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ANIMAL PHYSIOLOGY

Arctic Teleost Fishes with Canceled Accelerated Senescence Program Are a Potential Source of Stress Protectors and Cancer Drugs

V. V. Ziuganov and E. G. Popkovich

Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow, 119991 Russia
e-mail: valeryz@orc.ru

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Abstract—A new approach to the development of natural cancer drugs from hydrobionts selected for longevity and stress resistance is analyzed. The accelerated senescence program was successfully canceled in holarctic stickleback *Gasterosteus aculeatus* by crossing the marine and freshwater forms in ponds and selection of hybrids. Prolongation of fish life was accompanied by an increased production of the antistress exocrine secretion by the renal epithelium of male sticklebacks (glycoproteins, peptides, and mucoids), required for successful egg development. Trials of the biodrug prepared from this secretion on salmons with epithelioma, guinea pigs with affected skin, and mice with transplantable tumors demonstrated a good therapeutic effect of the biologic.

INTRODUCTION

The world never stops to search for new drugs from natural organisms that have no side effects on the immune system. Extraction of drugs from aquatic organisms, hydrobionts, is given particular attention. Most hopes are pinned to hydrobiont-produced substances efficiently neutralizing microbes and parasites or poisoning or repelling predators. Bioactive substances with anticancer, anti-inflammatory, antimicrobial, and antiviral activities (lipids, mucopolysaccharides, peptides, and glycoproteins) have been recently isolated from oysters, mussels, *Mercenaria*, as well as other mollusks, crabs, shrimps, trepangs, polychaetes, leeches, sea squirts, frogs, and many fishes including halibut, sharks, mullets, loach, and seahorses (Pilat and Ivanov, 2002; *Medical Active…*, 2004). Medical significance of seahorses of the genus *Hippocampus* (Gasterosteiformes) is widely known; they are used in traditional medicine of Southeast Asia to treat cardiovascular, metabolic, and sexual disorders as well as certain cancers (www. sea horse project). At the same time, few know the medicinal potential of close relatives of seahorses, sticklebacks (Gasterosteidae), with an amazing capacity for wound healing (Ziuganov, 1991). Stickleback oil was successfully used in the blockaded Leningrad during the war to treat wounds and burns, since it contains carotene and vitamin A, which accelerate regeneration and wound healing (Gerbil'skii and Evropeitseva, 1945).

Sticklebacks have an amazing capacity to release proteins to the environment similar to fibroin filaments of spiders or byssus of oysters. Secondary epithelial cells in the kidney of spawning males release a 200 kDa sticky glycoprotein, which is, together with some other light proteins and mucoid substances, a component of glue used to build a spawning nest from algae (Jakobsson *et al.*, 1999; Jones *et al.*, 2001).

In this work, a possible medicinal significance of sticklebacks was analyzed. The biodrug largely composed of the renal secretion of sticklebacks was tested on salmons with epithelioma, guinea pigs with affected skin, and mice bearing transplantable Ehrlich carcinoma.

MATERIALS AND METHODS

Experimental animals and material collection. Selection fish with canceled accelerated senescence program were used. Field experiments on producing fish with such biological properties were described in details elsewhere (Ziuganov, 1983, 1988, 1991, 1995; Ziuganov *et al.*, 1987; Ziuganov and Bugaev, 1988; Ziuganov and Kalyuzhin, 2004; Ziuganov and Tashenov, 2004). Glue-producing stickleback spawners were caught with a landing net in ponds and maintained in 100 l aquaria in the pond littoral zone. Duckweed was given as a material for nest building. Males were induced for glue production by the spawn extract and ovarian liquid. Glue was collected with a Pasteur pipette from the urogenital foramen of living males (a noninvasive method). In some cases, males were anesthetized with 0.1% phenoxyethanol (Fluka), sacrificed, and the urinary bladder (containing the glue) was dissected using forceps and ophthalmic scalpel. Another noninvasive method of glue collection included its squeezing from the nest composed of sand and duck-

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weed leaves through a 500 μm mesh. The glue collected by two different methods under field conditions was stored in Eppendorf tubes for several hours in a portable cooler and then frozen at –16°C.

