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MORPHOGENESIS

Cytological State of Gonads and Level of Thyroid and Sex Steroid Hormones in Black Sea Trout *Salmo trutta labrax* Pall.

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Abstract—Cytological state of the gonads and hormonal state of hatchery Black Sea trout before differentiation into resident and anadromous forms (parr) at an age of 15 months have been examined. It has been shown that the hormonal changes associated with the choice of life strategy in the Black Sea trout females and males are pronounced to different degrees. As compared with the resident and anadromous individuals, the female parr display a low rate of oogenesis and similar hormonal status, while characteristic of the male parr are an intermediate rate of spermatogenesis, a low level of thyroid hormones and estradiol, and a medium testosterone level. As has been found, the undifferentiated Black Sea trout individuals predominantly develop into the resident form.

Keywords: Black Sea trout, within-population differentiation, parr, gametogenesis, thyroid hormones, sex steroid hormones

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INTRODUCTION

Within a population, the *Salmo trutta labrax* juveniles differentiate into the resident and anadromous forms at age 1+ in both wildlife and hatchery (Pavlov et al., 2010). The differentiation in the hatchery Black Sea trout as a rule takes several months (Pavlov et al., 2010, 2012), which is determined by pronounced physiological differences between individuals in one generation. A generation at an age of 15 months contains three forms, namely, the initial form (lacking any external signs of differentiation), i.e., parr, and two further developed forms (displaying external differences), i.e., resident and anadromous forms (Pavlov et al., 2014). Unlike the phenotypically developed individuals, the parr are smaller in size and have a grayish coloration with an olive tint separated by black vertical stripes along their body (Pavlov et al., 2010).

Pavlov et al. (2014) have demonstrated that the Black Sea trout resident form at an age of 15 months has more intensive spermatogenesis but slower oogenesis as compared with the anadromous form. The thyroxine and testosterone levels in the Black Sea trout blood is significantly higher in the individuals with intensive gametogenesis, while the concentration of triiodothyronine in the resident form is considerably lower as compared with the anadromous form (Pavlov et al., 2014). The degree of gonad development, rate of gametogenesis, and the levels of thyroid and sex steroid hormones in the parr have not been assessed so far. The knowledge of these specific features will make it possible to determine whether parr individuals are ready to differentiate and, presumably, to predict the direction of differentiation.

The goal of this work was to examine the state of gonads and the levels of sex steroid hormones (testosterone and estradiol) and the hormones involved in energy metabolism (thyroxine and triiodothyronine) in the parr at an age of 15 months and compare these data with the earlier examined resident and anadromous individuals of the same age.

MATERIALS AND METHODS

The work was conducted in July 2012 at the Adler Trout Breeding Plant (Krasnodar krai, Russia). The parr have been compared with the juveniles that already have selected either anadromous or resident strategy according to biological, cytomorphological, and biochemical parameters. The fish of the same generation were sampled on the same date (Pavlov et al., 2014).

The Black Sea trout parr at an age of 15 months were kept in a water-flow concrete tank at a stocking density of 400–500 individuals/m^3 and a water temperature of 10.7–13.2°C. The fish were fed BioMar feed. The individuals to be analyzed were placed into a box (1.2 × 1.2 × 1.2 m) installed in the same tank 24 h before sampling to prevent their feeding. This is necessary to standardize the EIA data. To provide a meaningful comparison of the data, the same 31 individuals (20 females and 11 males) were assayed.

Histological slides were made according to standard protocols (Romeis, 1953) using Medite (Germany) semiautomatic histological equipment, namely, TPC-15 histological processing device, TES-99 embedding system, and Meditome M530 microtome. The sections with a thickness of 3–5 μm were successively stained with Ehrlich's hematoxylin and eosin. Longitudinal sections of the cranial part of the gonads were photographed with a Keyence Biorevo BZ-9000 (Japan) motorized microscope. The maturity stages of gonads and the periods of spermatogenesis and oogenesis are given according to Murza and Khristoforov (1991) and Chmilevskii (2003).

