The structure of colonial interna in *Sacculina polygena* (Crustacea: Cirripedia: Rhizocephala)

Valeria V. Isaeva, Andrey I. Shukalyuk, Anna V. Trofimova, Olga M. Korn, and Alexey V. Rybakov

*Abstract.*—We performed short-term cultivation of alive interna fragments of *Sacculina polygena* Lützen & Takahashi, 1997 isolated from the organism of its host, the coastal crab *Hemigrapsus sanguineus* (De Haan) and studied the histology of parasitic interna and their relationships with the gut and gonads of the host. It is shown that the colonial interna of *S. polygena* comprises an extensive trophic system (roots and trunks) and a reproductive system represented by numerous primordia of externae at different stages of development. We observed asexual reproduction (blastogenesis) in the interna beginning with the development of numerous nuclei, dense aggregations of non-differentiated embryonic cells surrounded by a simple epithelium; later on each nucleus develops a primordium of a trophic module. An ovary with paired colleteric glands, paired male receptacles, and mantle with inner cavity and mantle opening arise in the anlagen of externae prior to their emerging to body surface of the crab, i.e., in the interna. The process of oogenesis begins in the primordium of the ovary; in late primordial externae we have found vitellogenic oocytes. Cells performing functions of ingestion, treatment, and accumulation of nutrients are located in the distal parts (rootlets) of the trophic system; the cells filled with nutrients then release the latter into root lumen, where the nutrients are conveyed toward the primordia and, later on, adult externae. Thus, we have visualized the colonial structure of *S. polygena* interna comprising numerous elements of reproductive and trophic systems.

*Introduction*

The life cycle of parasitic rhizocephalans includes a developmental stage that takes place inside host organism, the interna stage, and the subsequent stage of development of one or numerous externae, which perform the function of sexual reproduction, on the outside, on the body surface of the host. The larval development of rhizocephalans is very similar to that in typical free-living cirripedes and comprises a series of pelagic naupliar instars finally resulting in the development of a cypris larva. However, the subsequent metamorphosis of rhizocephalans takes place in entirely different manner, compared with other Cirripedia, and results in the development of a peculiar parasitic organism lacking all the external features and even the organization as a whole (Bauplan) of arthropods. Female cypris larvae inject into the organism of the future host either a particular larval stage, the vermicion, or a few so-called embryonic cells, which later on give rise to a primordial interna (Glenner et al., 2000). Male larvae introduce, in one way or another, the cells of male sexual line into the developing externa; in most rhizocephalans these cells develop in particular organs, male receptacles, of the externa (Høeg, 1992; Høeg & Lützen, 1993, 1995; Kas'yanov et al., 1997a, 1997b, 1998, 1999). The complication of
life cycle due to the parasitic mode of life leads in some species of the families Peltogastriidae, Sacculinidae and Duplorbidae and in all representatives of Thompsoniidae and Polysaccidae to the development of colonial organization. The latter arises as a result of asexual reproduction (budding) of internae followed by the development of numerous externae (Høeg, 1992; Høeg & Lützen, 1993, 1995; Lützen & Jespersen, 1992). Information is available also about asexual reproduction at endoparasitic stage of life cycle in Sacculina polygenea: numerous nuclei (early primordial externae) have been found in the interna and in one case it was found that two nuclei were interconnected to each other via a common root system (Takahashi & Lützen, 1998). The alive in vitro observations of artificially cultured interna fragments in S. polygenea allowed us to visualize directly the interconnections of two–three nuclei to each other (Isaeva et al., 1999).

The purpose of our project was to study the organization of interna in S. polygenea both in vitro and on histological sections, to distinguish cell and tissue systems of the parasite, to reveal the morphogenesis of numerous primordial externae, and to examine the condition of the gonads in the host crab, Hemigrapsus sanguineus, that are seriously affected by endoparasitic roots of S. polygenea.

