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Morphological differences in the skin of marble trout *Salmo marmoratus* and of brown trout *Salmo trutta*

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Abstract: Despite being genetically very closely related, the marble trout *Salmo marmoratus* and the brown trout *Salmo trutta* exhibit marked phenotypic differences, particularly with regard to skin pigmentation. Histological analysis of skin from the head and gill cover of differently aged individuals of the two species was carried out in order to characterize differences in skin structure. The basic structure of skin of the individuals studied corresponded with that described for other salmonids, though the head epidermis was somewhat thicker in *S. marmoratus* than in *S. trutta*, thickening with age in both species. Numerous secretory goblet cells and sporadic secretory sacciform cells were observed in the upper and middle part of the epidermis in both species. Melanophores were present in both species only in the dermis, and were bigger in *S. marmoratus* and present at lower average density than in *S. trutta*, and more or less constant across all age classes. In adult *S. marmoratus* with fully established marble pigmentation, light areas at low density with small (i.e. aggregated) melanophores were present, while in *S. trutta* melanophores were more uniformly distributed. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 2, 255–262)*

Key words: melanophore, epidermis, dermis, color pattern, pigmentation

Introduction

Differences in skin color or skin color pattern, both at the inter- and intra-specific level, are characteristic of salmonid fishes, where species-specific color patterns are fully established at the adult stage. In earlier stages, the phenotypes tend to be very similar, composed of dark vertical spots on the flanks, known as parr marks [1, 2]. Such variations in skin color pattern between species and life-stage are primarily dependent upon differences in the morphology, density and distribution of chromatophores in the skin defined as morphological color changes [3, 4]. Morphological color changes have a fundamental and long-lasting impact on external coloration, as opposed to quick and reversible physiological color changes.

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Recently, Leclercq et al. [3] proposed a distinction of morphological color changes into two types: ultimate and proximate. Ultimate morphological color changes are associated with the transition between two life stages such as larvae–juvenile and juvenile–adult. The formation of color pattern in teleosts at a given developmental stage is under strong genetic control [5–7]. In contrast, proximate morphological color changes refer to the morphological modulations of a given life-stage skin color in response to variations in different environmental factors (nutrition, solar-radiation, background adaptation) and social interactions. [3].

Both ultimate and proximate morphological changes are very common in salmonid fishes [3, 8, e.g. 9], including species of the genus *Salmo*. Of the numerous *Salmo* species recognized by Kottelat and Freyhof [10], the marble trout, *Salmo marmoratus* Cuvier, 1829, is one of the most distinctive. It is characterized by a distinctive marbled color pattern (Figure 1) and is native to the North Adriatic river systems, (i.e. the Po river system in Italy and the Soča river system in Slovenia and Italy). Phenotypically similar trout have been described also in some rivers of

Table 1. Body-size parameters of fish. Values represent mean ± SD (n = 3)

Age [years]	Average body weight [g]		Average size SL [cm]	
	S. marmoratus	*S. trutta*	*S. marmoratus*	*S. trutta*
0+	2.6 ± 0.3	2.1 ± 0.1	6.4 ± 0.2	5.9 ± 0.2
1+	70.5 ± 8.8	70.0 ± 17.3	18.0 ± 0.5	17.9 ± 1.0
2+	413.3 ± 80.2	138.7 ± 20.6	32.6 ± 2.3	23.8 ± 1.4
3+	623.3 ± 197.3		36.4 ± 3.3	

SL — standard length

the western Balkans that flow into the Adriatic Sea. The northern populations of *S. marmoratus* are also morphologically distinct from other *Salmo* species and subspecies [11].

The present study aimed to characterize skin in *S. marmoratus* and compare it to skin in the phylogenetically closely related brown trout, *Salmo trutta* L., in order to determine differences between these species in skin structure and secretory cells population, and the morphology, density and distribution of chromatophores in marble trout that cause its distinctive color pattern.

