ISSN 1995-4255, Contemporary Problems of Ecology, 2018, Vol. 11, No. 3, pp. 331–341. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © V.S. Artamonova, M.V. Ponomareva, V.V. Ignatenko, A.A. Makhrov, 2018, published in Sibirskii Ekologicheskii Zhurnal, 2018, No. 3, pp. 366–377.

Gonadal Development of Diploid and Triploid Pink Salmon (*Oncorhynchus gorbuscha*) from the White Sea

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Received November 25, 2017; in final form, December 25, 2017

Abstract—Only males (fully mature and failing to mature) and intersexes have been found among triploids of pink salmon. Possible mechanisms of genetic sex determination in pink salmon are discussed. The possibility of using triploids to regulate sizes of artificial pink salmon populations is also discussed. Triploids of pink salmon are deemed inefficient to use in aquaculture. Delayed maturation is observed in some diploid females reared in farming cages.

Keywords: delayed maturation, intersexes, sex, triploid, gonads, thermal shock, pink salmon, *Oncorhynchus gorbuscha*

DOI: 10.1134/S1995425518030034

INTRODUCTION

The origin of the sex-determination mechanism is one of the main problems of the modern theory of evolution. In particular, it is still not clear how well-differentiated sex chromosomes typical for higher vertebrate animals have been formed. Therefore, the attention of researchers is increasingly attracted by the mechanisms that regulate the sex ratio in lower vertebrates, in particular, in fish.

Modern methods of genome analysis have not fully disclosed the mechanisms of sex determination in model species of lower vertebrates, but they have led researchers to the important conclusion that the development of certain sexual characters in ontogeny is determined by the achievement of a threshold value of some sex-determination factor (review: Heule et al., 2014).

Substances (usually synthetic hormones) that can either suppress or stimulate the expression of genes responsible for sex formation, thereby redefining the genotypic sex of males or females, have long been used in fish-breeding to produce monosex fishes, although the direct mechanisms of action of these substances are as a rule unknown (Pandian, 2012). At the same time, an approach associated with a change in the number and/or ratio of chromosomal loci encoding sex-determination factors is needed to determine the contribution of the genetic component, whose expression is regulated in fact.

Salmonids (Salmonidae) are particularly attractive as an object of experiments on genome reorganization, since this is one of the groups of organisms where genetic mechanisms for sex determination are in the nascent stage. The origin of this family was accompanied by tetraploidization, and the formation of genera and species was accompanied with chromosome fusion (Frolov, 2000; Phillips and Rab, 2001). In some cases it seems that the simultaneous pairwise fusion of several nonhomologous chromosomes occurred (Zelinsky and Makhrov, 2002; Makhrov, 2017), resulting in a violation of sex-determination mechanisms, so the sex chromosomes evolved again (Phillips, 2013).

Moreover, sex chromosomes cannot be detected at all in karyological studies in most salmonids, particularly in pink salmon (*Oncorhynchus gorbuscha*) (review: Phillips, 2013), although DNA sequences originally found only in males of this species are known (Devlin et al., 2001; Brunelli and Thorgaard, 2004; Phillips et al., 2007). At the same time, as it turned out later, in some populations of pink salmon the mismatches of phenotypic sex to genotypic are frequent (up to 81.5% in males), provided the sequences are sex-specific (Brykov et al., 2010a, 2010b). From the all above, it follows that the genetic mechanism of sex determination in pink salmon is still very imper-

fect, and thus it can serve as a model object for studying the formation of this mechanism.

As was already mentioned, it is necessary to change the number and/or ratio of chromosomal loci encoding sexual factors and assess the impact of such changes on the phenotypic sex of individuals to elucidate genetic mechanisms of sex formation. One such approach may be the production of triploids. There are evidences of a change in sex ratio in artificially produced fish triploids: they we reported to be often dominated by males (review: Pandian, 2012). However, the effects of triploidy on the sex ratio in pink salmon are still unknown.

Meanwhile, the study of pink salmon triploids has, in particular, a certain practical significance, since such fish can theoretically be sterile (reviews: Gomel'skii and Grunina, 1988; Makhrov et al., 2014; Thresher et al., 2014) and therefore do not die at the age of two, like diploids.

