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Ontogeny of the sodium pump in embryos of rockfish of the genus *Sebastes*

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Synopsis

The purpose of the present investigation was to determine at what stages of embryonic development the sodium pump (Na +, K + -activated ATP phosphohydrolase, EC 3.6.1.3) appears and whether the ontogeny of the sodium pump plays a role in the matrotrophic viviparity of *Sebastes*. Early larval stages (stages 1–14) had embryonic tissues nearly devoid of Na, K-ATPase activity. After epiboly, tissues from embryos taken from both species, *S. schlegeli* and *S. taczanowskii,* had significant levels of enzymatic activity coincident with the appearance of neuronal tissue in the head fold (stages 15–20). Maximal levels of specific activities of the enzyme were reached with the onset of the vascular circulation and the opening of the buccal cavity together with maturation of the midgut and hindgut regions of the intestinal tract (stages 26 and 28). Ouabain, a specific inhibitor of Na, K-ATPase, was used to measure survival of embryos placed in different seawater concentrations under in vitro conditions. The most sensitive stages to external ouabain were 26–30. These findings support the hypothesis that the intestinal tract is functional prior to gestation and could be transporting important nutritive material found in maternal ovarian fluid.

Introduction

Extracellular fluid regulation in adult and juvenile marine teleosts is dependent upon the osmoregulatory function of two extrarenal organs, the gill and the intestinal tract (Conte 1969, Hickman & Trump 1969, Kirsch et al. 1981, Alderdice 1988). The midgut region of the adult gastrointestinal tract counterbalances the integumental osmotic body water loss by permitting epithelial absorption of water and salt to occur after the fish begins drinking seawater. Simultaneously, the gill arch eliminates the excess salt from the ingested seawater by allowing chloride secretory cells of the gill filaments to secrete sodium chloride to the outside environment.

It is logical to assume that marine teleost larvae would regulate extracellular fluids by a similar style and employ extrarenal structures for achieving ionic and osmotic balance. Unfortunately, developmental patterns are not known for such extrarenal structures as the gut and gill, which show the functional interdependence between embryonic extrabranchial chloride cells and ionic function of the developing gill filaments (Alderdice 1988).

In transporting epithelia, such as that lining the midgut and gill filaments, the sodium pump [ouabain-sensitive, sodium and potassium ion coupled

transport ATPase or Na, K-ATPase] represents the major driving force for the transepithelial transport of ions, organic molecules and water (Rossier et al. 1987). Fish gill and intestinal epithelia are rich in Na, K-ATPase enzymatic activity. In addition, the localization of Na, K-ATPase enzyme over the transporting epithelial cell surface is asymmetrically distributed and therefore gives the cell a functional polarity. In fact, the polarity of the epithelial sodium pump allows for vectorial transport of solute movement, which in turn controls volume and composition of extracellular fluids.

In oviparous species of teleost fishes, gametes are shed from the body, ovarian cavity or both, and fertilization together with embryogenesis occurs in the outside environment. In viviparous species, fertilization of gametes and development of the embryos and larvae occur within the ovary. Lecithotrophic viviparity is primarily dependent upon yolk material, stored in the egg following oogenesis, being sufficient to complete embryo development after fertilization. However, in matrotrophic viviparity, growth and development of the late embryonic stages are dependent upon maternal nutrition in addition to that stored in yolk platelets provided during oogenesis. Past research (Boehlert & Yoklavich 1984, Boehlert et al. 1986) on the reproductive mode exhibited by *Sebastes melanops* and *S. schlegeli* has given evidence that these species exhibit matrotrophic viviparity; females apparently provided nutrition during gestation of the embryo long after organogenesis but soon after the functional differentiation of the gut. This delayed gestation provides a time period allowing the maternal nutritional mechanism to act. In this case, the development of the intestinal transport epithelia along with the de novo synthesis of the sodium pumps probably is essential for nutritional uptake. For example, larvae at the late stages would initiate the drinking reflex and begin swallowing maternal ovarian fluid entering the buccal cavity. Following passage of this organically rich saline from the buccal cavity through the esophagus and stomach cavities, the fluid would enter into the lumen of the intestinal tract for assimilation. Upon reaching the midgut regions, the absorptive epithelium would transport water, ions and also nutrients, such as amino acids and proteins (Shimizu et al. 1991).

The purpose of this study was to determine at what stages of embryonic development the sodium pump appears and whether the ontogeny of the sodium pump plays a role in the matrotrophic viviparity of *Sebastes*.

Materials and methods

Experimental animals and developmental staging

White-edged rockfish, *S. taczanowskii*, were obtained by gill-netting or hook-line angling from the Usujiri shore near Hakodate, Hokkaido. Immediately after capture, fish were transported and placed in a 1-ton tank with flowing seawater and held until the start of the experiment. Kurosoi, *S. schlegeli*, were obtained from either captive brood stock at the Hokkaido Institute of Mariculture in Shikabe or were collected in Otobe in the Sea of Japan in May-June 1985. Fish were held in indoor tanks (1.8 × 1.8 × 1.1 m) with flowing seawater until the start of the experiment.

