BULLETIN OF MARINE SCIENCE, 57(2): 313–327, 1995

DIGESTIVE ENZYMES IN PARALARVAL CEPHALOPODS

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ABSTRACT

Fourteen enzymes involved in digestion (esterases, glycosidases and peptidases) were localized by histochemical methods in planktonic paralarvae belonging to four families of cephalopods: Octopodidae, Bolitaenidae (Octopods), Ommastrephidae and Enoploteuthidae (Oegopsid squids). The high protease activity and very low or histochemically undetectable amylasic activity indicate a carnivorous diet suggesting that the diet of paralarvae resembles that of adults. The digestive gland displays the highest enzyme activities which agrees with the key role of the gland in the digestive processes of cephalopods. In particular, the gland appears to be the main source of the proteolytic enzymes found in the posterior digestive tract. The high acid phosphatase activity, DAP II and acetyl-glycosaminidase activities, typically lysosomal, point to intracellular digestive processes in the gland. The posterior salivary glands are as well developed in squids as in octopods and they display several enzyme activities, most notably a high proteolytic activity. This could indicate that the salivary glands would be more involved in the digestive processes in paralarval squids than in adults where they are mostly poison glands. In all of the specimens studied, the whole digestive system appears to be already developed and able to digest prey. The high level of alkaline phosphatase activity of the skin suggests active exchanges with the external medium. It seems possible therefore that nutrients could be absorbed through the skin and provide a part of the energy necessary to the young cephalopods.

Many cephalopods, mostly squids and octopods, have early developmental stages that are planktonic, and they differ from later stages in habit, habitat and morphology. Young and Harman (1988) have proposed the term "paralarva" for these stages. These tiny and fragile organisms often are found dead in freshly collected plankton or they survive only a few minutes after capture. Oceanic species have not been successfully cultured, despite some attempts (Hamabe, 1962; Boletzky et al., 1973; Balch et al., 1985; Young et al., 1985; Arnold and O'Dor, 1990). Moreover, it is very difficult even to identify these stages because detailed systematic studies have not been done for most systematic groups or geographical areas. The results of the workshop on early juvenile cephalopods, held in Banyuls-sur-Mer, France, in 1985, give, where possible, distinguishing characters and keys to paralarval identifications, at least to the generic level (Sweeney et al., 1992). The very few studies available on paralarvae of oceanic cephalopods are concerned mostly with systematics, ecology and gross anatomy, and almost nothing has been done on other aspects of their biology.

The paralarval stage is most critical in the life history of cephalopods. It is a period of high mortality which could be related, in part, to the transition from embryonic feeding (yolk absorption) to prey capture (Vecchione, 1987). Thus a study of the digestive capability (functional morphology of digestive systems and digestive enzymes) of these early stages appeared promising, in spite of the difficulties in obtaining live material suitable for physiological studies. The year-round occurrence of paralarvae of several families of oceanic, neritic and benthic cephalopods off Fort Pierce, Florida, and the knowledge of their distribution, indicated that enough live material could be collected to support such a study.

Research on the enzymes involved in digestion gives much information about potential diet, digestive capability, the role of different organs in digestion and absorption, and the relative importance of intracellular and extracellular digestion.

However, in such tiny (1–7 mm in mantle length) and often rare specimens, it is very difficult to obtain enough extracts for in vitro measurements. Therefore we decided to use in situ methods to study and characterize several enzymes involved in digestion. These methods allow a precise localization of the enzyme activities at the tissue or cellular level, and they are particularly useful in tiny organisms (Southward and Southward, 1968; d'Hondt and Boucaud-Camou, 1982, 1983). This approach also has proven to be successful in analyzing the functions of the different parts of complex organs such as the digestive gland of Bivalvia (Mathers, 1973; Palmer, 1979; Boucaud-Camou et al., 1985a; Henry et al., 1991), and it also has been used to study the physiology of the digestive system of *Sepia officinalis* (Boucaud-Camou, 1969, 1974, 1982). The recent development of very specific synthetic substrates for peptidases allowed us to investigate a relatively large range of enzymes. We investigated hydrolases likely to be involved in processes of digestion and absorption, such as non-specific esterases (ubiquitous in plant and animal tissues), membrane-bound enzymes such as alkaline phosphatase (always found in cell membranes where active transport takes place), aminopeptidase M and dipeptidylaminopeptidase IV, and lysosomal enzymes, involved in intracellular digestion processes, such as acid phosphatase, N-acetylglycosaminidase, β-glucuronidase, dipeptidyl aminopeptidases I (cathepsin C) and II and cathepsin B. We also looked for amylase and non-specific proteases, all good indicators of the type of diet, as well as for the endopeptidases chymotrypsin and trypsin.

Material and Methods

Specimens.—This research was conducted at the Smithsonian Marine Station at Link Port, Fort Pierce, Florida. The live specimens were collected during six cruises (10/15, 10/24, 11/16, 11/26, 12/11 in 1990; 01/08 in 1991) offshore of Fort Pierce in the Florida Current (a tributary of the Gulf Stream) at 15.3, 17.4, 18.8, 19.3 and 19.8 nm offshore (approximately 27°28'N, 079°54'W). During each cruise five to seven 15-min oblique open net plankton tows were made with a 500-μm mesh, 1-m plankton net.

