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Seasonal and starvation-induced changes on gonads and lipid reserves of the digestive gland of *Nucella lapillus* (Caenogastropoda)

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This study was conceived to evaluate seasonal changes in the lipid reserves of the digestive gland of the carnivorous intertidal caenogastropod Nucella lapillus*, using a stereological approach. Volume density of lipid droplets in the digestive gland, digestive gland weight and volume were assessed in animals collected in March, June, September and December on the Portuguese coast. Gonad development was evaluated to detect any relationship between lipid content in the digestive gland and the reproductive cycle. The quantitative light microscopic analysis demonstrates that lipid droplets are a major component of the digestive gland. In males, the digestive gland and its lipid reserves were quite stable without significant variations throughout the year. In females, the percentage of digestive gland volume occupied by lipid droplets was higher in June and December, coinciding with the highest values of digestive gland volume. Due to the conjugation of these two factors, in June and December the total amount of lipids in the digestive gland was substantially increased in females. In both males and females a relationship between the development status of the gonad and the lipid reserves of the digestive gland was not evident. However, significant differences in the digestive gland lipid reserves were detected between males and females in June and December, pointing to a sex related effect on lipid reserves. To evaluate the use of lipid reserves as energy source in* N. lapillus*, the consumption of digestive gland lipids was followed during a starvation experiment.*

Keywords: Gastropoda, stereology, reproduction, lipids, digestive gland, microscopy

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INTRODUCTION

The dogwhelk *Nucella lapillus* (Linnaeus, 1758) is a common species of the intertidal zone on both sides of the North Atlantic, and the Portuguese coast is its southern distribution limit in Europe (Hughes, 1972; Crothers, 1985). This carnivorous gastropod lives on rocky shores alongside mussels and barnacles, which are its main preys (Crothers, 1985). Populations of *N. lapillus* suffered a great decline in some places due to imposex and female sterility caused by tributyltin (TBT) contamination, but populations are recovering after the ban of TBT-containing antifouling paints (Morton, 2009; Galante-Oliveira *et al.*, 2011). This species has been intensively investigated to evaluate the effects of TBT on the female reproductive system of caenogastropods (Følsvik *et al.*, 1999; Santos *et al.*, 2005; Plejdrup *et al.*, 2006), but can also be valuable as a model in other studies about marine gastropods.

Molluscs are influenced by numerous environmental factors such as temperature, food availability and pollutants, which can induce variations in metabolism and nutrient reserves (Da Silva & Zancan, 1994; Leung & Furness, 2001; Pazos *et al.*, 2003). Lipid and glycogen reserves can be accumulated in the digestive gland when food is abundant and consumed to sustain the animal during periods of food shortage or hibernation (Dimitriadis & Hondros, 1992) and to supply energy and nutrients required for reproduction and growth (Barber & Blake, 1991; Le Pennec *et al.*, 2001; Litaay & De Silva, 2003).

The digestive gland of gastropods is a major organ of the digestive system, formed by a vast number of blind ending digestive tubules. It is involved in extracellular and intracellular digestion of food material, absorption of nutrients, storage of lipids, glycogen and minerals, and also plays a major role in detoxification (Voltzow, 1994). The epithelium of digestive tubules is formed by digestive and basophilic cells, although additional minor cell types have been reported in *N. lapillus* (Dimitriatis & Andrews, 2000) and some other gastropod species (Franchini & Ottaviani, 1993; Kress *et al.*, 1994).

The basophilic cells are typical protein secreting cells. These pyramidal shaped cells contain large amounts of rough endoplasmic reticulum, a well-developed Golgi complex and accumulate secretory vesicles. They seem to be responsible for the secretion of digestive enzymes that undertake the extracellular digestion of food. The digestive cells have a columnar shape and are the most abundant. These cells carry out an intense endocytic activity and contain a large number of heterolysosomes, in which the digestion of food materials is completed. Lipid droplets and glycogen granules were found in the cytoplasm of digestive cells, but smaller amounts of these reserve substances can also be present in basophilic cells (Lobo-da-Cunha, 1999, 2000; Dimitriatis & Andrews, 2000).