Materials and Methods

The material was collected at “Vostok” Marine Biological Station of the Institute of Marine Biology FEB RAS, in Vostok Bay, Sea of Japan, during June–August 1997–1999. We studied internae of Sacculina polygenea Lützen & Takahashi, 1997 parasitizing the crab Hemigrapsus sanguineus (De Haan). To perform alive and histological observations we isolated large fragments of S. polygenea interna from the host crabs and carefully dissected the primordia of externae and other parts of the interna. In our observations we used 57 sacculinized crabs, while altogether 78 crabs H. sanguineus has been examined. As a medium for in vitro short-term (4–24 h) cultivation of S. polygenea interna fragments we used centrifuged hemolymph of healthy crabs H. sanguineus added with some antibiotics (Isaeva et al., 1999). The in vitro observations of the interna were performed using a “Telalav” inverted microscope. For histological purposes, the interna fragments isolated from the host’s organism were fixed with Bouin fluid, embedded in paraffin and stained by hematoxylin-eosin. To prepare semi-thin sections the material was fixed with 4% glutaraldehyde, postfixed with osmium tetroxide and stained with methylene blue.

Results

Level of sacculinization, localization of interna and pathological effects in the host crab gonads

Among 78 examined specimens of Hemigrapsus sanguineus, 47 carried 1–7 (most often 1–2) externae; furthermore, in at least 16 crabs without externae (9 of them showed scars left by perished externae) we have found well developed internae of the parasite. Thus, the fraction of crabs parasitized by Sacculina polygenea in the coastal zone of Vostok Station exceeded 80%.

The developed internal part of the parasite, the interna, is located in the hemolymph of body cavity of sacculinized crabs and is clearly visible among the tissues and organs of the crab due to its yellow coloration. The interna is fastened around the posterior region of the host’s gut (Fig. 1 A) making up a dense socket surrounding the hindgut (Fig. 1 B). The bulk of the interna is rather compact, the extending roots are mostly localized around the digestive (hepatopancreas and hindgut) and reproductive systems of the
host crab; we did not observed rootlets penetrating into the limbs of the crab. The branching rootlets of interna seem to show several successive dichotomic divisions; the branching is rather chaotic and shows no regular pattern.

It turned almost impossible to isolate the whole intact interna from the organism of the host crab as it is intimately associated with both the gut and the gonads of the host.

The lesion of gonads by penetrating rootlets of the sacculina leads to sterilization of host crabs that was observed in both sexes. The ovaries of female crabs were mostly heavily affected: the ovary wall turned destroyed and rootlets (distal portions of the trophic system) penetrated into the ovary so that the oocytes of the crab were scattered in-between the rootlets being in direct contact with the trophic system of the sacculina (Figs. 1 C, D). The surprising thing is that only a few oocytes showed degeneration (Fig. 1 D). They were destroyed, resorbed and seemed to be utilized by the trophic system of sacculina.

The degeneration of testes in
Fig. 2. A, Pathologically changed testis of a host crab with lobes of testis and rootlets of trophic system of *S. polygenea*, *in vitro*; T: lobes of crab testis; R: rootlets; scale = 100 μm. B, Same as fig. A, a histological section; scale = 100 μm. C, Spermatids of a host crab in the testis penetrated by parasitic rootlets; scale = 10μm. D, A primordium of an externa and transport trunks of *S. polygenea*; PE: primordium of externa; TT: transport trunks; scale = 100 μm. E, Transport canals of the trophic system; E: epithelium of the canals; UN: unstructured mass of nutrients; PE: primordium of externa; scale = 100 μm. F, Distal canals (rootlets) of trophic system in *S. polygenea*; BC: basophilic non-differentiated cells; CD: cell debris, remnants of destroyed cells in the lumen of the canal; scale = 10 μm.
sacculinized male crabs showed somewhat different pattern. The testis is also surrounded by dense mass of trophic system rootlets (Figs. 2 A, B), however the testis wall usually remains almost intact. The pathological effects are revealed as numerous lobes arising on the testis wall. The observations on live interna fragments of _S. polygenea_ isolated together with the gonads of _H. sanguineus_ clearly demonstrated well pronounced hypertrophic changes in crab testes including the numerous lobes arising on testis wall (Fig. 2 A) and even the development of a kind of adenoma. The spermiogenesis in the sacculinized male crab is suppressed; on the other hand, the spermatogenesis up to the development of spermatids seem to be not affected by the parasite (Fig. 2 C).

**Structure of interna**

Trophic system. The interna of _S. polygenea_ is differentiated into two major systems: a trophic system and a reproductive system. The trophic system, in turn, comprises distal branching rootlets (Figs. 1 A, B) extending throughout the hemocoel of crab host and transport trunks (Figs. 2 D, E) interconnecting mature externae and their primordia with the roots.

