color perception in general have also been shown to affect female choice

(ENDLER and HOUDE 1995; GRETHER *et al.* 2001; HUGHES *et al.* 2013; KODRIC-BROWN 1985).

The formation of the guppy pigment pattern has attracted the attention of geneticists for almost a century. Many color traits of male guppies are highly heritable and transmitted through the paternal line, which might at first seem surprising considering the variable coloration of male guppies found in natural populations (DZWILLO 1959; LINDHOLM and BREDEN 2002; TRIPATHI *et al.* 2008; WINGE 1922; WINGE 1927). The guppy, which has an XX (female)/XY (male) sex determination system with n=23 chromosomes, was one of the first organisms for which sex-linked inheritance of a trait, the male phenotype oculatus, was shown (SCHMIDT 1920). In 1922 and 1927, Winge described 18 color loci, of which three and nine were strictly linked to the X and differentiating Y chromosome (linkage group 12), respectively. Five of the remaining six were either located on the X or the Y chromosome and one of the genes, *zebrinus*, was shown to be autosomal. Since then, many more loci directing single or groups of male color traits, as well as loci required for the development of guppy pigment cells, have been described (LINDHOLM and BREDEN 2002). Until my work on this subject, however, none of the genes underlying guppy pigment pattern formation had been molecularly identified.

Positional cloning of zebrafish pigmentation genes demonstrated that a plethora of genes modulating, for instance, migration, proliferation, and differentiation of pigment cell precursors, is required for teleost pigment pattern formation (PARICHY and SPIEWAK 2015; SINGH and NÜSSLEIN-VOLHARD 2015). Zebrafish undergo a complex pigment pattern metamorphosis during the transition from the early larval to the juvenile stage and recent studies found that inhibitory and stimulating interactions between melanophores, xanthophores, and iridophores are required for the formation of the adult stripe pattern (SINGH and NÜSSLEIN-VOLHARD 2015). Likewise, complex interactions between pigment cell types might contribute to the natural variation of guppy ornaments.

Previous studies suggest that the male color pattern of the guppy is directed by one or several male-advantageous genes that are genetically linked to the male sex-determining locus on the non-recombining distal region of the Y chromosome, as well as by genes located on the pseudoautosomal region of the sex chromosomes and on the autosomes (DZWILLO 1959; GOODRICH *et al.* 1944; KHOO *et al.* 1999a; KHOO *et al.* 1999b; KHOO *et al.* 1999c; LINDHOLM and BREDEN 2002; PHANG *et al.* 1999; TRIPATHI *et al.* 2008; TRIPATHI *et al.* 2009b; WINGE 1922; WINGE 1927; WINGE and DITLEVSEN 1947). Recombination between the guppy X and Y chromosomes is rare and probably restricted by diversification (LISACHOV *et al.* 2015; TRIPATHI *et al.* 2009a). The genetic nature of most guppy color traits is highly complex, as either several loci with additive and/or suppressive effects are involved in the development of each trait, or the traits are tightly linked to the sex-determining locus, which could not be identified yet (TRIPATHI *et al.* 2008; TRIPATHI *et al.* 2009b). Single-locus recessive inheritance, however, has been shown for a number of guppy color phenotypes in which certain types, or subpopulations, of pigment cells are absent, e.g., for the golden, blond, and blue phenotypes (DZWILLO 1959; GOODRICH *et al.* 1944; HASKINS and DRUZBA 1938; WINGE and DITLEVSEN 1947). These spontaneous mutants provide the unique opportunity to correlate phenotypic differences with genetic alterations at a single locus, an approach that has been widely used to investigate pigment pattern formation in zebrafish. To analyze how a specific mutation affects the male guppy color pattern, the substantial amount of background variation caused by different alleles at various color loci distributed throughout the genome needs to be controlled. Crossing a mutation of interest into inbred guppy strains and comparing the phenotypes of mutant and wild-type male siblings helps to minimize the amount of variation derived from loci other than the investigated one.

As the transcriptome (SHARMA *et al.* 2014) and genome (KÜNSTNER *et al.*, in preparation) of the guppy have recently been sequenced, fine mapping of color loci in addition to candidate gene approaches can now be carried out to identify the genes that underlie guppy pigment pattern development.

2 Objectives of this work

My doctoral research focused on the morphology and developmental genetics of the guppy pigment pattern. I used transmission electron microscopy, candidate gene approaches, double-digest restriction site-associated DNA sequencing (ddRADseq) in combination with quantitative trait locus (QTL) mapping, and extensive phenotypic analyses to address the following questions:

i) Which types of pigment cells are present in guppy skin? Are these, as previously described, melanophores, xanthophores, iridophores, and leucophores?
ii) How are the pigment cells organized within the male ornaments?
iii) Which mutations underlie the golden, blue, and blond phenotype?
iv) How do the mutations in *golden*, *blue*, and *blond* affect the guppy newborn, adult male and female reticulate, and male-specific color pattern? What does this reveal about the development of the guppy pigment pattern?

3 “Multiple pigment cell types contribute to the black, blue, and orange ornaments of male guppies (*Poecilia reticulata*)”

Verena A. Kottler, Iris Koch, Matthias Flötenmeyer, Hisashi Hashimoto, Detlef Weigel, and Christine Dreyer, 2014

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Synopsis

Previously, four types of pigment cells were described in guppy skin (FUJII 1966; FUJII and TAGUCHI 1970; GOODRICH *et al.* 1944; GUNDERSEN and RIVERA 1982; TAKEUCHI 1975; TAKEUCHI 1976). However, how these pigment cells are organized within male skin, thereby forming the spots and stripes seen in adult males, has hardly been investigated. Knowing which pigment cell types are present in the ornaments of guppy males and where they are located is crucial to understanding male pigment pattern formation, which might require interactions between different pigment cell types.

To identify the types of pigment cells and their distributions in male skin, I chose to investigate the male ornaments of the three inbred wild-type guppy strains Cumaná (ALEXANDER and BREDEN 2004), Quare6 (REZNICK and ENDLER 1982), and Maculatus (SCHMIDT 1920; WINGE 1922) with transmission electron microscopy (TEM). Males of these strains develop a central black and orange spot below the dorsal fin during puberty, which provided the opportunity to compare the pigment cell distribution within these spots between the three strains. In addition, I analyzed the composition of the Cumaná-specific blue spot and orange-black lining of the tail fin, the Quare6-specific posterior black spot and tail fin color pattern, and the Maculatus-specific black and white color pattern on the dorsal fin. We hypothesized that several pigment cell types contribute to each trait, even to the color patterns that appear to consist of only one type of pigment cell, e.g., the black and orange spots.

Analysis of the TEM images revealed the presence of melanophores, xanthophores, and iridophores in the dermis and hypodermis of the investigated males. Consistent with previous studies that identified superficial dendritic and more deeply located corolla melanophores in guppy skin (GOODRICH *et al.* 1944; WINGE and DITLEVSEN 1947), we also found melanophores in the epidermis. Some xanthophores contained roundish vesicles, which are most likely filled with carotenoids, as has been described previously for the xanthophores of the guppy and the xanthophores of other species (OBIKA 1993; OBIKA and MEYER-ROCHOW 1990; TAKEUCHI 1975). We could not identify any leucophores, as information on the morphology of these cells is sparse. By investigating female skin under incident light conditions, I confirmed that melanophores, xanthophores, and iridophores are also present in adult guppy females.

We found that at least two types of pigment cells, one of them always iridophores, contribute to each male ornament. The pigment cell distribution within the central black and orange spot was conserved between the strains; in all males, the black spots were composed of hypodermal iridophores and melanophores and dermal melanophores, and the orange spots of hypodermal iridophores and xanthophores and dermal xanthophores. The Cumaná blue spot contained melanophores and iridophores in the hypodermis and iridophores in the dermis. These observations are in sharp contrast with the situation in zebrafish, in which the pigment cells forming the stripes are restricted to the hypodermis (HIRATA *et al.* 2003; HIRATA *et al.* 2005). We concluded from our study that iridophores might be much more important for guppy pigment pattern formation than previously thought, as they are in close proximity to melanophores and xanthophores in all of the investigated ornaments and interactions between iridophores and other pigment cell types during color pattern formation have recently been detected in zebrafish (FROHNHÖFER *et al.* 2013; KRAUSS *et al.* 2013; LANG *et al.* 2009; PATTERSON and PARICHY 2013; SINGH *et al.* 2014).

Contributions

VAK and CD conceived and designed the experiments. VAK took the pictures under incident light conditions and fixed the fish. IK prepared the samples for TEM. VAK, IK, and MF took the TEM images. VAK, IK, MF, and HH analyzed the TEM data. VAK wrote the paper; IK, MF, HH, DW, and CD provided helpful comments on the manuscript. DW oversaw the experimental design and data analysis.

License

Multiple Pigment Cell Types Contribute to the Black, Blue, and Orange Ornaments of Male Guppies (*Poecilia reticulata*)

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Abstract

The fitness of male guppies (*Poecilia reticulata*) highly depends on the size and number of their black, blue, and orange ornaments. Recently, progress has been made regarding the genetic mechanisms underlying male guppy pigment pattern formation, but we still know little about the pigment cell organization within these ornaments. Here, we investigate the pigment cell distribution within the black, blue, and orange trunk spots and selected fin color patterns of guppy males from three genetically divergent strains using transmission electron microscopy. We identified three types of pigment cells and found that at least two of these contribute to each color trait. Further, two pigment cell layers, one in the dermis and the other in the hypodermis, contribute to each trunk spot. The pigment cell organization within the black and orange trunk spots was similar between strains. The presence of iridophores in each of the investigated color traits is consistent with a key role for this pigment cell type in guppy color pattern formation.

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Introduction

The spectacular orange, yellow, white, and black along with the blue to green iridescent colors of male guppies (*Poecilia reticulata*) have attracted the attention of biologists and hobby breeders for almost a century [1–5]. The guppy is a small live-bearing freshwater fish native to northeastern South America. Guppy populations have been studied most extensively on the island of Trinidad, where male coloration, as well as other traits, such as body shape and life history characteristics, covary with predation intensity [6,7].

Mate choice experiments have demonstrated that guppy females, which are camouflaged by an inconspicuous reticulate pattern [4,8], prefer males with high amounts of orange and iridescent pigments [6,9,10]. Both orange and iridescent ornaments can indicate a male's current physical condition and genetic quality. The orange spots contain two types of pigments, carotenoids, which are obtained from the food, mainly from unicellular algae, and pteridines, which are synthesized de novo [11,12]. Orange pigments therefore reflect a male's foraging efficiency and ability to synthesize pteridines [11–13]. Pteridine production within the orange spots of wild guppy males varies with carotenoid availability; for instance, males produce less pteridines in habitats in which carotenoids are scarce, leading to a relatively constant pteridines to carotenoids ratio, and hence orange hue, across populations [11,12]. A recent study has shown that female guppy preference for this specific orange hue causes this pattern [14]. Iridescent ornaments increase the risk of being noticed by predators and hence provide information on a male's capability to evade these [6,9,15]. Males also intensify their black pigmentation during courtship, which might emphasize orange areas [6,16]. The amount and size of male ornaments is highly heritable and a substantial portion is inherited in a Y-linked manner from the father [2,5,8,17,18]. Studies have demonstrated that guppy females favor males with rare or novel color patterns over males with familiar phenotypes, suggesting that negative frequency-dependent selection contributes to the maintenance of male color polymorphisms within guppy populations [19–23].

While the selection pressures driving male color patterns have been well studied, little is known about the morphology of male ornaments. Five pigment cell types have been described in the skin of the guppy: black melanophores, orange to yellow xanthophores, red erythrophores, blue to green iridescent iridophores (Figure 1), and white leucophores [4,8,24–28]. The pigment organelles of melanophores, xanthophores, and erythrophores contain light-absorbing pigment colors, namely eumelanin and carotenoids/pteridines, respectively [11,29]. The thin guanine crystals found in organelles within iridophores produce glittering blue, green, and silvery structural colors by thin film interference and refraction of incident light waves [26,28,30]. Leucophores appear whitish by scattering light in various directions; their pigment granules might contain uric acid [28,30–32].

The precursors of vertebrate chromatophores migrate from the neural crest to various regions within the body [33,34]. There is

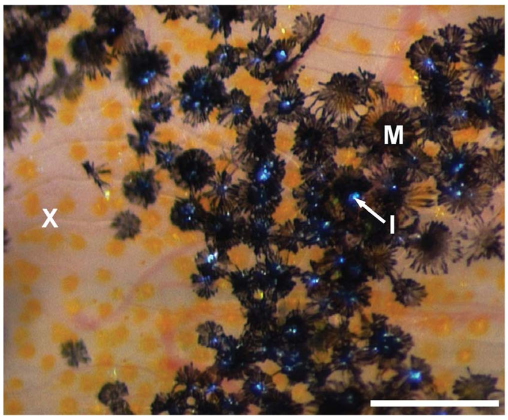

Figure 1. Pigment cell types of the guppy. Xanthophores (X), melanophores (M) and iridophores (I) on the dorsal side of a guppy female shown under incident light. Leucophores could not be identified. Scale bar: 200 µm.
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increasing evidence that both short- and long-range interactions between different types of pigment cells are required for proper migration, differentiation, and survival of their precursors. For example, during zebrafish (*Danio rerio*) pigment pattern development, iridophores stimulate xanthophore precursors to migrate to the prospective interstripe regions, but inhibit melanoblast localization to these areas, which then accumulate in the adjacent stripes [35]. Similarly, zebrafish xanthophores promote stripe development by short-range inhibitory and long-range stimulating interactions with melanophores [36–40]. Xanthophore-melanophore interactions are also crucial for the development of the male-specific pattern of the guppy, as male guppies lacking xanthophores due to a mutation in *colony-stimulating factor 1 receptor a* (*csf1ra*) also have severe melanophore localization defects [8]. To unravel such interactions in the guppy, it is critical to understand how different pigment cell types are organized within the male ornaments, which can be assessed best by transmission electron microscopy (TEM) [41–44]. Previous TEM studies on guppy pigment cells focused on the identification of different chromatophore types in the tail fins of adult males and on the development of lateral iridophores on the trunk [24–28]. The guppies investigated in these prior studies were obtained from pet shops. Unfortunately, the precise position of the ornaments on the guppy body was not documented, making it difficult to relate these TEM images to the macroscopically visible ornaments.

Here, we describe the pigment cell distribution, for which we subsequently use the term ultrastructure, within the blue, black, and orange trunk spots and fin color patterns of male wild-type guppies from three genetically divergent strains. TEM revealed that several chromatophore types contribute to each ornament. We could, however, not identify any leucophore. Our comprehensive study on pigment cell distribution in the skin of male guppies provides a foundation from which the natural variation in the placement and expression of male ornaments can be studied.

Results and Discussion

Identification of chromatophore types

We investigated the pigment spots and fin color patterns of male Cumaná, Quare6, and Maculatus guppies. Cumaná guppies are derived from a wild population in Venezuela [45]; the inheritance of the black and orange ornaments on the dorsal fin, the blue iridescent spot on the trunk, and the ventral black margin of the caudal peduncle is linked to the male Y chromosome (Figure 2A, 2B) [17,18,46]. Quare6 guppies are descendants of fish from the Quare river on Trinidad [47]. Male Quare6 guppies display roundish black and orange spots on their body and older males often develop brilliant color patterns on the tail fin (Figure 2C, 2D) [8,17]. Maculatus guppies have been bred in captivity by researchers and hobby breeders for almost a hundred years [1,48]; their central black and orange spots on the trunk in combination with the black spot on the dorsal fin are Y-linked (Figure 2E, 2F) [1]. The black in the dorsal fin of Maculatus males is usually surrounded by whitish pigments (Figure 2E) (VAK and CD, unpublished data and [48]).

We analyzed the ultrastructure of the central orange and central black spot near the gonopodium, as these two spots are present in all three strains despite their considerably different male ornaments (Figure 2) [8]. Additionally, we included the Cumaná blue iridescent spot and some typical color fin patterns of the three strains into our analysis (Figure 2). Our aim was to identify the different types of pigment cells, to clarify how they are organized within the skin, and to determine whether the ultrastructure of the central orange and black spots are similar in these strains.

Previously, two types of melanophores were described in wild-type guppies: dendritic ones, which are located on top of the scales, and corolla ones, which are located more deeply in the skin [4,8]. When guppy scales are removed, dendritic melanophores associated with them are usually detached as well (VAK, unpublished data and [5]). Consistently, we detected melanophores in the epidermis and dermis covering the scales as well as in the dermis and hypodermis beneath the scales by TEM (Figures 3A, 4). The differentiation or survival of the superficial dendritic melanophores of the guppy depends on the type III receptor tyrosine kinase Kita, as there are less dendritic melanophores in the guppy *kita* mutant golden [3–5,8]. The corolla melanophores in the deeper dermis and hypodermis were frequently associated with iridophores (Figure 1; shown in more detail in Figure 4). A subpopulation of these melanophores of the guppy, which appears early in development, depends on Kita as well [8]. As all males were euthanized with tricaine before fixation, the melanosomes within the melanophores should be mostly dispersed in our samples [49].

We found iridophores in both dermal and hypodermal skin layers (Figures 3B, 4, 5, 6A, 6B, 6D, S1, S2A-C, S3). They contain stacked guanine crystals, called 'reflecting platelets' [28,31]. The crystals usually are lost during sample preparation for TEM, leaving empty spaces that appear inflated in the TEM images (Figures 3B, 4, 5, 6A, 6B, 6D, 7B, 7E, 7G, S1, S2A-C, S3) [26]. The color produced by the iridophores highly depends on the orientation of the platelets relative to each other and the epidermis, as discussed in more detail below [26,30,31]. The number and distance between platelets, and the thickness of the cytoplasm influences their reflection as well [26,30,31]. We did not try to measure the size of the reflecting platelets or the thickness of the cytoplasm between them, as we hardly found any intact ones and the samples were affected by cytoplasmic shrinkage due to sample preparation [50]. The size of the platelets also varied greatly depending on their orientation with respect to the plane of section (Figures 3B, 4, 5, 6A, 6B, 6D, 7B, 7E, 7G, S1, S2A-C, S3).

Xanthophores were identified by the xanthosomes, which are roundish pigment organelles that appear clear or of medium electron density and are approximately 0.5 µm in diameter (Figures 3C, 3D, 4, 5, 7B-G, S1, S2) [27,32,42,43,51,52]. Xanthophores were found in both the dermis and hypodermis (Figures 3C, 3D, 4, 5, S1, S2). The proliferation and/or dispersion

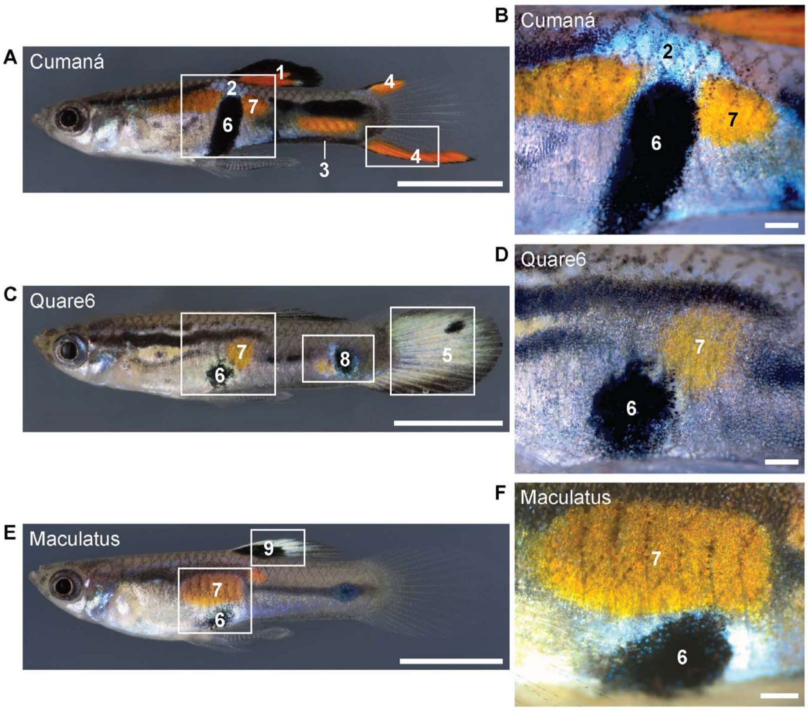

Figure 2. Phenotypes of male Cumaná, Quare6, and Maculatus guppies. (A,C,E) Lateral aspects of adult males taken under incident light conditions. White rectangles indicate details enlarged in (B,D,F) and Figures 6 and 7. Traits are labeled with numbers according to their appearance in the text: 1, Cumaná black and orange ornaments on the dorsal fin; 2, Cumaná blue iridescent spot; 3, Cumaná ventral black margin of the caudal peduncle; 4, Cumaná orange-black lining of the tail fin; 5, Quare6 tail fin color pattern; 6, central black spot; 7, central orange spot; 8, Quare6 posterior black spot on caudal peduncle; 9, Maculatus black spot and whitish ornaments on the dorsal fin. We investigated the ultrastructure of traits 2, 4 (orange part), 5, 6, 7, 8, and 9 (whitish part). Scale bars: (A,C,E) 0.5 cm; (B,D,F) 500 µm.
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of these cells during guppy development depends on signaling through the type III receptor tyrosine kinase Colony-stimulating factor 1 receptor a (Csf1ra) [8]. Previously, yellow to orange xanthophores and red erythrophores were described in guppy skin [4,8,27]. The discrimination between xanthophores and erythrophores, however, is solely based on the apparent color, not on structural differences [30]; therefore some authors have called them xantho-erythrophores [9,53]. We use the term xanthophore here.

Some xanthophores contained clusters of small, light-appearing vesicles or granules in addition to the considerably larger xanthosomes (Figures 3C, 3D, 5, 7C, S2A, S2B, S2D). We found such clusters in some xanthophores within the central orange spot of males from all three strains and in some xanthophores within the orange part of the orange-black margin of the Cumaná tail fin (Figures 3C, 3D, 5, 7C, S2A, S2B, S2D). It has been previously speculated that these clusters might be involved in carotenoid accumulation within the xanthophores [27]. Carotenoid droplets with an approximate diameter of 0.1 to 0.3 µm have been described in medaka (*Oryzias latipes*) and the teleost *Trematomus bernacchii* [52,54]. The organelles that we observed had an approximate diameter of 0.14 µm (Figures 3C, 3D, 5, 7C, S2A, S2B, S2D). Whether all guppy xanthophores contain such small additional organelles or whether these clusters are associated with a special developmental stage or location of the xanthophores is unknown.

The leucophores that have been previously described in the guppy contained globular leucosomes with a diameter of approximately 0.5 to 0.8 µm. Unfortunately, the study describing these leucosomes did not include any xanthophore images for comparison [28]. The leucosomes of medaka and the killifish *Fundulus heteroclitus* are supposed to be of approximately the same size as the ones of the guppy [32,55]. We did not observe any other cells resembling chromatophores beside the described melanophores, iridophores, and xanthophores. Interestingly, some larval chromatophores of medaka contain reddish pigment that they lose during further development, thereby becoming whitish leucophores [32,56]. This raises the possibility that some organelles can contain several pigment types and might change in the course of development [56,57]. Moreover, pigment cells containing two types of pigment organelles, so-called 'dichromatic chromatophores', have been described in some vertebrates. Examples of such chromatophores are the cyano-erythrophores of the mandarin fish (*Synchiropus splendidus*) and the erythro-iridophores of the diadema pseudochromis (*Pseudochromis diadema*) [58,59]. The organelles that we observed within the guppy xanthophores resemble leucosomes except for their smaller size. Further studies will elucidate whether they are carotenoid granules or leucophore-specific organelles containing uric acid.

Ultrastructure of Cumaná blue spot

While the iridescent areas of Quare6 and Maculatus males are largely diffuse, Cumaná males always have a distinct bluish iridescent spot below the dorsal fin (Figure 2A, 2B) [17,18,46]. Under certain light conditions, especially when seen from above, this spot might also appear whitish (data not shown). Inspection of

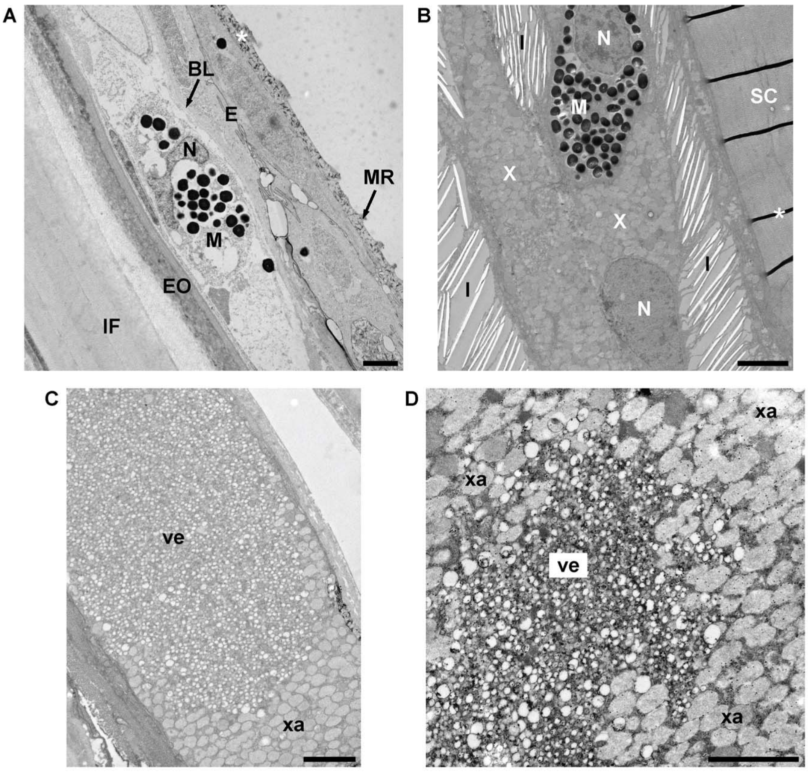

Figure 3. TEM images of guppy chromatophore types. (A) Melanophore on top of a scale in the dermis. Melanophores can be recognized by their dark-appearing pigment organelles, the eumelanin-containing melanosomes. (B) Melanophores, xanthophores, and iridophores in the hypodermis of the central orange spot of a Maculatus male. (C,D) Dermal xanthophores within the central orange spot of a Cumaná male. BL, basal lamina demarcating the boundary between the epidermis and dermis; E, epidermis; EO, external osseous layer of scale; I, iridophore; IF, internal fibrillary plate of scale; M, melanophore; MR, microridges of the epidermis; N, nucleus; SC, stratum compactum of dermis; ve, small vesicles or granules described in the text; X, xanthophore; xa, xanthosomes. Asterisks exemplarily mark artifacts caused by sample preparation; inflated empty spaces within iridophores are not marked. Individuals from which images (B-D) were taken were post-fixed with osmium tetroxide. Scale bars: 2 µm.
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TEM images revealed that this spot is formed by two sheets of iridophores, one of which is located in the stratum spongiosum of the dermis and the other in the hypodermis (Figure 4). Just below the hypodermal iridophores, on top of the muscles, we found melanophores whose appendices frequently protruded into the iridophore layer (Figure 4). The melanophores did not form a complete sheet; in some areas the iridophores were in direct contact with the underlying muscle layer (Figure 4). Melanophores were also present within the dermal iridophore layer (data not shown). The hypodermal as well as the dermal iridophore sheet contained some xanthophores, too (Figure 4 and data not shown). The reflecting platelets of the iridophores appeared to be randomly distributed and were tilted in different directions at some locations, whereas they looked more organized at other locations (Figure 4).

A previous study on the development of iridophores on the lateral trunk of fancy guppies reported that all reflecting platelets had an angle of approximately 15–30° relative to the surface of the fish, thought to account for the blue-green reflection with a wavelength of 496 nm [26]. The light blue coloration of the common surgeonfish (*Paracanthurus hepatus*) is derived from a double layer of iridophores in which the reflective platelets are oriented almost in parallel relative to the fish surface; the iridophores are located on top of melanophores [60]. Ordered iridophores above melanophores have also been observed in the blue skin of the blue-green damselfish (*Chromis viridis*) and the lizard *Plestiodon latiscutatus* [44,61]. In contrast, randomly arranged reflecting platelets usually seem to produce a whitish coloration, e.g. in the white spots of the domino damsel (*Dascyllus trimaculatus*) [41]. We found that both disordered and ordered reflecting platelets are present within the bluish to whitish spot of Cumaná males. The appearance of this spot is dynamic and depends on the angle of the incident light and the movement of the fish. The bluish coloration is presumably derived from the platelets that are arranged in parallel, while the whitish color comes from the disordered platelets. Interestingly, melanophores contribute to the ultrastructure of the blue ornament of the Cumaná guppy like in the common surgeonfish, the blue-green damselfish, and *P. latiscutatus*. Even within the stratum compactum, a melanophore was found in close contact with an iridophore (Figure 4B). We suspect that the melanophores modulate the reflection of the iridophores.

Ultrastructure of central orange spot

We detected large accumulations of xanthophores in the stratum spongiosum of the dermis and hypodermis within the Cumaná central orange spot (Figures 5, S1). These xanthophores frequently contained clusters of the small vesicles or granules described above (Figure 5). Between the xanthophores in the hypodermis were numerous iridophores, with reflecting platelets aligned in parallel (Figures 5B, S1). They were arranged slightly

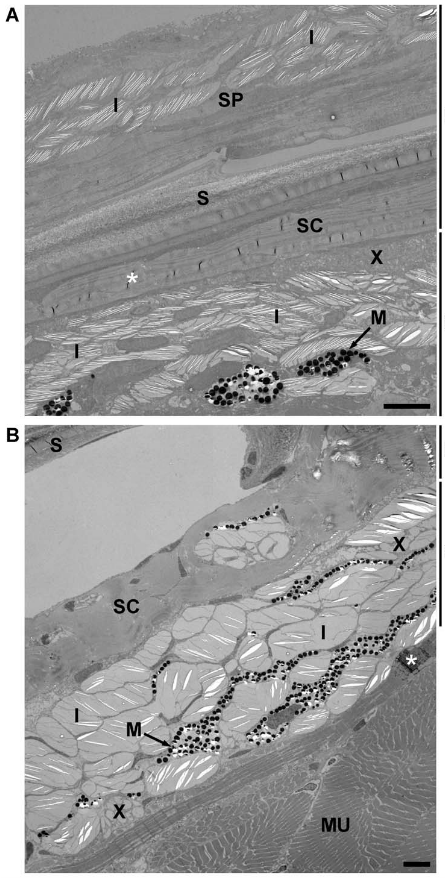

Figure 4. Ultrastructure of Cumaná blue spot. (A,B) TEM images of Cumaná blue spot. An image of the blue spot taken under incident light conditions is shown in Figure 2B (trait 2). Dermal iridophores and hypodermal iridophores and melanophores contribute to the spot. The epidermis was detached during sample preparation in (A). D, dermis; H, hypodermis; MU, muscle; S, scale; SP, stratum spongiosum of dermis. For other abbreviations see Figure 3. Individual from which image (A) was taken was post-fixed with osmium tetroxide. Scale bars: 5 µm.
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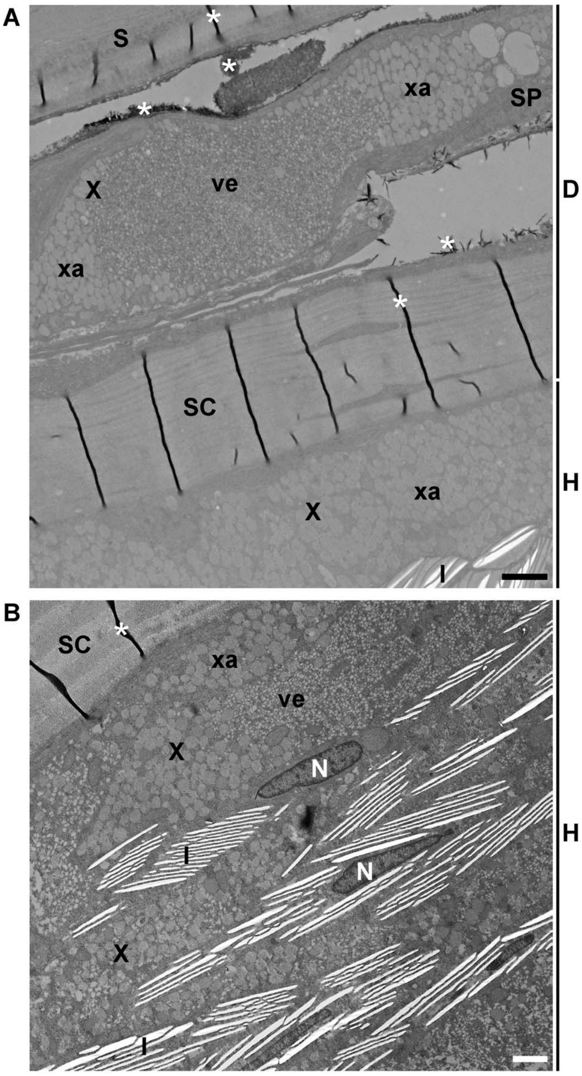

Figure 5. Ultrastructure of Cumaná central orange spot. (A,B) TEM images of Cumaná central orange spot. An overview image of the pigment cell distribution is shown in Figure S1. An image of the central orange spot taken under incident light conditions is shown in Figure 2B (trait 7). Dermal xanthophores and hypodermal xanthophores and iridophores contribute to the spot. For abbreviations see Figures 3 and 4. Individuals from which images were taken were post-fixed with osmium tetroxide. Scale bars: (A) 2 µm; (B) 1 µm.
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obliquely relative to the epidermis (Figure S1). Additionally, some melanophores, as well as iridophores with a more random arrangement of platelets, were present in the hypodermis (data not shown). We also found some iridophores and melanophores in the dermis (Figure S1 and data not shown). The ultrastructure of the central orange spot of Quare6 and Maculatus males appeared similar, except that we found none or only very few xanthophores and iridophores in the dermis (Figure S2A, S2C).

In general, the orange areas of Cumaná males appear darker and more intense than the orange areas of Quare6 and Maculatus males (Figure 2 and data not shown). As all of our fish are fed with *Artemia*, hence uptake *Artemia* carotenoids, the reasons for this must be intrinsic. Our results indicate that thick xanthophore layers in both the dermis and hypodermis cause the intense orange coloration of Cumaná males. The central orange spots of Quare6 and Maculatus males seem to contain fewer xanthophores as the more superficial xanthophore layer is mostly absent. This might be the reason why the spots of Quare6 and Maculatus males appear yellower than the ones of Cumaná males. Alternative explanations are that the production of pteridines varies between the strains as shown for other guppy populations [14], or that the uptake and metabolism of the *Artemia* carotenoids differs, leading to a different pteridines to carotenoids ratio. So far, only the pteridines synthesized by male guppies from Trinidad, which are drosopterins, have been positively identified [11]. As we analyzed the ultrastructure of the central orange spot of only four Cumaná males, more individuals need to be investigated to clarify whether indeed all Cumaná males have an additional superficial xanthophore layer.

Large reflecting platelets that are ordered in parallel to the epidermis and are not underlain with melanophores usually produce silvery, mirror-like reflections [30,42,62]. The numerous iridophores in the hypodermis of the male central orange spot of

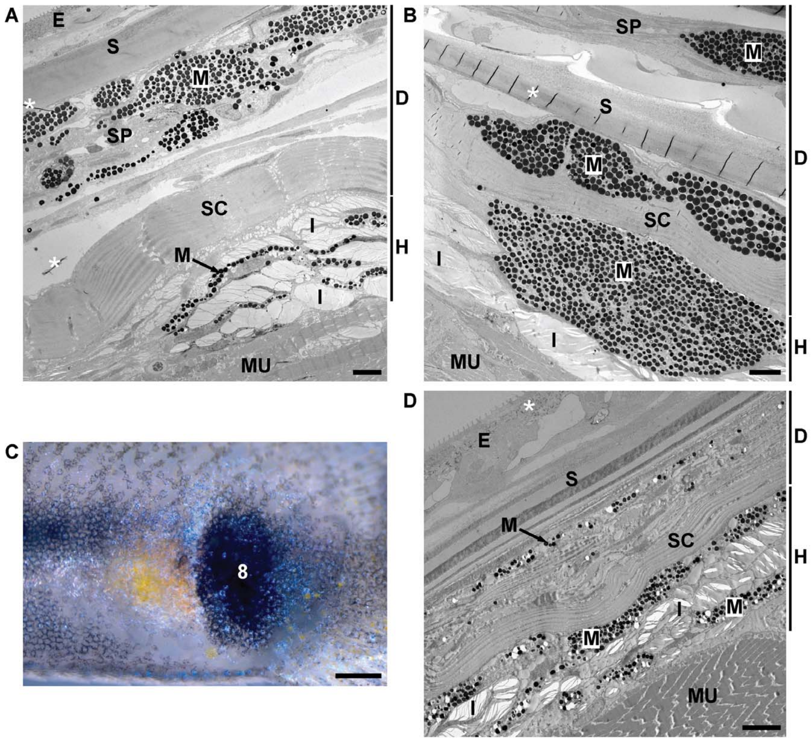

Figure 6. Ultrastructure of Cumaná central black spot and Quare6 posterior black spot. (A,B) TEM images of Cumaná central black spot. An image of the central black spot taken under incident light conditions is shown in Figure 2B (trait 6). Melanophores in the dermis and melanophores and iridophores in the hypodermis contribute to the spot. (C) Detail image of area boxed in Figure 2C showing Quare6 ornaments on the lateral caudal peduncle. Image was taken under incident light conditions. 8, posterior black spot. (D) TEM image of Quare6 posterior black spot. Individual from which image (B) was taken was post-fixed with osmium tetroxide. For abbreviations see Figures 3 and 4. Scale bars: (A,B,D) 5 μm; (C) 500 μm.
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the guppy form reflective sheets. These might reflect light that has not been absorbed or reflected before by the xanthophores, which would make the male orange ornaments shinier and probably more attractive for females by combining orange with iridescence coloration [6,9]. The observed differences in iridophores might also contribute to the color differences of the orange areas.

The ultrastructure of the central orange spot superficially resembles the one observed in the yellowish interstripes of zebrafish, yet the zebrafish xanthophores and iridophores are distributed exclusively in the hypodermis [42]. Whether xanthophore-iridophore interactions like the ones in zebrafish are required for the formation of the central orange spot of the guppy still needs to be investigated [35].

Ultrastructure of central black spot

We found two layers of melanophores within the central black spot of Cumaná, Quare6, and Maculatus males, which were located in the stratum spongiosum of the dermis and in the hypodermis (Figures 6A, 6B, S3). The melanophores were very large, occasionally up to 100 μm in diameter. The ones in the hypodermis were intermingled with iridophores, whose reflecting platelets seemed to be randomly arranged (Figures 6A, 6B, S3). Some xanthophores were scattered in the melanophore layers as well (data not shown).

Since iridophores cause the whitish belly coloration of fish including the guppy ([41] and data not shown), we considered the possibility that iridophores are merely present within the central black spot because this ornament is located on the lateral part of the belly. To test this, we investigated the ultrastructure of the posterior black spot located on the caudal peduncle of Quare6 males (Figures 2C, 6C, 6D). Like in the central black spot, dermal melanophores and hypodermal melanophores and iridophores were present (Figure 6D), suggesting that iridophores are components of the black spots of guppy males independent of their location. Iridophores also contribute to the black and white eye spots seen on the caudal peduncle of some guppy strains, e.g. the BDZW1 strain, by forming a light circle around the black spot [8].

Ultrastructure of fin color patterns

Patterns on the dorsal and tail fins vary greatly between the three guppy strains considered here (Figure 2). We found xanthophores and iridophores in the orange part of the orange-black margin of the Cumaná tail fin and the whitish-yellow part of the dorsal fin of Maculatus males (Figure 7A-E). The reflecting platelets appeared to be randomly oriented (Figure 7B, 7E). While xanthophores were more abundant in the orange Cumaná tail fin margin, iridophores were more frequent in the white ornament on the Maculatus dorsal fin (Figure 7B, 7C, 7E). The xanthophores within the orange Cumaná tail fin margin frequently contained the small vesicles or granules beside xanthosomes (Figure 7C). The white part of the Maculatus dorsal fin also contained few melanophores (Figure 7D and data not shown). Xanthophores and iridophores are also associated with each other in the light stripe regions of zebrafish fins [43]. Iridophores in the orange tail fin margin of Cumaná males might enhance the orange signal by increasing reflection. We found scattered iridophores, xanthophores, and melanophores in the tail fin of Quare6 males (Figure 7F, 7G). All fin pigment cells appeared to be located in hypodermal tissue similar to the situation in zebrafish fins [43].

Conclusions and Outlook

Our study demonstrates that at least two of the three types of pigment cells contribute to each of the investigated ornamental traits of Cumaná, Quare6, and Maculatus guppy males, suggesting that complex interactions between different chromatophore types both may be involved in establishing color patterns and enhance color signals in these strains. Notably, the ultrastructure of the central orange and black spots of Cumaná, Quare6, and Maculatus males is very similar, despite Cumaná and Quare6 guppies being derived from geographically distant populations that may have been separated for almost a million years [63]. More individuals from other populations need to be investigated to confirm that the ultrastructure of these spots is indeed conserved within the guppy. Additionally, TEM in combination with spectrophotometry may help clarify the relationship between ultrastructure and spectral characteristics like hue, saturation, and lightness of guppy ornaments in the future. It would be especially interesting to investigate whether natural guppy populations that differ in the production of drosopterins and hence orange coloration [14] also show differences in the ultrastructure of their orange ornaments.

We have also shown that the pigment cells within the trunk ornaments form thick sheets in the dermis and hypodermis. This

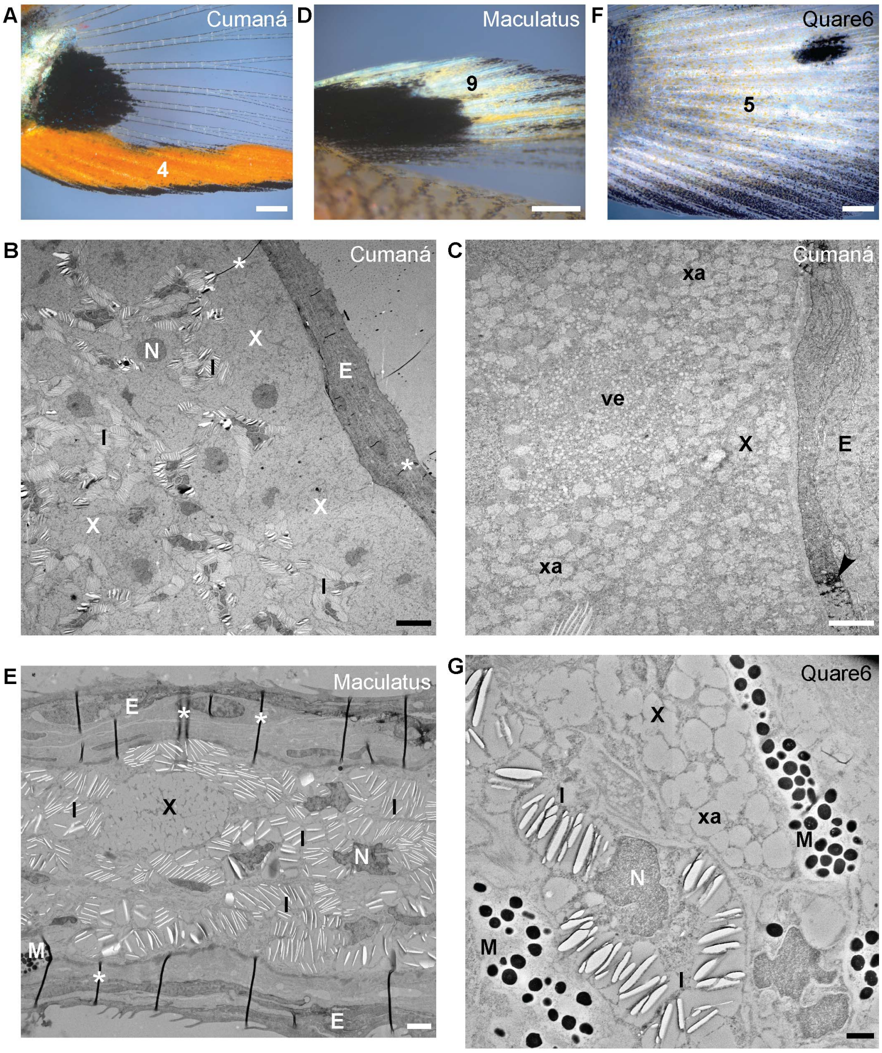

Figure 7. Ultrastructure of fin color patterns. (A,D,F) Detail images of regions boxed in Figure 2A, 2C, and 2E taken under incident light conditions. (D) and (F) are from the individual shown in Figure 2E and 2C, respectively. (A) is from a Cumaná male different from the one shown in Figure 2A. (B,C,E,G) TEM images. (A) Cumaná orange-black margin of tail fin (trait 4). The ultrastructure of the orange part is shown in (B) and (C). (D) Maculatus dorsal fin ornaments (trait 9). The ultrastructure of the whitish part is shown in (E). (F) Quare6 tail fin pattern (trait 5). The ultrastructure of the whitish area is shown in (G). For abbreviations see Figures 3 and 4. Individual from which image (C) was taken was post-fixed with osmium tetroxide. Scale bars: (A,D,F) 500 µm; (B) 5 µm; (C,G) 1 µm; (E) 2 µm.
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contrasts with the situation in zebrafish trunk stripes, in which the chromatophores are restricted to the hypodermis [42,43]. While we could easily identify melanophores, xanthophores, and iridophores in all males, we were not able to confirm the presence of leucophores. Future studies testing the dispersion-aggregation response of guppy chromatophores might reveal whether pigment cells showing a reaction opposite to that of melanophores and xanthophores exist in the three investigated guppy strains. Such a response would be typical for leucophores [64].

Iridophores were a major component of all ornaments, even of ornaments perceived as orange or black. The close association of iridophores with melanophores, and of iridophores with xanthophores, raises the question whether iridophores might interact with xanthophores and melanophores during color pattern formation in male guppies, as has been shown for zebrafish [35]. Because iridophores are transparent and reflective, their photographic documentation highly depends on reproducible light conditions. This might be the reason why previous studies on guppy pigment mutants focused only on melanophore and xanthophore defects. Our study suggests that it is crucial to consider iridophores as well, which might attract melanophores and xanthophores to the locations where spots arise during male color pattern formation. Depending on the location, iridophores might also repulse xanthophores or melanophores, or influence their survival [35]. It would be most interesting to investigate whether iridophores accumulate at the sites of prospective black and orange spots in juvenile males before melanophores and xanthophores appear. If this were the case, it would support a model in which subtle differences in iridophore migration and differential interactions of iridophore populations with other chromatophore types modulate male color pattern formation, ultimately leading to the extraordinary variation of male guppy ornaments.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the German Protection of Animals Act (§ 11 Abs. 1 Nr. 1 a und b TierSchG); all experiments were permitted by the Regierungspräsidium Tübingen (approval ID 35/9185.46). Fish were euthanized using 0.1% (w/v) tricaine (ethyl 3-aminobenzoate methanesulfonate salt) solution pH 7.

Guppy strains and rearing conditions

All guppies were reared at 25°C in a 12-hour light and dark cycle and fed six days a week with *Artemia*. The Cumaná and Quare6 strains originated from individuals that were collected in Venezuela near Cumaná and in the Quare river on Trinidad in 2003 [45,47]. The laboratory strain Maculatus was first described comprehensively in 1922 [1]. The males that we used in our study were at least four months old.

Imaging under incident light conditions

Images of males were taken with a Canon EOS 10D digital camera with a Canon Macro Lens EF 100 mm. A Leica MZFLIII dissecting microscope connected to a Zeiss AxioCam HRc color camera was used to visualize details of the male ornaments; images were processed with AxioVision Software Release 4.7.2. The brightness and contrast of some images was adjusted with Adobe Photoshop Software version 12.1.