Species from four families of cephalopods (octopods and oegopsid squids) were studied: Octopodidae, *Octopus* (cf. *vulgaris*) ML (mantle length): 1.7 to 2.6 mm; Bolitaenidae, *Japetella diaphana*, ML: 1.6 to 2 mm; several Ommastrephidae including type B′ of Roper and Lu (1978) ML: 2.4 to 4.6 mm and Enoploteuthidae, *Abralia* (probably *A. veranyi*), ML: 3.4 to 7 mm. The Ommastrephidae and *Octopus* species are of interest to fisheries, while the paralarvae of *Japetalla* and *Abralia* had never been studied for anatomy or physiology.

Preservation.—Most of the specimens were sorted on board from the plankton samples and immediately fixed. A few additional specimens were found back at the laboratory in the chilled plankton samples, 2 to 4 h after their capture. The enzyme activities were protected and the structures were preserved using the following methods:

Substrate-film methods. The substrate film method (Daoust, 1965) was used to detect amylase and protease. The animals were deep-frozen on dry ice in one drop of a 12% sucrose 0.4 M cacodylate buffer pH 7.4 and stored at −18°C. The specimens were embedded in Tissue-Tek (Miles) then immediately freeze-sectioned at 12 μm in an AO Reichert "Histostat" cryotome.

The fresh-frozen sections were placed directly on the substrate film and incubated after drying a few minutes on a slide-warmer at 37°C. Control sections were incubated after short treatment in 95°C distilled water.

Gelatin films were prepared for protease tests according to Chrétien (1965) and blackened with India ink instead of carbon. Starch films were prepared for amylase tests and stained after incubation with iodine following the method of Shear and Pearse (1963). The incubations were performed at 37°C for 10 to 60 min with the slides covered by a pH 7 phosphate buffer for amylase and a pH 7.4 phosphate buffer for protease. Digested clear areas on the film mark the sites of enzyme activity. The time of incubation is a good indicator of the activity of the enzyme.

Precipitation methods. A precipitation method was used for testing all other enzymes: simultaneous coupling with naphthylamide or naphthol substrates and a diazonium salt.

The animals were fixed for 2 to 3 h in cold 4% formalin (freshly prepared from paraformaldehyde in a 0.4 M cacodylate buffer pH 7.4 containing 10% sucrose), then rinsed in three baths of the

cacodylate buffer with 12% sucrose; they were kept at 5°C for at least 24 h before sectioning. The enzyme activities were still demonstrable after a fortnight.

The embedding in Tissue-Tek and freeze sectioning were carried out just as for the substrate-film methods. The frozen sections (12 μm) were placed on gelatin-coated microscope slides and dried at 37°C on a slide warmer. Each assay used 5 mg of substrate (naphthol or naphthylamide compound) dissolved in 0.1 ml of dimethylformamide or acetone, 5 ml of buffer and 5 mg of fast blue BB salt (Sigma). The mixture was filtered on ice and used immediately. For peptidases, we used 4-methoxy-β-naphthylamide substrates, introduced by Smith and van Frank (1975). The incubations (from 15 to 45 min) were performed at 37°C. Control sections were incubated in the same conditions and same mixture without the substrate. After incubation, the slides were rinsed in distilled water and mounted in glycerin jelly. They were examined immediately because of the unstable nature of some of the azo dyes.

The enzymes studied by the precipitation method are listed below, along with the substrates and buffers used: Non-specific esterases: α-naphthyl acetate (Sigma), 0.1 M phosphate buffer pH 7.4 (Gomori, 1952 modified by Lojda et al., 1979); Alkaline phosphatase: α-naphthyl phosphate (Merck) (Pearse, 1953), 0.1 M tris buffer pH 9.2 (Pearse, 1953); Acid phosphatase: α-naphthyl phosphate (Merck), 0.1 M acetate buffer pH 5.0 (Grogg and Pearse, 1953); Acetylglycosaminidase: naphthol AS-BI N-acetyl-β-D-glucosaminide (Sigma), 0.1 M cacodylate buffer pH 5.0 (Lojda et al., 1979); β-glucuronidase: naphthol AS BI-β-D-glucuronic acid (Sigma), 0.1 M cacodylate buffer pH 5.0. (Lojda et al., 1979); Chymotrypsin: naphthol AS propionate (Sigma) 0.1 M phosphate buffer pH 7.4 (Lagunoff, 1967); Trypsin: N-CBZ-glycyl-glycyl-arginine-4-methoxy-β-naphthylamide HCl (Bachem) and Na-benzoyl-DL-arginine-β-naphthylamide (Sigma), 0.1 M phosphate buffer pH 7.0 (Gossrau 1981); Aminopeptidase M: L-leucyl-methoxy-β-naphthylamide (Sigma), phosphate buffer 0.1 M pH 7.0 (Moore et al., 1980); Dipeptidyl aminopeptidase I (DAP I): L-prolyl-L-arginine-4-methoxy-β-naphthylamide (Bachem), 0.1 M cacodylate buffer pH 5.0 (Lojda et al., 1979); Dipeptidyl aminopeptidase II (DAP II): L-lysyl-L-alanine and L-lysyl-L-proline-4-methoxy-β-naphthylamide (Bachem), 0.1 M cacodylate buffer pH 5.0 (Lojda et al., 1979); Dipeptidyl aminopeptidase IV (DAP IV): glycyl-L-proline-4-methoxy-β-naphthylamide (Bachem), 0.1 M phosphate buffer pH 7.4 (Lojda et al., 1979) and Cathepsin B: N-Cbz-L-arginyl-L-arginine-4-methoxy-β-naphthylamide (Bachem) 0.1 M phosphate buffer pH 6.0 and N-Cbz-valyl-lysyl-lysyl-arginine-4-methoxy-β-naphthylamide (Bachem), 0.1 M cacodylate buffer pH 5.0.

