
EVALUATION OF DEVELOPMENT
STABILITY

Effect of Thyroid Hormones on the Development of Asymmetric Pigment Patterns in Teleost Fish: Experimental Data on the Example of *Amatitlania nigrofasciata* (Cichlidae) and *Poecilia wingei* (Poeciliidae)

D. V. Prazdnikov*

Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Moscow, 119071 Russia

*e-mail: pdyfish3409@rambler.ru

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Abstract—Thyroid hormones (THs) are the most important regulators of coloration ontogeny in teleosts. It has been shown experimentally that alterations in the hormonal status due to prolonged treatment with a biologically active form of THs, triiodothyronine, causes changes in the development of adult pigment patterns in bilaterally symmetrical *Amatitlania nigrofasciata* and *Poecilia wingei*. Induced hyperthyroidism in experimental fishes leads to an acceleration of coloration ontogeny and an increase in the frequency of asymmetry in various elements of the melanistic pattern. The revealed dependence of the development of asymmetric pigment patterns on the activity of the thyroid axis in fishes with varying degrees of metamorphic coloration transformations offers the prospect for further experimental studies of the role of the TH-signaling pathway in the evolution of teleosts.

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INTRODUCTION

Teleosts have the greatest variety of pigment patterns and types of chromatophores among vertebrates. Eight types of chromatophores that differ in chemical composition and reflecting abilities were found in the fish: melanophores (black–brown), iridophores (metallic iridescence), leucophores (shiny white–yellow), xanthophores (yellow–orange), erythrophores (red), cyanophores (blue), erythro–iridophores (reddish violet), and erythro–cyanophores (red–blue) (Fujii, 1993; Scharl et al., 2015). The adult pigment pattern of teleost fishes is formed as a result of complex interactions of different cell lines of chromatophores, integument tissues, the nervous system, and endocrine regulation at various stages of ontogeny.

Thyroid hormones (THs) are the most important regulators of the ontogeny of teleosts, affecting the activity of a wide range of tissues and processes (Janz, 2000; Blanton and Specker, 2007). The biologically active form of THs, triiodothyronine (T_3), controls complex cascades of target genes through interaction with specific TH receptors (transcription factors belonging to the nuclear receptor superfamily) and acts as a direct activator of the expression of many genes (Basset et al., 2003; Cheng et al., 2010; Grøntved et al., 2015). T_3 coordinates the ontogeny of various lines of pigment cells and regulates the transcriptional activity of the genes responsible for the forma-

tion of the adult pigment pattern of fish (McMenamin et al., 2014; Guillot et al., 2016; Saunders et al., 2019). Moreover, THs are involved in many pleiotropic processes in fish, including metamorphic transformations and the development of symmetric and asymmetric morphological structures (McMenamin and Parichy, 2013; Campinho et al., 2018; Campinho, 2019).

The effects of T_3 on the variability of the pigment patterns in various species of teleosts were previously shown (Yoo et al., 2000; Clement et al., 2001; McMenamin et al., 2014; Prazdnikov and Shkil, 2019a). In addition, in cichlid fishes (Cichlidae), it was found that the alterations in the hormonal status leads to change in the type of coloration ontogeny and an increase in phenotypic variability (Prazdnikov and Shkil, 2019a, 2019b). However, the effects of THs on the development of asymmetric pigment patterns in bilaterally symmetrical fish remain practically unstudied.

The purpose of this work is to evaluate the effects of THs on the development of asymmetric pigment patterns in two phylogenetically distant species of teleosts, grown under conditions of hyperthyroidism.