Transmission electron microscopy

The ultrastructure of each ornamental trait was investigated in four Cumaná, three Quare6, and three Maculatus males. Sample preparation for transmission electron microscopy was similar to [65]. Briefly, guppy males were sacrificed and fixed in 100 mM PO_4 buffer pH 7.2 containing 4% formaldehyde and 2.5% glutaraldehyde at 4°C overnight. The fixated fish were dissected into small pieces according to the regions of interest. Subsequently, some of the samples were post-fixed with 1% osmium tetroxide on ice for one hour. All samples were then stained with 1% aqueous uranyl acetate at 4°C for one hour in the dark. The samples were dehydrated in a graded series of ethanol/water concentrations; subsequently, a graded series of epon/araldite resin (Araldite 502/Embed 812 Kit, EMS) in propylene oxide was used for embedding. Ultra thin sections of 70–100 nm of the samples were taken along the longitudinal axis of the fish using a Leica Ultracut UCT microtome. Specimens were examined in a FEI Tecnai G^2 Spirit transmission electron microscope operating at 120 kV. Images were taken with a Gatan Ultrascan 4000 camera at maximum resolution using the manufacturer's software. Adobe Photoshop Software version 12.1 was used to adjust the brightness and contrast of some images.

Identification of skin layers

Skin layers were named according to [66].

Measurements of organelle size

Diameters of the organelles found in clusters within xanthophores were measured with ImageJ 1.47 (rsbweb.nih.gov/ij/). We measured the diameter at the widest part of 100 organelles and calculated the average. These measurements are just an approximation, as the organelles were cross-sectioned at different planes.

Supporting Information

Figure S1 Overview TEM image of Cumaná central orange spot. For abbreviations see Figures 3 and 4. Individual from which image was taken was post-fixed with osmium tetroxide. Scale bar: 10 μm.
(TIF)

Figure S2 Ultrastructure of Quare6 and Maculatus central orange spots. (A,B) TEM images of Quare6 central orange spot. (B) is an enlarged detail of (A) showing xanthosomes and vesicles or granules within a xanthophore as described in the text. (C,D) TEM images of Maculatus central orange spot. (D) shows xanthosomes and vesicles or granules within a xanthophore. The epidermis was lost during sample preparation. Images of the Quare6 and Maculatus central orange spots taken under incident light conditions are shown in Figure 2D and 2F (trait 7), respectively. For abbreviations see Figures 3 and 4. Individual from which images (A) and (B) were taken was post-fixed with osmium tetroxide. Scale bars: (A) 2 μm; (B,D) 1 μm; (C) 5 μm.
(TIF)

Figure S3 Ultrastructure of Quare6 and Maculatus central black spots. (A) TEM image of Quare6 central black spot. (B) TEM image of Maculatus central black spot. Images of the Quare6 and Maculatus central black spots taken under incident light conditions are shown in Figure 2D and 2F (trait 6), respectively. For abbreviations see Figures 3 and 4. Individuals from which images were taken were post-fixed with osmium tetroxide. Scale bars: 10 μm.
(TIF)

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Author Contributions

Conceived and designed the experiments: VAK CD. Performed the experiments: VAK IK MF. Analyzed the data: VAK IK MF HH. Wrote the paper: VAK. Provided helpful comments on the manuscript: IK MF HH DW CD. Oversaw the experimental design and data analysis: DW.

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Figure S1

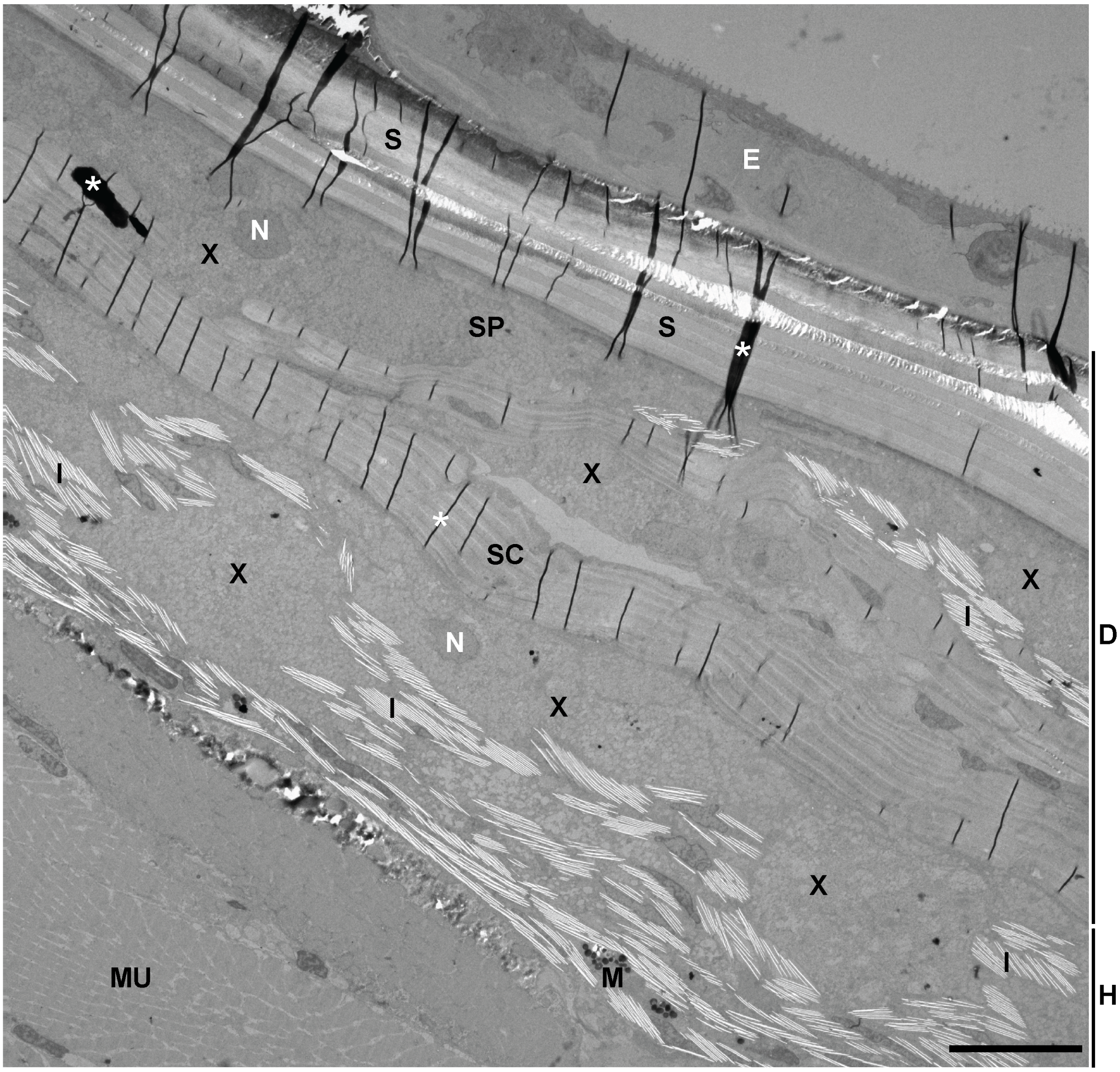

Figure S2

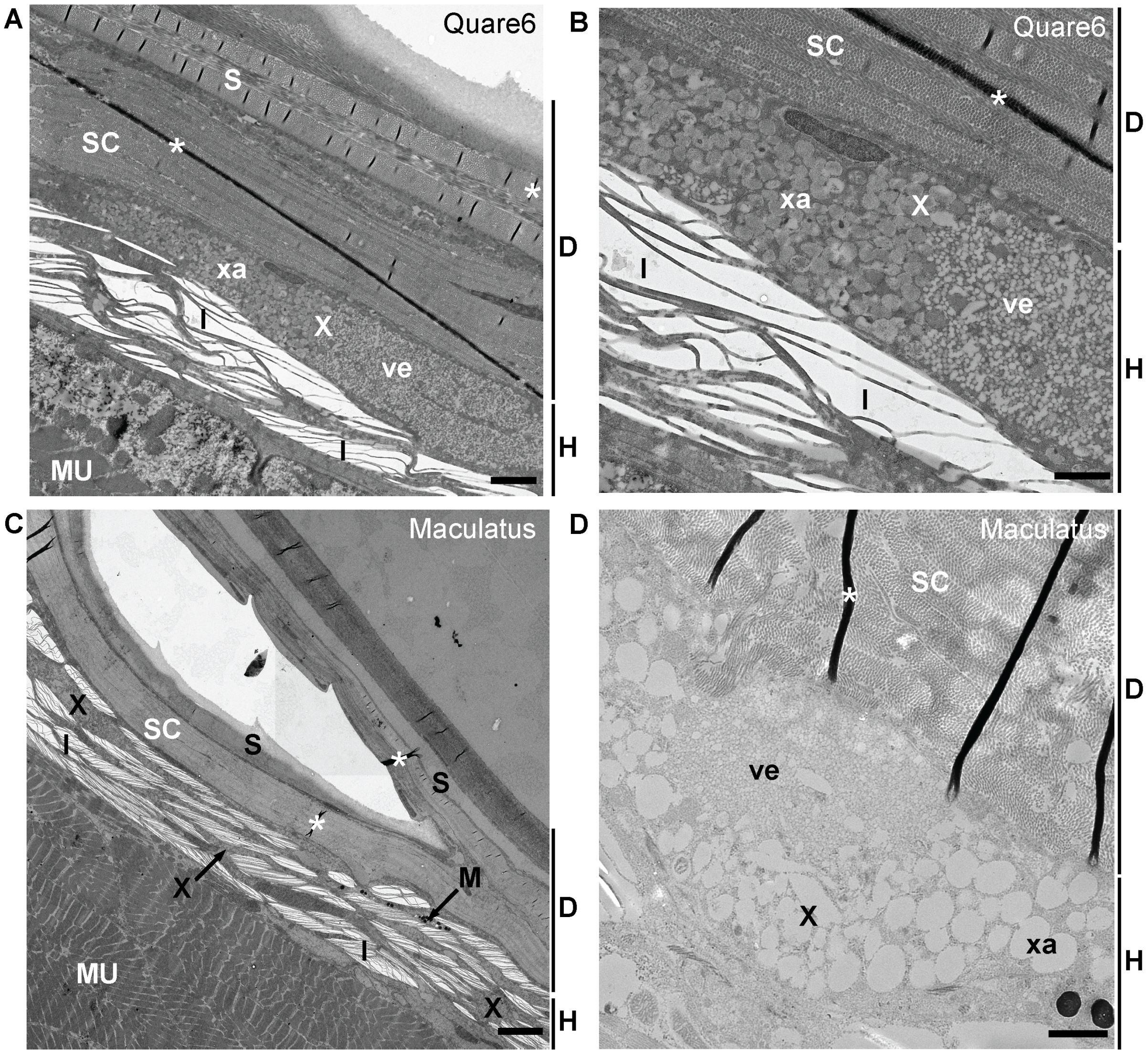
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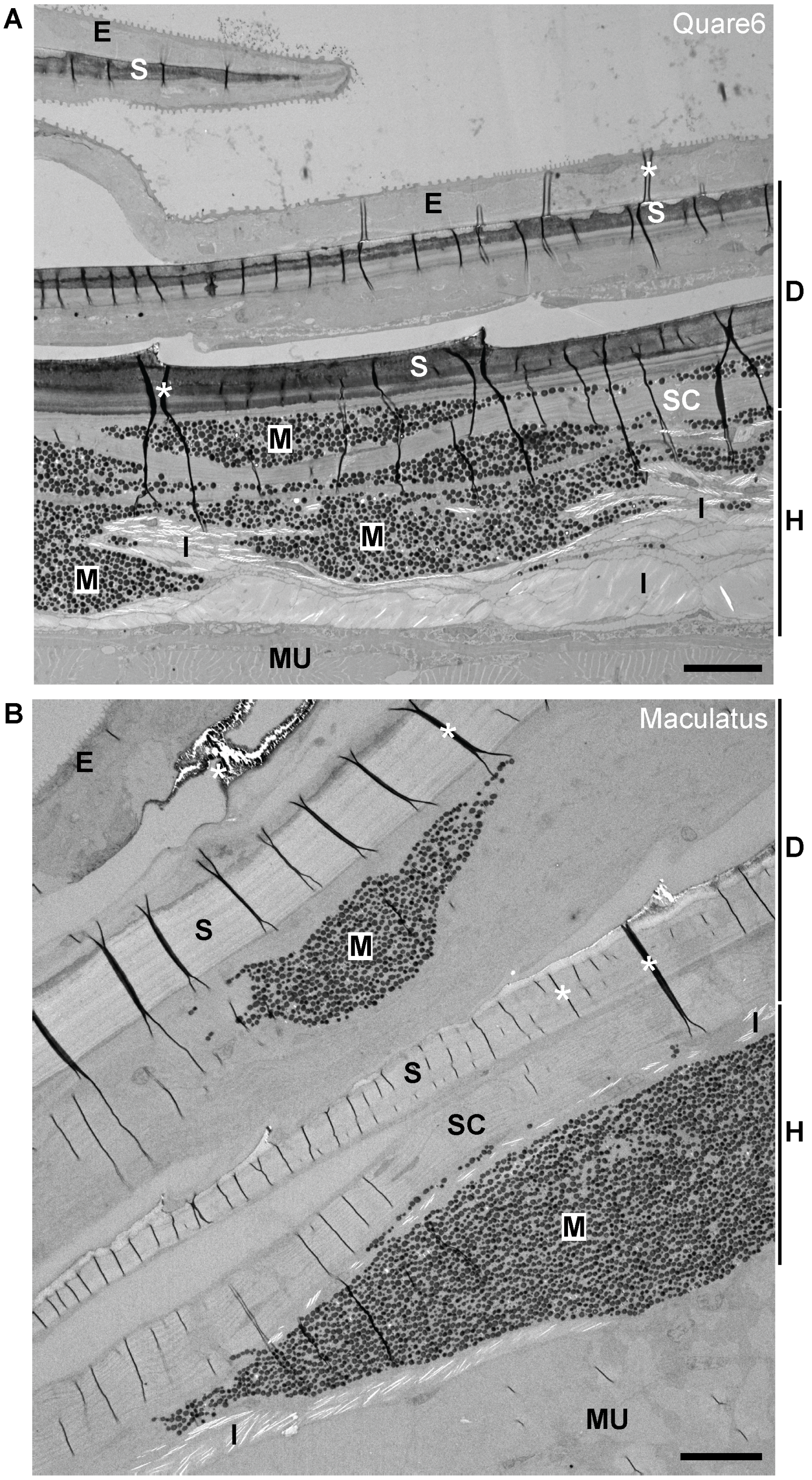
Figure S3



4 “Pigment pattern formation in the guppy, *Poecilia reticulata*, involves the Kita and Csf1ra receptor tyrosine kinases”

Verena A. Kottler, Andrey Fadeev, Detlef Weigel, and Christine Dreyer, 2013

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Synopsis

To study the genetics of guppy pigment pattern formation, I chose to investigate three spontaneous guppy color mutants, *golden*, *blue*, and *blond*, whose autosomal recessive, single-locus inheritance had previously been described (DZWILLO 1959; GOODRICH *et al.* 1944; HASKINS and DRUZBA 1938; WINGE and DITLEVSEN 1947). I selected these mutants as all of them have a distinct, easily recognizable phenotype in which either the melanophores or xanthophores are severely affected (DZWILLO 1959; GOODRICH *et al.* 1944; HASKINS and DRUZBA 1938; WINGE and DITLEVSEN 1947). These mutants provided the opportunity to explore how mutations in single loci affect the development of guppy pigment cells and pattern formation in general. This chapter refers to the *golden* and *blue* mutants; for *blond*, see section 5 on page 67.

I used a candidate gene approach to identify the mutations underlying the golden and blue phenotype. The phenotypes of golden and blue guppies resemble the ones of *sparse* and *panther* zebrafish, which are caused by mutations in *kita* and *csf1ra*, respectively (PARICHY *et al.* 2000b; PARICHY *et al.* 1999; PARICHY and TURNER 2003). Early metamorphic melanophores of zebrafish depend on Kita, while later-developing metamorphic melanophores and most xanthophores require Csf1ra for their development (PARICHY *et al.* 2000b; PARICHY *et al.* 1999; PARICHY and TURNER 2003). Sequencing of the guppy *kita* and *csf1ra* orthologs revealed that loss-of-function mutations in these genes are responsible for the golden and blue phenotype.

We pursued several approaches to investigate how these mutations affect the pigment pattern of newborn and adult guppies. Examination of the color pattern of wild-type and *kita* mutant guppies at different time points revealed that two temporally and genetically distinct melanophore populations, one depending on Kita and the other independent of Kita, contribute to the adult reticulate pattern. The newborn pigment pattern of the guppy requires Kita, but Kita-independent melanophores contribute to the pattern while the fish grow up. Melanophore counts demonstrated that both melanophore populations are independent of Csf1ra. Csf1ra, however, is required for the development of guppy xanthophores. To study the effects of the mutations on the male-specific pattern of the guppy, I crossed the mutations into the inbred Cumaná (ALEXANDER and BREDEN 2004), Guanapo (REZNICK and ENDLER 1982), Quare6 family II 215-3 (TRIPATHI *et al.* 2008), and Quare6 (REZNICK and ENDLER 1982) strains. By using a backcross scheme, we ensured that all N2 males whose phenotypes were compared had an X chromosome of the highly inbred laboratory strain BDZW1 (DZWILLO 1959) and an essentially identical Y chromosome, as crossing over between the guppy X and Y chromosomes is rare (TRIPATHI *et al.* 2009a). This made sure that most of the observed variation between the closely related wild-type and mutant males was derived from the different *kita* and *csf1ra* alleles. We categorized all color traits and analyzed first in wild-type and then in mutant males whether they were present, absent, or diffuse. If a color trait was present, we noted its location. CD and I analyzed the patterns independently; we obtained similar results.

The crosses revealed that the *csf1ra* loss-of-function mutation not only results in the absence of all orange traits but also severely disturbs the male-specific melanophore pattern. This suggests that Csf1ra stimulates black pattern formation, most likely via xanthophore-melanophore interactions as has been described in zebrafish (MAHALWAR *et al.* 2014; PARICHY and TURNER 2003; SINGH and NÜSSLEIN-VOLHARD 2015; YAMANAKA and KONDO 2014). We also found that certain black traits require Kita for proper formation.

Taken together, our results demonstrate that melanophores developing at different time points and differing in their genetic requirements for Kita and Csf1ra are essential for guppy color pattern formation.

Contributions

VAK and CD conceived and designed the experiments. VAK performed all experiments, took care of the crosses, and analyzed the data; VAK and CD both analyzed the male patterns. VAK and AF conducted the epinephrine experiment; VAK counted the melanophores. VAK wrote the manuscript. AF, DW, and CD provided helpful comments on the manuscript. DW oversaw the experimental design and data analysis.

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Pigment Pattern Formation in the Guppy, *Poecilia reticulata*, Involves the Kita and Csf1ra Receptor Tyrosine Kinases

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ABSTRACT Males of the guppy (*Poecilia reticulata*) vary tremendously in their ornamental patterns, which are thought to have evolved in response to a complex interplay between natural and sexual selection. Although the selection pressures acting on the color patterns of the guppy have been extensively studied, little is known about the genes that control their ontogeny. Over 50 years ago, two autosomal color loci, *blue* and *golden*, were described, both of which play a decisive role in the formation of the guppy color pattern. Orange pigmentation is absent in the skin of guppies with a lesion in *blue*, suggesting a defect in xanthophore development. In *golden* mutants, the development of the melanophore pattern during embryogenesis and after birth is affected. Here, we show that *blue* and *golden* correspond to guppy orthologs of *colony-stimulating factor 1 receptor a* (*csf1ra*; previously called *fms*) and *kita*. Most excitingly, we found that both genes are required for the development of the black ornaments of guppy males, which in the case of *csf1ra* might be mediated by xanthophore–melanophore interactions. Furthermore, we provide evidence that two temporally and genetically distinct melanophore populations contribute to the adult camouflage pattern expressed in both sexes: one early appearing and *kita*-dependent and the other late-developing and *kita*-independent. The identification of *csf1ra* and *kita* mutants provides the first molecular insights into pigment pattern formation in this important model species for ecological and evolutionary genetics.

THE guppy (*Poecilia reticulata*) is thought to be among the most color-polymorphic vertebrates (Endler 1983). Male guppies have an outstanding degree of variation in their ornamental patterns, which are shaped by a complex interplay between natural and sexual selection in wild populations. Along with introduction experiments, studies on guppy life-history traits, mate choice behavior, and predator–guppy as well as guppy–environment interactions have demonstrated that guppy populations can adapt rapidly to new environments (for an overview, see Magurran 2005). The guppy is therefore a prime model organism for the study of "evolution in action."

Despite our wealth of knowledge about the ecological importance of coloration, the genes and developmental pathways underlying guppy pigment pattern formation are unknown. Both forward and reverse genetic studies are hampered by the fact that guppies are livebearers with internal fertilization, an average gestation period of 3–4 weeks, and a relatively small brood size (Houde 1997). The genetic basis of sex determination is highly variable within the Poeciliid family, to which the guppy belongs. The guppy itself has incipient X and Y chromosomes that include a nonrecombining part (Traut and Winking 2001). Only males develop highly polymorphic ornaments during puberty, which are under hormonal control (Houde 1997). The genetic analysis of male guppy ornaments first attracted attention >80 years ago, when Winge described a total of 18 putative ornamental loci, of which 17 showed sex-linked inheritance and 9 were strictly Y-linked (Winge 1922, 1927). Many more pigment pattern loci, which can be Y-linked, X-linked, XY-linked, or autosomal, have since been described (Lindholm and Breden 2002). Ornamental traits linked to the sex

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Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.151738/-/DC1.
Data for File S1 and File S2 are available at ftp://ftp.tuebingen.mpg.de/ebio/csf1ra_kita_mutants
Sequence data from this article have been deposited under GenBank accession nos. KC143122 (*csf1ra*); KC143123 (*csf1ra blue* allele); KC143124 (*kita*); KC143125 (*kitla*); and KC143126
(part of *kita* genomic locus in *golden* mutants).

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chromosomes are typically expressed only in males, but females can develop some male color patterns when treated with testosterone (Clemens *et al.* 1966; Lindholm and Breden 2002). An analysis of quantitative trait loci (QTL) has confirmed that most male color traits are controlled by multiple genes, including genes on autosomes (Tripathi *et al.* 2009b). In contrast to the sex-specific genes, several autosomal color factors behave as ordinary Mendelian recessive genes and are expressed in both sexes (Goodrich *et al.* 1944; Dzwillo 1959; Lindholm and Breden 2002).

The pigment pattern of the guppy consists of three to four different types of neural crest-derived chromatophores: black melanophores, yellow/orange to reddish xanthophores, blue iridescent iridophores, and, possibly, white leukophores (Takeuchi 1976; Tripathi *et al.* 2008). Guppy embryos are staged according to the differentiation of their eyes. In the middle-eyed stage, the retina is fully pigmented and the first melanophores differentiate on the head above the midbrain (Martyn *et al.* 2006). More melanophores appear during the late-eyed stage and form dark stripes along the lateral midline, on the back, and on the belly (Martyn *et al.* 2006). In the very late-eyed stage shortly before birth, a rhombic reticulate pattern consisting of melanophores emerges on the trunk (Martyn *et al.* 2006). It has also been referred to as a ground, diamond, or camouflage pattern (Goodrich *et al.* 1944; Martyn *et al.* 2006; Tripathi *et al.* 2008). All pigment cell types are present in wild-type embryos at birth and contribute to the newborn pattern (Figure 1A); *e.g.*, the yolk is partially covered by iridophores and melanophores, while xanthophores are widely dispersed (Martyn *et al.* 2006).

The reticulate pattern of very late-eyed embryos and newborn guppies appears to persist into adulthood (Tripathi *et al.* 2008). This pattern is expressed in both sexes, but becomes overlain in males by male-specific ornaments (Figure 1, B–E). Two different melanophore types occur in adult wild-type guppies: large, roundish corolla and more heterogeneously shaped dendritic melanophores (Goodrich *et al.* 1944). The reticulate pattern is composed of corolla melanophores in deep skin layers, which are additionally arranged irregularly over the whole body, whereas dendritic melanophores are distributed superficially and are associated with the scales (Figure 1E) (Goodrich *et al.* 1944; Winge and Ditlevsen 1947).

Two autosomal color loci that are expressed in male and female guppies are *blue* and *golden* [also known as *fredlini* (Haskins and Druzba 1938; Winge and Ditlevsen 1947) and not to be confused with the zebrafish (*Danio rerio*) *golden* locus (Lamason *et al.* 2005)]. The mutations in both genes occurred spontaneously and act recessively (Goodrich *et al.* 1944; Dzwillo 1959). *Blue* mutants lack orange pigmentation in their skin, indicating a xanthophore defect (Figure 1) (Dzwillo 1959).

In *golden* mutants, the development of the melanophore pattern during embryogenesis and after birth is affected (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and Ditlevsen 1947). Golden guppies of both sexes lack melanophores in the skin at birth and have only a few peritoneal melanophores above the swim bladder (Figure 1A), but eventually develop an incomplete reticulate pattern, which gives them a "coarsely mottled appearance" (Goodrich *et al.* 1944; Winge and Ditlevsen 1947). Corolla melanophores are restricted to the reticulate pattern in *golden* mutants, which also have only a very few dendritic melanophores (Figure 1E) (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and Ditlevsen 1947). *golden* mutant females have only half of the normal number of melanophores in total (Goodrich *et al.* 1944). *golden* mutant males develop male-specific ornaments (Figure 1, B and C) (Haskins and Druzba 1938).

Among teleost fish, pigment pattern formation has been most extensively studied in zebrafish. Zebrafish undergo a complex pigment pattern metamorphosis during the transition from the embryonic/early larval to the juvenile/early adult stage (Johnson *et al.* 1995; Parichy and Turner 2003a,b; Kelsh *et al.* 2009; Parichy *et al.* 2009; Budi *et al.* 2011). The two type III receptor tyrosine kinases encoded by *kita* and its ancient paralog *colony-stimulating factor 1 receptor a* (*csf1ra*; previously called *fms*) (Braasch *et al.* 2006) play key roles during the establishment of the adult zebrafish pigment pattern: early metamorphic melanophores require *kita* for their development, while late metamorphic melanophores depend on *csf1ra* and *endothelin receptor b1a* (*ednrb1a*) (Parichy *et al.* 1999, 2000a,b; Parichy and Turner 2003b). *Csf1ra* is also crucial for xanthophore development (Parichy *et al.* 2000a; Parichy and Turner 2003b). In other teleost species, the functions of *kita* and *csf1ra* are less well understood; comparative studies including interspecies hybrids suggest some functional diversification of both receptor tyrosine kinases even within the *Danio* genus (Quigley *et al.* 2005; Parichy 2006; Mills *et al.* 2007).

Here, we present evidence that *golden* and *blue* correspond to guppy orthologs of *kita* and *csf1ra*. Both an early *kita*-dependent and a later-appearing *kita*-independent melanophore population contribute to the adult reticulate pattern in this species. In contrast to zebrafish, *csf1ra* is not required for the development of the late-appearing *kita*-independent melanophores. *Csf1ra*, however, is essential for xanthophore development and the formation of the male-specific melanophore pattern, which also requires *kita*.

Materials and Methods

Fish husbandry

All fish were maintained at 25° in a 12-hr light and dark cycle and fed 6 days a week with *Artemia*. No more than eight fish were kept per 1.5-liter tank. We used virgins for crosses, as guppy females are capable of storing sperm.

Strains

Guppies of the following inbred strains were used in this study; available phenotypes other than wild-type are shown in parentheses: Maculatus (MAC) (golden) (Tripathi *et al.*

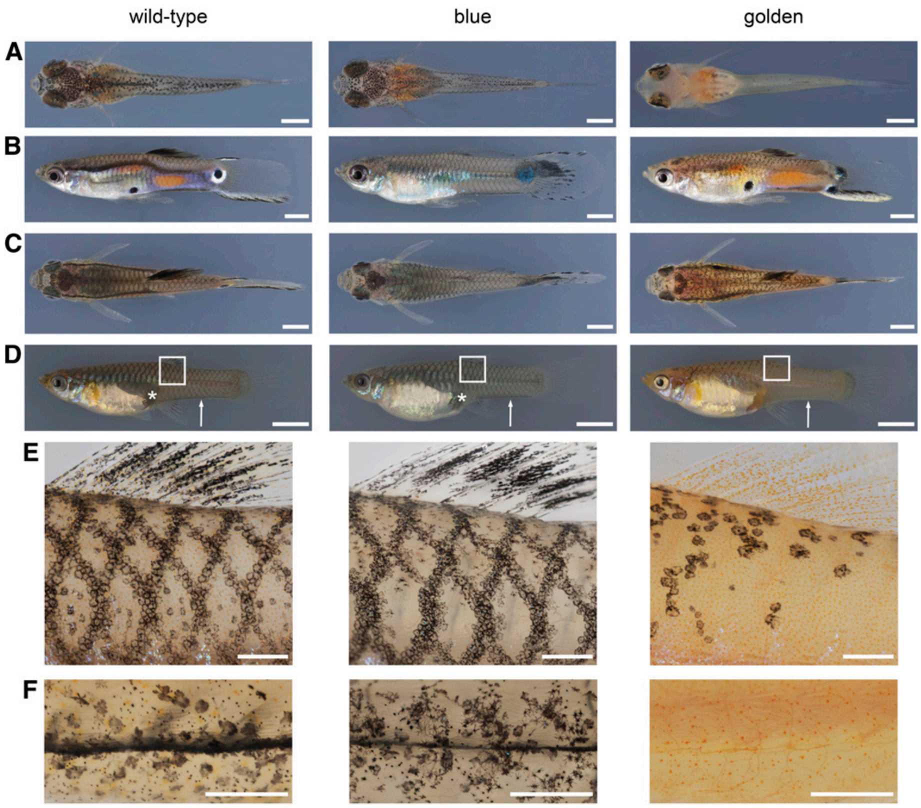


Figure 1 Blue and golden phenotypes. (A) Dorsal aspect of newborns. (B) Lateral aspect of adult males. (C) Dorsal aspect of adult males. (D) Lateral aspect of adult females. (E) Details of areas boxed in D showing the reticulate pattern. (F) Ventral view of the caudal peduncle of females (indicated by white arrows in D). *golden* mutants of both sexes lack a ventral black stripe and have only a few melanophores on the anterior head, including the choroid of the eyes. Golden females lack the female pigment spot above the anal fin (white asterisks in D). Individuals shown are from the BDZW1 (wild type, golden) and BDZW2 (blue) background. Bars: (A) 1 mm; (B and C) 2 mm; (D) 5 mm; (E and F) 0.5 mm.

2008); BDZW1 (golden, golden blue) and BDZW2 (blue only) (Dzwillo 1959); Armatus (golden) (Winge 1927); Guanapo and Quare6 (Reznick and Endler 1982); Quare6 family II 215-3 (Tripathi *et al.* 2008); and Cumaná (Alexander and Breden 2004). Maculatus, BDZW1, BDZW2, and Armatus are domesticated strains that have been bred in captivity for >50 years [in our laboratory since 2003 (Maculatus, BDZW1) and 2011 (Armatus, BDZW2)]; the others are derived from wild individuals caught in Trinidad (Guanapo and Quare rivers) and Venezuela (Cumaná region). Quare and Cumaná guppies were obtained in 2003 and Lower Guanapo (Twin Bridge) fish in 2009. All guppy strains are kept separately in small groups, usually consisting of two to three females and four to five males per tank and are allowed to reproduce freely, except for the Guanapo fish that are inbred by brother–sister mating.

blue was found in two strains that likely both were derived from the original population of *blue* mutants described by Dzwillo in 1959. We renamed them BDZW1 and BDZW2 for clarity. BDZW1 was obtained under the name "Blau" and comprised individuals heterozygous for *golden* and *blue*; BDZW2 ("Dzwillo 1959 Blau") fish were all homozygous for *blue*.

Phylogenetic analyses

Only unambiguously annotated sequences were used for the analyses (ORF begins with a start codon; number of exons are as predicted for other species; and introns begin and end with splice sites). Sequences were aligned with Clustal Omega (v1.1.0) (Goujon *et al.* 2010; Sievers *et al.* 2011). Maximum-parsimony and maximum-likelihood phylogenetic trees were constructed by the PhylipParsimony algorithm (Felsenstein 1989) via the SplitsTree4 (v4.12.8) interface (Huson and Bryant 2006) and by PhyML (Guindon *et al.* 2010), respectively. The topologies of the maximum-likelihood trees were not fully resolved (therefore not shown), but, as the maximum-parsimony trees, they suggest that the sequenced guppy ORFs are most similar to *kita*, *kit ligand a* (*kitla*), and *csf1ra* of other teleost species.

Complementary DNA analyses

We used polymerase chain reaction (PCR) to amplify genes of interest from first-strand complementary DNA (cDNA). Total RNA was extracted from 10 to 15 pooled early to very-late eyed embryos with TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was then directly used for first-strand cDNA synthesis using PrimeScript Reverse Transcriptase (TaKaRa) and the oligo(dT) primer 5′-ATTCTAGAGGCCGAGGCGGCCGACATGT(18)VN-3′. We added 1 U/μl SUPERaseIn RNase Inhibitor (Ambion) to each reaction. First-strand cDNA was used as template for PCR, which was carried out with Advantage 2 Polymerase Mix (Clontech) according to the manufacturer's instructions. PCR program was 10 cycles touchdown [95° for 15 sec, Tm (melting temperature) of primers (lower one) + 5° −0.5°/cycle for 30 sec, 68° for 2–5 min) followed by 27 cycles without touchdown (95° for 15 sec, Tm for 30 sec, 68° for 2–5 min]. Elongation time was adapted to the length of the expected product (~1 min/kb). Primer sequences and methods used for full-ORF amplification of candidate genes are listed in Supporting Information, Table S1. PCR products were analyzed by gel electrophoresis, purified with MinElute Gel Extraction Kit (QIAGEN), and subsequently cloned into pGEM-T Easy vector (Promega) following the manufacturer's instructions. Plasmid DNA was purified with Wizard Plus SV Minipreps DNA Purification System (Promega) according to the instruction manual and sequenced.

To investigate whether the *kita* transcripts V1–V6 cosegregate with the golden phenotype, we prepared first-strand cDNA from adult individuals as described before. Total RNA for cDNA synthesis was extracted separately from liver tissue of the parental male and the parental female and from pooled liver tissue of seven wild-type N2 fish and 11 golden N2 fish. Liver tissue was used as total RNA isolated from liver usually is of very high quality (personal observation). We used primers in exon 5 (forward: 5′-GATGCTGGGAGT TACAAATGCGTAG-3′) and exon 9 (reverse: 5′-AAACAGT ATGTAGGCTTGCTCTCC-3′) for PCR amplification with Advantage 2 Polymerase Mix (Clontech) and cloned the products into pGEM-T Easy vector (Promega). We sequenced the purified plasmid DNA of 24 colonies per parent and N2 pool to identify wild-type and *golden* mutant *kita* transcripts.

Real-time quantitative PCR

Total RNA for real-time quantitative PCR was prepared as described above from skin of adult wild-type, golden, and blue females that were 6–9 months old. Following DNaseI treatment, first-strand cDNA was prepared from 830 ng of total RNA primed with oligo(dT)$_{18}$ using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. First-strand cDNA was diluted 10-fold for real-time quantitative PCR that was conducted with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) according to the instruction manuals provided by the manufacturers. PCR program was 95° for 3 min, 40 times (95° for 10 sec, 60° for 10 sec, 72° for 5 sec), and 95° for 10 sec. Expression of *csf1ra*, *csf1rb*, *kita*, and *kitb* was determined by using three biological replicates with three technical repetitions each. Expression of the target genes was normalized to *glyceraldehyde-3-phosphate dehydrogenase* expression. Standard deviation and normalized expressions ($\Delta\Delta C_q$) were calculated with CFX Manager software. Primer sequences and efficiencies (Pfaffl 2001; Vandesompele *et al.* 2002) are given in Table S2.

Genomic DNA analyses

Genomic DNA was prepared with DNeasy Blood and Tissue Kit (Qiagen) from trunk tissue of adult guppies. We used 100 ng of DNA per PCR reaction. If not mentioned otherwise, Advantage 2 Polymerase Mix (Clontech) was used to carry out the PCRs. *Kita*^{insert} was first amplified using a forward primer in exon 6 (5′-TGTCTCTGAACGTTAG CATGGAG-3′) and a reverse primer in exon 7 (5′-ACACG GAGAAGTTCTGCTTTACC-3′) of *kita* (elongation time: 5 min). To test whether *kita*^{insert} and the golden phenotype are associated, we designed PCR assays for *kita*^{insert} and *kita*^{wt} using Phusion High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions. Details can be found in Table S3. PCR products were analyzed by gel electrophoresis. PCR products of *csf1ra*^{wt} and *csf1ra*^{indel} were purified with 0.2 U/μl FastAP Thermosensitive Alkaline Phosphatase (Fermentas) and 2 U/μl exonuclease I (Fermentas) (37° for 15 min, 85° for 15 min) and subsequently sequenced. Primer sequences are given in Table S3.

Sequence analysis

Purified plasmid DNA and PCR products were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a DNA Analyzer ABI Prism 3730XL (Applied Biosystems). Sequences were analyzed using the Staden package (Pregap4 v1.5 and Gap4 v4.10; http://staden.sourceforge.net/) and inspected manually. Exon–intron structures were predicted according to the gene structure of *kitla*, *kita*, and *csf1ra* of other teleost species (for species, see Table S1; exon–intron structure was inferred from http://www.ensembl.org).

Imaging

Photos of whole embryos or details of adult fish were taken with a Leica MZFLIII dissecting microscope connected to a Zeiss AxioCam HRc color camera and processed with AxioVision Software Release 4.7.2. Photos of fish after birth and adult fish were taken with a Canon EOS 10D digital camera using a Canon Macro Photo Lens MP-E 65 mm or Canon Macro Lens EF 100 mm. Adult males were at least 4 months old to ensure that their pigment pattern was fully developed. All photos were taken under incident light conditions. Fish were anesthetized in 0.1% (w/v) tricaine (ethyl 3-aminobenzoate methanesulfonate salt) solution (pH 7) before imaging. The background of most images was equalized with Adobe Photoshop software version 12.1; all original images are available upon request.

Analysis of melanophores

To analyze melanophore pattern development, photos of the same fish were taken every 3 days (day 1–40 after birth). Each fish was kept separately in a 1.5-liter tank. Fish were briefly anesthetized in 0.04% tricaine solution before imaging. Their siblings were kept as control; none of the imaged fish showed retarded development. Newly arising melanophores were detected by comparing consecutive images to each other.

To analyze the number of melanophores, fish were immersed in standard E2 solution (Nusslein-Volhard and Dahm 2002) containing 2.4 mg/ml epinephrine for ~5 min to contract melanosomes. Afterward, fish were anesthetized, and the right side under the dorsal fin, as well as the complete fish, were imaged. Melanophores were counted manually utilizing Adobe Photoshop software version 12.1. The number of melanophores was plotted against the area in square millimeters using Microsoft Excel for Mac 2011 version 14.2.3.

Results

Identification of golden as kita:

We used a candidate gene approach to identify *golden* at the molecular level. Based on a comparison with zebrafish pigment pattern mutants, we investigated two potential candidates for *golden*, *kita*, and *kitla* (also called *stem cell/steel factor*). Kitla is the ligand for the Kita receptor, and together the two are required for melanophore migration and survival in zebrafish (Hultman *et al.* 2007). While only one copy of *kit* and *kitl* exists in mouse and humans, two copies of each gene are present within the Teleostei. These copies can be considered as “ohnologs” since they are derived from ancestral *kit* and *kitl* genes that were duplicated during the teleost-specific whole-genome duplication event (Mellgren and Johnson 2005; Braasch *et al.* 2006; Hultman *et al.* 2007). Mutations in zebrafish *kita*, as in *golden*, greatly reduce melanophore number (Parichy *et al.* 1999). *Kita* is located on guppy autosomal linkage group 4 (Tripathi *et al.* 2008). *Kitla* has not been mapped so far.

To amplify the guppy orthologs of *kita* and *kitla*, we used rapid amplification of cDNA ends (Table S1). Phylogenetic analyses demonstrated that the obtained full-ORF sequences are orthologous to *kita* (GenBank accession no. KC143124) and *kitla* (GenBank accession no. KC143125) of other teleost species (Figure 2A and Figure S1). We also identified a potential guppy *kitb* ortholog by performing BLAST searches of zebrafish *kitb* against a preliminary genome and transcriptome assembly of the guppy (E. Sharma, A. Künstner, B. A. Fraser, M. Hoffmann, V. A. Kottler, G. Zipprich, D. Weigel, and C. Dreyer, unpublished data) (Figure 2A) (Altschul *et al.* 1990). We could not determine whether a guppy *kitlb* ortholog exists, as BLAST yielded no significant results.

Golden mutant guppies did not have any obvious polymorphisms in the *kitla* ORF. In contrast, among cDNAs from golden fish of the MAC and BDZW1 backgrounds, we found a total of six different *kita* splice variants, none of which was wild type. Most of these variants contained inserts with a length of 17, 36, or 53 bp and/or lacked parts of or the complete exon 6 (Figure 2B and Table S4). Exons 7 and 8 were additionally absent in two variants (Figure 2B and Table S4). In all cases, these alterations cause frameshifts and truncate the ORF. These *kita* variants are likely nonfunctional since the encoded proteins would lack the transmembrane, juxtamembrane, and split kinase domains, all of which are required for normal protein function (Mol *et al.* 2003).

Analysis of genomic DNA indicated that *kita* exon 6 of *golden* mutants has an insertion of 1678 bp. This insertion consists of two potential short exons of 17- and 36-bp length that surround a 1625-bp intron delineated by 5′ GT-AG 3′ canonical splice sites (Figure 2C). The splice sites within the novel intron seem to be recognized by the spliceosome and lead to mis-splicing of the *kita* pre-messenger RNAs (mRNAs) in the *golden* mutants. Depending on which splice sites are used, this leads to short insertions or removal of parts of exon

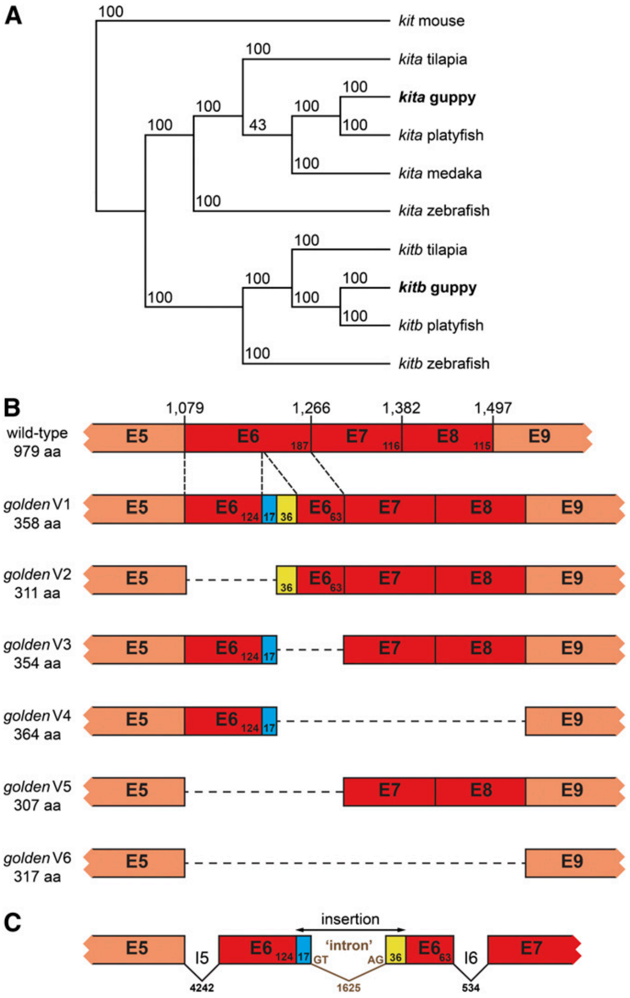


Figure 2 *golden* is an ortholog of *kita*. (A) Maximum-parsimony phylogenetic tree of *kita* and *kitb* ORF sequences. Mouse *kit* was used as an outgroup. Bootstrap support values from 100 replicates are shown. Accession numbers of sequences are the following: guppy *kita*, KC143124; medaka (*Oryzias latipes*) *kita*, ENSORLT00000000707; mouse (*Mus musculus*) *kit*, NM_021099; platyfish (*Xiphophorus maculatus*) *kita*, ENSXMAT00000013579; platyfish *kitb*, ENSXMAT00000017731; tilapia (*Oreochromis niloticus*) *kita*, ENSONIT00000003735; tilapia *kitb*, XM_003452144; zebrafish *kita*, NM_131053; and zebrafish *kitb*, NM_001143918. Guppy *kitb* sequence is available upon request. (B) Exons 5–9 of *kita* cDNAs sequenced from wild-type and *golden* mutant [variants (V) 1–6] backgrounds; exons affected by splicing defects are shown in dark red. Length in amino acids (aa) of the respective predicted protein is shown on the left. Numbers above wild type refer to the wild-type *kita* cDNA sequence deposited at GenBank (KC143124) and demarcate the last nucleotide of each exon. (C) Part of *kita* genomic locus in *golden* mutants ($kita^{insert}$) (GenBank accession no. KC143126). Numbers refer to base pairs. Exons (E), introns (I), and inserted sequence are not drawn to scale.

6 or of exons 6–8 from the mature transcript. Comparison with a preliminary genome assembly of the guppy (A. Künstner, E. Sharma, B. A. Fraser, M. Hoffmann, V. A. Kottler, D. Weigel, and C. Dreyer, unpublished data) suggests that >50 copies similar to the 1678-bp insertion, which all include a long terminal repeat of ~300 bp, exist (data not shown).

To confirm that *kita*^{insert} corresponds to *golden*, we tested for genetic linkage. Forty *golden* mutant fish from the MAC, BDZW1, and Armatus (ARM) backgrounds were homozygous for *kita*^{insert} and complementation test crosses between the strains demonstrated allelism (Figure S2B and S1C). In 28 wild-type MAC, BDZW1, and ARM individuals, only *kita*^{wt} could be detected. This indicates that *kita*^{insert} was likely introduced into several guppy strains by breeders because of the golden coloration. Next, we investigated whether *kita*^{insert} is also linked to the golden phenotype in a segregating backcross N2 population: we crossed a *golden blue* double-mutant female of the BDZW1 strain to a wild-type male from the Guanapo river in Trinidad; F_1 males were then backcrossed to produce $X^{BDZW1}X^{BDZW1}/X^{BDZW1}Y^{GU}$ N2 individuals. Forty-six golden and golden blue N2 fish were homozygous for *kita*^{insert}, while 12 wild-type and blue fish were heterozygous *kita*^{insert}/*kita*^{wt}. In addition, we could amplify only *kita*^{insert} variants from pooled cDNA of *golden* mutant N2 offspring. Taken together, all of these results make it very likely that guppy *golden* is allelic to *kita*.

Identification of blue as csf1ra:

We hypothesized *csf1ra* to be a candidate for *blue*, since *blue* mutants lack xanthophores as do *csf1ra* mutants of zebrafish (Dzwillo 1959; Parichy *et al.* 2000a). *Csf1ra* and *csf1rb* ohnologs have been identified in several teleost species (Braasch *et al.* 2006). *csf1ra* has previously been mapped to guppy autosomal linkage group 10 (Tripathi *et al.* 2008).

Rapid amplification of cDNA ends was used to amplify the guppy *csf1ra* ortholog (Table S1). Phylogenetic analyses showed that the sequenced cDNA is closely related to *csf1ra* of other teleost species (Figure 3A). We also identified a presumptive guppy *csf1rb* ortholog within a preliminary genome and transcriptome assembly of the guppy (E. Sharma, A. Künstner, B. A. Fraser, M. Hoffmann, V. A. Kottler, G. Zipprich, D. Weigel, and C. Dreyer, unpublished data) (Figure 3A).