Results

The digestive tract of cephalopods is U-shaped. The descending branch is the anterior part that consists of the buccal mass, the oesophagus, the crop (in octopods) and the stomach (situated posteriorly but morphologically anterior; it is lined with cuticle and involved in the initial breakdown of food). The posterior part which includes the caecum and the intestine is lined with glandular and ciliated epithelium. Final digestion and absorption take place in the caecum and, at least for some species, in the digestive gland (Boucaud-Camou and Boucher-Rodoni, 1983). The oesophagus, intestine, stomach and caecum open into the vestibule, developed to a greater or lesser degree, according to the ordinal groups (Mangold and Bidder, 1989). The ducts of the anterior and posterior salivary glands open into the buccal mass, while the ducts of the large digestive gland, covered with the digestive duct appendages, open into the caecum.

The enzyme activities were tested in the main digestive organs, that is, in the stomach and caecum for the digestive tract per se, and the posterior salivary glands, the digestive gland and the digestive duct appendages for the glandular organs. In oegopsid squids, where the vestibule is very well developed, we refer to the stomach, caecum and vestibule as the "digestive tract." The detailed results of the analyses for the four families are presented in Tables 1 (Octopodidae), 2 (Bolitaenidae), 3 (Ommastrephidae), and 4 (Enoploteuthidae). Because of the minute size of the paralarvae, the whole animal generally was sectioned, so we observed enzyme activities in organs other than those that we systematically investigated.

We worked on a relatively heterogenous material in terms of size and species, but we did not observe significant differences in the location or nature of enzymes

Table 1. Hydrolase activities in the main digestive organs of paralarval *Octopus* (Octopodidae). PSG: posterior salivary glands, DG: digestive gland, DDA: digestive duct appendages, St.: stomach, Caec: caecum, +++: high activity, ++: medium activity, +: low activity (estimation based on the intensity of the staining for the precipitation method and on the time of digestion of the substrate for the substrate-film method).

	PSG	DG	DDA	St	Caec
Esterases (non-spec)	+	++	++	+++	+++
Alkaline phosphatase	–	+/++	–	–	++
Acid phosphatase	–/++	+++	++	–	++
Amylase	–	–	–	–	+
Acetyl-glycosaminidase	++	++	–	++	–
Glucuronidase	–	–	–	–	–
Proteases (non-spec)	+	+++	–	++	+/++
Chymotrypsin	+++	++	–	+++	++
Trypsin	–	–	–	–	–
AMP M	–	–	–	–	–
DAP I	–	–	–	–	–
DAP II	–	++	–	–	+
DAP IV	–	+	–	–	–
Cathepsin B	–	–	–	–	–

within specimens of the same genus. In particular, we did not notice differences correlated to size.

Esterases.—Non-specific esterase activities occurred in the digestive system of all species investigated (Tables I–4). High levels of activity were found in the epithelia of the digestive tract (stomach, caecum, and, in octopods, crop) (Tables 1–4 and Figs. 1, 2) and in the internal epithelium of the suckers. The activity in the digestive gland was localized in small granules scattered in the glandular cells. The internal epithelium of the digestive duct appendages generally displayed esterase activity. Esterase activity also was found in the central nervous system (and probably corresponds to cholinesterase) and in the muscles, especially in a layer in the middle of the mantle wall and in the mantle epithelium (Fig. 2).

High alkaline phosphatasic activity occurred in the epithelia of the vestibule

Table 2. Hydrolase activities in the main digestive organs of paralarval Bolitaenidae. PSG: posterior salivary glands, DG: digestive gland, DDA: digestive duct appendages, St: stomach, Caec: caecum, +++: high activity, ++: medium activity, +: low activity (estimation based on the intensity of the staining for the precipitation method and on the time of digestion of the substrate for the substrate-film method).

	PSG	DG	DDA	St	Caec
Esterases (non-spec)	+/++	+/++	–	++	++
Alkaline phosphatase	–	+++	–	+	++
Acid phosphatase	+	+++	++	–	++
Amylase	–	–	–	–	–
Acetyl-glycosaminidase	+	++	+	++	+
Glucuronidase	–	+	–	–	–
Proteases (non-spec)	–	+++	–	++	–
Chymotrypsin	+++	++	–	+++	+++
Trypsin	–	–	–	–	–
AMP M	–	–	–	–	–
DAP I	–	–	–	–	–
DAP II	–	++	–	–	–
DAP IV	–	–	–	–	–

Table 3. Hydrolase activities in the main digestive organs of paralarval Ommastrephidae. PSG: posterior salivary glands, DG: digestive gland, DDA: digestive duct appendages, DT: digestive tract, +++: high activity, ++: medium activity, +: low activity (estimation based on the intensity of the staining for the precipitation method and on the time of digestion of the substrate for the substrate-film method).

	PSG	DG	DDA	DT
Esterases (non-spec)	+	++	+++	++/+++
Alkaline phosphatase	−	++	++	+++++
Acid phosphatase	+	+++	−	−/+
Amylase	−	−	−	−
Acetyl-glycosaminidase	+++	+	+	+
Glucuronidase	−	+	−	+
Proteases (non-spec)	+++	+++	−	+++
Chymotrypsin	−	+	−	−
Trypsin	−	−	−	−
AMP M	−	−	−	++
DAP I	−	−	−	−
DAP II	−	++	−	+
DAP IV	−	−	−	−

and caecum (Tables 1–4 and Fig. 3), especially at the apical part of the epithelial cells. Alkaline phosphatase was found in the digestive gland, with various levels of activity (Tables 1–4, and Figs. 3, 5). The highest activity occurred at the luminal border of the epithelial cells of the digestive gland (Fig. 4). Alkaline phosphatase activity also was displayed by the internal epithelium of the digestive duct appendages of the oegopsids (Tables 3, 4). High activities also were found in the skin of some specimens (Fig. 5).