The epithelium of distal canals of the trophic system includes at least two types of cells: non-differentiated cells with very basophilic cytoplasm, large nucleus and nucleolus (these seem to be cambial cells) and differentiated cells containing vacuoles comprising accumulated nutrients (lipids and inclusions of other nature), which might be distinguished in live internae, on histological preparations and on osmified semi-thin sections. The contents of the vacuoles, together with the remnants of cytoplasm, might be observed inside the distal canals of the trophic system (Fig. 2 F); while in the lumen of large trunks, one can see a homogeneous unstructured mass (Fig. 2 E).

The lining of these transport canals interconnecting the trophic system and the developing primordia of externae and mature externae is made up of a folded epithelium; the cells containing trophic inclusions are absent there (Fig. 2 E).

Reproductive system. Numerous primordia of future externae are located inside the major portion of interna that is arranged around the posterior part of host's gut, close to the border between cephalothorax and abdomen (Figs. 3 A, C). There, in direct vicinity to the crab's gut, one can retrace all morphogenesis stages of the externa primordia.

The earliest primordia arise as dense aggregations of non-differentiated embryonic cells characterized by compact, very basophilic cytoplasm and a large nucleus containing a large clearly noticeable nucleolus (Fig. 3 B). In this portion of interna one also can see isolated embryonic cells showing the same morphology. A characteristic morphological marker of later primordia is the presence of an epithelized vesicle enclosing the mass of non-differentiated cells of the future visceral sac, the nucleus, surrounded by primordial perisomatic cavity (Figs. 3 C, D). The nuclei are characterized by intensive mitotic cell divisions: inside a nucleus one can see 6–8 mitoses in a single field of vision (Fig. 3 D).

The **primordial externae of _S. polygenea_** are usually arranged in groups constituted of several primordia at either similar or different stages of development, from early nucleus to almost completely developed primordium of externa (Figs. 3 A, C, E).

In the course of subsequent development the external cell layer of nucleus is epithelialized (Figs. 3 C, E, F); each primordium splits into two portions, one (containing the inner cell mass of the nucleus) then forms the externa, another makes up the rootlets of the trophic system (Fig. 3 F). In both the cases one may observe typical epithelial morphogeneses through
Fig. 3. A, Early primordia in the interna of *S. polygenea*; IE: intestinal epithelium of the host crab; scale = 100 μm. B, A detail of fig. A, at greater magnification; ESC: embryonic stem cells; NU: nucleus; scale = 20 μm. C, More advanced primordia in the interna of *S. polygenea*; G: gut of the host crab; IE: intestinal epithelium of the host crab; NU: nucleus; scale = 100 μm. D, A nucleus (a detail of fig. C) at greater magnification; PC: perivisceral cavity; E: epithelium layer; ICM: internal cell mass; scale = 20 μm. E, The primordia of externae connected to each other, *in vitro*; scale = 100 μm. F, A primordium of externa with developing primordium of trophic system; PE: primordium of externa; PT: primordium of trophic system; scale = 50 μm.
development of folds, invaginations and evaginations of epithelial surfaces (Fig. 3 F). Both the primordia at this stage are enclosed by a common epithelial envelope and have no contact with the hemolymph of the host crab (Fig. 3 F). The germinal primordium (primordium of the ovary) in each future externa is represented at this stage by an aggregation of cells showing the characteristic morphology of non-differentiated embryonic cells: they have intensively basophilic cytoplasm, large nucleus and a nucleolus.

Later on, subsequent processes of epithelial morphogenesis take part in the primordial externae and all the structures develop that are characteristic of an externa ready to emerge. In dissected primordial externae 1–2 mm in diameter that are still inside the interna, one can see branched ovary (Figs. 4 A, B) and mantle surrounding the latter, with pronounced mantle cavity and mantle opening plugged by epithelium, covered by cuticle (Fig. 4 B). The mantle opening opens only after the virgin externa emerges to the outside, onto the ventral surface of host crab. In the branched ovary we revealed cells of female germinal line; in late primordia of externae, right before the emergence onto the body surface of host crab, one can see oocytes at different stages of development, from the most early to vitellogenic (Fig. 4 C). Between the lobes of the branched ovary there are striated muscle fibers (Fig. 4 C) and connective tissue. At the same time paired receptacles (Figs. 4 A, D) and colleteric glands (Fig. 4 E) appear in the primordium. Such a differentiated primordial externa, as well as the primordium of a miniature trophic system connected with the former, is already emerged (everted) from the epithelial cover enclosing the primordium earlier on, into the hemal cavity of host crab; beginning from this moment the newly arisen elements of trophic system seem to start functioning.