Blue mutant guppies carry a complex change in exon 17 of *csf1ra*, with a deletion of 5 bp and an insertion of 7 bp (Figure 3B). This indel causes a frameshift and truncates the ORF. The predicted protein lacks part of the second half of the split kinase domain, which is required for the activity of type III receptor tyrosine kinases (Yarden and Ullrich 1988; Mol *et al.* 2003). A similar mutation in zebrafish, *fms*^{j4blue}, inactivates *csf1ra* and leads to loss of xanthophores (Parichy *et al.* 2000a).

We used the same cross as above to test for linkage of *csf1ra* and *blue*. Forty-six blue and golden blue N2 fish were homozygous for *csf1ra*^{indel}, while 12 wild-type and golden N2 individuals tested were heterozygous *csf1ra*^{indel}/*csf1ra*^{wt}. Consistent with this, 24 *golden blue* mutant guppies of the BDZW1 strain were homozygous for *csf1ra*^{indel}, whereas only *csf1ra*^{wt} could be identified in 12 wild-type and golden fish of the same population. Another *blue* strain obtained from a hobby breeder, BDZW2, was also homozygous for the *csf1ra*^{indel} allele, and complementation test crosses to BDZW1 confirmed allelism (Figure S2A). *blue* is therefore likely to be the guppy ortholog of *csf1ra*, with the same *csf1ra*^{indel} mutation present in both strains tested.

In zebrafish, *csf1ra* promotes the migration of the xanthophore precursors from the neural crest (Parichy *et al.* 2000a), and both embryonic and metamorphic xanthophore populations require *csf1ra* activity (Parichy and Turner 2003b). To investigate whether *csf1ra* mutant guppies entirely lack xanthophores, we thoroughly inspected 7-month-old golden blue and blue fish that shared the same grandparents. In contrast to a previous study (Dzwillo 1959), we found a small number of xanthophores in 19 of 20 *golden blue* mutant individuals and in 5 of 17 *blue* mutants (Figure 3, C and C′). Most of the xanthophores were arranged on scale margins close to the dorsal fin. We could not detect any xanthophores on the lateral or ventral side of adult golden blue or blue fish or in blue embryos and newborns (we here refer to guppies as newborns 1–3 days after birth). A small number of xanthophores can also be found in zebrafish larvae, but not in adults, which are homozygous for *salz*^{tl41a} or *pfeffer*^{tm36b}, two alleles that fail to complement *fms*^{j4blue} (Maderspacher and Nusslein-Volhard 2003).

Expression of kita, csf1ra, and their ohnologs in female skin

We could detect *kita*, *kitb*, *csf1ra*, and *csf1rb* expression in the skin of adult wild-type, golden, and blue females by Reverse Transcriptase-PCR (data not shown). To assess whether Kitb and Csf1rb might be upregulated to compensate for the loss of Kita and Csf1ra function in the *golden* and *blue* mutants, respectively, we investigated the expression levels of these genes and their a-paralogons by real-time quantitative PCR. We found that *csf1ra* expression is downregulated in the skin of *blue* mutant females compared with the wild type, while there was no significant difference in the expression levels of *csf1rb* (Figure S3). In zebrafish, *csf1ra* is expressed in the xanthophore, macrophage, and osteoclast lineages (Parichy *et al.* 2000a). Our data suggest that the guppy ortholog of *csf1ra* is expressed in guppy xanthophores, as the almost complete absence of these cells in blue skin coincides with a low *csf1ra* expression level. Additionally, the blue transcript of *csf1ra* might undergo nonsense-mediated mRNA decay triggered by the premature termination stop codon. In contrast, the expression levels of *kita* and *kitb* were not significantly different in golden skin compared with wild type, respectively (Figure S3), which suggests that the golden *kita* transcripts are not subjected to nonsense-mediated decay. In conclusion, no significantly elevated expression of the *kit* and *csf1r* b-paralogons could be detected by real-time quantitative PCR in the skin of *golden* and *blue* mutants (Figure S3). Yet we cannot

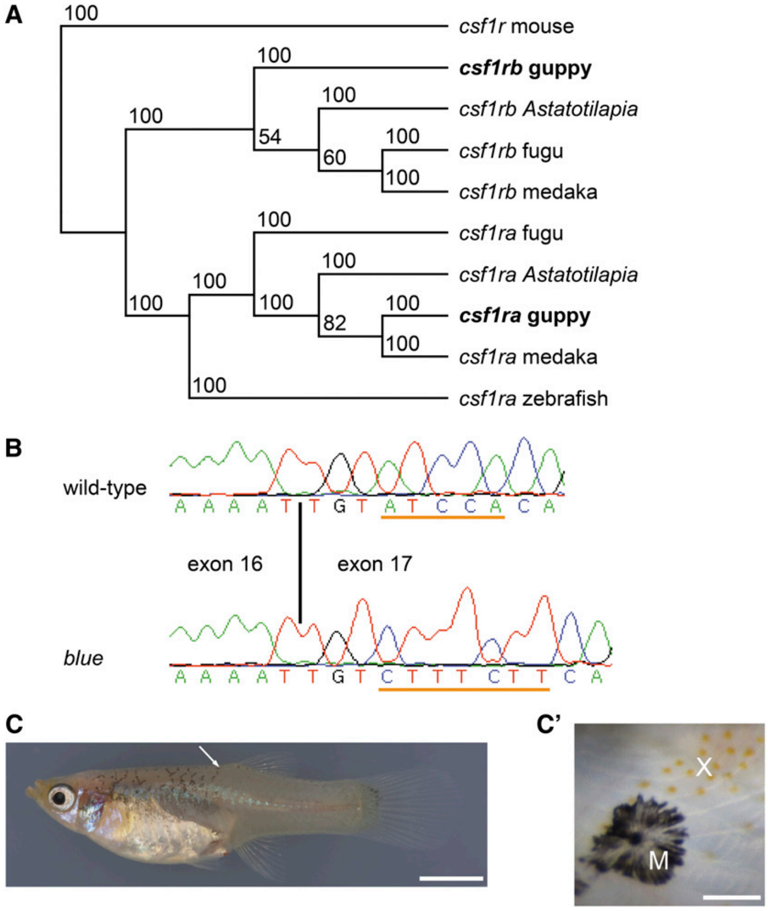


Figure 3 *blue* is an ortholog of *csf1ra*. (A) Maximum-parsimony phylogenetic tree of *csf1ra* and *csf1rb* ORF sequences. Mouse *csf1r* was used as an outgroup. Bootstrap support values from 100 replicates are shown. Accession numbers of sequences are the following: *Astatotilapia burtoni csf1ra*, DQ386648; *A. burtoni csf1rb*, DQ386647; fugu (*Takifugu rubripes*) *csf1ra*, U63926; fugu *csf1rb*, AF411927; guppy *csf1ra*, KC143122; medaka *csf1ra*, ENSORLT00000006111; medaka *csf1rb*, XM_004076731; mouse *csf1r*, NM_001037859; and zebrafish *csf1ra*, AF240639. Guppy *csf1rb* sequence is available upon request. (B) Partial sequence electropherograms of cDNAs from wild-type and *blue* mutant fish, which carry a deletion of 5 nt in exon 17 (underlined in wild-type sequence) that simultaneously contains a 7-nt insertion (underlined in *blue* sequence). The length of the predicted protein produced by *blue* mutants is 794 aa, with the last 12 new. The wild-type protein is 978 aa long. The first nucleotide of exon 17 corresponds to nucleotide 2392 in the 3084-bp wild-type *csf1ra* sequence (GenBank accession numbers of cDNAs: wild-type, KC143122; *blue*, KC143123). (C) *golden blue* mutant female; white arrow points to detail shown in C′. (C′) Small patch of xanthophores (X) and melanophore (M) on the back close to the dorsal fin of the female. Variation in the number of xanthophores was high in *blue* and *golden blue* mutant fish. Xanthophores were abundant and evenly distributed in wild-type and *golden* mutant females (Figure 1, E and F). Bars: (C) 5 mm; (C′) 50 μm.

exclude that less efficient salvage pathways mediated by Kitb and Csf1rb compensate for the loss of Kita and Csf1ra function in the mutants based on these findings.

Contribution of distinct melanophore populations to the adult reticulate pattern

Kita-dependent and -independent metamorphic melanophores contribute to the adult pigment pattern in zebrafish (Parichy *et al.* 1999; Parichy and Turner 2003b). Additionally, Kita activity is required for the dispersal of melanoblasts from the neural crest in zebrafish embryos (Parichy *et al.* 1999). To investigate whether temporally and genetically distinct melanophore populations exist in the guppy, we examined *golden* mutant guppies at different developmental stages.

First, we explanted wild-type and golden embryos 12 days after last parturition, which corresponds to the middle-eyed stage, at which the first melanophores differentiate in the skin above the midbrain in wild-type embryos (Martyn *et al.* 2006). *golden* mutant embryos lacked melanophores on the head and the trunk at this stage (Figure 4A). Second, we investigated the development of the pigment pattern between the 1st and the 40th day after birth by taking photographs of the same individuals every 3 days.

Immediately after birth, *golden* mutants had few patches of peritoneal melanophores, as described previously (Goodrich *et al.* 1944; Winge and Ditlevsen 1947). Additionally, we found some melanophores in their skin and close to the neural tube (Figure 4B). Four and 7 days after birth, few scattered melanophores were present close to the dorsal midline and on the head of *golden* mutants (Figure 4C and File S1). After 10 days, melanophores had become more abundant on the dorsal part of *golden* mutant fish and formed an incipient reticulate pattern, which became quite prominent after 16 days (Figure 4, D and E). The formation of the reticulate pattern seemed to be completed ~22 days after birth in the mutants (Figure 4, F and G), although we detected a few newly arising melanophores even in 40-day-old golden individuals (File S1). This suggests that two melanophore populations that are temporally and genetically distinct contribute to the adult reticulate pattern: one that develops early and depends on Kita and one that appears late and is independent of Kita. In wild-type fish, melanophores were abundant and distributed over the whole body from the day of birth onward (Figure 4, B–G).

Adult *golden* mutant females have about half the number of skin melanophores of wild-type females (Goodrich *et al.* 1944) and lack the pigment spot by the anal fin (Figure 1, D and E). Additionally, the amount of superficial dendritic melanophores associated with the scales is strongly reduced (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and

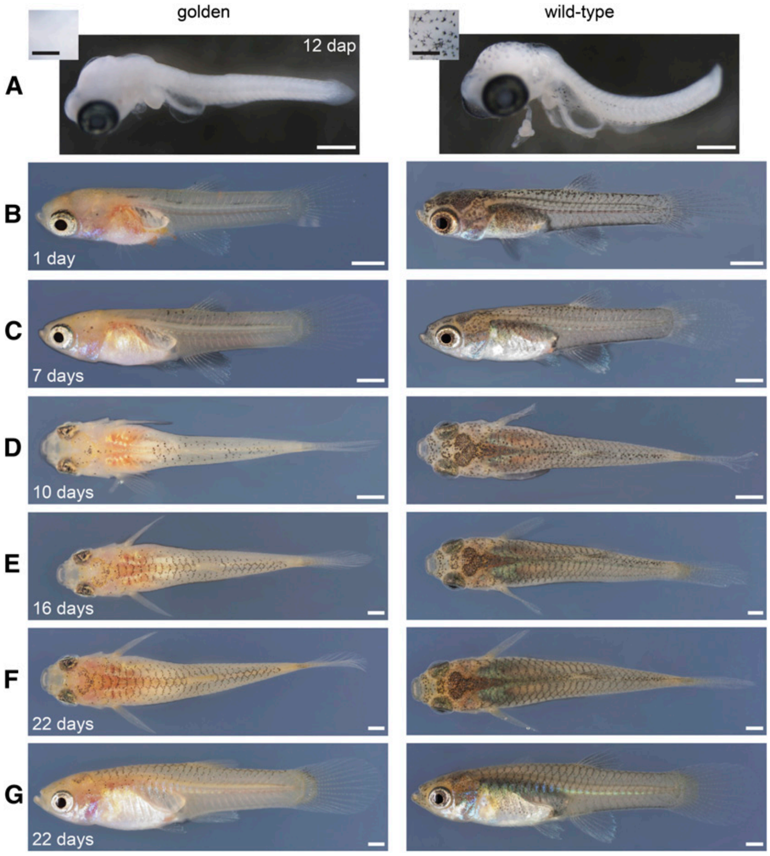


Figure 4 Melanophore pattern development in BDZW1 wild-type and *golden* mutant fish. (A) Embryos explanted 12 days after last parturition (dap). Yolk sacs were removed and embryos were fixed overnight at 5° in 4% paraformaldehyde and 1% dimethyl sulfoxide. Insets: Dorsal aspect of the midbrain region with individual melanophores apparent in wild type. (B, C, and G) Lateral, (D–F) dorsal aspects of the same females over a 3-week time course (days are after birth). All images taken, including the ones of males, can be found in File S1. Bars: (A) 500 μm, (insets) 250 μm; (B–G) 1 mm.

Ditlevsen 1947), and black pigment is scarce on the choroid of the eyes as well as on the anterior head in golden fish, indicating that these pigmentation traits depend on Kita (Figure 1, B–E). The arrangement of the melanophores along the scales seems to be independent of Kita, as the size of the reticulate pattern (diameter of rhombi) was similar in golden and wild-type fish (data not shown). Onset of puberty in golden and wild-type males was observed between day 19 and 28 (File S1); the development of the reticulate pattern in golden males was similar to that of golden females (File S1).

csf1ra-independent melanophore populations

In zebrafish, early *kita*-dependent metamorphic melanophores are independent of Csf1ra, whereas late-differentiating *kita*-independent melanophores require Csf1ra and Ednrb1a activity for their differentiation (Parichy *et al.* 2000a,b; Parichy and Turner 2003b). *kita csf1ra* double-mutant zebrafish lack almost all melanophores because of the strong additive effect of mutations in these two genes (Parichy *et al.* 2000a). Since we identified a *kita*-dependent and -independent melanophore population in the guppy, we asked whether any of them requires *csf1ra*.

Inspection of embryos and newborns revealed no major differences between the *blue* mutant and wild-type melanophore patterns (Figure S4A), although we cannot exclude that a small subset of the early appearing melanophores depends on Csf1ra signaling since we could not count these early cells (see Figure S4 legend). To investigate whether the late-differentiating melanophores depend on *csf1ra*, we compared an area below the dorsal fin in golden and golden blue adult females (Figure S4, E and E′). We found that both single- and double-mutant fish have similar numbers of melanophores per area (Figure S4F). Therefore, unlike in zebrafish, mutations in *kita* and *csf1ra* have no additive effect on the reticulate pattern of the guppy, which is further supported by the observation that the reticulate pattern of *blue* mutant guppies develops as in the wild type until at least day 40 after birth (Figure S4, A–D; File S1). Consequently, the late-appearing melanophores of the guppy reticulate pattern are independent of both Kita and Csf1ra signaling.

Requirement of Kita and Csf1ra signaling for male-specific ornaments

Guppy male-specific pigmentation patterns vary tremendously within and among populations, yet the within-population variance decreases considerably with inbreeding (personal observation). To investigate the roles of Kita and Csf1ra in male color pattern formation, we compared the ornamental patterns of related wild-type and mutant males. We crossed *golden blue* mutant BDZW1 females with wild-type males from the inbred wild-derived Cumaná (CUM), Guanapo (GU), Quare6 family II 215-3 (QUII), and Quare6

(QU) strains. The phenotypically wild-type F_1 males were backcrossed to *golden blue* mutant BDZW1 females to produce a N2 generation (Figure 5). As a result of the backcross, the grandfather's Y chromosome was combined with an X chromosome of the BDZW1 strain in all N2 males. Since recombination frequency of sex chromosomes is comparatively low in male meiosis (Tripathi *et al.* 2009a), this experimental design minimized the amount of pattern variation caused by X chromosomes derived from different strains, thereby allowing us to study the influence of the mutant autosomal genes on the pattern directed by a given Y chromosome. The number of male offspring derived from each cross is given in Table 1, and all images of the backcrosses can be found in File S2.

For our analysis, we focused on the most prevalent characteristic traits of each cross, as seen on the grandfather and its wild-type male offspring (Figure 6, Figure 7, Figure 8 and Figure 9; Figure S5). Furthermore, we tried to homologize the male ornaments of the different strains based on their color, shape, and approximate positions. A summary of all traits and generalization of our results is shown in Figure 9, which should facilitate tracking of single traits within the complex male patterns. We are, however, aware of the fact that superficially similar-looking traits need neither be directed by the same developmental pathways nor be derived from the same putative cell precursor pool.

Regardless of the origin of the Y chromosome, *blue* mutant N2 males lacked all orange color traits, indicating that Csf1ra activity is also required for the dispersal or differentiation of male-specific xanthophores (Figure 6, Figure 7, and Figure 9). In addition, the location and shape of the black male-specific ornaments were modified in the mutants.

Previous studies of the male-specific color pattern of the Cumaná guppy have shown that the blue iridescent spot on the trunk close to the dorsal fin, the combination of black and orange on the dorsal fin, and the ventral black lining of the caudal peduncle constitute strictly Y-linked traits (Figure 6A and Figure 9) (Tripathi *et al.* 2008, 2009a,b). The male-specific ventral black lining is more pronounced than the ventral black stripe of both sexes described in Figure 1F. Typically, two thicker black horizontal stripes, an anterior and a posterior one, are present on the trunk of Cumaná males (Figure 6A and Figure 9). The orange-black lining of the tail fin, which is often more pronounced on the ventral margin, is another male-specific Cumaná trait (Figure 6A and Figure 9). The prominent ventral black spot on the tail fin of many Cumaná males (Figure 6A and Figure 9; Figure S5) is very likely directed by one or more dominant factors located in the pseudo-autosomal region of the Cumaná sex chromosomes (Tripathi *et al.* 2008).

Most Cumaná male-specific traits were fully developed in the grandfather, in all F_1, and in all wild-type N2 males (Figure 6, A–C; for the ventral black spot on the tail fin, see Figure S5). Of the *golden* mutant N2 males, all but one showed all of these traits as well (Figure 6D and Figure 9; the pattern of the one exceptional individual might be the outcome of a rare recombination event). Comparisons between wild-type and golden males revealed that (i) *golden* mutants had less black on the dorsal fin; (ii) the ventral black lining of the caudal peduncle was shifted upward and was often discontinuous in golden fish; (iii) the posterior black stripe of *golden* mutants was shifted downward and was often discontinuous (Figure 6D and Figure 9). In addition, a concise posterior orange spot on the caudal peduncle was absent in 94% of the *golden* mutants (Figure 6D). The anterior black stripe, the anterior orange spot, the blue iridescent spot, the orange-black lining, and the ventral black spot on the tail fin were not obviously changed by the mutation in *kita* (Figure 6D and Figure 9). The *blue* mutant N2 males not only lacked all orange traits, but also lost the black components of the orange-black lining of the tail fin, whereas the ventral black spot persisted (Figure 6E and Figure 9). Other defects of the *blue* mutant fish were the following: (i) the anterior black stripe was absent or very diffuse; (ii) the posterior black stripe was shifted downward and seemed more diffuse, and small incoherent patches of melanophores were often found on the dorsal trunk; (iii) in only 43% of the blue males a comparatively small blue iridescent spot was visible close to the dorsal fin (Figure 6E and Figure 9). The phenotype of the *golden blue* mutant N2 males resembled the *blue* mutant fish, but they showed fewer melanophores on the dorsal fin and trunk (Figure 6F).

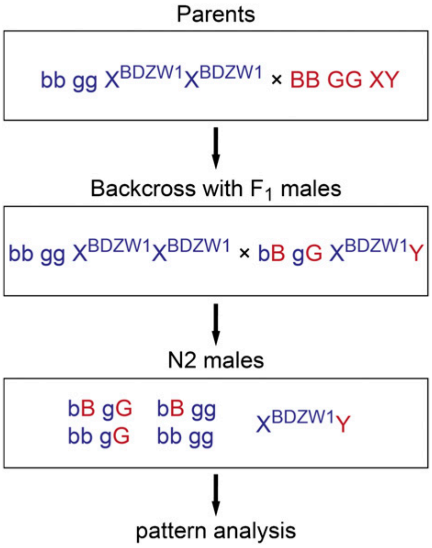


Figure 5 Crossing scheme that was used in the case of the CUM, GU, QUII, and QU strains. The color patterns of the wild-type, golden, blue, and golden blue N2 males were analyzed. *blue* (b) and *golden* (g) are located on different autosomes and can therefore recombine freely. "B" and "G" indicate wild-type alleles. BDZW1 and MAC males were crossed with females of the same strain (for details see Figure 8).

The pattern of the Guanapo grandfather was characterized by several black crescents forming a labyrinthine pattern in proximity to the gonopodium (Figure 6G and Figure 9). The melanophores on the proximal and distal parts of the tail fin were concentrated on the ventral half of the fin, and an

Table 1 Male offspring obtained from each cross

Male parent	F_1	N2			
		Wild type	Golden	Blue	Golden blue
Cumaná (CUM)	55	39	33[a]	52	53
Guanapo (GU)	30	24	25	18	21
Quare6 family II 215-3 (QUII)	34	16	16	11	10
Quare6 (QU)	43	17	18	25	14
		F_2			
		Wild type		Golden	
BDZW1	24	56		18	
Maculatus (MAC)	12	30		13	

[a] Actual number was 34, but one male had a BDZW1 instead of a CUM-like color pattern (see text).

orange spot was located close by (Figure 6G and Figure 9). The anterior and posterior black horizontal stripes, the ventral black lining of the caudal peduncle, and the anterior and posterior orange spots on the trunk appeared weaker compared with the Cumaná male (Figure 6G). The posterior black stripe approximately demarcated the lateral midline in the Guanapo grandfather.

All of the F_1 and wild-type N2 males showed the Guanapo traits described above, but only 41% of the wild-type males had an anterior orange spot (Figure 6, H and I). Furthermore, the F_1 and wild-type N2 males tended to have more complex black labyrinthine ornaments close to the gonopodium than the Guanapo grandfather, suggesting that cofactors derived from the autosomes or X chromosome of the BDZW1 strain modulate this trait (Figure 6, H and I). The black pigment pattern of the *golden* mutant N2 males was substantially changed in the following ways: (i) black pigment in the dorsal fin was reduced; (ii) the labyrinthine black ornaments close to the gonopodium were mostly lost; (iii) black pigment in the proximal part of the tail fin was concentrated dorsally; (iv) anterior and posterior black stripes were often discontinuous; and (v) the ventral black lining was shifted upward (Figure 6J and Figure 9). The orange ornaments persisted, and an anterior orange spot was found in 32% of the golden N2 males (Figure 6J and Figure 9). The *blue* mutant N2 males had an even more severe phenotype: (i) only one to two round black spots were present on the trunk that were in most individuals not located near the gonopodium; (ii) as in golden N2, the black in the proximal part of the tail fin was located dorsally, and several males had black tail-fin margins; and (iii) the anterior black stripe was absent or diffuse (Figure 6K and Figure 9). The *golden blue* mutant N2 males had fewer melanophores on the fins and trunk than the *blue* mutants (Figure 6L).

The pigmentation traits of the Quare6 family II 215-3 grandfather are shown in Figure 7A. Compared with the Cumaná grandfather, the black pigment on the dorsal fin and the ventral black lining of the caudal peduncle of the Quare6 family II 215-3 grandfather appeared weak. It had a black spot associated with its anterior black horizontal stripe, which we found in 83% of its wild-type and in 56% of its golden N2 male offspring (Figure 7, A–D). We found that *golden* mutant N2 males of this backcross had (i) less black on the dorsal fin, (ii) a more diffuse posterior black horizontal stripe, (iii) an upwardly shifted or no ventral black lining of the caudal peduncle, and (iv) a slightly upwardly shifted central black spot (Figure 7D and Figure 9). In *blue* mutant N2 males, black ornaments on the trunk were mostly reduced to a few spots, and in some individuals a spot appeared at an unusual position just behind the operculum (Figure 7E).

Fewer black ornaments on the dorsal fin were also observed in *golden* mutant males of the highly inbred BDZW1 and Maculatus strains (Figure 8). The black spot in the dorsal fin of the Maculatus strain is considered to be a strictly Y-linked trait (Winge 1922), which illustrates that the expression of such traits nevertheless depends on autosomal cofactors. Wild-type males of the BDZW1 strain have a creamy-black margin of the tail fin and a prominent black-and-white eye spot on the caudal peduncle (Figure 8, A–C, and Figure 9). The colors of the creamy-black tail-fin margin were intermingled or switched in *golden* mutant BDZW1 males, and, instead of the eye spot, they had black spots at a more dorsal position on the trunk or ventral position on the tail fin (Figure 8, D–F, and Figure 9; occasionally two spots were present). Hence, the position of the eye spot is more variable in *golden* mutant BDZW1 males compared with the wild type. The posterior black horizontal stripe of golden BDZW1 males was often diffuse (Figure 8, D–F, and Figure 9), while it was curved, absent, or diffuse in golden Maculatus males (Figure 8, G, K, and L, respectively, and Figure 9). The ventral black lining of the caudal peduncle was shifted upward (BDZW1, Figure 8, D–F, and Figure 9) or absent (MAC, Figure 8, G, K, and L, and Figure 9). *golden* mutant Maculatus males showed dorsal or ventral black spots on the tail fin as well, which were only rarely seen in wild-type males (Figure 8, G–L).

Discussion

How the extreme variation in colorful ornaments satisfies the conflicting demands of predator evasion and mate attraction in male guppies has fascinated scientists for almost a century (Winge 1922, 1927; Lindholm and Breden 2002; Magurran 2005). Importantly, despite the extreme interindividual differences, many color traits are highly heritable, and sons greatly resemble their fathers (Winge 1922, 1927; Dzwillo 1959; Lindholm and Breden 2002). A better understanding of these issues requires better knowledge of the ontogeny of guppy pigmentation. This has unfortunately been challenging, due to the intrinsic difficulties of working with a livebearer for which many standard techniques that can be utilized in model organisms are not available. Here, we have exploited the power of forward genetics to advance the understanding of pigmentation in guppies. We have discovered that mutations in the two type III receptor tyrosine kinase genes *kita* and *csf1ra* underlie the guppy *golden* and *blue* phenotype, respectively, and have studied the effects of the mutations on both the reticulate pattern shared by females and males

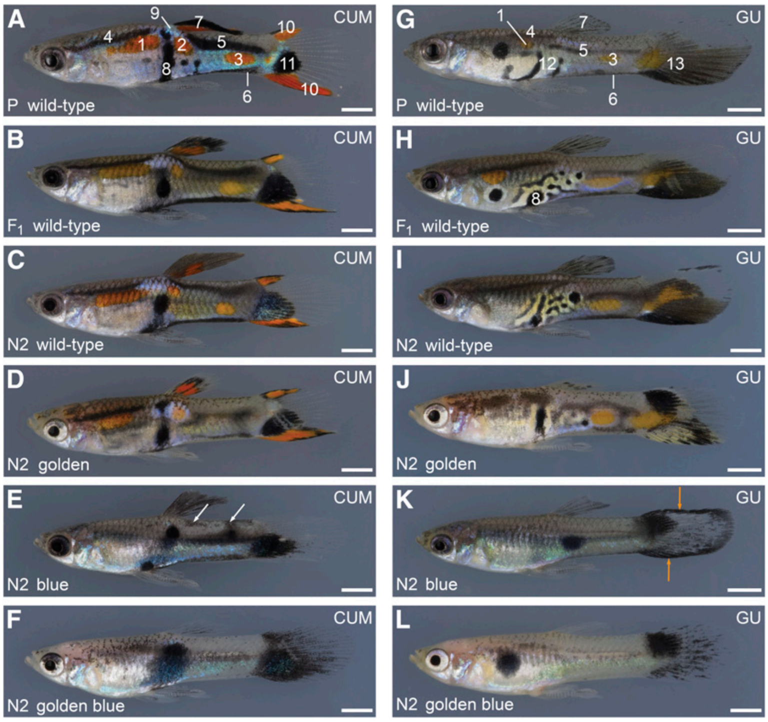


Figure 6 Ornaments in *golden* and *blue* mutant males from the Cumaná and Guanapo backgrounds. (A–F) Cross between a *golden blue* mutant BDZW1 female and a wild-type Cumaná male (A). Representative F_1 (B) and N2 males with different phenotypes (C–F) are shown. Cumaná traits are highlighted in A. White arrows in E show incoherent patches of melanophores described in the text. (G–L) Cross between a *golden blue* mutant BDZW1 female and a wild-type Guanapo male from a F_5 Guanapo laboratory inbreeding population (G). Representative F_1 (H) and N2 males with different phenotypes (I–L) are shown. Guanapo traits are highlighted in G and H. I–L lack the anterior orange spot. Orange arrows in K show the black margins of the tail fin described in the text. Traits are labeled with numbers that correspond with numbers used in Figure 9: 1, 2, and 3: anterior, central, and posterior orange spot; 4 and 5: anterior and posterior black horizontal stripes; 6: ventral black lining of caudal peduncle; 7: black pigment on dorsal fin (in CUM in combination with orange); 8: central black spot near gonopodium; 9: blue iridescent spot; 10: orange-black lining of tail fin; 11: ventral black spot on tail fin; 12: black crescents forming a labyrinthine pattern close to the gonopodium; and 13: orange spot and ventrally concentrated melanophores on tail fin. N2 males shown for each backcross are siblings or cousins. All photos taken from the backcrosses can be found in File S1. Bottom left in each panel: generation (P, grandfather; F_1; N2) and phenotype. Top right in each panel: Y chromosome origin. Bars: 2 mm.

and the male-specific ornaments. We found that the mutations in *kita* and *csf1ra* have strong effects on the expression of male-specific color patterns. In general, the mutation in *kita* made none or only minor changes in orange ornaments and affected the male melanophore pattern more subtly and in a more reproducible manner than the mutation in *csf1ra*. The salient feature of the *csf1ra* mutant males was the absence of all orange traits, with concomitant severe changes in black ornaments.

In many teleost species, including zebrafish, medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), and Japanese flounder (*Paralichthys olivaceus*), larvae and adults differ in their pigmentation patterns (Johnson *et al.* 1995; Parichy and Turner 2003a,b; Lynn Lamoreux *et al.* 2005; Kelsh *et al.* 2009; Yamada *et al.* 2010; Budi *et al.* 2011; Greenwood *et al.* 2012). Our study shows that the camouflage reticulate pattern of newborn guppies is not yet completely developed and that it is fully elaborated only during the first month after birth. Absence of most melanophores in embryonic and newborn *golden* mutants suggests that Kita is essential for the differentiation of an early melanophore population. A second melanophore population arises after birth and remains restricted to the dorsal half of the body in *golden* mutant fish, indicating that Kita signaling is not required for the differentiation of this melanophore subpopulation, yet that it might be essential for its proper migration. Alternatively, the migratory behavior of this subpopulation might be normal in *golden* mutants, with the later-differentiating melanophores enhancing the camouflage in wild-type fish on the dorsal side only. This could be investigated in the future by selective labeling and tracing of the *kita*-independent population or by finding another mutation that eliminates this population. Independently of these details, we conclude that the guppy has an early-appearing *kita*-dependent and a later-developing partially or fully *kita*-independent melanophore population and that both populations are required to form the non-sex-specific reticulate pattern. Whole-mount *in situ* hybridization experiments turned out to be extremely difficult in guppy embryos due to their size and very low permeability (U. Martyn, and C. Dreyer, unpublished data), but could potentially help to determine the developmental time point at which the first melanophores differentiate in guppy embryos, especially as the melanization of these melanophores might be delayed in a livebearing fish like the guppy. The tracking of putative pigment cell precursors for adult ornaments, however, would require analysis of serial sections of specimens from early embryogenesis to puberty.

Our study suggests that Kita functions have at least partially been conserved across teleosts. The last common ancestor of zebrafish and guppies lived probably >300 million years ago (Kasahara *et al.* 2007); nevertheless, the adult pigment pattern of both species depends on an early *kita*-dependent and a late *kita*-independent melanophore population, and loss-of-function mutations in *kita* decrease the amount of melanophores, including scale melanophores, in both species (Haskins and Druzba 1938; Goodrich *et al.* 1944; Winge and Ditlevsen 1947; Parichy *et al.* 1999; Parichy *et al.* 2000a). Yet the teleost-specific whole-genome duplication likely also facilitated subfunctionalization and

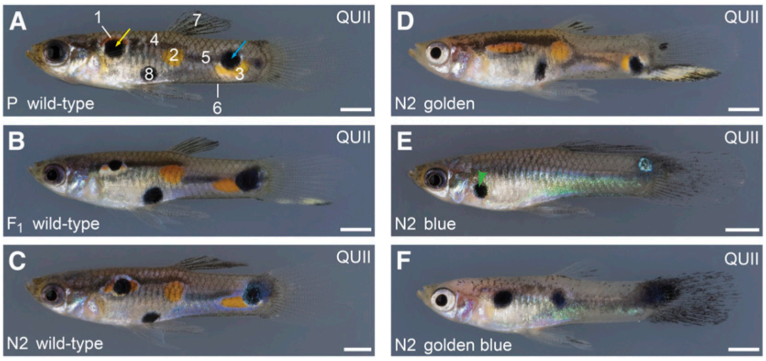


Figure 7 Ornaments in *golden* and *blue* mutant males from the Quare6 family II 215-3 background. Cross between a *golden blue* mutant BDZW1 female and a wild-type Quare6 family II 215-3 male (A). Representative F_1 (B) and N2 males with different phenotypes (C–F) are shown. Quare traits are highlighted in A. Yellow arrow points to black spot associated with anterior black horizontal stripe. Blue arrow points to black spot close to the tail fin that was not included in the analysis as its location was highly variable in wild-type fish. The green arrowhead in E shows a black spot close to the operculum described in the text. Traits are labeled with numbers corresponding with numbers used in Figure 6 and Figure 9. N2 males shown are cousins. All photos from this backcross and from the Quare6 backcross that was very similar regarding the male patterns can be found in File S2. Bottom left: generation (P, grandfather; F_1; N2) and phenotype. Bars: 2 mm.

phenotypic diversification, as revealed in different *Danio* species (Braasch *et al.* 2006; Mills *et al.* 2007).

An early xanthophore population contributes as regularly spaced cells to the reticulate pattern of guppy juveniles and adults (Figure 1, E and F). Our study shows that these non-sex-specific xanthophores depend on Csf1ra signaling. The presence of isolated dorsal clusters of xanthophores in *blue* mutant fish suggests that Csf1ra activity might not be absolutely required for differentiation, but for proliferation and dispersal of guppy xanthoblasts. We do not know yet when during ontogeny *csf1ra* acts and whether different xanthophore populations exist, but we showed that Csf1ra is not required for the formation of the non-sex-specific black reticulate pattern of the guppy. In contrast, *csf1ra* mutant zebrafish cannot form a complete non-sex-specific stripe pattern (Parichy *et al.* 2000a; Parichy and Turner 2003b).

Guppies are conspicuously sexually dimorphic in their pigmentation, and mate-choice experiments have demonstrated that females prefer males with pronounced orange and blue iridescent ornamentation (Endler 1983; Kodric-Brown 1985). Males are also able to intensify their black ornaments while courting (Endler 1983). Crosses between *golden blue* mutant females with male guppies of geographically and genetically diverse origins (Willing *et al.* 2010) gave us the opportunity to study the influence of Kita and Csf1ra loss-of-function on the diverse male-specific patterns of the guppy. The males originated from West Trinidad (Guanapo), East Trinidad (Quare), and Venezuela (Cumaná). The latter two strains had previously been used for genetic mapping and QTL analysis (Tripathi *et al.* 2009b). Our study demonstrates that the male-specific xanthophores of the guppy, whose development is induced during puberty like the one of male-specific melanophores and iridophores, also depend on *csf1ra* and that loss of Csf1ra and Kita function substantially changes the formation of male ornaments.

Comparison between wild-type and golden males showed that black stripes and spots appeared ectopically in *golden* mutants, although in a manner that varied between populations and individuals; *e.g.*, the ventral melanophores on the Guanapo tail fin were shifted to a more dorsal position, and *golden* mutant Maculatus males had novel black spots on their tail fins. In the BDZW1 strain, the arrangement of the creamy-black tail-fin margin, which might involve iridophores (personal observation), appeared reversed. In contrast, loss of Kita function did not change the dorso-ventral arrangement of the orange-black lining of the tail fin of golden N2 from the Cumaná cross. Interestingly, the marginal black components of the Cumaná tail-fin ornaments were lost together with the orange in *blue* mutants, whereas the major black spot on the tail fin persisted. In zebrafish, *kita* is expressed in melanoblasts, while *csf1ra* acts nonautonomously via short- and long-range xanthophore–melanophore interactions to promote melanophore migration and death during adult stripe formation (Parichy *et al.* 1999; Parichy and Turner 2003b; Nakamasu *et al.* 2009; Inaba *et al.* 2012). As transplantation experiments are not yet possible in the guppy, we could not determine whether Kita and Csf1ra act cell-autonomously or non-cell-autonomously during male pattern formation. However, as downregulation of *csf1ra* expression coincides with the absence of almost all xanthophores in the skin of blue fish, it is likely that *csf1ra* acts cell-autonomously within guppy xanthophores. Kita is an early melanoblast marker not only in zebrafish, but also in mice, and therefore most likely is expressed in guppy melanophores as well (Kelsh *et al.* 2009). Our observations suggest that some pattern elements in guppy males depend on coordinate expression of different cell types and that the formation of some of these pattern elements requires interactions between, or joint contribution from, different cell types. For example, xanthophore–melanophore interactions might underlie the formation of the orange-black lining of the tail fin of Cumaná males, but not the development of the black spot on the tail fin. Kita might directly affect the migration and/or survival of a subset of male melanophores. Terminal deoxynucleotidyl transferase-dUTP Nick End Labeling (TUNEL) assays could reveal whether cell apoptosis plays a role during male pattern formation in the guppy.

Comparisons between wild-type and blue offspring of the backcrosses suggest that compact black spots can form in absence of Csf1ra, most probably without interactions between

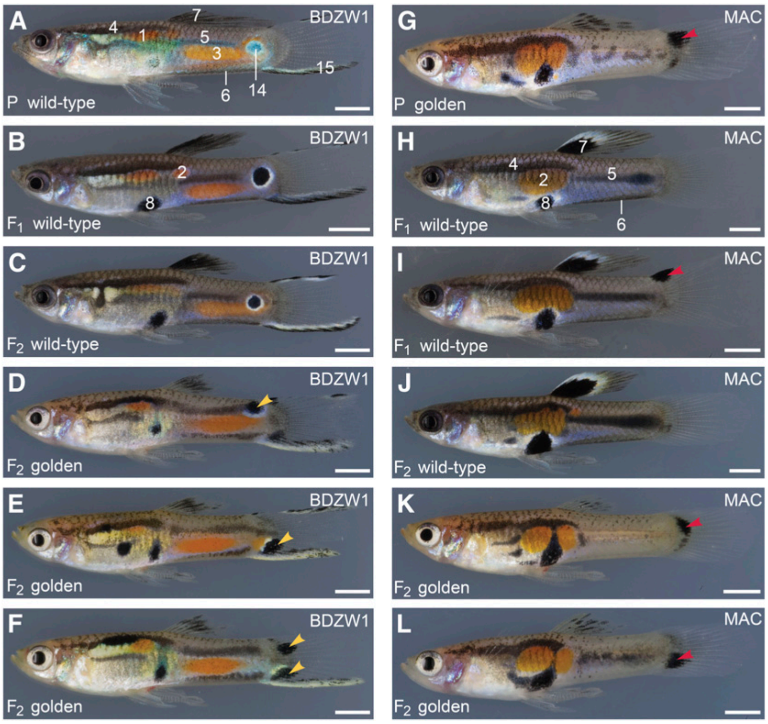


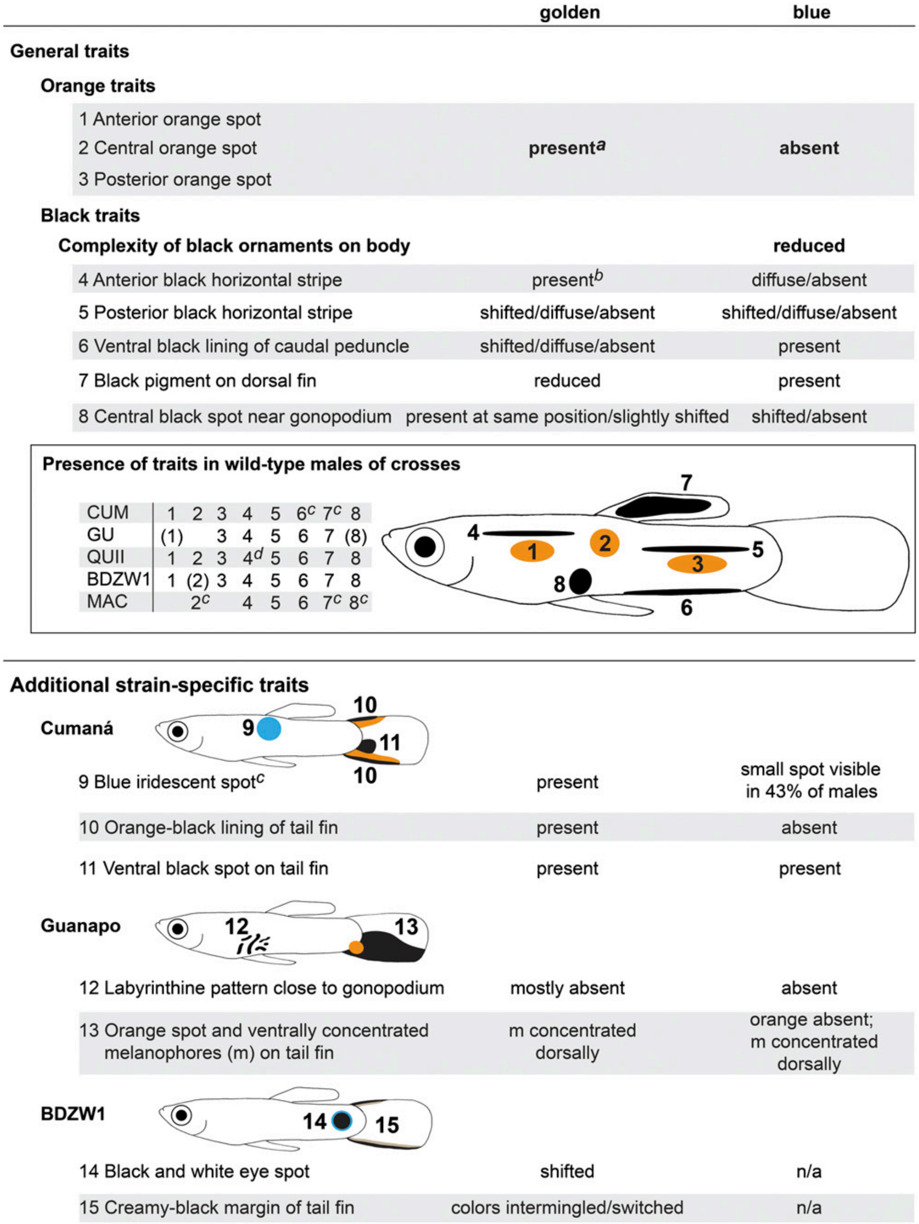
Figure 8 Ornaments in *golden* mutant males from the BDZW1 and Maculatus backgrounds. (A–F) Cross between a *golden* mutant BDZW1 female and a wild-type BDZW1 male (A). F_1 siblings were crossed to produce a F_2 generation. Representative F_1 (B) and F_2 males (C–F) are shown. BDZW1 traits are highlighted in A and B. All wild-type F_2 and 92% of the wild-type F_1 males had a central black-and-white eye spot on the caudal peduncle. Instead of this eye spot, one-half of the *golden* mutant F_2 males had a spot located more dorsally on the trunk, while the other half had a spot at a more ventral position, mostly on the tail fin (yellow arrowheads in D and E). Three males had two spots (yellow arrowheads in F). (G–L) Cross between a wild-type Maculatus female and a *golden* mutant Maculatus male (G). F_1 siblings were crossed to produce a F_2 generation. Representative F_1 (H and I) and F_2 males with different phenotypes (J–L) are shown. Maculatus traits are highlighted in H. Red arrowheads in (G, I, K, and L) indicate untypical black spots on the tail fin. Only one of the F_1 males showed such a black spot (I). It was not seen in any of the wild-type F_2 males, but it was present in 85% of the *golden* mutant F_2 males (K and L). Traits are labeled with numbers corresponding with numbers in Figure 6 and Figure 9. Additional traits to those shown in Figure 6: 14, black-and-white eye spot; 15, creamy-black margin of tail fin. F_2 males shown for each cross are siblings or cousins. Bottom left: generation (P, grandfather; F_1; F_2) and phenotype. Top right: Y chromosome origin. Bars: 2 mm.

xanthophores and melanophores, yet the final positions of these spots appear unpredictable. The labyrinthine ornaments close to the gonopodium in Guanapo males provide an example of interacting genetic cofactors in that backcross and potentially also of interactions between different cell types. Compared with wild type, these ornaments were greatly reduced in complexity in *golden* mutant N2 and lost in *blue* and *golden blue* mutant N2. Since only faint yellow pigment is seen in this area in wild-type fish, the contribution of xanthophores and their interactions is here hard to assess. The loss of the labyrinthine ornaments in *golden* mutant N2 might be explained by a reduced migratory potential of the *kita*-independent melanophores or their precursors in golden N2. We can, however, not exclude that a *kita*-dependent subpopulation of late melanophores contributes to this complex trait in wild-type fish.

golden blue double-mutant guppies from four different backcrosses always had less total black than *blue* mutants, but much more than zebrafish *kita csf1ra* double mutants (Parichy 2006). This might reflect different requirements for Kita and Csf1ra signaling: while xanthophores enhance the survival of adult stripe melanophores in zebrafish (Parichy and Turner 2003b), the survival of male hormone-induced melanophores in the guppy might not require xanthophores; yet the ability of these melanophores to form some of the complex traits might depend on interactions with these cells.

Taken together, we conclude that at least three temporally and genetically distinct melanophore populations occur in the guppy: first, a *kita*-dependent population differentiating during embryogenesis; second, a partially or fully *kita*-independent population mostly differentiating after birth; and third, a male-specific melanophore population whose differentiation, migration, and proliferation might be induced by testosterone during puberty. It remains to be resolved where the precursors of these male-specific pigment cells reside and whether they might be derived from the same pool as the late non-sex-specific *kita*-independent melanophores. Only a few recent publications have addressed the routes and fates of pigment cell precursor pools destined for delayed differentiation in other vertebrates (Watanabe *et al.* 2008; Adameyko *et al.* 2009; Yamada *et al.* 2010; Budi *et al.* 2011). While the embryonic and early larval pigment pattern of zebrafish is formed by melanoblasts that are derived directly from the neural crest, later-appearing metamorphic melanophores of zebrafish develop from post-embryonic extrahypodermal pigment cell precursors, which migrate to the hypodermis during pigment pattern metamorphosis (Budi *et al.* 2011). These precursors are associated with nerves and depend on Erbb3b and Tubulin α 8-like 3a signaling (Budi *et al.* 2011). Improvement of *in vitro* culture methods of guppy embryos (Martyn *et al.* 2006) may facilitate the ability to treat, and to subsequently raise, explanted guppy embryos with an ErbB inhibitor, which might reveal whether ErbB signaling is required to establish melanophore stem cells in the guppy as well.

