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Uptake and utilization of ^{14}C-glycine by embryos of *Sebastes melanops*

Mary M. Yoklavich[1] & George W. Boehlert[2]
[1] *Alaska Fisheries Science Center, NOAA-NMFS, 7600 Sand Point Way NE, Seattle, WA 98115-0070, U.S.A. Present address: Moss Landing Marine Laboratories, P.O. Box 450, Moss Landing, CA 95039, U.S.A.*
[2] *Southwest Fisheries Center Honolulu Laboratory, NOAA-NMFS, 2570 Dole St., Honolulu, HI 96822-2396 and Joint Institute for Marine and Atmospheric Research, University of Hawaii, 1000 Pope Rd., Honolulu, HI 96822, U.S.A.*

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Synopsis

The ability of embryos of the viviparous scorpaenid *Sebastes melanops* to take up nutrients from an exogenous substrate was demonstrated by incubating embryos at various stages of development (18–30 days after fertilization) in ^{14}C-labeled glycine for 24 h. Uptake was highest for embryos at the latest stages (28–30 days) and increased at a linear rate during the incubation period. Nutrient uptake was not time dependent in embryos at the early stages (18–22 days). Nutrient utilization by *S. melanops* embryos was measured by the oxidation of ^{14}C-labeled glycine to $^{14}CO_2$. The amount of respired $^{14}CO_2$ by the oldest embryos increased significantly at a linear rate over the 24 h incubation period. There was no evidence of nutrient utilization by the youngest embryos. The developmental changes we observed in the uptake and utilization of exogenous glycine are supported by our previous findings that the oldest embryos have fully developed mouths and guts, and require additional nutrition from intraovarian sources at this stage of development.

Introduction

The extent of maternal-embryonic relationships in viviparous fishes has been thoroughly reviewed by Wourms et al. (1988). Internal fertilization and embryonic gestation are common to all viviparous fishes. Among the viviparous teleost fishes, an evolutionary trend has formed in the maternal-embryonic trophic relationship, ranging from primitive lecithotrophy, in which embryonic nutrition is supplied endogenously from yolk stored in the egg, to highly advanced forms of matrotrophy, in which a significant amount of energy is transferred via specialized trophic structures from the mother to the offspring during embryonic development.

Until recently, members of the viviparous genus *Sebastes* were considered lecithotrophic, depending solely upon energy from yolk reserves for embryonic development (Balon 1981, Wourms 1981). Although a systematic study of the maternal contribution to embryonic development has yet to be accomplished in this speciose group, it now has been suggested that embryos of black rockfish, *S. melanops* (Boehlert & Yoklavich 1984), kurosoi, *S. schlegeli* (Boehlert et al. 1986), and copper rockfish, *S. caurinus* (Dygert & Gunderson 1991) derive additional energy from a source other than yolk. Evidence for matrotrophy in these species includes a marked discrepancy between the energy available in the yolk and the amount catabolically expended by the gestating embryos. Additionally, ultrastructural studies of the hindgut of *Sebastes*

embryos just before hatching indicate a functional gut and digestion of protein (Shimizu et al. 1991).

Amino acids, proteins and lipids are probable components of exogenous nutrition supplied to developing embryos of viviparous fishes. Uptake of proteins occurs in the trophotaeniae of embryonic goodeids (Lombardi & Wourms 1985), and absorption of amino acids has been demonstrated in embryonic poeciliids (Grove & Wourms 1982) and clinids (Veith 1979). This may be the case for *S. melanops* embryos, because total nitrogen levels remain constant while proteins decrease over development, indicative of supplemental nitrogenous nutrition (Boehlert & Yoklavich 1984). Degeneration of eggs and embryos during intraovarian development and maternal secretions from the ovarian epithelium are possible sources of histotrophe in *Sebastes* (Boehlert & Yoklavich 1984, Boehlert et al. 1986).

The objectives of the present study are (1) to demonstrate, through in vitro experiments with a radiolabeled amino acid, the direct uptake and use of nutrients from an exogenous substrate by *S. melanops* embryos at various stages of development, and (2) to attempt to identify the site of uptake in the embryos by autoradiography.

Sebastes melanops occupies shallow, coastal water from Baja California to the Aleutian Islands (Hart 1973). Females give birth typically from January through March (Laroche & Richardson 1980). The duration of gestation is 37 days at 10° C; hatching occurs about 5 days prior to parturition. Newly extruded, pelagic larvae of *S. melanops* are relatively small (notochord length = 4.5 mm), with little remaining yolk, yet have fully pigmented eyes, a functional mouth and gut and are ready to initiate feeding.