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Seasonal variations of lipids were studied in a few species of gastropods (Da Silva & Zancan, 1994; Litaay & De Silva, 2003; Morais *et al.*, 2003), but quantitative seasonal studies of lipid reserves in the digestive gland of marine carnivorous gastropods are lacking. Thus, the present work was conceived to investigate changes in the lipid reserves of the digestive gland of *N. lapillus* throughout the year in males and females. Gonad development was also assessed to detect any relationship between variations of lipid content and the reproductive cycle. To evaluate the use of lipid reserves as an energy source in *N. lapillus*, the consumption of digestive gland lipids was followed during a starvation experiment.

MATERIALS AND METHODS

Animal collection and body parameters

Male and female specimens of *Nucella lapillus* with a mean shell height of 2.35 cm (standard deviation (SD) 0.14) and 2.56 cm (SD 0.15), respectively, were collected in March, June, September and December during low tides in Cortegaça (Portugal) 40°56′24″N 8°39′40″W. Animals were processed in the laboratory shortly after collection. Imposex was not detected in the females used in this study. The gonadosomatic index (GSI), digestive gland index and digestive gland volume were calculated in animals collected in these seasons. All indices were based on body weight without shell (on average, shell weight corresponds approximately to 70% of the total weight of the animals used in this study). The shell was carefully removed and the soft body of each animal was weighted; afterwards the gonad–digestive gland complex was detached and weighted. The gonad (which could not be extracted as a single piece) was carefully removed from the digestive gland under a stereoscopic microscope and the isolated digestive gland weighted. Gonad weight was determined by subtracting the weight of the isolated digestive gland from the weight of the gonad–digestive gland complex, and used to calculate the GSI. The weight of the digestive gland was used to calculate the digestive gland index (DGI).

$$\mathrm{GSI}(\%) = \frac{\text{gonad weight}}{\text{body weight without shell}} \times 100$$

$$\mathrm{DGI}(\%) = \frac{\text{digestive gland weight}}{\text{body weight without shell}} \times 100$$

The volume of the digestive gland was estimated by the Scherle method (Scherle, 1970). For this purpose, the isolated organ was suspended by a thin string inside a beaker with a 0.9% NaCl solution on a precision scale. The volume of the organ was then calculated based on the weight of the displaced 0.9% NaCl solution (1.0048 $g \cdot ml^{-1}$).

Sample processing for microscopy

Pieces of digestive gland and gonad were fixed for 2 hours with 2.5% glutaraldehyde, diluted in 0.2M cacodylate buffer pH 7.4 with 5% sucrose. After washing in the same buffer, fragments were post-fixed with 2% OsO_4 buffered with cacodylate, dehydrated in ethanol and embedded in Epon. For light microscopy, semithin sections (2 μm thick) were stained with methylene blue and azure II. Unstained semithin sections of the digestive gland were used for determination of lipid droplets volume density (V_V) in each season and in both genders. Semithin sections of testis and ovary were used to assess gonad development stage.

Histochemistry and cytochemistry

Light microscopy techniques for the detection of polysaccharides (PAS) and lipids (Sudan black), based on the procedures described by Ganter & Jollès (1970), were applied to semithin sections (2 μm thick) of digestive gland fragments fixed and embedded in Epon as described above. The embedding medium was not removed from the sections before staining.

PAS REACTION

Semithin sections were treated with 1% periodic acid for 15 minutes, washed with ultrapure water, stained with Schiff reagent for 30 minutes, washed with ultrapure water and mounted with DPX.

SUDAN BLACK

Semithin sections were treated with 0.04% hydrogen peroxide for 10 minutes to remove OsO_4, washed with ultrapure water and stained with a saturated solution of Sudan black in 70% ethanol for 3 minutes. After washing in 70% ethanol and in ultrapure water, sections were mounted with Epon.

