The Effect of Sampling Methods on the Apparent Constituents of Ink from the Squid *Sepioteuthis australis*

F. Madaras · J. P. Gerber · F. Peddie · M. J. Kokkinn

Abstract Results of experiments conducted on ink recovered from the squid *Sepioteuthis australis* indicate that there is no epinephrine or protein naturally present in the ink as it would be ejected *in vivo*. Protein content was effectively zero when ink was syringed from the duct end of the ink sac of freshly killed animals. By contrast, there were proteins in samples collected from dead specimens where ink was collected by a stripping method. From these samples, a single large molecular weight protein was identified as having tyrosinase activity. Digestion of syringed ink did not yield signs of melanin-bound proteins. Analysis of supernatants after centrifugation of squid ink consistently revealed the presence of DOPA, dopamine, and taurine, whereas epinephrine and nor-epinephrine were recorded from what was believed to be contaminated ink. Histological investigations of the ink sac revealed a compartmentalised glandular structure distal to the duct end. Closer observation of the glandular tissue showed that compartments increased in size as they matured and moved further into the lumen. It was concluded that the presence of epinephrine and tyrosinase (or a related protein) in the ink of *S. australis* could be attributed to rupturing of basal glandular compartments or contamination from other sources during the extraction process.

Key Words *Sepioteuthis australis* · Ink sac · Ink · Protein · Tyrosinase · DOPA · Dopamine · Taurine

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Introduction

Cephalopod ink has been a subject of study for a variety of reasons. Since it contains eumelanin, it has provided a system for studying melanogenesis (e.g., Ortonne et al., 1981; Schraermeyer, 1994; Palumbo et al., 1997 and Palumbo, 2003). Early propositions for the bioactivity of the non-melanin fraction of cephalopod ink (Macginitie and Macginitie, 1968; Fox, 1976) led to analyses that documented the presence of tyrosinase (Prota et al., 1981), peptidoglycans (illexins) (Takaya et al*.*, 1994), L-DOPA, dopamine (Lucerno et al., 1994), and taurine (Derby et al., 2007).

Perhaps the most intensively studied cephalopod ink comes from the common cuttlefish, *Sepia officinalis* (Linnaeus 1758), which is distributed from the North Sea southwards towards the west coast of South Africa, including the Mediterranean Sea (Bakhayokho, 1983; Roper et al., 1984). Much of this work originated from the inspiration of R.A. Nicolaus in Naples (Prota, 2000). However, little is known about whether or not the ink is representative of other species.

Sepioteuthis australis (Quoy and Gaimard 1832)*,* the southern calamari, is a common in-shore squid widely distributed along coastal areas of southern Australia and northern New Zealand (Winstanley et al., 1983). The species is the subject of both recreational as well as commercial fishing, and thus was freely available for study. In common with most other cephalopods, *S. australis* uses inking as a defensive mechanism, which may also serve as an alarm signal between conspecifics (Gilly and Lucero, 1992; Wood et al., 2008).

A pilot project in our laboratory was designed to compare the non-melanin components of *S. australis* ink with published information from other cephalopods. Initial

mass spectrometric analysis of ink supernatant indicated high levels of epinephrine in ink stripped from the intact ink sacs of dead specimens (Fig. 1). Since there were no previous reports of significant levels of epinephrine in cephalopod ink, we concluded that the finding was either a significant novel discovery or an artifact resulting from contamination by autolysis or the sampling method.

Catecholamines, such as dopamine, nor-epinephrine, and 5-hydroxytryptamine were documented from the nervous tissue of cephalopods as far back as 1971 (Juorio, 1971). Subsequently, epinephrine has been found in the haemolymph of the curled octopus *Eledone cirrhosa* (Lacoste et al., 2001) and also reported from other tissues such as nerves in trace amounts (Springer et al., 2005).

Consequently, the observation of an epinephrine spike in the ink of *S. australis* was of interest. The possibility was raised that epinephrine in its ink may have evolved as an allelochemical against vertebrate predators with respiratory surfaces exposed to sea water.

Of the other non-melanin components of cephalopod ink, the presence of the enzyme tyrosinase commonly is reported (e.g., Prota et al., 1981; Derby, 2007) and was suggested as the component responsible for anti-tumour activity in mammalian cells (Takaya et al., 1994; Naraoka et al., 2000). However, the physiological or behavioral significance of ejecting tyrosinase into sea water in response to a predator or as a signal of alarm to conspecifics is difficult to explain.