Materials and methods

Adult *S. melanops* were captured from November 1984 to January 1985 by hook and line in 15–30 m of water off Oregon and transported alive to Oregon State University's Hatfield Marine Science Center, Newport, Oregon. The fish were held in circular 700 l tanks (1.2 m diameter) with running seawater at ambient temperature (about 10° C). Prior to experimentation, females were catheterized, and developmental stages of their embryos were determined using criteria described in Yamada & Kusakari (1991). Three females (fish No. 18, 141 and 19), examined on 31 January 1985, were gravid; embryos from each female were assigned developmental stages 21, 22 and 28 respectively.

Experiments on nutrient uptake and utilization were conducted on 4 and 6 February 1985 in a thermally controlled room at 10° C. Embryos were removed from anesthetized (MS-222) females by gentle catheterization, staged and placed into Gilson respirometer flasks (15 ml capacity) containing 4 ml of fish saline (Forster & Hong 1958) that was isosmotic with ovarian fluid (342 mOsM kg^{-1}). The saline contained 0.495 μCi ml^{-1} of ^{14}C-glycine (New England Nuclear, lot No. 533-25). Four replicate flasks, each containing an average of 191 embryos (range = 119–324), were used for each female. A filter paper wick and 0.100 ml of 10% potassium hydroxide (KOH) were placed in the sidearm or center well of each flask to capture $^{14}CO_2$ respired from glycine utilization. The flasks were stoppered and gently shaken during experiments. A control flask with no embryos was used to determine blank values of $^{14}CO_2$ production.

One flask of embryos from each female was terminated at 2, 4, 8 and 24 h after embryos were introduced to the radiolabeled saline. The filter paper and KOH were transferred into a scintillation vial with 10 ml of Ready-Solv EP fluor (Beckman). The radiolabeled saline was removed, and the flask and embryos were rinsed twice with a larger volume of fresh, unlabeled saline to remove surface contamination of the label.

Three replicates of 20 embryos were removed from the unlabeled saline, placed on a glass fiber filter and blotted from underneath to remove excess saline. The filter and embryos were digested overnight at 60° C in a scintillation vial with 1.0 ml of tissue solubilizer (Beckman BTS). After cooling to room temperature and the addition of 0.1 ml glacial acetic acid to reduce chemiluminescence, the dissolved embryonic tissue was diluted with 10 ml of fluor, dark adapted and assayed for radioactivity in a Beckman LS 8000 liquid scintillation

counter. Experimental controls included (1) embryos not exposed to labeled saline but handled identically to the radioactive ones, and (2) blank glass fiber filters wetted with the wash saline from an experimental flask.

Activity (counts per minute) was corrected for counting efficiency by developing a quench curve with standard, calibrated samples of ^{14}C treated similarly to experimental samples; counting efficiency averaged 86.5%. Corrected activity was expressed as disintegrations per minute (DPM); glycine uptake per embryo was calculated as (total DPM – control DPM) (20 embryos)$^{-1}$ (0.3873×10^{-6} μg glycine DPM^{-1}). Utilization of glycine was measured as (total $^{14}CO_2$ DPM – control DPM) (No. embryos)$^{-1}$.

Additional embryos from the flasks were prepared for autoradiography. They were embedded in histological resin (LR White), sectioned sagittally at 1 μm and affixed to microscope slides. The slides were dipped in Kodak NTB-2 nuclear track emulsion, thoroughly dried and exposed in light tight boxes for 2–21 days at room temperature. The autoradiographs were developed in Kodak D-19 developer for 2 min, fixed for 5 min in Kodak fixer, rinsed for 20 min and stained in hematoxylin and eosin. Sections of embryos were examined at 1000 × magnification for location of exposed silver grains.