DETECTION OF POLYSACCHARIDES BY ELECTRON MICROSCOPY

Ultrathin sections collected on gold grids were treated with 1% periodic acid for 30 minutes, washed in ultrapure water and treated for 2 hours with a solution of 0.2% thiosemicarbazide in 20% acetic acid. After being washed in 10% acetic acid and in water, the sections were treated with a 1% silver proteinate solution for 30 minutes and finally washed in water (Thiéry, 1967). Ultrathin sections were observed in a JEOL 100CXII transmission electron microscope, operated at 60 kV.

Stereology

In each season, the V_V of lipid droplets in the digestive gland (percentage of the gland volume occupied by lipid droplets) was estimated in 5 males and 5 females. For each animal, 5 digestive gland fragments were randomly selected and a semithin section was obtained from each one. On average, 7 systematically randomly selected fields were photographed in each section with a light microscope using the 100 × objective. For each animal, lipid droplets' V_V was estimated by point counting using an acetate sheet with a printed grid over the photographs (Royet, 1991):

$$V_v(\%) = \frac{\text{number of points on lipid droplets}}{\text{total number of points over the digestive gland}} \times 100$$

Starvation experiment

A group of animals collected in June were kept in a 50 l seawater aquarium (35‰ salinity) with an average temperature of 20°C. Animals were analysed after 30 and 45 days of starvation. Volume density of lipids in the digestive gland, digestive gland index and volume were evaluated as reported for the

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Table 1. Parameters used to obtain the indices for the seasonal study. Each value is an average of 5 animals and the standard deviation is given in parentheses. DG, digestive gland.

Month	Gender	Body weight without shell (mg)	DG weight (mg)	DG volume (mm^3)	Volume of lipids in DG (%)	Volume of lipids in DG (mm^3)	Gonad weight (mg)
March	Male	488 (104)	61 (20)	52.8 (17.6)	13.3 (6.3)	7.0 (2.3)	67 (30)
	Female	791 (136)	105 (48)	92.1 (40.9)	14.6 (7.6)	13.4 (6.0)	32 (37)
June	Male	666 (95)	96 (11)	80.3 (14.9)	19.6 (7.0)	15.7 (2.9)	126 (35)
	Female	918 (244)	214 (58)	191.5 (52.8)	29.4 (2.8)	56.3 (15.5)	24 (8)
September	Male	858 (110)	170 (77)	148.9 (65.8)	13.0 (3.7)	19.4 (8.6)	21 (21)
	Female	781 (153)	114 (42)	100.5 (39.7)	14.9 (3.6)	15.0 (5.9)	15 (9)
December	Male	958 (276)	125 (34)	117.5 (34.4)	20.6 (10.5)	24.2 (7.0)	157 (73)
	Female	1164 (232)	246 (51)	221.5 (45.0)	24.1 (1.7)	53.4 (10.9)	20 (12)

seasonal study. Data obtained with animals freshly collected in June for the seasonal study were considered as corresponding to zero days of starvation. For this experiment only females were considered.

Statistics and calculations

Each value is the mean of 5 animals and the SD was calculated with STATISTICA 9.0. Averages and SD of the parameters used to obtain the indices for the seasonal and starvation studies are given in Tables 1 and 2, respectively. For each season and gender, the mean value of lipid droplets V_V and the digestive gland volume (DGV) were used to estimate the total volume of lipids in the digestive gland (VL):

$$VL(mm^3) = \frac{DGV \times V_v}{100}$$

Since the animals were not all of the same size and weight, for comparative purposes, this value was divided by the animal weight without shell, giving the volume of lipid droplets in the digestive gland per gram of body ($mm^3.g^{-1}$).