Material and methods

Salmo marmoratus individuals from four age-classes (0+, 1+, 2+ and 3+ years) were collected from Tolminka fish farm (46°11´7´´N, 13°44´17´´E) and *S. trutta* individuals (0+ and 1+ years) from Bled fish farm (46°22´13´´N, 14°5´15´´E) and Brinta stream (2+) (46°13´16´´N, 13°39´27´´E), Slovenia. Skin samples from *S. marmoratus* (n = 12; three per age-class) and *S. trutta* individuals (n = 9; three per age-class) from across the age range were taken for histological analysis (Table 1).

Skin samples from the dorsal part of the head and gill cover were fixed in 10% buffer formalin and subsequently transferred and stored in 70% ethanol. The samples from the head, usually used for the study of the fish epidermis, were embedded in paraffin wax and cut, perpendicular to the surface, into 7-μm-thick slices. Hematoxylin-eosin, Mallory and Papanicolaou methods were used for general histological staining.

The thickness of head epidermis (A) was determined as the average value of thickness at 25 random places on each sample. The proportion of secretory cells (B) in the epidermis was assessed using morphometric point network (with a density of 10 μm × 10 μm) applied to 10 × magnification of histological cross-sections of epidermis. Approximately 1,000–1,500 points were evaluated in each sample. The absolute values (S_a) of secretory cells in epidermis tissue was determined from the equation $S_a = A \times B/100$. For detailed methodological description of morphometric point network, see Halačka et al. [12].

For accurate determination of glycoprotein content in goblet secretory cells, special histochemical staining with Alcian blue at pH 2.5 (acid glycoproteins) or pH 1.0 (only sulphated acid glycoproteins) [13] and PAS (neutral and acid sialated glycoproteins) [14] was used, including combinations of staining routinely used for examination of these cells in fish epidermis [15, 16].

Skin-strips from the gill-cover proved to be the most suitable to determine the distribution of melanophores. The strips were transferred to xylene and unfolded between two microscope slides. The number of melanophores in 16 squares each of 1 mm^2 was counted to estimate melanophore density. The maximal diameter of melanophores was determined as the average of the maximal diameter of the ten biggest melanophores from each square.

Statistical analysis was performed using general linear model (GLM) procedures of SAS package [17]. Differences between species and age-classes were determined by a two-way analysis of variance and the Tukey–Kramer multiple comparisons test.

Results

In *S. marmoratus,* the absolute thickness of epidermis ranged from 31.0 μm to 178.0 μm, average 102.08 ± ± 47.62 μm, while in *S. trutta* the epidermis was thinner (ranging from 25.0 μm to 139.0 μm, average 75.56 ± ± 39.92 μm). The absolute thickness of epidermis in both species increased with age ($p < 0.05$) (Table 2). Mean absolute thickness of epidermis in *S. marmoratus* varied from 34.7 ± 4.0 μm (fingerlings) to 150.3 ± ± 24.4 μm (3+ years). Over the period from 0+ to 2+ years, epidermis of *S. trutta* thickened by approximately 82.7 ± 25.8 μm ($p < 0.05$). Relative thickness of epidermis in both species decreased with age ($p < 0.05$) (Table 2). Significant difference in relative thickness of epidermis between species was observed at age 2+.

Two types of morphologically identical secretory cells — goblet and sacciform — were present in the

Figure 1. *Salmo marmoratus* from river Soča with its distinctive marble color pattern and *Salmo trutta* from stream Brinta

epidermis of both species (Figure 2). Goblet cells, containing a variable mix of neutral and acid (sialated, sulphated and non-sulphated) glycoproteins, were observed in epidermis of both *S. marmoratus* and *S. trutta*. However, a low absolute volume of goblet cells (*S. marmoratus*, 8.0 ± 2.0; *S. trutta*, 6.0 ± ± 0.0) was found in fingerlings of the two species (Table 2). The volume of goblet cells increased with age: a three-fold increase was found in *S. marmoratus* ($p < 0.05$), while in *S. trutta* the increase was four-fold ($p < 0.05$). The relative amount of goblet cells varied with age in *S. marmoratus*. In *S. trutta,* the relative volume of goblet cells increased gradually with age, from 13.0 ± 4.4 to 24.7 ± 6.1. Sacciform cells were present sporadically in both species, but in contrast to the granular-filamentous secretion of goblet cells, these were homogenous, non-alcianophilic (Figure 2) and PAS negative, staining pink-purple in hematoxylin-eosin, evidence of the presence of proteins.

