4-21-2005

Are Nutrients assimilated by juveniles of the brittle star Amphipholis squamata (Echinodermata: Ophiuroidea)?

Elizabeth A.G. Whitehill '05
Illinois Wesleyan University

Are nutrients assimilated by juveniles of the brittle star *Amphipholis squamata* (Echinodermata: Ophiuroidea)?

presented by

Elizabeth A.G. Whitehill
Department of Biology
Illinois Wesleyan University

21 April 2005

Approved for Research Honors by:

William Jaeckle, Project Advisor

Bruce Criley

Laura Moore

Loni Walker

Michael Young

Are nutrients assimilated by juveniles of the brittle star *Amphipholis squamata* (Echinodermata: Ophiuroidea)?

Elizabeth A.G. Whitehill
Department of Biology
Illinois Wesleyan University

22 April 2005

Abstract

Over 50 species of brittle stars (Echinodermata: Ophiuroidea) are known to brood their developmental stages (embryos and larvae) through metamorphosis in internal structures called bursae (Hyman, 1955). In some of these brooding ophiuroids, the source of the organic material necessary to support the growth and development of the embryos is somewhat obscure because the amount of material required is not maternally supplied in the egg. A species that follows this example is *Amphipholis squamata* (Delle Chiaje). Previous studies (Fontaine and Chia 1968) have shown that the larvae of *A. squamata* are able to acquire the organic material needed to sustain growth from simple carbohydrates and amino acids dissolved in the surrounding environment. However, the amount of nutrition provided by these molecules is probably not enough to sustain the growth that the larvae exhibit before they are released as juveniles. This study examined the uptake of high molecular weight organic molecules (polysaccharides and polypeptides), sources that are more likely to provide sufficient energy for growth. The data collected suggest that the juveniles do not readily absorb polysaccharides but do actively take up polypeptides which could then be broken down and used for growth.

Introduction

Invertebrates (animals without backbones) that live in marine environments exhibit two basic reproductive strategies. Some species release their gametes (eggs and sperm) into the open water through a process called free-spawning. In other species, fertilization (sperm/egg fusion) occurs internally within the female's body. Both reproductive patterns have theoretical advantages: internal fertilization increases the likelihood of the sperm meeting the egg, and free-spawning in the water column removes the necessity of a male coming into physical contact with a female of the same species. However, the gametes and fertilized eggs of free-spawners are a

favorite food of many animals that live in the sea. Eggs can be fertilized in the water column and develop without parental care or they can be fertilized and retained inside of the parent's body in a process called brooding. Brooded fertilized eggs may grow and develop without additional maternal nutrient input in a condition called viviparity, or, in addition to nutrients present in the egg, offspring can develop without the supplemental maternal nutrition (ovoviviparity). In general, viviparous animals require a greater amount of parental care and this strategy increases the amount of energy that the parent must direct into reproduction per offspring. The trade-off for increased parental care is an increased survival of offspring adulthood. Due to the increased energy requirement, viviparous species tend to produce smaller numbers of large young. Conversely, free-spawners, species have young that develop without any parental care, produce a greater number of eggs since the amount of energy directed to each offspring is much lower due to the reduction in parental care. The production of a greater number of eggs is thought to offset the higher mortality rate experienced during development.

Brittle stars (Class Ophiuroidea) are invertebrates and members of the phylum Echinodermata. They are generally small and consist of a central disc surrounded most often by five moveable arms (Ruppert and Barnes 1994). They get their food by deposit or filter feeding, scavenging, or hunting. Food particles are consumed through a set of jaws leading into a blind-ending gut (Ruppert and Barnes 1994). Respiration is accomplished through internal sacs called bursae that open to the outside on either side of each arm; there are ten total (see Fig. 1 for a generalized diagram of ophiuroid internal anatomy). Seawater is circulated through each bursa via the ciliated epidermis that lines the duct that leads from the bursa to the exterior of the animal. This circulation is believed to facilitate gas exchange across the bursae. Gametes are also released into the bursae where eggs are fertilized, and the fertilized eggs either exit through the

bursal slits leading to the external environment or are held within the bursae. For these species with bursal brooding, the embryos first develop into a larva, referred to as a "vestigial pluteus" (Fell 1946, Walker and Lesser 1989). The vestigial pluteus is morphologically distinct from the adult but then undergoes a metamorphosis to a juvenile resembling the adult of the species. The juveniles remain in the bursae until a time when they are large enough to live independently (Byrne 1991).

While developing in the bursae, the larvae either receive nutrition from the yolk stores provided within the egg or from additional sources. Turner and Dearborn (1979) examined the amount of organic material (biomass) in juveniles of the brooding ophiuroid *Ophionotus hexactis*, as a function of size (disc diameter). They found a positive logarithmic relationship:

$$\log \hat{Y}=2.77 (\log X) - 0.390$$

where $\hat{Y}$ is the estimated dry weight (mg) and X is the disc diameter (mm), (r^2 not given) between the dry weight and the disc diameter of the juveniles. The biomass is determined from dry weight. Turner and Dearborn (1979) concluded that the biomass of a juvenile *O. hexactis* is several orders of magnitude greater than that found in the eggs, suggesting that nutrients provided in the egg could not provide all organic material necessary for development and larval growth.

The ophiuroid *Amphipholis squamata* is a brooding, burrowing ophiuroid that lives primarily within the sediment. *A. squamata* produces mature eggs throughout the year which are released one at a time from each gonad into a bursa and are fertilized by sperm brought into the bursae. Larvae are brooded until the juveniles are mature enough to live independent of the adult (Fell 1946). Several individuals at different developmental stages can be found in a bursa at any one time (Fell 1946, Walker and Lesser 1989, pers. obs.).

Like *O. hexactis*, *A. squamata* does not seem provide sufficient nutrients in the egg to sustain the growth and development of larvae and juveniles. The eggs of *A. squamata* are on average 100 μm in diameter (Walker and Lesser 1989) and the juveniles eventually exhibit a diameter of about 800 μm when they exit the bursae (Fell 1946). Turner and Dearborn (1979) state that the disc diameter of a young ophiuroid should be a good indicator of the dry weight of the individual; the same is true for the egg's diameter and dry weight (Jaeckle 1995). The organic weight of a juvenile with a disc diameter of 800 μm is approximately 77 μg (Turner and Dearborn 1979). According to Jaeckle (1995), the organic dry weight of an egg is given by the equation:

$$\hat{Y} = 1.06\,X + 6.07$$

($r^2 = 0.972$) where $\hat{Y}$ is the dry organic weight (μg) and X is the egg's volume (mm^3). The dry organic weight of an average egg from *A. squamata* is estimated to be 6 μg. A fertilized egg therefore undergoes an almost 13-fold increase in biomass during its growth and development within the bursae. Based on these calculations an egg of this species cannot provide enough nutrition to produce a juveniles, resulting in an energetic paradox.