Normality of distribution and homogeneity of variance was confirmed for all data using STATISTICA 9.0. Bifactorial and one-way analyses of variance (ANOVAs) were applied to the different sets of data, and the Tukey honestly significant difference (HSD) test was applied when necessary.

RESULTS

Seasonal study

Lipid droplets were very abundant in the digestive gland of *N. lapillus* and could be seen in semithin sections as brown spots because they were strongly osmiophilic (Figure 1A), and could also be visualized using Sudan black staining (Figure 1B). However, Sudan black staining of lipid droplets in semithin sections was only possible after treatment with hydrogen peroxide to remove the attached osmium tetroxide that prevents the linkage between dye and lipids. Nevertheless, the stereological study of lipid droplets was made with unstained semithin section, because in the black and white images used for this purpose the osmiophilic lipid droplets were very conspicuous (Figure 1C). Lipids were by far the major reserve substance in the digestive gland of *N. lapillus*. Glycogen reserves did not seem to be important in this case, because the digestive gland cells were not stained by PAS reaction and just a few sparse glycogen granules were observed by transmission electron microscopy after a cytochemical reaction that stains polysaccharides.

To detect an eventual relationship between lipid storage in the digestive gland and reproductive status, a brief study of the gonads was carried out using semithin sections. Since imposex was not visible in females, the results of this study seem not to be affected by TBT contamination. Females with a matured orange colour ovary containing a large number of eggs in advanced vitellogenesis were captured only in March (Figure 1D, E). However, other females also collected in March were extremely immature with a very reduced and pale ovary (Figure 1F). In the other seasons, all of the collected females presented an immature ovary with oocytes in early stages of development containing reduced amounts of lipid reserves (Figure 1G). Light microscopy observation of testes (Figure 2A–D) showed that in September the male gonad is in a state of rest with very few spermatozoa present in the seminiferous tubules (Figure 2C), contrasting with the large number of spermatozoa during the rest of the year, especially in March (Figure 2A).

For a quantitative evaluation of the reproductive status the GSI was assessed (Figure 3A). Using a bifactorial ANOVA, significant differences were obtained between males and females of the same seasons ($F = 8.99$, $P < 0.001$). It was evident that for most of the year the GSI was significantly higher in males than in females ($P < 0.01$), except in September when the GSI was at its lowest value for males (Figure 3A), being significantly different ($P < 0.01$) from the GSI of males in the other seasons. The most mature females

Table 2. Parameters used to obtain the indices for the starvation study. Each value is an average of 5 females and the standard deviation is given in parentheses. DG, digestive gland.

Days of starvation	Body weight without shell (mg)	DG weight (mg)	DG volume (mm^3)	Volume of lipids in DG (%)	Volume of lipids in DG (mm^3)
0	918 (244)	214 (58)	191.5 (52.8)	29.4 (2.8)	56.3 (15.5)
30	1167 (129)	193 (43)	179.7 (39.6)	31.5 (4.4)	56.6 (12.5)
45	788 (67)	106 (7)	100.2 (3.6)	18.4 (6.5)	18.4 (0.7)

