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THE RELATIONSHIP BETWEEN SEAWEED DIET AND PURPLE INK PRODUCTION IN *APLYSIA DACTYLOMELA* RANG, 1828 (GASTROPODA: OPISTHOBRANCHIA) FROM NORTHEASTERN BRAZIL

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ABSTRACT *Aplysia dactylomela* is a large marine opisthobranch gastropod, which inhabits shallow tropical shoreline regions, eats red and green algae, and releases a purple ink when disturbed. Many functions have been proposed for this secretion and although there is no consensus on this, some of its constituents are believed to be derived from the red algal diet, it may provide the snail with a substantial survival advantage. *A. californica* only produces ink when it ingests red seaweeds. In some locations of the Northeastern Coast of Brazil, *A. dactylomela* is seen feeding only on green seaweeds, and yet it releases the ink. The aim of this work is to investigate this contradiction by studying the feeding habits of *A. dactylomela* and assessing the relationship between the algal diet and the purple ink. Feeding habits were investigated by field observation and by analysis of gut contents. Purple ink production was monitored by histologic analysis of the ink gland from sea hares kept in water tanks, fed with either red or green seaweeds. Composition and protein profile of the purple ink also were studied. Homologies between seaweed components and the purple ink were sought for by immunodiffusion techniques. Our findings are that the sea hare *A. dactylomela*, likewise other *Aplysia* species, needs to consume red seaweeds to be able to secrete the purple ink. The proteins of the ink seem to be synthesized by the sea hare itself and are not obtained directly from the diet, as is the case for the ink pigments.

KEY WORDS: *Aplysia dactylomela*, purple ink, ink gland, seaweed diet

INTRODUCTION

Aplysia dactylomela is a large marine opisthobranch gastropod which inhabits shallow tropical shoreline regions, eats red and green algae, and lays large numbers of fertilized eggs in string-like gelatinous masses close to the sea surface. It is hermaphroditic and nocturnally active, but may be exposed to sunlight as it rests during the day (Carefoot 1987). Sea hares probably are best known because of the purple ink they release when disturbed. This ink is secreted from the ink gland located on the edge of the mantle shelf, and the ability to produce purple ink is reported to be associated with a red seaweed- containing diet (Coelho et al. 1998). Of the 37 species of sea hare from the *Aplysia* genus, 30 can secrete purple ink (Nolen et al. 1995). Many functions have been proposed for this secretion, such as camouflage, alarm signal, pheromone, aposematism (use of color patterns by prey animals to signal their distastefulness to predators), bile excretion, predator deterrent, and cue of danger (Johnson & Willows 1999). Probably the ink has more than one role in the biology of sea hares. Although there is no consensus on the biologic function of the ink, it is known that some of its constituents are derived from red algae in the diet (Prince et al. 1998), and it provides the snail with a substantial survival advantage. Recent work by Prince et al. (1998) on the ink glands of *A. californica* has improved our understanding of ink gland structure and processing and secretion of purple ink.

In addition to studies of the ecologic and biologic aspects of chemical defense in sea hares, special attention also has been paid to the isolation and characterization of new bioactive substances from the purple ink. Thus, many bioactive substances have been isolated from the purple ink, including proteins with antibacterial activity in *A. punctata* (Nistratova et al. 1992), antitumor and citolytic activities in *Dolabella auricularia*, (Kisugi et al. 1989, Yamazaki et al. 1989a) and citolytic and antibacterial activities in *A. kurodai* (Yamazaki et al. 1989b, Yamazaki et al.1990). In Brazil, antibacterial and hemagglutinating activities were described for the purple ink of *A. dactylomela* (Melo et al. 1998; Melo et al. 2000).