Cytological state of the gonads was assessed according to the intensity of gametogenesis, that is, according to nucleoplasmic ratio (NPR), calculated as the ratio of the oocyte nuclear to the cytoplasmic areas in a section cutting the cell immediately near its central part. The oocyte at the stages of previtellogenesis and vitellogenesis considerably increases in size mainly at the expense of the cytoplasm rather than the nucleus (Murza and Khristoforov, 1991; Makeeva, 1992). Correspondingly, the significance of NPR during this period decreases with cell growth. The state of testicles was assessed according to the quantitative ratio of various gamete types per unit section area (1 mm^2). The Image J v. 1.46r software was used for cell count and the measurements necessary for computing NPR.

The concentrations of sex steroid (testosterone and estradiol-17β) and thyroid (thyroxine, T4, and triiodothyronine, T3) hormones were assayed in the blood serum. The blood was intravitally sampled in the morning after dawn (6:40 a.m. to 8:20 a.m.) when the hormone concentrations are the highest (Ganzha et al., 2015). The fish were bled intravitally form the caudal vein behind the anal fin with a 1–2-mL syringe; the sample volume varied from 200 to 750 μL depending on the individual size. The blood was transferred into sterile tubes and left vertically to settle for 30–40 min at room temperature. After clotting, the blood samples were centrifuged for 7 min at 4000 rpm in a Minispin (Eppendorf, Germany). The resulting serum was placed into sterile tubes to store in a freezer at –20°C. EIA was conducted in ST-3L (Elmi, Latvia) microplate thermostated shaker, StatFax 303 Plus semiautomatic immunoplate photometer, StatFax 3100 (Awareness Technology, Unites States) microplate washer, and the corresponding DRG (Germany) reagent kits. The concentrations of hormones in each serum sample were determined in triplicate to use their mean for further computations. The data on individual characteristics were processed using ANOVA and cluster analysis (according to Ward) as well as Student's *t*-test, Student's *t*-test for fractions, and Fisher test.

RESULTS

The female parr had a length of 13.2 ± 1.01 (11.2–15.2)[1] mm and a weight of 22.7 ± 5.42 (13.4–35.0) g and the male parr had a length of 13.3 ± 1.24 (11.2–14.9) cm and 22.4 ± 5.47 (12.0–30.4) g, respectively. The differences in these characteristics between the females and males are statistically insignificant (Student's and Fisher tests, $p > 0.05$)

The parr body length (*AC*) and weight are smaller in a statistically significant manner ($p < 0.01$, Student's *t*-test) as compared with the anadromous and resident forms of the same age (Pavlov et al., 2014). The ANOVA data demonstrate that the fish body length and weight depend in a statistically significant manner ($p < 0.001$) on their phenotypic form in the parr as well as in the individuals that have already settled their life strategies and that these dependences are different for the females and males within the same form ($p < 0.05$).

The share of males in sample decreased from the resident variant (77.7%) through anadromous individuals (51.4%) to parr (35.5%). The resident individuals differ in this characteristic from the remaining forms in a statistically significant manner (Student's *t*-test, $p \leq 0.015$).

Cytological state of the gonads. The parr ovaries are at stage II of maturity; they are semitransparent, slightly yellowish, and thickened along their entire length. The gametes are represented by previtellogenic oocytes, considerably varying in their size, which is determined by their asynchronous development. The same ovary contains both small cells with a diameter from 15 μm, only entering the stage of protoplasmic growth, and a few large oocytes (diameter, 200–250 μm), with vacuolated cytoplasm peripheral zone. The small cells contain a small amount of uniformly strained cytoplasm. In the abundant medium-sized cells, the cytoplasm contains clearly visible peripheral zones housing RNA, which are more intensively stained by hematoxylin. These zones are absent in the large oocytes. Both oocyte qualitative and quantitative compositions are similar in the Black Sea trout parr, resident, and anadromous individuals (Pavlov et al., 2014).

The oocyte NPR in the parr gonads amounts to 0.36 ± 0.008, varying from 0.11 to 0.88 (Fig. 1), which is significantly higher as compared with the resident and anadromous forms. Over 41% of all oocytes in the parr ovaries display an NPR ≤ 0.3, which is characteristic of more developed gametes. These shares are higher in the resident and anadromous forms, amounting to 45.7 and 59.1%, respectively; these differences are statistically significant ($p < 0.01$) according to Student's *t*-test for shares. The results suggest that the gonads in the parr develop somewhat less intensively as compared with the resident and anadromous forms.

