An examination of the role of the digestive gland of two loliginid squids, with respect to lipid: storage or excretion?

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The role of the digestive gland, with respect to non-structural lipid, was examined using proximal analysis, histochemistry and quantitative histological techniques in the tropical loliginid squids *Sepioteuthis lessoniana* (Lesson) and *Photololigo* sp. The digestive gland of both species was characterized by large and numerous lipid droplets in the apical portion of the digestive cells and very few in the basal portion. The apical lipid droplets were released into the lumen of the gland and subsequently rapidly removed. Despite the numerous large apical lipid droplets, the lipid concentration in the digestive glands of *S. lessoniana* and *Photololigo* sp. was lower than that reported for most squid species. There was no relationship between lipid concentration and stage of digestion, suggesting that lipid is not stored in the gland after a meal. There was also no relationship between lipid concentration and the sex of an individual or stage of reproductive maturity, suggesting that these squids are not storing lipid in the digestive gland for use in fuelling reproductive maturation or providing an energy source for oocytes. I believe this study is the first to combine proximal analysis and quantitative histological techniques to examine the role of the squid digestive gland with respect to non-structural lipids. The results indicate that the digestive gland of these tropical loliginid squids is excreting, not storing, excess dietary lipid.

Keywords: squid; digestive gland; lipid; storage; excretion

1. INTRODUCTION

Cephalopods have been described as resembling fish on a functional level (Packard 1972), differing from other molluscs in the relative complexity of their anatomy and physiology. This has allowed them to occupy, along with fish, many marine habitats (Packard 1972). Despite functional similarities to fish, cephalopods are limited by their molluscan heritage. This has resulted in a specialization in short life cycles and rapid continuous growth due to energetic constraints (O'Dor & Webber 1986). One of the constraints on cephalopods is the limited ability to digest, metabolize and store lipid (O'Dor & Webber 1986). Instead, protein and carbohydrate are the main metabolic substrates of cephalopods (Suryanarayanan & Alexander 1971; Hochachka *et al.* 1975; O'Dor *et al.* 1984). This inability to handle lipid efficiently is limiting because fats have a high ratio of energy content to weight and are important in the energy budget of many marine organisms, including fish (Weatherley *et al.* 1987). In teleosts, lipid concentrations greater than 5% of the total wet weight of the fish are common (Love 1980) and the liver is a major storage site (Lovern 1964; Love 1980; Weatherley *et al.* 1987). In contrast, cephalopods generally have less than 5% of their body weight as lipid (O'Dor & Webber 1986).

The only site of significant deposits of non-structural lipids in cephalopods is the voluminous digestive gland (Voogt 1983). This organ primarily synthesizes digestive enzymes and performs absorptive and excretory functions

(Boucher-Rodoni *et al.* 1987). The digestive gland of cephalopods is characterized by apparent functional stages of a single cell type, the digestive cell (Boucher-Rodoni *et al.* 1987). The histological appearance of the digestive gland of loliginid squids differs from that of other cephalopods, although the ultrastructure of the digestive cell is comparable to that of other species and undergoes the same stages of activity (Boucher-Rodoni & Boucaud-Camou 1987).

In many marine animals, lipid deposits are used during reproductive maturation by providing an energy source for the production of vitellogenic oocytes (Clarke *et al.* 1994), and as an energy source during periods of starvation (Lovern 1964). The exact nature of energy storage in cephalopods has long been referred to as a biochemical mystery (Hochachka *et al.* 1975). Despite considerable advances in this area, there is still a limited understanding of the energy reserves in these animals (O'Dor *et al.* 1984). Relatively large lipid deposits in the digestive gland have led to suggestions that it is involved in energy storage (Boucaud-Camou & Boucher-Rodoni 1983), comparable to the liver of teleosts (Castro *et al.* 1992).

Lipid levels in the digestive glands of the cuttlefish *Sepia officinalis* (Boucher-Rodoni & Mangold 1988; Castro *et al.* 1992) and the octopus *Octopus vulgaris* (O'Dor *et al.* 1984; Boucher-Rodoni & Mangold 1985, 1988) decrease in the very early stages of starvation. However, this is not conclusive evidence that the digestive gland is an energy store because lipid concentration would also decrease at the

Figure 1. Optical micrograph of a tubule in the digestive gland of *Sepioteuthis lessoniana*. Scale bar: 40 μ m. ad, apical lipid droplet; bd, basal lipid droplet; bm, basement membrane; dc, digestive cell; ld, lumen lipid droplet; L, lumen.

beginning of starvation if the majority of lipid in the digestive gland was excreted from the gland as waste. The use of energy reserves by squid during starvation, which have greater metabolic demands than cuttlefish and octopods (O'Dor & Wells 1987), has not been examined directly.