On the northeastern Coast of Brazil, there is an abundance of seaweed species that support the great biodiversity of this tropical region. On some beaches there is a predominance of green seaweeds (mainly *Ulva fasciata*), whilst on others, red seaweeds prevail. On the beaches dominated by green seaweed, *A. dactylomela* is seen feeding mainly on them and yet releases the purple ink when disturbed. This fact could be intriguing, considering that other *Aplysia* species need to consume red seaweeds to be able to produce the ink. To solve this apparent contradiction this study investigates the feeding habits of *A. dactylomela* and assesses the relationship between the algal diet and the purple ink.

MATERIALS AND METHODS

Determination of Feeding Habits

Feeding habits of *A. dactylomela* were observed in 2 beaches of Ceará State, Northeast of Brazil, between August 2000 and July 2001. One beach was particularly rich in the green seaweed *Ulva fasciata* (beach 1, 38°38′48″W and 3°41′24″S); whereas, the other was densely rich in the red species, particularly *Hypnea musciformis* and *Gracilaria* spp. (beach 2,39°25′45″W and 3°22′18″S). Feeding habits of sea hare were examined monthly during daytime at low tide by observing the seaweed species consumed in the field

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and by dissecting under a stereomicroscope the gut contents of the sea hares and capturing the images with a digital camera (Sony–Mavica, Japan).

Histologic Analysis of the Ink Gland

Specimens of *A. dactylomela* were collected during low tide and transported to the laboratory in a container with seawater. Some specimens were de-inked by squeezing them gently for a few minutes outside the water and then kept in seawater tanks for 15 days with unialgal diets (*U. fasciata* or *Gracilaria* sp.) to observe whether there was purple ink production. At the end of this period, sea hares were anaesthetized by storage in a refrigerator and then dissected carefully to remove the ink gland.

For light microscopy, ink gland samples were fixed with Bouin mixture (saturated picric acid solution, 40% formaldehyde, glacial acetic acid (15: 5:1 v/v) and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (Junqueira & Junqueira, 1983) and with bromophenol blue for detection of proteins (Pearse 1960). Histologic sections were observed under a microscope, and photographs were taken.

Chemical Composition of Purple Ink

Purple ink was analyzed for contents of water, protein (total nitrogen), reduced carbohydrate, lipid, and ash. For the water determination, 1g of ink was dehydrated in an oven at 100°C to 110°C to constant weight. Total nitrogen was determined in samples of freeze-dried purple ink by micro-Kjeldahl digestion (Baethgen & Alley 1989). The content of reduced carbohydrate was determined according to Dubois et al. (1956). Lipid content was determined by n-hexane Soxleht extraction, and ash was quantified by heating 1g of ink in a muffle furnace at 620°C for 18 h.

Amino Acid Analysis

Dry purple ink (1 mg) was hydrolyzed in 1 mL 6 M HCl with 1% phenol (w/v), in a sealed glass tube under N_2, at 110°C for 22 h. After hydrolysis, HCl and phenol were removed by evaporation, and the residue was analyzed in a Biochrom 20 (Pharmacia–LKB) amino acid analyzer.

Electrophoretic Profile of Ink Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the presence of beta-mercaptoethanol according to the method of Laemmli (1970). Proteins were stained with silver nitrate (Blum et al. 1987). Bovine serum albumin (66.0 kDa), egg albumin (45.0 kDa), porcine pepsin (34.7 kDa), bovine β-lactoglobulin (18.4 kDa), and egg lysozyme (14.3 kDa) were used as standards (Sigma Co., USA).

Seaweed Protein Extraction and Determination

Proteins were extracted from fresh samples of the green seaweed *U. fasciata* and the red species *H. musciformis* and *Gracilaria* sp. The samples were ground with a mortar and pestle with the following buffers at 50 mM: Glycine-HCl, pH 2.6; Tris-HCl, pH 7.0; and sodium borate, pH 9.0. The extracts of *U. fasciata* and *H. musciformis* were prepared at the proportion of 1:3 (m/v) and that of *Gracilaria* sp. at 1:5 (m/v). The extracts were filtered through a nylon tissue and centrifuged at 15,000 *g* for 10 min at 4°C. The supernatants (crude extracts) had the protein content determined according to Bradford (1976) with bovine serum albumin (BSA) as the standard (purchased from Sigma Co., USA).