The parr testicles are at intermediate maturity stage II–III and are prevalently represented by two cell types, namely, type A spermatogonia and more mature cells smaller in size and type B spermatogonia (Fig. 2a). In addition to spermatogonia, the most developed testicles contain primary spermatocytes, whereas type A

[1] Hereinafter, the mean and its error are shown in front of parenthesis and minimum and maximum values, inside the parentheses.

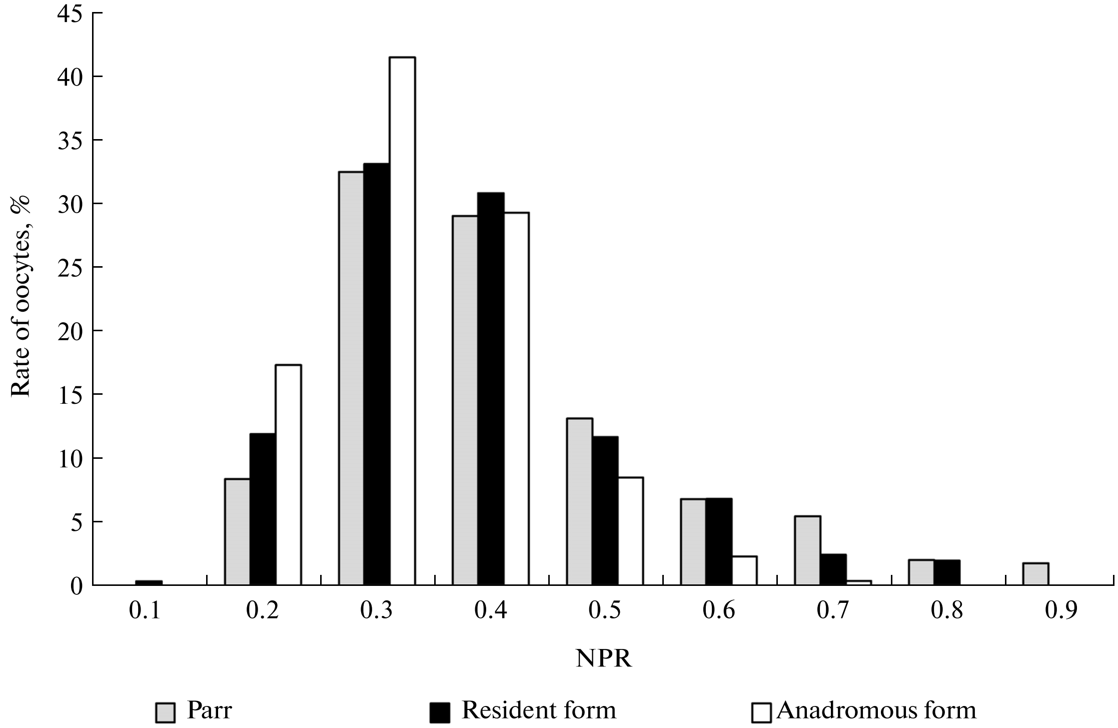

Fig. 1. Distribution of the oocytes according to the nucleoplasmic ratio (NPR) in the gonads of the Black Sea trout (*Salmo trutta labrax*) at an age of 15 months (hereinafter, the data on the resident and anadromous forms are according to Pavlov et al., 2014).

spermatogonia in such gonads are solitary. Of the 11 individuals examined for cytological state of the gonads, one fish differed from the remaining parr individuals as well as the resident and anadromous forms: the major part of its gametes was represented by spermatocytes: 1 mm^2 area of the section contained 5854 spermatocytes, 3934 spermatogonia A, and 117 spermatogonia B (Fig. 2b). The spermatocytes counts in the gonads of this individual considerably exceeded the maximum values in both the anadromous (1163 cells) and resident (3499 cells) form of the same age. The cytological state of such a gonad corresponds to maturity stage III. In order to obtain more adequate results on spermatogenesis, the data for this individual were discarded from statistical processing when comparing three Black Sea trout forms.

Type B spermatogonia, characteristic of the beginning of cell growth, are the most abundant in the parr gonads. The number of these cells per unit area in the parr is somewhat higher as compared with the anadromous form (statistically insignificant differences) but is by almost 20% smaller than in the resident form ($p < 0.05$). The parr also display an intermediate level of early sperm cells, type A spermatogonia ($p < 0.05$). Except for the testicles of maturity stage III in one individual, the gonads of the remaining parr individuals have hardly any spermatocytes; recalculated per unite area, the counts of these cells are minimal in all three compared forms ($p < 0.01$).