This study examined the role of the digestive gland, with respect to lipid, in the tropical loliginid squids *Sepioteuthis lessoniana* and *Photololigo* sp. (formerly called *Photololigo chinensis*, see Yeatman & Benzie (1994)). Using evidence based on lipid concentration and the position, density and size of lipid droplets, it was concluded that the digestive gland is involved in lipid excretion.

2. MATERIALS AND METHODS

(a) Specimen collection

Seventy-eight *Photololigo* sp. (50.9–161.0 mm dorsal mantle length, DML) were collected from waters around Townsville, Australia, between May 1994 and June 1996 using paired otter trawls. Ninety-five *Sepioteuthis lessoniana* were collected between January 1995 and August 1996, 82 from Townsville waters and 13 from waters around Brisbane, Australia. Juvenile *S. lessoniana* (19.9–37.5 mm DML) were collected by dip and purse seine nets. Adults (98.4–238.3 mm DML) were captured using 'squid jigs' in the Townsville region and tunnel-nets in the Brisbane region.

All individuals were sexed and assigned a reproductive maturity category: 0 (juvenile) to 5 (mature adult) using Lipinski's scale (Lipinski 1979). The digestive glands were weighed and retained for biochemical and histological analysis. The digestive state of each animal at capture was assessed using a stomach fullness index for *S. lessoniana* (0, empty to 5, completely full) and for *Photololigo* sp. using stomach weight. To remove the effect of animal size from stomach weight, the ratio of stomach weight to total body weight was calculated.

(b) Biochemical processing

A portion of the digestive gland was weighed before and after freeze-drying to obtain water content. Tissue was then homogenized in 5 ml of 100 mM NaCl solution. Lipid concentration was determined by a chloroform/methanol extraction (Mann &

Gallagher 1985) and quantified colorimetrically. Duplicates were run for each individual squid. Absorbance was converted to micrograms of lipid via a standard curve constructed using tripalmitin. Lipid content was expressed as a percentage of the total digestive gland wet weight (wwt).

(c) Histological processing

A portion of the digestive gland was removed from each individual (with the exception of the Townsville juveniles and Brisbane adults of *S. lessoniana*) and fixed in a formalin/acetic acid/calcium chloride solution (FAACC) for 48 h, then transferred to 70% ethanol for a further 24 h. Tissue was dehydrated in a graded ethanol series, cleared in toluene, then infiltrated and impregnated with paraffin wax. Sections were cut at 5 μ m, stained with Mallory–Heidenhain and mounted on slides with dibutylphthalate/polystyrene/xylene (DPX).

(d) Quantitative histological analysis

Standard histological processing removes lipids. However, using the oil red O technique on frozen sections (Lillie & Ashburn (1943), in Bayliss High (1984)), the clear vacuoles observed in the paraffin wax sections were identified as lipid droplets. The density and size of the lipid droplets in the digestive glands of both *S. lessoniana* and *Photololigo* sp. were measured using an image analysis system consisting of an Olympus BX50 microscope linked to a computer via a high-resolution Sony XTC camera. Images were captured using Imascan software, with SigmaScan software used to measure the size of the lipid droplets. Thirty individuals (juveniles and adults) of each species were examined for lipid droplets at two random sites within the digestive gland. When present, the number and position of the droplets, the basal portion of digestive cell, and the apical portion of digestive cell or lumen (figure 1), were noted and the diameter of each droplet measured. The density of lipid droplets for each position was calculated as the number of droplets per square millimetre of the histological section. The actual sizes of the lipid droplets were determined by analysis of the measured diameters using the Schwartz–Saltykov stereological method (Schwartz 1934, in Underwood 1970; Saltykov (1958), in Underwood (1970)).