Anti-Purple Ink Polyclonal Antibody

Anti-purple ink polyclonal antibody was developed in a 3-month-old albino rabbit, which was immunized by intramuscular injection with 1 mg of purple ink, previously dialyzed and freeze-dried and then dissolved in 1 mL of sterile saline solution containing incomplete Freund adjuvant, 1:1 v/v (Sigma Co.). Booster injections were given without adjuvant subcutaneously on the 14th, 21st, 28th, and 35th days after the primary injection. Immune serum was obtained by blood sampling from the marginal ear vein on the 21st, 28th, and 35th days.

Immunodiffusion Assay

Immunologic relationships between protein extracts from seaweeds and the purple ink were assessed by the Ouchterlony double diffusion method (Hudson & Hay 1989). The assays were done in 1% agar plates prepared with 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl and 0.02% sodium azide. After solidification of the agar, wells were made with a Pasteur pipette, and 30 μL of each seaweed extract was applied into each well and tested against the anti-purple ink antibodies applied in the central well. After incubation at room temperature for 48 hours, the gels were washed with 150 mM NaCl, dried, and stained with Coomassie brilliant blue for visualization of the precipitation arcs.

RESULTS

Feeding Habits

Sea hares from beach 1 were frequently surrounded by the green alga *U. fasciata*, and to a much less extent by red alga species (*Gracilaria* spp. and *H. musciformis*). In beach 2, however, the snails were surrounded by red algae, mainly *H. musciformis,* and some brown species. The analysis of gut contents from specimens collected showed that the snails ate mainly those alga species surrounding them. Those from beach 1 had gut contents comprised of approximately 70% *U. fasciata* (and traces of red species); whereas, those from beach 2 contained over 90% *H. musciformis* (photography not included).

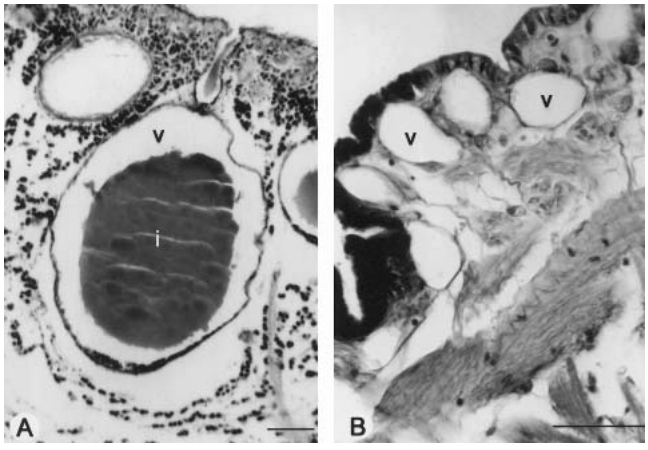

Figure 1. Light micrographs of ink glands in red-seaweed-fed (A) and green-seaweed-fed *Aplysia dactylomela* (B). Vesicles (v) were full of ink (i) in red-seaweed-fed sea hares and empty in green-seaweed-fed. Scale bars: A, B, 100 μm.

TABLE 1.

Chemical analysis of the purple ink from *Aplysia dactylomela.*

Components	% Dry Basis
Total protein (N × 6.25)	64.87 ± 2.56
Reduced carbohydrate	9.07 ± 1.84
Lipid	2.20 ± 0.40
Ash	2.72 ± 0.20

Values are means ± standard deviation of at least triplicate analyses.

Histologic Analysis of the Ink Gland

Histologic analysis showed that ink gland tissues from animals whose gut contained mainly green algae had most vesicles empty. The same was observed with the animals kept on the *U. fasciata* diet in the laboratory tank. By contrast, the ink glands of animals from beach 2, which consumed mainly red alga species, had most vesicles full (Fig. 1).

