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 polymorphisms within guppy populations [19–23].

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 pteridine to carotenoid 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
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 Cumana 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 kiaa 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, 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, 7E, 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].

We investigated the pigment spots and fin color patterns of male Cumana, Quare6, and Maculatus guppies. Cumana guppies are derived from a wild population in Venezuela [45]; the inheritance
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 Cumana 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 Trenatomus bernachii [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 cyan-o-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 Cumana blue spot

While the iridescent areas of Quare6 and Maculatus males are largely diffuse, Cumana 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
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 Plestodon laticaudatus [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. laticaudatus. 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
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 Cumana 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 Cumana 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 Cumana 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 Cumana males, more individuals need to be investigated to clarify whether indeed all Cumana 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
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 Cumana, 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 um 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 Cumana 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 Cumana 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 Cumana 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 Cumana 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 Cumana, 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 Cumana, Quare6, and Maculatus males is very similar, despite Cumana 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

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**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 Cumana male different from the one shown in Figure 2A. (B,C,E,G) TEM images. (A) Cumana 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 [64]. 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 und b TierSchG); all experiments were permitted by the Regierungspäsidium Tubingen (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 Cumanán and Quare strains originated from individuals that were collected in Venezuela near Cumanán 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 M2/FLII 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 Cumanán, 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 PO4 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 G² 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 ImagemJ 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 Cumanán 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.

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.

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.

Figure S4 Ultrastructure of Cumanán central black spot. (A) TEM image of Cumanán central black spot. (B) TEM image of Cumanán central black spot.
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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.