Krohn, in 1851 (as cited in Fell 1946), hypothesized that developing larvae of *A. squamata* received organic nutrients from the parent through an embryonic attachment (stalk) to the bursa. As an alternate hypothesis, Russo (1891) suggested that the larvae fed on cells sloughed off the bursal epithelium (as cited in Fell, 1946). Nearly 100 years later, Fell (1946) discounted Krohn's stalk hypothesis, citing that the stalk lacks the amount of vascular tissue one would expect of a nutritive structure. In addition, Fell demonstrated that nutrients for young larvae could not be provided by sloughed-off bursal cells because a mouth connecting the digestive system to the exterior does not form until metamorphosis. Russo's hypothesis could

hold true for the juveniles, however. Fell (1946) concluded that the nutrition for embryonic growth probably comes from the mother from vascular sinuses formed in bursal walls only when larvae are present and that larvae can only survive outside of the bursae when supplied with rich organic molecules. Utilizing a periodic acid-Schiff (PAS) reaction, glycoproteins, glycolipids, polysaccharides, and some fatty acids have been identified surrounding these sinuses, indicating a possible source of maternally-provided nutrition (Byrne 1994).

Fontaine and Chia (1968) elaborated on Fell's (1946) hypothesis by exposing *A. squamata* individuals to ^{3}H-glycine and D-glucose-6-^{3}H in seawater at concentrations of 6 and 50 μM, respectively. They found that the larvae developing in the bursae accumulated the label. Within the adult, the epidermis, tube feet, and muscles of the jaw of the adult also accumulated these nutrients. In the young larvae, only the epidermis assimilated the nutrients, but spatial distribution of label in juveniles was very similar to that of the adults. This evidence suggests that the uptake of nutrients in juveniles occurs similarly to nutrient uptake in adults. From this evidence, Fontaine and Chia hypothesize that uptake of dissolved organic material (DOM) may account for the nutrients needed to fuel growth and development after yolk stores have been depleted. I therefore hypothesize that DOM could be provided either from the surrounding seawater circulated through the bursae for respiratory purposes or from the mother via the bursal sinuses.

My criticism of Fontaine and Chia's (1968) study concerns the high concentrations of glycine and glucose used during their study. Generally, the concentration of dissolved free amino acids in seawater is less than 1 μM (Williams 1975 as cited in Wright and Manahan 1989). The authors therefore utilized six times the concentration of glycine and 50 times the glucose found in seawater. However, Henrichs and Farrington (1979, as cited in Wright and Manahan

1989) state that the concentrations of amino acids in pore water of sediment are 10 to 100 μM. *A. squamata* lives within the sediment, so it is possible that these animals obtain nutrition by the transport of dissolved amino acids. Rates of transport of carbohydrates are very low for larvae, in the femtometers $hour^{-1}$ range, even when exposed to high substrate concentrations. The absorption of carbohydrates into the epidermal epithelium of marine invertebrate larvae is energetically insignificant (W.B. Jaeckle, pers. comm.).

Another possible source of nutrients for larvae and juveniles is the subcuticular bacteria that coexist on *A. squamata* (Fig. 2). The bacteria may secrete organic molecules which may be consumed via absorption by the developing larvae. These bacteria can take up most amino acids (potentially concentrating the amino acids for use by the embryos) and secrete lipopolysaccharides (Walker and Lesser 1989). Larvae and juveniles may also ingest the bacteria directly via endocytosis (Walker and Lesser 1989). Walker and Lesser (1989) found these bacteria associated with developing embryos and in involutions of the bursal epithelial cells lying beneath the cuticle in adults. In addition, adults of a congeneric species of marine ophiuroid, *Amphipholis gracillima*, are able to absorb the extracellular polymeric secretions (EPS) released by bacteria growing on and within surrounding sediment (Hoskins et al. 2003). The ability of this closely-related brittle star to absorb nutrients secreted by bacteria suggests that *A. squamata* may also be able to absorb nutrients provided in a similar way by the subcuticular bacteria. However, there are conflicting reports as to whether larvae can develop in a bacteria-free environment (Fell 1946, Johnson 1972 as cited in Walker and Lesser 1989). The bacteria are therefore a possible source of nutrients, although whether or not their presence is vital to larval growth and development remains obscure.

High molecular weight molecules are a likely source of nutrition provided maternally or via DOM in seawater. These molecules are readily available as DOM and they provide more energy per molecule than a single molecule of glucose or an amino acid. The carbon atoms of organic molecules are used for energy; amino acids are used both as a source of energy and as building blocks for protein synthesis that is needed for growth. High molecular weight organic compounds would provide more carbon molecules than amino acids or simple sugars, and thus might be able to provide enough energy for development. Also, the proportion of DOM that is comprised of high molecular weight polymeric molecules may have been underestimated in the past (Lesser and Walker 1992). We designed this study in order to test the hypothesis that polysaccharides and polypeptides were a possible source of nutrition for the developing larvae and juveniles of *A. squamata*. We anticipated that the larvae would assimilate these organic molecules, as they are a likely source of nutrients required to complete embryonic and larval development.

Materials and Methods

Animals

Adult *Ophioderma* sp. were provided by the Marine Biological Laboratory in Woods Hole, MA and kept in aquaria at Illinois Wesleyan University in Bloomington, Illinois in artificial seawater. Preliminary experiments (on adults) occurred in September and October 2004.

Aquarium-cultured *Amphipholis squamata* adults were obtained from Dr. Claudia Mills (University of Washington) and Dr. Richard Emlet (University of Oregon). Individuals were stored at approximately 16°C in (artificial) saltwater aquaria at Illinois Wesleyan University.

Detection of macromolecule assimilation

In order to remove phenol, which is toxic to the animals, from the iron dextran (Sigma D-8517), it dialyzed in a 10K Slide-a-Lyzer in bacteria- and contaminant-free water overnight. Using known dilutions of the iron dextran (pre-dialysis), a standard curve was created in order to determine an accurate concentration of the experimental solution. Absorbance was measured at 800 nm, outside the range of phenol's absorbance. The dialyzed iron dextran was then diluted to approximately a 2 mg/mL solution with seawater that had been filtered with a 0.2 μm filter (particles larger than 0.2 μm will not pass through the filter).