Concentrations of thyroid and sex steroid hormones. According to ANOVA, the concentrations of triiodothyronine and estradiol-17β in the parr, as well as in resident and anadromous forms, depend in a statistically significant manner ($p < 0.05$) on the particular form and this dependence is different for the females and males within the same form. The testosterone concentration in the blood depends on the sex and form in a statistically significant manner ($p < 0.01$). Any statistically significant dependences of T4 concentration on sex and form have not been found.

The average level and variation in the thyroid hormones in the female parr are higher as compared with the males: $\sigma = 2.4$ and $\sigma = 0.5$ for T3, respectively, and $\sigma = 2168$ and $\sigma = 715$ for T4 (Fig. 4). The differences in variation in the concentrations of these hormones in females and males are statistically significant according to the Fisher test ($p < 0.01$).

Of all the examined Black Sea trout forms, the male parr display the lowest triiodothyronine level in the blood and its variation (the differences in variation are statistically significant according to the Fisher test, $p < 0.01$). In the parr, the triiodothyronine concentration is higher in females than in males versus the resident form, where the concentration in males is higher than in females.

The testosterone concentrations in the male parr and resident form are significantly higher as compared with the females (statistically significant according to Stu-

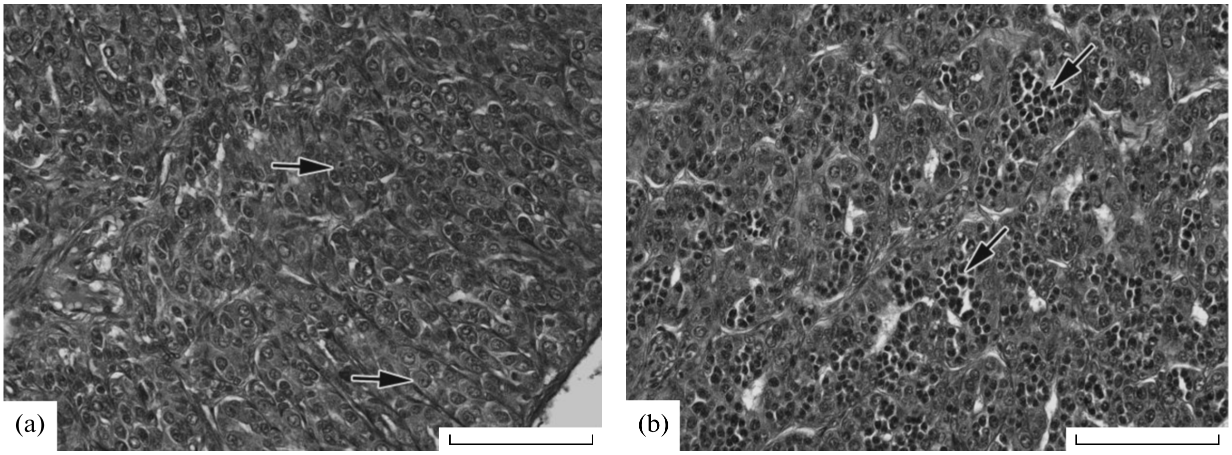

Fig. 2. Gametes in the testicles of the Black Sea trout parr at an age of 15 months: (a) type B spermatogonia in a cyst (arrows) and (b) the cysts with numerous primary spermatocytes; scale bar, 50 μm.