Results

Sebastes melanops embryos were at developmental stages 24.5, 25 and 30 on 4 February (experiment 1); based on the stage duration (Boehlert & Yoklavich 1984), the time since fertilization was 18.1, 18.8 and 27.4 days respectively (Table 1). Embryos from the same females had progressed approximately one stage by 6 February (experiment 2). Glycine uptake was variable but positive in all experiments (Table 2). Competition for or depletion of ^{14}C-glycine in the substrate was unlikely, with an average of only 0.6% of the radioactivity removed (as evident from the combined activity in the embryonic tissue and CO_2 fractions). Values of glycine uptake in experiment 1 were significantly higher ($p < 0.01$) than in experiment 2 based on a three-way analysis of variance (ANOVA) testing for the effects of individual fish (i.e. stage of embryonic development), incubation time and experiment on glycine uptake, and post-hoc multiple comparisons. Uptake rates were significantly higher ($p < 0.01$) for the embryos from fish number 19, at stages 30 and 31 during experiment 1 and experiment 2 respectively. No significant difference was found in uptake rates between fish numbers 18 and 141, whose embryos were at similar developmental stages. The overall relationship of uptake with time was not significant ($p = 0.398$). To further test the relationship between incubation time and glycine uptake in embryos from different fish, the uptake values within experiments were standardized by fish (mean = 0, SD = 1), thereby allowing for the combination of data from both experiments. Correlation coefficients between incubation time and glycine uptake were significant ($p < 0.01$) only for the fish (No. 19) with embryos at the most advanced developmental stages, suggesting that glycine uptake increased with time only for these embryos (Fig. 1).

Glycine utilization was measured as $^{14}CO_2$ production, and these experiments suggested slightly different results. For embryos younger than developmental stage 30, subtraction of the control $^{14}CO_2$

Table 1. Developmental stages of *Sebastes melanops* embryos used for nutrient-uptake experiments at 10° C. Time since fertilization at each stage was estimated from Boehlert & Yoklavich (1984).

Developmental stage	Time since fertilization (days)	Stage characteristics
24.5 and 25	18.1 18.8	Eyes partially pigmented; gut formed; heart beats; pectoral fin buds
26	20.4	Liver development
30	27.4	Mouth formed and open; caudal fin rays; gut continuous and open; hindgut with amorphous material
31	29.3	Pre-hatching; peristaltic movement of gut

values resulted in negative mean estimates of $^{14}CO_2$ production (Table 3). In the experiments with embryos at the late stages, however, respired $^{14}CO_2$ increased significantly with time, indicating that these embryos metabolically used glycine (Fig. 2).

Absolute identification of a site of nutrient uptake in our study of *S. melanops* embryos remains problematic. Autoradiographs of embryonic tissue containing radioactive glycine and exposed for 2–21 days were largely negative; no visible image of reduced silver grains was produced systematically in association with areas of radioactivity. Some activity of carbon-14 was evident as isolated silver grains along the exterior surface of the epidermis in a few sections of the finfold in the nape and head regions of embryos at stage 25 following 24 h of incubation (Fig. 3). Although the nuclear emulsion (NTB2) is recommended to detect low-energy isotopes such as carbon-14 by light microscropy (Gahan 1972), the amount of radioactive glycine incorporated into a 1 μm section of tissue may be insufficient to produce a positive, discernible image within a reasonable exposure time. Because NTB2 exhibits a high efficiency level in detecting radiation, increased sensitivity is associated with a greater tendency for high background fog levels; consequently, only grain densities well above background can be identified as bonafide autoradiographs.

Table 2. Uptake of ^{14}C-glycine by embryos of *Sebastes melanops*. Values of glycine uptake are means of three replicates; values in parentheses are standard deviations.

Fish No.	Developmental stage	Time (h)	Glycine uptake ($\times 10^{-6}$ μg embryo^{-1})	
Experiment 1				
18	24.5	2	53.495	(36.167)
18	24.5	4	19.432	(2.496)
18	24.5	8	23.202	(2.845)
18	24.5	24	29.344	(6.228)
141	25	2	28.218	(5.835)
141	25	4	59.918	(30.534)
141	25	8	119.031	(71.149)
141	25	24	40.728	(8.593)
19	30	2	218.863	(134.482)
19	30	4	81.496	(62.033)
19	30	8	116.714	(89.571)
19	30	24	298.204	(332.064)
Experiment 2				
18	25.5	2	12.548	(1.317)
18	25.5	4	12.338	(1.889)
18	25.5	8	12.348	(2.072)
18	25.5	24	44.526	(4.816)
141	26	2	6.048	(1.059)
141	26	4	13.874	(2.027)
141	26	8	15.504	(8.363)
141	26	24	53.356	—
19	31	2	9.153	(0.846)
19	31	4	27.046	(7.356)
19	31	8	43.771	(36.190)
19	31	24	57.488	(5.039)