Adult *Ophioderma* sp. were incubated for 7, 12, and 24 hours in a 2 mg/mL iron dextran solution. After each incubation period, animals were removed, rinsed in filtered seawater and placed in a 1 $MgCl_2$: 1 seawater solution for 30 minutes in order to cause muscle relaxation. Before dilution, the concentration of $MgCl_2$ was 66 ppt (parts per thousand). The animals were then preserved in phosphate-buffered 10% formaldehyde for a minimum of 24 hours. Once fixed, the specimens were then rinsed in contaminant-free water and the bursae were removed from the animal. The bursae were subsequently incubated for one hour in a 3:2 solution of 1% HCl and 2% potassium ferrocyanide. The iron reacts with the ferrocyanide to yield an insoluble blue reaction product (iron(III)-hexacyanoferrate(II)) that is readily observed with a compound microscope. After exposure to the acidic ferrocyanide solution, tissue was rinsed to remove any remaining solution and to stop the reaction. Tissue used for whole mounts was then dehydrated for 20-30 minutes with increasing concentrations of ethyl alcohol (15, 30, 50, 70, 95, and 100%) and cleared in xylene. The animals were then placed on a glass slide in Permount and a coverslip was added. Control animals not exposed to iron dextran were treated in an identical manner to the experimental specimens.

Adult and juvenile *A. squamata* were exposed to an iron dextran or ferritin (from Sigma, F-4503) solution (both 2 mg/mL) for 24 hours. Juveniles were excised from the parent's bursa prior to incubation. The ferritin solution was not dialyzed because it did not contain phenol. After removing the adults and juveniles from the solutions they were treated as described above for *Ophioderma* sp. Adults and were preserved in either phosphate-buffered 2.5% glutaraldehyde or 10% formaldehyde for a minimum of 24 hours. Exposure to the acidic ferrocyanide solution and subsequent dehydration were as described above. Adults were dissected prior to dehydration and surveyed for juveniles using dissecting and compound microscopes.

Detection of the label in tissue

A number of animals from the above experimental treatments were also infiltrated with and placed in plastic (EMbed 812, EMS Co., hereafter EPON) in order to determine where in the adult body iron dextran might be absorbed and if any brooded larvae or juveniles would also assimilate the polysaccharide. These individuals were exposed to iron dextran for 24 hours and subsequently incubated in the acidic ferrocyanide solution. They were then placed in an ethylene diamine tetracetate (EDTA) solution in order to dissolve the calcium carbonate skeleton within the animal (as described in Dietrich and Fontaine 1975). The EDTA solution was changed approximately every 12 hours until examination using polarized light indicated that all calcium carbonate within the animal's body had dissolved. The animals were then dehydrated as described above.

Once in the 100% ethanol, the animals were transferred to propylene oxide (PO) for a total of 10 minutes (two changes of fluid at five minutes each) and then transferred to a 2 PO: 1 EPON solution. This solution was changed to a 1 PO: 1 EPON solution after approximately 24

hours, and then to a 1 PO: 2 EPON solution after another 24 hours. The PO was then allowed to evaporate and then the animals were embedded in pure EPON and put in an oven at 58°C until the resin had hardened. The tissue was subsequently sectioned (1 μm thick) using a diamond knife with a Sorvall MT-IIB ultramicrotome. The sections were stained with a 1% acid fuscin stain which stains cell nuclei.

Fluorescence Microscopy

Juveniles were placed in solutions of bovine serum albumin (BSA, mol wt 66,430; Sigma A-9771; 0.66 mg/mL) and dextran (average mol wt 77,000, Sigma FD-70S; 2 mg/mL) which were both labeled with fluorescien isothiocyanate (FITC) for 2, 4, 8, 12, and 24 hours. Juveniles were also incubated in 0.2 μm filtered seawater and served as experimental controls. Juveniles were viewed with a Nikon E600 microscope equipped for epifluorescence. FITC fluorescence was examined using a 450-490 nm band pass excitation filter, a 505 nm dichroic beam splitter, and a 520 nm long pass barrier filter. Digital images were taken using a Nikon DS-5M digital camera.

Table 1 gives an overview of the trials that were run.

Results

Detection of macromolecule assimilation

Examination of the bursae of *Ophioderma* sp. incubated in iron dextran for 7, 12, and 24 hours revealed that the bursal epithelium of this species took up iron dextran from solution (Figure 3A). Part of the bursal epithelium of the control animals (unexposed to iron dextran) turned blue as well when exposed to acidic ferrocyanide, suggesting that at least some of the color observed in the exposed species may be an artifact, thus some of the data obtained might have been a false positive. However, some of the blue color was found in the calcium carbonate

($CaCO_3$) ossicles within the tissue, but the blue ossicles cannot explain the distinct particles seen within the bursal epithelia in the 7, 12, and 24 hour incubation data (Figure 3B-D).

Juveniles of *A. squamata* removed from the bursae and incubated in iron dextran and ferritin developed blue epithelia after the one hour incubation in the acidic ferrocyanide solution. The animals exposed to iron dextran for 24 hours had some blue coloration along their epithelia, but it was difficult to discern if the blue was intracellular; there were no visible vesicles containing the reaction product (Figure 4A). When incubated in ferritin, however, the epithelia of the juveniles did assimilate the protein. Defined vacuoles filled with the reaction product are evident in whole mounts of the animals (Figure 4B). However, juveniles that were removed from the parental bursae after exposure to iron dextran and ferritin did not have cells containing the blue reaction product. Initially these juveniles appeared blue, but the coloration was lost as the animals were dehydrated. Because the reaction product is essentially insoluble once taken up by cells, this change in color was probably caused by the loss of the reaction product that was loosely attached to the epithelium (adsorbed) during the changing of the surrounding solution. In addition, controls failed to develop a blue color after incubation in the acidic ferrocyanide solution.

Detection of the label in tissue

The results of the whole mount individuals exposed to iron dextran were similar in the tissues embedded in EPON (data not shown). Except for a few blue particles attached to the animal's epithelium, there was no blue coloration present in the sections (data not shown). The lack of extracellular polymer uptake is also observed in the adult bursae—there was no reaction to the acidic ferrocyanide solution in any of the tissue sectioned. We therefore concluded that iron dextran was not absorbed by any of the cells of the adult in the sectioned tissue.

Fluorescence

The pattern of absorption by juveniles incubated in the fluorescein-labeled organic polymers paralleled the data collected from the juveniles incubated in iron dextran and ferritin. The controls (in seawater; Figure 5) were slightly less fluorescent than the juveniles incubated in the in the FITC-dextran (Figure 6) throughout the course of the timed experiments. In the FITC-dextran juveniles, there was no evidence of any vesicle formation in the epithelial cells. However, the epithelial cells took up FITC-BSA into discrete vesicles (Figure 7) after as few as two hours. The vesicles were evident throughout the rest of the 24 hour time trial.