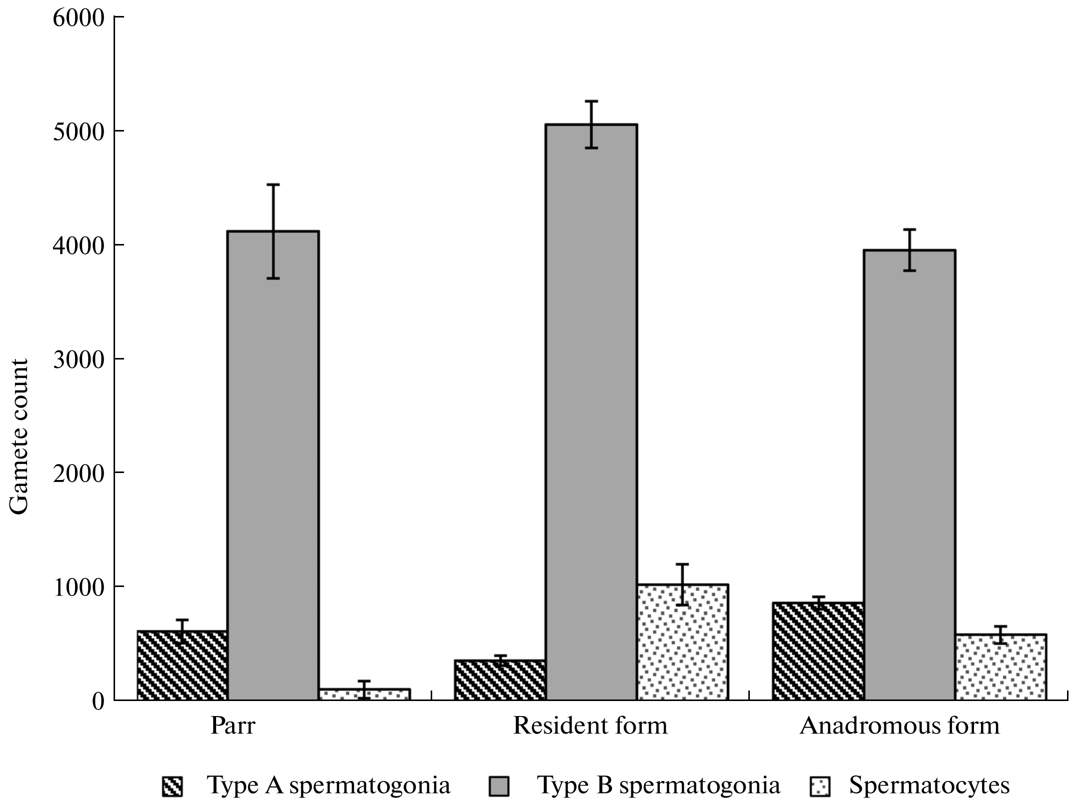

Fig. 3. Ratio of gametes of different types in the testicles of the Black Sea trout *Salmo trutta labrax* at an age of 15 months; (|) standard deviation.

dent's *t*-test, $p < 0.01$; Fig. 5). The resident males displayed the highest average testosterone level; the differences are statistically significant relative to all anadromous females and males ($p < 0.05$). The variation in the concentration of this hormone in the blood is statistically significantly ($p < 0.05$ according to the Fisher test) lower in the anadromous males ($\sigma = 0.26$) as compared with the resident males ($\sigma = 0.48$) and parr ($\sigma = 0.33$). The testosterone concentration in the blood serum and its variation in the females of different phenotypic forms do not differ in a statistically significant manner ($p > 0.05$).

The male parr displayed the lowest average estradiol-17β concentration ($p < 0.01$), while the resident males displayed the highest (Fig. 5). The variation in the estradiol-17β concentrations in the blood serum of Black Sea trout individuals belonging to different