High acid phosphatasic activity occurred in the digestive gland of all the animals investigated (Tables 1–4 and Figs. 6, 7). This activity was localized in the large vacuoles (Fig. 7) and also scattered in small granules within the glandular cells. Acid phosphatase activities generally occurred in the caecal epithelium, and to a lesser extent in the internal epithelium of the digestive duct appendages (Tables 1, 2, 4).

Table 4. Hydrolase activities in the main digestive organs of paralarval *Abralia* (Enoploteuthidae). PSG: posterior salivary glands, DG: digestive gland, DDA: digestive duct appendages, DT: digestive tract, +++: high activity, ++: medium activity, +: low activity (estimation based on the intensity of the staining for the precipitation method, and on the time of incubation for the substrate-film method).

	PSG	DG	DDA	DT
Esterases (non-spec)	+	+	++	++/+++
Alkaline phosphatase	+	−/++	+++	−/+++
Acid phosphatase	++	+++	−	++/+++
Amylase	−	−	−	−
Acetyl-glycosaminidase	+++	++	+	+/++
Glucuronidase	−	−	−	−
Proteases (non-spec)	++	+	−	+++
Chymotrypsin	−	++	−	++
Trypsin	−	−	−	−
AMP M	−	−	−	−
DAP I	−	−	−	−
DAP II	−	+++	−	−
DAP IV	−	−	−	−

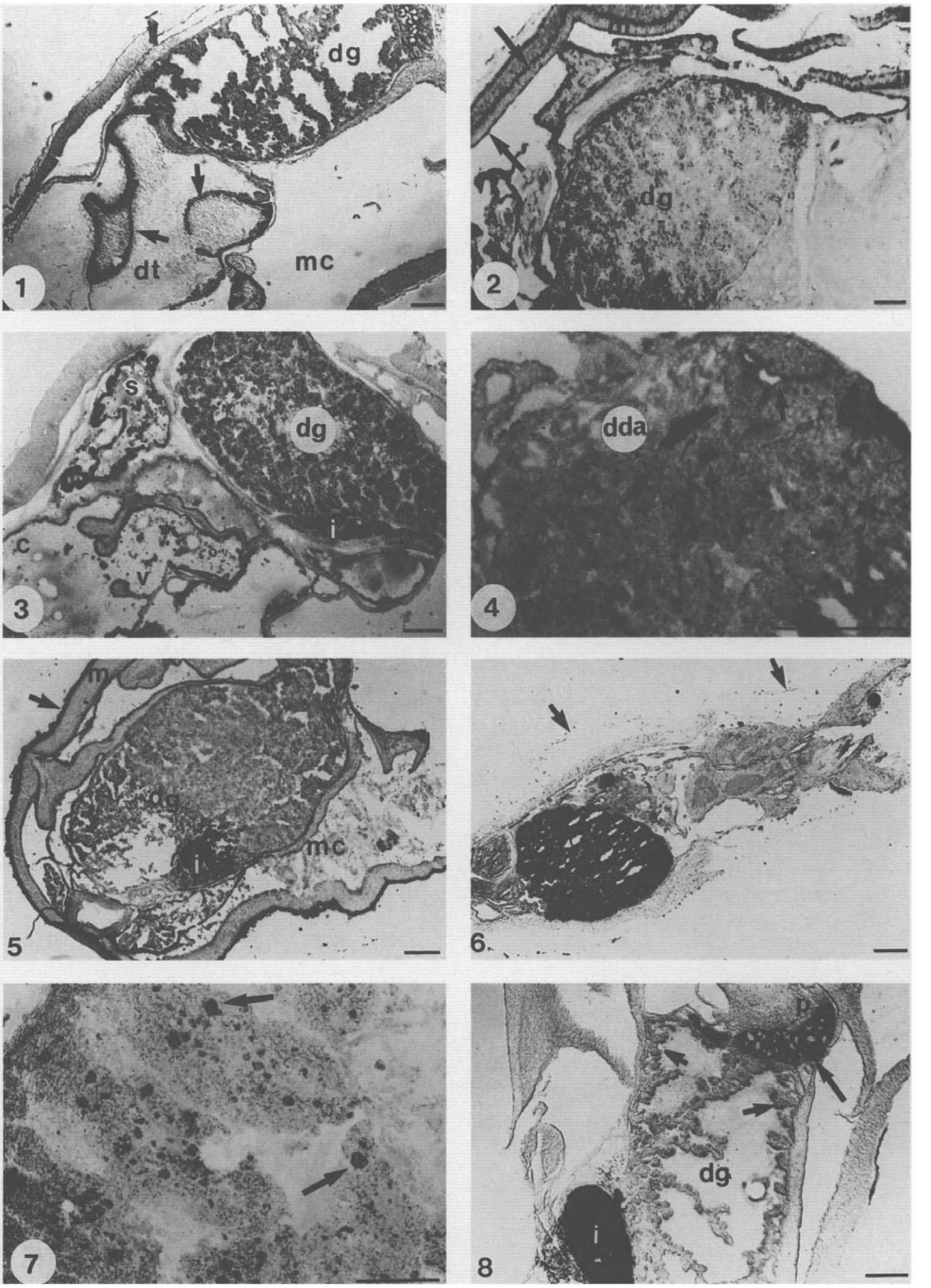

Figure 1. Esterase activity in a longitudinal section of *Abralia*. ×29. The epithelium of the digestive tract (caecum and vestibule) displays a high activity (arrows).