In stickleback, regulatory mutations in *kitla* are associated with the light coloration of gills and ventral skin in several freshwater populations (Miller *et al.* 2007). This indicates that differential distribution of Kitla can lead to



	golden	blue
General traits		
Orange traits		
1 Anterior orange spot 2 Central orange spot 3 Posterior orange spot	**present^{a}**	**absent**
Black traits		
Complexity of black ornaments on body		**reduced**
4 Anterior black horizontal stripe	present^{b}	diffuse/absent
5 Posterior black horizontal stripe	shifted/diffuse/absent	shifted/diffuse/absent
6 Ventral black lining of caudal peduncle	shifted/diffuse/absent	present
7 Black pigment on dorsal fin	reduced	present
8 Central black spot near gonopodium	present at same position/slightly shifted	shifted/absent

Presence of traits in wild-type males of crosses

CUM	1	2	3	4	5	6^{c}	7^{c}	8
GU	(1)		3	4	5	6	7	(8)
QUII	1	2	3	4^{d}	5	6	7	8
BDZW1	1	(2)	3	4	5	6	7	8
MAC		2^{c}		4	5	6	7^{c}	8^{c}

Additional strain-specific traits	golden	blue
Cumaná		
9 Blue iridescent spot^{c}	present	small spot visible in 43% of males
10 Orange-black lining of tail fin	present	absent
11 Ventral black spot on tail fin	present	present
Guanapo		
12 Labyrinthine pattern close to gonopodium	mostly absent	absent
13 Orange spot and ventrally concentrated melanophores (m) on tail fin	m concentrated dorsally	orange absent; m concentrated dorsally
BDZW1		
14 Black and white eye spot	shifted	n/a
15 Creamy-black margin of tail fin	colors intermingled/switched	n/a

Figure 9 Summary and generalization of the results regarding the male-specific pattern. Both Csf1ra and Kita are required for the formation of male ornaments of the guppy. The black pigment on the dorsal fin can be a distinct patch or diffuse. Numbers in parentheses refer to traits that were not present in all wild-type males of the specific strain. Traits that were too variable in wild-type males were not included into the analysis. More details are described in the text and shown in Figure 6, Figure 7, and Figure 8 and in Figure S5. ^{a}Exception: a concise posterior orange spot was absent in 94% of the golden N2 males with a Y^{CUM} background. ^{b}Exception: often discontinuous in golden N2 males with a Y^{GU} background. ^{c}Y-linked in respective strain. ^{d}Associated with a black spot in 83% of the wild-type males with a Y^{QUII} background.

distinct pigmentation patterns in natural populations. In contrast to the receptor Kita, the functions of Csf1ra seem to be less conserved even among species in the genus *Danio*, as indicated by limited complementation of *csf1ra* loss of function in interspecies hybrids (Quigley *et al.* 2005). Furthermore, a study with haplochromine cichlids has suggested that positive selection has acted on *csf1ra*, which is expressed in the yellow egg spots of these fish (Salzburger *et al.* 2007). QTL mapping with higher marker density may reveal whether or not Kita and Csf1ra, and/or their ligands, also affect natural variation of guppy ornaments. For this purpose, we are generating a denser genetic map of the guppy based on Restriction-site Associated DNA (RAD) markers. Combined with the ongoing whole-genome sequencing, these experiments will further enhance our efforts to unravel the network of genetic factors that cooperatively maintain the highly complex male ornaments of the guppy.

Acknowledgments

We thank Harald Auer for the donation of the ARM and BDZW2 guppy strains; Axel Meyer for the BDZW1 guppy strain; David Reznick for guppies from the Quare and Guanapo rivers; Christopher Dooley for general advice on zebrafish pigment pattern mutants; Tobias Langenecker for assistance with real-time quantitative PCR; Gertrud Scheer, Alexandra Schnell, and Philipp Vollmer for guppy images; Richard Neher for help with data analysis; Joffrey Fitz for technical support; and Bonnie Fraser, Felicity Jones, and Axel Künstner for helpful suggestions on the manuscript. This work was supported by a Gottfried Wilhelm Leibniz Award of the Deutsche Forschungsgemeinschaft and by funds from the Max Planck Society to D.W.

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GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.151738/-/DC1

Pigment Pattern Formation in the Guppy, *Poecilia reticulata*, Involves the Kita and Csf1ra Receptor Tyrosine Kinases

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DOI: 10.1534/genetics.113.151738

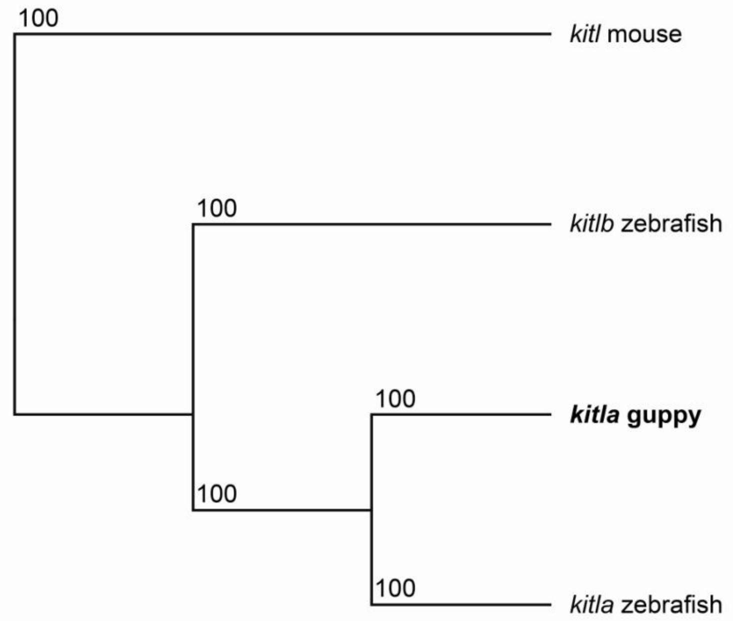

Figure S1. Maximum parsimony phylogenetic tree of *kitl* ORF sequences. Mouse *kitl* was used as an outgroup. Bootstrap support values from 100 replicates are shown. Accession numbers of sequences: guppy *kitla*, KC143125; mouse *kitl*, NM_013598; zebrafish *kitla*, AY929068; zebrafish *kitlb*, AY929069.

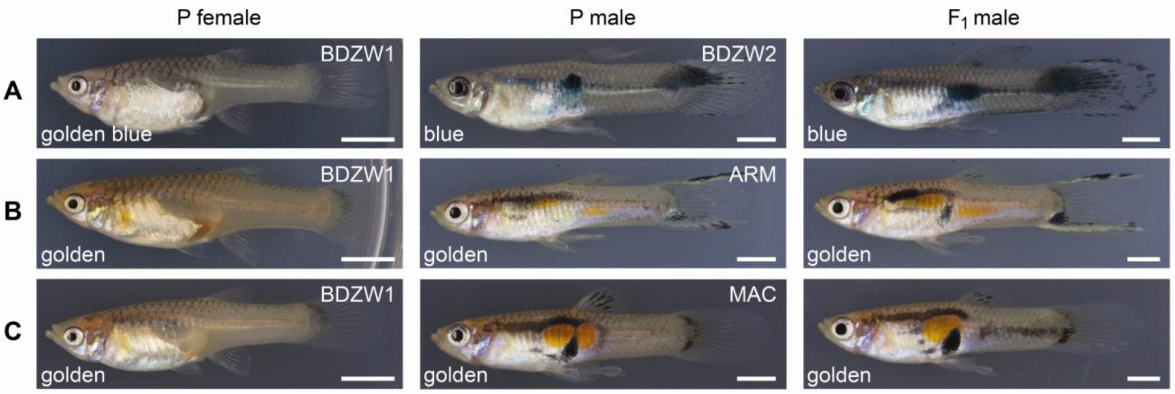

Figure S2. Complementation analyses.
(A) Non-complementation of the blue phenotype. All F_1 from a cross between a golden blue BDZW1 female and a blue BDZW2 male were blue. (B,C) Non-complementation of the golden phenotype. All F_1 from crosses of golden BDZW1 females to golden ARM and MAC males were golden. Parents (P) and representative F_1 male are shown for each cross. Scale bars: males 2 mm; females 5 mm.

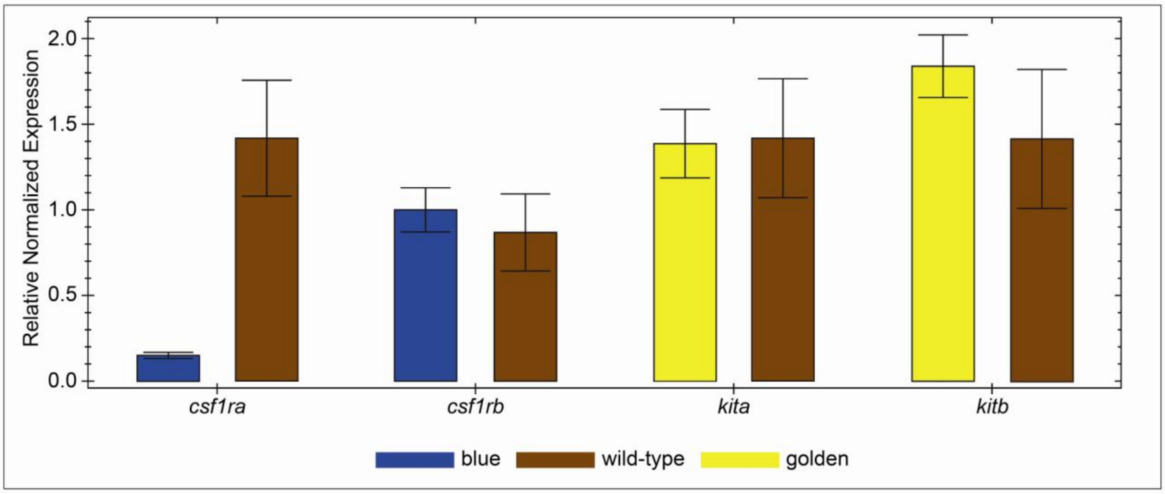

Figure S3. Expression levels of *csf1ra*, *csf1rb*, *kita*, and *kitb* in adult female skin.
Normalized expressions ($\Delta\Delta C_q$) of the genes in wild-type, golden, and blue female skin are shown. Expression levels were determined by real-time quantitative PCR using three biological replicates (one replicate refers to skin of one female) with three technical repetitions each. Expression was normalized to *gapdh* expression. Primer efficiencies were: *gapdh*, 85.3%; *csf1ra*, 96.4%; *csf1rb*, 97.6%; *kita*, 98.2%; *kitb*, 95%.

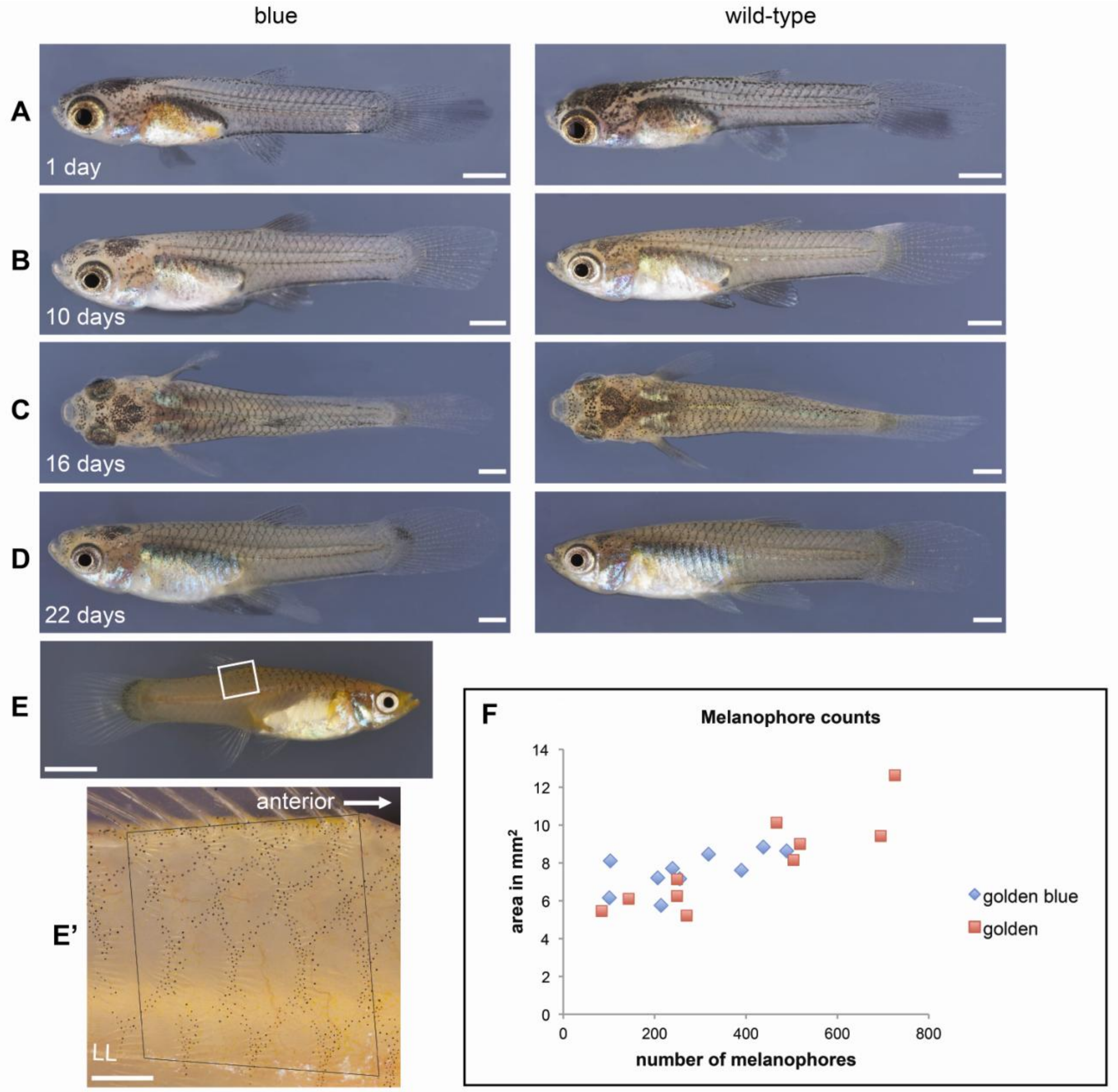

Figure S4. Melanophore pattern development in wild-type and *blue* mutant fish.
(A-D) Photos of the same males over a three-week time course (days are after birth). The blue fish has a BDZW2, the wild-type fish a BDZW1 background. The amount of melanophores of *blue* mutant and wild-type newborns was not compared, as variation in melanophore number is high between guppy strains (unpublished observation). (E') Detail of area boxed in (E); black rectangle indicates area below dorsal fin in which melanophores were counted. (F) Melanophore counts in 10 *golden* and 10 *golden blue* mutant females. The number of melanophores is strongly correlated with the size of the area. All fish shared the same grandparents and were approximately one year old. LL: Lateral line. Scale bars: (A-D) 1 mm; (E) 5 mm; (E') 1 mm.

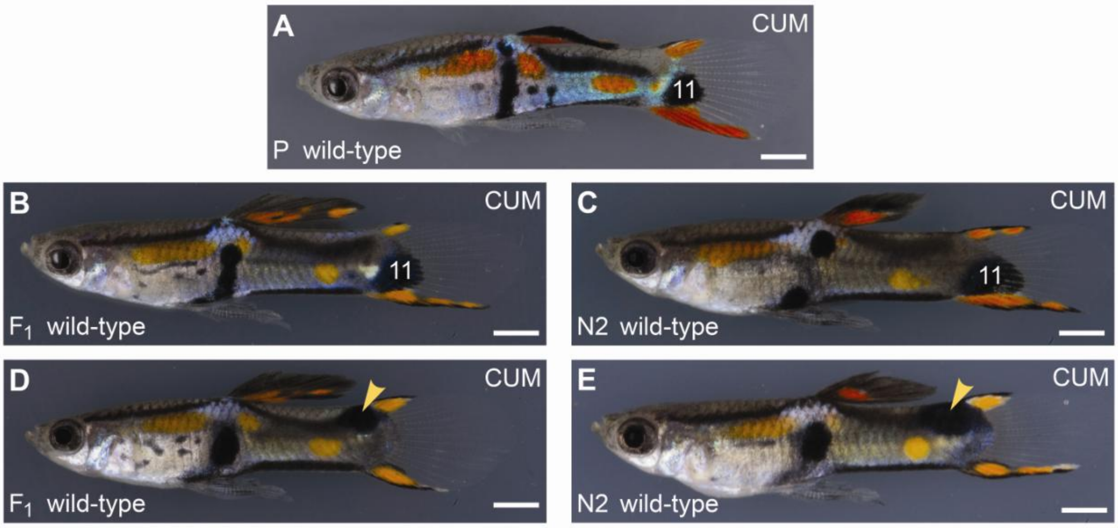

Figure S5. Inheritance of the ventral black spot on the tail fin of the Cumaná strain. (A) Grandfather, (B) F_1, and (C) N2 males with a ventral black spot on the tail fin (11). (D) F_1, and (E) N2 males with a dorsal black spot on the caudal peduncle (yellow arrowheads). The black spot on the tail fin was present in 33 of 55 F_1 males; all other males had a black spot on the caudal peduncle. This suggests that cofactors exist that modulate this trait, which might be derived from autosomes or the X chromosome of the BDZW1 strain in this case. All N2 males had the spot at the same position as their father. (C) and (E) are the sons of (B) and (D), respectively. P, grandfather. Scale bars: 2 mm.

File S1

Photos tracking the development of the melanophore pattern after birth.

Image names include image number and days after birth at which the image was taken. Additional pictures with higher magnification of some of the fish are available upon request. Note that the gestation time in the guppy varies; although all fish were imaged at identical time points after birth, they might not be in the exact same developmental stage. We did not take any photos from the dorsal aspect during the first week after birth, as most fish did not survive this at this age. Dataset was deposited at: ftp://ftp.tuebingen.mpg.de/ebio/csf1ra_kita_mutants/

File S2

Photos of males taken from backcrosses to investigate the influence of the golden and blue mutations on the male-specific ornaments.

Cross20_BDZW1_CUM: cross between a golden blue BDZW1 female and a wild-type Cumaná male. Seven F1 males were backcrossed to golden blue BDZW1 females (BC1 to 7 pairs) to produce a N2 generation. The color pattern of one of the golden males (cross20_F1fBC6_whitem_0488) resembled the one of the BDZW1 strain rather than the one of the Cumaná strain, which might be the outcome of a rare recombination event. Cross21_BDZW1_GU: cross between a golden blue BDZW1 female and a wild-type Guanapo male. Four F1 males were backcrossed to golden blue BDZW1 females (BC1 to 4 pairs) to produce a N2 generation. Cross23_BDZW1_QU: cross between a golden blue BDZW1 female and a wild-type Quare6 male. Four F1 males were backcrossed to golden blue BDZW1 females (BC1 to 4 pairs) to produce a N2 generation. Cross24_BDZW1_QUII: cross between a golden blue BDZW1 female and a wild-type Quare6 family II 215-3 male. Four F1 males were backcrossed to golden blue BDZW1 females (BC1 to 4 pairs) to produce a N2 generation. Image names include cross number, generation, phenotype, sex, and identification number. P, grandparents; BCs, backcrosses; m, male; f, female; F1fBC1, F1 offspring (here called N2 generation) from BC1 pair; wtm, wild-type male; whitem, golden male; bluem, blue male; ghostm, golden blue male. Dataset was deposited at: ftp://ftp.tuebingen.mpg.de/ebio/csf1ra_kita_mutants/

Table S1 Methods used for full-ORF amplification of pattern formation candidate genes

<table>
<tr><th>Gene</th><th>Origin of partial sequences</th><th>Primers for RACE PCR</th><th>Primers used for full-ORF amplification by PCR</th><th>Primers used for internal sequencing of full-ORF clones</th></tr>
<tr><td><i>Kitla</i></td><td>Consensus primers:
Forward: 5’-AAGTT GGATACGCGTCTG TGTCC-3’
Reverse: 5’-CTTCC ACACMAGCAGGA AWA-3’</td><td>3’-RACE PCR primers (forward):
5’-CTCATCAGCTTCGTTGCCAA GTC-3’ (reaction 1)
5’-CACTGCTGGCCCTAATATTC CTG-3’ (reaction 2)</td><td>5’ UTR forward: 5’-ATTGGATAT GTGCACACAGGATGATGAC-3’
3’ UTR reverse: 5’-GTTTGTCTT TCACCTTTGAACACCAACAG-3’</td><td>-</td></tr>
<tr><td colspan="5">We amplified a short piece of the guppy ortholog of <i>kitla</i> by using consensus primers that were chosen based on an alignment of <i>kitla</i> of other teleost species[a]. We then designed guppy-specific <i>kitla</i> primers for RACE PCR; however, the 5’-RACE PCR failed. To obtain a part of the guppy <i>kitla</i> 5’ UTR, we ordered forward consensus primers in the 5’ UTR of <i>kitla</i> based on an alignment of <i>kitla</i> sequences of other teleost species[b]. 5’-ATTGGATATGTGCACACAGGATGATGAC-3’ was used successfully in combination with guppy-specific <i>kitla</i> reverse primers. Subsequently, we used this primer in combination with a reverse primer in the 3’ UTR for full-ORF amplification.</td></tr>
<tr><td><i>Kita</i></td><td><i>Kita</i> marker sequence (Tripathi et al., 2008)</td><td>3’-RACE PCR primers (forward):
5’-CAGAGCTGAAGGTCCTCAGTT ACTTGG-3’ (reaction 1)
5’-TCAGCACCTGTGAGCTAAAGG AGAAC-3’ (reaction 2)</td><td>5’ UTR forward: 5’-AGAGCT ACACCAGCTTGACCAC-3’
3’ UTR reverse: 5’-CGTAAC ATCACAGGCACTTAGAGG-3’</td><td>5’-GACAGTAAAGAAAGTCC CTTTAGGTCC-3’ (forward)
5’-GGAACTCCCACTGGTGAT TGTAG-3’ (reverse)
5’-GACAAGTCGTCCTCATCG AACATCTC-3’ (reverse)</td></tr>
<tr><td colspan="5">We used a partial <i>kita</i> sequence to design primers for RACE PCR. The 5’-RACE PCR failed; to obtain a part of the guppy <i>kita</i> 5’ UTR, we ordered forward consensus primers in the 5’ UTR of <i>kita</i> based on an alignment of <i>kita</i> sequences of other teleost species[c]. 5’-CTSYAGGAC AACAGCATGYTTG-3’ was used successfully in combination with guppy-specific <i>kita</i> reverse primers. Subsequently, we designed primers in the UTRs for full-ORF amplification.</td></tr>
<tr><td><i>Csf1ra</i></td><td>Transcriptome database</td><td>5’-RACE PCR primers (reverse):
5’-CTATCCGTACATATTCATCTG TCTCC-3’ (reaction 1)
5’-CAGTGTAGAAGCACTTGTAC GTTCC-3’ (reaction 2)</td><td>5’ UTR forward: 5’-CCTCCTTCA AGCTGAAGACATC-3’
3’ UTR reverse: 5’-ACCGACTCA GCCTTGAACTG-3’</td><td>5’-GATCTGAGGTGTGAAGG CAAC -3’ (forward)
5’-CCTTCACAACATAGTTGG AGTCATTC-3’ (reverse)
5’-GTCTAGACATCCAGAGCA TCCTGAC-3’ (forward)</td></tr>
</table>

We detected an incomplete *csf1ra* transcript in our guppy transcriptome database (SHARMA, E., A. KÜNSTNER, B. A. FRASER, M. HOFFMANN, V. A. KOTTLER, G. ZIPPRICH, D. WEIGEL, and C. DREYER, unpublished data) and carried out a nested 5'-RACE PCR to obtain the missing 5' end of the wild-type *csf1ra* transcript. Based on the full-length cDNA sequence of *csf1ra*, we designed primers in the UTRs for full-ORF amplification.

Rapid amplification of cDNA ends (RACE)

First-strand cDNA for 5'/3'-RACE PCR was prepared with BD SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions. The RACE PCR product of reaction 1 was diluted 50-fold to serve as template in reaction 2 (nested RACE PCR). Reverse (5'-RACE PCR) and forward (3'-RACE PCR) gene-specific primers were combined with the forward (5'-RACE PCR) and reverse (3'-RACE PCR) primer of the BD SMART PCR cDNA Synthesis Kit, respectively. Both PCR reactions were carried out with Advantage 2 Polymerase Mix (Clontech) as described before.

Accession numbers

[a] GenBank EU218897 (stickleback, *Gasterosteus aculeatus*); Ensembl ultracontig72 (medaka, *Oryzias latipes*)
[b] GenBank EU218897 (stickleback), AB285216 (fugu, *Takifugu rubripes*), AY929068 (zebrafish, *Danio rerio*), FJ907418 (goldfish, *Carassius auratus*); Ensembl GSTENT10006132001 (tetraodon, *Tetraodon nigroviridis*)
[c] Ensembl MEDAKA1:4:1429800:1459632:1 (medaka), FUGU4:scaffold_13:1529100:1545623:1 (fugu)

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Table S2 List of primers used for real-time quantitative PCRs

Gene	Primers	Efficiency
Gapdh	Forward: 5’-ACATCAAGAAGGTTGTGAAAGCTG-3’ Reverse: 5’-ATCAAAGATGGAGGAGTGAGAATC-3’	85.3%
Csf1ra	Forward: 5’-AACTGGAGGAGGAGCAGGTAATC-3’ Reverse: 5’-GTGACACTTAGGCTTGTCATACG-3’	96.4%
Csf1rb	Forward: 5’-TTGACGAGTGACATGTTGCCTC-3’ Reverse: 5’-ATCATCATCCTCTTCTTCTGCTCTG-3’	97.6%
Kita	Forward: 5’-AAGAGAGCTGCAGATGGTGAC-3’ Reverse: 5’-CGTCAGCTTCGAGGTACAGG-3’	98.2%
Kitb	Forward: 5’-ACCAAGAACGTGTACCTGACTC-3’ Reverse: 5’-CTACACACAGAGCTAAGCCTCC-3’	95.0%

Table S3 List of primers used for genomic PCRs

Gene	Primers	Purpose	Remarks
Kita	Forward exon 6: 5’-TGTCTCTGAACG TTAGCATGGAG-3’ Reverse in 36 bp ‘exon’ of insert: 5’-AAGCTTGTCAAGGAATCTGGTATGG-3’	Amplification of *kita*^{insert} (quick PCR test)	
Kita	Forward exon 6: 5’-TGTCTCTGAACGTTAGCATGGAG-3’ Reverse exon 7: 5’-ACACGGAGAAGTTCTGCTTTACC-3’	Amplification of *kita*^{wt} (quick PCR test)	Elongation time of 2 min not sufficient to amplify *kita*^{insert}
Csf1ra	Forward exon 16: 5’-ACATTGACGACCTGCTGAGATTC-3’ Reverse exon 17: 5’-CCTTCACAACATAGTTGGAGTCATTC-3’	Amplification of *csf1ra*^{wt} and *csf1ra*^{indel}	

Table S4 Sequences and description of six *kita* splice variants found in *golden* mutants

Sequences of variants (V)[a]	Description	Predicted protein
>V1 gatgctgggagttacaaatgcgtagccggaaacgacaaagggaacagtacgatgtccgtgtggctggacgtt tatgaacgggggtttatcagctcagcagaaatccacaacagaaccttccacgtccgcgtcggcgacagtttgt ctctgaacgttagcatggaggcgtatccaaagccgcgcgccgcctcctggagcttgtctgttactaccaataat ctcacatacccataccagattccttgacaagcttcatgggcgagaacctcagaaacaccagcgaccacgtcat caccacgcacagcagggactacagctacatcagcgagttgaggctggtgcggctcaaagcgacagaaggtg gcatttataccttcgaagcctccaacggcgacgcggcggtaaagcagaacttctccgtgtttgttatcagtaag cctgcgatcatagatcatgggggcccagtggatggacaggtgcactgtgtggctgaaggctaccctgcccccc agatcaaatggtactactgcgagaagcatgctgtcaggtgctccctgcaaaagaacgccacccaggaggagc gcagcgtcatgaccgtcatgtcgggaagcaccagcttcgggaagagggtggagagctgggtcaacgtcaga aaacagttcagtactctggagtgcgtcgccaccggggacggagagcaagcctacatactgttt	17+36 bp extra	358 aa, with the last 15 new
>V2 gatgctgggagttacaaatgcgtagccggaaacgacaaagggaacagtacgatgtccgtgtggctggacgtt tatgatctcacatacccataccagattccttgacaagcttcatgggcgagaacctcagaaacaccagcgacca cgtcatcaccacgcacagcagggactacagctacatcagcgagttgaggctggtgcggctcaaagcgacag aaggtggcatttataccttcgaagcctccaacggcgacgcggcggtaaagcagaacttctccgtgtttgttatc agtaagcctgcgatcatagatcatgggggcccagtggatggacaggtgcactgtgtggctgaaggctaccct gccccccagatcaaatggtactactgcgagaagcatgctgtcaggtgctccctgcaaaagaacgccacccag gaggagcgcagcgtcatgaccgtcatgtcgggaagcaccagcttcgggaagagggtggagagctgggtcaa cgtcagaaaacagttcagtactctggagtgcgtcgccaccggggacggagagcaagcctacatactgttt	lacks 124 bp of exon 6, 36 bp extra	311 aa, with the last 9 new
>V3 gatgctgggagttacaaatgcgtagccggaaacgacaaagggaacagtacgatgtccgtgtggctggacgtt tatgaacgggggtttatcagctcagcagaaatccacaacagaaccttccacgtccgcgtcggcgacagtttgt ctctgaacgttagcatggaggcgtatccaaagccgcgcgccgcctcctggagcttgtctgttactaccaatact acatcagcgagttgaggctggtgcggctcaaagcgacagaaggtggcatttataccttcgaagcctccaacg gcgacgcggcggtaaagcagaacttctccgtgtttgttatcagtaagcctgcgatcatagatcatgggggccc agtggatggacaggtgcactgtgtggctgaaggctaccctgccccccagatcaaatggtactactgcgagaa gcatgctgtcaggtgctccctgcaaaagaacgccacccaggaggagcgcagcgtcatgaccgtcatgtcggg aagcaccagcttcgggaagagggtggagagctgggtcaacgtcagaaaacagttcagtactctggagtgcgt cgccaccggggacggagagcaagcctacatactgttt	17 bp extra, lacks 63 bp of exon 6	354 aa, with the last 11 new
>V4 gatgctgggagttacaaatgcgtagccggaaacgacaaagggaacagtacgatgtccgtgtggctggacgtt tatgaacgggggtttatcagctcagcagaaatccacaacagaaccttccacgtccgcgtcggcgacagtttgt ctctgaacgttagcatggaggcgtatccaaagccgcgcgccgcctcctggagcttgtctgttactaccaatagt gctccctgcaaaagaacgccacccaggaggagcgcagcgtcatgaccgtcatgtcgggaagcaccagcttcg ggaagagggtggagagctgggtcaacgtcagaaaacagttcagtactctggagtgcgtcgccaccggggac ggagagcaagcctacatactgttt	17 bp extra, lacks 63 bp of exon 6 and complete exons 7 and 8	364 aa, with the last 21 new
>V5 gatgctgggagttacaaatgcgtagccggaaacgacaaagggaacagtacgatgtccgtgtggctggacgtt tatgctacatcagcgagttgaggctggtgcggctcaaagcgacagaaggtggcatttataccttcgaagcctc caacggcgacgcggcggtaaagcagaacttctccgtgtttgttatcagtaagcctgcgatcatagatcatggg ggcccagtggatggacaggtgcactgtgtggctgaaggctaccctgccccccagatcaaatggtactactgcg agaagcatgctgtcaggtgctccctgcaaaagaacgccacccaggaggagcgcagcgtcatgaccgtcatgt cgggaagcaccagcttcgggaagagggtggagagctgggtcaacgtcagaaaacagttcagtactctggag tgcgtcgccaccggggacggagagcaagcctacatactgttt	lacks complete exon 6	307 aa, with the last 5 new
>V6 gatgctgggagttacaaatgcgtagccggaaacgacaaagggaacagtacgatgtccgtgtggctggacgtt tatggtgctccctgcaaaagaacgccacccaggaggagcgcagcgtcatgaccgtcatgtcgggaagcacca gcttcgggaagagggtggagagctgggtcaacgtcagaaaacagttcagtactctggagtgcgtcgccaccg gggacggagagcaagcctacatactgttt	lacks complete exons 6, 7, and 8	317 aa, with the last 15 new

[a] Forward primer in exon 5 and reverse primer in exon 9 of *kita* were used for amplification; primer sequences are given in MATERIALS AND METHODS.

5 “*Adenylate cyclase 5* is required for melanophore and male pattern development in the guppy (*Poecilia reticulata*)”

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Synopsis

Melanophore development is highly complex and can be modulated at various levels, for instance, during melanoblast migration, proliferation, and differentiation. Melanophore appearance also changes when the melanosomes within the cells disperse or aggregate. The melanophores of blond guppies seem small, suggesting that *blond* is required for guppy melanophore differentiation or melanosome dispersal (GOODRICH *et al.* 1944). We found that *blond* corresponds to the guppy ortholog of *adcy5* by using ddRADseq and QTL mapping. Blond guppies are homozygous for a loss-of-function mutation in *adcy5*.

Adenylate cyclases are membrane-bound proteins that regulate the intracellular level of the second messenger cyclic adenosine monophosphate (cAMP) (BUSCA and BALLOTTI 2000; DUMAZ and MARAIS 2005; FUJII 2000; SUNAHARA and TAUSSIG 2002). In mouse, Adcy5 is involved in melanoma growth (DE LORENZO *et al.* 2014). To find out whether the mutation in *adcy5* affects melanosome dispersion or melanophore differentiation in the guppy, I designed several experiments. First, melanophore counts demonstrated that melanophores are not reduced in number in blond guppies. Second, treatment of blond and wild-type skin with chemicals increasing the intracellular level of cAMP, inspection of fully aggregated melanophores, semi-thin sections, and TEM images revealed that the melanophores of blond guppies are much smaller than the ones of wild-type fish. Adcy5 is therefore required for melanophore differentiation in the guppy.

The loss-of-function mutation in *adcy5* had a pronounced effect on the orange traits of the males of our F_2 mapping population, which was surprising, as Adenylate cyclases have not been previously found to be involved in pigment pattern formation. I assessed the presence and absence of all orange traits in the males of the F_2 mapping population and measured the size of the present traits. We found that Adcy5 is required for the formation per se of some orange traits and strongly affects the size of most of the orange ornaments. This suggests that in the guppy not only xanthophores are required for black ornament formation but also normally sized melanophores are required for orange trait formation.

In conclusion, our study reveals that Adcy5 is required for the differentiation of all melanophores of the guppy, irrespective of their Kita/Csf1ra-dependence or -independence, and establishes *adcy5* as a novel pigment pattern gene.

Contributions

VAK and CD conceived and designed the experiments. VAK, MH, and ES prepared the library for ddRADseq. AK analyzed the ddRADseq data and performed the QTL analysis. TL carried out and analyzed the RT-qPCR experiment. VAK fixed the fish for TEM; IK prepared the semi-thin and TEM sections. VAK, IK, and MF took the TEM images; IK took the pictures of the semi-thin sections. VAK performed all other experiments, crossed the fish, and analyzed the data. VAK wrote the manuscript; all authors provided helpful comments. DW oversaw the experimental design and data analysis.

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Note

As Table S3 sheet 1 and Table S4 in their current form are too small to be read when printed, larger versions of these tables can be found in the Appendix of this work.

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Adenylate cyclase 5 is required for melanophore and male pattern development in the guppy (*Poecilia reticulata*)

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GenBank accession numbers of guppy sequences

KP222266, KP222267, KP222268, KP222269, KP222270, KP222271, KP222272, KP222273, KP222274, KP222275

European Nucleotide Archive (ENA) accession number of RAD sequencing data

PRJEB7924

Dataset S1

ftp://ftp.tuebingen.mpg.de/ebio/adcy5_mutant/

SUMMARY

Guppies (*Poecilia reticulata*) are colorful fish that have attracted the attention of pigmentation researchers for almost a century. Here, we report that the blond phenotype of the guppy is caused by a spontaneous mutation in the guppy ortholog of *adenylate cyclase 5* (*adcy5*). Using double digest restriction site-associated DNA sequencing (ddRADseq) and quantitative trait locus (QTL) mapping, we linked the blond phenotype to a candidate region of 118 kb, in which we subsequently identified a 2-bp deletion in *adcy5* that alters splicing and leads to a premature stop codon. We show that *adcy5*, which affects lifespan and melanoma growth in mouse, is required for melanophore development and formation of male orange pigmentation traits in the guppy. We find that some components of the male orange pattern are particularly sensitive to loss of Adcy5 function. Our work thus reveals a function for Adcy5 in patterning of fish color ornaments.

SIGNIFICANCE

Our study demonstrates the power of ddRADseq based QTL mapping in the guppy, *Poecilia reticulata*, a species with limited genetic resources, but of wide interest to pigmentation research. A

mutation in *adcy5* strongly affects guppy melanophore development, suggesting that this isoform of Adenylate cyclase plays an important role in the development of vertebrate melanophores. Adcy5 knockout delays the growth of mouse melanoma cells *in vitro*; our discovery suggests that it would be informative to investigate *adcy5* mutants of fish melanoma models, e.g., of the guppy relative *Xiphophorus*.

Keywords

Guppy, pigmentation, ddRADseq, QTL mapping, Adenylate cyclase, *adcy5*

Running Title

Guppy *adcy5* pigmentation mutant

INTRODUCTION

Males of the livebearing guppy (*Poecilia reticulata*) are famous for their variable pigment patterns that consist of dazzling spots and stripes that can be black, yellow to red, whitish, or greenish to blue. Guppy populations on Trinidad have been intensively investigated with respect to variation of male color and life-history traits, which are subject to both natural and sexual selection and thus offer outstanding opportunities to study evolutionary processes in real-time (Endler, 1983; Endler, 1995; Magurran, 2005). The ornaments of male guppies are formed by neural crest-derived melanophores, xanthophores, and iridophores that are arranged in thick layers in the dermis and hypodermis (Fujii, 1966; Fujii and Taguchi, 1970; Gundersen and Rivera, 1982; Kottler et al., 2013; Kottler et al., 2014; Sauka-Spengler and Bronner-Fraser, 2008; Takeuchi, 1975; Takeuchi, 1976). Female guppies, which lack conspicuous colors, are camouflaged by a net-like reticulate pattern that covers the whole body (Goodrich et al., 1944; Kottler et al., 2013; Kottler et al., 2014; Tripathi et al., 2008). This pattern is composed of large, corolla melanophores interspersed with xanthophores and iridophores (Figure 1A) (Goodrich et al., 1944; Kottler et al., 2013; Kottler et al., 2014). A second type of melanophores, called dendritic, is associated with more superficial skin layers close to the scales (Figure 1A, 1B) (Goodrich et al., 1944; Kottler et al., 2013; Kottler et al., 2014). Dendritic and some corolla melanophores are distributed irregularly over the whole body of males and females (Figure 1A) (Goodrich et al., 1944). In males, the reticulate pattern is partially covered by the male-specific ornaments that are induced by hormonal cues during puberty (Goodrich et al., 1944; Kottler et al., 2013; Kottler et al., 2014; Tripathi et al., 2008). The presence of a fourth chromatophore type, leucophores, could not be confirmed in a recent study (Kottler et al., 2014).

Guppy pigmentation has been studied since the 1920s, when Schmidt and Winge described several ornaments that were linked to the male Y chromosome, which contains the sex-determining locus (Schmidt, 1920; Sharma et al., 2014; Tripathi et al., 2009; Winge, 1922; Winge, 1927). Since then, guppy researchers and hobby breeders have described a wealth of spontaneous color variants that differ in male coloration and/or the characteristics of the reticulate pattern (for an overview see (Lindholm and Breden, 2002)). Even so, our understanding of guppy pigment pattern development is limited, especially regarding its genetic control, as only two genes that are crucial for guppy pigment pattern formation have been identified. Both encode type III receptor tyrosine kinases. *Kita* (*golden*) is required for the development of early melanophores that form the adult reticulate pattern together with late *kita*-independent melanophores (Kottler et al., 2013). The other, *colony-stimulating factor 1 receptor a* (*csf1ra*; *blue*), is essential for normal xanthophore development (Kottler et al., 2013). Additionally, mutations in both genes affect the male-specific melanophore pattern, which might be partially mediated by xanthophore-melanophore interactions (Kottler et al., 2013).

The mutations in *kita* and *csf1ra* mainly affect the presence and location of certain guppy melanophore populations. In contrast, the appearance of all melanophores in the skin seems to be altered by a mutation in *blond*, which was first described 70 years ago (Goodrich et al., 1944). Guppies homozygous for the autosomal recessive allele *blond* appear yellowish, as the large, corolla-shaped melanophores are replaced by small, punctate melanophores in this mutant (Figure 1C, 1D, 1D', 1E, 1F, 1F') (Goodrich et al., 1944). We mapped *blond* using a reduced representation sequencing method, double digest restriction site-associated DNA sequencing (ddRADseq), in combination with quantitative trait locus (QTL) analysis. With only 141 F_2 individuals, we narrowed the *blond* interval to a 118 kb region on linkage group (LG) 2, in which we subsequently identified the causative mutation in *adenylate (adenylyl) cyclase 5* (*adcy5; ac5*). The analysis of the blond phenotype highlights the importance of Adcy5 for guppy melanophore development and the formation of orange male pattern traits. The phenotypic consequences of a mutation in *adcy5* are of great interest, as Adcy5 is involved in the growth of melanoma and other types of cancer in mammals (De Lorenzo et al., 2014).

RESULTS

Mapping of *blond* using ddRADseq

We obtained two strains of blond guppies, EnUlmBL and EnKABL, and the wild-type progenitor of one of the strains, EnUlmWT, from guppy hobby breeders (Figure 1C, 1D, 1D', 1E, 1F, 1F'). Test crosses demonstrated allelism of *blond*^{EnUlmBL} and *blond*^{EnKABL} (Figure S1). A previous study found a correlation between a deletion in the ORF of guppy *melanocortin 1 receptor* (*mc1r*) and a light phenotype resembling blond; however, this could not be confirmed in a F_2 generation (Tezuka et al., 2011). *Mc1r* did not carry any polymorphisms that were associated with the blond phenotype in our fish (data not shown).

To take an unbiased approach, we sought to identify the genomic region(s) linked to *blond* using ddRADseq and QTL mapping. Because *blond* had been shown to be an autosomal recessive mutation (Goodrich et al., 1944), we crossed a wild-type Quare3-2 female with a blond EnUlmBL male and subsequently intercrossed two of their phenotypically wild-type F_1 offspring to obtain a F_2 mapping population. For ddRADseq library preparation, the genomic DNA of the grandparents, of the F_1 pair, and of 33 blond and 108 wild-type F_2 fish was digested with *Pst*I, a restriction enzyme with a 6-bp recognition site, and *Mse*I, which has a 4-bp recognition site. Fragments were ligated with barcoded adapters and size-selected. A single sequencing library was analyzed on the Illumina HiSeq 2000 platform. Reads were mapped to the guppy reference genome assembly (Künstner et al., in preparation). In total, 21,448 homozygous single nucleotide polymorphisms (SNPs) were discovered between the grandparents, of which 7,394 were recovered in 134 of the F_2 individuals with sufficient coverage (Table S1).

Interval mapping was performed using Haley-Knott regression to take missing genotype data at a putative QTL into account (Broman et al., 2003). We found linkage of the blond phenotype to a single region of the genome on LG2 (Figure 2A). Closer examination of this region showed that the markers were distributed unequally along this LG. Therefore, we narrowed down the region most likely to contain *blond* by manually inspecting the markers and their LOD scores. The three markers with the highest LOD scores (LOD 30.9; 30.9; 30.8) were imputed by the regression method and were located between the actual SNP markers 31,213 (SNP A) and 31,226 (SNP B) (Figure 2B). All wild-type F_2 fish were either heterozygous or had the allele of the wild-type grandmother at SNP A. Only five wild-type F_2 individuals were homozygous for the allele of the blond grandfather at SNP B. All blond fish were homozygous for the allele of the grandfather at both positions. This indicated that *blond* is most likely closely linked to SNPs A and B. SNP A and B both mapped to scaffold_24 on LG2 of the guppy genome and were estimated to be approximately 118,017 bp apart from each other.

Comparison of the 118 kb sequence against the NCBI nr/nt database revealed that it comprises the ORFs of three genes, *signal transducing adaptor molecule 2* (*stam2*), *protein tyrosine phosphatase-like member b* (*ptplb*), and *adcy5* (Figure 2B).

Blond corresponds to the guppy ortholog of *adcy5*

Sanger sequencing of cDNAs from EnUlmWT and EnUlmBL embryos did not identify any obvious mutations that might disrupt the gene function of *stam2* and *ptplb* in blond fish (GenBank accession numbers: KP222266 and KP222267). Because *adcy5* has 21 exons with a 3,642 bp ORF (*adcy5* transcript variant X1, GenBank accession number: XM_008432387), we attempted separate amplification of the 5' and 3' portions of the gene. We separately obtained information for exons 1 to 7 and 21 from genomic DNA of adult fish (GenBank accession numbers: KP222269, KP222270, KP222271, KP222272, KP222273, KP222274, KP222275) and information for exons 8 to 20 from embryonic cDNA (GenBank accession number: KP222268). Comparison with the guppy reference genome demonstrated that the amplified sequences were almost identical to the predicted guppy *adcy5* transcript variant X1, which is most similar to *adcy5* of other fish species (Figure 2C). In nine of the 10 teleost genomes that are available on ENSEMBL, only one copy of *adcy5* is annotated (ENSEMBL v78). Comparison of the guppy *adcy5* transcript variant X1 to the guppy genome assembly identified *adcy5* on LG2 as well as the more distantly related *adcy6* on LG7.

Blond fish are homozygous for a 2-bp deletion affecting the last bp of exon 1 and the first bp of intron 1 (Figure 2D) (GenBank accession numbers: KP222269 and KP222270). The deletion disrupts the donor splice site with the canonical sequence GT at the beginning of intron 1, which is over 46 kb long in the guppy. This intron is 79,346 bp long in zebrafish (*Danio rerio*) and 95,339 bp in medaka (*Oryzias latipes*) (ENSEMBL v78). The deletion is predicted to lead to a splicing defect of intron 1, as well as a frameshift and premature stop of translation. The 2-bp deletion is perfectly linked to the blond phenotype in our mapping cross, with all 33 blond individuals being homozygous for the deletion allele, while 33 F_2 siblings with a wild-type appearance showed the expected 2:1 (21:12) ratio of heterozygous to homozygous wild-type Quare3-2 genotypes. We also genotyped 20 phenotypically wild-type and 20 blond fish of the EnUlm strain, which were homozygous for the wild-type and deletion allele, respectively. Finally, 20 blond EnKABL fish obtained from a different hobby breeder were also homozygous for *adcy5*^{del}. This suggests that guppy hobby breeders crossed *adcy5*^{del} into different guppy strains in the past because of the conspicuous blond coloration; this would be similar to what happened in the case of the golden phenotype (Kottler et al., 2013). Alternatively, the EnUlm and EnKABL strains might be derived from a common 'ancestral' strain into which *adcy5*^{del} was introduced or in which it originated, as the ornaments of males of both strains resemble the ones described for 'Endler's guppy' (Alexander and Breden, 2004).

Using quantitative PCR after reverse transcription (RT-qPCR), we found that *adcy5* expression is strongly reduced in blond skin (Figures 2E, S2A), indicating that the mutant transcript is subject to nonsense-mediated decay. The expression of *stam2* and *ptplb*, in contrast, is not reduced in the skin of blond fish (Figure S2A). In zebrafish larvae, *adcy5* is expressed in the whole body, but is slightly enriched in the head and brain (Peng et al., 2009). RT-qPCR revealed that *adcy5* is at least as highly expressed in female guppy brain as in female guppy skin, suggesting that Adcy5 fulfills multiple functions in various regions within the body (Figure S2B).

Taken together, these results provide good evidence that the blond phenotype is caused by a mutation in the guppy ortholog of *adcy5* and demonstrate that *adcy5* is expressed in guppy skin and brain.