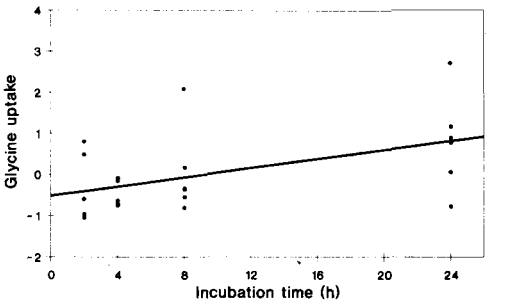

Fig. 1. Glycine uptake as a function of incubation time for developmental stages 30–31 in embryos from *Sebastes melanops* (fish No. 19). Data within each experiment were standardized (mean = 0, SD = 1) prior to analysis. The relationship (solid line) is described by the linear regression: Glycine uptake = − 0.533 + 0.056 (time); N = 24 and r = 0.506.

Discussion

Although our results do not cover a wide range of developmental stages, they suggest a fundamental difference between the *S. melanops* embryos at stages 24–26 and 30–31. Glycine uptake in embryos at the early stages was not significantly related to incubation time, and no glycine utilization was documented. Both uptake and utilization of glycine in embryos at the late stages, however, were time dependent (Fig. 1, 2). The most significant developmental event in the intervening stages is the opening of the mouth, which generally occurs near developmental stage 28 (Yamada & Kusakari 1991). Associated with this event, amorphous material has been noted in the hindgut lumen of *S.*

Table 3. Production of $^{14}CO_2$ (disintegrations per minute) by embryos of *Sebastes melanops*. Values are based on a single estimate per time period.

Fish No.	Time (h)	$^{14}CO_2$ production (DPM embryo^{-1})	
		Experiment 1	Experiment 2
18	2	− 0.761	− 0.070
18	4	− 0.937	0.346
18	8	− 1.970	− 0.898
18	24	− 1.639	− 2.054
141	2	− 1.428	− 0.355
141	4	− 0.344	− 0.352
141	8	− 0.540	− 0.056
141	24	− 3.704	-
19	2	0.622	1.595
19	4	1.336	2.209
19	8	0.219	3.323
19	24	14.336	8.455

schlegeli (Shimizu & Yamada 1980, Boehlert et al. 1986), *S. melanops* (Boehlert & Yoklavich 1984) and *S. caurinus* (Stahl-Johnson 1984) embryos at late stages.

Several structural and functional adaptations for nutrient transfer prior to opening of the mouth have developed in viviparous teleost embryos. Absorption of nutrients from embryotrophe takes place across the embryonic epidermis during early development and via the epithelium of fins and gut during later stages in some species of clinids (Veith 1980, Cornish & Veith 1986). More advanced absorptive structures include the trophotaeniae, which are extensions of the embryonic gut in goodeids (Turner 1937, Lombardi & Wourms 1985), and spatulate median fins in embiotocids (Webb & Brett 1972, Dobbs 1975). *Sebastes* embryos have no obvious structures for nutrient uptake or respiration. However, intracellular digestion of a nitrogenous-based histotrophe has been suggested from histological examination of the hindgut epithelium in late-stage embryos of *S. schlegeli* (Boehlert et al. 1986) and *S. melanops* (Boehlert & Yoklavich 1984). Evidence includes a hypertrophied hindgut epithelium with cells characterized by apical microvilli and numerous supranuclear, eosinophilic inclusions (Fig. 4a). Similar inclusions have been described in the hindgut epithelium of healthy,

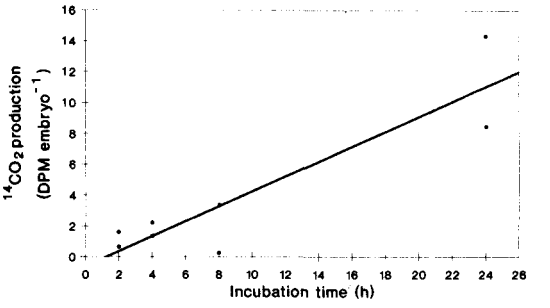

Fig. 2. Production of $^{14}CO_2$ as a function of incubation time by developmental stages 30–31 in embryos from *Sebastes melanops* (fish No. 19). Data from two experiments are combined. The relationship (solid line) is described by the linear regression: $^{14}CO_2$ production = − 0.580 + 0.483 (time); N = 8 and r = 0.909.