Melanophores were present only in the dermis, predominantly just under the epidermis (Figure 2). Mean density of melanophores in fingerlings and yearlings was significantly lower in *S. marmoratus* than in

Table 2. Absolute and relative thickness of epidermis and absolute and relative amount of secretory cells in head epidermis. Values represent mean ± SD (n = 3)

Age [years]	Absolute thickness [μm]		Relative thickness		Goblet cells (absolute)		Goblet cells (relative)	
	M	T	M	T	M	T	M	T
0+	34.7 ± 4.0^{a}	28.0 ± 3.6^{a}	13.6 ± 1.9^{a}	13.3 ± 1.1^{a}	8.0 ± 2.0^{a}	6.0 ± 0.0^{a}	22.3 ± 4.7	13.0 ± 4.4
1+	94.7 ± 8.1^{b}	88.0 ± 15.1^{b}	1.4 ± 0.3^{b}	1.3 ± 0.2^{b}	23.7 ± 2.9^{ab}	18.0 ± 1.0^{ab}	25.3 ± 0.6	21.0 ± 8.5
2+	128.7 ± 18.6^{bc}	110.7 ± 25.6^{b}	0.3 ± 0.1^{cA}	0.8 ± 0.2^{bB}	30.3 ± 11.2^{b}	27.7 ± 10.4^{b}	23.7 ± 7.6	24.7 ± 6.1
3+	150.3 ± 24.4^{c}		0.2 ± 0.0^{c}		25.7 ± 8.5^{ab}		16.7 ± 3.2	

M-*S. marmoratus*; T-*S. trutta*. ^{abc}Different letters within column denote significant differences ($p < 0.05$) between age-classes; ^{AB}Different letters in row denote significant differences ($p < 0.05$) between species

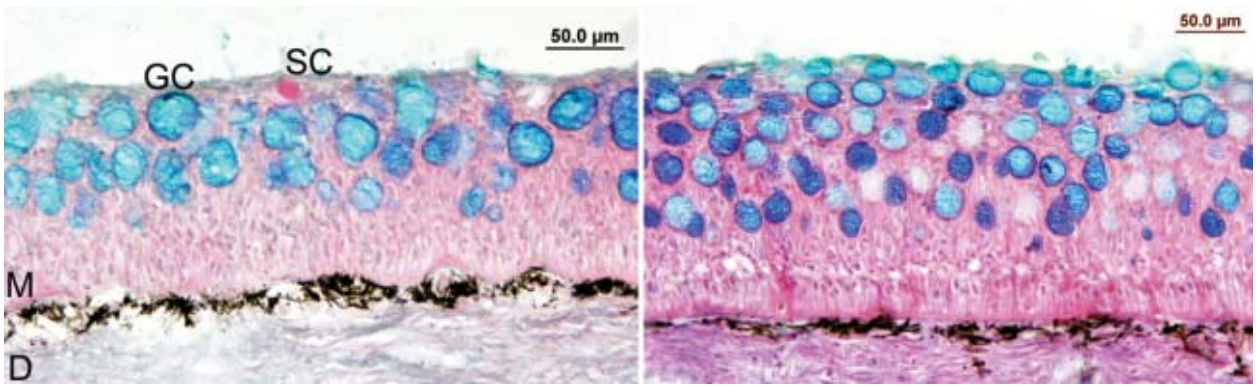

Figure 2. Head skin, section perpendicular to surface, from *S. trutta* (left) and *S. marmoratus* (right) stained with Alcian blue at pH 2.5. Different intensity of blue coloration shows variable composition of glycoprotein content. GC — goblet cell; SC — solitary sacciform cell; M — melanophore; D — dermis

Table 3. Density and diameter of melanophores in gill cover dermis of *S. marmoratus* (M) and *S. trutta* (T) of different age-classes. Values represent mean ± SD (n = 3)