Table 1. Results of the three-factor orthogonal ANOVA examining differences in lipid content of the digestive gland (%wwt) with species, sex and reproductive maturity category

(SS, sum of squares; d.f., degrees of freedom; MS, mean squares; *F*, *F*-value; *p*, *p*-value, significant if $p < 0.05$.)

source of variation	SS	d.f.	MS	<i>F</i>	<i>p</i>
residual	3.48	108	0.03		
species	0.42	1	0.42	12.98	0.000
sex	0.01	1	0.01	0.21	0.650
reproductive category	0.12	4	0.03	0.90	0.464
species by sex	0.01	1	0.01	0.33	0.569
species by reproductive category	0.14	4	0.04	1.12	0.352
sex by reproductive category	0.16	4	0.04	1.24	0.298
species by sex by reproductive category	0.17	4	0.04	1.35	0.257

(e) Statistical analysis

A three-factor orthogonal ANOVA was used to examine differences in the lipid content of the digestive gland (%wwt), the density and the size of lipid droplets. The lipid concentration of the gland was compared between species (*S. lessoniana* and *Photololigo* sp.), sex (juvenile, female and male) and sexual maturity category (0–5). The density and size of lipid droplets were compared between species, sex and position of the lipid droplet (basal portion of digestive cell, apical portion of digestive cell and lumen). The data were transformed ($\log_{10}$) to avoid violating the assumption of homogeneity of variances. A 'post hoc' Tukey's honestly significant difference (HSD) test was used to explore significant terms in the models. The correlation coefficients (*r*) indicated the nature of the association between percentage of lipid in the digestive gland (wwt) and the stomach weight-to-total body weight ratio for *Photololigo* sp. and the stomach fullness index for *S. lessoniana*.

3. RESULTS

The amount of lipid present in the digestive glands of *Sepioteuthis lessoniana* and *Photololigo* sp. was not a function of either the sex of the individual or the stage of reproductive maturity (table 1). There was also a poor correlation between the lipid content of the digestive gland and the stomach fullness index for *S. lessoniana* ($r=0.075$, $n=78$, $p=0.508$) and the stomach weight-to-total body weight ratio for *Photololigo* sp. ($r=-0.027$, $n=57$, $p=0.841$). There was a significant difference between the lipid content of the digestive glands of the two species (table 1), with *S. lessoniana* containing almost twice as much lipid (9.8 ± 0.5 s.e.% wwt, $n=80$) as that of *Photololigo* sp. (5.7 ± 0.3 s.e.% wwt, $n=69$).

The density of lipid droplets in the digestive gland differed as a function of an interaction between species, sex and position in the digestive gland (table 2). In both species the apical region of the digestive gland cells contained the majority of the lipid droplets, ca. 80%, with the density comparable between the sexes in this region

Table 2. Results of the three-factor orthogonal ANOVA examining differences in the density (number mm^{-2}) of lipid droplets in the digestive gland epithelium with species, sex and position of the droplet

(SS, sum of squares; d.f., degrees of freedom; MS, mean squares; *F*, *F*-value; *p*, *p*-value, significant if $p < 0.05$.)

source of variation	SS	d.f.	MS	<i>F</i>	<i>p</i>
residual	26.24	348	0.08		
species	3.06	1	3.06	40.56	0.000
sex	0.09	2	0.05	0.62	0.536
position	37.79	2	18.89	250.54	0.000
species by sex	1.41	2	0.70	9.34	0.000
species by position	1.79	2	0.90	11.89	0.000
sex by position	0.28	4	0.07	0.93	0.447
species by sex by position	1.43	4	0.36	4.75	0.001

(figure 2a,b). There was a twofold greater density of apical droplets in the gland of *S. lessoniana* compared with that of *Photololigo* sp. (figure 2a,b). *Sepioteuthis lessoniana* juveniles had densities of lipid droplets in the basal portion of digestive cells and the lumen ca. 50% lower than those of the adults (figure 2a). Conversely, *Photololigo* sp. juveniles on average had densities of lipid droplets in the basal portion of digestive cells and the lumen approximately twice those of the adults (figure 2b).

There was a significant difference in the average lipid droplet size for the three positions in the digestive gland for both species (table 3). Droplets in the basal region of digestive cells were ca. 50% smaller than those of the apical and lumen regions (figure 3). Lumen droplets were on average the largest of the three positions, being ca. 10% larger than those of the apical region of the digestive cells (figure 3). The variation in the size of the droplets was also a function of the interaction between the species and sex of the individual (table 3). *Sepioteuthis lessoniana* males and females had larger lipid droplets than those of *Photololigo* sp., with juveniles of both species having similar sized droplets and displaying the greatest variability in lipid droplet size (figure 4). Females of both species had droplets ca. 20% larger than those of males (figure 4).