Role of Adcy5 in melanophore differentiation and/or growth

The reticulate pattern of adult guppies consists of early Kita-dependent and later-developing Kita-independent corolla melanophores (Figures 1A, S3A, S3A') (Goodrich et al., 1944; Kottler et al., 2013). Dendritic melanophores, which are more variable in shape, are distributed irregularly over the whole body, as are some corolla melanophores (Figures 1A, 1B, S3A, S3A') (Goodrich et al., 1944). The phenotype of *kita* mutant guppies is characterized by a reduced number of melanophores; both dendritic and ventral corolla melanophores are hard to find (Figure S3B, S3B') (Goodrich et al., 1944; Kottler et al., 2013). The density of melanophores per mm^2 does not differ significantly between blond and wild-type fish (Mann-Whitney U=61, n_1=n_2=10, P=0.4 two-tailed) (Table S2 and (Goodrich et al., 1944)), indicating that the mutation in *adcy5* does not affect melanophore fate specification or proliferation. All melanophores of blond fish, however, appear smaller; only very few melanophores are slightly dendritic in shape (Figure S3C, S3C'). *Adcy5/kita* double mutant guppies (cream) exhibit an additive phenotype without any detectable dendritic melanophores (Figure S3D, S3D'). This suggests that the slightly dendritic melanophores seen in blond guppies correspond to the dendritic melanophores of wild-type fish.

The blond phenotype is already apparent at birth, however at this stage not as pronounced as in adult fish: some melanophores on the head of blond newborns resemble corolla ones and the melanophores located laterally on the trunk are somewhat dendritic, but smaller than those of wild-type newborns (Figure S4A, S4A', S4B, S4B'). Corolla melanophores could not be identified on the head of adult blond guppies, pointing to Adcy5 being required for the maintenance of melanophore shape after birth (Figure S4C, S4D). Two major possibilities how Adcy5 might influence the shape of the melanophores are: I.) Adcy5 might be required for melanosome dispersion; punctate melanophores would then appear small because their melanosomes are permanently aggregated; or, II.) Adcy5 might promote melanophore differentiation and/or growth; the punctate melanophores would then actually be smaller in size.

As a physiological mechanism of background adaptation, zebrafish larvae aggregate their melanosomes in bright light and disperse their melanosomes in the dark (Fujii, 2000; Peng et al., 2009). Ethanol disturbs this response in zebrafish by stimulating melanosome dispersion even in bright light conditions (Peng et al., 2009). The zebrafish *adcy5* mutant *fantasma*, however, fails to disperse its melanosomes when treated with ethanol and also shows reduced behavioral sensitivity to this drug (Peng et al., 2009). Cultured melanophores of *fantasma* zebrafish disperse their melanosomes upon treatment with the Adenylate cyclase activator forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), which both increase the intracellular level of cAMP, but fail to maintain the dispersion when forskolin and IBMX are removed (Peng et al., 2009). To test whether the punctate melanophore phenotype of blond guppies might be caused by a failure to maintain melanosome dispersion, we treated melanophores of the caudal peduncle of wild-type and blond female guppies with forskolin and IBMX. Almost all melanophores of wild-type fish had reacted to the treatments after 10 min by dispersing their melanosomes (Figure 3A, 3A', 3B, 3B'). In contrast, only a few melanophores of blond fish showed a reaction after 10 min, which were presumably dendritic melanophores, as they were associated with the scales and were not corolla-shaped (Figure 3C, 3C', 3D, 3D' and data not shown). Many of the melanophores of blond fish remained punctate, suggesting that these correspond to the corolla melanophores of wild-type fish (Figure 3C, 3C', 3D, 3D' and (Goodrich et al., 1944)). Even after 39 min of forskolin and 28 min of IBMX treatment, the melanosomes of blond fish still showed no visible reaction or were only slightly more dispersed than after 10 min, suggesting that a delayed response of the melanosomes of blond fish does not cause the observed differences. As not all melanophores of blond fish could be stimulated to disperse their melanosomes, or some of the melanophores are too small to show an easily visible reaction, our results indicate that a failure to maintain melanosome dispersion alone cannot explain the blond phenotype.

Inspection of fully aggregated melanophores revealed that blond melanophores appear much smaller, suggesting that the melanophores of blond and wild-type fish differ in cell size or melanosome content (Figure 4A, 4B). We therefore used semi-thin sections to investigate the possibility that the melanophores of blond fish might be smaller than the ones of wild-type fish or might show a melanosome defect. The semi-thin sections, which enabled us to trace single melanophores from ventral to dorsal within the tail fin tissue, revealed that the melanophores of blond fish appear much smaller throughout (Document S1).

As aggregated melanosomes might let the cells appear small and we were not able to discern the cell membranes of the melanophores in the semi-thin sections, we used transmission electron mircoscopy (TEM) to further investigate the dimensions of the melanophores. We found that the melanosomes of blond fish appear normal, as does the eumelanin within the melanosomes (Figure 4C-F). The melanosomes are homogenously distributed in wild-type and blond melanophores, with only some narrow cell protrusions being free of melanosomes (Figure 4C-F). In all TEM sections, however, the blond melanophores outlined by the cell membranes are clearly smaller than wild-type melanophores, suggesting that the punctate phenotype of the blond melanophores is a consequence of their smaller size (Figure 4C-F).

Taken together, we conclude that Adcy5 is required for melanophore differentiation and/or growth in the guppy, although we cannot exclude that Adcy5 also has a direct role in melanosome movement.

Role of Adcy5 in male pigment pattern formation

Mutations in the type III receptor tyrosine kinases Kita and Csf1ra affect pigment cell differentiation as well as the formation of male-specific color traits (Kottler et al., 2013). We therefore investigated whether the loss-of-function mutation in *adcy5* might also have an effect on male ornaments besides altering the morphology of the melanophores. Blond males seem to lack black spots and stripes (Figures 1C, 1E, S1B, S1D, 5A, 5B) (Dataset S1). Close inspection of blond skin, however, showed that black spots and stripes are present, but hard to see because of the small melanophore size (Figure 5A’, 5B’). Measurement of the total area of the orange ornaments on the body and, separately, on the tail fin of 40 wild-type and 40 blond F_2 males derived from five heterozygous F_1 pairs revealed that, on average, only 3.6% of the body and 5.7% of the tail fin of blond males are covered by orange ornaments, while on average 9.7% of the body and 10.6% of the tail fin of wild-type males are overlain with orange ornaments (Table S3). This suggests that the mutation in *adcy5* affects the formation or size of the orange ornaments.

To investigate in detail how the orange ornaments are affected, we first characterized the orange pattern elements of male wild-type guppies in the F_2 population (Dataset S1). Wild-type males usually have an anterior orange spot, which is located ventral of the anterior black horizontal stripe (Figure 5A, 5C) (Dataset S1). The anterior orange spot is always in front of an imaginary line connecting the anterior attachments of the dorsal and pelvic fins (Figure 5A, 5C) (Dataset S1). Behind the central black spot, but ventral of the posterior black horizontal stripe, are one or two, rarely three, posterior orange spots, which we call posterior orange spot complex (Figure 5A, 5B, 5C) (Dataset S1). Sometimes, one or two orange spots are present dorsal of the posterior black horizontal stripe and lateral line, which we named dorsal orange spot complex (Figure 5A, 5C) (Dataset S1). The tail fin of most males is characterized by a dorsal and ventral orange-black margin, which is occasionally comprised of more than one element (dorsal and ventral orange margin complexes) (Figure 5A, 5B, 5C) (Dataset S1). Although the black spots and stripes are not visible on whole-body photographs of blond fish, the orange pattern elements of blond males could be easily identified based on their positions (Figure 5B).

First, we investigated whether the mutation in *adcy5* might affect the formation *per se* of one, or several, of the orange pattern elements by analyzing pictures of 74 wild-type and 74 blond F_2 males derived from 10 heterozygous F_1 pairs. For each male, we noted which orange pattern traits are present and then counted the number of elements of which each of the traits consists. All 74 wild-type males have an anterior orange spot, but only 11% of the blond males, indicating that Adcy5 promotes the formation of this spot (Table S4). In 24% of the blond males, the posterior orange spot complex is absent, while only 3% of the wild-type males lack this trait (Table S4). Likewise, 22% of the blond males have no dorsal orange margin on the tail fin, which is always present in wild-type males (Table S4). Interestingly, the ventral orange margin of the tail fin is composed of more than two elements in 30% of the blond males, but only in 1% of the wild-type males, suggesting that the margin tends to break up into smaller spots in blond fish (Table S4). Second, we compared the size of the orange ornaments of the 40 wild-type and 40 blond individuals mentioned above to find out whether Adcy5 might also affect the extent of the ornaments (Table S3). If the posterior orange spot complex is present, its size relative to the entire body area is significantly smaller in blond than in wild-type males ($P<0.001$) (Table S3). The sizes of the dorsal and ventral orange margin complexes in relation to the tail fin area are also significantly smaller in blond males ($P<0.001$) (Figure 5D and Table S3). The size of the dorsal orange spot complex in relation to the body area does not differ significantly between blond and wild-type fish ($P=0.1$) (Table S3). Taken together, these results suggest that Adcy5 strongly affects the size of all male orange ornaments, except for the dorsal orange spot complex. Additionally, Adcy5 promotes spot formation *per se*, especially of the anterior orange spot.

DISCUSSION

Guppies are beautifully colored fish that are famous for adapting quickly to habitats differing in, e.g., predation pressure and nutrient availability (Magurran, 2005). The development of the highly complex guppy pigment pattern is still poorly understood. Using genotyping-by-sequencing, we found that the blond phenotype of the guppy is caused by a mutation in *adcy5*. The mutation in this gene strongly affects the phenotype of the melanophores and the development of the male pattern. Blond is the third guppy pigment mutant for which the underlying mutation has been identified (Kottler et al., 2013).

RADseq is a cost-efficient method to interrogate a large number of genetic markers (Baird et al., 2008; Catchen et al., 2013; Hohenlohe et al., 2010; Jones et al., 2013). In fish, it has been used to map regions of the genome linked to pigmentation differences in African cichlids and to sex-determination in zebrafish and Atlantic halibut (*Hippoglossus hippoglossus*) (Anderson et al., 2012; Henning et al., 2014; O'Quin et al., 2013; Palaiokostas et al., 2013). A study on the white fur variant of the tiger (*Panthera tigris tigris*) represents one of the few examples in which the causal SNP, a missense mutation in *solute carrier family 45 member 2* (*slc45a2*), was pinpointed using a combination of RADseq, whole-genome sequencing, and genome-wide association (Xu et al., 2013).

Our mapping results demonstrate that ddRADseq in combination with QTL mapping can on its own be an efficient method for highly accurate mapping, especially when a high-quality reference genome is available. Although we recovered only about a third of the grandparental markers in the F_2 individuals, the number of markers was sufficient to identify a candidate region for *blond*. Importantly, we used eight barcodes to tag the DNA of each grandparent during library preparation, which ensured a high coverage of the grandparental RAD tags. The high coverage allowed us to reliably call the homozygous markers for the grandparents, which is crucial for subsequent QTL mapping. Our results suggest that a F_2 mapping population comprising 100 to 200 individuals can be sufficient to map a single-locus trait (Rowan et al., 2011; Schneeberger and Weigel, 2011) and that the LOD scores are highly informative by giving precise information on the location of the causative mutation.

The mutation in *adcy5* strongly affects the guppy pigment pattern, most noticeably the appearance of the melanophores. Upon activation by G protein-coupled receptors, Adenylate cyclase (Adcy) catalyzes the synthesis of the intracellular second messenger cAMP from ATP (Busca and Ballotti, 2000; Dumaz and Marais, 2005; Fujii, 2000; Sunahara and Taussig, 2002). Ten different isoforms of Adcy have been reported in mammals, of which nine are membrane-bound (Adcy1 to Adcy9) and one is soluble (Adcy10) (Sunahara and Taussig, 2002). Teleosts appear to lack Adcy4 and Adcy10, as the genes encoding these isoforms are not annotated in any of the 10 teleost genomes hosted on ENSEMBL v78. Surprisingly little is known about the identity of the Adcy isoforms that act within melanophores. Cyclic AMP plays an important role during physiological color change in fish, i.e., melanosome dispersion (high cAMP level) and aggregation (low cAMP level) (Andersson et al., 2003; Fujii, 2000; Richardson et al., 2008). Cultured melanophores of the zebrafish *adcy5* mutant *fantasma* disperse their melanosomes when grown in presence of forskolin and IBMX, which increase the intracellular level of cAMP (Peng et al., 2009). When forskolin and IBMX are removed, however, cAMP levels drop, leading to melanosome aggregation (Peng et al., 2009). Treatment of the dispersed melanophores with an Extracellular-signal Regulated Kinase (ERK) inhibitor showed that ERK is to some extent required for melanosome aggregation in zebrafish melanophores (Peng et al., 2009). The MAPK (Mitogen-Activated Protein Kinase)/ERK pathway is activated during both melanosome dispersion and aggregation in *Xenopus* melanophores and presumably mediates the microtubule-dependent component of melanosome movement (Deacon et al., 2005). During aggregation, the deactivation of Adcy, e.g., by the melatonin receptor, leads to a decrease of cAMP, which reduces PKA activity (Andersson et al., 2003; Deacon et al., 2005). Inhibition of PKA in this situation stimulates the MAPK/ERK pathway (Andersson et al., 2003; Deacon et al., 2005). Interestingly, transient activation of ERK is much higher during melanosome aggregation than during melanosome dispersion in *Xenopus* melanophores (Deacon et al., 2005).

In contrast to what has been reported in zebrafish, our results suggest that the punctate phenotype of the melanophores of *adcy5* mutant guppies is primarily caused by a defect in melanophore differentiation and/or growth and not in melanosome dispersion. Most melanophores of blond fish did not react to the forskolin and IBMX treatments or their reaction was not visible because of their small size. Only superficial dendritic melanophores dispersed their melanosomes during our experiments, which confirms that indeed two different populations of melanophores, corolla and dendritic ones, need to be distinguished in the skin of adult guppies (Goodrich et al., 1944). Already 70 years ago, it was reported that the dendritic melanophores of blond guppies respond to various stimuli, while the punctate melanophores show almost no reaction (Goodrich et al., 1944). Further investigations using semi-thin sections and TEM demonstrated that blond melanophores are smaller than wild-type ones, suggesting that Adcy5 is required for melanophore growth in the guppy. In zebrafish, a reduction in melanophore size has been described in morphants with reduced activity of *paired box 3* (*pax3*), which is required for xanthophore differentiation in this species (Minchin and Hughes, 2008).

Most likely, Adcy5 affects guppy melanophore development via the cAMP/PKA and/or the MAPK/ERK pathways. In melanophores, the G protein-coupled receptor Mc1r activates Adcy upon binding an activating ligand, e.g., α-Melanocyte Stimulating Hormone (α-MSH) (Busca and Ballotti, 2000). An increase in cAMP leads to the activation of PKA, which phosphorylates and thereby activates an array of substrates, among them cAMP-Response Element Binding (CREB) transcription factors in the nucleus (Busca and Ballotti, 2000). CREBs bind to cAMP Response Elements (CRE), e.g, in the promoter of the *microphthalmia-associated transcription factor* (*mitf*), thereby activating expression of *mitf* and of other genes involved in melanophore development and melanin synthesis (Busca and Ballotti, 2000; Cheli et al., 2010). Up-regulation of the cAMP pathway also activates the MAPK/ERK pathway in mouse melanoma cells in a PKA-independent manner via Ras and B-Raf (Busca et al., 2000; Busca and Ballotti, 2000; Dumaz and Marais, 2005). B-Raf mediates the activation of the ERK activator MEK (MAPKK, MAP kinase kinase), thereby inducing melanophore proliferation and differentiation (Busca et al., 2000; Busca and Ballotti, 2000; Dumaz and Marais, 2005).

Analysis of the male guppy ornaments revealed that the *adcy5* mutation affects the formation and size of the orange pattern traits. Blond males more often lacked the anterior orange spot, the posterior orange spot complex, and the dorsal orange margin complex on the tail fin. The size of all orange pattern traits is significantly smaller in blond males, except for the dorsal orange spot complex. The ventral orange margin is composed of more than two elements in about a third of the blond males, but only in 1% of the wild-type males. These results suggest that male guppy xanthophores, which differentiate during puberty and are under hormonal control, require Adcy5 to form normally sized spots and stripes. Inspection of blond and wild-type female skin did not reveal any prominent differences in the number of xanthophores (data not shown). However, we often noticed orange areas in the skin of blond males that appeared to be loose clusters of xanthophores, indicating that the male-specific xanthophores of blond males are sometimes scattered instead of densely packed at the sites where spots are present in wild-type fish (Figure 5B). This suggests that Adcy5 stimulates orange trait formation in the guppy, either by leading xanthophores to prospective orange trait locations, or by promoting their accumulation at these sites. Adcy5 could affect the xanthophores either directly by being expressed in the cells or indirectly via the melanophores by xanthophore-melanophore interactions. Absence of functional xanthophore-melanophore interactions presumably causes the disturbed melanophore pattern of male guppy *csf1ra* mutants, which lack most xanthophores (Kottler et al., 2013). Especially the color patterns on the tail fin margins seem to depend on such interactions (Kottler et al., 2013). As the development of *adcy5*-deficient melanophores is impaired, they might not be able to properly interact with the male-specific xanthophores. Recent studies in zebrafish and other *Danio* species, however, have demonstrated that pigment patterning relies on interactions not only between xanthophores and melanophores, but also between iridophores, xanthophores, and melanophores (Mahalwar et al., 2014; Patterson et al., 2014; Patterson and Parichy, 2013; Singh et al., 2014). *Adcy5*^{del}-mediated iridophore defects could therefore also be responsible for the observed differences in orange pigmentation, especially as iridophores are in close contact with xanthophores in the hypodermis within the male orange spots (Kottler et al., 2014). The orange traits of the blond males are differently affected by the *adcy5* mutation, suggesting that the formation of some male orange ornaments, e.g., of the anterior orange spot, depends more heavily on Adcy5-mediated cues than the formation of others.

In summary, we have discovered that a mutation in a gene encoding a specific Adcy isoform can lead to a pigmentation phenotype in fish. The phenotype of *adcy5* mutant guppies is striking and suggests that Adcy5 plays an important role during melanophore development. Knockout of *adcy5* has been shown to extend the lifespan of mice by protecting them from cardiomyopathy, oxidative stress, and osteoporosis, which is mediated by the activation of the MAPK/ERK pathway (Yan et al., 2007). Moreover, a recent study has demonstrated that Adcy5 knockout in mouse decreases the risk of age-related tumor development and delays the growth of melanoma cells in cell culture by reducing tumor proliferation and angiogenesis (De Lorenzo et al., 2014). Identification of an *adcy5* mutant of the fish melanoma model *Xiphophorus*, or introduction of an *adcy5* mutation into the medaka melanoma model system, might make it possible to test whether *adcy5* knockout delays melanoma growth *in vivo* in the future (Patton et al., 2010).

METHODS

Guppy strains

Wild-type and blond EnUlm (EnUlmWT, EnUlmBL) and blond EnKABL guppies were acquired from guppy hobby breeders in 2008. The male ornaments of both strains resemble the ones described for 'Endler's guppy' (Alexander and Breden, 2004). Double mutant cream fish were obtained in the F_2 generation of a cross between an EnKABL female and a golden Maculatus male (Kottler et al., 2013). Quare3-2 fish were derived from wild individuals caught in Trinidad in 2003 (Quare river). All guppies were fed six days per week with *Artemia* and maintained at 25°C in a 12-hour light and dark cycle. For all crosses, virgin females were used.

Imaging

Pictures of whole fish were taken with a Canon EOS 10D digital camera (Canon Macro Lens EF 100 mm) under incident light conditions. All details were taken with a Zeiss Axiozoom.V16 connected to a Zeiss AxioCam HRc. Images were processed and stacked with ZEN pro (ZEN 2012 blue edition) software (Zeiss). If necessary, brightness was adjusted with ADOBE PHOTOSHOP SOFTWARE v12.1.

Mapping cross

A wild-type Quare3-2 female was crossed with a blond EnUlmBL male. Full-sib crosses between F_1 fish were set up to obtain a F_2 mapping population. The phenotype of all F_2 fish (wild-type or blond) was determined; a dissecting microscope was used to phenotype the females. We obtained a total of 592 F_2 in our mapping cross of which 444 were wild-type and 148 were blond, confirming Mendelian single-locus inheritance (exact 3:1 ratio). For ddRADseq library preparation, 141 F_2 individuals were used.

Library preparation for ddRADseq

The ddRADseq library was prepared as described by (Poland et al., 2012). Genomic DNA of the grandparents, one F_1 pair, and 141 F_2 individuals derived from this pair (33 blond, 108 wild-type) was digested with the restriction enzymes *Pst*I and *Mse*I and barcoded. Eight different barcodes were used for each grandparent and one barcode for each of the other individuals (159 barcodes in total) (Poland et al., 2012). After multiplexing and PCR amplification (12 cycles), the PCR product was size-selected on a 2% low range agarose gel (Bio-Rad). Fragments of the insert size range 120 – 220 bp were excised and purified for sequencing. The library was sequenced on the Illumina HiSeq 2000 platform (100 bp read length, single-end). ENA accession number of sequencing data: PRJEB7924.

Genotyping and QTL mapping

Sequencing libraries were retrieved and demultiplexed using the IMPORT command in SHORE version 0.8unstable (rev. 8112-2171) (Ossowski et al., 2008) allowing for 2 mismatches in the barcode. Barcodes and adapter sequences were trimmed from the sequences. Raw reads were converted to FASTQ files using the CONVERT command as implemented in SHORE. Quality filtering was done using default cutoffs in SHORE (chastity violation 57, quality violation 3, quality cutoff read trimming of 5). Chastity filters were used to remove reads that resulted from overlapping clusters. Remaining high quality reads were mapped to the reference genome (RefSeq ID GCF_000633615.1) using BWA version 0.6.2 (Li and Durbin, 2009) with default parameters. Mapping results were enhanced by local realignment using GATK version 1.6-13 (McKenna et al., 2010). SNP detection was performed using GATK UNIFIEDGENOTYPER (default parameters) (McKenna et al., 2010). The resulting data was scanned for missing information and only SNPs were kept with a maximum of 50 missing individuals. Afterwards, genotypes were assigned to each individual.

Interval mapping with a single QTL model was performed using Haley-Knott regression with a model for binary traits as implemented in R/qtl version 1.31-9 (Broman et al., 2003). Significance LOD thresholds were determined using 1,000 permutations across the whole genome. Statistical analyses were performed using R Version 3.0.1 (R Development Core Team, 2008). The sequencing data of the F_1 pair was not required and therefore not used for the mapping.

PCR of cDNA for Sanger sequencing

Total RNA was extracted from 10 to 15 pooled embryos using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was used for first-strand cDNA synthesis as described in (Kottler et al., 2013), which was directly used for PCR. PCR was performed with *Taq* DNA Polymerase and ThermoPol Buffer (New England BioLabs) according to the manufacturer's instructions. PCR fragments were analyzed by gel electrophoresis, extracted with MinElute Gel Extraction Kit (Qiagen) and cloned into pGEM-T Easy vector (Promega). Plasmid DNA was purified and sequenced as described in (Kottler et al., 2013). Sequences were aligned with the STADEN package (PREGAP4 v1.5 and GAP4 v4.10; http://staden.sourceforge.net/). Primer sequences: *stam2*, forward 5'-TAATGGAGAAGGAGGAAGAGACG-3', reverse 5'-AGAAGAGTGTAGCTGAAGGTCTGC-3'; *ptplb*, forward 5'-AGTAGTGAGTAGCGGTGATCTTGC-3', reverse 5'-CTCTCATGTGAGCCGTTCTGAG-3'; *adcy5*, forward 5'-GCCTACTTGAAGAGGAGCAACATAG-3', reverse 5'-TCGGTATTCCAGTGTGTAGCTG-3'.

PCR of genomic DNA for Sanger sequencing

Genomic DNA of adult guppy trunk tissue was prepared with DNeasy Blood and Tissue Kit (Qiagen). PCR, cloning of PCR products, sequencing, and analysis of sequences was carried out as described above. Exons were annotated according to the predicted guppy *adcy5* transcript variant X1 (GenBank accession number XM_008432387) and the guppy genome (RefSeq ID GCF_000633615.1). Primer sequences: exon1, forward 5'- CGAGTCAACCTTGTGACTGAATAG-3', reverse 5'-TCAACAAGCTGTCGGTTTAGACTC-3'; exons 2 to 3, forward 5'-GCTGATAGGAATAGAACAACAACG-3', reverse 5'-CTTCGTTCATATCCGTGTTCATC-3'; exon 4, forward 5'-CACGTTAAGCTTCTCAACTTCACC-3', reverse 5'-TTCTAGTCTTCCTGTATGTCTGTAAGG-3'; exons 5 to 7, forward 5'-TTGCTTCTCGGTTAATACCATAACG-3', reverse 5'-CCTCAGCCTTACTCAATGTCAAG-3'; exon 21, forward 5'-CGTCTCTACTTCAACACACCTGAG-3', reverse 5'-AGGTCTGTAAGCCGTCTCGTTAC-3'.

Phylogenetic analysis

Zebrafish (zf), medaka (me), and Amazon molly (*Poecilia formosa*) (Am) coding sequences of *adcy1*, *adcy2*, *adcy3*, *adcy5*, *adcy6*, *adcy7*, *adcy8*, and *adcy9* were downloaded from http://ensembl.org (ENSEMBL v78). *Adcy4* and *adcy10* sequences were not available. If two or more copies of one gene were annotated, we randomly downloaded one of the copies. A maximum-parsimony phylogenetic tree was constructed as described in (Kottler et al., 2013) using SPLITSTREE4 v4.12.8. ENSEMBL accession numbers: zf *adcy1a*, ENSDART00000099790; zf *adcy2a*, ENSDART00000084264; zf *adcy3*, ENSDART00000110365; zf *adcy5*, ENSDART00000124333; zf *adcy6a*, ENSDART00000087654; zf *adcy7*, ENSDART00000129156; zf *adcy8*, ENSDART00000087823; zf *adcy9*, ENSDART00000101030; me *adcy1a*, ENSORLT00000016127; me *adcy2b*, ENSORLT00000018050; me *adcy3*, ENSORLT00000003094; me *adcy5*, ENSORLT00000012233; me *adcy6*, ENSORLT00000008754; me *adcy7*, ENSORLT00000017521; me *adcy8*, ENSORLT00000008052; me *adcy9*, ENSORLT00000010411; Am *adcy1a*, ENSPFOT00000026834; Am *adcy2a*, ENSPFOT00000016987; Am *adcy3*, ENSPFOT00000022024; Am *adcy5*, ENSPFOT00000017087; Am *adcy6*, ENSPFOT00000011917; Am *adcy7*, ENSPFOT00000015564; Am *adcy8*, ENSPFOT00000002789; Am *adcy9*, ENSPFOT00000000724. Guppy *adcy5*, coding sequence of GenBank accession number XM_008432387.

Expression studies in the skin and brain of adult wild-type and blond females

Total RNA was extracted with TRIzol (Invitrogen) from the skin and brains of adult wild-type females and the skin of adult blond females. All females were nine to 10 months old. The wild-type skin and brains were derived from different individuals. For first-strand cDNA synthesis, 800 ng or 1 µg of total RNA was primed with oligo(dT)$_{18}$ (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). The first-strand cDNA was diluted 10-fold to a total volume of 200 µl of which 2 µl were used as template for real-time quantitative PCR (RT-qPCR). RT-qPCR was carried out with Maxima SYBR Green qPCR Master Mixes (Thermo Scientific) using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The performed protocol was: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s, followed by 72 °C for 7 min. Three biological replicates with three technical repetitions each were used to determine the expression of *adcy5*, *stam2*, and *ptplb*; *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) or *eukaryotic translation elongation factor 1 alpha a* (*eef1aa*) expression was utilized to control for differences in RNA input and in the efficiency of the cDNA synthesis reaction. Relative quantification (Pfaffl, 2001) was carried out for all primer pairs taking into account the individual primer efficiencies obtained by means of serial dilution (factor 5). Standard deviation was calculated across the biological replicates. Primer sequences: *gapdh*, forward 5’-ACATCAAGAAGGTTGTGAAAGCTG-3’, reverse 5’-ATCAAAGATGGAGGAGTGAGAATC-3’; *eef1aa*, forward 5’-AAGCCCATGGTGGTGGAGCCCTTC-3’, reverse 5’-CTTCTCTGCAGCCTTGGTTGTCTTTC-3’; *adcy5* primer pair 1, forward 5’-GATCCAGGTGACGGCTGATC-3’ (spans exon 20 - exon 21 junction), reverse 5’-TCATCATCTCTCCTTTGCCTTTGAC-3’ (exon 21); *adcy5* primer pair 2, forward 5'-CGGATGATGGACCAGATGAAATAC-3' (exon 19), reverse 5'-ACCGGGCCAATGTTTAGACC-3' (spans exon 19 - exon 20 junction); *stam2*, forward 5'-GCCTGGACCGCTGCATTCAATAC-3', reverse 5'-GAGCTTGGGTCATGTACTGGGAGTTC-3'; *ptplb*, forward 5'- GGCCCAGCACCTTCTTCCTCTG-3', reverse 5'-TCCCCAACAAGTACAACTTCTCCTTC-3'. Primer efficiencies when *gapdh* was used as reference: *gapdh*, 87%; *adcy5* primer pair 1, 109%; *adcy5* primer pair 2, 99%. Primer efficiencies when *eef1aa* was used as reference: *eef1aa*, 89%; *adcy5* primer pair 1, 94%; *adcy5* primer pair 2, 87%; *stam2*, 100%; *ptplb*, 89%.

Melanophore counts

To count the number of melanophores, 10 wild-type and 10 blond females were sacrificed by immersing them in E2 solution (Nüsslein-Volhard and Dahm, 2002) containing 0.1% (w/v) tricaine (ethyl 3-aminobenzoate methanesulfonate salt) and 2.4 mg/ml epinephrine. The females were at least eight months old. Pictures were taken as described in (Kottler et al., 2013). Melanophores were counted manually using IMAGEJ 1.49j (imagej.nih.gov/ij).

Melanosome dispersion experiments

Stock solutions of forskolin and IBMX were prepared in DMSO according to the manufacturer's instructions (Sigma-Aldrich). Adult female wild-type and blond guppies were kept in light for 24 hours to stimulate melanosome aggregation. For each experiment, a fish with the respective phenotype was anesthetized on ice and sacrificed by decapitation. A piece of the caudal peduncle was cut out with a scalpel and washed briefly in physiological saline solution (Logan et al., 2006). Subsequently, the piece was transferred to a single well whose bottom was covered with 1% agarose and covered by 25 µM forskolin or 100 µM IBMX in saline solution (concentrations according to (Peng et al., 2009)). The first stack of images was taken immediately using a Zeiss AxioZoom.V16 connected with a Zeiss AxioCam HRc. The second stack of images was taken after 10 min, providing sufficient time for melanosome dispersal (Logan et al., 2006). Additional stacks were taken after 39 min (blond, forskolin) and 28 min (blond, IBMX). The light of the microscope was switched on during the whole experiment.

Semi-thin sections and transmission electron microscopy

For the semi-thin sections, ventral tail fin tissue of wild-type and blond males was embedded as described in (Kottler et al., 2014). Sections of 300 nm of the samples were taken approximately every 2 µm from ventral to dorsal using a Leica Ultracut UCT microtome. Sections were dried at 60°C for one minute and stained with a solution containing 0.5% toluidine blue and 1% sodium tetraborate for one minute at room temperature. Sections were examined with a Zeiss Axioplan 2 microscope; images were taken with a Zeiss AxioCam MRm camera and processed with AxioVision Software. Transmission electron microscopy was conducted as described in (Kottler et al., 2014).

Analysis of male ornaments

The total areas of the left body side and tail fin and the size of the orange traits on the left side were measured with IMAGEJ 1.49j (imagej.nih.gov/ij). All males were at least three months old when the pictures were taken. Analysis and plotting of the results was carried out with MICROSOFT EXCEL FOR MAC 2011 v14.4.5 and APPLE NUMBERS v3.5 (Mac OS X).

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AUTHOR CONTRIBUTIONS

VAK and CD conceived and designed the experiments. VAK, MH, and ES prepared the library for ddRADseq. AK analyzed the ddRADseq data and performed the QTL analysis. TL carried out and analyzed the RT-qPCR experiment. IK and MF prepared the semi-thin sections and conducted the TEM. VAK performed all other experiments and analyzed the data. VAK wrote the manuscript; all authors provided helpful comments. DW oversaw the experimental design and data analysis.

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FIGURE LEGENDS

Figure 1. Guppy melanophore types and pigmentation pattern of wild-type and blond fish.

(A) Corolla melanophores forming the reticulate pattern (red arrowheads), irregularly distributed corolla melanophores (yellow arrows), and superficial dendritic melanophores (white arrows) on the caudal peduncle of a wild-type EnUlmWT female. (B) Detached scale of an EnUlmBL female highlighting the dendritic melanophores that are located in the skin above the scales. The melanophores in (B) were treated with 25 µM forskolin. Corolla and dendritic melanophores can only be unambiguously distinguished from each other when the skin layer containing the melanophore is known (Kottler et al., 2014). Male (C) and female (D) wild-type EnUlmWT guppies; male (E) and female (F) blond EnUlmBL fish. The phenotype of EnKABL guppies is very similar to (E,F). (D’,F’) Details of areas boxed in (D) and (F), respectively, but of different individuals, showing the reticulate pattern on the caudal peduncle. The melanophores of blond guppies appear smaller. Scale bars: (C,D,E,F) 0.5 cm; (A,B,D',F') 0.5 mm.

Figure 2. *Blond* corresponds to the guppy ortholog of *adcy5*.

(A) QTL mapping results based on 7,394 markers. Blue and orange dashed lines mark the significance thresholds p=0.01 and p=0.5 (1,000 permutations). Some markers with high LOD scores mapped to UN (unassigned) scaffolds; these scaffolds presumably belong to LG2. Structural variants and mis-assigned markers may contribute to the irregularity of the QTL peak. (B) Diagram of the mapping interval. Zero wild-type individuals were homozygous for the allele of the blond grandfather at SNP A (rank 1 of all wild-type individuals). Five wild-type individuals were homozygous for the allele of the blond grandfather at SNP B (rank 2 of all wild-type individuals). (C) Maximum-parsimony phylogenetic tree of fish *adcy* genes. Bootstrap support (100 replicates) was 100 except for the clades for which values are shown. (D) Partial sequence electropherograms of genomic DNA from wild-type and blond fish. The 2 bp-deletion at the exon 1/intron 1 border (dashed orange line) of *adcy5* is indicated. (E) Relative normalized expression of *adcy5* in adult female blond skin. Measured fold change in expression between wild-type and blond was 8.3 for primer pair 1 and 6.3 for primer pair 2. *Gapdh* was used as reference gene.

Figure 3. Melanosome dispersion experiments.

Treatment of caudal peduncle pieces of wild-type and blond females with forskolin and IBMX. Only few melanophores of the blond fish showed a visible reaction to the chemicals. These cells were presumably superficial dendritic melanophores (red arrows in (C',D')). The more deeply located corolla melanophores showed no visible response (blue arrows in (C',D')). Scale bars: 0.5 mm.

Figure 4. Size measurement of melanophores.

(A,B) Fully aggregated melanophores of wild-type and blond adult females below the dorsal fin. Melanosomes were aggregated with epinephrine. (C-F) Transmission electron microscopy images of wild-type and blond melanophores in the tail fin. Red arrowheads outline the cell membranes of the melanophores in (E); one of the melanophores has a melanosome-free protrusion. N, nucleus. Scale bars: (A,B) 1 mm; (C-F) 2 µm.

Figure 5. Analysis of male pigment patterns.

(A) Wild-type and (B) blond F_2 male of the mapping cross. The color traits mentioned in the text are indicated and are also shown schematically in (C). Only black ornaments necessary for the identification of the orange traits were considered. (A',B') details of areas boxed in (A) and (B), respectively, but of different individuals. The punctate melanophores of blond fish are cumulated at the sites where black spots and stripes can be seen in wild-type fish. Red arrowhead in (B) marks scattered xanthophores (see Discussion). (D) Measurement of the area of the dorsal orange margin complex in 40 blond and 40 wild-type individuals. 1, Anterior black horizontal stripe; 2, anterior orange spot (always anterior of the red imaginary dashed line connecting the attachments of the dorsal and pelvic fins (C)); 3, central black spot; 4, posterior orange spot complex; 5, posterior black horizontal stripe; 6, dorsal orange spot complex; 7, dorsal orange margin complex; 8, ventral orange margin complex. Scale bars: (A,B) 0.5 cm; (A',B') 1 mm.

SUPPORTING INFORMATION

Figure S1. Complementation test.

Cross between a blond EnKABL female (A) and a blond EnUlmBL male (B). Both female (C) and male (D) F_1 fish are blond. P, parents. Scale bars: 0.5 cm.

Figure S2. Expression of *adcy5*, *ptplb*, and *stam2* in the skin and brain.

(A) Relative normalized expression of *adcy5*, *ptplb*, and *stam2* in the skin of adult blond females. *Eef1aa* was used as reference gene. *Adcy5/1*, *adcy5* primer pair 1; *adcy5/2*, *adcy5* primer pair 2. (B) Relative expression of *adcy5* in adult wild-type female brain normalized to the expression of *adcy5* in adult wild-type female skin. *Eef1aa* was used as reference gene. These results demonstrate that *adcy5* is expressed in guppy brain, but cannot represent a precise estimate of the expression differences between brain and skin, as no optimal reference gene for this analysis could be found. We tested several potential reference genes (Olsvik et al., 2005); the expression of most genes differed greatly between brain and skin. Of these, *eef1aa* was most consistent; however, a difference of 2.7 cycles between skin (first) and brain tissue remained in the RT-qPCR.

Figure S3. Comparison of pigmentation phenotypes among different mutants.

Pictures of the lateral side below the dorsal fin of wild-type, golden (*kita* mutant), blond (*adcy5* mutant), and cream (*kita/adcy5* double mutant) females. (A’,B’,C’,D’) are details of (A,B,C,D), respectively. Cream guppies show an additive phenotype; their melanophores are punctate and located only on the dorsal half of the body. Red arrowheads in (A’), corolla melanophores of the reticulate pattern; yellow arrows in (A’), irregularly distributed corolla melanophores; white arrows in (A’), dendritic melanophores; black arrows in (C’), slightly dendritic melanophores. Scale bars: 0.5 mm.

Figure S4. Phenotypes of wild-type and blond newborns.

Dorsal aspects of the head of newborn (A,B) and female adult (C,D) guppies. (A’,B’) Lateral aspects of the area below the dorsal fin of newborn fish. Scale bars: 0.5 mm.

Table S1. Number of markers used for QTL mapping.

Number of markers found on each LG in the F_2 generation of the mapping cross.

Table S2. Melanophore counts.

Area below the dorsal fin (Kottler et al., 2013) in mm^2 and number of melanophores within the area of 10 blond and 10 wild-type (wt) females.

Table S3. Measurement of orange trait sizes of 40 blond and 40 wild-type F_2 males.

Sheet 1 contains the measurements and analyses, sheet 2 the plots of the results, and sheet 3 the results of Mann-Whitney *U* tests. The areas of the elements of the posterior and dorsal orange spot complexes and the dorsal and ventral orange margin complexes were measured from anterior to posterior. Wt, wild-type.

Table S4. Count of orange traits and number of complex elements for 74 blond and 74 wild-type F_2 males.

Analyses are at the bottom of the table. 0, trait not present; 1, trait consists of one element; etc. Wt, wild-type.

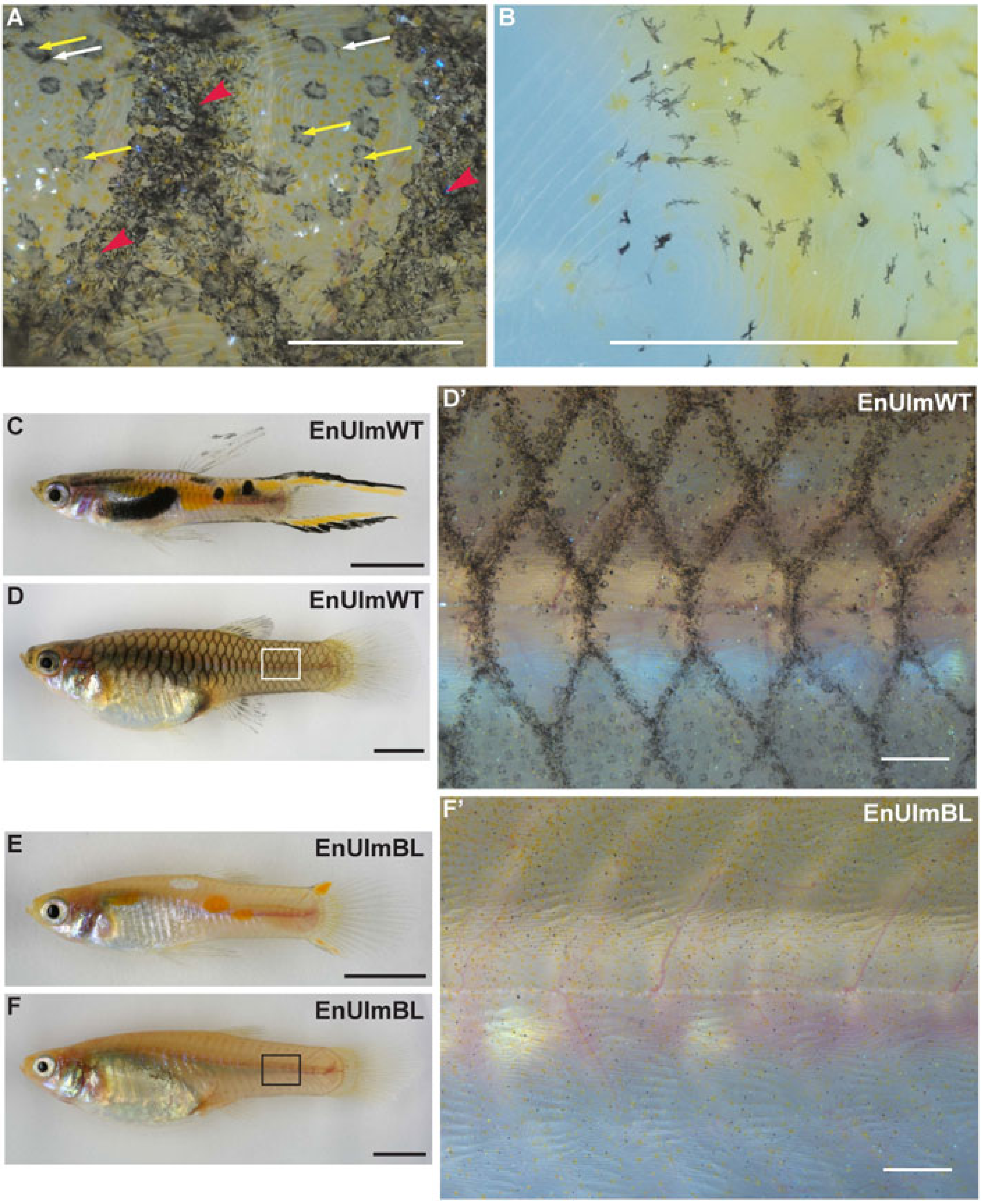
Document S1. Semi-thin sections of wild-type and blond male tail fin tissue.

The tissue was sectioned from ventral (section 1) to dorsal (section 51). Distance between semi-thin sections is approximately 2 µm. MG5, wild-type tissue; MG2, blond tissue.

Dataset S1. Pictures of wild-type and blond fish of the mapping cross.

The pictures of the grandparents (P), F_1 males, F_1 pairs, and male F_2 individuals of our mapping cross can be downloaded at: ftp://ftp.tuebingen.mpg.de/ebio/adcy5_mutant/

M, male; f, female; wtm, wild-type male; blondm, blond male.