Age [years]	Mean density/mm^2		Min. and max. density/mm^2				Max. diameter of melanophores [μm]	
	M	T	M		T		M	T
			Min	Max	Min	Max		
0+	19.0 ± 8.5^{A}	75.3 ± 27.3^{B}	6.3 ± 2.5$^{a, A}$	32.0 ± 11.4^{A}	45.3 ± 4.5^{B}	83.3 ± 6.1$^{ab, B}$	310.3 ± 88.6	239.3 ± 69.8
1+	19.0 ± 5.6^{A}	71.7 ± 35.5^{B}	6.0 ± 6.9$^{a, A}$	31.7 ± 7.6^{A}	57.3 ± 27.2^{B}	101.7 ± 27.8$^{a, B}$	382.0 ± 49.1	234.3 ± 47.6
2+	19.3 ± 3.0	45.7 ± 19.2	9.0 ± 2.0^{a}	35.0 ± 7.0	35.7 ± 19.8	49.3 ± 20.8^{b}	413.7 ± 95.8	260.0 ± 74.6
3+	20.7 ± 5.5		0.0 ± 0.0^{b}	38.0 ± 5.0			485.7 ± 24.4	

^{abc}Different letters within column denote significant differences ($p < 0.05$) between age-classes; ^{AB}Different letters in row denote significant differences ($p < 0.05$) between species

S. trutta (Table 3). In *S. trutta* only, the mean density of melanophores was higher ($p > 0.05$) in fingerlings and yearlings than in older age-classes. The maximal density of melanophores in fingerlings and yearlings was significantly lower in *S. marmoratus* than in *S. trutta*. In older (3+) *S. marmoratus* individuals, minimal density fell to zero in light areas, corresponding to the form of the marble color pattern. Minimal and maximal densities were higher in *S. trutta*. However, differences in maximal and minimal density were observed in areas with spotted coloration. The maximal diameter of melanophores in *S. marmoratus* was larger ($p > 0.05$) than in *S. trutta*, particularly in older sexually mature individuals (Table 3).

Melanophores in the dermis of *S. marmoratus* and *S. trutta* appeared in four different physiological states (Figure 3). The marble color pattern in *S. marmoratus* (fully established when an individual reaches sexual maturity at 2-3 years of age; see Figures 4 and 5) consists of both light and dark areas. Melanophores in a completely aggregated state (average diameter, 50 μm) predominate in light areas, with distance between melanophores of about 300 μm. Areas without melanophores were also observed (Figures 4, 5). Three different physiological states of melanophores were found in dark areas (Figure 3); (1) melanophores in margins between light and dark areas, (2) melanophores in black areas with completely dispersed state, and (3) maximally dispersed melanophores in gray areas with a light centre. In both black and gray areas, the distance between melanophores was similar to that in light areas — from 200 to 400 μm. The diameter of melanophores in dark areas was approximately ten-fold larger than that of melanophores in light areas (Table 3).

Melanophores in an aggregated/dispersed state predominated in the dermis of *S. trutta* (Figure 6A). The diameter of melanophores varied from 100 to 200 μm, with an average distance between them of 200 μm. Melanophores in a completely dispersed state and with a maximum diameter of 250 μm were present exceptionally in black spots (Figure 6B).

Discussion

Although *S. marmoratus* and *S. trutta* are phylogenetically very closely related [18, 19] — indeed *S. marmoratus* is often considered to be a subspecies of *S. trutta* — the results of our study show perceptible differences in skin structure between these two species, especially regarding the shape and distribution of melanophores. The phenotypic differences between the species are inherited, and therefore the marble color pattern formation can be categorized as an ultimate morphological change as defined by Leclercq et al. [3].

The skin of the individuals of *S. marmoratus* and *S. trutta* studied corresponds in its basic structure to that described for other salmonids. The epidermis is somewhat thicker in *S. marmoratus* than in *S. trutta*, and in both species gradually increases with age. Relative thickness in both species decreases with age. The measured values are similar to those for other salmonids [20–24], though direct comparison was not possible due to methodological differences or missing supplementary information. Among central European species, significantly thicker head skin epidermis is found in some cyprinids (*Carassius carassius* (L.) 240 μm, *Cyprinus carpio* (L.) 400 μm) or Lotidae (*Lota lota* (L.) 360 μm) [25] than in salmonids. On the other hand, epidermis of similar thickness to *Salmo* is characteristic of small cyprinids: *Cottus gobio* (L.) (150 μm), *Barbatula barbatula* (L.) and *Gobio gobio* (L.) (100 μm) [25].