In the laboratory, thawed glue was filtered through a 0.45 μm membrane by centrifugation at 3700 *g* for 10 min to remove cells and foreign particles. A fraction of the biodrug was frozen again while the rest was lyophilized to a powder. The content of the glue organic matter was determined at the Russian Federal Research Institute of Fisheries and Oceanography (Moscow). The concentration of solids was 9.1 mg/ml; carbohydrates, 3.0 mg/ml; lipids, 0.64 mg/ml; total nitrogen, 1.60 mg/ml; nonprotein nitrogen, 1.26 mg/ml; protein nitrogen, 0.34 mg/ml; and mineral matter, 2.1 mg/ml. A detailed biochemical analysis of proteins, peptides, and amino acids was then carried out at the Institute of Genetics and Selection of Industrial Microorganisms (Moscow). Protein concentration was 0.37 and 0.27 mg/ml according to the method of Bradford (1976) and amino acid analysis, respectively.

The protein from stickleback glue included all 20 proteinogenic amino acids (except tryptophan): Asp.a/Asp, 0.0419 mg/ml; Thr, 0.0181 mg/ml; Ser, 0.0136 mg/ml; Glu.a/Glu, 0.0310 mg/ml; Pro, 0.45–0.0135 mg/ml; Gly, 0.0103 mg/ml; Ala, 0.0104 mg/ml; Val, 0.0160 mg/ml; Ile, 0.0128 mg/ml; Leu, 0.0181 mg/ml; Tyr, 0.0111 mg/ml; Phe, 0.0119 mg/ml; His, 0.0162 mg/ml; Lys, 0.0161 mg/ml; Arg, 0.0152 mg/ml; Cys, 0.0122 mg/ml; Met, 0.0016; and total amino acids, 0.270 mg/ml.

The glue was subjected to ultrafiltration through a UM5 membrane. The resulting fraction contained no proteins, included the same proteinogenic amino acids (0.0288 mg/ml in total), and represented the peptides and free amino acids of the mucus. The glue also demonstrated proteolytic activities of a metalloproteinase, acid proteinase, and aminopeptidase.

Denaturing electrophoresis of the mucus proteins after Laemmli (1970) (10% polyacrylamide gel) demonstrated the presence of over 10 proteins from 23 to >200 kDa. Figure 1 shows the results of electrophoresis of 20 and 100 μl mucus (lanes *2* and *3*, respectively). Lane *1* shows the molecular weight standards. The gel was stained with Coomassie R.

The glue of wild type sticklebacks, which are 4–5 cm in length and die after spawning, is hard to collect due to low production. The glue of large selected long-lived sticklebacks rich in biologically active substance (Ziuganov, 2005) was used in experiments as a wound healing, antistress, and antitumor drug. This biodrug was registered under the name Arktika+ by the Ministry of Health of the Russian Federation. After a trial for toxicological, microbiological, physicochemical, and clinical safety, the drug was given the sanitary–epidemiological certificate no. 77.99.11.915.D.001282.03.02.

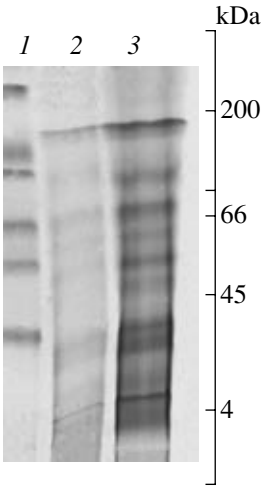

Fig. 1. Electrophoretic analysis of stickleback mucus proteins; lane *1*, molecular weight standards; lane *2*, 20 μl mucus; lane *3*, 100 μl mucus; coomassie staining.

RESULTS

Tests on Atlantic salmon parr with tumors. Epidermal papillary tumor (epithelioma) is found in parr of Atlantic salmon (*Salmo salar*) in northwest Russia from 1970s (Rudikov *et al.*, 2000). High disease incidence (50%) and death rate (30–90%) of affected fish at the age of 1–4 years is observed in salmon fisheries. Water contamination with carcinogens, cocarcinogens (nitrosamine, benzopyrene, etc.), and viral agents (such as herpesvirus) are considered as the factors of tumor formation. The incidence of epithelioma is 1.5% in adult fish (Ziuganov and Kalyuzhin, 2004).