A
B
C
EnUlmWT
D
EnUlmWT
D'
EnUlmWT
E
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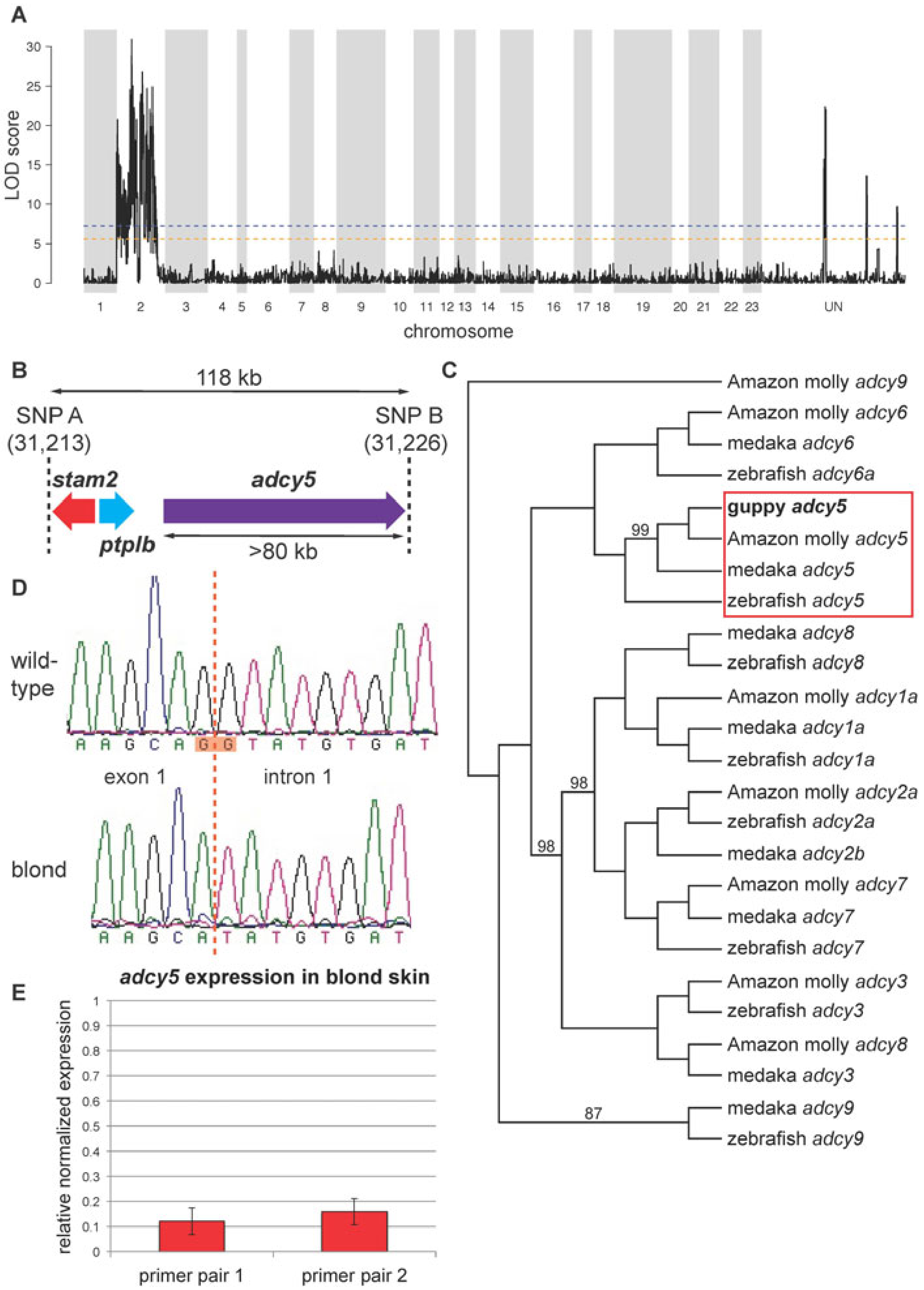
F
EnUlmBL
F'
EnUlmBL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25
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10
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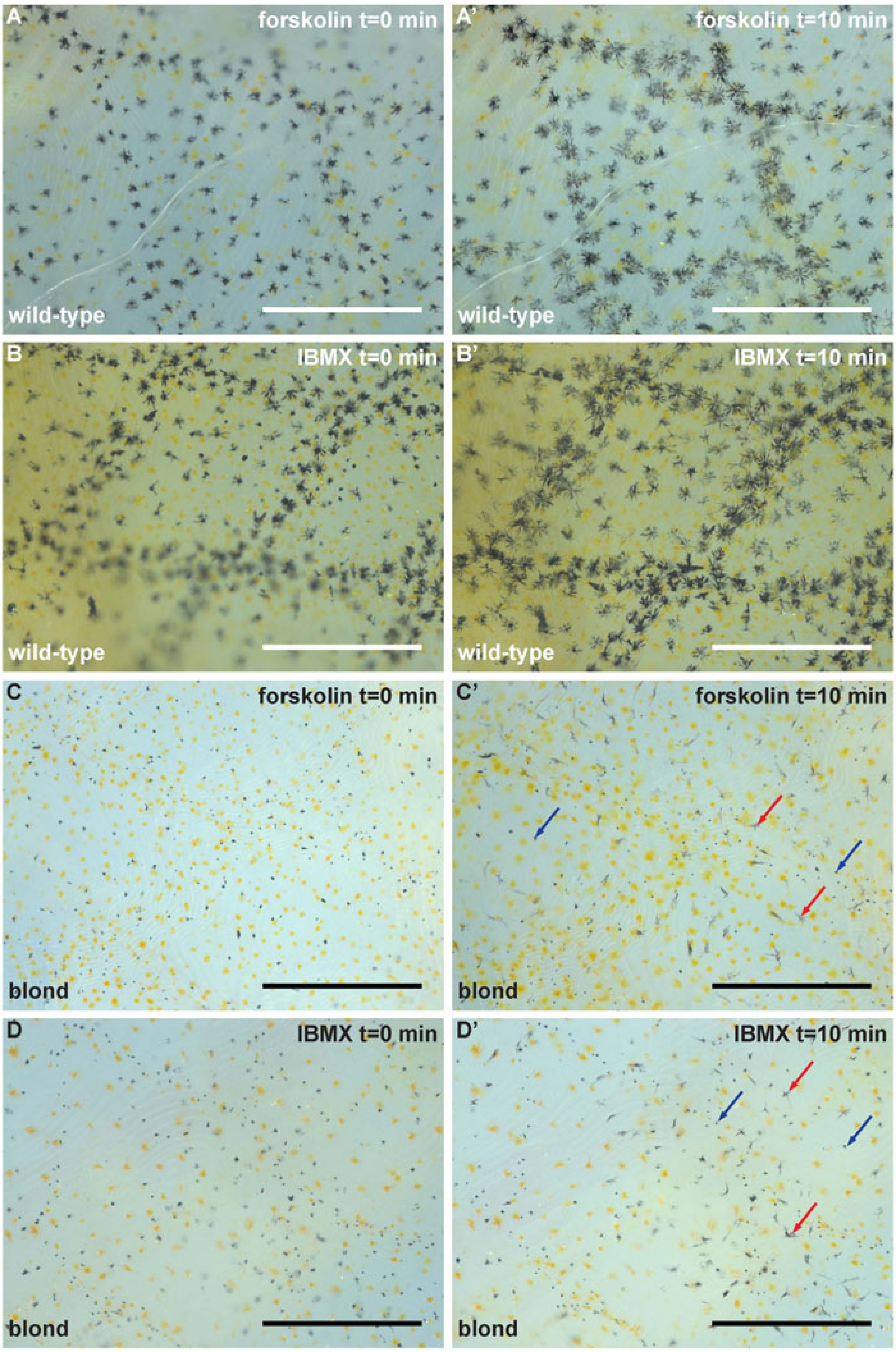
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 UN
chromosome
B
118 kb
SNP A
(31,213)
SNP B
(31,226)
stam2
ptplb
adcy5
>80 kb
C
Amazon molly adcy9
Amazon molly adcy6
medaka adcy6
zebrafish adcy6a
99
guppy adcy5
Amazon molly adcy5
medaka adcy5
zebrafish adcy5
medaka adcy8
zebrafish adcy8
Amazon molly adcy1a
medaka adcy1a
zebrafish adcy1a
98
Amazon molly adcy2a
zebrafish adcy2a
medaka adcy2b
98
Amazon molly adcy7
medaka adcy7
zebrafish adcy7
Amazon molly adcy3
zebrafish adcy3
Amazon molly adcy8
medaka adcy3
87
medaka adcy9
zebrafish adcy9
D
wild-type
A A G C A G G T A T G T G A T
exon 1
intron 1
blond
A A G C A T A T G T G A T
E
adcy5 expression in blond skin
relative normalized expression
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
primer pair 1
primer pair 2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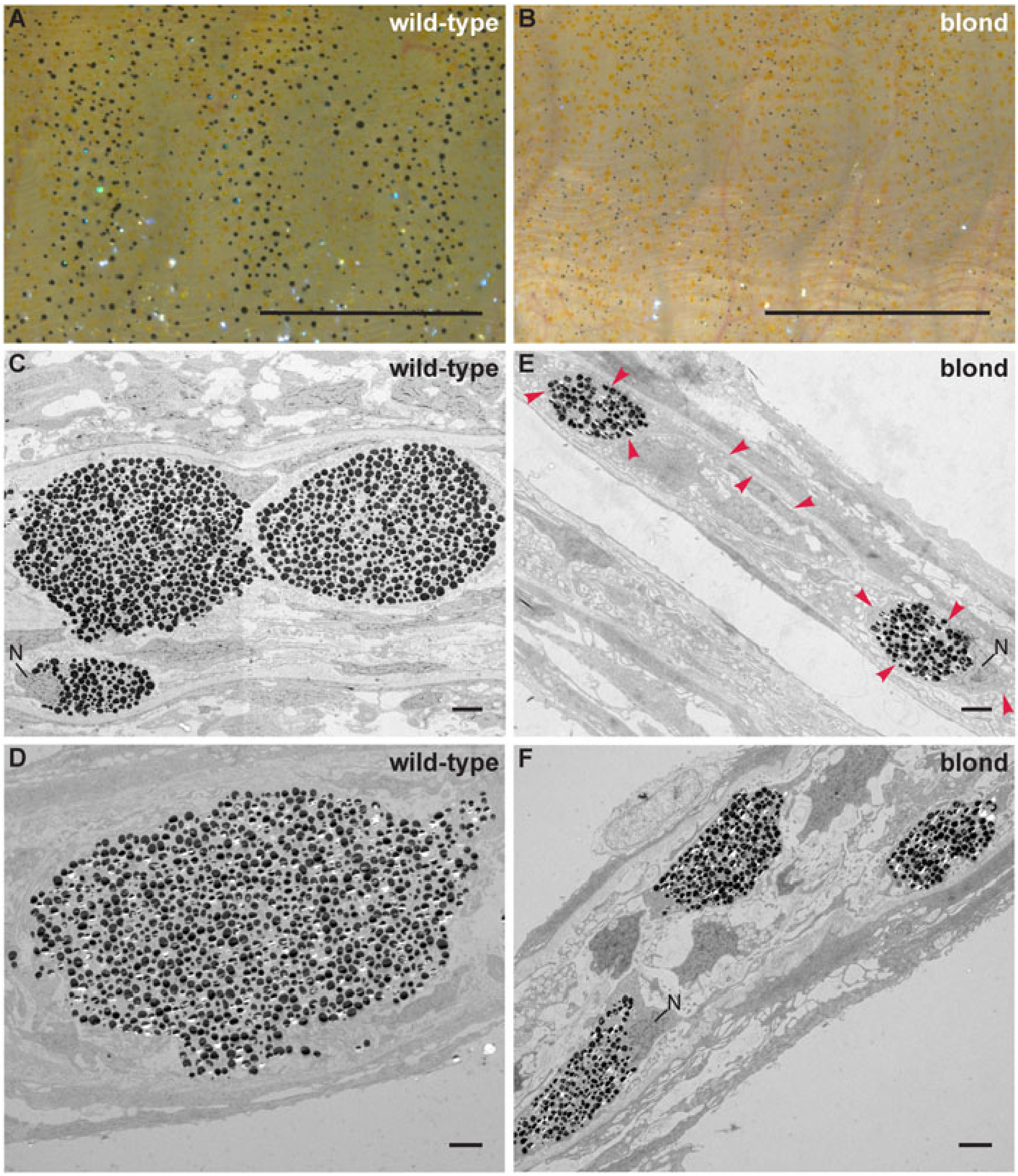
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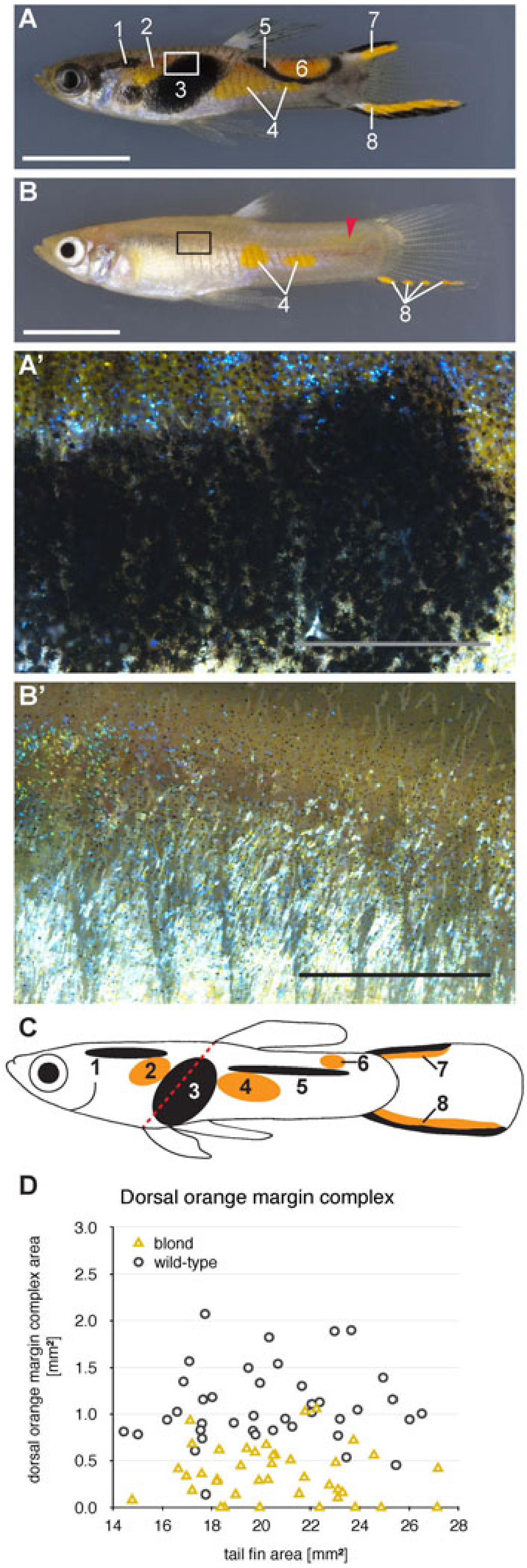
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6
7
8
D
Dorsal orange margin complex
blond
wild-type
dorsal orange margin complex area [mm²]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
14
16
18
20
22
24
26
28
tail fin area [mm²]

Figure S1

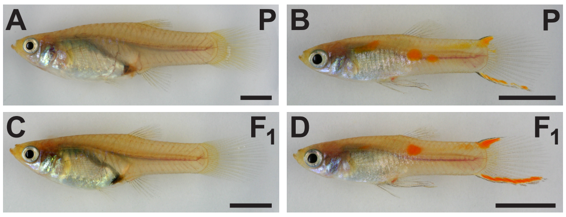


Figure S2

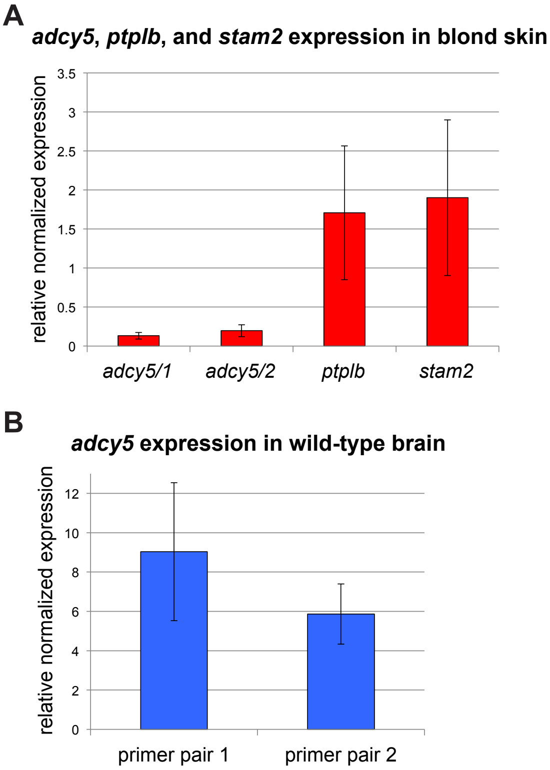


Figure S3

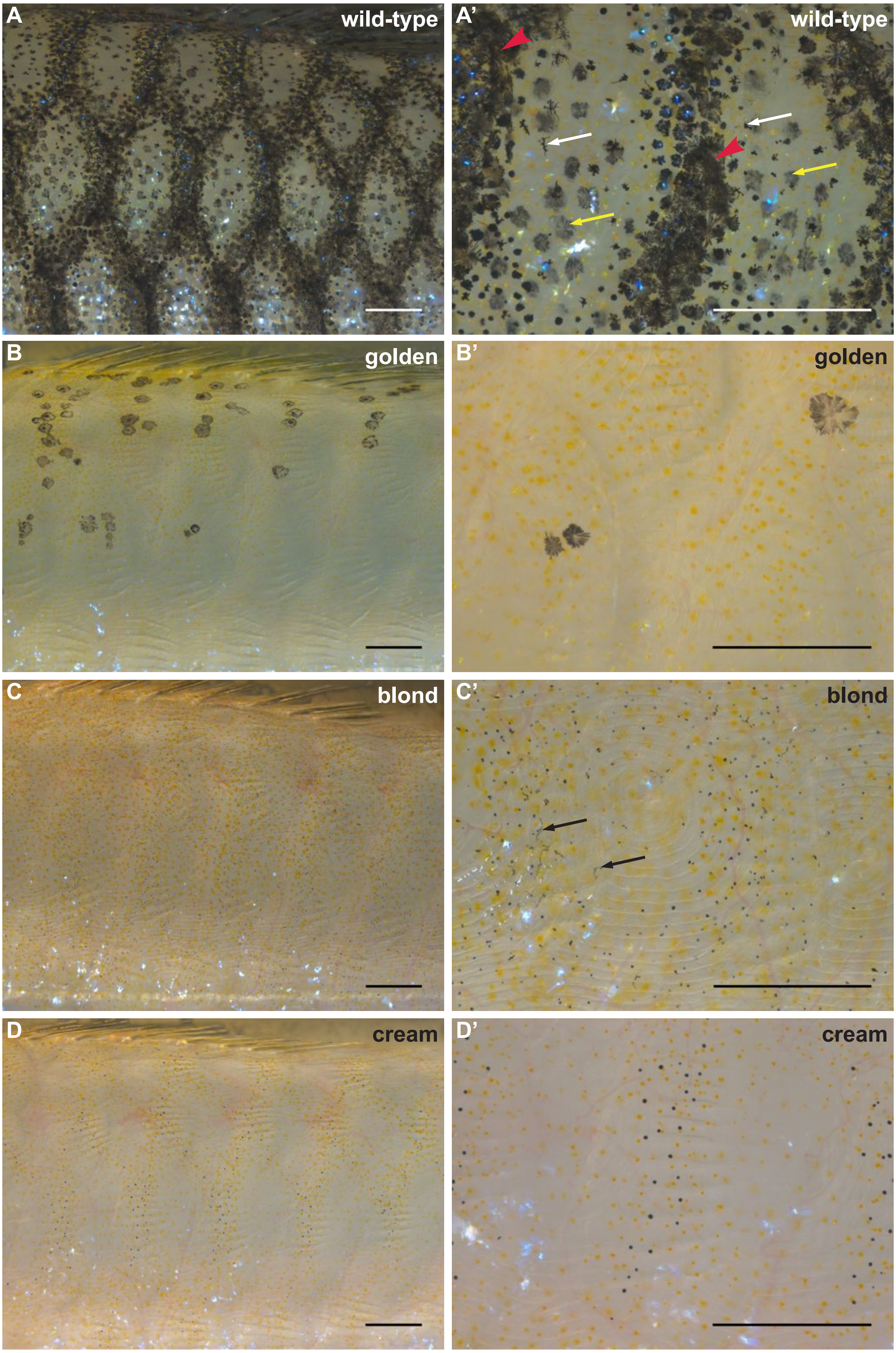

A
wild-type
A'
wild-type
B
golden
B'
golden
C
blond
C'
blond
D
cream
D'
cream

Figure S4

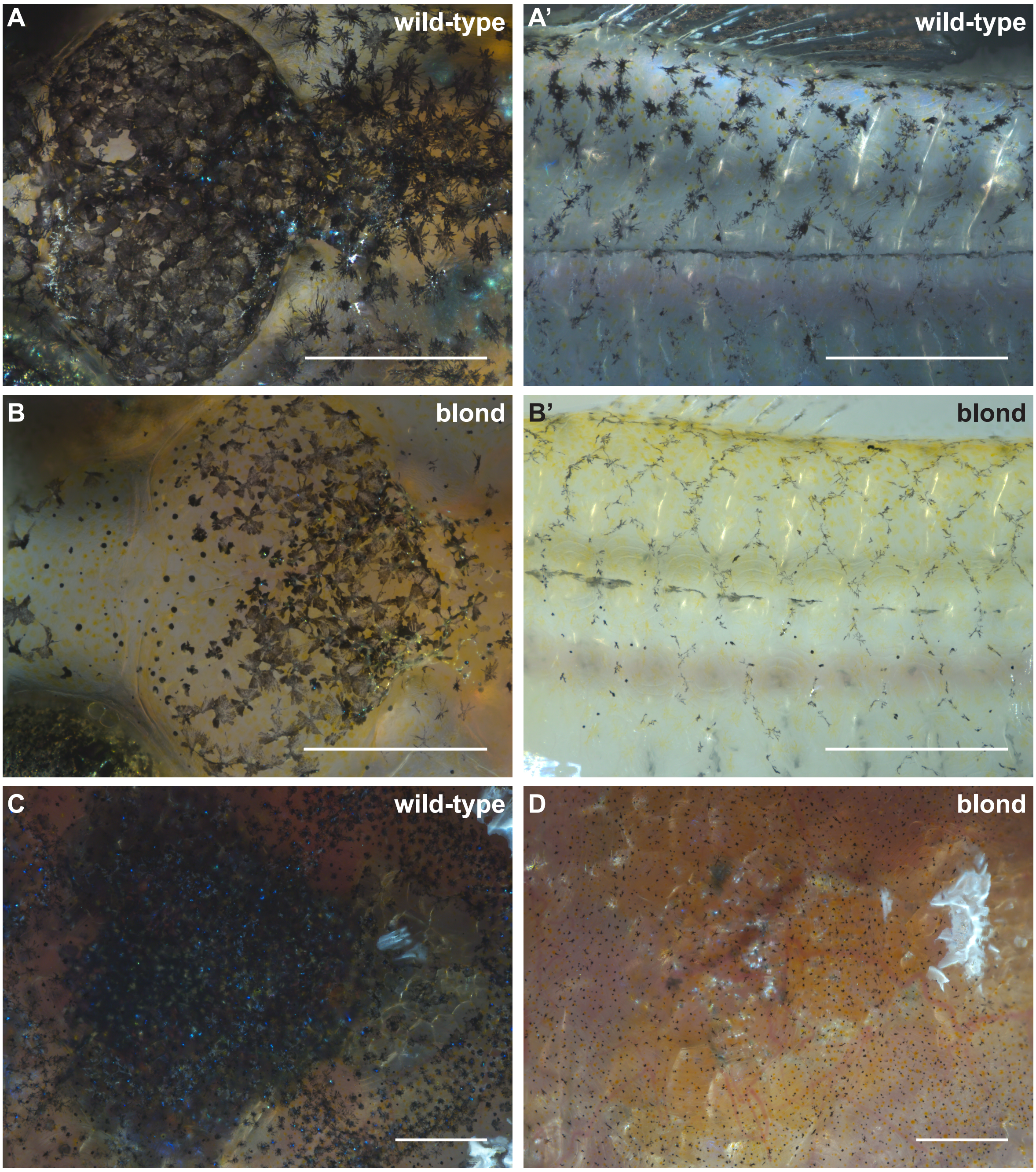


Table S1

Linkage group	Number markers
1	248
2	551
3	329
4	284
5	283
6	348
7	257
8	351
9	355
10	354
11	362
12	205
13	302
14	291
15	315
16	342
17	170
18	213
19	467
20	194
21	288
22	228
23	264
Un	393
Total	7394

Table S2

individual	**area in mm2**	**number of melanophores**	**number of melanophores/mm2**
blond female 1	4.90	1319	269.46
blond female 2	5.39	1064	197.40
blond female 3	8.73	2195	251.37
blond female 4	7.26	1987	273.80
blond female 5	7.22	1613	223.53
blond female 6	5.39	1438	266.64
blond female 7	6.77	1433	211.76
blond female 8	6.60	1431	216.75
blond female 9	7.52	1608	213.72
blond female 10	6.40	1390	217.29
wt female 1	7.89	1837	232.97
wt female 2	6.32	1324	209.39
wt female 3	9.33	2411	258.36
wt female 4	5.79	1538	265.45
wt female 5	6.40	1749	273.20
wt female 6	8.84	2126	240.58
wt female 7	6.60	1775	268.78
wt female 8	6.33	1350	213.34
wt female 9	8.81	2325	263.84
wt female 10	5.54	1479	267.11
		Average blond:	234.17
		Average wt:	249.30
		Average overall:	241.74
		Median blond:	220.41
		Median wt:	261.10
		Median overall:	245.98

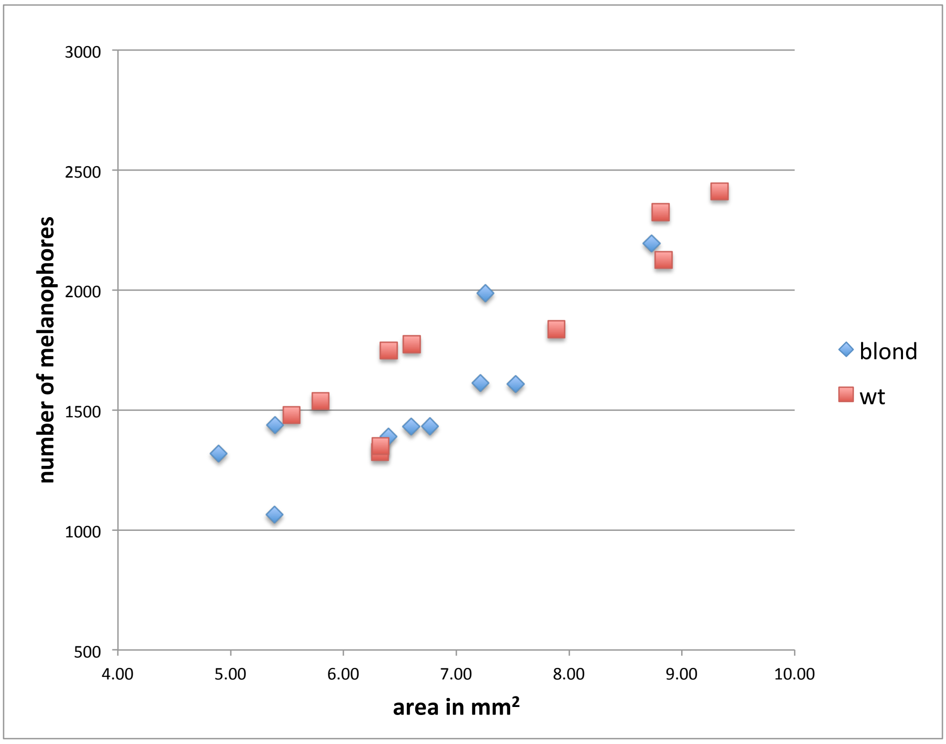
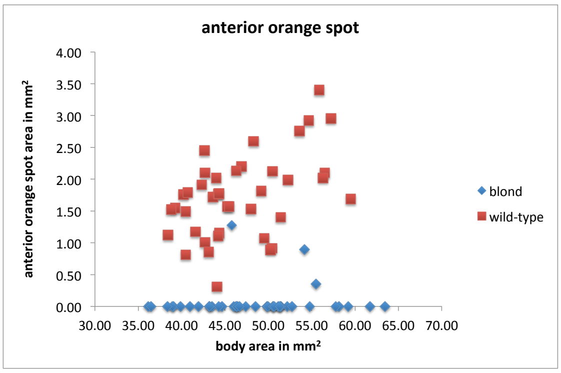
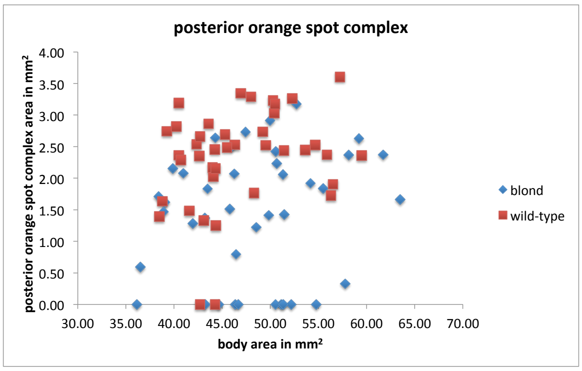


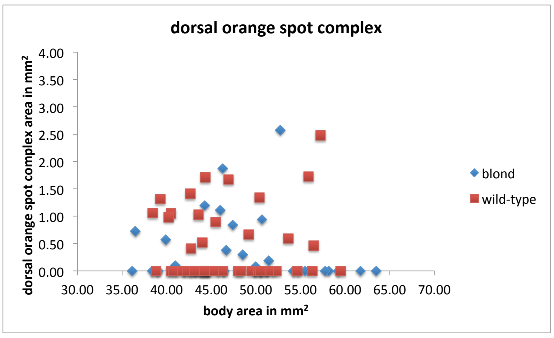
Table S3 sheet 1

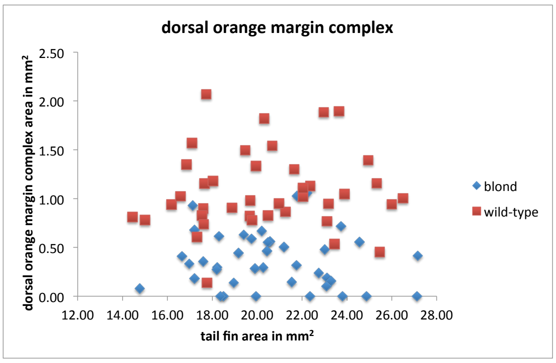
individual	body area in mm2	anterior orange spot in mm2	anterior orange spot area in relation to body area (%)	posterior orange spot element 1 in mm2	posterior orange spot element 2 in mm2	sum posterior orange spot elements in mm2 = posterior orange spot complex area	posterior orange spot complex area in relation to body area (%)	dorsal orange spot element 1 in mm2	dorsal orange spot element 2 in mm2	sum dorsal orange spot elements in mm2 = dorsal orange spot complex area	dorsal orange spot complex area in relation to body area (%)	sum orange on body in mm2	percentage orange on body	tail fin area in mm2	dorsal orange margin element 1 in mm2	dorsal orange margin element 2 in mm2	dorsal orange margin element 3 in mm2	dorsal orange margin element 4 in mm2	sum dorsal orange margin elements in mm2 = dorsal orange margin complex area	dorsal orange margin complex area in relation to tail fin area (%)	ventral orange margin element 1 in mm2	ventral orange margin element 2 in mm2	ventral orange margin element 3 in mm2	ventral orange margin element 4 in mm2	ventral orange margin element 5 in mm2	sum ventral orange margin elements in mm2 = ventral orange margin complex area	ventral orange margin complex area in relation to tail fin area (%)	sum orange on tail fin in mm2	percentage orange on tail fin
cross9_F2_frompair2_blondm_5680	39.07	0.00	0.00	1.62	0.00	1.62	4.15	0.00	0.00	0.00	0.00	1.62	4.15	17.12	0.73	0.20	0.00	0.00	0.93	5.43	0.94	0.00	0.00	0.00	0.00	0.94	5.50	1.87	10.93
cross9_F2_frompair2_blondm_6070	58.15	0.00	0.00	2.37	0.00	2.37	4.07	0.00	0.00	0.00	0.00	2.37	4.07	27.12	0.00	0.00	0.00	0.00	0.00	0.00	1.16	0.00	0.00	0.00	0.00	1.16	4.27	1.16	4.27
cross9_F2_frompair2_blondm_6259	49.83	0.00	0.00	1.41	0.00	1.41	2.83	0.00	0.00	0.00	0.00	1.41	2.83	21.78	0.86	0.17	0.00	0.00	1.03	4.71	0.67	0.10	0.00	0.00	0.00	0.77	3.54	1.80	8.25
cross9_F2_frompair2_blondm_6261	46.01	0.00	0.00	2.50	0.00	2.50	5.44	0.84	0.27	1.11	2.41	3.61	7.85	20.57	0.50	0.06	0.00	0.00	0.56	2.72	0.33	0.09	0.11	0.00	0.00	0.53	2.59	1.09	5.31
cross9_F2_frompair2_blondm_6523	40.97	0.00	0.00	2.08	0.00	2.08	5.08	0.10	0.00	0.10	0.24	2.18	5.32	17.20	0.18	0.00	0.00	0.00	0.18	1.05	0.53	0.05	0.04	0.00	0.00	0.62	3.59	0.80	4.64
cross9_F2_frompair2_blondm_6525	51.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	22.75	0.24	0.00	0.00	0.00	0.24	1.04	1.04	0.13	0.00	0.00	0.00	1.16	5.10	1.40	6.14
cross9_F2_frompair2_blondm_7489	43.47	0.00	0.00	1.83	0.00	1.83	4.22	0.00	0.00	0.00	0.00	1.83	4.22	18.22	0.30	0.00	0.00	0.00	0.30	1.63	0.59	0.00	0.00	0.00	0.00	0.59	3.23	0.89	4.86
cross9_F2_frompair2_blondm_7562	41.96	0.00	0.00	1.28	0.00	1.28	3.05	0.00	0.00	0.00	0.00	1.28	3.05	20.27	0.27	0.03	0.00	0.00	0.29	1.45	0.74	0.00	0.00	0.00	0.00	0.74	3.66	1.04	5.11
cross9_F2_frompair2_blondm_7583	36.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.77	0.08	0.00	0.00	0.00	0.08	0.53	0.73	0.13	0.00	0.00	0.00	0.86	5.80	0.94	6.34
cross9_F2_frompair2_blondm_7585	38.91	0.00	0.00	1.47	0.00	1.47	3.77	0.00	0.00	0.00	0.00	1.47	3.77	17.59	0.36	0.00	0.00	0.00	0.36	2.03	0.61	0.11	0.00	0.00	0.00	0.72	4.07	1.07	6.10
cross9_F2_frompair2_blondm_7739	46.44	0.00	0.00	0.79	0.00	0.79	1.71	0.00	0.00	0.00	0.00	0.79	1.71	18.96	0.14	0.00	0.00	0.00	0.14	0.73	0.82	0.05	0.00	0.00	0.00	0.86	4.54	1.00	5.27
cross9_F2_frompair2_blondm_7741	46.27	0.00	0.00	2.07	0.00	2.07	4.48	1.87	0.00	1.87	4.05	3.95	8.53	18.50	0.00	0.00	0.00	0.00	0.00	0.00	0.84	0.24	0.00	0.00	0.00	1.08	5.83	1.08	5.83
cross9_F2_frompair2_blondm_8012	54.76	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	23.81	0.00	0.00	0.00	0.00	0.00	0.00	0.34	0.23	0.15	0.00	0.00	0.72	3.00	0.72	3.00
cross9_F2_frompair2_blondm_8013	61.72	0.00	0.00	2.37	0.00	2.37	3.84	0.00	0.00	0.00	0.00	2.37	3.84	23.74	0.72	0.00	0.00	0.00	0.72	3.02	0.71	0.24	0.00	0.00	0.00	0.96	4.03	1.67	7.05
cross9_F2_frompair2_blondm_8014	45.79	1.27	2.78	1.52	0.00	1.52	3.31	0.00	0.00	0.00	0.00	2.79	6.09	18.20	0.28	0.00	0.00	0.00	0.28	1.51	0.64	0.18	0.00	0.00	0.00	0.82	4.50	1.09	6.01
cross9_F2_frompair2_blondm_left_5676	59.21	0.00	0.00	1.53	1.10	2.63	4.44	0.00	0.00	0.00	0.00	2.63	4.44	24.87	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.08	0.06	0.08	0.04	0.48	1.93	0.48	1.93
cross9_F2_frompair3_blondm_5668	43.18	0.00	0.00	1.37	0.00	1.37	3.17	0.00	0.00	0.00	0.00	1.37	3.17	19.75	0.59	0.00	0.00	0.00	0.59	2.98	1.02	0.00	0.00	0.00	0.00	1.02	5.19	1.61	8.16
cross9_F2_frompair3_blondm_7144	63.48	0.00	0.00	0.94	0.72	1.66	2.62	0.00	0.00	0.00	0.00	1.66	2.62	22.35	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.07	0.00	0.00	0.00	0.12	0.51	0.12	0.51
cross9_F2_frompair3_blondm_7146	54.17	0.89	1.65	1.92	0.00	1.92	3.54	0.00	0.00	0.00	0.00	2.81	5.19	27.16	0.42	0.00	0.00	0.00	0.42	1.53	0.97	0.00	0.00	0.00	0.00	0.97	3.58	1.39	5.11
cross9_F2_frompair3_blondm_7625	49.95	0.00	0.00	2.91	0.00	2.91	5.83	0.08	0.00	0.08	0.16	2.99	5.99	19.90	0.29	0.00	0.00	0.00	0.29	1.43	0.11	0.00	0.00	0.00	0.00	0.11	0.56	0.40	1.99
cross9_F2_frompair5_blondm_6426	39.88	0.00	0.00	2.15	0.00	2.15	5.40	0.57	0.00	0.57	1.43	2.73	6.83	17.20	0.68	0.00	0.00	0.00	0.68	3.94	0.71	0.00	0.00	0.00	0.00	0.71	4.10	1.38	8.04
cross9_F2_frompair5_blondm_6432	44.65	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.48	0.45	0.11	0.00	0.00	0.55	2.70	0.90	0.21	0.00	0.00	0.00	1.11	5.41	1.66	8.11
cross9_F2_frompair5_blondm_6755	50.56	0.00	0.00	2.42	0.00	2.42	4.79	0.00	0.00	0.00	0.00	2.42	4.79	21.75	0.32	0.00	0.00	0.00	0.32	1.46	0.85	0.00	0.00	0.00	0.00	0.85	3.91	1.17	5.37
cross9_F2_frompair5_blondm_8017	57.77	0.00	0.00	0.33	0.00	0.33	0.56	0.00	0.00	0.00	0.00	0.33	0.56	23.11	0.19	0.00	0.00	0.00	0.19	0.82	0.28	0.22	0.35	0.24	0.00	1.09	4.72	1.28	5.54
cross9_F2_frompair5_blondm_8018	52.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.21	0.67	0.00	0.00	0.00	0.67	3.31	0.65	0.47	0.00	0.00	0.00	1.12	5.55	1.79	8.86
cross9_F2_frompair6_blondm_6278	44.27	0.00	0.00	2.64	0.00	2.64	5.96	1.20	0.00	1.20	2.70	3.84	8.66	19.41	0.46	0.03	0.14	0.00	0.63	3.24	1.35	0.00	0.00	0.00	0.00	1.35	6.97	1.98	10.21
cross9_F2_frompair6_blondm_6279	51.44	0.00	0.00	1.42	0.00	1.42	2.77	0.18	0.00	0.18	0.36	1.61	3.12	22.22	0.72	0.04	0.16	0.15	1.06	4.75	0.92	0.21	0.00	0.00	0.00	1.13	5.06	2.18	9.81
cross9_F2_frompair6_blondm_6291	52.72	0.00	0.00	3.17	0.00	3.17	6.02	2.58	0.00	2.58	4.89	5.75	10.91	24.57	0.56	0.00	0.00	0.00	0.56	2.27	0.71	0.16	0.04	0.00	0.00	0.90	3.67	1.46	5.94
cross9_F2_frompair6_blondm_6392	50.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	19.94	0.00	0.00	0.00	0.00	0.00	0.00	0.52	0.15	0.00	0.00	0.00	0.67	3.35	0.67	3.35
cross9_F2_frompair6_blondm_6394	43.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	18.30	0.53	0.09	0.00	0.00	0.61	3.36	0.78	0.09	0.00	0.00	0.00	0.87	4.75	1.48	8.11
cross9_F2_frompair6_blondm_6395	51.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	21.19	0.51	0.00	0.00	0.00	0.51	2.39	0.65	0.10	0.00	0.00	0.00	0.75	3.54	1.26	5.93
cross9_F2_frompair6_blondm_6408	38.39	0.00	0.00	1.71	0.00	1.71	4.45	0.00	0.00	0.00	0.00	1.71	4.45	16.64	0.41	0.00	0.00	0.00	0.41	2.46	0.61	0.15	0.00	0.00	0.00	0.76	4.55	1.17	7.01
cross9_F2_frompair6_blondm_6409	46.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	19.17	0.44	0.00	0.00	0.00	0.44	2.31	0.33	0.11	0.00	0.00	0.00	0.44	2.29	0.88	4.60
cross9_F2_frompair6_blondm_6818	50.69	0.00	0.00	1.29	0.95	2.23	4.41	0.94	0.00	0.94	1.86	3.18	6.26	23.01	0.40	0.08	0.00	0.00	0.48	2.07	0.90	0.10	0.00	0.00	0.00	0.99	4.32	1.47	6.39
cross9_F2_frompair7_blondm_6360	47.40	0.00	0.00	2.73	0.00	2.73	5.75	0.37	0.47	0.84	1.77	3.57	7.52	20.43	0.46	0.00	0.00	0.00	0.46	2.27	1.31	0.00	0.00	0.00	0.00	1.31	6.43	1.78	8.70
cross9_F2_frompair7_blondm_6375	36.49	0.00	0.00	0.59	0.00	0.59	1.63	0.72	0.00	0.72	1.98	1.32	3.61	18.36	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.19	0.21	0.00	0.00	0.57	3.12	0.57	3.12
cross9_F2_frompair7_blondm_6449	48.53	0.00	0.00	1.22	0.00	1.22	2.52	0.30	0.00	0.30	0.61	1.52	3.13	21.54	0.15	0.00	0.00	0.00	0.15	0.67	0.25	0.12	0.08	0.00	0.00	0.45	2.08	0.59	2.75
cross9_F2_frompair7_blondm_6451	51.31	0.00	0.00	2.06	0.00	2.06	4.02	0.00	0.00	0.00	0.00	2.06	4.02	23.29	0.16	0.00	0.00	0.00	0.16	0.67	0.06	0.12	0.15	0.04	0.00	0.36	1.54	0.52	2.21
cross9_F2_frompair7_blondm_6491	46.68	0.00	0.00	0.00	0.00	0.00	0.00	0.38	0.00	0.38	0.81	0.38	0.81	16.97	0.33	0.00	0.00	0.00	0.33	1.95	0.04	0.06	0.07	0.07	0.00	0.24	1.41	0.57	3.37
cross9_F2_frompair7_blondm_6492	55.51	0.35	0.63	1.84	0.00	1.84	3.31	0.00	0.00	0.00	0.00	2.19	3.94	23.09	0.10	0.00	0.00	0.00	0.10	0.45	0.29	0.20	0.13	0.01	0.00	0.62	2.70	0.73	3.15
sum			5.06				117.14				23.27		145.47							72.89							154.50		227.39
sum/40			**0.13**				**2.93**				**0.58**		**3.64**							**1.82**							**3.86**		**5.68**
individual	**body area in mm2**	**anterior orange spot in mm2**	**anterior orange spot area in relation to body area (%)**	**posterior orange spot element 1 in mm2**	**posterior orange spot element 2 in mm2**	**sum posterior orange spot elements in mm2 = posterior orange spot complex area**	**posterior orange spot complex area in relation to body area (%)**	**dorsal orange spot element 1 in mm2**	**dorsal orange spot element 2 in mm2**	**sum dorsal orange spot elements in mm2 = dorsal orange spot complex area**	**dorsal orange spot complex area in relation to body area (%)**	**sum orange on body in mm2**	**percentage orange on body**	**tail fin area in mm2**	**dorsal orange margin element 1 in mm2**	**dorsal orange margin element 2 in mm2**	**dorsal orange margin element 3 in mm2**	**dorsal orange margin element 4 in mm2**	**sum dorsal orange margin elements in mm2 = dorsal orange margin complex area**	**dorsal orange margin complex area in relation to tail fin area (%)**	**ventral orange margin element 1 in mm2**	**ventral orange margin element 2 in mm2**	**ventral orange margin element 3 in mm2**	**ventral orange margin element 4 in mm2**	**ventral orange margin element 5 in mm2**	**sum ventral orange margin elements in mm2 = ventral orange margin complex area**	**ventral orange margin complex area in relation to tail fin area (%)**	**sum orange on tail fin in mm2**	**percentage orange on tail fin**
cross9_F2_frompair2_wtm_5683	40.49	0.81	2.01	1.89	1.30	3.19	7.88	1.05	0.00	1.05	2.60	5.06	12.48	19.66	0.82	0.00	0.00	0.00	0.82	4.17	1.03	0.00	0.00	0.00	0.00	1.03	5.25	1.85	9.42
cross9_F2_frompair2_wtm_5684	39.26	1.55	3.94	2.75	0.00	2.75	6.99	0.85	0.47	1.32	3.35	5.61	14.28	14.44	0.81	0.00	0.00	0.00	0.81	5.62	1.06	0.00	0.00	0.00	0.00	1.06	7.31	1.87	12.93
cross9_F2_frompair2_wtm_5685	40.49	1.49	3.68	1.62	0.74	2.36	5.84	0.00	0.00	0.00	0.00	3.85	9.52	18.88	0.91	0.00	0.00	0.00	0.91	4.79	1.09	0.11	0.00	0.00	0.00	1.20	6.37	2.11	11.16
cross9_F2_frompair2_wtm_5686	43.58	1.72	3.94	2.86	0.00	2.86	6.57	1.03	0.00	1.03	2.35	5.61	12.86	21.26	0.87	0.00	0.00	0.00	0.87	4.07	1.14	0.00	0.00	0.00	0.00	1.14	5.34	2.00	9.41
cross9_F2_frompair2_wtm_6071	49.53	1.07	2.16	2.52	0.00	2.52	5.09	0.00	0.00	0.00	0.00	3.59	7.25	22.96	1.89	0.00	0.00	0.00	1.89	8.21	0.84	0.19	0.00	0.00	0.00	1.02	4.45	2.91	12.66
cross9_F2_frompair2_wtm_6260	50.41	0.92	1.82	2.72	0.31	3.03	6.01	0.99	0.35	1.34	2.66	5.29	10.49	23.11	0.77	0.00	0.00	0.00	0.77	3.32	0.74	0.11	0.00	0.00	0.00	0.85	3.69	1.62	7.02
cross9_F2_frompair2_wtm_6521	52.27	1.99	3.80	3.26	0.00	3.26	6.24	0.00	0.00	0.00	0.00	5.25	10.04	23.90	1.05	0.00	0.00	0.00	1.05	4.38	1.19	0.00	0.00	0.00	0.00	1.19	4.99	2.24	9.37
cross9_F2_frompair2_wtm_6522	54.66	2.92	5.34	2.53	0.00	2.53	4.63	0.00	0.00	0.00	0.00	5.45	9.97	20.97	0.95	0.00	0.00	0.00	0.95	4.52	1.49	0.00	0.00	0.00	0.00	1.49	7.10	2.44	11.62
cross9_F2_frompair2_wtm_6524	59.49	1.69	2.84	2.36	0.00	2.36	3.97	0.00	0.00	0.00	0.00	4.05	6.81	26.50	1.00	0.00	0.00	0.00	1.00	3.79	1.09	0.00	0.00	0.00	0.00	1.09	4.11	2.09	7.90
cross9_F2_frompair2_wtm_6526	56.50	2.10	3.72	1.91	0.00	1.91	3.37	0.46	0.00	0.46	0.82	4.47	7.91	23.45	0.54	0.00	0.00	0.00	0.54	2.28	1.04	0.00	0.00	0.00	0.00	1.04	4.43	1.57	6.71
cross9_F2_frompair2_wtm_6806	44.33	1.16	2.61	1.25	0.00	1.25	2.82	0.00	0.00	0.00	0.00	2.41	5.43	17.60	0.90	0.00	0.00	0.00	0.90	5.10	0.76	0.00	0.00	0.00	0.00	0.76	4.32	1.66	9.43
cross9_F2_frompair2_wtm_6807	45.29	1.57	3.46	2.69	0.00	2.69	5.94	0.00	0.00	0.00	0.00	4.26	9.41	22.04	1.02	0.00	0.00	0.00	1.02	4.63	1.07	0.00	0.00	0.00	0.00	1.07	4.83	2.09	9.46
cross9_F2_frompair2_wtm_6809	38.79	1.52	3.93	1.63	0.00	1.63	4.21	0.00	0.00	0.00	0.00	3.16	8.14	15.00	0.78	0.00	0.00	0.00	0.78	5.21	0.06	0.41	0.00	0.00	0.00	0.47	3.15	1.25	8.36
cross9_F2_frompair2_wtm_6838	46.92	2.20	4.68	3.35	0.00	3.35	7.13	1.22	0.46	1.67	3.56	7.21	15.37	21.65	1.30	0.00	0.00	0.00	1.30	6.01	1.28	0.00	0.00	0.00	0.00	1.28	5.91	2.58	11.92
cross9_F2_frompair2_wtm_6839	48.00	1.53	3.19	1.67	1.62	3.29	6.85	0.00	0.00	0.00	0.00	4.82	10.04	22.36	1.13	0.00	0.00	0.00	1.13	5.04	0.60	0.00	0.00	0.00	0.00	0.60	2.69	1.73	7.73
cross9_F2_frompair2_wtm_6840	56.30	2.02	3.59	1.72	0.00	1.72	3.06	0.00	0.00	0.00	0.00	3.74	6.65	25.46	0.45	0.00	0.00	0.00	0.45	1.78	0.52	0.20	0.00	0.00	0.00	0.72	2.82	1.17	4.60
cross9_F2_frompair3_wtm_6366	50.26	0.89	1.76	3.23	0.00	3.23	6.43	0.00	0.00	0.00	0.00	4.12	8.19	19.47	1.50	0.00	0.00	0.00	1.50	7.68	1.10	0.00	0.00	0.00	0.00	1.10	5.63	2.59	13.32
cross9_F2_frompair3_wtm_6410	48.30	2.60	5.37	1.77	0.00	1.77	3.66	0.00	0.00	0.00	0.00	4.36	9.03	16.18	0.94	0.00	0.00	0.00	0.94	5.80	0.91	0.00	0.00	0.00	0.00	0.91	5.59	1.84	11.39
cross9_F2_frompair3_wtm_6411	41.59	1.18	2.83	0.93	0.55	1.48	3.56	0.00	0.00	0.00	0.00	2.66	6.39	17.54	0.83	0.00	0.00	0.00	0.83	4.71	0.03	0.00	0.00	0.00	0.00	0.03	0.15	0.85	4.86
cross9_F2_frompair3_wtm_6412	44.08	0.31	0.70	2.03	0.00	2.03	4.60	0.00	0.00	0.00	0.00	2.34	5.30	16.85	1.27	0.08	0.00	0.00	1.35	8.01	0.92	0.00	0.00	0.00	0.00	0.92	5.47	2.27	13.48
cross9_F2_frompair5_wtm_6292	51.42	1.40	2.73	2.44	0.00	2.44	4.75	0.00	0.00	0.00	0.00	3.85	7.48	24.94	1.39	0.00	0.00	0.00	1.39	5.58	1.25	0.21	0.00	0.00	0.00	1.45	5.83	2.84	11.40
cross9_F2_frompair5_wtm_6293	40.23	1.76	4.37	2.82	0.00	2.82	7.01	0.98	0.00	0.98	2.45	5.56	13.82	17.09	1.57	0.00	0.00	0.00	1.57	9.17	1.48	0.00	0.00	0.00	0.00	1.48	8.67	3.05	17.84
cross9_F2_frompair5_wtm_6294	50.48	2.12	4.21	2.08	1.10	3.18	6.30	0.00	0.00	0.00	0.00	5.30	10.50	20.48	0.83	0.00	0.00	0.00	0.83	4.03	1.45	0.00	0.00	0.00	0.00	1.45	7.10	2.28	11.13
cross9_F2_frompair5_wtm_6295	40.70	1.79	4.41	1.27	1.02	2.29	5.62	0.00	0.00	0.00	0.00	4.08	10.03	16.59	1.02	0.00	0.00	0.00	1.02	6.17	1.06	0.00	0.00	0.00	0.00	1.06	6.38	2.08	12.55
cross9_F2_frompair5_wtm_6296	44.25	1.77	4.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.77	4.00	17.76	0.06	0.08	0.00	0.00	0.14	0.78	0.04	0.09	0.17	0.11	0.00	0.40	2.25	0.54	3.04
cross9_F2_frompair6_wtm_6276	44.22	1.11	2.50	1.58	0.88	2.46	5.55	0.00	0.00	0.00	0.00	3.56	8.06	17.65	1.16	0.00	0.00	0.00	1.16	6.55	1.00	0.00	0.00	0.00	0.00	1.00	5.68	2.16	12.23
cross9_F2_frompair6_wtm_6277	42.69	1.01	2.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.01	2.36	19.76	0.78	0.00	0.00	0.00	0.78	3.94	0.65	0.00	0.00	0.00	0.00	0.65	3.29	1.43	7.23
cross9_F2_frompair6_wtm_6280	46.30	2.13	4.60	2.53	0.00	2.53	5.46	0.00	0.00	0.00	0.00	4.66	10.06	19.94	1.33	0.00	0.00	0.00	1.33	6.69	1.15	0.00	0.00	0.00	0.00	1.15	5.75	2.48	12.44
cross9_F2_frompair6_wtm_6281	42.63	2.45	5.75	2.35	0.00	2.35	5.52	1.17	0.24	1.41	3.31	6.21	14.57	20.32	1.55	0.27	0.00	0.00	1.82	8.95	1.01	0.08	0.00	0.00	0.00	1.08	5.33	2.90	14.28
cross9_F2_frompair6_wtm_6282	45.49	1.57	3.44	2.49	0.00	2.49	5.47	0.89	0.00	0.89	1.96	4.95	10.88	20.68	1.54	0.00	0.00	0.00	1.54	7.45	1.27	0.31	0.00	0.00	0.00	1.58	7.63	3.12	15.07
cross9_F2_frompair6_wtm_6289	55.88	3.40	6.08	2.37	0.00	2.37	4.24	1.72	0.00	1.72	3.08	7.49	13.40	23.64	1.90	0.00	0.00	0.00	1.90	8.01	2.12	0.10	0.00	0.00	0.00	2.22	9.39	4.12	17.40
cross9_F2_frompair6_wtm_6393	44.00	2.02	4.58	2.17	0.00	2.17	4.93	0.52	0.00	0.52	1.18	4.71	10.69	18.02	1.18	0.00	0.00	0.00	1.18	6.55	0.79	0.10	0.00	0.00	0.00	0.90	4.97	2.08	11.52
cross9_F2_frompair6_wtm_6404	42.33	1.91	4.52	1.74	0.80	2.54	6.00	0.00	0.00	0.00	0.00	4.45	10.52	17.73	2.07	0.00	0.00	0.00	2.07	11.68	1.41	0.00	0.00	0.00	0.00	1.41	7.98	3.48	19.65
cross9_F2_frompair6_wtm_6405	42.71	2.10	4.92	2.67	0.00	2.67	6.24	0.41	0.00	0.41	0.96	5.18	12.12	22.05	1.11	0.00	0.00	0.00	1.11	5.02	1.13	0.03	0.00	0.00	0.00	1.16	5.27	2.27	10.29
cross9_F2_frompair7_wtm_6361	43.11	0.85	1.98	1.00	0.33	1.33	3.08	0.00	0.00	0.00	0.00	2.18	5.05	17.32	0.61	0.00	0.00	0.00	0.61	3.50	0.82	0.00	0.00	0.00	0.00	0.82	4.76	1.43	8.25
cross9_F2_frompair7_wtm_6362	49.20	1.82	3.69	0.05	2.69	2.74	5.56	0.67	0.00	0.67	1.36	5.22	10.61	19.69	0.98	0.00	0.00	0.00	0.98	4.98	1.09	0.00	0.00	0.00	0.00	1.09	5.54	2.07	10.53
cross9_F2_frompair7_wtm_6363	53.61	2.76	5.15	2.45	0.00	2.45	4.57	0.59	0.00	0.59	1.10	5.80	10.82	25.33	1.16	0.00	0.00	0.00	1.16	4.57	0.46	0.00	0.00	0.00	0.00	0.46	1.81	1.62	6.38
cross9_F2_frompair7_wtm_6364	57.24	2.96	5.16	3.61	0.00	3.61	6.30	0.88	1.60	2.48	4.33	9.04	15.80	26.00	0.67	0.27	0.00	0.00	0.94	3.62	0.95	0.20	0.00	0.00	0.00	1.15	4.41	2.09	8.03
cross9_F2_frompair7_wtm_6365	44.31	1.77	4.00	2.16	0.00	2.16	4.86	1.32	0.40	1.71	3.86	5.64	12.73	23.18	0.95	0.00	0.00	0.00	0.95	4.09	0.78	0.02	0.00	0.00	0.00	0.79	3.42	1.74	7.51
cross9_F2_frompair7_wtm_6373	38.45	1.12	2.92	1.40	0.00	1.40	3.63	1.06	0.00	1.06	2.76	3.58	9.30	17.61	0.74	0.00	0.00	0.00	0.74	4.18	1.47	0.08	0.00	0.00	0.00	1.55	8.79	2.29	12.97
sum			146.73				199.94				41.70		388.37							214.63							207.85		422.48
sum/40			**3.67**				**5.00**				**1.04**		**9.71**							**5.37**							**5.20**		**10.56**