Along with DOPA, dopamine, and taurine, the presence of proteins in cephalopod ink is widely accepted. For example, Linh Tran et al. (2006) emphasised the claim that melanins *in vivo* are strongly bound to a protein host and that sample preparation frequently removes the protein, leaving subsequent studies to focus on the chromophore alone. Similarly, Meredith et al. (2006) suggested that the term melanin should 'encompass both the chromophore and the associated protein'. The presence of proteins within the eumelanin structure also has been suggested by Meng and Kaxiras (2008).

Two major obstacles have stood in the way of describing fully the chemical structure of the melanin complex. It has a monotonous absorbance pattern in the visible and near UV part of the spectrum. It is insoluble in all useful solvents,
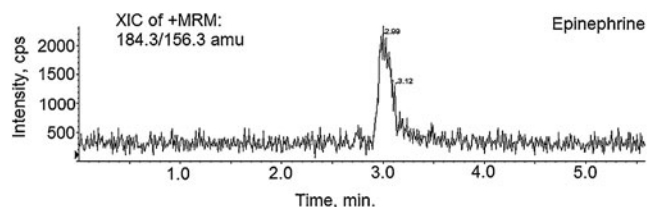
and thus, it defies structural analysis (Linh Tran et al., 2006).

Our initial discovery of a spike of epinephrine, along with a question about the biological justification for ejecting tyrosinase with ink led to this investigation. The aim was, therefore, to measure the levels of epinephrine and protein in the ink of *S. australis* specimens. Because of a suspicion that the epinephrine in our initial sample may have been an artifact, we undertook to compare the ink recovered by methods that minimized the chance of contamination from ink sac tissues (Syringed ink from freshly caught specimens), with that recovered by more traditional procedures (Stripping Method).

Histological studies of the ink sac were performed, along with comparative analyses of ink sampled from *S. australis* by each procedure.

Methods and Materials

Histological Structure of the Ink Sac The ink sacs of five *S. australis* were dissected from freshly-caught specimens and fixed in buffered formalin. They were infiltrated subsequently with paraffin wax, and a series of transverse and longitudinal histological sections was cut and stained with haematoxylin and eosin. Sections were viewed under the microscope, and representative views were photographed for analysis.

Ink Sampling Ink samples from specimens of *S. australis* were collected for comparison by two different methods:

Syringed Ink Method. Squid were caught along the South Australian coast, placed 'live' on a bed of ice, and anaesthetized with a $MgCl_2$ spray (Messenger et al., 1985) before being killed by decapitation across the edge of the mantle (i.e., posterior to the pleuro-visceral ganglion) to avoid stimulation of ink release. The mantle cavity was opened immediately along its ventral surface, and the proximal end of the ink sac was freed from the surrounding tissue. The duct end of the ink sac was cut off with sharp scissors and held open with forceps before the ink was carefully drawn from the distal lumen with a 1 ml syringe fitted with a yellow (200 µl) automatic pipette tip. The sample then was transferred to an Eppendorf tube, taking care not to touch the outside surface of the syringe onto the lip of the tube. Samples were held on ice until they were stored at −80°C.

Attempts to run this difficult collection protocol rendered only 6 ink samples with a sufficient volume of uncontaminated ink from approximately 30 squid caught. Many squid had emptied their ink sacs prior to

Fig. 1 Chromatogram of epinephrine in diluted ink from the ink sac of *Sepioteuthis australis*

sampling attempts, or it proved impossible to introduce the tip of the syringe into the ink sac without contamination from external fluids. Problems also arose from the tendency of the pipette tip to adhere to the inside of the sac unless the fluid was drawn up very slowly. However, sufficient material was removed from stored material near the duct of the ink sac for analysis, representing uncontaminated ink. Of course, the nature of the ink could change once it was ejected into sea water, but that was not the focus of this study.

Milked Ink Method. Fresh squid were purchased within a day of being caught and held on ice (not frozen) by arrangement with Valente Seafoods, Adelaide, Australia. Dead animals were taken to the laboratory on ice, and the ink was extracted as soon as possible by the following method. The mantle cavity was opened along its ventral surface and the entire ink sac dissected out. The duct end was cut off and held over an Eppendorf collection tube while the ink was 'milked' by running forceps down its length, taking care not to introduce external fluids. Samples then were held on ice until stored at −80°C.

These samples represent ink contaminated with exudates from cells after autolysis, contents of the ink gland chambers, and possibly materials from the external surface of the ink sac.

Determination of Epinephrine, DOPA, Dopamine, and Taurine HPLC grade acetonitrile was obtained from Biolab (Australia). DL-DOPA, DL-dopamine, taurine, epinephrine/adrenaline, and ammonium acetate were purchased from Sigma-Aldrich (Australia). Water was purified for HPLC analysis by using a Milli-RQ ultrapure Water System (Millipore, USA).