In addition to the imperfection of the genetic mechanism of sex determination, pink salmon is also interesting because the life cycle of representatives of this species is exactly 2 years. Between there are two lines of pink salmon—even and odd—there is almost complete reproductive isolation. These lines are even sometimes called sibling species. The pink salmon of the even line spawns in the autumn of even years. In the spring of the next (odd) year, the fry leaves for feeding in the sea and spends the summer, winter, and one more summer there. In the autumn of the even year, this generation of pink salmon returns to the rivers for spawning and, after spawning, dies due to irreversible physiological and morphological rearrangements of the organism which accompany maturation. The pink salmon of the odd line has a similar life cycle, but spawns in odd years. Moreover, there is not only prezigotic but also a certain postzigotic reproductive isolation between the lines: although descendants from the artificial hybridization of pink salmon of two lines survive in the sea and are even fertile, the descendants of these hybrids do not return from the sea (Gharrett and Smoker, 1991; Gharrett et al., 1999).

The spawning rivers of pink salmon are located in the northern part of the basin of the Pacific Ocean and adjacent areas of the Arctic Ocean basin: in Alaska (Babaluk et al., 2000) and in Siberia from Chaun Bay to the Lena River (Chereshnev and Kirillov, 2007). However, since 1956, pink salmon has been introduced into the rivers of the European North, and now it is also widespread in the basins of the White and Barents seas (Zubchenko et al., 2004; Dorofeyeva et al., 2004; Hesthagen and Sandlund, 2007; Witkowski and Głowacki, 2010; Veselov et al., 2016).

Like any alien species, pink salmon is a potential threat to the ecosystems of the rivers of the White and the Barents sea basins (Zubchenko et al., 2004). Its appearance in the rivers of Scotland caused real panic. For example, *The Times* on September 25, 2015 published an article entitled "Native Salmon Threatened by Invaders Bred in Cold War." On the other hand, pink salmon has become an important commercial target for the inhabitants of the White Sea coast. The dosed release of sterile triploid pink salmon, whose life span is not limited to 2 years, from fish hatcheries could become a compromise between the conservation of natural ecosystems and the need to support the people inhabiting the White Sea region. This is even more important because the triploids of some other salmonids have higher growth rates than matured diploids (review: Artamonova and Makhrov, 2015). However, data on the development of gonads and the growth rate of pink salmon triploids are not complete.

Pink salmon triploids were obtained in a number of experiments (Utter et al., 1983; Chernenko, 1985; Benfey et al., 1989; Joyce et al., 1994; Teplitz et al., 1994), but triploid pink salmon was never grown to an adult state. Such a task was posed in a study by Benfey et al. (1989), but both pink salmon diploids and triploids died before the diploids ripened due to a water failure in this experiment.

The aim of this study is to study the sex ratio and the state of gonads of pink salmon triploids and diploids during the maturing of diploids. The task of the study is to evaluate the influence of triploidization on the development of gonads in pink salmon. The practical task is to study the effectiveness of triploidization as a method of sterilizing pink salmon and assess the possibility of using pink salmon triploids in aquaculture.

MATERIALS AND METHODS

The pink salmon caught in the Keret' River (the western coast of the White Sea) in August 2013 (in accordance with Permission no. 782013031972) were used as breeders. The eggs received from 15 females August 26, 2013, was fertilized by sperm from 6 males. To determine the average mass of the egg, the eggs of each female were sampled.

A thermal shock was carried out after fertilization. Its conditions varied in different sets of eggs (the time after fertilization was 10, 12, and 15 min; the time of the thermal shock was also 10, 12, and 15 min; the temperature of the thermal shock was from +28 to +30°C). Three control sets of fish eggs at this time remained in a water temperature corresponding to the water temperature in the river (+16°C).

The eggs were incubated at the fames in the flowing water for a period of 36–41 days (the average incubation period was 470 degree days). The water temperature decreased relatively smoothly from +16 to +14°C during the first 27 days, and then, in subsequent days, more sharply from +14 to +7.5°C. Hatching of the larvae was prolonged.