MATERIALS AND METHODS

As experimental species of fish, we used *Amatitlania nigrofasciata* (Günther, 1867) (Perciformes, Cichlidae, Cichlasomatinae) and *Poecilia wingei* Poeser,

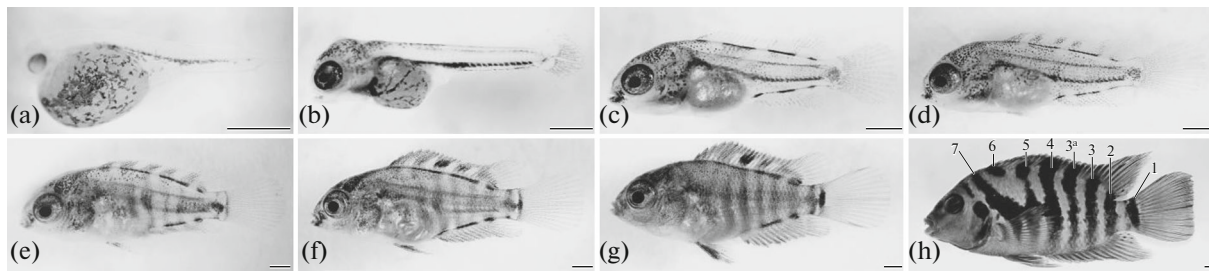


Fig. 1. The development of the pigment pattern in *Amatitlania nigrofasciata*: (a, b) early larval stage; (c, d) late larval stage; (e, f, g) juvenile stage; (h) adult. The numerals denote the numbers of vertical bars. Scale: 1 mm.

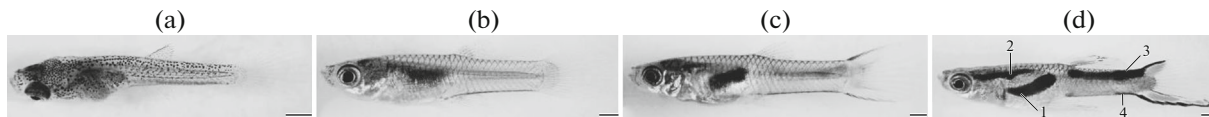


Fig. 2. The development of juvenile (a–c) and adult (d) pigment patterns in male *Poecilia wingei*. The numerals denote the melanistic pattern elements. Scale: 1 mm.

Kempkes and Isbrücker, 2005 (Cyprinodontiformes, Poeciliidae, Poeciliinae). The choice of these species of model fish was caused by the participation of the main types of chromatophores in the formation of coloration, the varying degrees of metamorphic transformations of pattern (Figs. 1, 2), and the important role of the pigment patterns in life and evolution. In addition, the above species are convenient for experimental work, as they are relatively simple to raise, can be bred easily in aquaria, and are short-cycle fish species.

For the experiment with *A. nigrofasciata*, a clutch obtained by spawning a pair of fish was used. Starting from the early larval stage (Fig. 1a), they were divided into two groups (80 individuals per group), which were reared under different hormonal regimes before the formation of adult coloration: (i) the control group with a natural TH status and (ii) the hyperthyroid group with a high T_3 level. For the experimental work with *P. wingei*, offspring from females giving birth on the same day were taken and randomly divided into two equal groups (74 individuals each), which were also reared in two hormonal regimes (natural hormonal status and hyperthyroidism) up to the formation of the adult pigment pattern. Changes in the TH status were induced by the traditional methods (Brown, 1997; Prazdnikov and Shkil, 2019a). Hyperthyroidism in model fish was induced by the addition of the active form of the TH—3,5,3'-triiodo-L-thyronine (T_3) (Sigma-Aldrich, China) into the aquarium water up to the concentration 0.15 $\mu\text{g}/\text{mL}$. The concentration of T_3 was chosen in such a way as to provoke changes in the rate and timing of the development of the pigment pattern, but not cause a significant increase in mortality or severe physiological stress in the experimental groups. Every other day, 1/3 of the

water from each aquarium was replaced. The necessary amounts of T_3 were added to the water up to the predetermined concentrations. The other conditions of the experiment (temperature, aeration, the stocking density of fish, feeding, light regime, background of the aquarium walls, etc.) were the same for all the groups.

In all groups of fish, we registered the time of formation of the definitive pigment pattern: for *A. nigrofasciata*, the days post-fertilization (dpf), and for *P. wingei*, the days after birth (adb), since the species belongs to ovoviviparous fish. Adult fish were photographed with a Canon EOS 100D digital camera (Japan) in a narrow aquarium equipped with a ruler (1 mm division). Each individual was photographed from two sides. The standard length (SL, mm) of adult fish was measured on the photographs.