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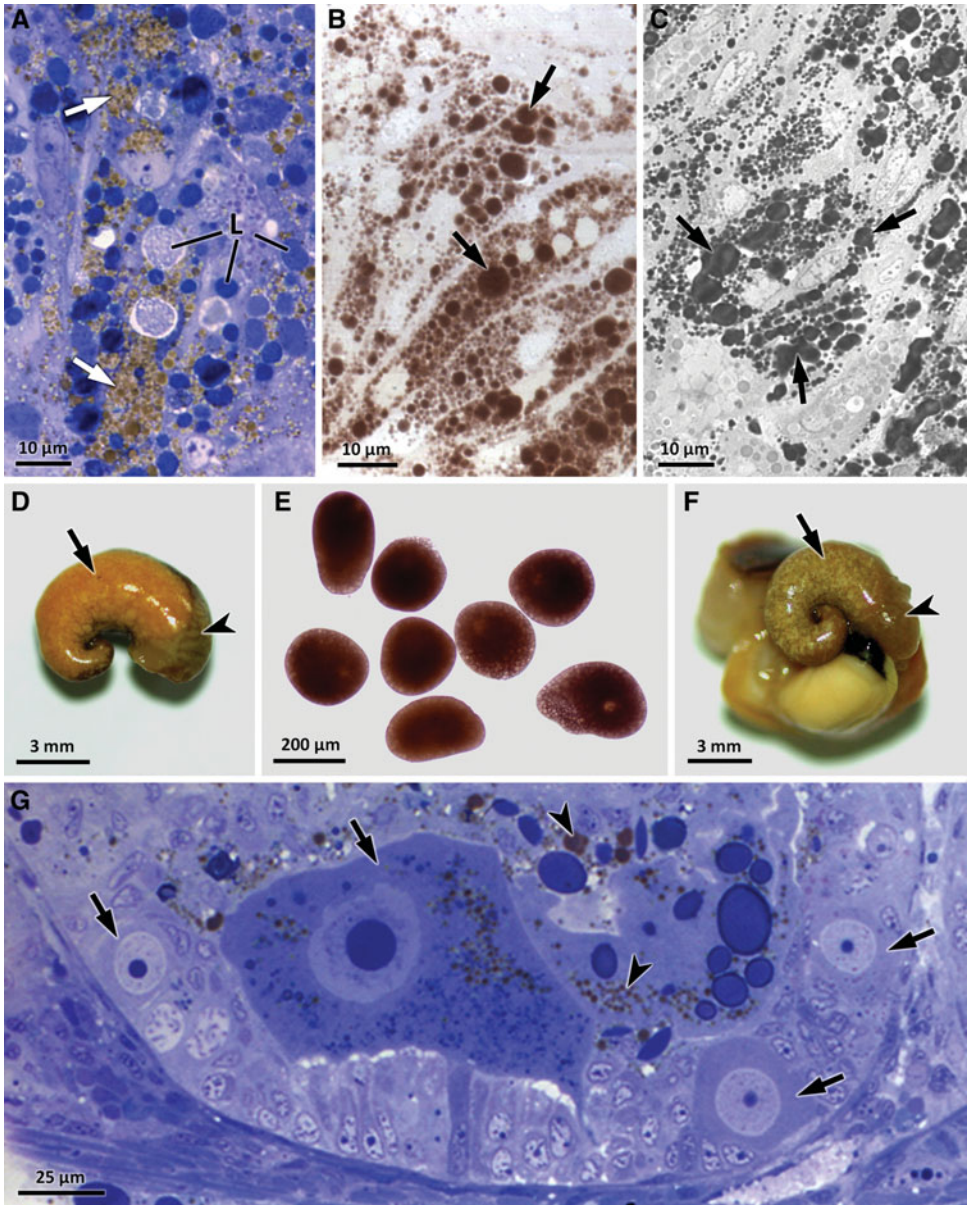

Fig. 1. Digestive gland and ovary of *Nucella lapillus*: (A) semithin section of the digestive gland stained with methylene blue and azure II, showing many osmiophilic lipid droplets (arrows) and lysosomes (L) in digestive cells; (B) lipid droplets stained by Sudan black (arrows) in a semithin section of the digestive gland; (C) the osmiophilic lipid droplets (arrows) are conspicuous in unstained semithin sections of the digestive gland; (D) mature ovary (arrow) attached to the digestive gland (arrowhead); (E) eggs at advanced vitellogenesis; (F) immature ovary (arrow) attached to the digestive gland (arrowhead); (G) semithin section of an immature ovary containing oocytes at different development stages (arrows). Some osmiophilic lipid droplets are visible in oocytes that have started to accumulate reserves (arrowheads).

had a GSI around 10% and were collected in March, but others also collected in March were very immature with a GSI of 1% or even less. In the other seasons, female GSI was low (Figure 3A), but due to the large variability in March seasonal GSI differences were not significant for females ($P > 0.05$). The values of GSI were well correlated with gamete maturation. Oocytes in advanced vitellogenesis were only found in females with the higher GSI, and males with low GSI contained very few sperm cells.