Thus, inside each single interna we can see numerous module elements of reproductive and trophic systems of different size and at different stages of development.

Discussion

*Sacculina polygenea* was described recently from the coastal crab *Hemigrapsus sanguineus* (De Haan, 1835) in Japan (Lützen & Takahashi, 1997). The invasion level of crabs in different coastal areas of Japan ranges within wide limits, from 1.1–1.7% in Tomioka Bay to 41.6–79.6% in Inuki Bay of Oyano Island (Yamaguchi et al., 1994; Takahashi & Lützen, 1998). We also registered very great level of invasion (more than 80%) in Peter the Great Bay, Sea of Japan, close to Vostok Marine Biological Station.

*General morphology of Sacculina and effects on the host*

The terms “externa” and “interna” were introduced into practical usage by Yves Delage (1883) to distinguish the internal (“Sacculine interne”) and external (“Sacculine externe”) portions of *Sacculina carcini*, a common parasite of the European green crab, *Carcinus maenas*. Delage also introduced the term “nucleus” to designate the compact mass of cells forming the visceral mass of the primordial externa. Glenner et al. (2000) preferred to replace this “confusing” term with the more neutral “globular body”.

In *S. polygenea* the interna is relatively compact, its roots do not penetrate into the limbs of host crab, like Delage has shown for *S. carcini* (Delage, 1884) on his classic drawings, now reproduced in almost all manuals for invertebrate zoology. No regular branching pattern is observed; the structures like “lamp brushes” of peltogastrids (Høeg, 1982; Høeg & Lützen, 1995; Bresciani & Høeg, 2001) obviously are absent.

The gonad lesions in the crabs
Fig. 4. A, A primordium of externa in vitro; MO: mantle opening; RE: receptacles; scale = 100 μm. B, A sagittal section of a primordium of externa; MC: mantle cavity; OV: branched ovary; MO: mantle opening; scale = 100 μm. C, Oocytes in the ovary of primordial externa; M: striated muscle fibers of the ovary; scale = 50 μm. D, Frontal section of a primordial externa; OV: ovary; MC: mantle cavity; RE: receptacles; scale = 100 μm. E, Canals of colleteric gland at a histological section; CG: colleteric gland; OV: ovary; scale = 100 μm.
Fig. 5. Schematic drawing of a parasitic colony in *S. polygenea* showing early primordia of the reproductive system (left frame), a primordial externa and adjoining parts of the trophic system (right frame; connection of the primordium with external epithelial layer not shown); CD: cell debris, remnants of destroyed cells in the lumen of rootlet canal; E: epithelia of interna; ESC: embryonic stem cells; EX: adult externa; G: gut of host crab; IE: intestinal epithelium of host crab; JE: juvenile externa just emerged onto the body surface of host crab; NM: unstructured mass of nutrients; NU: nucleus; PE: primordium of externa; R: trophic system rootlets; TM: trophic module of externa; TT: transport trunks.

*Carcinus maenas* and *C. mediterraneus* caused by *S. carciini* result in suppression of spermatogenesis and vitellogenesis; it was shown that the roots of sacculina penetrate into the ovary of female crabs and come in contact with oocytes, while in male crabs a hypertrophy of testis was observed (Rubiliani et al., 1980). We observed similar changes in gonads of *H. sanguineus* infected by *S. polygenea*. The gonads of the host crab seem to represent a rich source of nutrients for the trophic system of the sacculina interna. Despite the pathological changes in the testes of host crabs parasitized by *S. polygenea*, the spermatogenesis still takes place, like it was reported earlier on for craboids

*Paralithodes platypus* parasitized by rhizocephalan *Briarosaccus callosus* (Meyer, 1990). However, according to our results, the spermatogenesis only proceeds up to the appearance of spermatids, whereas the spermiogenesis is suppressed (or perhaps spermiogenesis is simply arrested at the stage reached when the parasite exerts its action).