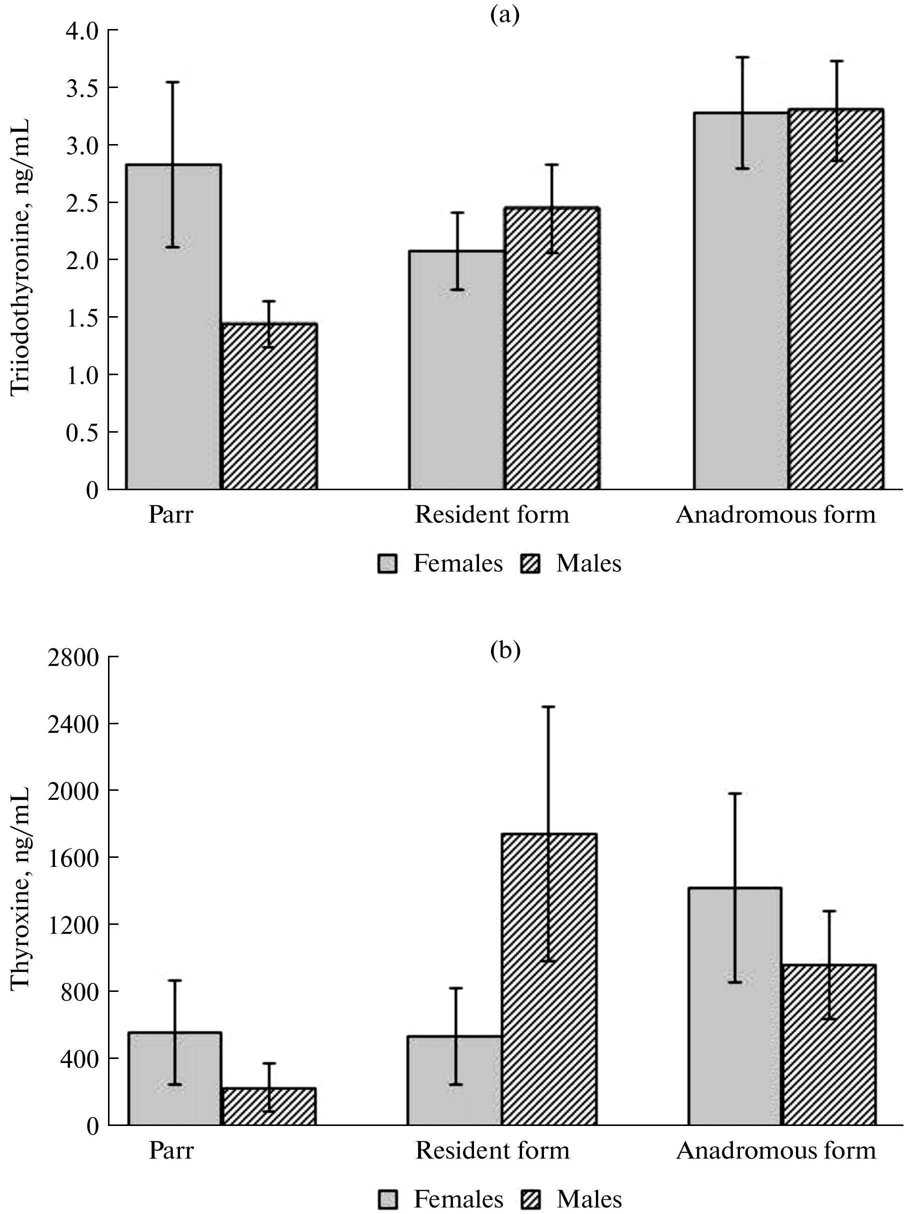

Fig. 4. Concentrations of thyroid hormones in the blood of female and male Black Sea trout at an age of 15 months; the error bar denotes standard error of mean.

forms does not differ in a statistically significant manner ($p > 0.05$). In the parr, the concentration of this hormone is higher in females than in males versus the anadromous and resident forms, where the males display a higher concentration as compared to females.

Analysis of the correlation between the analyzed hormones according to Spearman has detected statistically significant ($p < 0.05$) rather pronounced dependences between thyroxine and triiodothyronine (r_s = 0.58) as well as between the concentrations of estradiol-17β and thyroid hormones (r_s = 0.43 for both T3 and T4).

Cluster analysis (Fig. 6) has shown that the Black Sea trout resident females and female parr (25% of the maximum distance) form the most compact clusters according to the examined cytological and hormonal characteristics as well as the males of these forms (13%). These clusters unite at the level of 37%. The anadromous form is the most distant from the remaining ones.

DISCUSSION

The undifferentiated Black Sea trout juveniles (parr) at an age of 15 months differ from the individuals already differentiated into phenotypic forms of the same age in the body length and weight, state and mat-