Chemical Composition of Purple Ink

The purple ink is composed of approximately 99.5% water. Purple ink proximal chemical analysis, on dry basis, is described in Table 1, being comprised mainly of proteins (over 60%). Regardless of diet, the ink had the same basic composition of protein, carbohydrate, lipid, and ash.

Amino Acid Composition

The results of amino acid composition (Table 2) showed that the purple ink contains high levels of acidic amino acids, glutamic, and aspartic acid and very low content of sulfur amino acids.

Electrophoretic Profile of Ink Proteins

The purple ink of sea hares from beaches 1 and 2 showed similar electrophoretic profiles in terms of mobility and intensity of protein bands (Fig. 2). Two prominent bands were observed at 60 and 45 kDa, several minor bands span the apparent molecular mass range 20–30 kDa, whilst others were approximately 15 and few below 14 kDa.

TABLE 2.

Amino acid composition of purple ink of *Aplysia dactylomela*

Amino Acid Residue	g of Amino Acid in 100 g of Dry Matter
Ala	6.03
Arg	5.07
Asx	11.77
Cys	1.43
Glx	11.95
Gly	6.48
His	1.12
Ile	3.68
Leu	7.10
Lys	4.70
Met	1.55
Phe	4.55
Pro	8.98
Ser	5.78
Thr	7.54
Trp	ND
Tyr	5.46
Val	7.59

ND = not determined.

Serological Relationship Between the Purple Ink and Seaweed Extracts

The immunodiffusion assay showed that the anti-purple ink antibodies reacted with the ink but did not recognize any seaweed component extracted either with acid, neutral or alkaline buffer (photography not included). This indicates, therefore, that no immunologic reactivity exists between components of algal diet and the purple ink.

DISCUSSION

This study describes the relationship between the seaweeds consumed by the sea hare *A. dactylomela* and its purple ink.

Although it is has been reported that the secretion of purple ink by sea hares is associated with their consumption of red seaweeds (Prince et al. 1998), specimens that consumed mainly green seaweeds (beach 1) also were seen to release the purple ink. This contradiction was solved when the analysis of gut contents showed the presence of traces of red seaweeds, indicating that minor consumption of red species was enough to support the ink production.