Discussion

The overall evidence of polysaccharide uptake presented in this study suggests that the juveniles of *A. squamata* are unable to assimilate dextran molecules, a carbohydrate that animals are unable to metabolize because it contains α1,6 linkages (W.B. Jaeckle, pers. comm.). The juveniles incubated in iron dextran for 24 hours did not show any blue reaction product intracellularly after exposure to the acidic ferrocyanide solution. The higher levels of background fluorescence in the juveniles incubated in FITC-dextran as compared to the controls are due to adsorption of the label. Increased fluorescence in these juveniles is therefore probably due to adsorption of the sugar. Because both iron dextran and FITC-dextran failed to be incorporated into the epithelia, it is likely that the juveniles of *A. squamata* are unable to absorb polysaccharides from solution. The data suggest that the juveniles might exclude use of polysaccharides as a source of DOM altogether, although we cannot positively conclude this given the data. The inability to consume one type of complex carbohydrate is, however, suggestive of an underlying pattern of nutrient procurement.

When presented with ferritin within the bursal space, the juveniles of *A. squamata* have shown the ability to assimilate the protein. Staining and fluorescence patterns indicate that these polypeptides were detected by the juveniles and incorporated into vesicles in the epithelial cells via pinocytosis or endocytosis. Because these results were observed with both ferritin and BSA, it is possible that this trend is true for all polypeptides that are of similar composition and size. Walker and Lesser (1989) hypothesized that symbiotic subcuticular bacteria absorb dissolved amino acids from solution and synthesize them into polypeptides which are subsequently excreted and consumed by the larvae of *A. squamata.* If their hypothesis is not rejected through further studies then it could explain why juveniles readily take polypeptides up from their surrounding environment. If the proteins secreted by bacteria have evolved as an important mechanism of nutrient acquisition in *A. squamata* juveniles, it is plausible that juveniles can remove a number of different types of proteins up from the surrounding seawater including BSA and ferritin. Interestingly, uptake of dissolved amino acids and polypeptides is common among marine invertebrates (Wright and Manahan 1989), and it may be likely that larvae and juveniles of *A. squamata* use the same endocytotic mechanism that other marine invertebrates use to absorb proteins from seawater.

The lack of blue coloration in the juveniles that were incubated in iron dextran and ferritin while inside the bursae is perplexing. The lack of reaction product in the epithelia of these individuals suggests that although juveniles outside of the adult may be able to readily assimilate nutrients from the surrounding seawater, this mechanism of nutrient acquisition may not be relevant in a natural setting. However, it is possible that the bursae may have been closed during incubation in the iron dextran, ferritin, or acidic ferrocyanide solution, thus remaining unavailable for assimilation by juveniles and larvae.

Although this study suggests sources of additional fuel for the growth and development of *A. squamata* offspring, it did not address the source of the polypeptides that were absorbed. Future work could elucidate the source of the polypeptides using tools such as autoradiography. Radiolabeled polysaccharides and polypeptides could also be used to find rates of uptake of these molecules which could confirm or refute the results presented here. Also, determining if the absorption of organic molecules differs when juveniles are incubated in solutions inside and outside the bursae would also be prudent, as the results of the trial I performed were inconclusive.

With the evolution of ophiuroids that brood their young from small eggs to much larger juveniles came the need to create a system of predictably delivering nutrients to the developing offspring. Without the delivery of additional carbon and nitrogen, the embryo could not sustain growth and development because its energy and protein stores would soon run out. Byrne et al. (2001) suggested that the development from zygote to a pre-metamorphic larva is not as metabolically taxing as once thought. However, with the large difference in organic weight between the egg and the post-metamorphic juvenile of *A. squamata*, it is unlikely that this overestimation could account for the energy an embryo needed by an embryo to develop and grow to term. The larvae and juveniles of *A. squamata* have an immediate source of the nutrients required for growth and development: the DOM dissolved in the seawater that is continually circulated throughout the bursae in which they live. Although it is difficult to explain the observed preference for polypeptides over polysaccharides, the results of this study show that the juveniles of *A. squamata* are capable of using some high molecular weight dissolved organic molecules as a source of nutrition for their growth and metabolic needs.

Acknowledgements

I would like to thank Dr. William Jaeckle for his assistance with my project, Dr. Claudia Mills at the Friday Harbor Laboratories and Dr. Richard Emlet at the Oregon Institute of Marine Biology for providing animals, and Drs. Bruce Criley, Laura Moore, Loni Walker, and Michael Young for reviewing this manuscript. Funding was partially provided by a grant from the Theta Rho chapter of the Beta Beta Beta biological society and the Illinois Wesleyan University student senate.

References

Brusca, R.C., Brusca, G.J. 1990. Invertebrates. Sunderland: Sinauer Associates, Inc. 922 p.

Byrne, M. 1991. Reproduction, development, and population biology of the Caribbean ophiuroid *Ophionereis olivacea*, a protandric hermaphrodite that broods its young. Marine Biology 111: 387-399.

Byrne, M. 1994. Ophiuroidea. In Harrison, F.W., Chia, F.-S. Microscopic anatomy of invertebrates. New York: Wiley-Liss. p 247-343.

Byrne, M., Selvakumaraswamy, P., Cisternas, P., Villinski, J.T., Raff, R.A. 2001. Evolution of maternal provisioning in ophiuroids, asteroids and echinoids. Pp. 171-175 in Echinoderm Research 2001. J.P. Feral and B. David, eds. Balkema Lisse. The Netherlands.

Dietrich, H.F., Fontaine, A.R. 1975. A decalcification method for ultrastructure of echinoderm tissues. Stain Technol. 50: 351-354.

Fell, H. B. 1946. The embryology of the viviparous ophiuroid *Amphipholis squamata* Delle Chiaje. Trans. Proc. R. Soc. N.Z. 75: 419-464.

Fontaine, A.R., Chia, F. 1968. Echinoderms: An autoradiographic study of assimilation of dissolved organic molecules. Science 161: 1153-1155.

Hoskins, D.L, Stancyk, S.E., Decho, A.W. 2003. Utilization of algal and bacterial extracellular polymeric secretions (EPS) by the deposit-feeding brittlestar *Amphipholis gracillima* (Echinodermata). Mar. Ecol. Prog. Ser. 247: 93-101.

Hyman, L. H. 1955. The Invertebrates, Vol. IV, Echinodermata. McGraw-Hill. New York. 763 p.

Jaeckle, W.B. 1995. Variation in the size, energy content and biochemical composition of invertebrate eggs: correlates to the mode of larval development. In: McEdward, L. editor. Ecology of marine invertebrate larvae. Boca Raton: CRC Press. p 49-77.

Lesser, M.P., Walker, C.W. 1992. Comparative study of the uptake of dissolved amino acids in sympatric brittle stars with and without endosymbiotic bacteria. Comp. Biochem. Physiol. 101B: 217-223.