4. DISCUSSION

Given low concentrations of lipid and no correlation between the amount of lipid and the sex, reproductive maturity or digestive state, it appears that most of the lipid in the digestive gland of *S. lessoniana* and *Photololigo* sp. is not stored. The low density of basal lipid droplets in the digestive cells further supports this. The digestive epithelium rests on a highly vascularized basement membrane, providing the potential for absorption of storage compounds from the basal region of the cells into the blood. Had lipid droplets occurred predominantly in the basal portion of the digestive cells, this would have suggested storage of lipid in these cells. However, the high density of large apical lipid droplets and their release from the digestive gland cells suggests that instead of being stored, the bulk of the lipid is eliminated from the gland and excreted as waste.

Figure 2. (a) *Sepioteuthis lessoniana*; (b) *Photololigo* sp. Density (number $\text{mm}^{-2} \pm \text{s.e.}$) of lipid droplets in the digestive gland for each position (basal portion of the digestive cells, apical portion of the digestive cells and the lumen) and sex (juvenile, female and male).

Despite the presence of large apical lipid droplets, the lipid concentration in the digestive glands of *S. lessoniana* (9.8% wwt) and *Photololigo* sp. (5.7% wwt) is lower than that of most squid species e.g. up to 40% wwt in *Todarodes pacificus* (Takahashi 1960, in O'Dor & Webber 1986), 28–31% wwt in *Illex argentinus* (Clarke *et al.* 1994) and 20.5–60.5% wwt in *Beryteuthis magister* (Hayashi & Yamamoto (1987), in Piatkowski & Hagen (1994)). The amount of lipid in the digestive glands of *S. lessoniana* and *Photololigo* sp. is more similar to that of *Sepia officinalis* (8–13% wwt) (Blanchier & Boucaud-Camou 1982, 1984).

There was no evidence that the variation in digestive gland lipid levels was a function of sex or the stage of reproductive maturation in *S. lessoniana* or *Photololigo* sp. The lipid content of the digestive gland of the ommastrephid squid *Todarodes pacificus* decreases with sexual maturity (Takahashi 1960, in O'Dor & Webber 1986), suggesting that lipid stores are mobilized to produce gonadic material. In general, however, there is little evidence supporting the role of digestive gland lipids in fuelling reproductive maturation and providing for storage materials in mature

Table 3. Results of the three-factor orthogonal ANOVA examining differences in the average size (μm) of lipid droplets in the digestive gland epithelium with species, sex and position of the droplet

(SS, sum of squares; d.f., degrees of freedom; MS, mean squares; *F*, *F*-value; *p*, *p*-value, significant if $p < 0.05$.)

source of variation	SS	d.f.	MS	<i>F</i>	<i>p</i>
residual	458.75	152	3.02		
species	78.99	1	78.99	26.17	0.000
sex	27.44	2	13.72	4.55	0.012
position	127.56	2	63.78	21.13	0.000
species by sex	38.20	2	19.10	6.33	0.002
species by position	7.83	2	3.92	1.30	0.276
sex by position	17.83	4	4.46	1.48	0.212
species by sex by position	8.69	4	2.17	0.72	0.580

oocytes (e.g. *Sepia officinalis*, Blanchier & Boucaud-Camou 1982, 1984; Castro *et al.* 1991; *Octopus tuelchis*, Pollero & Iribarne 1988; and *Illex argentinus*, Clarke *et al.* 1994). Instead, muscle proteins are used during sexual maturation in *Octopus vulgaris* (O'Dor & Wells 1978; O'Dor *et al.* 1984). Similarly, several squid species use mantle muscle as an energy source during reproduction (Jackson & Mladenov 1994; Moltschaniwskyj 1995).

Fluctuations of lipid levels in the digestive gland of *Sepia officinalis* are more closely related to diet than reproductive maturity (Blanchier & Boucaud-Camou 1984). Diet also appears to be the major factor in the lipid content of the digestive gland of the squid *Sthenoteuthis pteropus* in the eastern Atlantic Ocean (Abolmasova *et al.* 1990). Thus, the significant difference between the lipid concentrations in the digestive glands of *S. lessoniana* and *Photololigo* sp. may be a function of a difference between the diet of these two species. However, this cannot be qualified, as the dietary composition of neither of these two squids has been examined. Differences in nutrient use (O'Dor *et al.* 1984) and metabolism (Wells *et al.* 1983) between species can also result in differences in lipid concentration in the organs of cephalopods.