Goblet cells are the second most common cell type in teleost epidermis [26], and can be observed in the upper and middle layers in *S. marmoratus* and *S. trutta* (Figure 2). The number of goblet cells in salmonid

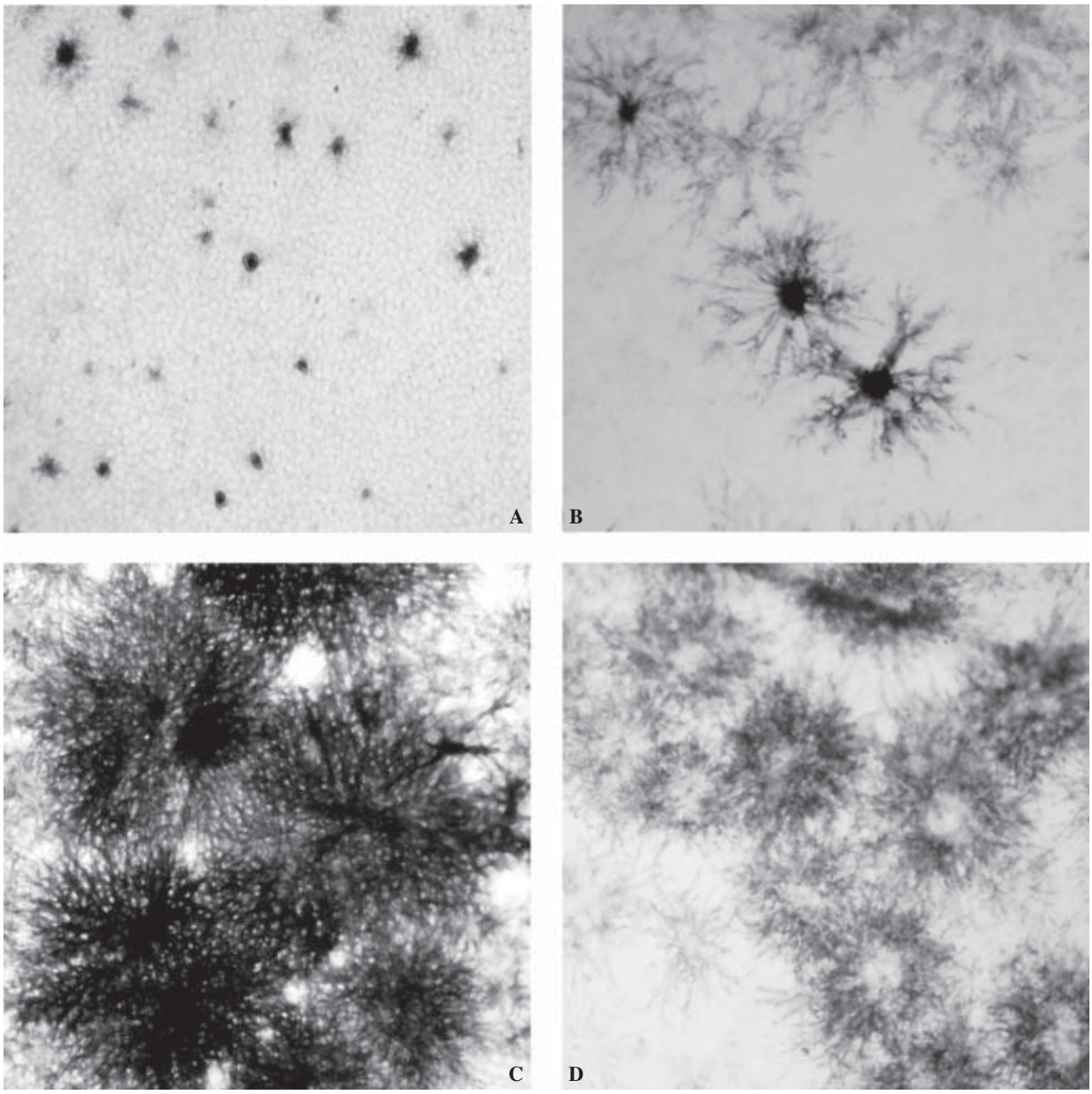

Figure 3. Different types of melanophore in gill cover dermis of *S. marmoratus* and *S. trutta*. **A.** Completely aggregated state; **B.** Aggregated/dispersed state; **C.** Completely full-area dispersed state; **D.** Maximally dispersed state with light center