Ruppert, E.E., Barnes, R.D. 1994. Invertebrate zoology. 6th ed. Fort Worth: Harcourt College Publishers. 1056 p.

Turner, R.L., Dearborn, J.H. 1979. Organic and inorganic composition of post-metamorphic growth stages of *Ophionotus hexactis* (E.A. Smith) (Echinodermata: Ophiuroidea) during intraovarian incubation. J. Exp. Mar. Biol. Ecol. 36: 41-51.

Walker, C.W., Lesser, M.P. 1989. Nutrition and development of brooded embryos in the brittlestar *Amphipholis squamata*: do endosymbiotic bacteria play a role? Marine Biology 103: 519-530.

Wright, S.H., Manahan, D.T. 1989. Integumental nutrient uptake by aquatic organisms. Annu. Rev. Physiol. 51: 585-600.

Table 1: Summary of Trials

Species	**Stage**	**Compound**	**Concentration**	**Duration of Exposure**
Ophioderma sp.	Adult	Iron dextran	2 mg/mL	7, 12, and 24 hours*
Amphipholis squamata	Juvenile removed from bursa	Iron dextran and ferritin	2 mg/mL	24 hours*
	Adult containing juveniles	Iron dextran and ferritin	2 mg/mL	24 hours*
	Adult	Iron dextran	2 mg/mL	24 hours†
	Juvenile removed from bursa	FITC-BSA	0.66 mg/mL	2, 4, 8, 12, and 24 hours§
	Juvenile removed from bursa	FITC-dextran	2 mg/mL	2, 4, 8, 12, and 24 hours§

* Used for whole mount
† Embedded into plastic
§ Observed living animals using a compound microscope

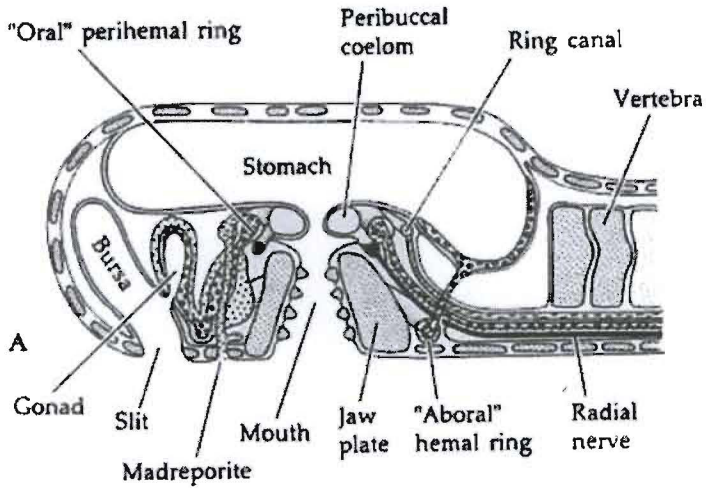

Fig. 1. Diagram of ophiuroid internal anatomy. "Slit" indicates bursal slit. From Brusca and Brusca 1990.

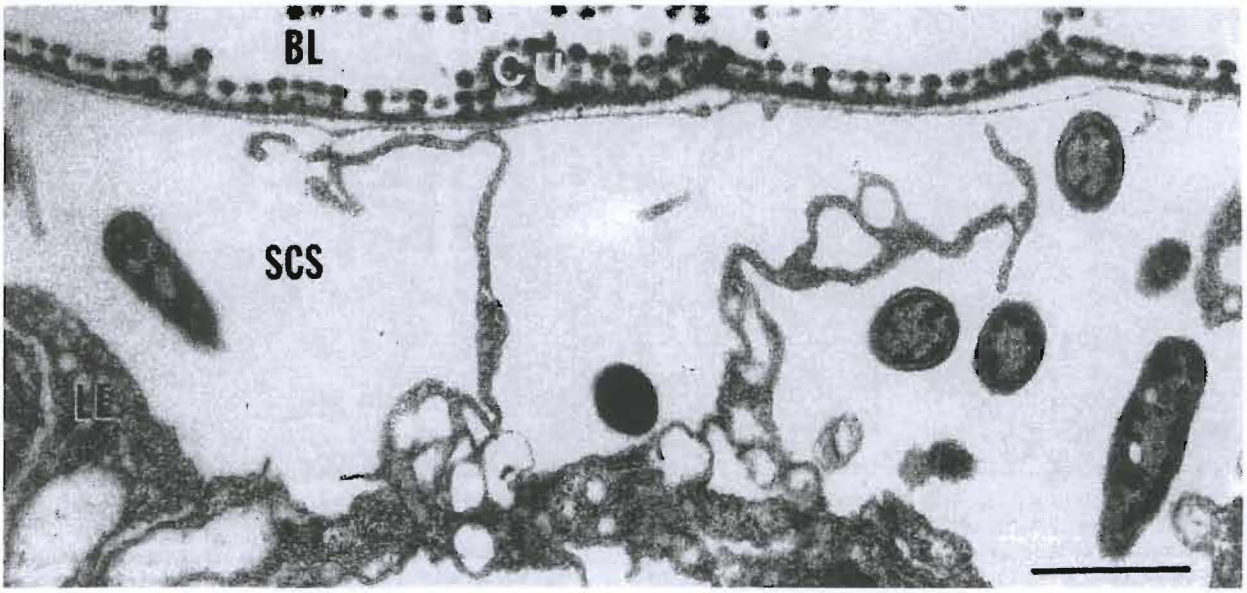

Fig. 2. *Amphipholis squamata*. From Walker and Lesser (1989; Fig. 5). Larval epidermis (LE), cuticle (CU) and subcuticular space (SCS) containing symbionts not located in specialized epidermal pits. BL: bursal lumen; scale bar = 1 μm.