Due to the fact that the main elements of the pigment pattern in model fishes mainly consist of melanophores, when analyzing the asymmetry of the pigment pattern, only melanistic elements on the right and left sides of the body were taken into account. To analyze the pigment pattern of *A. nigrofasciata*, we used the classification of melanistic elements proposed for Mesoamerican cichlids (Řičan et al., 2005; Prazdnikov and Shkil, 2019a), with allocation of eight postcranial vertical bars (Fig. 1h). Since the same melanistic pattern was noted in both genders, in males and females we took into account changes in bars, breaks and fusions on the left side of the body relative to the right side. In *P. wingei*, only males were used for analysis, since the females have a simple reticulate pattern of melanophores without additional elements. According to the classification of coloration elements of male *P. wingei* (Kottler et al., 2013), four melanistic

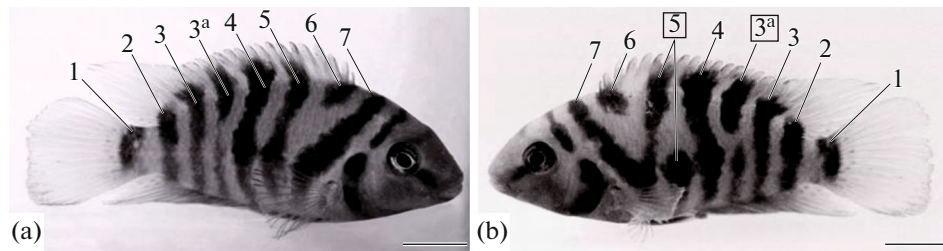


Fig. 3. The asymmetry of the pigment pattern (a, b) in the control group of *Amatiilania nigrofasciata*. The numerals denote the numbers of melanistic elements. The numerals in the frame denote the asymmetric elements of the pattern relative to the right side of the body. Scale: 10 mm.

elements were identified for analysis (Fig. 2d). In the pigment pattern of males, changes in the shape and position of melanophore patches and stripes on the left side relative to the right side of the body were taken into account.

In assessing the asymmetry, we used indicators and methods proposed for the analysis of animal asymmetry (Zakharov, 1987; Zakharov et al., 2001) with modifications that take into account the features of the traits analyzed in model objects. For assessing the asymmetry of the pigment pattern in the experimental groups of fish, we calculated (i) the frequency of asymmetric manifestation per individual (A_i), (ii) the average frequency of asymmetric manifestation per element of the pattern (A_{av}), and (iii) the relative frequency of occurrence of the asymmetric pattern element (A_r). A_i was calculated as the ratio of the number of individuals with an asymmetric pattern to the total number of all individuals in the group. A_{av} was calculated by the formula $A_{av} = (\sum E_p)/n$, where E_p is the number of asymmetric pattern elements in each individual divided by the number of pigment pattern elements used in the analysis, and n is the number of individuals in the group. A_r was calculated as the ratio of the number of individuals asymmetric by a given pattern element to the total number of asymmetric individuals in the group. In the analysis of asymmetry, we took into account changes in the melanistic pattern elements on the left side relative to the right side of the body.

RESULTS

The development of the pigment pattern in *A. nigrofasciata* is a complex process with distinct ontogenetic stages, pronounced by metamorphic transformations of the larval type of the pigment pattern into the adult type (Fig. 1). The larval pigment pattern consists of two horizontal melanophore stripes (Figs. 1b, 1c). The adult pattern begins to develop during the larval–juvenile transition, when sections of vertical melanophore bars are formed (Figs. 1d, 1e).

In the control group of *A. nigrofasciata*, the adult melanophore pattern with eight vertical bars on the trunk was finally formed by 100 dpf. Of the 79 individ-

uals studied, an asymmetric pattern was found in only one individual simultaneously with two vertical bars (Fig. 3).