In the seasonal study, the volume density (V_V) of lipid droplets ranged from a minimum average of 13.0% of the digestive gland volume in males collected in September to a maximum of 29.4% in females collected in June (Table 1; Figure 3B). However, according to bifactorial ANOVA there were no significant differences in lipid droplets' V_V between males and females of the same seasons ($F = 1.04$, $P > 0.05$). In males there were no significant seasonal variations of lipid droplets' V_V in the digestive gland ($P > 0.05$). However, one-way ANOVA showed that in females there were significant seasonal differences regarding lipid droplets' V_V ($F = 12.84$, $P < 0.001$), and according to the Tukey HSD test, the significant differences occurred between consecutive seasons ($P < 0.05$). Moreover, according to a one-way ANOVA, a seasonal variation was detected in the female digestive gland index ($F = 10.79$, $P < 0.001$) (Figure 3C), signifying that the weight of this organ in regard to total body weight without shell is significantly higher in June and December than in March and September

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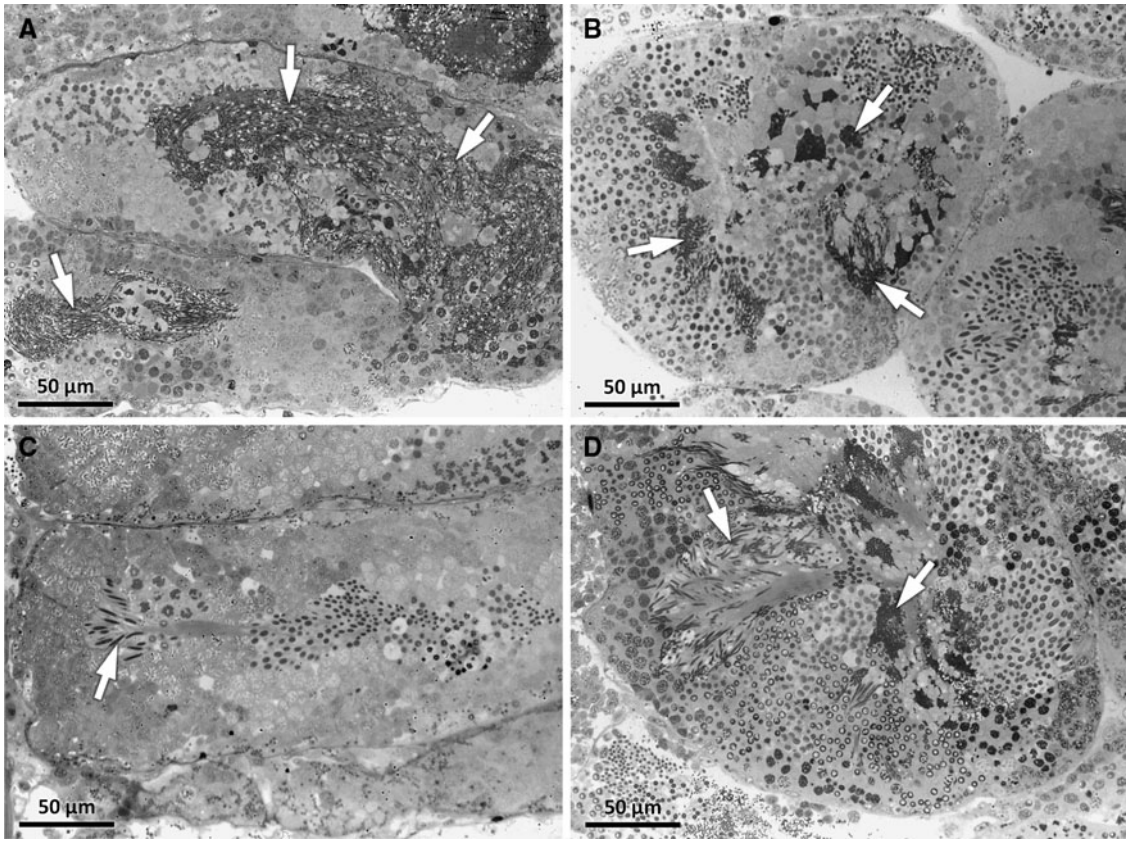