Ink sampled by either method (about 50 mg) was vortex mixed with water, centrifuged, and the supernatants diluted 10 times with water. These unwashed samples were analyzed directly. Additional samples were washed repeatedly with water to remove soluble material, and the recovered melanin was suspended in 10 volumes of water and stirred for 2 hr at ambient temperature. The extract was centrifuged, and aliquots of the supernatants were transferred to autosampler vials for analysis.

The analytical equipment consisted of a solvent delivery system comprising two Shimadzu LC-10ADVP pumps used isocratically, a DGU-14A degasser, and SIL-HTC auto sampler (Shimadzu, Japan) held at 10°C. The HPLC system interfaced with a triple stage quadruple mass spectrometer (API 3000, Applied Biosystems, Canada) via an electrospray source. Instrument control, data acquisition, and data analysis were performed with Analyst software V1.4.1 (Applied Biosystems). For each analyte, MRM

transitions were optimized in the positive mode by infusing the relevant reference compound; DOPA $298.3 \rightarrow 152.3$, dopamine $154.3 \rightarrow 137.3$, epinephrine $184.3 \rightarrow 166.3$, norepinephrine $170.3 \rightarrow 152.3$, and taurine $126.0 \rightarrow 108.0$. A Hilic C18 column (3×50 mm, Phenomenex) equipped with a matched guard was used with a mobile phase consisting of 30% acetonitrile and 10 mM aqueous ammonium acetate, delivered at a flow rate of 0.2 ml/min. The injection volume was 10 µl. Reference solutions were prepared fresh on the day.

Determining Protein in Ink Supernatants Mushroom tyrosinase, L-3, 4-Dihydroxyphenylalanine (L-DOPA), bovine pancreatic trypsin, subtilisin A, and pepsin were obtained from Sigma (St. Louis, MO, USA). SDS-PAGE was performed by using ready-made (NuPAGE) 4–12% (1.5 mm thick) SDS-acrylamide gels, MOPS-SDS running buffer, and SDS sample buffer. These were all obtained from Invitrogen Pty Ltd. Agarose was obtained from Amersham Biosciences Pty Ltd. All other chemicals used were of analytical grade.

To 300 µl of the ink sample, 100 µl of 50 mM Tris–HCl pH 8.0 was added. The mixture was vortexed at maximum speed for 30 sec and allowed to stand overnight at 4° C. The mixture was vortexed again for 30 sec and centrifuged at 13,000 rcf for 10 min. Fifty µl of the ink supernatant was mixed with SDS-PAGE sample buffer and incubated at 70° C for 10 min (all samples were non-reduced so that the tertiary structure of the proteins was preserved). Ten µl of the ink-SDS buffer incubate was loaded onto an SDS-acrylamide gel plate. The gel was run in a 1:20 dilution of MOPS running buffer made from a concentrated stock buffer solution. The gel was stained with a 0.1% (w/v) coomassie brilliant blue R-250 solution and de-stained in an acetic acid-water–methanol mixture (1:5:5).

Determining Electrophoretic Mobility of Proteins Present The method of Madaras et al. (2005) was used. Proteins in this method separate out on the basis of net protein electric charge, and the separation is not dependent on protein size. Electrophoresis was done by using a sodium barbitone buffer pH 8.6 where all the proteins that migrate towards the positive electrode possess a net negative charge. One µl of the Tris–HCl ink extract was loaded into an agarose gel plate sample well. Electrophoresis was performed by using 50 mM sodium barbitone buffer (pH 8.6, at ~2 Vcm^2). The gel was stained as described for SDS acrylamide gels. The stained protein bands were evaluated by the Gel-Pro Analyzer (Media Cybernetics USA) computer imaging software.

Enzyme Digestion of the Melanin Fraction Although no proteins could be detected in the Syringed Ink supernatant,

the possibility remained that some proteins were covalently linked to the melanin surface and were not detected in the supernatant or a melanin emulsion. To investigate this possibility, a series of experiments was performed using proteolytic enzymes to see whether proteins could be digested from the melanin. For this experiment, six ink samples from each group (Syringed or Milked Ink) were pooled (~600 µl) and washed repeatedly using un-buffered 0.15 M NaCl. The ink was suspended in the solution, vortexed at maximum speed for 30 sec, centrifuged at 13,000 rcf for 5 min, and the supernatant discarded. This process was repeated x 5. The washing process was designed to remove any freely soluble proteins present in the melanin ink leaving only the melanin particles.