In the hyperthyroid group of *A. nigrofasciata*, the development of the melanistic pigment pattern was accelerated in comparison with the control group. The development of adult elements of the pattern, vertical bars, was completed by 65 dpf. In majority of hyperthyroid fish was five bars on the trunk as a result of the absence or fusion in the pattern of bars 3^a, 5, 6 (Fig. 4). At the same time, heterochronies led to deviations in the development of fin structures and an increase in the frequency of asymmetry by melanistic elements of the pigment patterns (Fig. 4, Table 1). The highest relative frequency of occurrence among asymmetric pattern elements was in bar 3 ($A_r = 0.61$), which is represented in the pattern by various variants of breaks and fusions with vertical bar 2 ($A_r = 0.42$) (Figs. 4a–4f, 4n). The smallest relative frequency of asymmetry was observed in bars 4 ($A_r = 0.16$) (Fig. 4j) and 5 ($A_r = 0.06$) (Figs. 4d, 4t). Among asymmetric hyperthyroid *A. nigrofasciata*, in 29% of individuals we observed asymmetry simultaneously by two elements of the pigment pattern (Figs. 4a–4d, 4g–4j, 4m–4p).

In *P. wingei*, metamorphic transformations of the pigment pattern (Fig. 2) are less pronounced than in *A. nigrofasciata*. The larval pattern of *P. wingei* consists of melanophores that form a rhombic reticulate on the trunk (Fig. 2a). In females, throughout ontogeny, a melanophore reticulate pattern is retained, while in males, in addition to xanthophore elements, on this pattern additional melanophore elements are superimposed (Figs. 2b–2d): spot–stripe (element 1), anterior (element 2) and posterior (element 3) dorsal horizontal stripes, and the ventral lining of the caudal peduncle (element 4).

In the control group of *P. wingei*, the definitive melanophore pattern in males was finally formed by 95 adb. Asymmetry was observed only in one element of the pigment pattern—spot–stripe (Figs. 5a–5d).

In the hyperthyroid group of *P. wingei*, the ontogeny of the pigment pattern was accelerated compared to that of males from the control group; the formation of melanistic elements was completed by 60 adb.