The juveniles were grown in fresh water tanks for a year and a half, until May 2015, and then transported to cage farming of Chupa Bay (the western coast of the

White Sea). When released to the seawater, the fish had an average mass of 36.1 g.

The sea cages had a volume of 12 m^3; the stocking density was 250 individuals per 1 m^3. During breeding in the sea, the fish were hand-fed ad libitum three times a day with minced meat of the three-spined stickleback (*Gasterosteus aculeatus*). This small fish exceeds the number of other fish of the White Sea; its biomass reaches 1600 t (Ivanova et al., 2016).

The fully grown pink salmon (55 individuals) were taken for analysis on October 2–5, 2015. The fork length, mass, sex, and maturity of the gonads were determined for all individuals (Murza and Khristoforov, 1991). The blood was also smeared from all individuals for subsequently determining the maximum diameter of the erythrocyte nuclei; the gonads were fixed in Bouin's fluid for subsequent histological analysis. The absolute fecundity and mass of eggs were determined for three mature females. During material processing, the presence or absence of gonadal anomalies in the fish was recorded.

The ploidy of the pink salmon genome was determined by measuring the maximum diameter of the erythrocyte nuclei in blood smears stained with azure-eosin (Kalashnikova, 1981). A diameter of 50 or more cells was measured for each individual. The accuracy of this method makes it possible to identify triploid salmonids with a probability up to 100% (Benfey et al., 1984).

Histological preparations were done according to standard methods (Roskin, 1951; Romeis, 1948) using semiautomatic specialized histological equipment (Medite, Germany): a TPC-15 histoprocessor, TES-99 filling station, and Meditome M530 microtome. Sections 5 μm in thickness were stained with Ehrlich's hematoxylin and eosin. Photos of the gonad slices were made using a Keyence Biorevo BZ-9000 motorized microscope (Japan).

RESULTS

Optimal Conditions for Obtaining Pink Salmon Triploids

The survival rate of eggs from fertilization to hatching of the larvae was greater in the control sets in comparison with the experimental. The rate of hatched larvae was 68, 77, and 87% of the number of eggs laid for incubation for the three control sets. The survival of eggs in the experiment was mostly affected by the duration of the thermal shock: an increase of the duration of the heat effect from 10 to 15 min leads to the decreasing of this parameter by 2–5 times in other sets of eggs. An increase of the temperature of thermal shock from +28 to +30°C affected the survival of the eggs to a lesser extent and reduced the number of surviving embryos by no more than 1.5 times (on average, for 20%). The lengthening of the time interval that passed from the moment of fertilization to the moment of the beginning of the thermal shock from 10 to 15 min had no significant effect on the survival of the larvae. The best results (87% of the survival in the control) were obtained in the experiment set with the beginning of heat shock 12 min after fertilization, temperature + 28.5°C, and duration 10 min.

Determination of Genome Ploidy

The distribution of the maximum diameters of the erythrocyte nuclei for females and males that have a V stage of gonadal maturity and do not have deviations in the development of the reproductive system and for experimental fishes with undeveloped gonads (stage I or stage II) are shown in Fig 1. Fish with a third or fourth stage of gonad maturity were not used in standard distribution plotting. Based on literature data on the development of the reproductive system in salmonid diploids and triploids (Gomel'skii and Grunina, 1988; Benfey et al., 1989; Benfey, 1999), we assumed that individuals of the first group should be represented exclusively or predominantly by diploids, and those of the second group by triploids.

Indeed, it turned out that the distributions of the maximum diameter of the erythrocyte nuclei for both groups of individuals are normal; have average values of 7.04 and 9.37, respectively; and overlap insignificantly (Fig. 1).

A study of individual distributions showed that most fishes are clearly divided into two groups: with the modal value of the maximum diameter of the erythrocyte nuclei about 7 μm (diploid) and about 9.5 μm (triploids). However, six fish had average values of the maximum diameter of the erythrocyte nuclei intermediate between diploids and triploids, and may have been diploid–triploid mosaics, some of whose cells contained two haploid sets of chromosomes and some, three. Indirect evidence in favor of this assumption can be the deviation from the normal distributions of the one for the maximum diameters of the erythrocyte nuclei in two of these fish.