Table S3 sheet 2









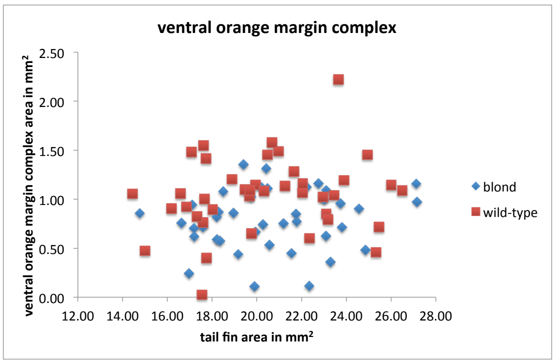


Table S3 sheet 3

Anterior orange spot in relation to body area:

Mann-Whitney U test all values:
$n_1=n_2=40$, $U=1589$, the difference between the two samples is highly significant ($P<0.001$, two-tailed test)

Mann-Whitney U test only values>0:
$n_1=40$, $n_2=3$, $U=109$, the difference between the two samples is marginally significant ($P<0.05$, two-tailed test)

Posterior orange spot complex area in relation to body area:

Mann-Whitney U test all values:
$n_1=n_2=40$, $U=1266$, the difference between the two samples is highly significant ($P<0.001$, two-tailed test)

Mann-Whitney U test only values>0:
$n_1=38$, $n_2=30$, $U=876$, the difference between the two samples is highly significant ($P<0.001$, two-tailed test)

Dorsal orange spot complex area in relation to body area:

Mann-Whitney U test all values:
$n_1=n_2=40$, $U=919.5$, the two samples are not significantly different ($P=0.2$, two-tailed test)

Mann-Whitney U test only values>0:
$n_1=17$, $n_2=13$, $U=150$, the two samples are not significantly different ($P=0.1$, two-tailed test)

Dorsal orange margin complex area in relation to tail fin area:

Mann-Whitney U test all values:
$n_1=n_2=40$, $U=1483$, the difference between the two samples is highly significant ($P<0.001$, two-tailed test)

Mann-Whitney U test only values>0:
$n_1=40$, $n_2=33$, $U=1203$, the difference between the two samples is highly significant ($P<0.001$, two-tailed test)

Ventral orange margin complex area in relation to tail fin area:

Mann-Whitney U test all values:
$n_1=n_2=40$, $U=1141$, the difference between the two samples is highly significant ($P<0.001$, two-tailed test)

Mann-Whitney U test only values>0:
all values are >0

Table S4

individual	anterior orange spot	posterior orange spot complex	dorsal orange spot complex	dorsal orange margin complex	ventral orange margin complex
cross9_F2_frompair11_blondm_7735	0	0	0	0	1
cross9_F2_frompair2_blondm_6070	0	1	0	0	1
cross9_F2_frompair2_blondm_7489	0	1	0	1	1
cross9_F2_frompair3_blondm_5668	0	1	0	1	1
cross9_F2_frompair5_blondm_6755	0	1	0	1	1
cross9_F2_frompair6_blondm_6824	0	1	0	1	1
cross9_F2_frompair11_blondm_7574	0	1	0	1	1
cross9_F2_frompair3_blondm_7146	1	1	0	1	1
cross9_F2_frompair4_blondm_left_5704	0	2	0	1	1
cross9_F2_frompair3_blondm_7625	0	1	1	1	1
cross9_F2_frompair5_blondm_6426	0	1	1	1	1
cross9_F2_frompair7_blondm_6360	0	1	2	1	1
cross9_F2_frompair8_blondm_7614	0	2	2	1	1
cross9_F2_frompair2_blondm_5680	0	1	0	2	1
cross9_F2_frompair2_blondm_7562	0	1	0	2	1
cross9_F2_frompair8_blondm_left_5672	1	1	1	2	1
cross9_F2_frompair6_blondm_6278	0	1	1	3	1
cross9_F2_frompair6_blondm_6392	0	0	0	0	2
cross9_F2_frompair1_blondm_left_5644	0	0	0	0	2
cross9_F2_frompair1_blondm_left_5648	0	1	0	0	2
cross9_F2_frompair3_blondm_7144	0	2	0	0	2
cross9_F2_frompair8_blondm_6271	0	2	0	0	2
cross9_F2_frompair8_blondm_7615	0	2	0	0	2
cross9_F2_frompair2_blondm_7741	0	1	1	0	2
cross9_F2_frompair10_blondm_6723	0	1	1	0	2
cross9_F2_frompair1_blondm_5485	1	1	1	0	2
cross9_F2_frompair2_blondm_6525	0	0	0	1	2
cross9_F2_frompair2_blondm_7583	0	0	0	1	2
cross9_F2_frompair5_blondm_8018	0	0	0	1	2
cross9_F2_frompair6_blondm_6395	0	0	0	1	2
cross9_F2_frompair6_blondm_6409	0	0	0	1	2
cross9_F2_frompair1_blondm_6265	0	0	0	1	2
cross9_F2_frompair11_blondm_6286	1	0	0	1	2
cross9_F2_frompair2_blondm_7585	0	1	0	1	2
cross9_F2_frompair2_blondm_7739	0	1	0	1	2
cross9_F2_frompair2_blondm_8013	0	1	0	1	2
cross9_F2_frompair6_blondm_6408	0	1	0	1	2
cross9_F2_frompair1_blondm_left_5666	0	1	0	1	2
cross9_F2_frompair4_blondm_7613	0	1	0	1	2
cross9_F2_frompair10_blondm_6828	0	1	0	1	2
cross9_F2_frompair2_blondm_8014	1	1	0	1	2
cross9_F2_frompair7_blondm_7626	0	1	1	1	2
cross9_F2_frompair4_blondm_7608	0	2	1	1	2
cross9_F2_frompair7_blondm_6528	0	1	2	1	2
cross9_F2_frompair5_blondm_6432	0	0	0	2	2
cross9_F2_frompair6_blondm_6394	0	0	0	2	2
cross9_F2_frompair1_blondm_left_5678	0	0	0	2	2
cross9_F2_frompair2_blondm_6259	0	1	0	2	2
cross9_F2_frompair10_blondm_6768	0	1	0	2	2
cross9_F2_frompair7_blondm_left_5694	1	1	1	2	2
cross9_F2_frompair6_blondm_6818	0	2	1	2	2
cross9_F2_frompair6_blondm_6279	0	1	1	4	2
cross9_F2_frompair2_blondm_8012	0	0	0	0	3
cross9_F2_frompair8_blondm_6456	0	0	0	0	3
cross9_F2_frompair1_blondm_6263	0	1	0	0	3
cross9_F2_frompair7_blondm_6375	0	1	1	0	3
cross9_F2_frompair1_blondm_left_5646	0	0	0	1	3
cross9_F2_frompair7_blondm_7628	0	1	0	1	3
cross9_F2_frompair4_blondm_left_5642	0	1	0	1	3
cross9_F2_frompair4_blondm_6437	1	1	0	1	3
cross9_F2_frompair2_blondm_6523	0	1	1	1	3
cross9_F2_frompair6_blondm_6291	0	1	1	1	3
cross9_F2_frompair7_blondm_6449	0	1	1	1	3
cross9_F2_frompair7_blondm_left_5692	0	1	1	1	3
cross9_F2_frompair4_blondm_7610	0	2	1	1	3
cross9_F2_frompair2_blondm_6261	0	1	2	2	3
cross9_F2_frompair3_blondm_7147	0	0	0	1	4
cross9_F2_frompair5_blondm_8017	0	1	0	1	4
cross9_F2_frompair7_blondm_6451	0	1	0	1	4
cross9_F2_frompair7_blondm_6492	1	1	0	1	4
cross9_F2_frompair6_blondm_6406	0	2	0	1	4
cross9_F2_frompair7_blondm_6491	0	0	1	1	4
cross9_F2_frompair7_blondm_left_5696	0	1	1	1	4
cross9_F2_frompair2_blondm_left_5676	0	2	0	0	5
cross9_F2_frompair6_wtm_6277	1	0	0	1	1
cross9_F2_frompair2_wtm_6521	1	1	0	1	1
cross9_F2_frompair2_wtm_6522	1	1	0	1	1
cross9_F2_frompair2_wtm_6524	1	1	0	1	1
cross9_F2_frompair2_wtm_6806	1	1	0	1	1
cross9_F2_frompair2_wtm_6807	1	1	0	1	1
cross9_F2_frompair3_wtm_6366	1	1	0	1	1
cross9_F2_frompair3_wtm_6410	1	1	0	1	1
cross9_F2_frompair6_wtm_6280	1	1	0	1	1
cross9_F2_frompair1_wtm_5487	1	1	0	1	1
cross9_F2_frompair1_wtm_6399	1	1	0	1	1
cross9_F2_frompair7_wtm_6493	1	1	0	1	1
cross9_F2_frompair11_wtm_6288	1	1	0	1	1
cross9_F2_frompair2_wtm_6839	1	2	0	1	1
cross9_F2_frompair3_wtm_6411	1	2	0	1	1
cross9_F2_frompair5_wtm_6294	1	2	0	1	1
cross9_F2_frompair5_wtm_6295	1	2	0	1	1
cross9_F2_frompair6_wtm_6276	1	2	0	1	1
cross9_F2_frompair6_wtm_6404	1	2	0	1	1
cross9_F2_frompair7_wtm_6361	1	2	0	1	1
cross9_F2_frompair1_wtm_left_5656	1	2	0	1	1
cross9_F2_frompair2_wtm_5686	1	1	1	1	1
cross9_F2_frompair2_wtm_6526	1	1	1	1	1
cross9_F2_frompair5_wtm_6293	1	1	1	1	1
cross9_F2_frompair7_wtm_6363	1	1	1	1	1
cross9_F2_frompair7_wtm_6450	1	1	1	1	1
cross9_F2_frompair7_wtm_6452	1	1	1	1	1
cross9_F2_frompair8_wtm_6454	1	1	1	1	1
cross9_F2_frompair8_wtm_6517	1	1	1	1	1
cross9_F2_frompair2_wtm_5683	1	2	1	1	1
cross9_F2_frompair7_wtm_6362	1	2	1	1	1
cross9_F2_frompair4_wtm_5681	1	2	1	1	1
cross9_F2_frompair4_wtm_6438	1	2	1	1	1
cross9_F2_frompair10_wtm_6725	1	2	1	1	1
cross9_F2_frompair11_wtm_6287	1	2	1	1	1
cross9_F2_frompair2_wtm_5684	1	1	2	1	1
cross9_F2_frompair2_wtm_6838	1	1	2	1	1
cross9_F2_frompair4_wtm_6439	1	1	2	1	1
cross9_F2_frompair7_wtm_6527	1	1	2	1	1
cross9_F2_frompair3_wtm_6412	1	1	0	2	1
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equals number of elements (0 means absent)

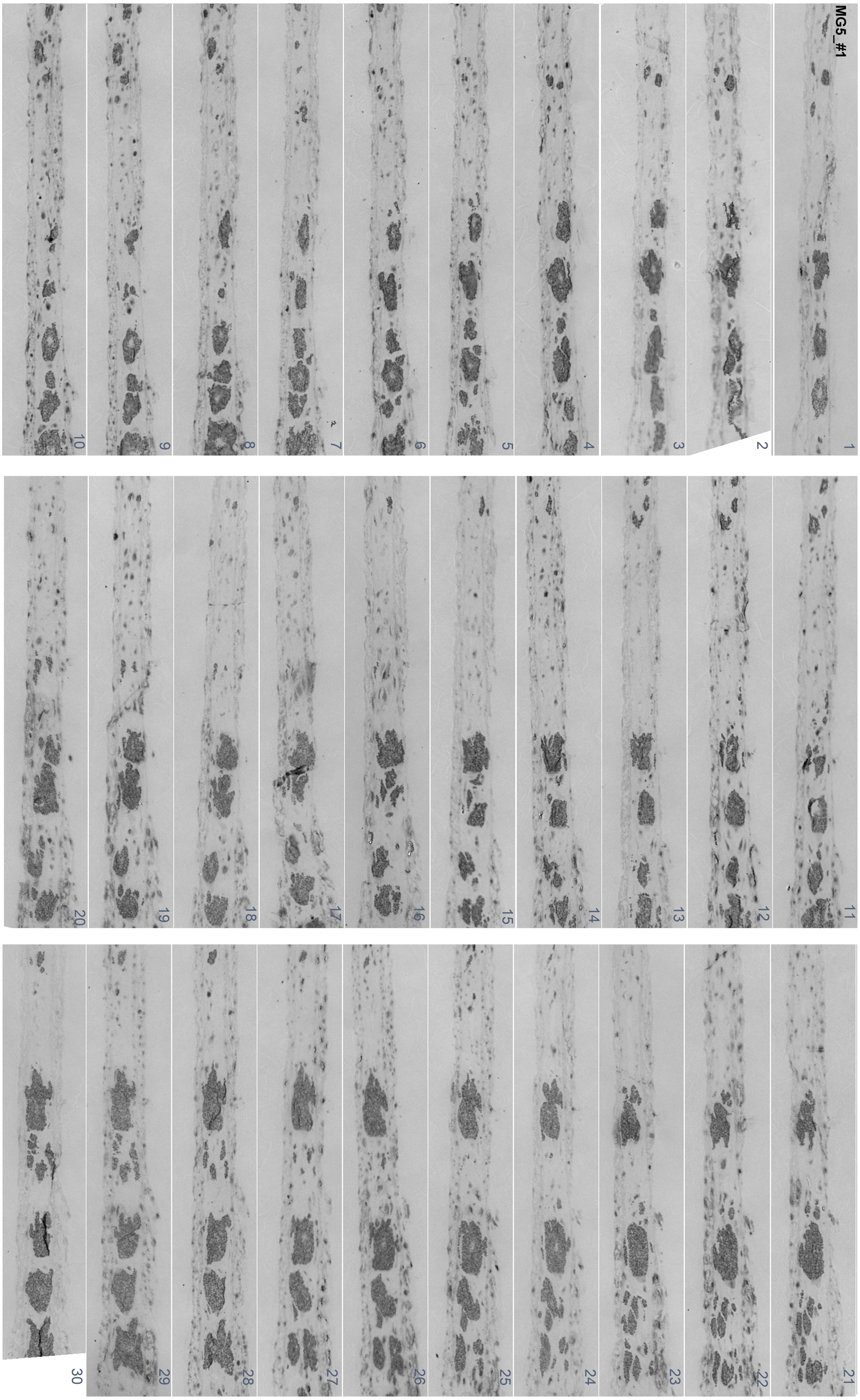
Anterior orange spot		
	present in %	absent in %
blond	10.81	89.19
wild-type	100.00	0.00

Posterior orange spot complex				
	present in %	absent in %	number of elements (if present) 1 in %	number of elements (if present) 2 in %
blond	75.68	24.32	82.14	17.86
wild-type	97.30	2.70	69.44	30.56

Dorsal orange spot complex				
	present in %	absent in %	number of elements (if present) 1 in %	number of elements (if present) 2 in %
blond	32.43	67.57	83.33	16.67
wild-type	44.59	55.41	72.73	27.27

Dorsal orange margin complex					
	present in %	absent in %	number of elements (if present) 1 in %	number of elements (if present) 2 in %	number of elements (if present) more than 2 in %
blond	78.38	21.62	77.59	18.97	3.45
wild-type	100.00	0.00	85.14	14.86	0.00

Ventral orange margin complex					
	present in %	absent in %	number of elements (if present) 1 in %	number of elements (if present) 2 in %	number of elements (if present) more than 2 in %
blond	100.00	0.00	22.97	47.30	29.73
wild-type	100.00	0.00	58.11	40.54	1.35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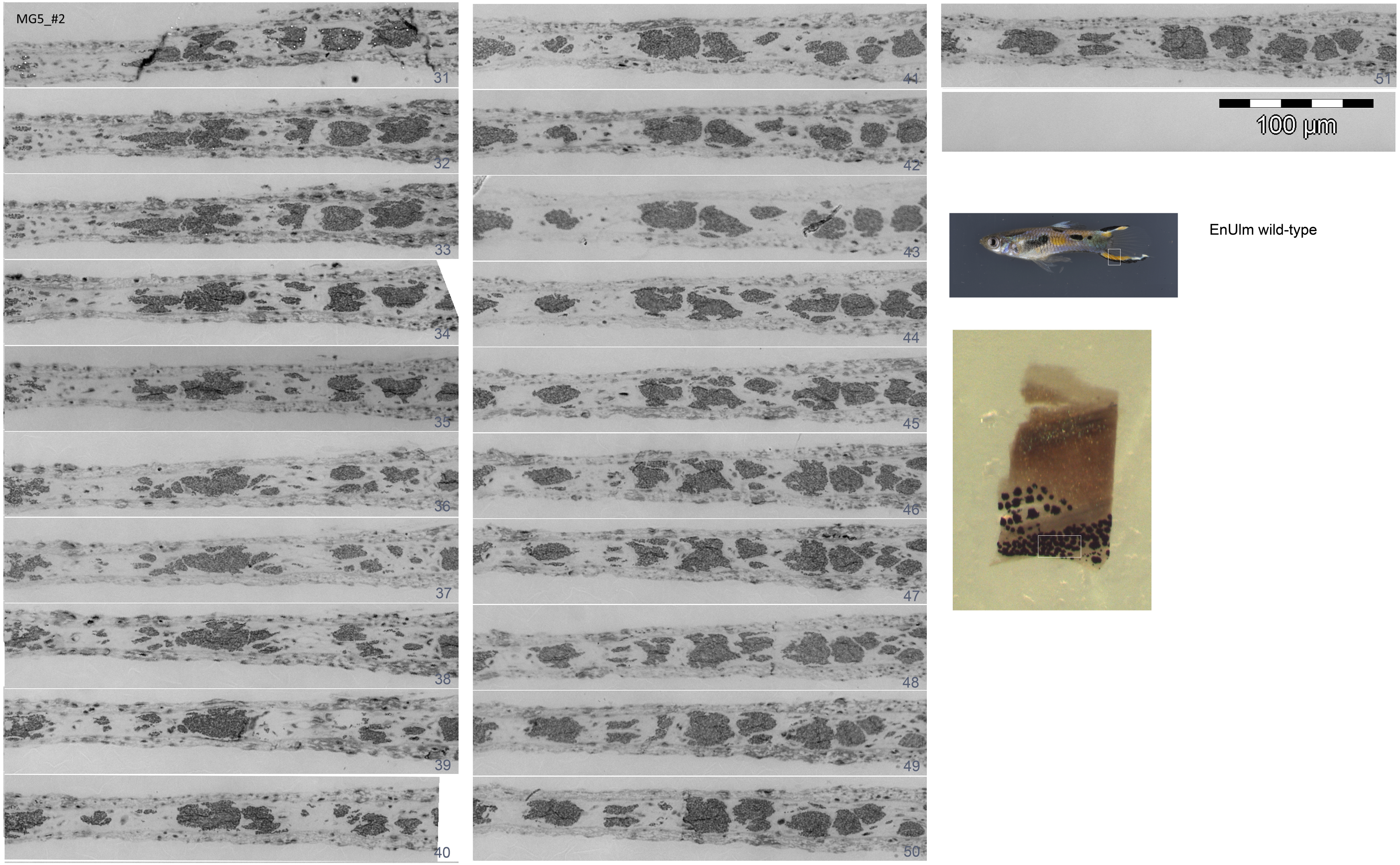
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100 µm
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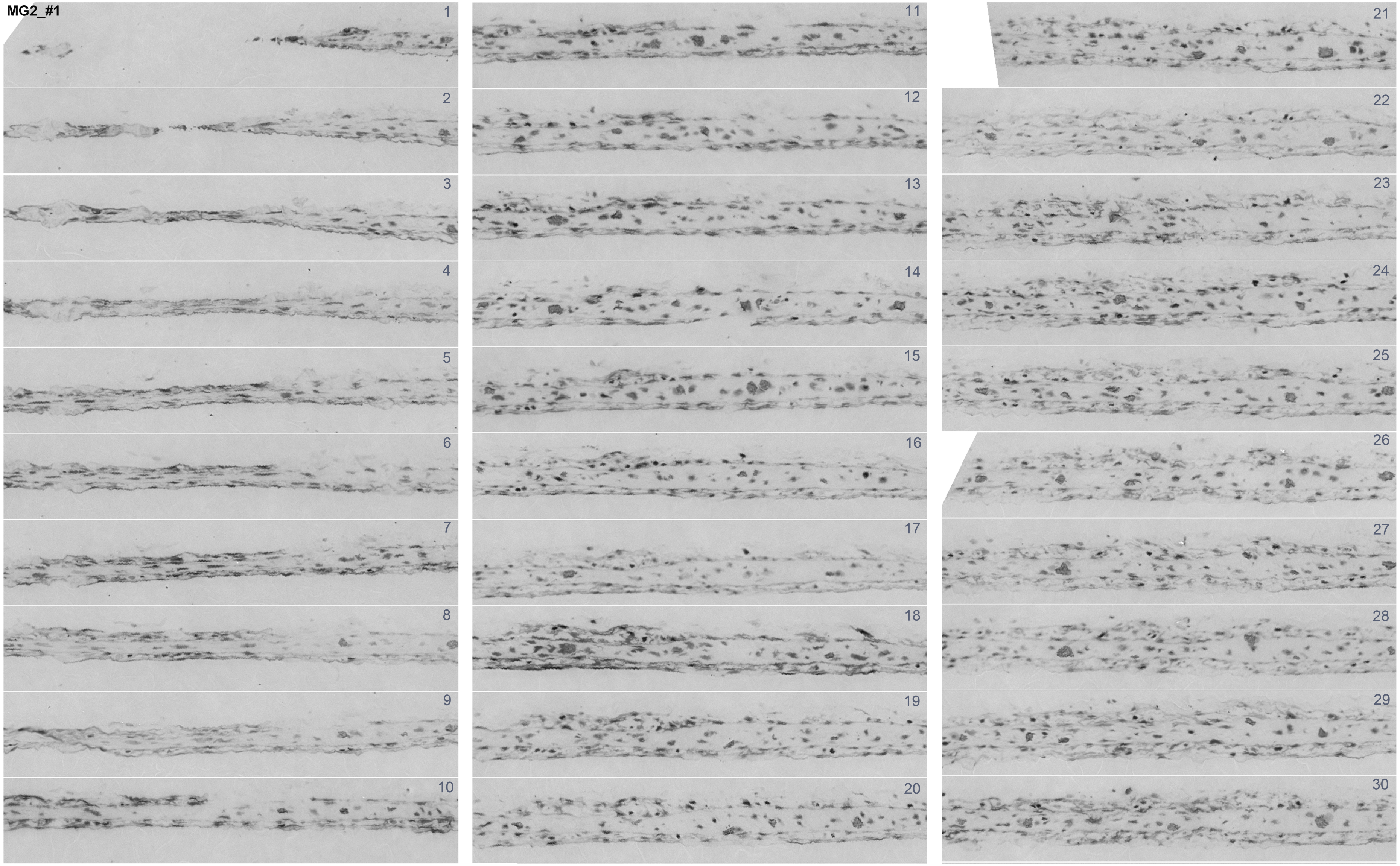
MG2_#1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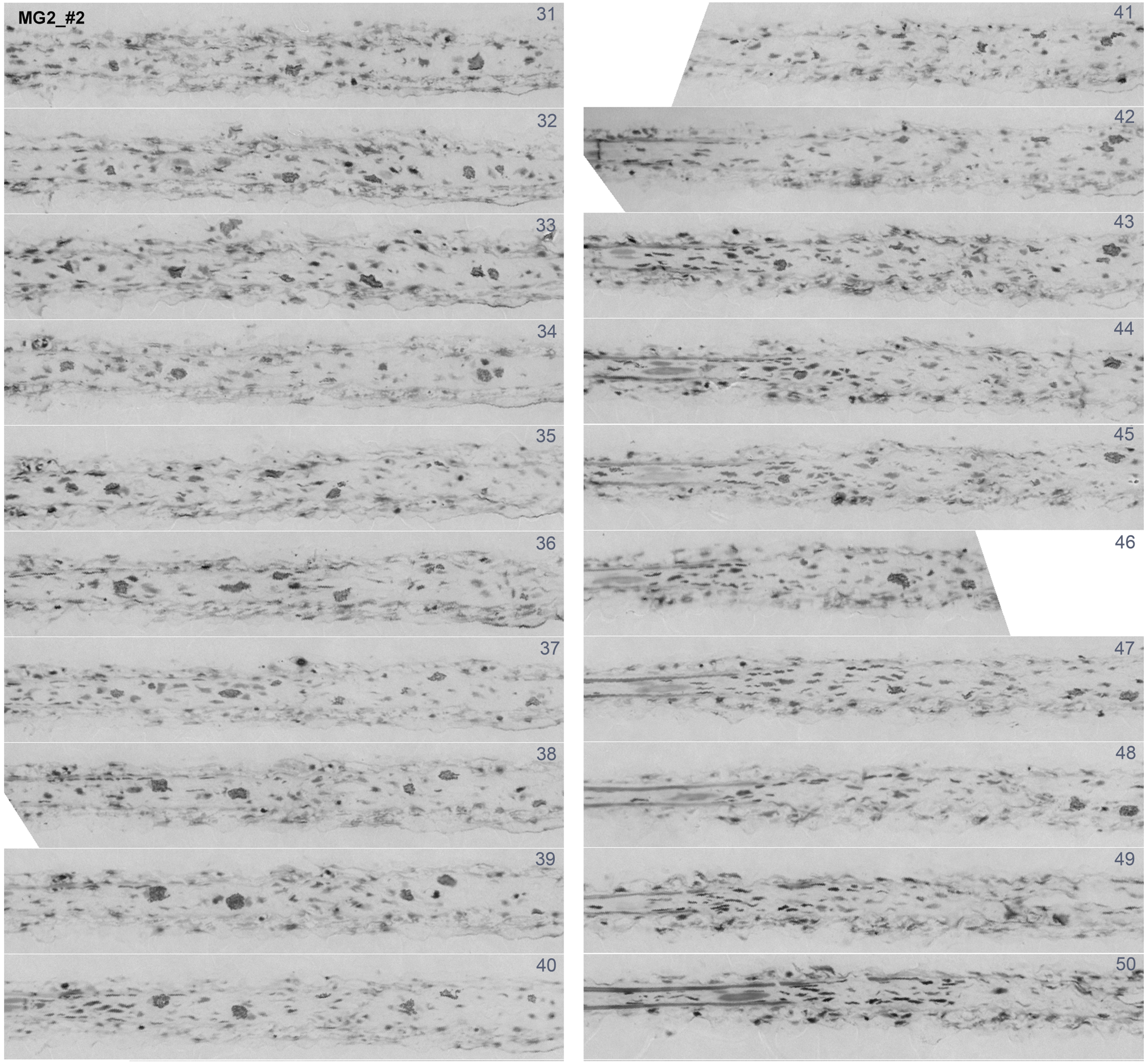
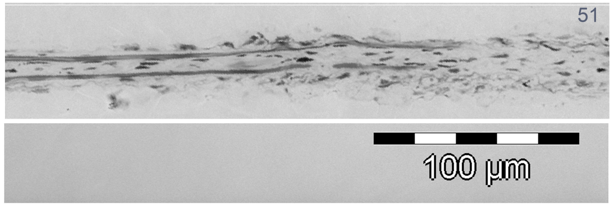
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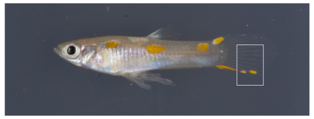
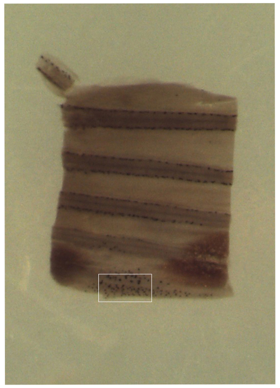


EnUlm blond



6 Discussion

The development of the polychromatic guppy pigment pattern is a fascinating field of research. To tackle questions such as "Where do the pigment cells of adult guppy males come from and which genes control their development?," knowledge is required about the distribution of the pigment cells in the skin and the different pigment cell populations forming the guppy newborn, adult reticulate, and male-specific color pattern. During my doctoral research, I explored the organization of pigment cells in guppy ornaments and identified three genes that are crucial for guppy color pattern formation. *Kita*, *csf1ra*, and *adcy5* are the first pigmentation genes of the guppy that have been molecularly identified and whose function has been studied.

The current knowledge on the molecular genetics of teleost pigment pattern formation is mostly derived from the analysis of zebrafish pigmentation mutants. Extensive studies investigating the functions of the genes underlying these mutants have demonstrated that zebrafish melanophores, xanthophores, and iridophores can be subdivided into several temporally and genetically distinct sub-lineages. Together with *kit ligand a* (*kitla*), the gene encoding the type III receptor tyrosine kinase Kita is essential for the formation and survival of zebrafish early metamorphic melanophores (DOOLEY *et al.* 2013; HULTMAN *et al.* 2007; PARICHY *et al.* 1999). Homozygous loss-of-function mutations in mouse *kit* are lethal, as they do affect not only pigmentation but also hematopoiesis (GEISSLER *et al.* 1988). Likewise, the platinum-colored variety of red fox (*Vulpes vulpes*) and some types of white horses (*Equus caballus*) are only viable when the mutations in *kit* are carried in a heterozygous state (HAASE *et al.* 2007; JOHNSON *et al.* 2015). In contrast, guppies and zebrafish homozygous for *kita* loss-of-function mutations are fully viable and fertile, presumably because two sub-functionalized paralogous *kit* loci are present in their genomes, which is a result of the teleost-specific whole-genome duplication (BRAASCH *et al.* 2009; BRAASCH *et al.* 2006; MELLGREN and JOHNSON 2005). Likewise, two loci of the ancient *kit* paralog *csf1r* can be found within the genomes of most teleosts, but zebrafish has lost *csf1rb* (BRAASCH *et al.* 2009; BRAASCH *et al.* 2006).

Csf1ra and its ligand Colony-stimulating factor (Csf1) are required for the migration and survival of zebrafish xanthoblasts and, non-autonomously, for the development of late metamorphic melanophores (LANG *et al.* 2009; MADERSPACHER and NÜSSLEIN-VOLHARD 2003; PARICHY *et al.* 2000b; PARICHY and TURNER 2003; PATTERSON and PARICHY 2013). Csf1ra is also involved in the development of the pigment pattern of other *Danio* species and is expressed in the orange egg spots of haplochromine cichlids (PATTERSON *et al.* 2014; QUIGLEY *et al.* 2005; SALZBURGER *et al.* 2007). Consistent with the fact that mammals lack xanthophores and the effects of *csf1ra* on melanophores are of an indirect nature, *csf1r* loss-of-function does not affect mouse pigmentation (DAI *et al.* 2002).

Adenylate cyclases, of which ten isoforms have been described in mammals, are involved in various processes, including physiological color change in fish and melanophore development (ANDERSSON *et al.* 2003; BUSCA and BALLOTTI 2000; FUJII 2000; RICHARDSON *et al.* 2008; SUNAHARA and TAUSSIG 2002). In mouse, Adcy5 loss-of-function protects against oxidative stress, decreases the risk of developing cancer, and delays the growth of melanoma cells in vitro (DE LORENZO *et al.* 2014; YAN *et al.* 2007). The zebrafish *adcy5* mutant *fantasma* shows reduced behavioral sensitivity to ethanol; *fantasma* melanophores fail to maintain melanosome dispersion in cell culture (PENG *et al.* 2009). An effect of the mutation on zebrafish pigment pattern formation has not been described yet.

In zebrafish, *kita* and *csf1ra* are expressed in melanoblasts and xanthoblasts, respectively, and act autonomously within these cells (PARICHY *et al.* 2000b; PARICHY *et al.* 1999). Expression of *adcy5* in zebrafish melanophores has not been investigated yet, but Adcy5 presumably directly influences the melanosome movement within these cells (PENG *et al.* 2009). As an in situ hybridization protocol still needs to be optimized in the guppy, it remains elusive whether *kita* and *adcy5* are expressed in guppy melanoblasts and *csf1ra* in guppy xanthoblasts, but at least for *kita* and *csf1ra* this seems highly likely considering the conserved functions of these genes (GEISSLER *et al.* 1988; HAASE *et al.* 2007; JOHNSON *et al.* 2015; KELSH *et al.* 2009; PARICHY *et al.*

1999; PATTERSON *et al.* 2014; QUIGLEY *et al.* 2005; SALZBURGER *et al.* 2007; YAMADA *et al.* 2010).

Recent studies on zebrafish pigment pattern development have shed light on the origin of the pigment cells forming the adult stripe pattern. In this context, pigment cells are often subdivided into embryonic/early larval ones that are derived "directly from the neural crest," and adult ones that originate from "stem cells" or "progenitor cells" (DOOLEY *et al.* 2013; HULTMAN and JOHNSON 2010; O'REILLY-POL and JOHNSON 2009; PARICHY and SPIEWAK 2015; SINGH and NÜSSLEIN-VOLHARD 2015; SINGH *et al.* 2014). The slightly misleading term "directly" here means that the precursors of the early pigment cells are restricted to their specific fates, migrate, and differentiate as melanophores, xanthophores, and iridophores while the neural crest is still present, hence very early in development (KELSH *et al.* 2009; SAUKA-SPENGLER and BRONNER-FRASER 2008). Stem cells are usually defined as cells that are able to self-renew and to give rise to various differentiated cell types, while the fate and ability for self-renewal of the stem cell-derived progenitor cells are already much more restricted (CRANE and TRAINOR 2006). Neural crest stem and progenitor cell establishment is a highly complex and poorly understood process, which involves partial, stepwise restriction of the developmental potential of the prospective stem/progenitor cells and migration of the cells to the sites within the body where they are maintained (CRANE and TRAINOR 2006; DUPIN and SOMMER 2012). At certain time points, e.g., during zebrafish pigment pattern metamorphosis and fin regeneration (O'REILLY-POL and JOHNSON 2009; PARICHY and SPIEWAK 2015; SINGH and NÜSSLEIN-VOLHARD 2015), the "dormant" stem/progenitor cells are activated and start to proliferate. Progenitor cells then migrate to their point of destination, e.g., in the skin, where they differentiate (PARICHY and SPIEWAK 2015; SINGH and NÜSSLEIN-VOLHARD 2015). Unfortunately, not much is known about the number and the self-renewal potential of zebrafish pigment stem/progenitor cells. It is also often unclear how many different cell types are derived from the stem/progenitor cell pools. In accordance with (PARICHY and SPIEWAK 2015), I here use the term "stem cell" to refer to "latent precursors that normally give rise to adult chromatophores," which includes both stem and progenitor cells. I am aware that, depending on the stem cell definition, these cells might not be considered "true" stem cells.

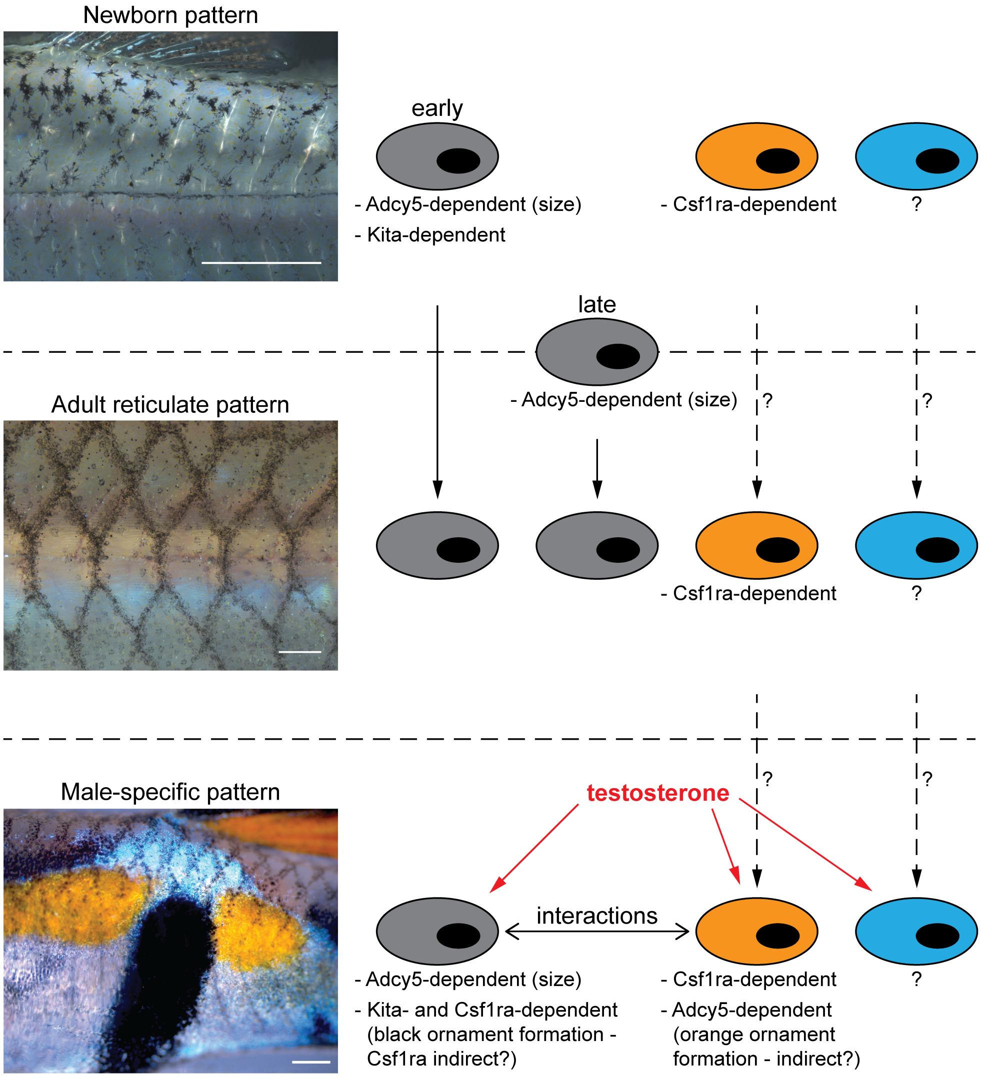


Figure 1. Summary of guppy pigment cell lineages identified in this work.

We found that three temporally and genetically distinct sub-lineages of melanophores, early Kita-dependent, late Kita-independent, and male-specific Kita- and Csf1ra-dependent ones induced by testosterone, contribute to guppy color pattern formation. The reticulate pattern consists of Kita-dependent and Kita-independent melanophores. The Kita-dependent melanophores also give rise to most of the adult dendritic and irregularly distributed corolla melanophores (these are heavily reduced in *kita* mutants, see Goodrich *et al.* 1944; Haskins and Druzba 1938; Winge and Ditlevsen 1947). We could not identify any genetically distinct xanthophore and iridophore lineages, as all xanthophores required Csf1ra for their development and we did not find any genes required for iridophore formation. Note that presumably several melanophore, xanthophore, and iridophore sub-lineages contribute to the male-specific pattern. Melanophores are shown in black, xanthophores in orange, and iridophores in blue. If no other explanation is given, "Kita-dependent" and "Csf1ra-dependent" implies that the development of the cell type per se depends on Kita and Csf1ra, respectively. Scale bars: 0.5 mm.

The metamorphic melanophores that contribute to the adult zebrafish stripe pattern are derived from ErbB-dependent embryonic stem cells that are established early in development in close proximity to the segmentally arranged dorsal root ganglia (BUDI *et al.* 2008; BUDI *et al.* 2011; DOOLEY *et al.* 2013; SINGH *et al.* 2014). The stem cells from which the early metamorphic melanophores are derived additionally require Kita-Kitla-signaling for their establishment or survival (DOOLEY *et al.* 2013). Late metamorphic melanophores, in contrast, indirectly depend on Csf1ra, but not on Kita, as interactions with xanthophores are required for their development (PARICHY *et al.* 2000b; PARICHY and TURNER 2003). Xanthophores have been shown to repulse and "chase" melanophores when coming into contact with them, but they also provide long-range cues that are required for melanophore survival at a certain distance (INABA *et al.* 2012; NAKAMASU *et al.* 2009; PARICHY and TURNER 2003).

Like the adult melanophores, zebrafish metamorphic iridophores are derived from stem cells associated with the dorsal root ganglia (SINGH *et al.* 2014). These stem cells are multipotent, as they also give rise to a small subset of melanophores, glia, and peripheral neurons (SINGH *et al.* 2014). Most metamorphic xanthophores, however, originate from embryonic xanthophores that persist into adulthood; their development is promoted by thyroid hormone and interactions with iridophores mediated by Csf1ra-Csf1-signaling (MAHALWAR *et al.* 2014; MCMENAMIN *et al.* 2014; PATTERSON and PARICHY 2013). In zebrafish mutants in which only one type of chromatophore persists, the remaining pigment cell type is distributed uniformly across the body, pointing to the fact that interactions between the different types of pigment cells are essential for the formation of the adult pattern (FROHNHÖFER *et al.* 2013; SINGH and NÜSSLEIN-VOLHARD 2015).

Although no pigment pattern metamorphosis has been described in the guppy, my work demonstrated that two temporally and genetically distinct melanophore sub-lineages contribute to the guppy newborn and adult reticulate pattern, an early Kita-dependent one and a late Kita-independent one (Figure 1). The pigment pattern of the guppy seems fundamentally different from the striped pattern of zebrafish, but nevertheless early and late melanophore populations differing in their requirements for Kita contribute to both patterns, suggesting that such melanophore populations might also contribute to the pigment patterns of other teleosts. In contrast to zebrafish, however, the late Kita-independent melanophores of the guppy are also independent of Csf1ra; the reticulate pattern of *csf1ra* mutant fish was not different from the one seen in wild-type fish. Furthermore, the xanthophores seen in newborns and adult females are distributed evenly, suggesting that they are not "patterned," i.e., dependent on interactions with other pigment cell types (SINGH and NÜSSLEIN-VOLHARD 2015). The formation of the guppy reticulate pattern therefore does not seem to depend on melanophore-xanthophore interactions, but interactions between melanophores and iridophores might still be involved in its formation. The analysis of the phenotype of the guppy *adcy5* mutant revealed that guppy melanophores do not need to be normally sized to form a complete reticulate pattern, which suggests that, if melanophore-iridophore interactions are required for the formation of this pattern, *adcy5* mutant melanophores are competent to interact with iridophores despite their small size. A guppy mutant lacking iridophores, e.g., a mutant similar to zebrafish *shady* (LOPES *et al.* 2008), would be very helpful to further study the formation of the guppy reticulate pattern, as its phenotype would reveal whether iridophore-melanophore interactions are needed for the development of the reticulate pattern. If not, it might be possible that the tissue environment provides the cues necessary for the formation of this pattern (KRAUSS *et al.* 2014; LANG *et al.* 2009; PATTERSON and PARICHY 2013).