Pregnant females of *S. taczanowskii* were anesthetized with ethyl 4-aminobenzoate, then the ovaries were carefully dissected to allow removal of the developing embryos. Seven lots of 100 embryos were obtained from a pregnant female containing a single brood of synchronously developing embryos. In pregnant females of *S. schlegeli*, the embryos were removed by gently suction into a silicon tube inserted through the genital aperture. Classification of the embryonic stages used in the study followed the protocol described by Yamada & Kusakari (1991). Excess ovarian fluid was removed by blotting embryos with dry filter paper. Embryos were either frozen or used fresh for enzymatic analysis. Fresh embryos were placed in small (1 ml) glass homogenizers and homogenized in a sucrose-imidazole-EDTA (SIE) medium. Tissue homogenates were stored at −40° C until chemical and enzymatic analyses could be performed.

Enzyme and ouabain-mortality analyses

Na + K-activated ATPase activity

The homogenizing medium (SIEs) used for *S. schlegeli* consisted of 0.25 M sucrose, 0.1 M imidazole buffer at pH 7.2 and 2 mM Na_2EDTA; the homogenizing medium (SIEt) used for *S. taczanowskii* consisted of 0.25 M sucrose, 0.02 M imidazole buffer at pH 6.8 and 6 mM Na_2EDTA. Fifty or one hundred embryos were added to 1.0 ml of SIE and homogenized for 1–2 min in a cold glass homogenizer. The homogenate was either stored in a freezer at − 40° C or used immediately for enzyme analysis.

The reaction mixture that gave optimal activity for *S. schlegeli* enzyme preparations consisted of 0.2 ml of salt solution (0.8 M NaCl, 0.2 M KCl), 0.2 ml of AMI solution (12.5 mM Na_2ATP, 25 mM $MgCl_2$, 250 mM imidazole, pH 7.2, with and without 12.5 mM ouabain) and 0.1 ml enzyme preparation. The reaction mixture was incubated at 37° C for 20 min and then stopped with 1.0 ml of 5 or 10% TCA reagent. Inorganic phosphate released from ATP was assayed by the method of Peterson et al. (1978). Protein was determined by the Peterson (1977) modification of Lowry et al. (1951) method by using serum albumin as the standard.

The reaction mixture that gave optimal activity for *S. taczanowskii* preparations consisted of 0.4 ml salt-substrate medium for *S. schlegeli* (337.5 mM NaCl, 162.5 mM KCl, 50 mM $MgCl_2$, 12.5 mM Na_2ATP, 250 mM imidazole, pH 7.0 with and without 2.5 mM ouabain) plus 0.1 ml of enzyme preparation. The reaction mixture was kept in an ice bath until the addition of enzyme, incubated at 37° C for 20 min and then stopped by adding 10 ml of 5% TCA reagent. Inorganic phosphate released from ATP was assayed by the method of Goldenberg & Fernandez (1966).

The specific activity of the sodium pump was determined as the difference between phosphate released in the absence and presence of ouabain. Activity was expressed as micromoles phosphate released per hour per mg protein or per 100 embryos.

Larval ouabain-mortality assays

Ouabain crystals (strophanthin G octahydrate, Sigma Chemical Co., St. Louis, MO) were added to each saline solution to a concentration of 1 mM. Sterilized seawater was used as the basic saline medium, and it was diluted with distilled water to 10, 33, 67% of full strength (100%) seawater. The associated osmolalities were 96, 304, 568 and 865 mosm · kg^{-1} respectively. Standard-sized petri dishes were filled with 20 ml of saline solution with and without ouabain. *Sebastes schlegeli* embryos at various stages of development were exposed in triplicate dishes; numbers of embryos per replicate ranged from 28 to 121. At select time intervals (24, 48 and 72 h), dead embryos were removed from the exposure dishes and counted.

Results

Embryonic Na, K-ATPase activity

The Na, K-ATPase activity in early developmental stages of both species was absent. However, as growth and development of the embryos proceeded, there occurred in the embryonic tissues an onset and elevation of Na, K-ATPase specific activities (Fig. 1). Apparently, all stages following epiboly have progressively higher enzyme levels. Unfortunately, fewer replicates were performed on *S. schlegeli* due to the lack of embryos, and the results of the enzymatic activities at the earliest stages were based upon only one aliquot of embryos. However, seven samples of embryos were available for enzymatic analysis at each stage of *S. taczanowskii* assayed. In *S. taczanowskii* it was found that among the embryo samples there appeared to be little enzymatic variability as shown by error bars. Thus, our interpretation of the developmental pattern for the Na, K-ATPase is based upon these data. For *S. taczanowskii* species there is an absence of Na, K-Atpase in the very early stages (stages 1–14) until well after epiboly. Following the appearance of the neuronal tissue in the head fold and the formation of the optic vesicle together with body somites (stages 15–20), the detectable levels