There were four males among the fish which could not be unequivocally attributed to diploids or triploids. The gonads of one of them, according to histological analysis, had I stage of maturity; the fork length was 251 mm and the mass was 140 g. The histological analysis of the gonads of the other two males showed that they were at stage II of maturity (there were visible cysts with spermatogonia of type B), fork length was 211 and 223 mm, and weight was 80 and 95 g. The stage of maturity of the fourth fish was determined only visually (III); the fork length of this male was 240 mm and weight was 140 g.

The fifth fish that could not be attributed to either pure diploids or pure triploids was a female that had stage V gonad maturity. Its fork length and mass were 310 mm and 345 g, respectively. It was unusual that, along with eggs an usual size, this fish also had abnormally large oocytes (Fig. 2).

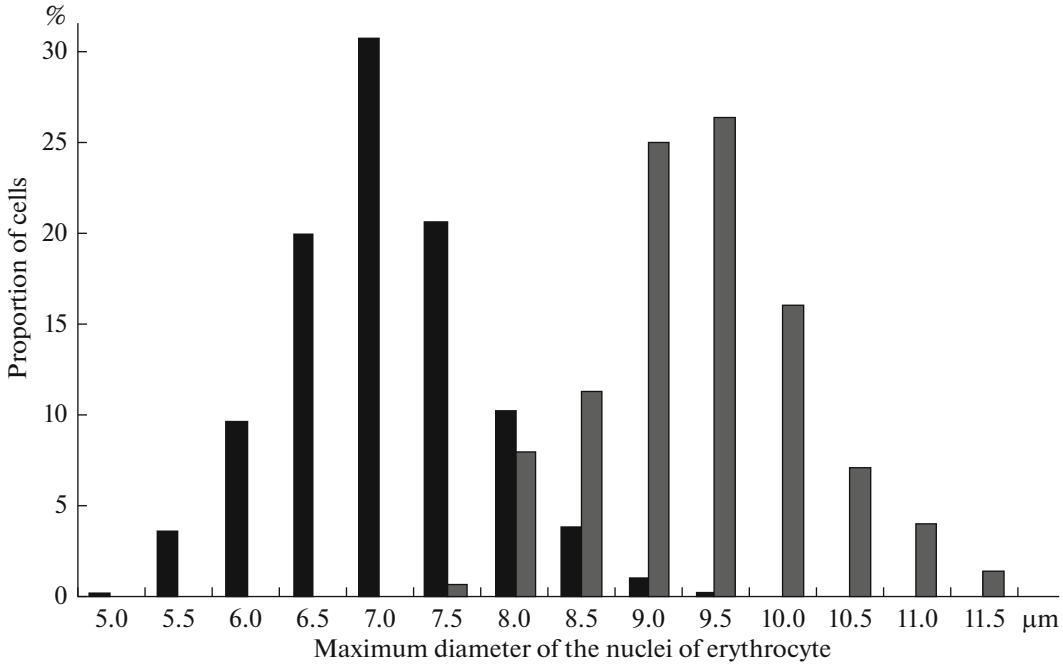

Fig. 1. Erythrocyte distribution in accordance with the maximum diameter of their nuclei for the individuals of pink salmon with gonads of the V stage of maturity (diploids, black bars) and fish with undeveloped gonads (gray bars, triploids).

Fig. 2. Fish eggs of chromosome mosaic (or individuals with a number of chromosomes intermediate between diploid and triploid). (*1*) Normal size oocytes; (*2*) large oocytes.

Histological analysis showed that the sixth fish was an intersex. There were two groups of five oocytes of early maturity stage III each and resorbing small oocytes of the earlier stage (II) in the slide. In addition, part of the gonad matured according to the male type: in the field of view was a group of ampoules filled with spermatocytes of orders I and II, which corresponds to early stage of maturity III. The fork length of this fish was 300 mm and the mass was 320 g.

Diploid Fish

Data on sex composition, stages of gonad maturity, length, and mass of studied fish are shown in Table 1.