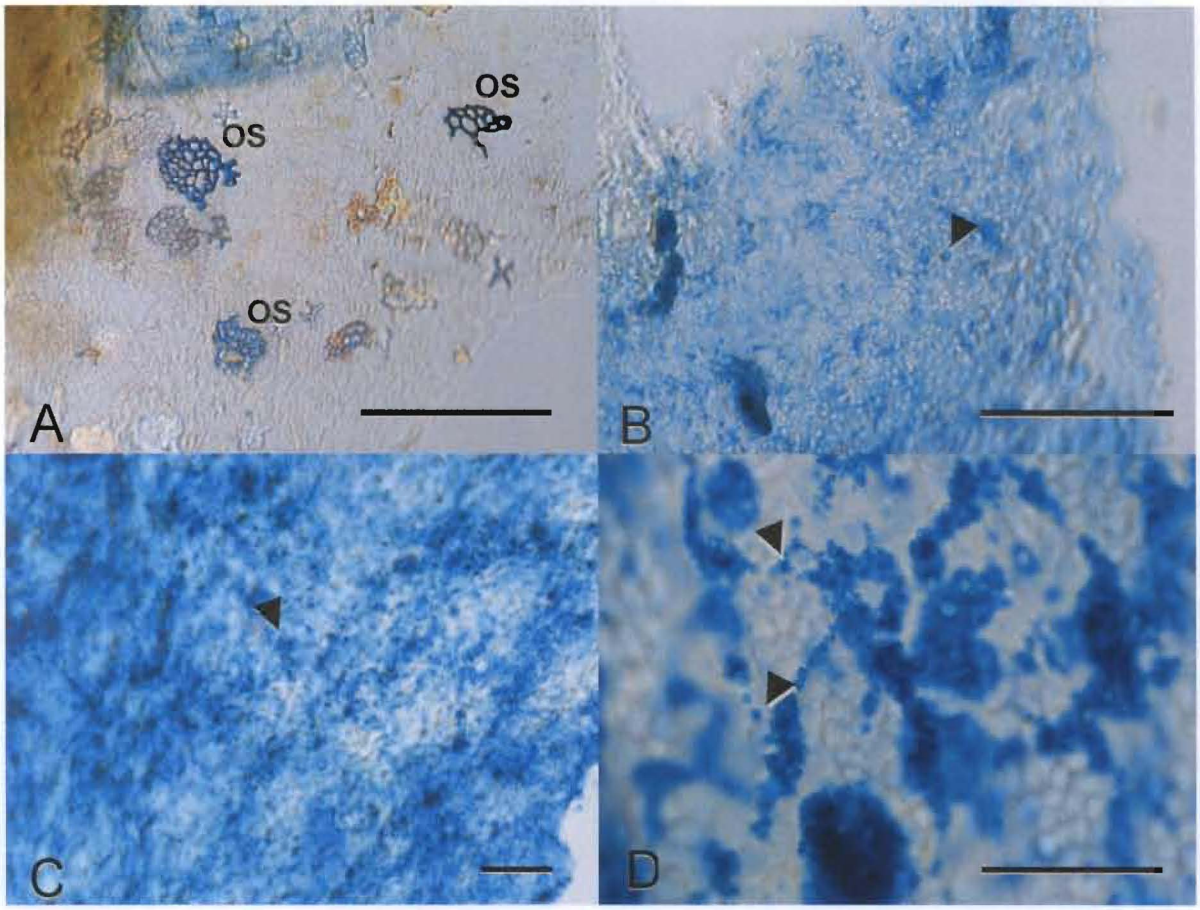

Fig. 3. *Ophioderma* sp. (A) Whole mount of bursal epithelium of control animal after incubation in acidic ferrocyanide solution for one hour. Ossicle (OS); x200. Scale bar is 50 _m. (B) Whole mount of bursal epithelium from animal incubated in ìron dextran (2 mg/mL) for 7h and subsequently exposed to acidic ferrocyanide. Note the particles of the blue reaction product (arrow) present in food vacuoles. Presence of these food vacuoles indicates that iron dextran is being absorbed. x200. Scale bar is 50 _m. (C) Animal incubated in iron dextran for 12h. Dark blue indicates cells/vacuoles x400. Scale bar is 5 _m. (D) Animal incubated in iron dextran for 24h. The food vacuoles in the cells are highly visible. x1000. Scale bar is 5 _m.

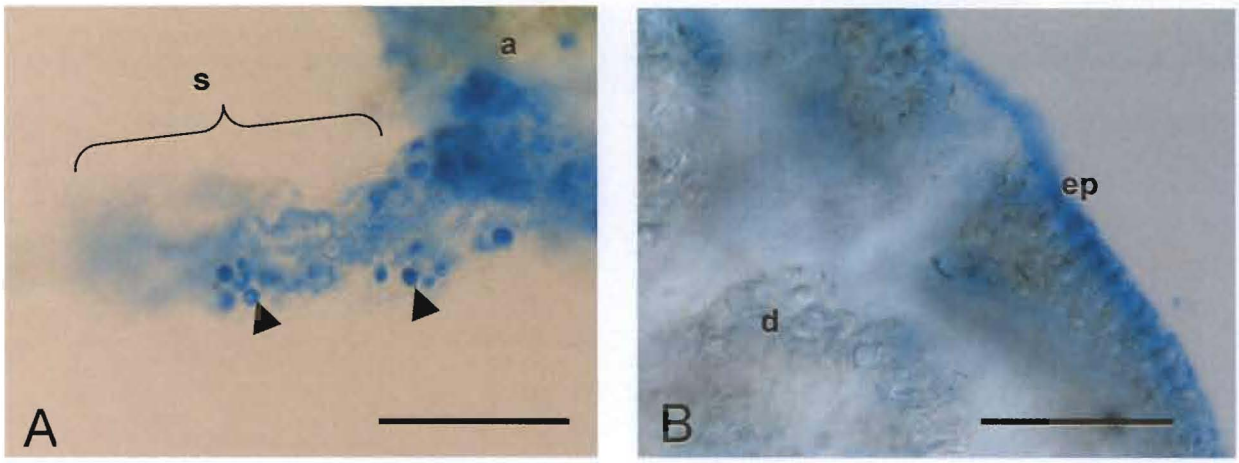

Fig. 4. Juveniles of *Amphipholis squamata*. (A) Arm spine (s) of juvenile removed from the genital bursa and incubated in ferritin for 24h. Labeled vesicles containing ferritin are readily visible in the cells in the spine of an arm (a). Vesicle containing blue reaction product indicated by arrows; x200. (B) Epithelium (ep) of juvenile removed from the genital bursa and incubated in iron dextran outside for 24h. Notice the generally blue epithelium lacks discrete vacuoles that would suggest that the animal is assimilating iron dextran. d: disc. x200. Scale bars are 50 _m.