Figure 2. Esterase activity in a longitudinal section of an *Octopus*. ×29. The arrows show the high activity in the middle muscle layer and in the inner epithelium of the mantle.

Figure 3. Alkaline phosphatase activity in a longitudinal section of a rhynchoteuthion. ×36. High AP activities are found in the digestive gland (apical part of the cells) and in the epithelia of the digestive tract (stomach, caecum and vestibule).

Glycosidases.—No amylase activity could be detected histochemically in most specimens, even after one hour of incubation (Tables 1–4). A very low activity, however, was noted in the caecum of one *Octopus* specimen.

High acetyl-glycosaminidase activities were found in the glandular cells of the posterior salivary glands (Tables 1–4 and Figs. 8, 9), in the digestive gland (Tables 1–4 and Figs. 8, 9) (localized in the large vacuoles) and in the epithelia of the digestive tract (Tables 1–4 and Fig. 9), especially in the anterior part, where the stomach displayed the highest activity (Fig. 10). Low levels of glucuronidase activity were found only in the vacuoles of the digestive gland of Bolitaenidae and Ommastrephidae (Tables 2, 3).

Peptidases.—High protease activity was displayed in the digestive gland of all species (Tables 1–3 and Fig. 11), except *Abralia* (Table 4) in which the lumen of the digestive tract (stomach, vestibule and caecum) showed the highest activity (Table 4 and Fig. 12).

High or medium protease activities generally were found in the lumen of the posterior salivary glands of oegopsids (Tables 3, 4 and Fig. 13), as well as in the lumen of the digestive tract (Tables 3, 4, and Fig. 12). This activity could not be correlated with trypsin (the two different substrates tested gave negative results). Positive results for chymotrypsin were found in the digestive gland of the two octopods (in the "boules," large secretory granules) and of *Abralia* (Tables 1, 2, 4), but not in the Ommastrephidae (Table 3). Moreover, we found chymotrypsin activity in the glandular cells of the buccal mass (Figs. 14, 15), in the secretory cells of the posterior salivary glands and inside the suckers in both octopods (Fig. 14), and in the oesophagus epithelium in *Octopus* (Fig. 14).

Membrane-bound exopeptidases, amino-peptidase M and DAP IV, were not detected in the digestive system (Tables 1–4) but were found with high activities in the neuropile of ganglia of the central nervous system. The lysosomal endo-peptidase DAP II was found in the digestive gland of all families (Tables 1–4 and Fig. 16), localized primarily in large vacuoles. Cathepsin B, another lysosomal peptidase, was tested with two different substrates in *Octopus,* without positive results (Table 1).

Discussion

Two preliminary remarks are necessary concerning the histochemical methods used in this work. First, a negative reaction does not necessarily mean that an enzyme is absent. Enzymes may be present in an inactive stage (zymogen), or

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Figure 4. Alkaline phosphatase activity in the digestive gland of a Bolitaenidae. ×116. The enzyme activity is localized at the luminal border of the digestive cells (arrows). The digestive duct appendages (dda) are negative.

Figure 5. Alkaline phosphatase activity in a longitudinal section of an *Octopus.* ×29. Note the high activity of the mantle epithelium especially the external epithelium (arrow).

Figure 6. Acid phosphatase activity in a longitudinal section of a Bolitaenidae. ×29. The digestive gland displays a very high activity (in black). Note the characteristic gelatinous coat around the body (arrows).

Figure 7. Acid phosphatase activity in the digestive gland of an *Octopus.* ×72. The activity is especially high in the large vacuoles (arrows).

Figure 8. Acetyl-glycosaminidase activity (arrows)in the posterior salivary glands and digestive gland of *Abralia* (longitudinal section). ×36. c: caecum; dda: digestive duct appendages; dg: digestive gland; dt: digestive tract; i: ink sac; m: mantle; mc: mantle cavity; p: posterior salivary glands; s: stomach; v: vestibule. Bar scale: 200 μm.