Table 1. Sex, maturity stage, length, and mass of diploid fish

Sex	Maturity stage	*N* (the number of individuals which underwent a histological analysis are given in parentheses)	Average fork length, mm (distribution limits are given in parentheses)	Average mass, g (distribution limits are given in parentheses)
Females	III	10 (5)	281.7 (258–305)	235.0 (170–285)
	IV	1	276	230
	V	5 (1)	273.4 (258–294)	256.4 (175–340)
Males	III	1 (1)	274	210
	V	18 (3)	261.7 (225–287)	191.9 (100–240)

Table 2. Sex, maturity stage, length, and mass of triploid fish

Sex	Maturity stage	*N* (the number of individuals which underwent a histological analysis are given in parentheses)	Average fork length, mm (distribution limits are given in parentheses)	Average mass, g (distribution limits are given in parentheses)
Males	I, II	5 (5)	243.2 (224–265)	137.0 (105–190)
	VI	6 (3)	244.7 (208–277)	148.3 (95–220)
Imtersexes	See the text	3 (3)	262.7 (227–300)	188.3 (115–305)

The females differed in stage of gonad maturity. Some females matured (their gonads were in the V stage); one female had stage of maturity IV. Absolute fertility was 249–566 with an average of 396 eggs. The weight of eggs varied from 0.102 to 0.115, with an average of 0.108 g. The mass of eggs in female breeders from the Keret' River—the parents of the studied fish—was higher. It varied from 0.123 to 0.172 g, with the average mass 0.146 g.

The maturity stage of the rest of the females was determined during autopsy as III. A histological analysis of the gonads of four individuals showed that they were in the late stage III; in one fish, the destroyed oocytes of early stage III were determined. In some females only single oocytes matured.

The gonads of one of the diploid males were in the third stage of maturity (according to the results of the histological analysis, early stage III); the left gonad of this fish was strongly shortened. The rest of the studied diploid males had flowing sperm (visually, the stage of maturity was defined as V). One diploid fish had milt, divided into lobes, and fused at the anus. A histological analysis of the gonads of three males of this group showed that in one fish they were at stage V of maturity, in the second they were at stage V–VI of maturity, and in the third they were at stage VI. Numerous residual spermatozoa were visible in the lumens of seminiferous ampoules of all three individuals.

Triploid Fish

Some of the triploid males did not mature. Visually, the stage of maturity of their gonads was determined in some as I and in others as II. Histological analysis confirmed these observations (Table 2). The color of all the fish of this group was silvery (Fig. 3).

The gonads of the rest of the triploid males were at the VI stage of maturity according to visual observations; in one of the fish they were with constrictions. All the fish of this group had obvious external signs of preparation for spawning: yellowish color and a hook on the jaw (Fig. 4).

There were no females among the triploids. Both fish, determined upon autopsy as females with gonads of stage II of maturity, turned out to be intersexes. Histological analysis showed that the gonads of these fish looked like the gonads of males at early stage III of maturity, but they had inclusions of oocytes, which also were in early stage III of maturity. In addition, another intersex was detected during histological analysis among fish visually defined as males with gonads of stage II of maturity (Fig. 5).

DISCUSSION

Features of Diploid Pink Salmon Maturing in Cages

It should be noted that diploid fish grown in cages were much smaller than pink salmon feeding in the sea. The males of the odd line of pink salmon spawn-

Fig. 3. Triploid male of pink salmon, stage II of maturity.