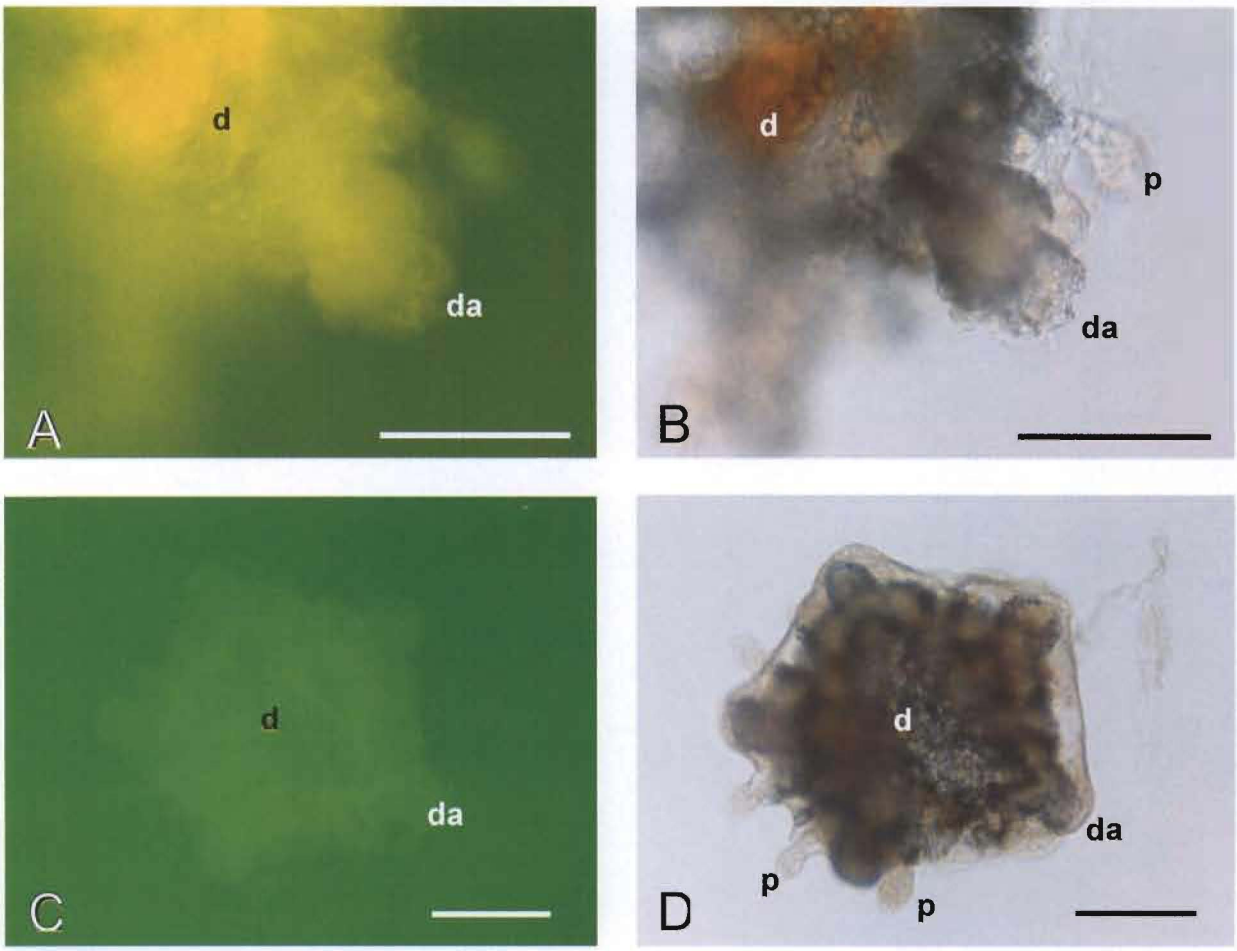

Fig. 5. *Amphipholis squamata*, control. Although no FITC was given to the control animals, there is a small amount of autofluorescence (Fig. 5A, 5C). However, note the absence of vesicles. (A) Control juvenile viewed using combined fluorescence and transmitted light microscopy incubated for 8h in seawater. Viewing with the transmitted light while under fluorescence creates the reddish coloring. Developing arm: da, disc: d; x200. (B) Animal in A viewed using brightfield illumination. Developing arm: da, disc: d, podium: p; x200. (C) Control animal incubated for 24h in seawater viewed using fluorescence microscopy. Developing arm: da, disc: d; x100. (D) Animal in C using brightfield illumination. Developing arm: da, disc: d, podium: p; x100. Error bars are 50 _m.