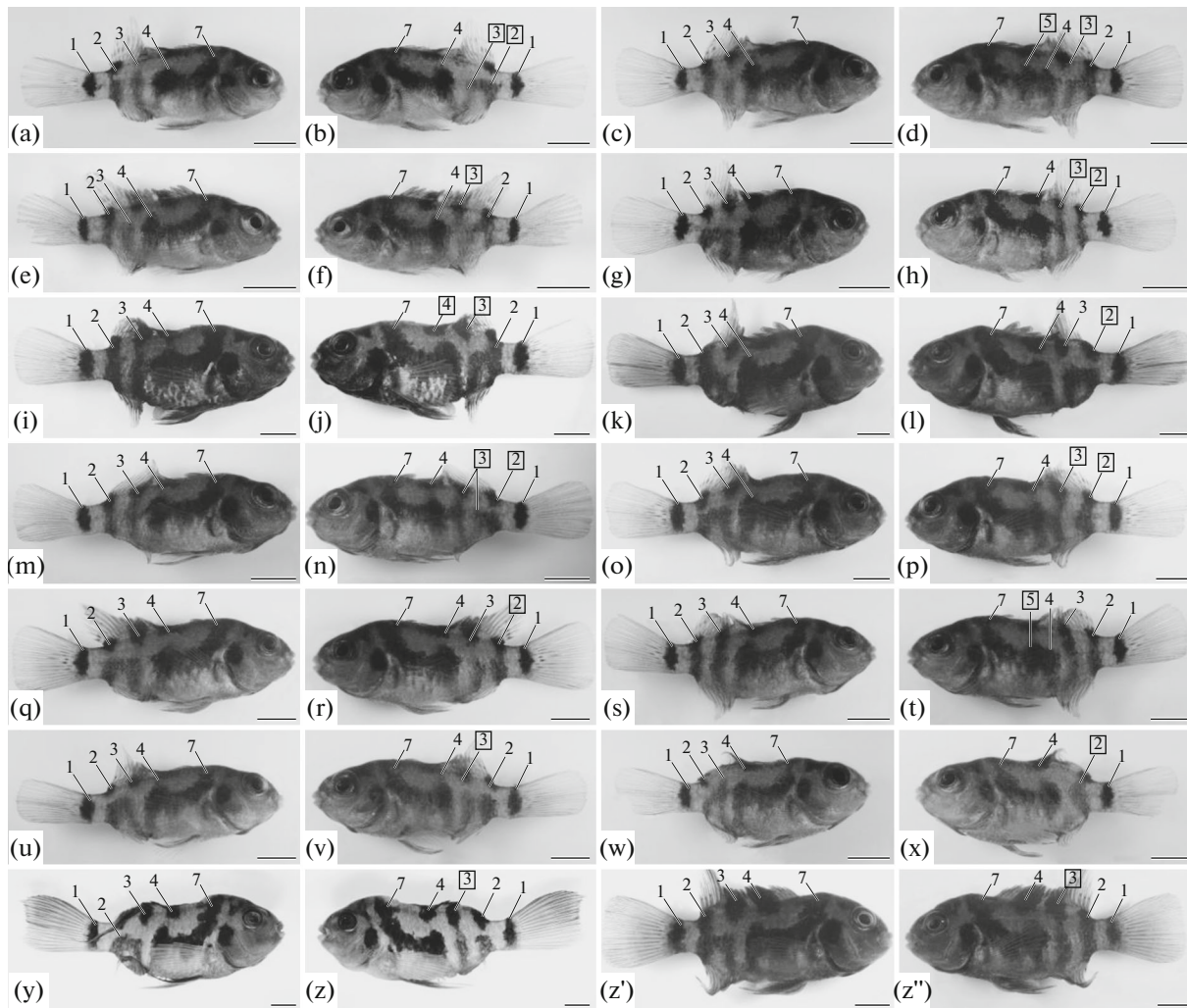


Fig. 4. The asymmetry of the pigment pattern (a–z'') in the hyperthyroid group of *Amatitlania nigrofasciata*. The numerals denote the numbers of melanistic elements. The numerals in the frame denote the asymmetric elements of the pattern relative to the right side of the body. Scale: 5 mm.

Moreover, in hyperthyroid males an increase in phenotypic variability and the frequency of asymmetry in the melanistic elements of the pigment pattern was observed (Fig. 6, Table 1). The highest relative frequency of asymmetry was in the first element of the pigment pattern ($A_r = 1$), which was transformed into a separate short stripe and spot, or spots of various sizes and shapes (Fig. 6), including eyespots (Figs. 6p, 6q). The smallest relative frequency of asymmetry was

by the anterior dorsal stripe ($A_r = 0.1$), which formed asymmetric breaks with other melanistic elements of the pattern (Fig. 6p). Among asymmetric hyperthyroid *P. wingei* in 40% of males, asymmetry was observed simultaneously in elements 1 and 3 of the pigment pattern (Figs. 6b, 6e–6j).

In two species of model fish in the composition of all analyzed coloration elements, in addition to the overwhelming population of melanophores, another

Table 1. The frequency of asymmetry of the pigment pattern in the experimental groups of *Amatitlania nigrofasciata* and *Poecilia wingei*

Species	Control group		Hyperthyroid group	
	A_i	A_{av}	A_i	A_{av}
<i>A. nigrofasciata</i>	0.01 ($n = 79$)	—	0.41 ($n = 75$)	0.10
<i>P. wingei</i>	0.04 ($n = 47$)	0.01	0.26 ($n = 38$)	0.12

A_i is the frequency of asymmetric manifestation per individual; A_{av} is the average frequency of asymmetric manifestation per element of the pattern; n is the number of individuals analyzed in the group.