The washed ink pellet was divided into two aliquots, and two different enzyme digests were prepared: one using 200 µl pepsin (0.05 mg/ml final concentration) at pH 1.0 in 0.1 M HCl, and the other using 200 µl trypsin (0.03 mg/ml) and subtilisin A (0.02 mg/ml final concentration) at pH 7.5 in tris-buffered saline pH 8.0. The washed melanin precipitate and enzyme mixtures were incubated at 37ºC for 4 h with occasional mixing. The digests then were centrifuged at 13,000 rcf for 10 min and examined for the presence of proteins by SDS-PAGE as described above.

As a positive control to demonstrate enzyme digestion, human albumin was used at 0.5 mg/ml concentration. The proteolytic enzyme concentrations and the conditions for the digestion of albumin were the same as those used for the melanin.

*Tyrosinase Assay T*yrosinase activity was assayed by the dopachrome method (Fling et al., 1963) as follows. The standard reaction mixture contained the substrate (5 mML-DOPA) in 0.1 M sodium phosphate buffer (pH 6.8), and 10 µl of the ink supernatant (as described above) were added to 990 µl of the L-DOPA substrate. The reaction took place in a micro cuvette with a path length of 1 cm, and the absorbance at 475 nm was monitored continuously for 10 min with a spectrophotometer (Shimadzu UV-1601) at 25º C. One unit of tyrosinase was defined as the amount of enzyme required to oxidize 1 µmol of L-DOPA per min under the above conditions, and calculated by using the molar extinction coefficient of dopachrome (3600 M−1 cm−1). For a positive control tyrosinase enzyme (mushroom> 1000 unit/mg) was used.

Results

Investigating the Histological Structure of the Ink Sac Histological sections revealed a mass of glandular tissue at the base of the ink sac, which sometimes extended over half way along the length of the lumen (Fig. 2). This mass
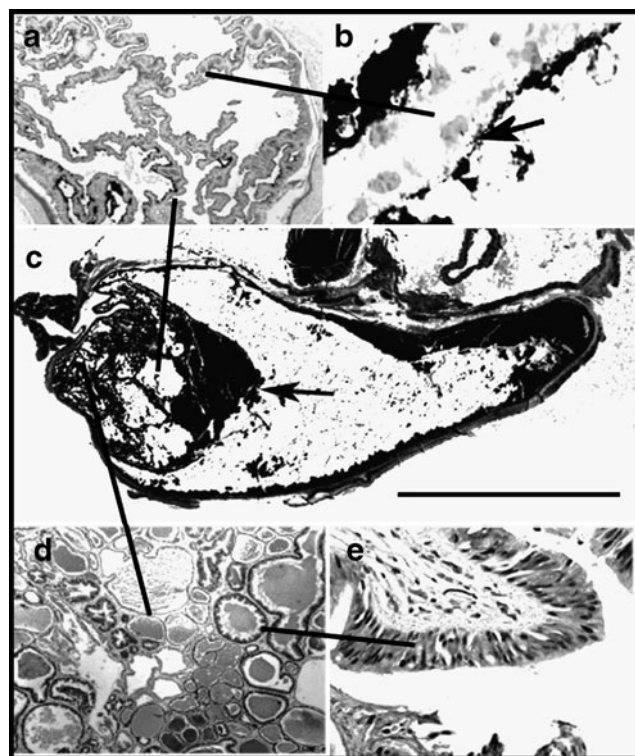

Fig. 2 Histological sections of the ink sac of *Sepioteuthis australis*. Legend: **c** Longitudinal section of whole ink sac (*arrow* indicates basal glandular area); Scale bar 10mm; **a** Melanin-producing reticulated tissue; **b** Melanin Granules in cells; **d** Mixed tissue (follicles and mucous ducts?) at the base of the gland; **e** Mucus-producing epithelium

seemed to comprise a series of compartments and ducts that increased in size as they matured and moved further into the lumen of the sac. The large spaces further away from the basal layer were filled with ink.

Three distinct histological elements were found within the glandular tissue: apparently enclosed follicles that contained light-staining material (Fig. 2d); ducts lined with mucus-secreting cells (Fig. 2e); and reticulated epithelial layers with melanin granules visible in the cytoplasm of cells (Figs. 2a and b). It was not possible, without detailed serial-sectioning, to positively determine whether the glandular tissue was either comprised of closed follicles or tubular glandular epithelium, or a combination of both (Fig. 2).

Determination of the Presence of Epinephrine, DOPA, Dopamine, and Taurine After centrifugation of the diluted Milked Ink, substantial amounts of DOPA and taurine were recorded consistently (mid to high ppb range). Since the initial viscosity of the collected ink samples ranged from liquid to paste for different specimens, and analysis was performed directly after dilution, no attempts were made to quantitate the catecholamines. Dopamine was present to a lesser extent, but varied depending on the individual

samples. In some ink isolates, significant amounts (low ppb range) of epinephrine and nor-epinephrine were found (Fig. 1). These levels fluctuated among ink samples, and often no trace of either analyte was found in ink samples from different animals.