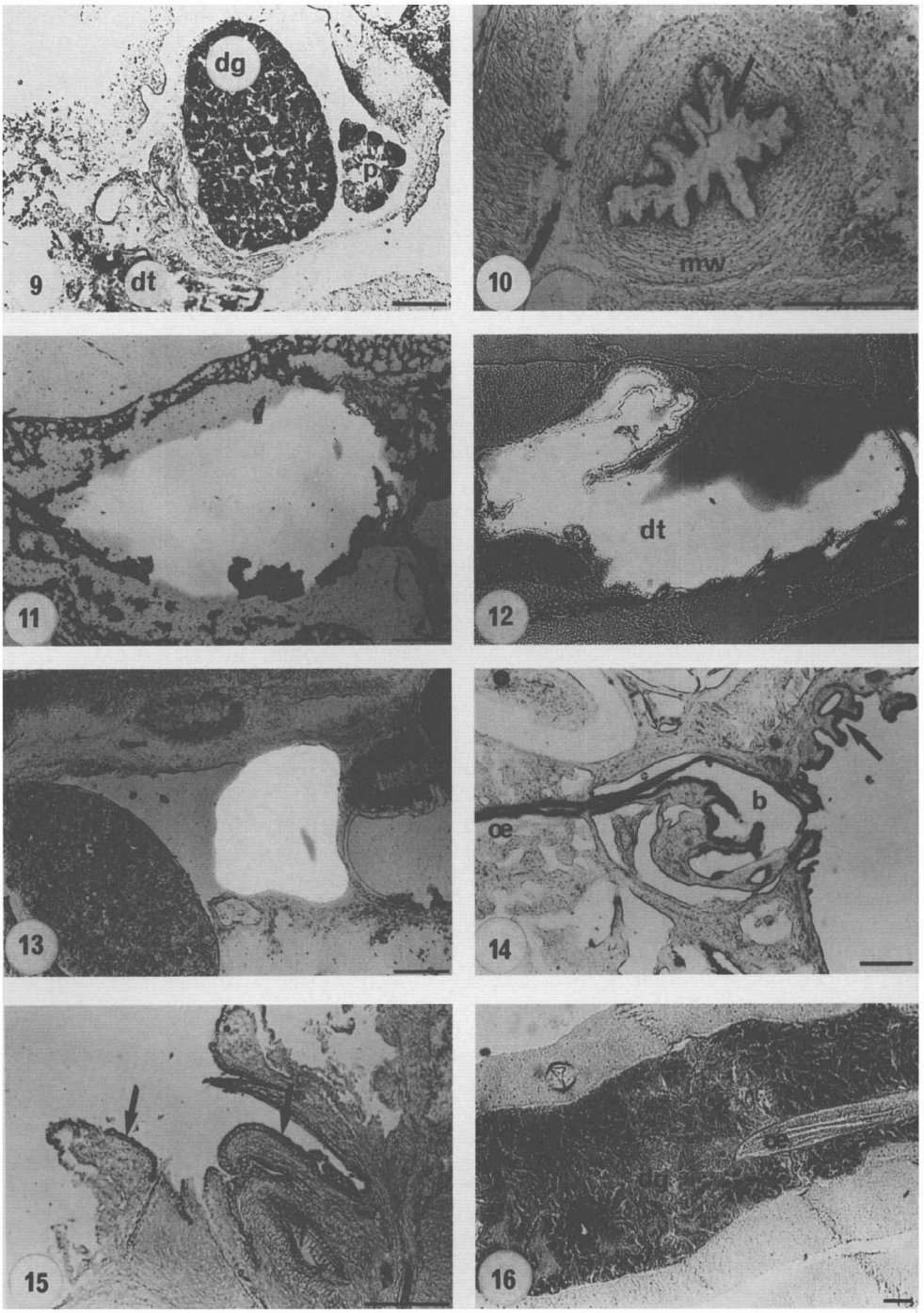

Figure 9. Acetyl-glycosaminidase activity in the posterior salivary glands, the digestive gland and the digestive tract of a rhynchoteuthion (longitudinal section). ×46.

Figure 10. High acetyl-glycosaminidase activity in the stomach epithelium (arrow) of an *Octopus* (transverse section). ×116.

they may not be concentrated enough in the tissues to give a positive reaction, especially with the substrate-film method. Second, some of the substrates potentially can be split by several enzymes. The large range of substrates used in this study, however, allows us to accept the results as reasonable. For example, the naphthol AS propionate used for chymotrypsin analysis is potentially split by esterases (Southward and Southward, 1968). So, the occurrence of the same localization of esterase and chymotrypsin could be confusing. In the paralarvae, however, localizations of esterases and chymotrypsin were found different (Tables 3, 4), thus indicating that esterases, in our material, do not split the naphthol AS propionate which could be considered specific for chymotrypsin activity. On the other hand, it is likely that in the nervous system, the same enzyme, a membrane-bound dipeptidase of optimum pH 7–7.5, gives positive results with leucyl-methoxy-β-naphthylamide and glycyl-prolyl-methoxy-β-naphthylamide. This enzyme could be the peptidyl-amino hydrolase characterized by Aniello and Strazzullo (1984).

The high non-specific esterase activity generally found in the digestive epithelia probably indicates a high metabolic activity of these epithelia. In the digestive gland, some of the esterase activity might be involved in lipid metabolism, as this gland is the only site of lipid storage in cephalopods. With the exception of *Abralia,* all the paralarvae examined had many neutral lipid droplets in their digestive glands. Our results on the localization of esterases in the paralarvae are in agreement with previous findings on adult "decapods" (*Sepia officinalis,* Boucaud-Camou, 1974).

Alkaline phosphatase is a membrane-bound enzyme involved in active transport. Thus it is not surprising to find this enzyme in the caecum and in the digestive gland, the two major absorptive organs (Boucaud-Camou and Boucher-Rodoni, 1983). Moreover, in the digestive gland, this activity is localized at the luminal border of the digestive cells, where numerous microvilli occur (Boucaud-Camou, 1972; Boucaud-Camou and Yim, 1980). We noticed some variation in activity which could be related to the stage of digestion; high activities probably indicate ongoing absorption at the time of fixation. Such changes correlated to digestion have been observed in the digestive gland of Bivalvia (Mathers, 1973; Palmer, 1979; Boucaud-Camou et al., 1985a; Henry et al., 1991).

The high acid phosphatase activity found in the digestive gland clearly reveals the intracellular digestive activity of the gland and marks all the lysosomes II (as well the autolysosomes which are the largest digestive vacuoles). The activity found in the caecal epithelium is in agreement with the results of Boucaud-Camou

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Figure 11. Proteolytic activity: marked by the clear digested area in the digestive gland of a Bolitaenidae (longitudinal section). ×46.

Figure 12. Proteolytic activity: clear digested area in the digestive tract of an *Abralia* (longitudinal section). ×29.

Figure 13. Proteolytic activity: clear digested area in the posterior salivary gland of a rhynchoteuthion (longitudinal section). ×46.

Figure 14. Chymotrypsin activity displayed by the epithelia of the buccal cavity, oesophagus and suckers (arrows) of an *Octopus* (longitudinal section). ×46.