References

1. Winge O (1922) One-sided masculine and sex-linked inheritance in Lebistes reticulatus. Journal of Genetics 12: 145–162.
2. Winge O (1927) The location of eighteen genes in Lebistes reticulatus. Journal of Genetics 16: 1–43.
3. Haskins CP, Drozja JP (1938) Note on anomalous inheritance of sex-linked color factors in the Guppy. Am Nat 72: 571–574.
4. Goodrich HB, Josephson ND, Trinka JP, Slate JM (1944) The cellular expression and genetics of two new genes in Lebistes reticulatus. Genetics 29: 384–592.
5. Winge O, Ditlevens E (1947) Colour inheritance and sex determination in Lebistes. Heredity 1: 65–63.
6. Endler JA (1983) Natural and sexual selection on color patterns in poeciliid fishes. Environmental biology of Fishes 9: 173–190.
7. Endler JA (1995) Multiple-trait coevolution and environmental gradients in guppies. Trends Ecol Evol 10: 22–29.
8. Kohler VA, Feiters A, Weigl D, Dreyer C (2013) Pigment Pattern Formation in the Guppy, Poecilia reticulata, Involves the Kit and Cafra Receptor Tyrosine Kinases. Genetics 194: 631–646.
9. Kodrim-Brown A (1985) Female preference and sexual selection for male coloration in the guppy (Poecilia reticulata). Behavioral Ecology and Sociobiology 17: 199–205.
10. Houde AE (1987) Mate choice based upon naturally occurring color-pattern variation in a guppy population. Evolution: 1–10.
11. Grether GF, Hudson J, Endler JA (2001) Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (Poecilia reticulata). Proceedings of the Royal Society of London Series B: Biological Sciences 268: 1245–1253.
12. Grether GF, Cummings ME, Hudson J (2005) Countergradient variation in the sexual coloration of guppies (Poecilia reticulata): drosopterin synthesis balances carotenoid availability. Evolution 59: 175–188.
13. Endler JA (1980) Natural selection on color patterns in Poecilia reticulata. Evolution 34: 76–91.
14. Deere KA, Grether GF, Sun A, Sinheimer JS (2012) Female mate preference explains countergradient variation in the sexual coloration of guppies (Poecilia reticulata). Proc Biol Sci 279: 1684–1690.
15. Endler JA (1987) Predation, light intensity and courtship behaviour in Poecilia reticulata (Pisces: Poeciliidae). Animal Behaviour 35: 1376–1383.
16. Brooks R (1996) Melanin as a visual signal amplifier in male guppies. Naturwissenschaften 83: 39–41.
17. Tripathi N, Hoffmann M, Dreyer C (2008) Natural variation of male ornamental traits in the guppy, Poecilia reticulata: a possible case of incipient speciation. Journal of Evolutionary Biology 12: 138–153.
18. Tripathi N, Hoffmann M, Weigel D, Dreyer C (2009) Linkage analysis reveals a novel genetic constraint in guppy pigmentation. Proc Biol Sci 276: 2195–2208.
19. Goda M, Fujii R (2001) Coloration and chromatophores of the domino damsel, Dascyllus trimaculatus. Zoolog Sci 18: 163–174.
20. Hirata M, Nakamura K, Kanemaru T, Shibata Y, Kondo S (2003) Pigment cell pattern formation by contact-dependent depolarization. Science 335: 657–660.
21. Hirata M, Nakamura K, Kondo S (2005) Pigment cell distributions in different tissues of the zebrafish, with special reference to the striped pigment pattern. Dev Dyn 234: 293–300.
22. Tanigami T, Hayagi K, Sugimoto M, Hasegawa M (2006) Ultrastructure of the dermal chromatophores in a lizard (Scincidae: Plestiodon latiscutatus) with conspicuous body and tail coloration. Zoolog Sci 23: 793–799.
23. Alexander HJ, Breeden F (2004) Sexual isolation and extreme morphological divergence in the Cumaná guppy, Poecilia reticulata, as revealed by the rapid-freezing and freeze-substitution method. Cell Tissue Res 319: 213–223.
24. Fujii R, Tagschi S (1976) Ultrastructure of nerve-melanophore relationships in the guppy, Lebistes reticulatus. Annot Zool Japon 40: 123–131.
25. Gundersen R, Rivera E (1982) An ultrastructural study of the development of the dermal iridophores and structural pigmentation in Poecilia reticulata (Peters). Journal of Morphology 172: 349–359.
26. Ploegmakers EL, Deelder JA, Van der Sluis J (1990) Pigment Cell Research 3: 33–37.
27. Menter DG, Ozawa M, Tatemoto K, Takeuchi JH (1986) Transmission electron microscopic study of the guppy, Lebistes reticulatus. Annot Zool Japon 50: 88–91.
28. Takeuchi JH (1975) Electron microscopic study on erythropoietic guppies. Lebistes reticulatus. Annot Zool Japon 45: 242–251.
29. Takeuchi JH (1976) Electron microscopy of two types of reflecting chromatophores (iridophores and leucophores) in the guppy, Lebistes reticulatus. Cells Tissues Organs 173: 17–27.
30. Braasch I, Scharl M, Vollrath JN (2007) Evolution of pigment synthesis pathways by gene and genome duplication in fish. BMC Evol Biol 7: 74.
57. Ofilpant LW, Hudon J (1993) Pteridines as reflecting pigments and components of reflecting organelles in vertebrates. Pigment Cell Research 6: 205–208.
58. Goda M, Fujii Y, Sugimoto M, Fujii R (2013) Novel Dichromatic Chromatophores in the Integument of the Mandarin Fish Synchiropus splendidus. Biol Bull 224: 14–17.
59. Goda M, Ohata M, Ikoma H, Fujii Y, Sugimoto M, et al. (2011) Integumental reddish-violet coloration owing to novel dichromatic chromatophores in the teleost fish, Pseudochromis diadema. Pigment Cell Melanoma Res 24: 614–617.
60. Goda M, Toyohara J, Visconti A, Oshima N, Fujii R (1994) The Blue Coloration of the Common Surgeonfish, Paracanthurus hepatus-I. Morphological Features of Chromatophores. Zoolog Sci 11: 527–535.
61. Fujii R, Kasukawa H, Miyaji K, Oshima N (1989) Mechanisms of Skin Coloration and Its Changes in the Blue-Green Damselfish, Chromis viridis. Zoolog Sci 6: 477–486.
62. Fujii R (1993) Coloration and chromatophores. The physiology of fishes: 535–562.
63. Magurran AE (2005) Evolutionary ecology: the Trinidadian guppy. New York: Oxford University Press. xi, 206 p.
64. Iga T (1976) The mode of action of potassium ions on the leukophores of a freshwater teleost, Oryzias latipes. Journal of Experimental Zoology 205: 413–421.
65. Harris MP, Rohner N, Schwarz H, Perathoner S, Konstantinidis P, et al. (2008) Zebrafish eda and edar mutants reveal conserved and ancestral roles of ectodysplasin signaling in vertebrates. PLoS Genet 4: e1000206.
66. Hawkes JW (1974) The structure of fish skin. Cell Tissue Res 149: 159–172.