The same observations were made when melanin was separated from the supernatant. Suspensions of melanin, purified to different extents, were allowed to stir for 2 hr. After centrifugation and analysis of the supernatants, the presence of all the previously mentioned compounds was evident. Interestingly, dopamine was released into solution at a much slower rate than DOPA.

By contrast, supernatant from Syringed Ink showed no measurable traces of epinephrine or nor-epinephrine. This result matched that found for the protein content of Syringed Ink. However, DOPA, dopamine, and taurine were detected consistently in all ink supernatants, or were found to desorb from separated melanin, regardless of the method used to collect the original ink (Fig. 3).

Determining the Presence of Protein in Ink Supernatants Figure 4 shows the non-reduced SDS-PAGE of Milked and Syringed ink samples. No proteins were found in the Syringed Ink extracted from 'live' animals, while the Milked Ink samples yielded strong indications of protein. The purpose of the SDS surfactant was to mobilize all proteins according to size by negating charge. From the

Fig. 3 Chromatograms of taurine, dopa and dopamine in ink collected by the Syringed method. The individual molecular ion→fragment mass transitions are shown separately and the scale varied according to each specific sample

result, it was concluded that the protein present fell into a narrow mass range, or was mainly a single component.

Determining the Electrophoretic Mobility of the Proteins Present in Ink Sample Supernatants Figure 5 shows the agarose gel electrophoresis and protein scan on the pooled ink supernatants for which protein bands previously had been detected by SDS-PAGE (Fig. 4). Proteins in this method separated out on the basis of the net protein electric charge, and the separation was not dependent on the protein size. There was only one major protein band visible. This result, in conjunction with the SDS-PAGE result, once again indicated that the major protein in the ink found in these samples was composed largely of one high molecular weight protein that carried a net negative charge at pH 8.6.

Enzyme Digestion of the Melanin Fraction of Ink to Release Bound Proteins Figure 6 shows the results of the protein digestion experiments on the washed ink melanin. No protein could be detected when the melanin was obtained from Syringed Ink. However, protein was detected in both the pepsin and trypsin-subtilisin digested melanin collected by the Milked Ink Method. This most likely is due to precipitated and denatured protein that could not be removed by the washing process. Once exposed to the enzymes, soluble protein-peptides were released. The positive control experiment showed that both pepsin and trypsin-subtilisin completely digested albumin during the 4 hr incubation, and that the enzymes themselves are not detectable on the SDS-PAGE gel at the concentration used in the digestion experiment (Fig. 7).

Tyrosinase Assay on the Ink Samples Table 1 shows the residual tyrosinase activity in the Syringed Ink samples compared with the Milked Ink samples. While none of the Syringed Ink samples showed tyrosinase activity, the Milked Ink showed values comparable to the positive control that used commercial tyrosinase.
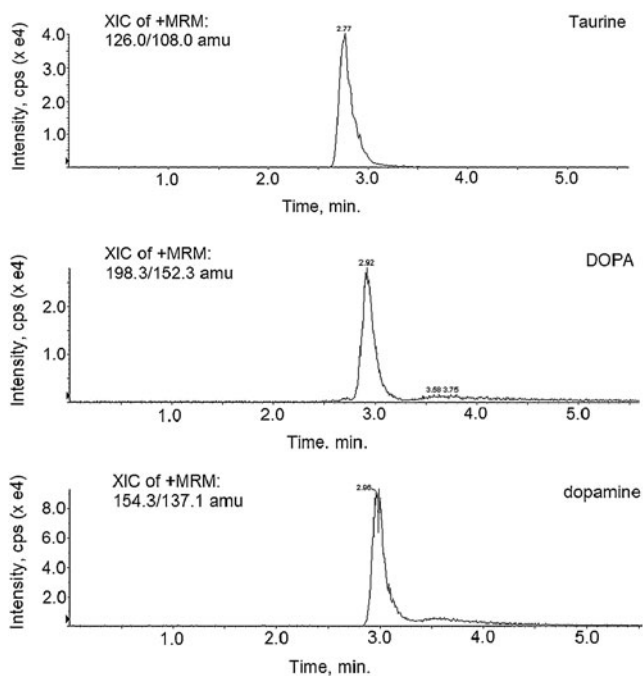
Discussion

The study of cephalopod ink involves several approaches. First, there is the biological approach, which seeks to understand the significance of inking behavior for the biology of a species. This may include an interest in the production of: 'smoke screens' and pseudomorphs (Bush and Robison, 2007), allelochemicals (Lucerno et al., 1994), or alarm signals to conspecifics (Wood et al., 2008). Recent elegant work has shown it to be a combination of these factors (Wood et al., 2010). The approach taken in the current study is, therefore, interested primarily in the nature of the ink as it would be ejected from the ink sac *in vivo*.