Fig. 2. Semithin sections of testes of male *Nucella lapillus* collected in March (A), June (B), September (C) and December (D). Spermatozoa (arrows) are most abundant in March and very scarce in September.

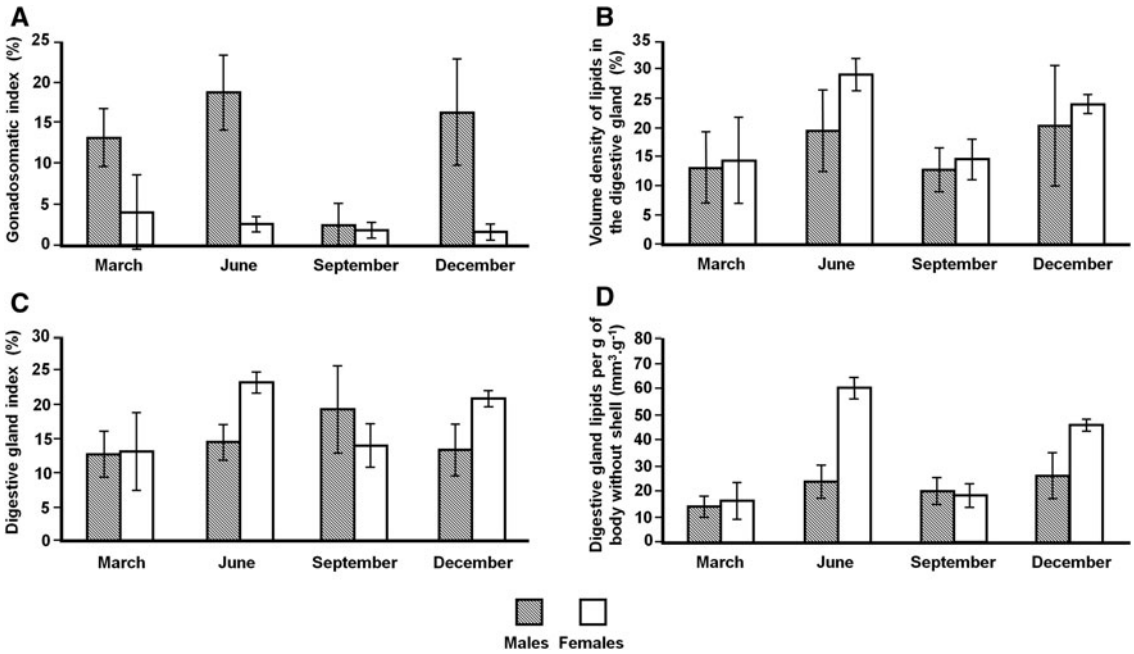

Fig. 3. Seasonal and sexual variations of the gonadosomatic index (A), volume density of lipid droplets in the digestive gland (B), digestive gland index (C) and volume of digestive gland lipid droplets per gram of body without shell (D). Standard deviation is represented by vertical lines above and below mean values.

($P < 0.05$). In males, significant seasonal differences were not detected in the digestive gland index ($P > 0.05$). Between genders, using a bifactorial ANOVA ($F = 6.89$, $P < 0.01$), significant differences were detected only in June ($P < 0.05$), when the digestive gland index had the highest values for females (Figure 3C).