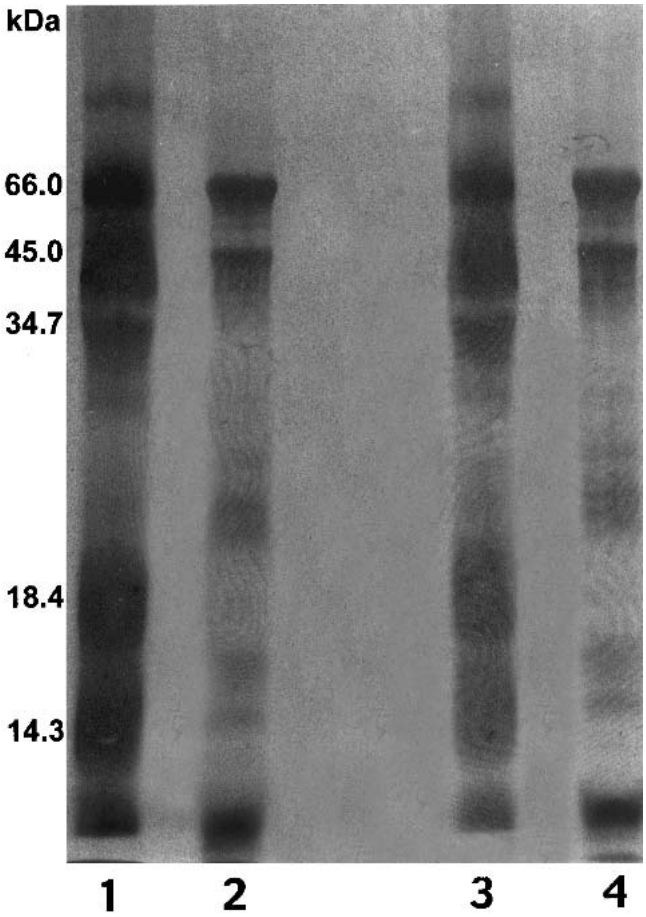

Figure 2. SDS-Polyacrylamide gel electrophoresis. Lanes 1 and 3, molecular mass markers; lane 2 purple ink from *Aplysia dactylomela* collected at beach 1(green seaweed rich) and lane 4 purple ink from specimens from beach 2 (red seaweed rich).

To prove that *A. dactylomela*, like *A. californica* (Prince et al. 1998; Coelho et al. 1998), only produces ink when it ingests red seaweeds; laboratory experiments were conducted with sea hares receiving the green seaweed *U. fasciata*, or the red *Gracilaria* sp., as the sole source of food. Our light microscopy studies of the ink gland of sea hares consuming only *U. fasciata* showed that most of the vesicles were devoid of purple ink; whereas, individuals that consumed red seaweeds had full vesicles in the ink gland. These observations confirm the necessity of red-seaweed consumption for *A. dactylomela*, and probably other sea hares of the same genus, to be able to secrete the purple ink.

The chemical analysis of the purple ink showed that, regardless of the main seaweed species consumed by the sea hare, the content of protein in the ink was the same. This could indicate that either diet, rich in green or red seaweeds, must be able to supply the amino acids necessary for ink protein synthesis. Our analysis of amino acid composition showed that the purple ink is rich in acidic amino acids and has very low content of sulfur amino acids, reflecting the amino acid composition of both seaweeds (Ramos et al. 2000). Likewise, the electrophoretic profile of the ink proteins was always the same, regardless of the sample origin (beach 1 or 2), time of the year, or sea hare age. The finding that the anti-ink antiserum did not recognize any protein of the seaweed extracts suggests that the sea hare itself synthesizes the ink proteins and that they are not obtained directly from the diet, as is the case for the ink pigments (Troxler et al. 1981; MacColl et al. 1990). Coelho et al. (1998) previously suggested that the pigment of *A. californica* purple ink is of algal origin, but the proteins are not. Thus, there seems to be no doubt about the origin of the ink substances (pigments and proteins) in *A. dactylomela*. Nevertheless, the roles in the purple ink have not yet been completely elucidated. Several biologic activities have been described *in vitro* for isolated proteins (Kisugi et al. 1989; Yamazaki et al. 1989a; Yamazaki et al. 1989b; Yamazaki et al. 1990; Nistratova et al. 1992; Melo et al. 1998; Melo et al. 2000) and pigments (Wessels et al. 2000) from sea hare ink, and all give support to a defense role.

CONCLUSIONS

The sea hare *A. dactylomela*, and possibly other *Aplysia* species, needs to consume red seaweeds to be able to secrete the purple ink. The pigment is from algal origin; whereas, the proteins are not. The protein content and composition seem to be always the same, regardless of the seaweed species consumed.

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LITERATURE CITED

Baethgen, W. E. & M. M. Alley. 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldalh digest. *Commun. Soil Sci. Plant Anal.* 20:961–969.

Blum, H., H. Bier & H. J. Gross. 1987. Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8: 93–99.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Carefoot, T. H. 1987. *Aplysia:* It's biology and ecology. *Oceanogr. Mar. Biol. Ann. Rev.* 25:167–284.

Coelho, L., J. Prince & T. G. Nolen. 1998. Processing of defensive pigment in *Aplysia californica*: acquisition, modification and mobilization of the red algal pigment r-phycoerythrin by the digestive gland. *J. Exp. Biol.* 201:425–438.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers & F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350–356.