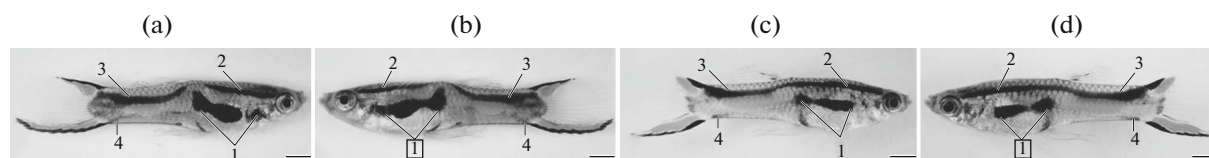


Fig. 5. The asymmetry of the pigment pattern (a–d) in the control group of *Poecilia wingei*. The numerals denote the numbers of melanistic elements. The numerals in the frame denote the asymmetric elements of the pattern relative to the right side of the body. Scale: 2 mm.

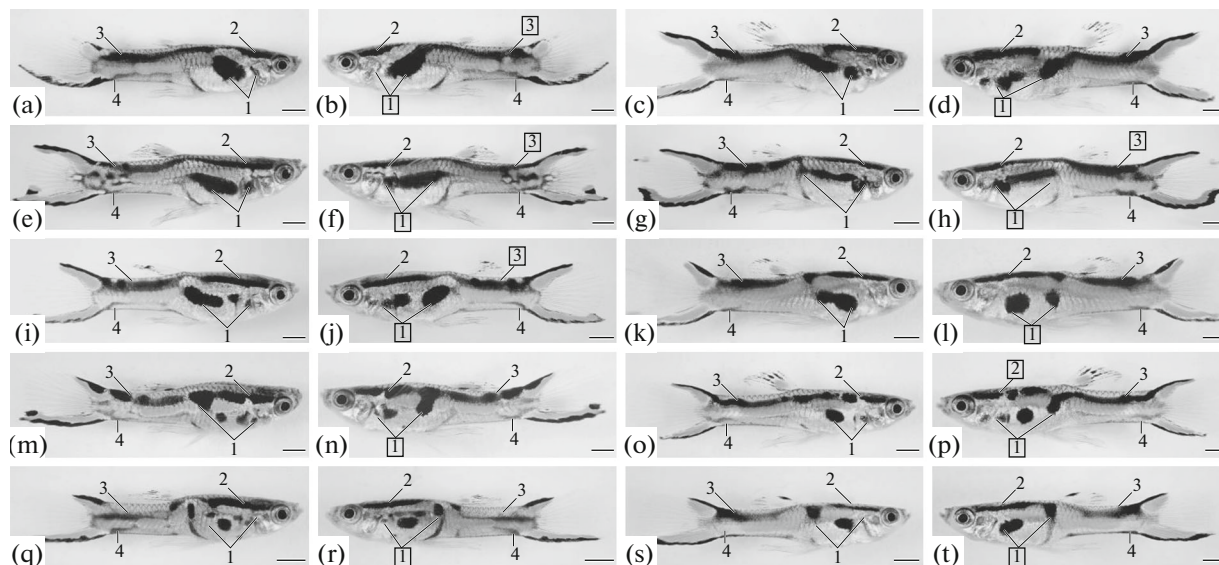


Fig. 6. The asymmetry of the pigment pattern (a–t) in the hyperthyroid group of *Poecilia wingei*. The numerals denote the numbers of melanistic elements. The numerals in the frame denote the asymmetric elements of the pattern relative to the right side of the body. Scale: 2 mm.

type of pigment cells was also included—iridophores, which are usually located under a layer of melanophores or on the periphery of melanistic elements.

DISCUSSION

Experimental data indicate the influence of TH on the development of asymmetric pigment patterns in the two model fish species. In *A. nigrofasciata* and *P. wingei* grown under hyperthyroidism, there was an accelerated coloration ontogeny and an increased frequency of asymmetry of the pigment pattern relative to fishes from the control group.