It was repeatedly noted that sacculinized crabs show morphological and behavioral feminization (Day, 1935; Hartnoll, 1967; Ritchie & Høeg, 1981). The invasion of the *S. polygenea* was also shown to be accompanied by changes in behavioral reactions in both sexes of crabs *H. sanguineus* (Takahashi et al., 1997)
and morphological modifications in the males (Yamaguchi & Aratake, 1997). Our results also provide evidences about clearly pronounced morphological feminization of male crabs parasitized by S. polygenea.

**Trophic and reproductive systems of interna**

As it was already noted in our previous short communication (Shukalyuk & Isaeva, 2000), one can distinguish two major systems in the interna of S. polygenea, a trophic system and a reproductive system. The histological examinations allowed us to distinguish the colonial structure of interna in S. polygenea, with numerous elements of reproductive and trophic systems at different stages of growth and development. Numerous externae located at the body surface of host crab are interconnected by trunks (the nutrient transport system) with the rootlets and the major mass of the interna, being components of the complex colony, which is schematically shown at Fig. 5.

The trophic system of the interna in S. polygenea is differentiated into distal rootlets, which seem to perform the function of nutrient ingestion from host crab hemolymph, their treatment and accumulation, and transport canals, the trunks, interconnecting mature externae and their primordia with the rootlets. In the distal regions of trophic system we revealed non-differentiated basophilic cells, probably stem cells, and differentiated cells performing the trophic function. It seems that these differentiated cells are similar to axial cells revealed by ultrastructural investigation in the rootlets of some other rhizocephalan species (Hubert et al., 1979; Payen et al., 1981; Høeg, 1992; Bresciani & Høeg, 2001). The granules of accumulated nutrients are released into the lumen of trophic system canals, also the shedding and destruction of the cells into fragments might be visible in the lumen of the canals. In all likelihood, the final process of cell destruction represents the apoptosis, a programmed cell death, because the morphological features of the moribund cells in the trophic system of S. polygenea, disintegration of cytoplasm into fragments and condensation of chromatin are characteristic of apoptosis processes studied on other organisms (Kerr et al., 1972; Jacobson et al., 1997). The similar stages of disintegration were revealed during ultrastructural study in trophic system cells of another representative of rhizocephalans, Peltogasterella gracilis (Shukalyuk et al., 2001), in this species cells filled with nutrients disintegrate making up the cavity and releasing the nutrients together with cell remnants into the canal lumen. The death of these cells provides the completion of their morphogenetic and trophic functions.

The reproductive system is represented in each interna by numerous primordia of externae at different stages of development. The primordial externae appear as nuclei, numerous compact clusters of non-differentiated embryonic cells surrounded by a layer of epithelium. Such a morphology and morphogenesis of early nuclei has been already described in other species of Rhizocephala (Pérez, 1928; Bocquet-Védrene & Parent, 1972; Høeg, 1982; Høeg & Lützen, 1995). In the same area, close to the early nuclei, there are also isolated cells showing morphology identical with the non-differentiated nucleus cells. They probably represent embryonic stem totipotent cells capable of forming the future nuclei. It seems that the reproductive capacity of interna in S. polygenea is very great or even almost unlimited providing successive generations of externae during several years.

Later primordium gives rise to the primordium of the trophic system. It seems that each primordial externa, being inter-
connected with the main trophic system of the interna, is also provided by a personal supplementary trophic module, the module that is the nearest to the developing externa. The organization and morphogenesis of the primordium, when the root system and epidermis of externa develop from epithelium, while the visceral sac develops from the nuclei are described, in more or less detail, for several species of solitary rhizocephalans (Smith, 1906; Day, 1935; Reinhard, 1942; Bocquet-Védrine & Parent, 1972; Rubiliani et al., 1980; Høeg, 1982, 1992); there are only minor disagreements in the interpretation of development succession of perisomatic and mantle (brood chamber) cavities in the course of nucleus development (Bocquet-Védrine & Parent, 1972). In the species with colonial organization of interna the processes of morphogenesis and cytodifferentiation were not studied until now.

It seems that the morphogenesis of each of the numerous primordia that later on give rise to both the externa and the trophic module connected with the former is similar to the differentiation of the only primordium in solitary rhizocephalans. We also have retraced the following differentiation of primordial externa; for this purpose we examined the primordia of future externae isolated from the tissues of internae. It is shown that even prior to the emerging of virgin externa onto the body surface of host crab it already has all tissue and organ systems differentiated including ovary with developing oogonia and oocytes at all stages of oogenesis up to vitellogenesis. The processes of gonad differentiation were studied only in a few species of Rhizocephala; it was known that the ovary develops from the cells of interna nucleus (Bocquet-Védrine, 1961; Høeg, 1982), however, no descriptions of gametogenetic processes in the interna were available.