At stage I, light nodes with rough surface appear on fish skin. At stage II, pocky lumps with paraffin consistency and fine granular surface are formed (Fig. 2). The number and size of the lumps can vary from single or multiple plaques to their conglomerates. They can be found on body back and sides, head, and pins. At late stage III, ulcers of different size are formed after tumor rejection, which, after a secondary infection, can be transformed to necrosuppurative inflammations of skin and muscle and eventually lead to degradation of deep tissues and caudal fin necrosis.

Trials of Arktika+ were carried out in 2000–2002 on 2-year-old salmon parr with stage II epithelioma at the Umba Fisheries in the Murmansk Region. Table 1 demonstrates a high curative effect: the survival rate of the parr with stage II disease increased from 7–11% (control) to 89–96% (tenfold on average). The rate of remission (subsiding of the symptoms) increased from 0% (control) to 85–89%. The results were reproduced for three years.

Tests for skin wound healing in guinea pigs Cavia porcellus and for toxicity for rats and mice were carried out in the Laboratory Testing Center of the Institute of

Fig. 2. Two-year-old Atlantic salmons affected by plaque neoplasms (epitheliomata) complicated by *Saprolegnia* infection (a) and recovered parr (b).

Beauty (Moscow) (protocol no. 101, March 3 2000). A 1 × 1 cm derma squares were cut out of 30 guinea pigs (the method of V.S. Peschanskii). Arktika+ treatment accelerated skin wound healing by 62 and 27% by days 8 and 12 after surgery, respectively, as compared to the control group. The drug decreased the healing time by 5 days, which amounted to 19.3% relative to the control group.

Based on these data as well as tests on albino mice ($n = 36$) and albino rats ($n = 30$) allowed the Institute to certify the absence of irritating or allergenic effect on the skin and mucous membranes as well as the absence of acute and subacute toxicity and cytotoxic effect on embryonic diploid human cells (protocol no. 104, February 2001). Overall, the Institute of Beauty certified that the drug has a pronounced wound healing effect and recommended to introduce it in curative cosmetics to accelerate skin regeneration.

Tests for anticancer activity of the biodrug in mice with implanted tumors were carried out at the Petrov Research Institute of Oncology (St. Petersburg). Twenty NMRI mice were intraperitoneally implanted with Ehrlich ascites carcinoma (10^6 cells). Starting from the next day, half of them received the biodrug in their drinking water (1:200 by volume) while the other half receiving pure water served as the control. The volume of consumed water was measured daily; it amounted to 3.5–4.5 ml per mouse per day in both groups. The day of animal death was recorded. Table 2 shows the biodrug-induced extension of the mean mouse lifespan.

According to the report of the head of the Oncology Department Prof. V.N. Anisimov, "All mice of the control group died 9 to 13 days after carcinoma inoculation. In the group of mice receiving the drug, 50% of mice survived day 13. The drug significantly increased the mean lifespan of mice by 16.7% (Table 2). Considering the peptide nature and low toxicity of the mucus, this preliminary result deserves serious consideration and suggests testing the mucus drug for anticancer activity on tumor lines of different histogenesis." Thus, the drug diluted 200-fold (!) demonstrated a significant curative effect in tumor-bearing mice.

Table 1. Trial of Arktika+ drug on two-year-old salmon *Salmo salar* parr with stage II epithelioma kept for 60 days in nurse ponds in the Umba Fisheries (Murmansk Region) in 2000–2002

Fish groups	Total number of fish survived by the end of the experiment (with the number of fish with animals in parenthesis)		
	2000	2001	2002
Untreated (control)	10 out of 100 (10)*	14 out of 200 (14)*	22 out of 200 (22)*
Treated with the drug (experiment)	96 out of 100 (14)*	187 out of 200 (24)*	178 out of 200 (19)*

Notes: * Differences between the compared groups (control–experiment) were significant at $p < 0.05$. Experimental fish were intramuscularly administered 0.1 ml drug (1 mg solid/10 g body weight or 0.1 g/1 kg) into the caudal peduncle using an insulin syringe three times a week for 2 months. Control fish were administered saline using the same schedule.

Table 2. Trial of Arktika+ drug on NMRI mice with implanted Ehrlich ascites carcinoma carried out at the Petrov Research Institute of Oncology (2004)

Group	Time of mice death, days after implantation									
Control (water)	9	10	10	10	12	12	13	13	13	12 ± 0.5*
Drug (1 : 200)	10	11	12	13	13	15	15	15	16	14 ± 0.6*

* Differences between the compared groups (control–experiment) were significant at $p = 0.03$.