The presence of large and numerous apical lipid droplets in these two tropical loliginids is consistent with information available for other loliginid squids (Bidder 1950; Boucher-Rodoni & Boucaud-Camou 1987), with suggestions that the large size of lipid droplets in the digestive gland of loliginids is the result of oil-rich diets (Boucher-Rodoni & Boucaud-Camou 1987). As with *S. lessoniana* and *Photololigo* sp., lipid in the apical portion of the digestive cells is eliminated from the digestive glands of *Loligo vulgaris* and *L. forbesii* along with the vacuoles it is contained in (Bidder 1950). Furthermore, in *L. vulgaris* and *L. forbesii* the occurrence of lipid in the digestive gland is not related to the stage of digestion (Bidder 1950). This also appears to be the case for *S. lessoniana*, with no relationship between lipid concentration in the digestive gland and the stomach fullness index, and also *Photololigo* sp., which had no relationship between lipid concentration in the gland and the stomach weight-to-total body weight ratio.

Figure 3. Mean size ($\mu\text{m} \pm \text{s.e.}$) of lipid droplets in the digestive gland for both species combined (*Sepioteuthis lessoniana* and *Photololigo* sp.) and for each position (basal portion of the digestive cells, apical portion of the digestive cells and the lumen).

After a meal, lipids begin to accumulate in the digestive glands of *Sepia officinalis* (Boucaud-Camou 1977) and *Octopus vulgaris* (O'Dor *et al.* 1984). This suggests that lipids are taken up in the digestive gland and stored until mobilized as an energy source or used for reproductive investment (O'Dor & Webber 1986). However, there is no direct evidence for lipid absorption in this organ (Boucher-Rodoni *et al.* 1987). Instead, in *S. officinalis* and *O. vulgaris* the caecum is the site for the absorption of lipids (Boucaud-Camou 1977; O'Dor *et al.* 1984). The large caecal sac (in squid the caecum is divided into the ciliated organ and the caecal sac), and perhaps the intestine, are likely to be the main organs of fat absorption in squid (Boucher-Rodoni & Boucaud-Camou 1987). In the case of *S. lessoniana* and *Photololigo* sp., it is possible that lipids arrive in the digestive gland via the blood, with lipids entering the basal portion of digestive cells. Most of the lipids then collect in the apical droplets, which are eventually released into the lumen for excretion.

The digestive glands of *L. vulgaris* and *L. forbesii* (Boucher-Rodoni & Boucaud-Camou 1987) and those of other cephalopods (Boucaud-Camou & Boucher-Rodoni 1983) have been shown to be a site of excretion with cellular wastes being packaged in the brown body vacuoles (Bidder 1957) and excreted from the cells. These brown body vacuoles are released into the lumen of the gland, passing first to the caecum and then to the intestine, from which they are eliminated in mucus strings contained in the faeces (Boucaud-Camou & Yim 1980). As is the case for *Sepia officinalis* (Boucaud-Camou & Boucher-Rodoni 1983), the lipid droplets found in the digestive glands of *S. lessoniana* and *Photololigo* sp. are often contained in the brown body vacuoles, which are present

Figure 4. Mean size ($\mu\text{m} \pm \text{s.e.}$) of lipid droplets in the digestive gland for each species (*Sepioteuthis lessoniana* and *Photololigo* sp.) and sex (juvenile, female and male).

in both species (J. M. Semmens, unpublished data), suggesting they may facilitate lipid excretion from this gland. Anecdotal evidence indicates that squid fed diets high in lipids produce oily faeces; however, the lipid content of faeces and its relationship to diet needs further investigation. Furthermore, the ability of cephalopods to metabolize absorbed lipids is relatively limited (Mommensen & Hochachka 1981). This suggests that large portions of dietary lipid will remain unused and may be stored for later use and/or excreted, as appears to be the case for most non-structural lipids in the digestive glands of *S. lessoniana* and *Photololigo* sp.

As the digestive gland is the only organ with significant lipid deposits in squid, the results of this study have important lifestyle implications for these animals. It can be suggested that perhaps, unlike many other marine organisms, they do not store and subsequently mobilize significant amounts of lipid for energetic, reproductive or somatic processes. Coupled with very limited carbohydrate stores, compared with that of other molluscs and fish (Storey & Storey 1983), this reinforces the idea of a 'live for today' metabolic approach for squid. Squid, and cephalopods in general, have a metabolism geared towards growth rather than the accumulation of energy stores (O'Dor & Webber 1986). The muscle protein laid down during growth can then be used as an energy source during starvation (e.g. O'Dor *et al.* 1984) and reproduction (e.g. Moltschaniwskyj 1995).

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