Figure 15. Chymotrypsin activity in the glandular cells of the buccal mass (arrows) of an *Abralia* (longitudinal section). ×72.

Figure 16. DAP II activity in the digestive gland of an *Abralia* (longitudinal section). ×29. b: buccal cavity; dg: digestive gland; dt: digestive tract; e: eye; i: ink sac; mw: muscular wall; oc: oesophagus; p: posterior salivary glands. Bar scale: 200 μm.

(1977), who found evidence of pinocytotic activity in the caecum of *Sepia officinalis.*

We could not detect any amylase activity, except minimally in the caecum of one *Octopus.* As noted above, this does not mean that there is no amylase activity, but, more likely, that the activity is too low to be detected histochemically by the only method available (substrate-film).

Acetylglycosaminidase is typically a lysosomal enzyme, and its localization in the vacuoles of the digestive gland confirms the intracellular digestive processes (autophagocytic as well as heterophagocytic) within the glandular cells. N-hexosaminidase has been purified from the digestive gland of *Octopus vulgaris* (Nakagawa et al., 1987). In the posterior salivary glands, this activity probably is involved in crinophagic processes that regulate the secretion. The activity found in all the epithelia lined with a cuticle probably is involved in cuticle reshaping, because the cuticle contains chitin (Hunt and Nixon, 1981), a polymer of acetylglycosamine.

The high proteolytic activity found in the digestive tract and digestive gland is in agreement with previous results obtained with the same techniques in adults and early young (Boucaud-Camou, 1969, 1974, 1982). However, we observed changes of the respective activity in the different organs. These changes probably are related to the stage of digestion, just as for example, Boucher-Rodoni (1982) found changes of proteolytic activity related to the stage of digestion in the digestive gland of *Sepia officinalis* and *Eledone cirrhosa.* The in situ proteolytic activity in the posterior salivary gland of decapod cephalopods reported here is a new finding. The only histochemical data are for the adult *Sepia* where the activity was rather low (Boucaud-Camou, 1969), although an in vitro activity has been detected in *Sepia officinalis* (Koueta and Boucaud-Camou, 1986). However, several in vitro studies have demonstrated high proteolytic activity in the posterior salivary gland of *Octopus vulgaris* (Sakaguchi, 1968; Morishita, 1972a; Morishita et al., 1974a, 1974b, 1974c).

The high proteolytic activity of the digestive gland appears to be a general feature in cephalopods and is clearly related to their carnivorous diet. Proteolytic activities were found in extracts of the digestive gland of *Sepia officinalis* (Romijn, 1935; Boucaud-Camou, 1974; Boucher-Rodoni, 1982), *Octopus vulgaris,* (Sawano, 1935; Sakaguchi, 1968; Morishita, 1978), *Eledone cirrhosa* (Boucher-Rodoni, 1982), *Loligo vulgaris* (Pignero and Rocca, 1969) and *Todarodes pacificus* (Kozlovskaya and Vaskovsky, 1970). In the digestive tract, the activity was localized in the lumen, thus confirming that the proteolytic activity found in the digestive tract of cephalopods is mainly related to the secretions of the associated glands (posterior salivary glands and digestive gland).

The proteolytic activity could be partly related to chymotrypsin. In octopods, we found a chymotrypsin-like activity in all parts of the digestive system. The posterior salivary glands and the digestive gland are the likely sources of this enzyme in the anterior and posterior parts of the digestive tract, respectively. The occurrence of chymotrypsin-like enzymes in the posterior salivary glands of octopods has been demonstrated previously; e.g., six chymotrypsin-like enzymes were found in the posterior salivary glands of *Octopus vulgaris* (Morishita, 1974b, 1974c, 1978). In *Sepia officinalis* and chymotrypsin-like activity has been found histochemically in the digestive gland (Boucaud-Camou, 1974, 1982). Chymotrypsin has been characterized in the digestive tract of *Sepia officinalis* (Rothe et al., 1970). On the other hand, trypsin was not found histochemically in any of the species we investigated. Negative results were also obtained with adult *Loligo forbesi* (E. Boucaud-Camou and R. Boucher-Rodoni, Univ. Caen, unpubl. results),

whereas trypsin-like activities were detected by in vitro methods in the digestive gland of *L. vulgaris* (Pignero and Rocca, 1969). The histochemical detection method is perhaps inadequate or not sufficiently sensitive.

DAP II found in the lysosomal system of the digestive gland is another enzyme involved in the intracellular digestive processes. Such an activity has been demonstrated in *Loligo forbesi* (E. Boucaud-Camou and R. Boucher-Rodoni, Univ. Caen, unpubl. results) and in the digestive gland of Bivalvia by the same histochemical method (Boucaud-Camou et al., 1985a; Henry et al., 1991). The glucuronidase activity found in the digestive gland also is characteristic of lysosomes.

The negative results obtained in testing for cathepsin B were more surprising, as this enzyme is known to occur in the digestive gland of *Octopus* (Morishita, 1972b, 1972c). However, the azo-coupling procedure has been said to be unsatisfactory to detect this cysteine proteinase (Van Noorden et al., 1987).