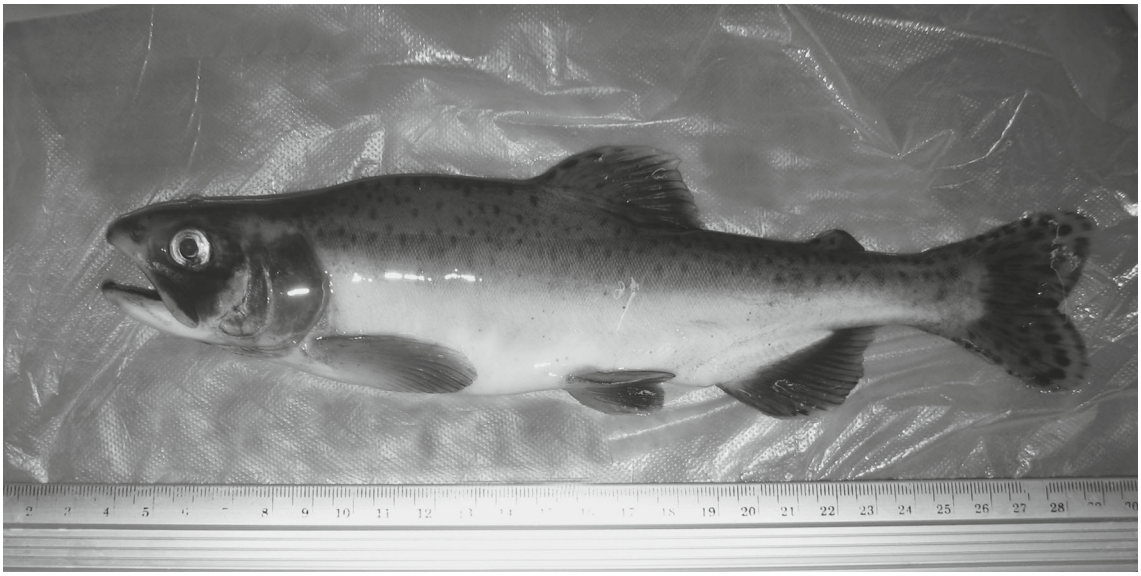

Fig. 4. Triploid male of pink salmon, stage VI of maturity.

ing in the Keret' River weighed 660–2850 g (average 1601.0 g) and had a fork length of 380–595 mm (average 493.0 mm). The females of this group weighed 660–2200 g (average 1317.4 g) and had a fork length of 395–550 (average 471.1) mm (V.S. Artamonova, D.K. Dirin, D.L. Laius, A.A. Makhrov, and Z.N. Yudina, unpublished data).

This could be explained by the fact that this set of fish was grown in small (2 × 2 m) pools with fresh water during the first year and a half, while in natural conditions the pink salmon of the White Sea of the odd line goes to feed in the sea by the first year of life, in the second half of May or in the middle of June (Veselov et al., 2016).

At the study period, in early October 2015, the gonads of some female diploids, descendants of pink salmon from the Keret' River, were in stages III and IV of maturity, while breeders entering this river by the beginning of October had already fully ended spawning.

The fact of the maturation delay of the pink salmon females during cage breeding is also noted in the literature. One female with a pronounced delay in the development of gonads was found among the 2-summer-old pink salmon grown in cages in the Kislaya Bay on the Kola Peninsula (Oganesyan and Kolechkin, 1995). Later, in February–March of the following year, matured females were identified among the fish of the same set of pink salmon which