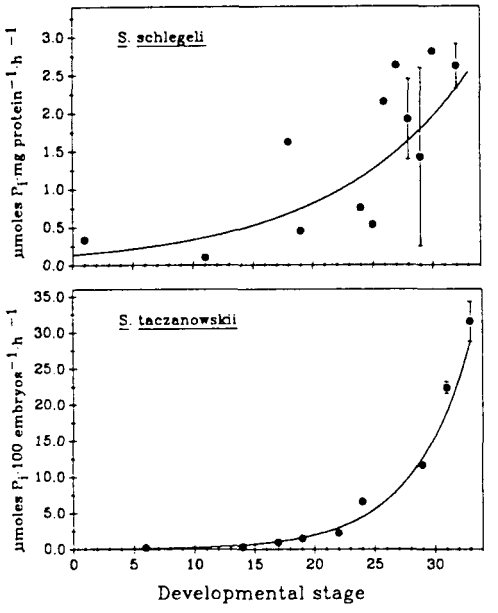

Fig. 1. Ontogeny of the sodium pump in two species of *Sebastes*. The difference in activities between absence and presence of ouabain are presented in two different ways for the different species. Data for *S. schlegeli* are fitted to the curve log (activity) = − 1.973 + 0.88 (stage), N = 12, r^2 = 0.583; all values are from a single determination except for stages 24 (2 determinations), 28 (3), 29 (2), and 32 (3). Data for *S. taczanowskii* are fitted to the curve log (activity) = − 3.441 + 0.206 (stage), N = 9, r^2 = 0.963; all values are the mean of seven replicates. Error bars represent 1 SD.

of enzyme activity increased substantially. However, the most dramatic increase in Na, K-ATPase specific activity occurred with the onset and establishment of the vascular circulation, the opening of the buccal cavity and the maturation of the midgut and hindgut regions of the intestinal tract (stages 26–28). These enzyme activity levels corresponded with the morphogenetic development of the post-abdominal body structures and remained relatively stable until hatching (stages 31–32), then increased dramatically. The high levels of enzyme activity in newborn larvae – somewhat higher than the hatching stages – might be related to the need to drink full-strength seawater.

Ouabain mortality during embryogenesis

The effect of ouabain, a specific inhibitor of Na, K-ATPase, on the survival of embryos placed in different external salines was measured. The ionic gradient and length of exposure impact the maintenance of osmotic equilibrium by the developing osmoregulatory organs at various stages of embryogenesis. Exposures to 10 and 100% seawater resulted in nearly 100% mortality of embryos at five different stages. Apparently 33 and 67% seawater constituted the more appropriate range for assaying embryonic osmoregulation and salt tolerance. Therefore, these salines were used to determine the effect of inhibiting the sodium pump with ouabain and its impact upon embryonic osmoregulation and salt regulation.

The earliest embryos tested (stages 1 and 24) were not affected by the presence or absence of 1 mM ouabain for exposure times of 24, 48 and 72 h at either 33 and 67% seawater. Longer exposure times of 48 and 72 h produced more variability among the control group mortalities, whereas the 24 h exposure time had relatively low mortalities at stages 24–30 as shown in Figure 2. In contrast, the sensitivity to external ouabain was observed to increase in stages 25, 28 and 30.

Discussion

Numerous studies on oviparous species of teleost fishes have shown that fertilized eggs and embryos can maintain osmotic gradients between the extracellular fluids and the external environment (Holliday & Jones 1965, 1967, Wiesbart 1968, Holliday 1969, Rudy & Potts 1969, Eddy 1974, Shen & Leatherland 1978, Tay & Garside 1978, Linden et al. 1979, Guggino 1980a, 1980b, Quast & Howe 1981). The major mechanism in the early life stages, such as from the zygote to the end of epiboly, transforms the perivitelline membrane from a semipermeable water barrier to one that is virtually impermeable (see review by Alderdice 1988). Called 'water hardening', this mechanism operates from the time of fertilization until the end of epiboly.