Hudson, L. & F. C. Hay. 1989. Practical Immunology. 3rd. ed. Oxford: Blackwell Scientific Publications. pp

Junqueira, L. & L. M. M. S. Junqueira. 1983. Técnica Básicas de Citologia e Histologia. Livraria e Editora Santos, São Paulo.

Kisugi, J., H. Ohye, H. Kamiya & M. Yamazaki. 1989. Biopolymers from marine invertebrates. X. Mode of action of an antibacterial glycoprotein, aplysianin E, from eggs of a sea hare, *Aplysia kurodai. Chem. Pharm. Bull.* 37:3050–3053.

Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T_4. *Nature* 227:680–685.

MacColl, R., J. Galivan, D. S. Berns, Z. Nimec, D. Guard-Friar & D. Wagoner. 1990. The chromophore and polypeptide composition of *Aplysia* ink. *Biol. Bull. Mar. biol. Lab. Woods Hole* 179:326–331.

Melo, V. M. M., A. M. Fonseca, I. M. Vasconcelos & A. F. F. U. Carvalho. 1998. Toxic, antimicrobial and hemagglutinating activities of the purple ink of the sea hare *Aplysia dactylomela* Rang, 1828. *Braz. J. Med. Biol. Res.* 31:785–791.

Melo, V. M. M., A. B. G. Duarte, A. F. F. U. Carvalho, E. A. Siebra & I. M. Vasconcelos. 2000. Purification of a novel antibacterial and haemagglutinating protein from the purple gland of the sea hare *Aplysia dactylomela* Rang, 1828. *Toxicon.* 38:1415–1427.

Nistratova, S. N., I. N. Mitskevich, M. E. Mirabetregalado & N. Josef. 1992. Inhibitory effect of purple ink produced by a mollusk *Aplysia punctata* on growth of Gram-negative marine bacteria. *Microbiol.* 61: 630–632.

Nolen, T. G., P. M. Johnson & C. E. Kicklighter. 1995. Ink secretion by the marine snail *Aplysia californica*. Enhances it's ability to escape from a natural predator. *J. Comp. Phisiol. A* 176:239–254.

Pearse, A. G. E. 1960. Histochemistry Theoretical and Applied, v. 2.: Jet. London: Churchill Ltda. pp

Prince, J., T. G. Nolen & L. Coelho. 1998. Defensive ink pigment processing and secretion in *Aplysia californica*: Concentration and storage of phycoerythrobilin in the ink gland. *J. Exp. Biol.* 201:1595–1613.

Ramos, M. V., C. O. Monteiro, R. A. Moreira & A. F. F. U. Carvalho. 2000. Amino acid composition of some Brazilian seaweed species. *J. Food Biochem.* 24:33–39.

Troxler, R. R., G. D. Offner & T. R. Capo. 1981. Structural studies on Aplysioviolin. *Biol. Bull. Mar. biol. Lab. Woods Hole* 161:339.

Wessel, M., G. M. Konig & A. D. Wright. 2000. New natural product isolation and comparison of the secondary metabolite content of three distinct samples of the sea hare Aplysia dactylomela from Tenerife. *J. Nat. Prod.* 63:920–928.

Yamazaki, M., K. Kimura, J. Kisugi, K. Muramoto & H. Kamiya. 1989a. Purification and characterization of a cytolytic protein from purple ink of the sea hare, *Dolabella auriculata. Chem. Pharm. Bull.* 37:2179–2182.

Yamazaki, M., S. Tansho, J. Kisugi, K. Muramoto & H. Kamiya. 1989b. Isolation and characterization of a novel cytolytic factor in purple ink of the sea hare *Aplysia kurodai. Cancer Res.* 49:3834–3838.

Yamazaki, M., H. Ohye, J. Kisugi & H. Kamiya. 1990. Bacteriostatic and cytolytic activities of purple ink from the sea hare. *Dev. Comp. Immunol.* 14:379–383.