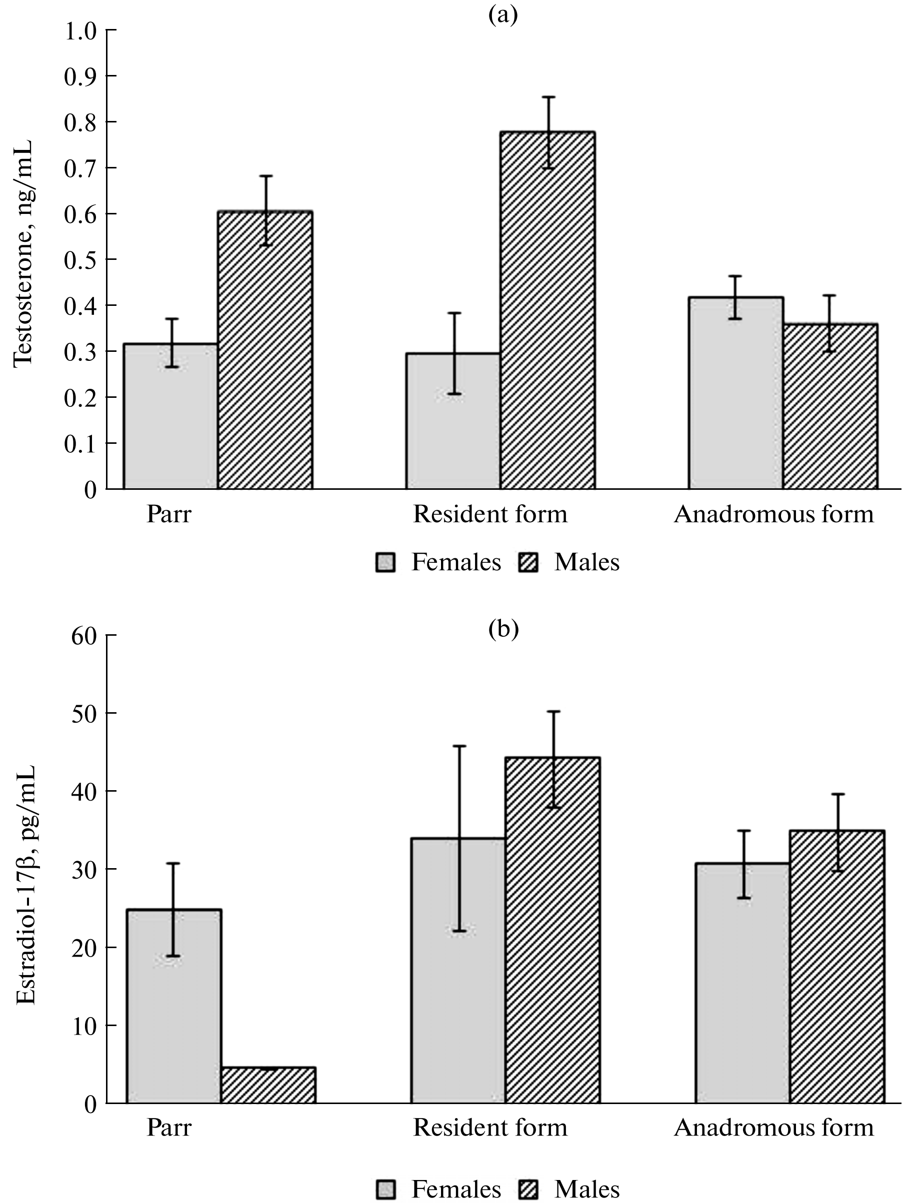

Fig. 5. Concentrations of testosterone and estradiol-17β in the blood of female and male Black Sea trout at an age of 15 months; the error bar denotes standard error of mean.

uration rate of their sex products, and hormonal characteristics.

The parr body length and weight are smaller as compared with the resident and anadromous forms, confirming earlier data (Pavlov et al., 2010). Pavlov et al. (2012) have shown that the statistically significant differences in the size and weight characteristics in generations of hatchery Black Sea trout appear as early as an age of 1.5–2.0 months, i.e., long before the moment when they select their life strategy. Low values of biological characteristics may suggest a high competition, which leads to insufficient nutrition of part of individuals or some other adverse farming conditions. Presumably, these causes are to be considered as the major factors for a delay in selection of the life strategy by the parr, since an individual needs to be physiologically prepared to make such a choice.

Characteristic of the parr are a slower oogenesis as compared with both the anadromous and resident forms. The fact that the intensity of oogenesis in the resident form is lower as compared with the anadromous form suggests that the female parr are closer in the state of their gonads to the resident form. The Black Sea trout male parr in the intensity of spermatogenesis are close to the anadromous form: they do not differ in type B spermatogonia and slightly yield to the