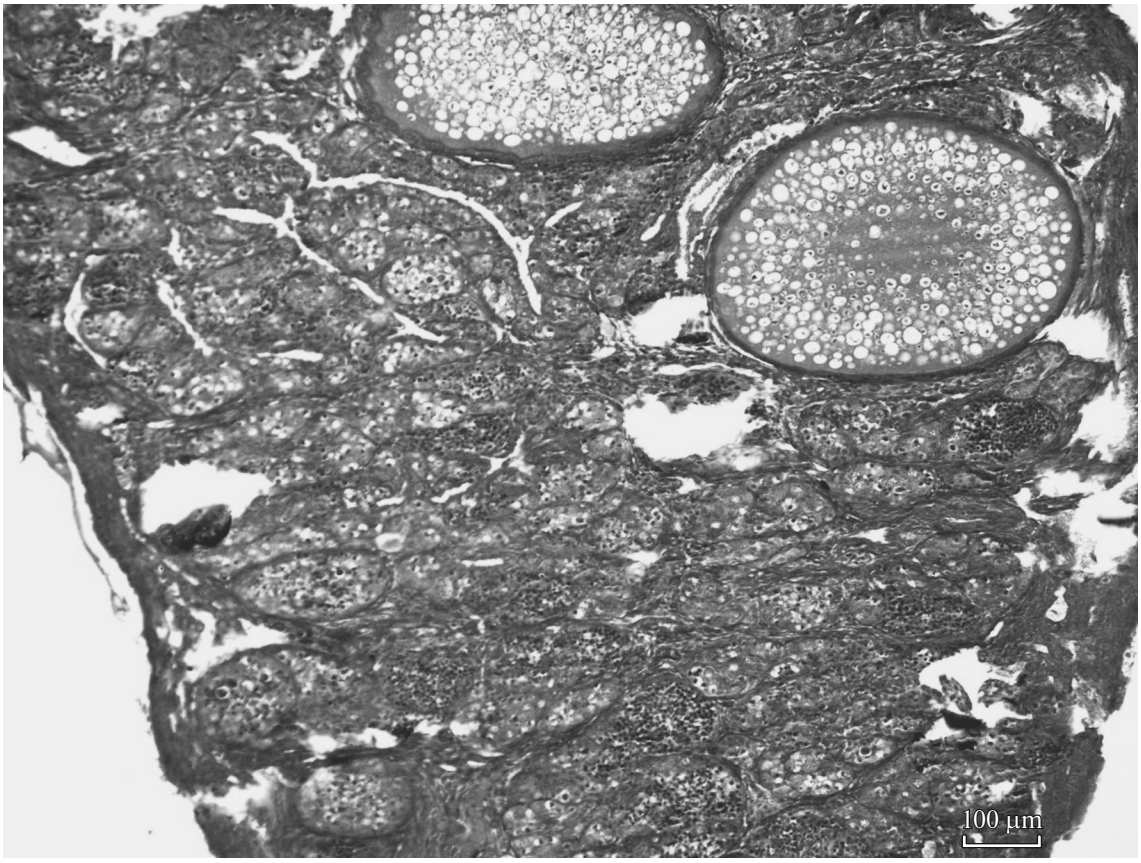

Fig. 5. Gonads of triploid intersex of pink salmon.

did not die in cages at the age of 2 and were left for wintering (Anokhina, 1999).

Similar data are also available for female pink salmon grown in tanks. Their maturation may occur later or even next year (Persov et al., 1966; Kwain, 1982; Sakun and Persov, 1984; Benfey et al., 1989; personal communications with A.N. Balanina and A.N. Ulyanova, Umba hatchery).

The mature delay in pink salmon males was also repeatedly observed. Therefore, for example, maturing male 2+ was caught in a lake in which juveniles of this species were released 2 years before (Bakshtanskii, 1962).

Matured 2+ old pink salmon are occasionally found in natural populations (review: Heard, 1991). They are found in a significant amount in the artificially formed pink salmon population of Lake Superior in North America (Wagner and Stauffer, 1980; Nicolette, 1984).

The average mass of fish eggs in matured fish was approximately 25% less than that of the breeders from which they were obtained. It is necessary to attribute this fact to the peculiarities of maturation of diploid female pink salmon in cages. In addition, the absolute fecundity of pink salmon grown in cages (on average 396 eggs) was significantly less than of the pink salmon of the parent generation: the total fecundity of female breeders ranged from 450 to 800 eggs, and the absolute fecundity of pink salmon caught near the Keret' river mouth range from 1230 to 2283 fish eggs with average value of 1833 eggs, according to the data (Zelennikov and Kuznetsova, 2003).

Note that specific features peculiar to small female of pink salmon, such as delayed maturity, reduced fecundity, and small size of fish eggs, are typical for small breeders of salmonids of other species, in particular, for Atlantic salmon (Makhrov et al., 2013).

Sex Composition and Maturation of Triploids

Only males and intersexes were found among the pink salmon triploids. Triploid females of pink salmon were mentioned (Benfey et al., 1989), but histological studies of gonads of these fish were not performed. Therefore, it is possible that these fish were also intersexes. This seems likely to be true, as a study conducted on another species of salmonid, rainbow trout (*Parasalmo mykiss*), has shown that extensive areas with spermatogenesis in the gonads of most triploid females appear with advancing age (Carrasco et al., 1998), although the nature of this the phenomenon is not yet clear. Predominantly or exclusively

triploid hybrids, which were obtained by interbreeding of some fish forms (Lamatsch et al., 2010; Pandian, 2012) and amphibians (Mezhzherin et al., 2010; Pruvost et al., 2015), are also males. This phenomenon has not yet been explained.

In our experiment, triploid males of pink salmon were represented either by individuals whose gonads did not develop (I or II stage of maturity) or by fish that did not practically differ from diploid males by its outer features. The latter agrees with the noted maturation of triploid males of pink salmon and the development of the so-called nuptial changes (Benfey et al., 1989).