The high protease activity and very low or histochemically undetectable amylasic activity suggest a carnivorous diet, indicative that the diet of paralarvae resembles that of adults. Boletzky (1974) came to the same conclusions from morphological studies of the prehensile organs (arms, tentacles, suckers). The observations of remains detected in the stomachs of paralarvae (Vecchione, 1991a, 1991b; Passarella and Hopkins, 1991) and the faunal composition of the plankton samples from off Fort Pierce show that crustacean larvae and copepods are most probably the major components of the diet. In any case, a difference exists between the digestive enzyme distribution of adults and young. Adult cephalopods have a rather high histochemically detectable amylase activity, while the young have none. The young seem especially equipped to digest proteins. In ommastrephid rhynchoteuthions the possibility of a microphagus (phytoplanktonic) diet has been proposed (O'Dor et al., 1985). Actually the strong proteolytic and weak amylolytic activities suggest (but not absolutely prove) carnivorous habits.

The digestive gland displays the highest enzyme activities, which agrees with the key role of the gland in the digestive processes of cephalopods. In particular, the gland appears to be the main source of the proteolytic enzymes found in the posterior digestive tract. The nature of these enzymes is not yet clear, but our results suggest that chymotrypsin-like enzymes are partly responsible for these activities. Other endopeptidases undoubtedly are involved, as well.

The high acid phosphatase activity, DAP II and acetylglycosaminidase activities, typically lysosomal, suggest intracellular digestive processes in the digestive gland. They were found primarily in large vacuoles in the digestive gland (brown-body vacuoles). These processes may be related either to autophagocytic or heterophagocytic types of digestion that have been demonstrated to occur in the digestive gland of *Sepia officinalis* (Boucaud-Camou and Yim, 1980). Studies of the infrastructure of the digestive gland of paralarval squids are in progress and should help to confirm the results of the enzyme study.

The posterior salivary glands are as well developed in squid as in octopod paralarvae and they display several enzymatic activities, most notably a high proteolytic activity. The lytic enzymes they contain could facilitate the penetration of the toxins into the tissues of the prey. Moreover, they are likely to play a role in digestion. Indeed, posterior salivary gland enzymes act in the anterior part of the digestive system in *Octopus* (Morishita, 1978), and this could be true for paralarvae in general. The proteolytic activity of the posterior salivary glands would facilitate and accelerate the breakdown of food in the anterior part of the digestive tract, enhancing the efficiency and speed of digestion which are known to be very high in young cephalopods (Boucher-Rodoni et al., 1987). The enzymatic activities that we have found at the level of the suckers in octopodan para-

larvae most likely come from the secretion of the posterior salivary glands. These enzymes could be involved in diffusion of the venom, or involved in some "external" digestive processes. The possibility of external digestion has been discussed for octopods (Boucaud-Camou and Boucher-Rodoni, 1983; Mangold and Bidder, 1989) in which predigestion actually seems limited to loosening muscle attachments. This is probably important to paralarvae as well.

In all the specimens we studied, even the smallest, the entire digestive system appears well developed and equipped with digestive enzymes. This contrasts with the development of young *Sepia officinalis,* the only species available for comparison. *Sepia officinalis* hatches from large eggs and the young are benthic. The paralarvae in our study, however, had a mantle length longer than the presumed size at hatching and no internal yolk was left. Therefore, they must have lived for some time in the plankton before they were collected. In *Sepia officinalis* the digestive activities begin with the ingestion of the first meal, usually within 3 days after hatching (Boucaud-Camou et al., 1985b). The hatchlings depend entirely on their yolk reserves before they start active feeding. These reserves are consumed within a fortnight. Squid paralarvae usually hatch from small eggs with small yolk reserves, so the endotrophic phase is short. O'Dor et al. (1986) calculated that the yolk is entirely consumed within 3 days in *Illex illecebrosus* paralarvae reared at 25°C. Such high temperatures occur in surface waters in the Gulf Stream even during the winter, so the paralarvae must be able to catch prey very soon after hatching. They have to overcome two major difficulties. First, food may be scarce. Second, paralarvae have to learn quickly to capture their prey efficiently. Boletzky (1974) noticed that planktonic young are well equipped to capture prey with their few, relatively large suckers. However, the tentacles of paralarvae may not be fully functional (Vecchione, 1981). Moreover, the denticulated beaks (Boletzky, 1974) and the well developed posterior salivary glands which secrete several lytic enzymes allow the prey to be subdued very rapidly. An additional feature that should help the paralarvae to survive the first critical stages after hatching is the high alkaline phosphatase activity of the skin which suggests active exchanges with the seawater. Vecchione and Hand (1989) noted that in *Loligo forbesi,* absorption of dissolved organic matter through the skin seems to be very beneficial during the first days of life. It seems possible therefore that nutrients could be absorbed through the skin and provide a part of the energy necessary to the young squids.

Probably some paralarvae cannot obtain enough food in their first days of life. These starving animals would become weak very quickly and would be unable to escape their predators. Perhaps that is the reason that we did not find starving animals in our samples. A comparative histological study of paralarvae is in progress on a relatively large range of specimens and will yield more information about the conditions of starvation.

ACKNOWLEDGMENTS

This work was carried out during a senior postdoctoral fellowship of the Smithsonian Institution at Link Port, Ft. Pierce, Florida, awarded to Eve Boucaud. Additional support was provided through a SMSLP research grant to C. Roper et al. We thank the director of the Smithsonian Marine Station, M. Rice, and the staff for help with collecting animals and in the laboratory: S. Reed, J. Piraino, H. Reichardt, W. Lee. We are very grateful to Dr. M. Vecchione, National Marine Fisheries Service, Systematics Laboratory, for help with identification of specimens and for valuable discussions. We thank Dr. K. Mangold and Dr. M. Vecchione for critical comments on the manuscript.

This paper is publication number 286 of the Smithsonian Marine Station at Link Port, Ft Pierce, Florida, U.S.A.

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DATE ACCEPTED: May 27, 1994.

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