epidermis varies seasonally, and with other factors such as infection or handling [15, 27, 28]. The relative and absolute amount of goblet cells in *S. marmoratus* and *S. trutta* determined in this study are similar to, and correspond with, values obtained by Knoz et al. [20] in adult *S. trutta* caught in non-spawning periods.

Melanophores in fish are in general mostly found in the dermis and sometimes in the epidermis [3]. In the present study on *S. marmoratus* and *S. trutta,* they were found only in the dermis, confirming a general characteristic of salmonid skin [20–22]. The absence of melanophores in epidermis has also been observed in other European freshwater fish — e.g. *Leuciscus idus* (L.), *Leuciscus cephalus* (L.), *Tinca tinca* (L.), *L. lota*, *G. gobio*, *Cottus poecilopus* (Heckel, 1837) — but absence or presence is not always consistent within genera: e.g. it is present in *Abramis ballerus* (L.), but not in *Abramis brama* (L.) or *Abramis bjoerkna* (L.) [25].

Dermal chromatophores are typically arranged in three or four contiguous layers, collectively referred

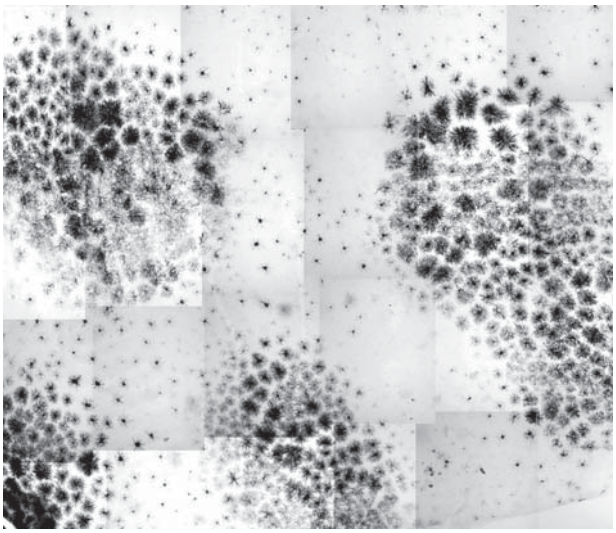

Figure 4. Skin from gill cover of *S. marmoratus* with light and dark areas

Figure 5. Dermis of gill cover, section perpendicular to the surface, border between light and dark areas in *S. marmoratus*

to as the dermal chromatophore unit [29], the outermost layer of which consists of xanthophores and erythrophores, the second layer iridophores, and the third layer melanophores. Most types of color change in fish involve changes in one or more components of the dermal chromatophore unit [30]. Morphological analysis of chromatophores in the skin of *S. trutta*, *Oncorhynchus mykiss* (Walbaum, 1792) and *Salvelinus fontinalis* (Mitchill, 1814) has revealed that melanophores are the most abundant chromatophore type [31]. They are very important for body coloration in salmonid fish and are responsible for the presence of black spots across the body [1, 32, 33]. The speed of change in coloration depends on whether the change is physiological (fast) or morphological (slow). Most studies on pigment cells have focused on short-term physiological changes, such as an adaptive coloration change in response to background, changing light conditions or physiological state [34–36]. Although much less studied, morphological color change that involves a decrease or an increase in the total number of pigment cells [37, 38] is also of great interest and could be relevant in the case of *S. marmoratus*. The present study revealed that melanophores are larger in *S. marmoratus*, and present at a lower average density that is more or less constant across all age classes, than in *S. trutta*. The maximum diameter of melanophores in *S. marmoratus* increases with age, while the density of melanophores is more or less the same, which most likely indicates that the marble color pattern formation is more the result of