DISCUSSION

Disabling the accelerated senescence program in fish increases the production of the antistress secretion. In a series of stickleback populations, absolutely all spawners die after the first reproduction act from accelerated senescence (progeria) at the age of 1 year, which is also typical for Pacific salmons *Oncorhynchus*, pink, chum, and sock-eyed salmons. Fish of this type (semelparous) die after spawning as a result of triggering a special biochemical program, in which the pro-

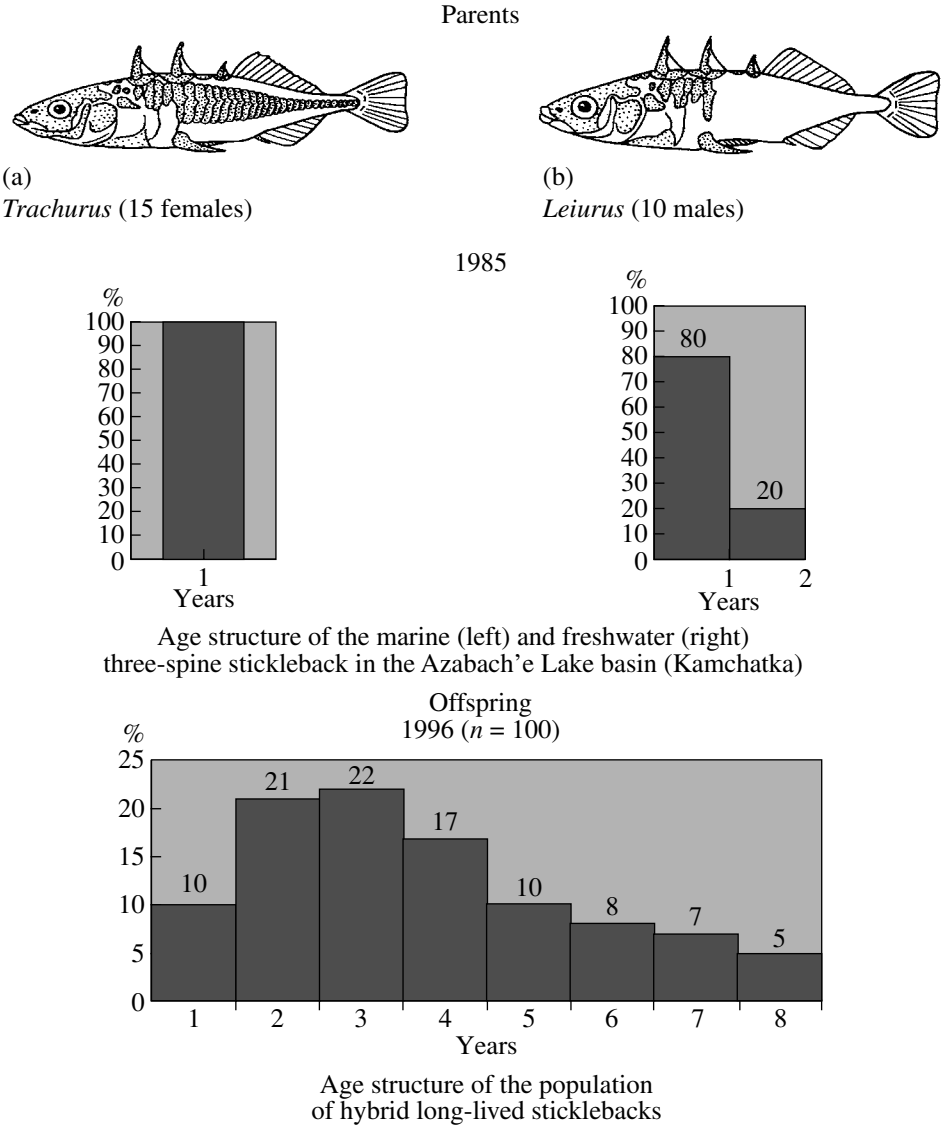

Fig. 3. Schematic long-term experiment on interspecific hybridization of three-spine sticklebacks (Ziuganov, 1988; Ziuganov and Tashenov, 2004). Group hybridization in a pond with semelparous parents with the limiting age of 1–2 years; controlled pollination of females of the marine *Trachurus* (a) by males of freshwater *Leiurus* (b) established fertile long-lived offspring with the limiting age of 7–8 years; parental phenotypes are indicated above.

duction of steroid hormones, particularly, stress hormone cortisol, plays the key role. Accelerated senescence is induced by the activation of the cascade hypothalamus–pituitary–adrenal cortex–high cortisol levels–thymus atrophy (accompanied by depressed immunity)–high blood sugar, fatty acids, cholesterol, and insulin–death of cardiac and renal infarction and insults.