Fig. 4 Shows the non-reduced SDS-PAGE on ink samples; A1 to A6 were independent Syringed Ink samples from 6 different animals and B1 to B6 were independent Milked Ink samples from 6 different animals. MW is the molecular weight standards used

Syringed Ink   **Milked Ink**
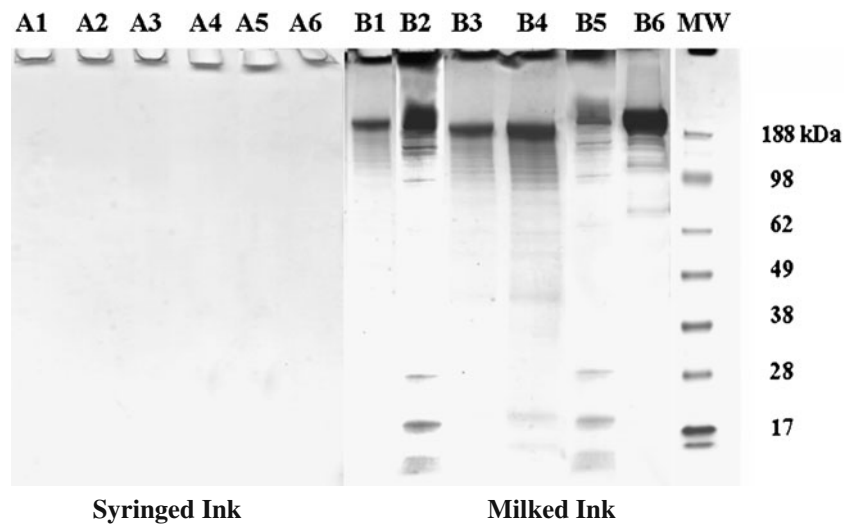
A second approach focuses on the cellular biochemical mechanisms of melanin synthesis where the intra- and extra-cellular location of enzymes and substrates is of interest (such as in melanosomes, etc.) (e.g., Palumbo et al., 1997; Palumbo, 2003). A third approach has no particular interest in cephalopod biology or melanin synthesis. Rather, its focus is on the utility of the ink for culinary or therapeutic purposes (Russo et al., 2003; Lei et al., 2007). Finally, the ink of cephalopods often is used in studies that attempt to determine the structure of melanin itself (Linh Tran et al., 2006; Meredith et al., 2006: Meng and Kaxiras, 2008).

However, despite the variety of perspectives taken in the study of cephalopod ink, no real attention has been given to the definition of its actual provenance. Because of the paucity of information about cephalopod ink sac structure

and histology, no distinction seems to have been made between material located within the glandular chambers of the ink sac and that stored in the ink sac lumen and ready for ejection.

The results of experiments conducted on the ink of *S. australis* indicated that there was no epinephrine or protein naturally present in the supernatant of ink carefully syringed from the duct end of the ink sac in freshly-killed animals (Syringed Ink). By contrast, there were proteins (and possibly epinephrine) in samples milked from the ink sacs of dead specimens.

The SDS-PAGE experiment showed that all the protein present fell within a narrow mass range, while agarose gel
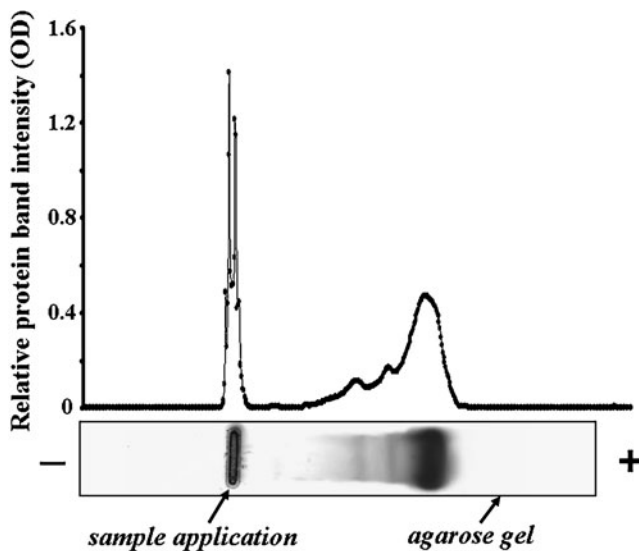