However, despite their external similarity to diploids, triploid males of pink salmon produce, apparently, inferior sperm and therefore are unable to produce offspring. In any case, this is true for salmonids of other species (review: Artamonova and Makhrov, 2015). At the same time, triploid males of Atlantic salmon (*Salmo salar*) and masu salmon (*Oncorhynchus masou*) are able to participate in spawning (Kitamura et al., 1991; Fjelldal et al., 2014). This means that triploid males with developed gonads can apparently, if necessary, be used to suppress the reproduction of pink salmon. The question of the possibility of the subsequent development of gonads in those males that did not mature in our experiment remains open. The age and size such fish can reach also remains unknown.

Nevertheless, as our experiment shows, pink salmon triploids are not of aquacultural interest, since they are represented only by males and intersexes, and most males during the spawning period of diploids undergo a physiological and morphological rearrangement after which the fish become unviable. Growing other triploid salmon, in particular, rainbow trout, is beneficial when it comes to obtaining a large commercial fish (for references, see Artamonova and Makhrov, 2015).

Intersexes and Mechanisms of Sex Determination of Pink Salmon

We found three intersexes among the triploids; one intersex was detected among fish that could not be reliably attributed to either diploids or triploids (perhaps it is a diploid–triploid mosaic or an individual with a number of chromosomes intermediate between diploid and triploid).

Among the breeders of pink salmon going to spawn in the rivers of the Pacific Ocean basin, intersexes were not described (Ivanova, 1956; Ievleva, 1968; Drozdov et al., 1981; Smirnov et al., 2011). It is known that all the larvae of pink salmon initially form ovaries, and only at the age of 30–123 days after hatching from the eggs ovaries changed into testes in males (Persov, 1965).

However, single oocytes were noted in the testes of two male pink salmons grown in cages in the White Sea during the experiment conducted by Knipovich Polar Research Institute of Marine Fisheries and Oceanography (Oganesyan, 1979). Similar intersexes were also found among wild pink salmon breeders in the Rynda River on the Kola Peninsula (Karachun, 1982).

On the other hand, triploids, as well as diploid–triploid mosaics, have been repeatedly identified in populations of pink salmon (Utter et al., 1983; Chernenko, 1991; Miller et al., 1994). Therefore, it is possible that the intersexes mentioned in literature were not diploids.

The fairly regular appearance of pink salmon intersexes outside their natural range and also during artificial breeding could theoretically be explained by the influence of certain environmental factors on the expression of the sex-specific genes of pink salmon. However, the fact that such individuals appear in large numbers among triploid fish, with the complete absence of females among the latter, mainly indicate the features of the sex determination mechanism in pink salmon.

The ratio of sexes in triploids observed in our experiment is possible only if the sex of pink salmon is determined by multicopy genes coding factors that contribute to the transformation of the individual into a male. Such genes should be present not only in the pink salmon Y chromosome, but also in its X chromosome, although in a smaller amount (Makhrov et al., 2018). Under these conditions, the expression of the sex-specific genes of the three X chromosomes can lead to a concentration of their products that will be sufficient to partially or completely redefine the sex of the triploid.

CONCLUSIONS

Only males and intersexes were determined among the pink salmon triploids. This allowed us to hypothesize the mechanisms of sex determination in this salmon species. According to this mechanism, the genes encoding the factors contributing to the transformation of the individual in the male are not only in Y, but also in X chromosome, although in smaller numbers. It is shown that the use of triploid males and intersexes in aquaculture is impractical. However, for a final assessment of the prospects of practical use of salmon triploids, it is necessary to get female triploids by hormonal sex over-determination of fish and to investigate their biological and reproductive characteristics. Such an experiment will also contribute to further revealing the mechanisms of sex determination in pink salmon.

ACKNOWLEDGMENTS

We are grateful to V.E. Gilepp, A.G. Laidinen, and A.D. Posin for their support in organizing the experiment and to E.D. Pavlov for help in conducting a histo-

logical analysis. This work was supported by the Russian Science Foundation, project no. 16-14-10001.

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Translated by T. Kuznetsova