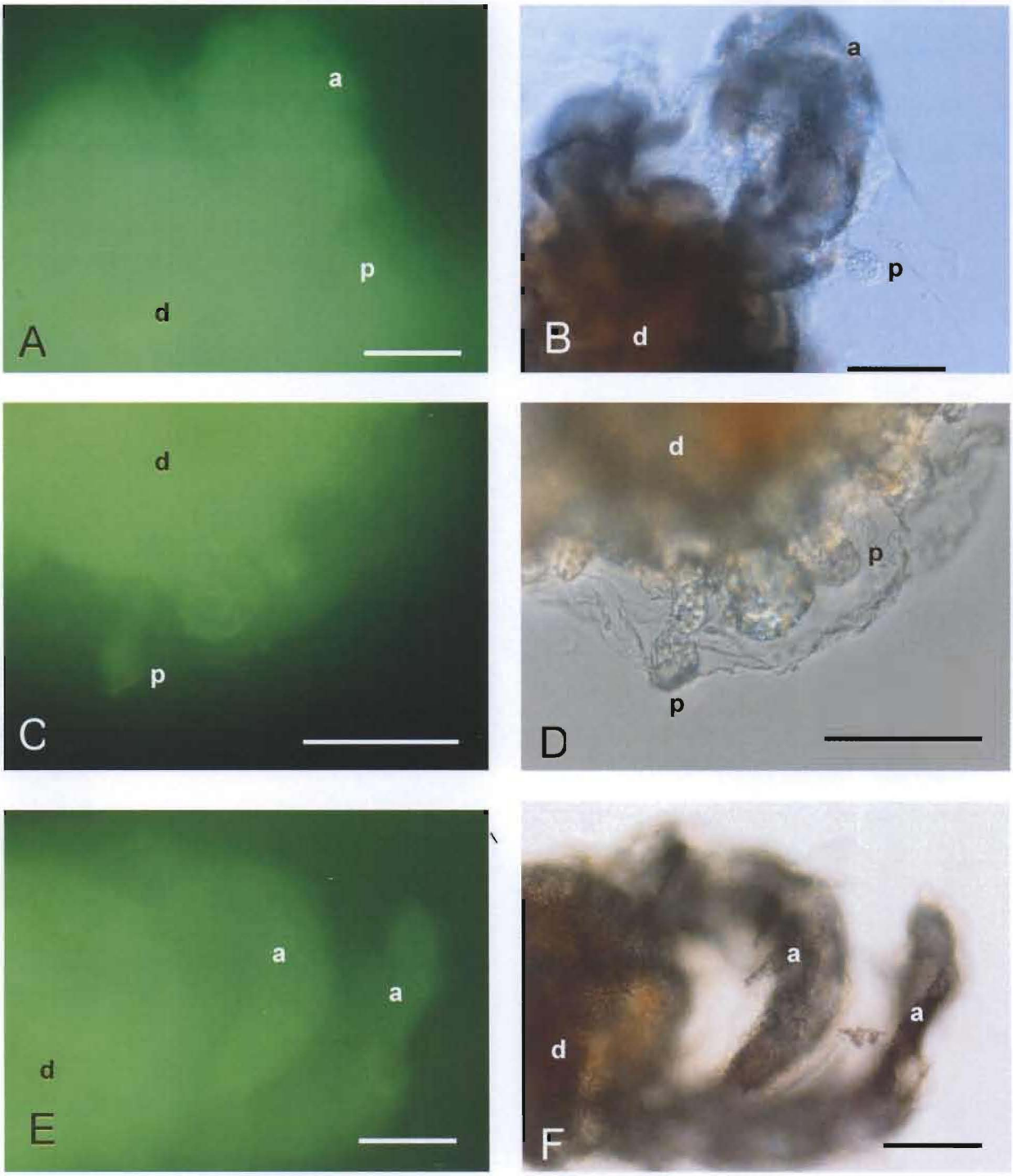

Fig. 6. Juveniles of *Amphipholis squamata* incubated in FITC-dextran. Fluorescent vesicles are not seen in any of these pictures, suggesting that the dextran is not being absorbed. (A) Juvenile exposed to FITC-dextran (2 mg/mL) for 2h viewed using fluorescence microscopy. Arm: a, disc: d, podium: p; x100. (B) Animal in A using brightfield illumination. Labels as in A; x100. (C) Juvenile exposed to FITC-dextran for 4h viewed using fluorescence microscopy. Disc: d, podium, p; x200. (D) Animal in C using brightfield illumination. Labels as in C; x200. (E) Juvenile exposed to FITC-dextran for 8h viewed using fluorescence microscopy. Arm, a, disc, d; x100. (F) Animal in E using brightfield illumination. Labels as in E. x100. Scale bars are 50 _m.

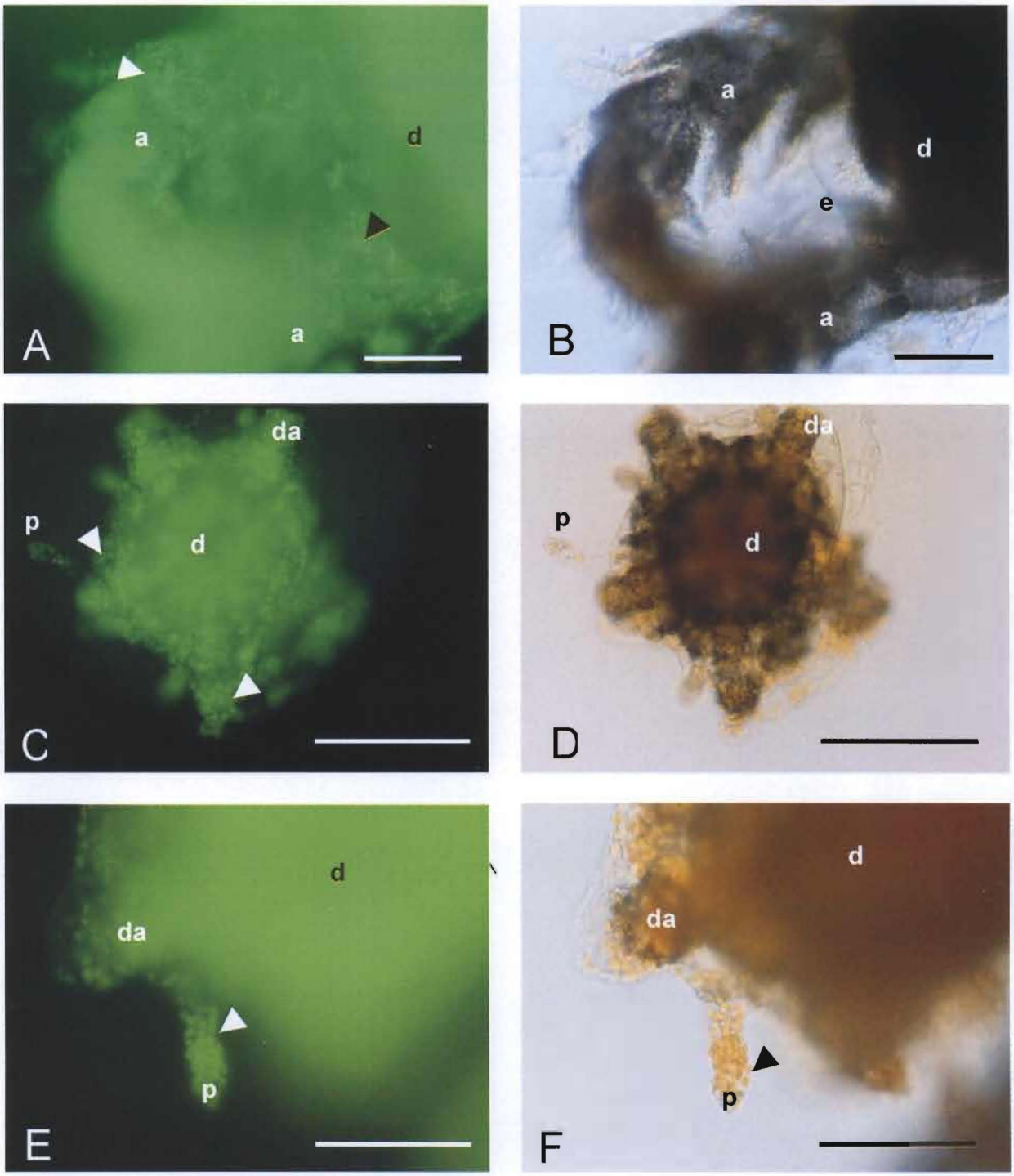

Fig. 7. Juveniles of *Amphipholis squamata* incubated in FITC-BSA. Vesicles containing FITC-BSA are present in all of these animals, indicating that the protein is being absorbed. (A) Juvenile incubated in FITC-BSA (0.66 mg/mL) for 2h; viewed using fluorescence microscopy. Arm: a, disc: d, vesicles indicated by arrow; x100. Scale bar is 50 _m. (B) Animal in A using brightfield illumination. Labels same as in A, edge of epidermis of disc, E; x100. Scale bar is 50 _m. (C) Juvenile incubated in FITC-BSA for 8h viewed using fluorescence microscopy. Developing arm: da, disc: d, podium: p; x20. Scale bar is 500 _m. (D) Animal in C using brightfield illuminatoin. Labeled as in C; x20. Scale bar is 50 _m. (E) Juvenile incubated in FITC-BSA for 24h viewed using fluorescence microscopy. Developing arm: da, podium: p, disc: d, arrow indicates vesicle; x200. Scale bar is 50 _m. (F) Animal in E using brightfield illumination. Labeled as in E; x200. Scale bar is 50 _m.