Fig. 5 The agarose gel electrophoresis and protein scan on the ink supernatant (samples B1 to B6 pooled) where protein bands were detected by the SDS-PAGE
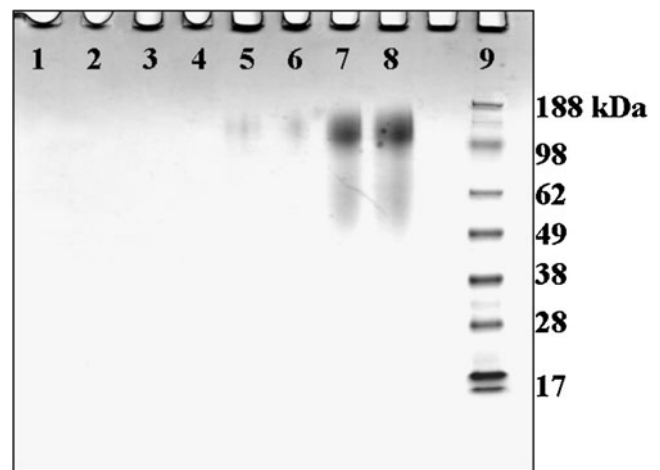

Fig. 6 Results of the protein digestion experiments on the washed ink melanin. Melanin samples from A1–A6 pooled and subsampled as 1–4 below. Melanin samples from B1–B6 pooled and subsampled as 5–8 below. Legend: Lanes *1* and *2*–Syringed Ink melanin after pepsin digest (pH 1.0); *3* and *4* Syringed Ink melanin after trypsin and substilisin digest (pH 7.5); *5* and *6* Milked Ink melanin after pepsin digest (pH 1.0); *7* and *8* Milked Ink melanin after trypsin and substilisin digest (pH 7.5); *9* Molecular Weight Standards
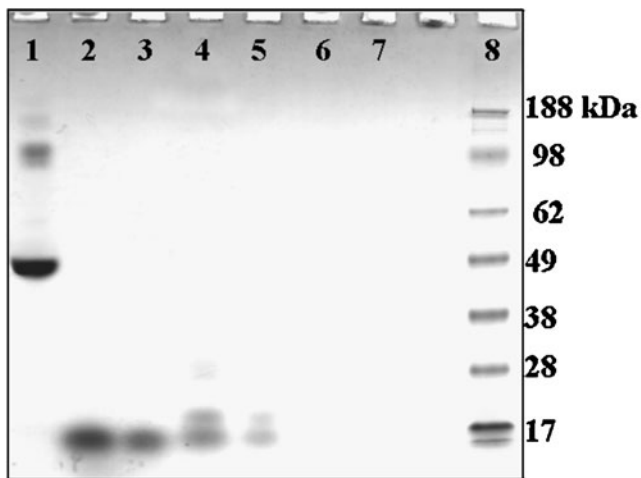
Fig. 7 Positive control for ink melanin protein digestion experiment. Legend: *1* - albumin (0.5 mg/ml); *2* and *3* albumin-pepsin digest (pH 1.0); *4* and *5* albumin-trypsin+substilisin digest (pH 7.5); *6* pepsin only (at the concentration used in the digestion experiment); *7* trypsin + substilisin only (at the concentration used in the digestion experiment). The enzymes alone are not detectable (at the concentration used in the digestion experiment), "8" molecular weight standards

electrophoresis, which separates proteins based on net electric charge, also indicated a single protein having an overall negative charge. Further analysis indicated that a single large molecular weight protein predominated in the non-melanin fraction of Milked Ink. Ink samples that contained this protein had strong tyrosinase enzyme activity.

In pursuit of proteins which may have been bound to the melanin fraction of the ink, and hence not evident in electrophoresis experiments, we performed digestion experiments to liberate such tightly bound species. No indication was found of the presence of bound proteins in the Syringed Ink samples. Hence, there was no detectable protein, either bound or free, in the ink carefully syringed from the duct area of the ink sac of freshly-killed *S. australis* specimens. In the case of Milked Ink, the presence of a protein that exhibited tyrosinase activity was most likely due to contamination from the rupture of glandular tissue at the base of the ink sac.

For comparison with other cephalopod studies, we analyzed ink supernatants for the presence of DOPA, dopamine, taurine, epinephrine, and nor-epinephrine. While traces of epinephrine and nor-epinephrine were recorded from Milked Ink samples, none was found in Syringed Ink collected from 'live' animals. On the other hand, in agreement with published reports (e.g., Lucerno et al., 1994), DOPA, dopamine, and taurine were consistently present in all ink samples analyzed, although in varying amounts. Exact quantification was not pursued, as the moisture content of the original ink samples varied from liquid to a paste consistency.