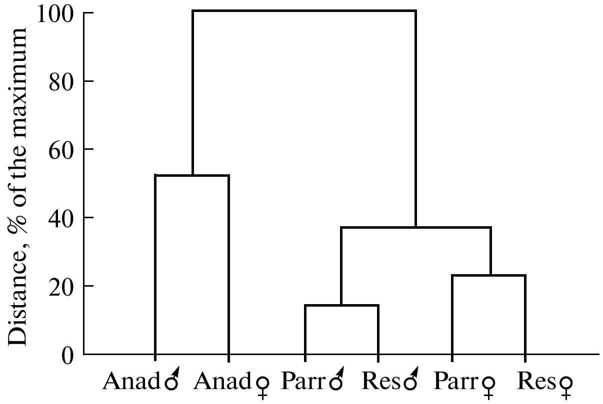

Fig. 6. Cluster analysis (according to Ward) of the hormonal (concentrations of triiodothyronine, T3; thyroxine, T4; testosterone; and estradiole-17β) and cytological (NPR and cell count per unit area) characteristics of the Black Sea trout *Salmo trutta labrax* at an age of 15 months: res, resident form and anad, anadromous form.

anadromous individuals in type A spermatogonia and spermatocytes. Correspondingly, the spermatogenesis in parr is slower than in the resident form. The only observed exception was one fish with its gonads at stage III of maturity. The testicles of this individual were closer in their development to the resident form but the spermatocyte counts in its gonads considerably exceeded the maximum value observed in the gonads of resident form. Note that this individual in the levels of sex steroid and thyroid hormones did not display any significant differences from the remaining examined parr individuals. It is known (Pavlov et al., 2010) that the wildlife Black Sea trout population contains dwarf individuals; in particular, the females and males at age 1+ that reached sexual maturity have been observed. Presumably, the found individual with intensive spermatogenesis may further develop into a dwarf male.

The parr and the individuals that had already chosen their life strategy differ in the quantities of some of the examined hormones. Most of the differences have been detected between the male parr and the remaining forms. In particular, the male parr displayed the lowest level of triiodothyronine and thyroxine in the blood as compared with the other examined forms. Note that the level of testosterone in the parr is noticeably higher as compared with the anadromous form but lower than in the resident form. The concentration of estradiol-17β in the blood in the male parr is sevenfold lower than in the anadromous males and ninefold lower than in the resident males. Such pronounced distinctions were undetectable in the female parr. However, statistically significant differences in the levels of triiodothyronine, testosterone, and estradiol in the blood were observed between the females and males within each form. Note that the qualitative hormonal differences between the females and males are less pronounced within the anadromous and resident forms. Such a specific position of the male parr in the level of hormones suggests an early formation of specific dwarf variant. An indirect confirmation of such a probability is the discovered male individual with the gonads at stage III of maturity.

Thus, the hatchery parr in their cytological and hormonal characteristics are closer to the resident form of the Black Sea trout as compared with the anadromous one. Cluster analysis demonstrates that the individuals that selected an anadromous life strategy form a separate group distant from both the resident form and parr, which is determined by larger physiological changes when preparing to parr–smolt transformation. The parr that delay with the choice of their life strategy will most likely differentiate into the Black Sea trout resident form. The sex ratios observed for the parr and resident fish also suggests this inference: the parr at an age of 15 months comprised 68% of females and 32% of males versus the resident form, with 24 and 76% females and males, respectively (the differences are statistically significant according to Student's t-test for fractions, $p = 0.0001$). The sex ratio changes at an age of 16 months: the rate of females decreases to 40% in the parr and increases to 33% in the resident form (Pavlov et al., 2010). Consequently, a small share of resident females with time is supplemented by the remaining female parr that selected a resident life strategy. In general, the following order of Black Sea trout form development is likely: the individuals having selected an anadromous life strategy are the first to emerge and males are the first to make their choice of resident life strategy as compared with females.

CONCLUSIONS

(1) As has been confirmed, the hatchery parr at an age of 15 months, which remain undifferentiated, have shorter body length and lower body weight as compared with the resident and anadromous forms. It has been shown that the parr differ in both the state and rate of gonad development and in the concentrations of thyroid and sex steroid hormones in the blood.

(2) Characteristic of the female parr is a low rate of oogenesis as compared with the resident and anadromous females and the similar levels of thyroid and sex steroid hormones.

(3) As compared with the males of differentiated forms, the male parr display intermediate spermatogenesis intensity and low levels of thyroid hormones and estradiol. The testosterone concentration in the male parr is noticeably higher as compared with the anadromous form but lower than in the resident form.

(4) The hatchery parr at an age of 15 months, which remain undifferentiated, will further mainly give the resident form.

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