As the reticulate pattern of the guppy becomes more elaborate after birth and female guppies grow during their whole life (REZNICK and ENDLER 1982; TRIPATHI *et al.* 2009b), not all of the melanophores contributing to this pattern can be derived directly from the neural crest. At least the Kita-independent melanophores that develop after birth and the melanophores replenishing the reticulate pattern during growth should be derived from stem cells. It is tempting to speculate that these stem cells are associated with the dorsal root ganglia as described in zebrafish, but this is difficult to test, as most methods established in zebrafish cannot be easily applied to a livebearing fish such as the guppy. Moreover, stem cells seem to be very rare; it was recently proposed that the adult melanophores and iridophores of zebrafish are derived from one stem cell each per segment (DOOLEY *et al.* 2013; SINGH *et al.* 2014). Whether the iridophores and xanthophores of newborn guppies persist into adulthood or are replaced by stem cell-derived iridophores and xanthophores remains elusive.

The development of the male-specific pigment pattern of the guppy is one of the most exciting aspects of guppy biology. Like the late Kita-independent melanophores, the male-specific melanophores, xanthophores, and iridophores might be derived from stem cells, whose reactivation, proliferation, migration, and differentiation could be induced by testosterone during puberty. As even guppy females develop some male color traits when treated with testosterone, and sometimes even naturally later in life under the influence of high endogenous testosterone levels (CLEMENS *et al.* 1966; MAGURRAN 2005; WINGE and DITLEVSEN 1947), these stem cells do not seem to be restricted to guppy males and might be established early in development. Not all of the male-specific pigment cells need to originate from stem cells, however, as some pigment cell types might persist into adulthood and could be reorganized and stimulated to form spots and stripes by interactions with the other pigment cell types. I therefore suggest that either all or a subset of the pigment cells of adult guppies are derived from either one or two dormant stem cell pools (ADAMEYKO *et al.* 2009; PARICHY and SPIEWAK 2015; YAMADA *et al.* 2010), which are set aside early in female and male development for later proliferation and differentiation. If the pigment cells of adult guppies originate from two stem cell pools, then one of them should provide the chromatophores required during growth and regeneration, while the other pool should be testosterone-responsive, generating the pigment cells of the adult male color pattern. However, it is also possible that only one stem cell pool gives rise to the pigment cells of both the reticulate pattern and the male-specific pattern, as stem cells are often multipotent and are able to react to contrasting stimuli by generating different cell types (ADAMEYKO *et al.* 2009; CRANE and TRAINOR 2006; DUPIN and SOMMER 2012). The fate of the stem cell derivatives could also depend on the tissue environment (KRAUSS *et al.* 2014; LANG *et al.* 2009; PATTERSON and PARICHY 2013).

As almost all xanthophores of the guppy depend on Csf1ra-signaling and the xanthophores of newborns and adult females are not "patterned," i.e., their Adcy5-dependence could not be investigated, we could only identify temporally distinct sub-lineages of xanthophores. In contrast, we could show that the male-specific melanophores of the guppy are not identical with the early or late melanophores contributing to the adult reticulate pattern, as they differ in their requirements for Kita and Csf1ra (Figure 1). The development per se of the male-specific melanophores does not depend on Kita, as *kita* mutant males develop black spots and stripes. These spots and stripes, however, often appeared diffuse, suggesting that Kita is required for "sharpening" the black ornaments, e.g., by promoting melanophore cohesion or apoptosis of ectopic melanophores. Moreover, we discovered that the male-specific melanophores strongly depend on Csf1ra signaling, as the black color patterns of *csf1ra* mutant males appear disorganized. This is presumably caused by the absence of the xanthophore-mediated cues, which are required for black trait formation. For instance, xanthophores within the orange part of the orange-black margin of the Cumaná tail fin might repel melanophores close by, but promote their survival or proliferation at a certain distance, leading to the formation of the distinct orange-black pattern.

Analysis of the phenotypes of wild-type and *adcy5* mutant males revealed that normally sized melanophores are required for orange trait formation (Figure 1), which suggests that *adcy5* mutant melanophores cannot provide all of the cues that are necessary for the xanthophores to form wild-type-like orange spots and stripes. Taken together, our results suggest that mutual interactions between xanthophores and melanophores are required for male guppy color pattern formation.

Unfortunately, we were not able to study iridophores in detail, as no guppy iridophore mutant was available. Our TEM study revealed that iridophores are in close contact with melanophores and xanthophores in the ornaments of guppy males. During zebrafish pigment pattern metamorphosis, iridophores are the first pigment cell type to form stripes; iridophores and their interactions with melanophores and xanthophores are therefore fundamentally important for zebrafish pigment pattern formation (PATTERSON and PARICHY 2013; SINGH and NÜSSLEIN-VOLHARD 2015; SINGH *et al.* 2014). Moreover, iridophores are also present in the orange egg spots on the anal fins of male haplochromine cichlids; they arrive at the prospective egg spot sites before the xanthophores (SANTOS *et al.* 2014). This is mediated by a gain of Four and a half LIM domain protein 2 expression in the haplochromine iridophores and suggests that iridophore-xanthophore interactions contribute to egg spot formation (SANTOS *et al.* 2014). Based on our observations, it seems likely that iridophores also play a key role in guppy male pigment pattern development, as they contribute to each trait and arrive early at the sites of prospective spot and stripe regions in juvenile males (personal observation).

Interestingly, we found that the male ornaments differ in their requirements for *kita*, *csf1ra*, and *adcy5*. While some spots and stripes were heavily affected by the loss-of-function mutations in these genes, others were not. This suggests that the male-specific melanophores, xanthophores, and iridophores of the guppy each can be subdivided into several genetically distinct sub-lineages, which presumably also differ in the extent to which they depend on interactions with other pigment cell lineages. Cis-regulatory changes affecting the expression of *kita* and *csf1ra* might contribute to the natural variation of male guppy color traits, as modifications of the Kita- and Csf1ra-signaling pathways have been shown to be involved in pigment pattern variation in sticklebacks, humans (*Homo sapiens*), and *Danio* species (GUENTHER *et al.* 2014; MILLER *et al.* 2007; PATTERSON *et al.* 2014).

In conclusion, my research gives insights into the complex genetic networks underlying guppy pigment pattern formation for the first time and paves the way for further in-depth studies on guppy pigment cell lineages. I found that Kita- and Csf1ra-signaling are required for male pattern formation, as are normally sized melanophores. To further study guppy pigment pattern formation and potential stem cells, I would next focus on investigating the role of the Endothelin and ErbB pathways in the guppy. Analysis of the zebrafish mutants *rose* and *karneol* and knockdown of *endothelin 3b* demonstrated that Endothelin-signaling is required for zebrafish iridophore development, while ErbB-signaling is essential for stem cell establishment (BUDI *et al.* 2008; DOOLEY *et al.* 2013; FROHNHÖFER *et al.* 2013; HULTMAN *et al.* 2009; KRAUSS *et al.* 2014; PARICHY *et al.* 2000a; SINGH *et al.* 2014). As a first step towards investigating Endothelin- and ErbB-signaling in the guppy, I would therefore try to identify mutations underlying iridophore-deficient guppy mutants like the "see thru" strain, some of which I would expect to affect components of the Endothelin pathway, and I would treat explanted guppy embryos with a commercially available ErbB inhibitor. For this, however, one would first need to obtain and successfully maintain iridophore-deficient guppy strains and to establish a protocol that makes it possible to raise very early explanted embryos in vitro (MARTYN *et al.* 2006). Furthermore, ddRADseq in combination with QTL mapping represents a promising approach that could be used to identify mutations underlying natural color variation in the guppy in the future.

Recently, high-quality assemblies of the female and male guppy genome have become available (KÜNSTNER *et al.*, in preparation) and analysis of the sequencing data will hopefully result in the identification of the pigmentation genes located at the male sex-determining locus. Together with mapping approaches and the analysis of other spontaneous guppy color mutants like the ones shown here, this will eventually lead to a much better understanding of the pathways that are required for the formation of the beautiful color patterns of male guppies, which have intrigued researchers for almost a hundred years.

7 References

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8 Appendix

8.1 Table S3 sheet 1

individual	body area in mm2	anterior orange spot in mm2	anterior orange spot area in relation to body area (%)
cross9_F2_frompair2_blondm_5680	39.07	0.00	0.00
cross9_F2_frompair2_blondm_6070	58.15	0.00	0.00
cross9_F2_frompair2_blondm_6259	49.83	0.00	0.00
cross9_F2_frompair2_blondm_6261	46.01	0.00	0.00
cross9_F2_frompair2_blondm_6523	40.97	0.00	0.00
cross9_F2_frompair2_blondm_6525	51.13	0.00	0.00
cross9_F2_frompair2_blondm_7489	43.47	0.00	0.00
cross9_F2_frompair2_blondm_7562	41.96	0.00	0.00
cross9_F2_frompair2_blondm_7583	36.15	0.00	0.00
cross9_F2_frompair2_blondm_7585	38.91	0.00	0.00
cross9_F2_frompair2_blondm_7739	46.44	0.00	0.00
cross9_F2_frompair2_blondm_7741	46.27	0.00	0.00
cross9_F2_frompair2_blondm_8012	54.76	0.00	0.00
cross9_F2_frompair2_blondm_8013	61.72	0.00	0.00
cross9_F2_frompair2_blondm_8014	45.79	1.27	2.78
cross9_F2_frompair2_blondm_left_5676	59.21	0.00	0.00
cross9_F2_frompair3_blondm_5668	43.18	0.00	0.00
cross9_F2_frompair3_blondm_7144	63.48	0.00	0.00
cross9_F2_frompair3_blondm_7146	54.17	0.89	1.65
cross9_F2_frompair3_blondm_7625	49.95	0.00	0.00
cross9_F2_frompair5_blondm_6426	39.88	0.00	0.00
cross9_F2_frompair5_blondm_6432	44.65	0.00	0.00
cross9_F2_frompair5_blondm_6755	50.56	0.00	0.00
cross9_F2_frompair5_blondm_8017	57.77	0.00	0.00
cross9_F2_frompair5_blondm_8018	52.19	0.00	0.00
cross9_F2_frompair6_blondm_6278	44.27	0.00	0.00
cross9_F2_frompair6_blondm_6279	51.44	0.00	0.00
cross9_F2_frompair6_blondm_6291	52.72	0.00	0.00
cross9_F2_frompair6_blondm_6392	50.53	0.00	0.00
cross9_F2_frompair6_blondm_6394	43.24	0.00	0.00
cross9_F2_frompair6_blondm_6395	51.35	0.00	0.00
cross9_F2_frompair6_blondm_6408	38.39	0.00	0.00
cross9_F2_frompair6_blondm_6409	46.33	0.00	0.00
cross9_F2_frompair6_blondm_6818	50.69	0.00	0.00
cross9_F2_frompair7_blondm_6360	47.40	0.00	0.00
cross9_F2_frompair7_blondm_6375	36.49	0.00	0.00
cross9_F2_frompair7_blondm_6449	48.53	0.00	0.00
cross9_F2_frompair7_blondm_6451	51.31	0.00	0.00
cross9_F2_frompair7_blondm_6491	46.68	0.00	0.00
cross9_F2_frompair7_blondm_6492	55.51	0.35	0.63
sum			5.06
sum/40			**0.13**

individual	body area in mm2	anterior orange spot in mm2	anterior orange spot area in relation to body area (%)
cross9_F2_frompair2_wtm_5683	40.49	0.81	2.01
cross9_F2_frompair2_wtm_5684	39.26	1.55	3.94
cross9_F2_frompair2_wtm_5685	40.49	1.49	3.68
cross9_F2_frompair2_wtm_5686	43.58	1.72	3.94
cross9_F2_frompair2_wtm_6071	49.53	1.07	2.16
cross9_F2_frompair2_wtm_6260	50.41	0.92	1.82
cross9_F2_frompair2_wtm_6521	52.27	1.99	3.80
cross9_F2_frompair2_wtm_6522	54.66	2.92	5.34
cross9_F2_frompair2_wtm_6524	59.49	1.69	2.84
cross9_F2_frompair2_wtm_6526	56.50	2.10	3.72
cross9_F2_frompair2_wtm_6806	44.33	1.16	2.61
cross9_F2_frompair2_wtm_6807	45.29	1.57	3.46
cross9_F2_frompair2_wtm_6809	38.79	1.52	3.93
cross9_F2_frompair2_wtm_6838	46.92	2.20	4.68
cross9_F2_frompair2_wtm_6839	48.00	1.53	3.19
cross9_F2_frompair2_wtm_6840	56.30	2.02	3.59
cross9_F2_frompair3_wtm_6366	50.26	0.89	1.76
cross9_F2_frompair3_wtm_6410	48.30	2.60	5.37
cross9_F2_frompair3_wtm_6411	41.59	1.18	2.83
cross9_F2_frompair3_wtm_6412	44.08	0.31	0.70
cross9_F2_frompair5_wtm_6292	51.42	1.40	2.73
cross9_F2_frompair5_wtm_6293	40.23	1.76	4.37
cross9_F2_frompair5_wtm_6294	50.48	2.12	4.21
cross9_F2_frompair5_wtm_6295	40.70	1.79	4.41
cross9_F2_frompair5_wtm_6296	44.25	1.77	4.00
cross9_F2_frompair6_wtm_6276	44.22	1.11	2.50
cross9_F2_frompair6_wtm_6277	42.69	1.01	2.36
cross9_F2_frompair6_wtm_6280	46.30	2.13	4.60
cross9_F2_frompair6_wtm_6281	42.63	2.45	5.75
cross9_F2_frompair6_wtm_6282	45.49	1.57	3.44
cross9_F2_frompair6_wtm_6289	55.88	3.40	6.08
cross9_F2_frompair6_wtm_6393	44.00	2.02	4.58
cross9_F2_frompair6_wtm_6404	42.33	1.91	4.52
cross9_F2_frompair6_wtm_6405	42.71	2.10	4.92
cross9_F2_frompair7_wtm_6361	43.11	0.85	1.98
cross9_F2_frompair7_wtm_6362	49.20	1.82	3.69
cross9_F2_frompair7_wtm_6363	53.61	2.76	5.15
cross9_F2_frompair7_wtm_6364	57.24	2.96	5.16
cross9_F2_frompair7_wtm_6365	44.31	1.77	4.00
cross9_F2_frompair7_wtm_6373	38.45	1.12	2.92
sum			146.73
sum/40			**3.67**

posterior orange spot element 1 in mm2	posterior orange spot element 2 in mm2	sum posterior orange spot elements in mm2 = posterior orange spot complex area
1.62	0.00	1.62
2.37	0.00	2.37
1.41	0.00	1.41
2.50	0.00	2.50
2.08	0.00	2.08
0.00	0.00	0.00
1.83	0.00	1.83
1.28	0.00	1.28
0.00	0.00	0.00
1.47	0.00	1.47
0.79	0.00	0.79
2.07	0.00	2.07
0.00	0.00	0.00
2.37	0.00	2.37
1.52	0.00	1.52
1.53	1.10	2.63
1.37	0.00	1.37
0.94	0.72	1.66
1.92	0.00	1.92
2.91	0.00	2.91
2.15	0.00	2.15
0.00	0.00	0.00
2.42	0.00	2.42
0.33	0.00	0.33
0.00	0.00	0.00
2.64	0.00	2.64
1.42	0.00	1.42
3.17	0.00	3.17
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	0.00
1.71	0.00	1.71
0.00	0.00	0.00
1.29	0.95	2.23
2.73	0.00	2.73
0.59	0.00	0.59
1.22	0.00	1.22
2.06	0.00	2.06
0.00	0.00	0.00
1.84	0.00	1.84

posterior orange spot element 1 in mm2	posterior orange spot element 2 in mm2	sum posterior orange spot elements in mm2 = posterior orange spot complex area
1.89	1.30	3.19
2.75	0.00	2.75
1.62	0.74	2.36
2.86	0.00	2.86
2.52	0.00	2.52
2.72	0.31	3.03
3.26	0.00	3.26
2.53	0.00	2.53
2.36	0.00	2.36
1.91	0.00	1.91
1.25	0.00	1.25
2.69	0.00	2.69
1.63	0.00	1.63
3.35	0.00	3.35
1.67	1.62	3.29
1.72	0.00	1.72
3.23	0.00	3.23
1.77	0.00	1.77
0.93	0.55	1.48
2.03	0.00	2.03
2.44	0.00	2.44
2.82	0.00	2.82
2.08	1.10	3.18
1.27	1.02	2.29
0.00	0.00	0.00
1.58	0.88	2.46
0.00	0.00	0.00
2.53	0.00	2.53
2.35	0.00	2.35
2.49	0.00	2.49
2.37	0.00	2.37
2.17	0.00	2.17
1.74	0.80	2.54
2.67	0.00	2.67
1.00	0.33	1.33
0.05	2.69	2.74
2.45	0.00	2.45
3.61	0.00	3.61
2.16	0.00	2.16
1.40	0.00	1.40

posterior orange spot complex area in relation to body area (%)	**dorsal orange spot element 1 in mm2**	**dorsal orange spot element 2 in mm2**
4.15	0.00	0.00
4.07	0.00	0.00
2.83	0.00	0.00
5.44	0.84	0.27
5.08	0.10	0.00
0.00	0.00	0.00
4.22	0.00	0.00
3.05	0.00	0.00
0.00	0.00	0.00
3.77	0.00	0.00
1.71	0.00	0.00
4.48	1.87	0.00
0.00	0.00	0.00
3.84	0.00	0.00
3.31	0.00	0.00
4.44	0.00	0.00
3.17	0.00	0.00
2.62	0.00	0.00
3.54	0.00	0.00
5.83	0.08	0.00
5.40	0.57	0.00
0.00	0.00	0.00
4.79	0.00	0.00
0.56	0.00	0.00
0.00	0.00	0.00
5.96	1.20	0.00
2.77	0.18	0.00
6.02	2.58	0.00
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	0.00
4.45	0.00	0.00
0.00	0.00	0.00
4.41	0.94	0.00
5.75	0.37	0.47
1.63	0.72	0.00
2.52	0.30	0.00
4.02	0.00	0.00
0.00	0.38	0.00
3.31	0.00	0.00
117.14		
2.93		

posterior orange spot complex area in relation to body area (%)	**dorsal orange spot element 1 in mm2**	**dorsal orange spot element 2 in mm2**
7.88	1.05	0.00
6.99	0.85	0.47
5.84	0.00	0.00
6.57	1.03	0.00
5.09	0.00	0.00
6.01	0.99	0.35
6.24	0.00	0.00
4.63	0.00	0.00
3.97	0.00	0.00
3.37	0.46	0.00
2.82	0.00	0.00
5.94	0.00	0.00
4.21	0.00	0.00
7.13	1.22	0.46
6.85	0.00	0.00
3.06	0.00	0.00
6.43	0.00	0.00
3.66	0.00	0.00
3.56	0.00	0.00
4.60	0.00	0.00
4.75	0.00	0.00
7.01	0.98	0.00
6.30	0.00	0.00
5.62	0.00	0.00
0.00	0.00	0.00
5.55	0.00	0.00
0.00	0.00	0.00
5.46	0.00	0.00
5.52	1.17	0.24
5.47	0.89	0.00
4.24	1.72	0.00
4.93	0.52	0.00
6.00	0.00	0.00
6.24	0.41	0.00
3.08	0.00	0.00
5.56	0.67	0.00
4.57	0.59	0.00
6.30	0.88	1.60
4.86	1.32	0.40
3.63	1.06	0.00
199.94		
5.00		

sum dorsal orange spot elements in mm2 = dorsal orange spot complex area	dorsal orange spot complex area in relation to body area (%)	sum orange on body in mm2
0.00	0.00	1.62
0.00	0.00	2.37
0.00	0.00	1.41
1.11	2.41	3.61
0.10	0.24	2.18
0.00	0.00	0.00
0.00	0.00	1.83
0.00	0.00	1.28
0.00	0.00	0.00
0.00	0.00	1.47
0.00	0.00	0.79
1.87	4.05	3.95
0.00	0.00	0.00
0.00	0.00	2.37
0.00	0.00	2.79
0.00	0.00	2.63
0.00	0.00	1.37
0.00	0.00	1.66
0.00	0.00	2.81
0.08	0.16	2.99
0.57	1.43	2.73
0.00	0.00	0.00
0.00	0.00	2.42
0.00	0.00	0.33
0.00	0.00	0.00
1.20	2.70	3.84
0.18	0.36	1.61
2.58	4.89	5.75
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.00	1.71
0.00	0.00	0.00
0.94	1.86	3.18
0.84	1.77	3.57
0.72	1.98	1.32
0.30	0.61	1.52
0.00	0.00	2.06
0.38	0.81	0.38
0.00	0.00	2.19
	23.27	
	0.58	

sum dorsal orange spot elements in mm2 = dorsal orange spot complex area	dorsal orange spot complex area in relation to body area (%)	sum orange on body in mm2
1.05	2.60	5.06
1.32	3.35	5.61
0.00	0.00	3.85
1.03	2.35	5.61
0.00	0.00	3.59
1.34	2.66	5.29
0.00	0.00	5.25
0.00	0.00	5.45
0.00	0.00	4.05
0.46	0.82	4.47
0.00	0.00	2.41
0.00	0.00	4.26
0.00	0.00	3.16
1.67	3.56	7.21
0.00	0.00	4.82
0.00	0.00	3.74
0.00	0.00	4.12
0.00	0.00	4.36
0.00	0.00	2.66
0.00	0.00	2.34
0.00	0.00	3.85
0.98	2.45	5.56
0.00	0.00	5.30
0.00	0.00	4.08
0.00	0.00	1.77
0.00	0.00	3.56
0.00	0.00	1.01
0.00	0.00	4.66
1.41	3.31	6.21
0.89	1.96	4.95
1.72	3.08	7.49
0.52	1.18	4.71
0.00	0.00	4.45
0.41	0.96	5.18
0.00	0.00	2.18
0.67	1.36	5.22
0.59	1.10	5.80
2.48	4.33	9.04
1.71	3.86	5.64
1.06	2.76	3.58
	41.70	
	1.04	

percentage orange on body	tail fin area in mm2	dorsal orange margin element 1 in mm2	dorsal orange margin element 2 in mm2	dorsal orange margin element 3 in mm2
4.15	17.12	0.73	0.20	0.00
4.07	27.12	0.00	0.00	0.00
2.83	21.78	0.86	0.17	0.00
7.85	20.57	0.50	0.06	0.00
5.32	17.20	0.18	0.00	0.00
0.00	22.75	0.24	0.00	0.00
4.22	18.22	0.30	0.00	0.00
3.05	20.27	0.27	0.03	0.00
0.00	14.77	0.08	0.00	0.00
3.77	17.59	0.36	0.00	0.00
1.71	18.96	0.14	0.00	0.00
8.53	18.50	0.00	0.00	0.00
0.00	23.81	0.00	0.00	0.00
3.84	23.74	0.72	0.00	0.00
6.09	18.20	0.28	0.00	0.00
4.44	24.87	0.00	0.00	0.00
3.17	19.75	0.59	0.00	0.00
2.62	22.35	0.00	0.00	0.00
5.19	27.16	0.42	0.00	0.00
5.99	19.90	0.29	0.00	0.00
6.83	17.20	0.68	0.00	0.00
0.00	20.48	0.45	0.11	0.00
4.79	21.75	0.32	0.00	0.00
0.56	23.11	0.19	0.00	0.00
0.00	20.21	0.67	0.00	0.00
8.66	19.41	0.46	0.03	0.14
3.12	22.22	0.72	0.04	0.16
10.91	24.57	0.56	0.00	0.00
0.00	19.94	0.00	0.00	0.00
0.00	18.30	0.53	0.09	0.00
0.00	21.19	0.51	0.00	0.00
4.45	16.64	0.41	0.00	0.00
0.00	19.17	0.44	0.00	0.00
6.26	23.01	0.40	0.08	0.00
7.52	20.43	0.46	0.00	0.00
3.61	18.36	0.00	0.00	0.00
3.13	21.54	0.15	0.00	0.00
4.02	23.29	0.16	0.00	0.00
0.81	16.97	0.33	0.00	0.00
3.94	23.09	0.10	0.00	0.00
145.47				
3.64				

percentage orange on body	tail fin area in mm2	dorsal orange margin element 1 in mm2	dorsal orange margin element 2 in mm2	dorsal orange margin element 3 in mm2
12.48	19.66	0.82	0.00	0.00
14.28	14.44	0.81	0.00	0.00
9.52	18.88	0.91	0.00	0.00
12.86	21.26	0.87	0.00	0.00
7.25	22.96	1.89	0.00	0.00
10.49	23.11	0.77	0.00	0.00
10.04	23.90	1.05	0.00	0.00
9.97	20.97	0.95	0.00	0.00
6.81	26.50	1.00	0.00	0.00
7.91	23.45	0.54	0.00	0.00
5.43	17.60	0.90	0.00	0.00
9.41	22.04	1.02	0.00	0.00
8.14	15.00	0.78	0.00	0.00
15.37	21.65	1.30	0.00	0.00
10.04	22.36	1.13	0.00	0.00
6.65	25.46	0.45	0.00	0.00
8.19	19.47	1.50	0.00	0.00
9.03	16.18	0.94	0.00	0.00
6.39	17.54	0.83	0.00	0.00
5.30	16.85	1.27	0.08	0.00
7.48	24.94	1.39	0.00	0.00
13.82	17.09	1.57	0.00	0.00
10.50	20.48	0.83	0.00	0.00
10.03	16.59	1.02	0.00	0.00
4.00	17.76	0.06	0.08	0.00
8.06	17.65	1.16	0.00	0.00
2.36	19.76	0.78	0.00	0.00
10.06	19.94	1.33	0.00	0.00
14.57	20.32	1.55	0.27	0.00
10.88	20.68	1.54	0.00	0.00
13.40	23.64	1.90	0.00	0.00
10.69	18.02	1.18	0.00	0.00
10.52	17.73	2.07	0.00	0.00
12.12	22.05	1.11	0.00	0.00
5.05	17.32	0.61	0.00	0.00
10.61	19.69	0.98	0.00	0.00
10.82	25.33	1.16	0.00	0.00
15.80	26.00	0.67	0.27	0.00
12.73	23.18	0.95	0.00	0.00
9.30	17.61	0.74	0.00	0.00
388.37				
9.71				

dorsal orange margin element 4 in mm2	sum dorsal orange margin elements in mm2 = dorsal orange margin complex area	dorsal orange margin complex area in relation to tail fin area (%)
0.00	0.93	5.43
0.00	0.00	0.00
0.00	1.03	4.71
0.00	0.56	2.72
0.00	0.18	1.05
0.00	0.24	1.04
0.00	0.30	1.63
0.00	0.29	1.45
0.00	0.08	0.53
0.00	0.36	2.03
0.00	0.14	0.73
0.00	0.00	0.00
0.00	0.00	0.00
0.00	0.72	3.02
0.00	0.28	1.51
0.00	0.00	0.00
0.00	0.59	2.98
0.00	0.00	0.00
0.00	0.42	1.53
0.00	0.29	1.43
0.00	0.68	3.94
0.00	0.55	2.70
0.00	0.32	1.46
0.00	0.19	0.82
0.00	0.67	3.31
0.00	0.63	3.24
0.15	1.06	4.75
0.00	0.56	2.27
0.00	0.00	0.00
0.00	0.61	3.36
0.00	0.51	2.39
0.00	0.41	2.46
0.00	0.44	2.31
0.00	0.48	2.07
0.00	0.46	2.27
0.00	0.00	0.00
0.00	0.15	0.67
0.00	0.16	0.67
0.00	0.33	1.95
0.00	0.10	0.45
		72.89
		1.82

dorsal orange margin element 4 in mm2	sum dorsal orange margin elements in mm2 = dorsal orange margin complex area	dorsal orange margin complex area in relation to tail fin area (%)
0.00	0.82	4.17
0.00	0.81	5.62
0.00	0.91	4.79
0.00	0.87	4.07
0.00	1.89	8.21
0.00	0.77	3.32
0.00	1.05	4.38
0.00	0.95	4.52
0.00	1.00	3.79
0.00	0.54	2.28
0.00	0.90	5.10
0.00	1.02	4.63
0.00	0.78	5.21
0.00	1.30	6.01
0.00	1.13	5.04
0.00	0.45	1.78
0.00	1.50	7.68
0.00	0.94	5.80
0.00	0.83	4.71
0.00	1.35	8.01
0.00	1.39	5.58
0.00	1.57	9.17
0.00	0.83	4.03
0.00	1.02	6.17
0.00	0.14	0.78
0.00	1.16	6.55
0.00	0.78	3.94
0.00	1.33	6.69
0.00	1.82	8.95
0.00	1.54	7.45
0.00	1.90	8.01
0.00	1.18	6.55
0.00	2.07	11.68
0.00	1.11	5.02
0.00	0.61	3.50
0.00	0.98	4.98
0.00	1.16	4.57
0.00	0.94	3.62
0.00	0.95	4.09
0.00	0.74	4.18
		214.63
		5.37

ventral orange margin element 1 in mm2	ventral orange margin element 2 in mm2	ventral orange margin element 3 in mm2	ventral orange margin element 4 in mm2
0.94	0.00	0.00	0.00
1.16	0.00	0.00	0.00
0.67	0.10	0.00	0.00
0.33	0.09	0.11	0.00
0.53	0.05	0.04	0.00
1.04	0.13	0.00	0.00
0.59	0.00	0.00	0.00
0.74	0.00	0.00	0.00
0.73	0.13	0.00	0.00
0.61	0.11	0.00	0.00
0.82	0.05	0.00	0.00
0.84	0.24	0.00	0.00
0.34	0.23	0.15	0.00
0.71	0.24	0.00	0.00
0.64	0.18	0.00	0.00
0.21	0.08	0.06	0.08
1.02	0.00	0.00	0.00
0.04	0.07	0.00	0.00
0.97	0.00	0.00	0.00
0.11	0.00	0.00	0.00
0.71	0.00	0.00	0.00
0.90	0.21	0.00	0.00
0.85	0.00	0.00	0.00
0.28	0.22	0.35	0.24
0.65	0.47	0.00	0.00
1.35	0.00	0.00	0.00
0.92	0.21	0.00	0.00
0.71	0.16	0.04	0.00
0.52	0.15	0.00	0.00
0.78	0.09	0.00	0.00
0.65	0.10	0.00	0.00
0.61	0.15	0.00	0.00
0.33	0.11	0.00	0.00
0.90	0.10	0.00	0.00
1.31	0.00	0.00	0.00
0.18	0.19	0.21	0.00
0.25	0.12	0.08	0.00
0.06	0.12	0.15	0.04
0.04	0.06	0.07	0.07
0.29	0.20	0.13	0.01

ventral orange margin element 1 in mm2	ventral orange margin element 2 in mm2	ventral orange margin element 3 in mm2	ventral orange margin element 4 in mm2
1.03	0.00	0.00	0.00
1.06	0.00	0.00	0.00
1.09	0.11	0.00	0.00
1.14	0.00	0.00	0.00
0.84	0.19	0.00	0.00
0.74	0.11	0.00	0.00
1.19	0.00	0.00	0.00
1.49	0.00	0.00	0.00
1.09	0.00	0.00	0.00
1.04	0.00	0.00	0.00
0.76	0.00	0.00	0.00
1.07	0.00	0.00	0.00
0.06	0.41	0.00	0.00
1.28	0.00	0.00	0.00
0.60	0.00	0.00	0.00
0.52	0.20	0.00	0.00
1.10	0.00	0.00	0.00
0.91	0.00	0.00	0.00
0.03	0.00	0.00	0.00
0.92	0.00	0.00	0.00
1.25	0.21	0.00	0.00
1.48	0.00	0.00	0.00
1.45	0.00	0.00	0.00
1.06	0.00	0.00	0.00
0.04	0.09	0.17	0.11
1.00	0.00	0.00	0.00
0.65	0.00	0.00	0.00
1.15	0.00	0.00	0.00
1.01	0.08	0.00	0.00
1.27	0.31	0.00	0.00
2.12	0.10	0.00	0.00
0.79	0.10	0.00	0.00
1.41	0.00	0.00	0.00
1.13	0.03	0.00	0.00
0.82	0.00	0.00	0.00
1.09	0.00	0.00	0.00
0.46	0.00	0.00	0.00
0.95	0.20	0.00	0.00
0.78	0.02	0.00	0.00
1.47	0.08	0.00	0.00

ventral orange margin element 5 in mm2	sum ventral orange margin elements in mm2 = ventral orange margin complex area
0.00	0.94
0.00	1.16
0.00	0.77
0.00	0.53
0.00	0.62
0.00	1.16
0.00	0.59
0.00	0.74
0.00	0.86
0.00	0.72
0.00	0.86
0.00	1.08
0.00	0.72
0.00	0.96
0.00	0.82
0.04	0.48
0.00	1.02
0.00	0.12
0.00	0.97
0.00	0.11
0.00	0.71
0.00	1.11
0.00	0.85
0.00	1.09
0.00	1.12
0.00	1.35
0.00	1.13
0.00	0.90
0.00	0.67
0.00	0.87
0.00	0.75
0.00	0.76
0.00	0.44
0.00	0.99
0.00	1.31
0.00	0.57
0.00	0.45
0.00	0.36
0.00	0.24
0.00	0.62

ventral orange margin element 5 in mm2	sum ventral orange margin elements in mm2 = ventral orange margin complex area
0.00	1.03
0.00	1.06
0.00	1.20
0.00	1.14
0.00	1.02
0.00	0.85
0.00	1.19
0.00	1.49
0.00	1.09
0.00	1.04
0.00	0.76
0.00	1.07
0.00	0.47
0.00	1.28
0.00	0.60
0.00	0.72
0.00	1.10
0.00	0.91
0.00	0.03
0.00	0.92
0.00	1.45
0.00	1.48
0.00	1.45
0.00	1.06
0.00	0.40
0.00	1.00
0.00	0.65
0.00	1.15
0.00	1.08
0.00	1.58
0.00	2.22
0.00	0.90
0.00	1.41
0.00	1.16
0.00	0.82
0.00	1.09
0.00	0.46
0.00	1.15
0.00	0.79
0.00	1.55

ventral orange margin complex area in relation to tail fin area (%)	sum orange on tail fin in mm2	percentage orange on tail fin
5.50	1.87	10.93
4.27	1.16	4.27
3.54	1.80	8.25
2.59	1.09	5.31
3.59	0.80	4.64
5.10	1.40	6.14
3.23	0.89	4.86
3.66	1.04	5.11
5.80	0.94	6.34
4.07	1.07	6.10
4.54	1.00	5.27
5.83	1.08	5.83
3.00	0.72	3.00
4.03	1.67	7.05
4.50	1.09	6.01
1.93	0.48	1.93
5.19	1.61	8.16
0.51	0.12	0.51
3.58	1.39	5.11
0.56	0.40	1.99
4.10	1.38	8.04
5.41	1.66	8.11
3.91	1.17	5.37
4.72	1.28	5.54
5.55	1.79	8.86
6.97	1.98	10.21
5.06	2.18	9.81
3.67	1.46	5.94
3.35	0.67	3.35
4.75	1.48	8.11
3.54	1.26	5.93
4.55	1.17	7.01
2.29	0.88	4.60
4.32	1.47	6.39
6.43	1.78	8.70
3.12	0.57	3.12
2.08	0.59	2.75
1.54	0.52	2.21
1.41	0.57	3.37
2.70	0.73	3.15
154.50		**227.39**
3.86		**5.68**

ventral orange margin complex area in relation to tail fin area (%)	sum orange on tail fin in mm2	percentage orange on tail fin
5.25	1.85	9.42
7.31	1.87	12.93
6.37	2.11	11.16
5.34	2.00	9.41
4.45	2.91	12.66
3.69	1.62	7.02
4.99	2.24	9.37
7.10	2.44	11.62
4.11	2.09	7.90
4.43	1.57	6.71
4.32	1.66	9.43
4.83	2.09	9.46
3.15	1.25	8.36
5.91	2.58	11.92
2.69	1.73	7.73
2.82	1.17	4.60
5.63	2.59	13.32
5.59	1.84	11.39
0.15	0.85	4.86
5.47	2.27	13.48
5.83	2.84	11.40
8.67	3.05	17.84
7.10	2.28	11.13
6.38	2.08	12.55
2.25	0.54	3.04
5.68	2.16	12.23
3.29	1.43	7.23
5.75	2.48	12.44
5.33	2.90	14.28
7.63	3.12	15.07
9.39	4.12	17.40
4.97	2.08	11.52
7.98	3.48	19.65
5.27	2.27	10.29
4.76	1.43	8.25
5.54	2.07	10.53
1.81	1.62	6.38
4.41	2.09	8.03
3.42	1.74	7.51
8.79	2.29	12.97
207.85		**422.48**
5.20		**10.56**

8.2 Table S4

individual	**anterior orange spot**	**posterior orange spot complex**
cross9_F2_frompair11_blondm_7735	0	0
cross9_F2_frompair2_blondm_6070	0	1
cross9_F2_frompair2_blondm_7489	0	1
cross9_F2_frompair3_blondm_5668	0	1
cross9_F2_frompair5_blondm_6755	0	1
cross9_F2_frompair6_blondm_6824	0	1
cross9_F2_frompair11_blondm_7574	0	1
cross9_F2_frompair3_blondm_7146	1	1
cross9_F2_frompair4_blondm_left_5704	0	2
cross9_F2_frompair3_blondm_7625	0	1
cross9_F2_frompair5_blondm_6426	0	1
cross9_F2_frompair7_blondm_6360	0	1
cross9_F2_frompair8_blondm_7614	0	2
cross9_F2_frompair2_blondm_5680	0	1
cross9_F2_frompair2_blondm_7562	0	1
cross9_F2_frompair8_blondm_left_5672	1	1
cross9_F2_frompair6_blondm_6278	0	1
cross9_F2_frompair6_blondm_6392	0	0
cross9_F2_frompair1_blondm_left_5644	0	0
cross9_F2_frompair1_blondm_left_5648	0	1
cross9_F2_frompair3_blondm_7144	0	2
cross9_F2_frompair8_blondm_6271	0	2
cross9_F2_frompair8_blondm_7615	0	2
cross9_F2_frompair2_blondm_7741	0	1
cross9_F2_frompair10_blondm_6723	0	1
cross9_F2_frompair1_blondm_5485	1	1
cross9_F2_frompair2_blondm_6525	0	0
cross9_F2_frompair2_blondm_7583	0	0
cross9_F2_frompair5_blondm_8018	0	0
cross9_F2_frompair6_blondm_6395	0	0
cross9_F2_frompair6_blondm_6409	0	0
cross9_F2_frompair1_blondm_6265	0	0
cross9_F2_frompair11_blondm_6286	1	0
cross9_F2_frompair2_blondm_7585	0	1
cross9_F2_frompair2_blondm_7739	0	1
cross9_F2_frompair2_blondm_8013	0	1
cross9_F2_frompair6_blondm_6408	0	1
cross9_F2_frompair1_blondm_left_5666	0	1
cross9_F2_frompair4_blondm_7613	0	1
cross9_F2_frompair10_blondm_6828	0	1
cross9_F2_frompair2_blondm_8014	1	1
cross9_F2_frompair7_blondm_7626	0	1
cross9_F2_frompair4_blondm_7608	0	2
cross9_F2_frompair7_blondm_6528	0	1
cross9_F2_frompair5_blondm_6432	0	0
cross9_F2_frompair6_blondm_6394	0	0
cross9_F2_frompair1_blondm_left_5678	0	0
cross9_F2_frompair2_blondm_6259	0	1
cross9_F2_frompair10_blondm_6768	0	1
cross9_F2_frompair7_blondm_left_5694	1	1
cross9_F2_frompair6_blondm_6818	0	2
cross9_F2_frompair6_blondm_6279	0	1
cross9_F2_frompair2_blondm_8012	0	0
cross9_F2_frompair8_blondm_6456	0	0
cross9_F2_frompair1_blondm_6263	0	1
cross9_F2_frompair7_blondm_6375	0	1
cross9_F2_frompair1_blondm_left_5646	0	0
cross9_F2_frompair7_blondm_7628	0	1
cross9_F2_frompair4_blondm_left_5642	0	1
cross9_F2_frompair4_blondm_6437	1	1

cross9_F2_frompair2_blondm_6523	0	1
cross9_F2_frompair6_blondm_6291	0	1
cross9_F2_frompair7_blondm_6449	0	1
cross9_F2_frompair7_blondm_left_5692	0	1
cross9_F2_frompair4_blondm_7610	0	2
cross9_F2_frompair2_blondm_6261	0	1
cross9_F2_frompair3_blondm_7147	0	0
cross9_F2_frompair5_blondm_8017	0	1
cross9_F2_frompair7_blondm_6451	0	1
cross9_F2_frompair7_blondm_6492	1	1
cross9_F2_frompair6_blondm_6406	0	2
cross9_F2_frompair7_blondm_6491	0	0
cross9_F2_frompair7_blondm_left_5696	0	1
cross9_F2_frompair2_blondm_left_5676	0	2
cross9_F2_frompair6_wtm_6277	1	0
cross9_F2_frompair2_wtm_6521	1	1
cross9_F2_frompair2_wtm_6522	1	1
cross9_F2_frompair2_wtm_6524	1	1
cross9_F2_frompair2_wtm_6806	1	1
cross9_F2_frompair2_wtm_6807	1	1
cross9_F2_frompair3_wtm_6366	1	1
cross9_F2_frompair3_wtm_6410	1	1
cross9_F2_frompair6_wtm_6280	1	1
cross9_F2_frompair1_wtm_5487	1	1
cross9_F2_frompair1_wtm_6399	1	1
cross9_F2_frompair7_wtm_6493	1	1
cross9_F2_frompair11_wtm_6288	1	1
cross9_F2_frompair2_wtm_6839	1	2
cross9_F2_frompair3_wtm_6411	1	2
cross9_F2_frompair5_wtm_6294	1	2
cross9_F2_frompair5_wtm_6295	1	2
cross9_F2_frompair6_wtm_6276	1	2
cross9_F2_frompair6_wtm_6404	1	2
cross9_F2_frompair7_wtm_6361	1	2
cross9_F2_frompair1_wtm_left_5656	1	2
cross9_F2_frompair2_wtm_5686	1	1
cross9_F2_frompair2_wtm_6526	1	1
cross9_F2_frompair5_wtm_6293	1	1
cross9_F2_frompair7_wtm_6363	1	1
cross9_F2_frompair7_wtm_6450	1	1
cross9_F2_frompair7_wtm_6452	1	1
cross9_F2_frompair8_wtm_6454	1	1
cross9_F2_frompair8_wtm_6517	1	1
cross9_F2_frompair2_wtm_5683	1	2
cross9_F2_frompair7_wtm_6362	1	2
cross9_F2_frompair4_wtm_5681	1	2
cross9_F2_frompair4_wtm_6438	1	2
cross9_F2_frompair10_wtm_6725	1	2
cross9_F2_frompair11_wtm_6287	1	2
cross9_F2_frompair2_wtm_5684	1	1
cross9_F2_frompair2_wtm_6838	1	1
cross9_F2_frompair4_wtm_6439	1	1
cross9_F2_frompair7_wtm_6527	1	1
cross9_F2_frompair3_wtm_6412	1	1
cross9_F2_frompair4_wtm_6440	1	2
cross9_F2_frompair7_wtm_6374	1	2
cross9_F2_frompair11_wtm_6285	1	1
cross9_F2_frompair2_wtm_6071	1	1
cross9_F2_frompair2_wtm_6809	1	1
cross9_F2_frompair2_wtm_6840	1	1
cross9_F2_frompair5_wtm_6292	1	1

cross9_F2_frompair6_wtm_6290	1	1
cross9_F2_frompair6_wtm_6407	1	1
cross9_F2_frompair1_wtm_5689	1	1
cross9_F2_frompair1_wtm_6266	1	1
cross9_F2_frompair8_wtm_6379	1	1
cross9_F2_frompair2_wtm_5685	1	2
cross9_F2_frompair1_wtm_6398	1	2
cross9_F2_frompair4_wtm_5682	1	2
cross9_F2_frompair7_wtm_6453	1	2
cross9_F2_frompair3_wtm_6413	1	1
cross9_F2_frompair6_wtm_6282	1	1
cross9_F2_frompair6_wtm_6289	1	1
cross9_F2_frompair6_wtm_6393	1	1
cross9_F2_frompair6_wtm_6405	1	1
cross9_F2_frompair7_wtm_6373	1	1
cross9_F2_frompair10_wtm_6724	1	1
cross9_F2_frompair4_wtm_6441	1	2
cross9_F2_frompair7_wtm_6365	1	1
cross9_F2_frompair10_wtm_6720	1	1
cross9_F2_frompair2_wtm_6260	1	2
cross9_F2_frompair1_wtm_6072	1	1
cross9_F2_frompair1_wtm_6267	1	1
cross9_F2_frompair8_wtm_6378	1	1
cross9_F2_frompair8_wtm_6455	1	1
cross9_F2_frompair6_wtm_6281	1	1
cross9_F2_frompair7_wtm_6364	1	1
cross9_F2_frompair5_wtm_6296	1	0

Anterior orange spot		
	present in %	absent in %
blond	10.81	89.19
wild-type	100.00	0.00

Posterior orange spot complex		
	present in %	absent in %
blond	75.68	24.32
wild-type	97.30	2.70

Dorsal orange spot complex		
	present in %	absent in %
blond	32.43	67.57
wild-type	44.59	55.41

Dorsal orange margin complex		
	present in %	absent in %
blond	78.38	21.62
wild-type	100.00	0.00

Ventral orange margin complex		
	present in %	absent in %
blond	100.00	0.00
wild-type	100.00	0.00

dorsal orange spot complex	dorsal orange margin complex	ventral orange margin complex
0	0	1
0	0	1
0	1	1
0	1	1
0	1	1
0	1	1
0	1	1
0	1	1
0	1	1
1	1	1
1	1	1
2	1	1
2	1	1
0	2	1
0	2	1
1	2	1
1	3	1
0	0	2
0	0	2
0	0	2
0	0	2
0	0	2
0	0	2
1	0	2
1	0	2
1	0	2
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1	2	2
1	4	2
0	0	3
0	0	3
0	0	3
1	0	3
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0	1	3
0	1	3
0	1	3

1	1	3
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1	1	3
1	1	3
2	2	3
0	1	4
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0	1	4
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2	1	1
2	1	1
0	2	1
0	2	1
0	2	1
1	2	1
0	1	2
0	1	2
0	1	2
0	1	2

0	1	2
0	1	2
0	1	2
0	1	2
0	1	2
0	1	2
0	1	2
0	1	2
0	1	2
1	1	2
1	1	2
1	1	2
1	1	2
1	1	2
1	1	2
1	1	2
1	1	2
2	1	2
2	1	2
2	1	2
0	2	2
0	2	2
0	2	2
1	2	2
2	2	2
2	2	2
0	2	4

equals number of elements (0 means absent)

number of elements (if present) 1 in %	number of elements (if present) 2 in %
82.14	17.86
69.44	30.56

number of elements (if present) 1 in %	number of elements (if present) 2 in %
83.33	16.67
72.73	27.27

number of elements (if present) 1 in %	number of elements (if present) 2 in %	number of elements (if present) more than 2 in %
77.59	18.97	3.45
85.14	14.86	0.00

number of elements (if present) 1 in %	number of elements (if present) 2 in %	number of elements (if present) more than 2 in %
22.97	47.30	29.73
58.11	40.54	1.35