actively feeding larvae of marine fish (O'Connell 1976, Theilacker 1978). Such inclusions were less numerous or entirely absent from the epithelial cells in the hindgut of *Sebastes* embryos at the early stages (Fig. 4b). Ultrastructural examination of hindgut epithelium of *S. melanops* embryos at stage 28, following incubation in the protein-tracer horseradish peroxidase (HRP), demonstrated ac-

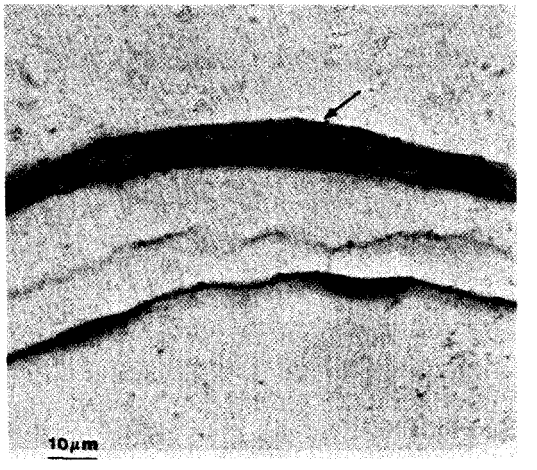

Fig. 3. Autoradiograph of a transverse histological section of dorsal finfold epidermis in the nape region of a *Sebastes melanops* embryo at stage 25 (about 19 days after fertilization). Note the black exposed grains on the surface of the epidermis (arrow).

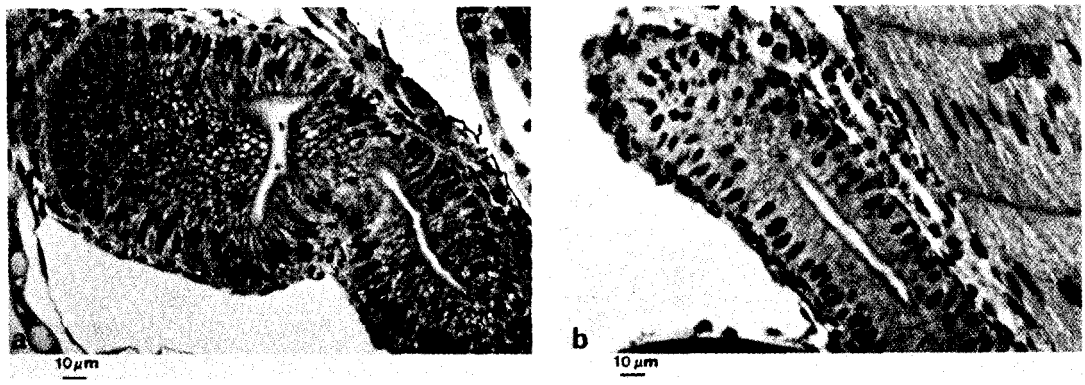

Fig. 4. Histological sections of hindgut tissue of embryos of *Sebastes melanops*: a – Cross section of the hindgut of an embryo at stage 30 (about 27 days post-fertilization). The expanded epithelium is characterized by intracellular, supranuclear, eosinophilic inclusions. b – Sagittal section of mid- and hindgut region dorsal to the yolk sac in an embryo at stage 25 (about 19 days post-fertilization).

tive uptake of high molecular weight substances (Shimizu et al. 1991).

The demonstrated uptake of glycine in embryos of *S. melanops* at the early stages is at odds with their lack of glycine utilization. Although certainly not definitive, the uncommon occurrence of exposed silver grains along the surface of the epidermis of embryos at the early stages suggests perhaps some of the radioactivity is due to superficial adsorption of the glycine and therefore remains metabolically unavailable. Alternatively, within the 24 h incubation period, nutrients possibly were being taken up but not metabolized appreciably by embryos at the early stages. Although the protein-tracer HRP generally was not incorporated into the epidermis of *S. melanops* embryos at stage 24 (Shimizu et al. 1990), a few outer epidermal cells contained inclusions of HRP and had well-developed apical microvilli; this suggests that some uptake of nutrients may occur at these sites in younger embryos.

A comparison of energy utilization by *S. melanops* embryos, based upon calorimetry and catabolism, indicated that a divergence between these estimates occurred at about developmental stage 28 and suggests that additional nutrition is required to continue development to parturition (Boehlert & Yoklavich 1984). This supports the developmental changes we have observed in both uptake and utilization of exogenous glycine by embryos at the late stages.

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