Heterochronic shifts in the ontogeny of *A. nigrofasciata* led to pronounced morphological consequences and an increase in phenotypic variability. The decrease in the number of vertical bars in the pigment pattern is associated with hormonally induced changes in the timing of the development of pigment cell lineages, which, in turn, caused changes in the type of coloration ontogeny, as was experimentally shown earlier (Prazdnikov and Shkil, 2019a). In hyperthyroid *A. nigrofasciata*, the highest relative frequency of

occurrence among asymmetric elements was observed in bars 3 and 2, which formed various combinations of fusions and/or breaks in the pigment pattern on both the right and left sides of the body. The asymmetry of these two bars is probably due to the later formation in coloration during the larval–juvenile transition (resolution of a broad median melanophore patch into bars) compared with other vertical bars and a greater “sensitivity” to increased level of TH (the migratory potential of melanoblasts in the skin).

In hyperthyroid male *P. wingei*, as well as in *A. nigrofasciata*, an increase in phenotypic variability was observed. Among the four melanistic elements of the pattern on the trunk, the greatest variability was in element 1 (spot–stripe). By this element asymmetry in all hyperthyroid males with asymmetric pigment patterns was observed.

We suggest that the high frequency of asymmetry by the melanistic elements of the adult pigment pattern in hyperthyroid fishes is associated with different levels of expression of melanophore pathway genes in the skin on the right and left sides of the body. It is known that in different species of flatfishes asymmet-

ric coloration develops during metamorphic transformations controlled by THs, when the bilaterally symmetrical larva transforms into an asymmetric juvenile (Campinho, 2019). Moreover, despite the fact that the unpigmented precursors of melanophores are located on both sides of the body, the adult melanophores differentiate only on the upper side, thereby causing asymmetric pigmentation (Watanabe et al., 2008; Yamada et al., 2010; Darias et al., 2013; Washio et al., 2013). Alterations of TH status during ontogeny of the pigment pattern of teleost fishes affects the times of appearance and morphogenetic behavior of various lines of chromatophores, including the total number of cells and the cascade of interactions, which ultimately leads to serious changes in the adult pigment patterns (McMenamin et al., 2014; Parichy and Spiewak, 2015; Prazdnikov and Shkil, 2019a; Saunders et al., 2019). Induced hyperthyroidism in *A. nigrofasciata* and *P. wingei* probably influenced bilateral migration, differentiation, and also the interaction between cell populations of melanophores and iridophores, which led to the development of asymmetric pigment patterns, including those with a transitional state of melanistic elements.

The asymmetry by melanistic coloration elements—spots and bars—was observed earlier in other species from the family Poeciliidae (Sheridan and Pomiankowski, 1997; Morris et al., 2005). It is assumed that the asymmetry by the pigment pattern in the population is maintained by sexual selection (Gross et al., 2007; Morris et al., 2012). In hyperthyroid male *P. wingei*, asymmetry occurs not only in melanistic elements, but also in elements of a yellow–orange pattern (spots and stripes) formed by xanthophores (unpublished data). It can be assumed that such asymmetric males may have an advantage during courtship by increasing their potential attractiveness when demonstrating coloration in front of females (exaggerating the quantity of the orange signal, including due to a combination with asymmetric melanophore elements). Previous studies on *Andinoacara rivulatus* (Cichlidae) have shown that hyperthyroidism, among other things, affects the development of secondary sexual characteristics and spawning behavior (Prazdnikov, 2018; Prazdnikov and Shkil, 2019b). All these data indicate that THs are able to regulate many ontogenetic processes in teleost fishes, including coordinate the work of other hormonal systems. However, the above assumptions require additional experimental investigations.

CONCLUSIONS

The development of the adult pigment pattern in *A. nigrofasciata* and *P. wingei* is coordinated by THs. Alterations in hormonal status caused by prolonged T_3 treatment lead to an acceleration of coloration ontogeny and an increase in the frequency of asymmetry in the melanistic elements of the adult pigment pattern.

The similarity of reactions on the alterations of the TH level in two phylogenetically distant species of experimental fish with varying degrees of metamorphic coloration transformations indicates common mechanisms for regulating the development of pigment patterns in bilaterally symmetrical fishes. Furthermore, the results obtained indicate the participation of THs in the coordination of bilateral migration of chromatophores, and also open up new perspectives for studying the role of the thyroid hormone signaling pathway in the evolution of coloration of Teleostei.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

ADDITIONAL INFORMATION

This article was translated by the author.

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