Asexual reproduction and coloniality

As it was already known, the interna of S. polygenea comprises numerous nuclei, which in one case were reported to be interconnected to each other. This allowed the authors to make a conclusion that there is asexual reproduction in the life cycle of this species (Takahashi & Lützen, 1998). The nuclei of S. polygenea are usually arranged in tight groups consisting of two or more bugs (Isaeva et al., 1999; Shukalyuk & Isaeva, 2000). Thus, in S. polygenea the asexual reproduction at embryonic stage of development (blastogenesis) results in development of colonial organization. The development of numerous nuclei from the epithelial wall of an early interna (young tumor) was also described in the solitary S. carcini (Rubiliani et al., 1980). Judging from the descriptions of Rubiliani with co-authors, after a lapse of time all these nuclei but one disappeared, the only retained nucleus then develops into the adult externa.

In all studied representatives of Peltogasteridae, Lernaeodiscidae and Sacculinidae the development of particular structures, so called "root follicles" is described inside the early interna (Pérez, 1931a, 1937; Bresciani & Høeg, 2001). In peltogastrids they represent bulbous terminal expansions clearly noticeable on parasite rootlets located within the cephalothorax of the host. Similar follicles, sessile or sitting on short stalks also arise along the rootlets. In Peltogaster the follicles usually are colorless and might easily be distinguished from the rest of the root system, which is usually green colored (Pérez, 1931b). Moreover, unlike the remainder parts of root system, the follicles are intensively stained by neutral red (Pérez, 1937; Bresciani & Høeg, 2001). The rootlets of sacculinids bear so called "flask-shaped follicles" (or "follicules lageniformes" according to Pérez). In live internae they
also are intensively stained by neutral red, but, unlike the follicles of peltogastrids, they are sitting on narrow stalks and each has on terminal surface a peculiar pocket made up of invaginated epithelium.

Pérez (1937) supposed that the root follicles represent growth zones. However, in all likelihood, the contents of the follicles represent nothing more nor less than secondary nuclei. In true colonial species, in particular *S. polygena*, they represent primordia of future externae, so the terminal invagination gradually develops into the mantle and visceral mass of the future externa. In this connection, it is pertinent to note that the root follicles are present not only in the colonial species, but in solitary rhizocephalans as well, in particular they have been revealed in the species of *Peltogaster, Septosaccus, Drepanorchis* and non-colonial *Sacculina* (Bresciani & Høeg, 2001). It seems that the development of numerous anlagen of secondary nuclei provides additional assurances that the life cycle would be successfully completed in case, for example, when developing externa dyes at early stage of development. Thus, we have every reason to suppose that all representatives of the families Sacculinidae, Peltogastridae and Lernaeodiscidae have strong prerequisites for the development of colonial organization. In non-colonial species with numerous anlagen of secondary nuclei the development of one, main, primordium of externa is probably accompanied by inhibition of the development of all secondary anlagen.

The budding (asexual reproduction) of interna in some species of Rhizocephala with colonial organization results in the development of a complex life cycle with alternating sexual and asexual generations, i.e., the metagenesis (Ivanova-Kazas, 1979; Kas'yanov et al., 1997b). A specific feature distinguishing the colonies of rhizocephalans from the colonies of coelenterates, ascidians and other colonial animals is that in the former species the colonies are entirely constructed of only female organisms, the male sex is reduced down to spermatogenic cells injected into the female externae by pelagic plankton male larvae.

Thus, our observations on live internae of *S. polygena* and histological studies allowed us to visualize the colonial organization of interna in this species comprising numerous elements of reproductive and trophic systems integrated into an organism of a higher rank, which is entirely lacking of major morphological characters and the whole Bauplan of arthropods.

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Addresses: (AVT) Department of Cytology and Histology, Far Eastern State University, Vladivostok 690000, Russia; (VVI, AIS, OMK & AVR) Institute of Marine Biology, Far East Division, Russian Academy of Sciences, Vladivostok 690041, Russia

E-mail: (AVR) avr@eastnet.febras.ru (VVI) ibmfebas@online.ru