When seeking an explanation for our results, we searched for information on the gross histological structure of other cephalopod ink sacs and were surprised to find just a single published paper (Wang et al., 2008). This indicated the presence of a glandular mass at the base of the ink sac of the cuttlefish, *Sepiella maindroni*. Wang et al. (2008) described the basal secretory tissue as being highly branched, extending into the basal lumen of the ink sac and containing blood vessels, most of which were capillaries.

By comparing the histological structure of the ink sac of *S. australis* with that of *S. maindroni* (Wang et al., 2008), it was possible to note an important structural difference. The glandular tissue in *S. australis*, rather than being entirely dendritic and exposed to the ink sac lumen, as described for *S. maindroni,* seemed also to consist of a number of compartments, separate from the lumen (Fig. 2).

Using the structure of the *S. australis* ink sac as a guide, we propose that the ink along with its cargo of allelo-chemicals is synthesized in the basal mixed-glandular compartments and then released into the lumen of the sac for storage, perhaps by a break in the thin inactive compartmental walls. Indications are that the mucus component of ink may be synthesized in separate tubular structures.

We suggest it is this essentially protein-free material that is expelled by the animal for defensive and signalling purposes. Further, from this work, it is possible to suggest that tyrosinase activity in the ink of *S. australis* is present only when the basal glandular tissue is damaged, either during the sampling procedure or by autolysis after death.

A complicating factor may arise from the similarity between tyrosinase and haemocyanin, which is reputed to display tyrosinase-like activity (Schoot Uiterkamp and Mason, 1973; Jaenicke and Decker, 2004). Reported tyrosinase activity of cephalopod ink may be due to the

Table 1 Residual tyrosinase activity in the ink samples a1 to a6 (syringed) and b1 to b6 (milked), compared with mushroom tyrosinase (10 μg)

Samples	Tyrosinase activity (units)	Samples	Tyrosinase activity (units)
A1	0	B1	16
A2	0	B2	30
A3	0	B3	23
A4	0	B4	27
A5	0	B5	15
A6	0	B6	55
		tyrosinase enzyme	36

One unit of tyrosinase was defined as the amount of enzyme required to oxidize 1 μmol of L-DOPA per min under the assay conditions (see "Methods"), which was calculated using the molar extinction coefficient of dopachrome (3600 M^{-1} cm^{-1})

presence of hemocyanin, which has been included in samples by the rupturing of vessels in the ink gland.

While other studies have consistently documented the presence of proteins in the ink of cephalopod species (Prota et al., 1981; Takaya et al., 1994; Naraoka et al., 2000; Derby, 2007), we have been unable to detect free proteins or those associated with the melanin itself in Syringed Ink from *S.australis*.

There are at least three possible explanations. First, there may be significant differences among cephalopod taxa with regard to ink secretion and composition. Second, sampling procedures to date may have ruptured glandular cells, thus releasing proteins with tyrosinase activity. Third, there may be well-hidden (buried) proteins deep within the melanin superstructure that are not accessible to proteolysis. The latter alternative seems unlikely in view of recent findings by Meredith et al. (2006) showing that melanin consists of layers of pi-stacked 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole oligomer sheets.

Regarding the presence of DOPA, dopamine, and taurine in the ink supernatant, there is clear consistency with the findings of others who have studied different cephalopod species (Lucerno et al., 1994; Fiore et al., 2004). Our recordings of epinephrine and nor-epinephrine in some samples of ink preserved from dead animals can almost certainly be ascribed to contamination either from autolysis of cells after death or from damage while stripping of well-vascularised glandular tissue.

In order to understand the role of chemicals associated with the ink of *S. australis* there is a need for further work such as that of Gilly and colleagues (Gilly and Lucero, 1992; Lucerno et al., 1994) as well as that of Wood et al. (2010) on the effects of cephalopod ink allelochemicals on conspecifics and other marine organisms. A good example of such investigations on another mollusc taxon is that conducted by Derby and associates using the ink of *Aplysia* (e.g., Derby, 2007; Derby et al., 2007).

It is the various approaches and extraction methods for studying the ink of cephalopods that leads to confusion. The term 'ink' then is ambiguous. In some cases, it is regarded as the product of an ink sac being homogenized in a Waring blender (e.g., Naraoka et al., 2000). In others, it is the contents of an ink sac being removed after careful dissection (Liu and Simon, 2003; Fiore et al., 2004). Finally, it may be the dark material with its accompanying allelochemicals that may be ejected by many cephalopods when under threat or stress, as for *S. australis* in this study.

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