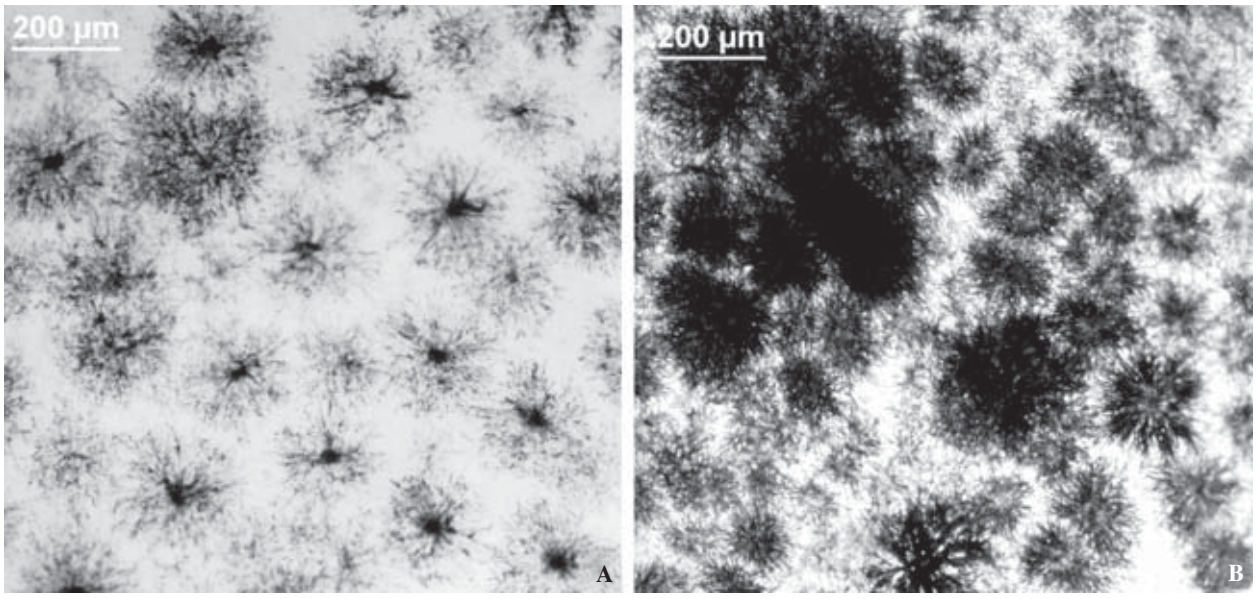

Figure 6. Skin from gill cover of *S. trutta*. **A.** Predominant melanophore distribution; **B.** Distribution of melanophores in black spots

hypertrophy than hyperplasia of pigment cells. In adult marbled *S. marmoratus* individuals, light areas with small (i.e. aggregated) melanophores at low density are present (marble color pattern; observed already in 2+ individuals, data not shown), while in *S. trutta* melanophores are smaller and more uniformly distributed, coinciding with the pigment pattern (i.e. black spots).

Apart from epidermal thickness, differences between the skin of *S. marmoratus* and *S. trutta* are restricted to differences in the characteristics of the dermal melanophores. It is assumed that physiological characteristics of the skin in the two species are similar.

Numerous studies have shown that fish skin is a markedly variable organ with great inter-generic or even inter-specific differences. However, most comparative studies have, due to the simplicity and high frequency of variable markers (epidermal thickness, presence and count of secretory cells and histology of their content), focused on the epidermis. Our study clearly shows the need to extend such examinations to the dermis.

It has been observed in *S. marmoratus* that the formation of the marble color pattern is dependent on the stage of development of an individual organism: the color change occurs primarily in the transition from juvenile to (immature) adult and, being an ancient adaptation, is likely to have a strong genetic component [3]. This marbling is created by the gradual loss or aggregation of melanophores in light areas and the extension of melanophores in dark areas, and is fully established before sexual maturation. The marble color pattern generated is very distinctive, and our analysis reveals that differences in the shape and distribution of melanophores account for its characteristic appearance. Studies of genes involved in the generation and disruption of the adult pigment patterns could reveal the origin and genetic background of the marble color pattern.

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