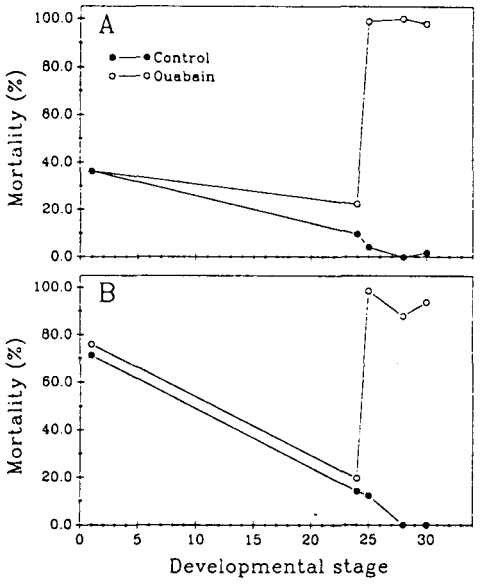

Fig. 2. Mortality rates in vitro for *Sebastes schlegeli* embryos at different developmental stages incubated in saline with and without 1 mM ouabain. Each point is the mean mortality rate of three replicates calculated after arcsine transformation: a – 304 mosm · kg^{-1} (33% seawater) saline, b – 568 mosm · kg^{-1} (67% seawater) saline.

From post-gastrulation to hatching, teleost fishes employ the mechanism of osmoregulation to compensate for fluid loss and utilize some type of osmoregulatory organ. Studies by Guggino (1980a, 1980b) have clearly shown that the osmotic water loss occurs along the embryonic integumentary surfaces. The fish embryo immediately initiates some type of drinking behavior to counterbalance the loss of fluid. In the killifish, *Fundulus heteroclitus,* the embryo has a measured drinking rate of 600 picoliters per mg protein per hour, as determined by the C^{14}-dextran method (Guggino 1980a, 1980b). Seawater does not enter through the mouth but rather through the branchial chambers that communicate anteriorly with the pharynx and posteriorly through pores connected to the perivitelline space. Excretion of excess salt is via ionic regulation that utilizes epithelial cells involved in active transport of ions. The site of extrusion of chloride ion has been identified as chloride cells in the epithelium lining the embryonic cavity and yolksac. After hatching occurs, the site of chloride secretion is mostly in the gill filaments. Unfortunately, measurement of the sodium pump enzyme has not been made in any of the larval stages. Hwang (1989) has reported the distribution and morphology of chloride cells in various teleost larvae.

Other oviparous species – in which the newly hatched embryos lack gill filaments and have the kidney represented only by the pronephric glomerulus and the gastrointestinal tract without either an oral or anal opening – have been shown to utilize other kinds of epithelial structures as pseudo-osmoregulatory organs. For instance, the skin reportedly is the major osmoregulatory organ for herring, *Clupea harengus* (Holliday & Jones 1965), plaice, *Pleuronectes platessa* (Holliday & Jones 1967), and sardine, *Sardinops sagax,* embryos (Lasker & Threadgold 1968), when placed in different levels of saline.

In contrast with the oviparous species, the number of studies on osmoregulation in embryos of viviparous species of teleosts is sparse (see review by Wourms et al. 1988). Since it has been shown by Veith (1979) that the follicular fluid is a benign osmotic environment in comparison with seawater, the protective function of the extraembryonic envelope, the chorion, may be unnecessary. Boehlert & Yoklavich (1984) have shown that the zona radiata was only 1.6% of the dry weight of the egg in *Sebastes melanops* and contrasts with the 15–33% found in oviparous marine teleosts (Blaxter & Hempel 1966, Robertson 1974). In *Sebastes,* the evidence obtained from the present study on the developmental staging of the membranous Na, K-ATPase suggests that osmoregulation occurs during the formation and opening of the mouth parts. The differentiation of the midgut is also accompanied by a dramatic increase in Na, K-ATPase specific activity, probably associated with the formation of microvilli and basolateral plications of the absorptive epithelium (Shimizu et al. 1991). The most perplexing observation from the present study is the finding that late stage embryos (stages 28–33) have the ability to be salt tolerant for short periods of time, but beyond 72 h, the osmoreg-

ulatory mechanism appears to become inefficient and salt tolerance cannot be sustained. One interpretation would be that the mechanism(s) controlling water and salt balance are not completely functional at these late stages. These findings are in agreement with the earlier work of Veith (1980), who compared the osmoregulatory ability of small and large embryos of the clinid, *Clinus superciliosus*. He showed that small embryos are capable of some degree of osmoregulation since they have good survival in 40–50% dilution of full-strength seawater. In 100% seawater, the small embryos exhibited high mortality, whereas the large embryos were fully functional and showed very few deaths. He concluded that water and ion fluxes across the embryonic exchange surfaces were too great in the small embryos to allow full regulation at the higher salinities. In the present study, it is clear that the sodium pump in *Sebastes* is playing an important role in osmoregulation. Although the ontogenetic pattern of the sodium pump in oviparous teleosts is poorly known, comparison with the viviparous *Sebastes* would be of interest since it would allow a greater degree of differentiating between the developmental role of the sodium pump in maternal nutrient uptake versus osmoregulation. Further research on this topic in *Sebastes* embryos should address the specific localization of the Na, K-ATPase in various transport type tissues.

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