Accelerated senescence of Pacific salmons and sticklebacks can be disabled by gonadectomy or adrenalectomy in juveniles. Such operation doubles their lifespan. Dil'man (1986) was among the first to notice nearly identical hormonal changes during senescence of semelparous fish and human. The difference is a much slower rate of changes in humans than in pink salmon. Recently Maldonado *et al.* (2002) demonstrated a striking similarity between the deposition of β-amyloid plaques in the brain neurons of salmon dying after spawning and the course of Alzheimer's disease in humans. Senescence and related systemic diseases are thought to result from overstrained rather than fading activity of the systems controlling energy processes, adaptation, and reproduction. In particular, the increasing activity decreases the responsiveness of the hypothalamus to the negative feedback signals (Todorov and Todorov, 2003).

While studying the genetics and evolution of sticklebacks, we began to think about a gerontological significance of this fish and its medical use 20 years ago. In 1985 we made an attempt to experimentally disable the program of post-reproductive death in holarctic sticklebacks by interspecific hybridization rather than castration and to extend the lifespan of fish normally living for 1 year (Ziuganov *et al.*, 1987; Ziuganov, 1988). Long term selection in natural water bodies (ponds in the subpolar regions of Karelia) with unusual environmental conditions allowed us to raise hybrid three-spined sticklebacks with high behavioral stress resistance and lifespan extended to 7–8 years (Ziuganov and Tashenov, 2004) (Fig. 3). Hence, monocyclic semelparous fish were transformed to polycyclic iteroparous ones, which die of old age after several acts of reproduction. Noteworthily, these large (up to 10 cm) long-lived fish with outstanding resistance to wounding as well as infectious and parasitic diseases, demonstrated a 150–200% increased production of the abovementioned secretion of renal glandular cells—glue (or mucus). This secretion is produced by all stickleback species. In addition to the main gluing function for the nest, to which the female lays the eggs and which is actively guarded by the male, the secretion can protect the eggs from infections, synchronize their development, protect the male guard from parasites, heal its wounds, and attract females as a pheromone (Ziuganov, 1991). Recently we demonstrated that fish can use is as an antidepressant. Eating the mucus allows sticklebacks to relieve confrontation stress on the spawning grounds and worried depression (Ziuganov and Tashenov, 2004). The selected long-lived fish proved less susceptible to diseases, more slowly aged, and lived for extra 5–7 years. Apparently, this resulted from a genetic heterosis, which improved the activity of hypothalamus and other subcortical brain structures and optimized the neuroendocrine regulation of stickleback spawning.

The mechanism of the biodrug action remains unclear. The fact is that the survival rate of eggs exposed to the secretion approaches 100%, which has been confirmed many times. At the same time, the survival rate of stickleback embryos in an aquarium with up to date equipment (filtration, aeration, water medication, etc.) is 30–40% at best and embryonic abnormalities are often observed (Ziuganov, 1991). The biodrug can stimulate the apoptosis of atypical and tumor cells. The discovery of apoptosis and confirmation of its applicability to all eukaryotic cells made clear that it could play an important role in tumor regression. This aspect is the subject of further inquiries. Our data on the recovery of certain tumor types by the exocrine secretion of hydrobionts transformed from semelparous to iteroparous confirms the hypothesis of Makrushin (2004) that carcinogenesis is an abnormal preparation of the organism to diapause preceding unfavorable environmental conditions. The Arktika+ biodrug reverses this preparation. According to Makrushin (2004), asexual semelparous reproduction was the evolutionary precursor of carcinogenesis, while tissue degradation accompanying asexual iteroparous reproduction was a precursor of senile involution.

Finally, the genome of three-spined stickleback is now actively studied at the Fred Hutchinson Cancer Research Center (United States). American colleagues have obtained interesting data on the stickleback genome (which is similar to the human genome by the number of linkage groups and genes) and now search for the genes determining tumor processes in vertebrates.

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