For females, the percentage of digestive gland volume occupied by lipid droplets (V_V) was higher in June and December,

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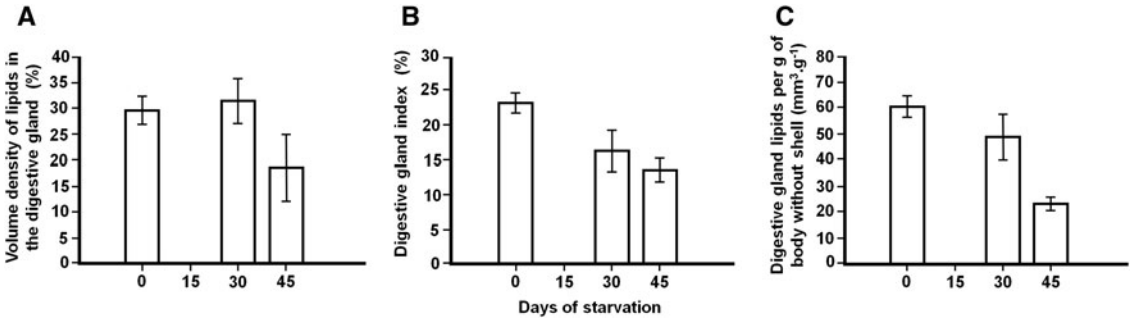

Fig. 4. Effects of starvation on volume density of lipid droplets in the digestive gland (A), digestive gland index (B) and volume of digestive gland lipid droplets per gram of body without shell (C). Standard deviation is represented by vertical lines above and below mean values.

coinciding with the highest values of digestive gland volume (Table 1). Due to the conjugation of these two factors, in June and December the total lipid content of the females digestive gland, expressed in mm^3, was substantially increased (Table 1). In order to compare animals with differences in body weight, the total volume of lipid droplets in the digestive gland was divided by the weight without shell (Figure 3D). The values obtained by this calculation are more relevant, because the variations in lipid droplets' V_V and the changes in the volume of the digestive gland are both considered. With this approach, the seasonal differences among females attained a higher magnitude (Figure 3D) and a stronger statistical significance according to a bifactorial ANOVA ($F = 22.90$, $P < 0.001$), with very significant differences between consecutive sampling seasons according to the Tukey HSD test ($P < 0.001$). In addition, significant differences between sexes emerge in June ($P < 0.001$) and December ($P < 0.001$), when lipid storage in the digestive gland reached the highest values for females (Figure 3D). However, even with this approach, in males the seasonal variations remained non-significant ($P > 0.05$).

Starvation experiment

Prolonged starvation induced alterations in the digestive gland, and significant differences in lipid droplets' V_V were detected using a one-way ANOVA ($F = 10.74$, $P < 0.01$). During the first 30 days the V_V of lipid droplets did not change significantly ($P > 0.05$), but 45 days of starvation substantially reduced the V_V of lipids in the digestive gland ($P < 0.01$) (Figure 4A). On the other hand, the digestive gland index decreased in a linear fashion from day 0 to day 45 (Figure 4B) presenting a significant variation according to one-way ANOVA ($F = 19.77$, $P < 0.001$), meaning that the digestive gland is continuously shrinking during starvation. Moreover, if the percentage of digestive gland volume occupied by lipid droplets (V_V), the total volume of this organ and body weight are taken into account (Table 2), it becomes clear that some digestive gland lipids were consumed during the first 30 days of starvation (Figure 4C). After 45 days of starvation, the lipid droplets in the digestive gland were reduced to almost 1/3 of the initial value (Figure 4C).

DISCUSSION

The quantitative morphological analysis of lipid droplets demonstrated that these are a major component of the digestive gland in *N. lapillus*. For males, the results clearly showed that the digestive gland and its lipid reserves are quite stable without significant variations throughout the year. On the other hand, a significant seasonal variation was detected in the digestive gland of females, in which lipids droplets are accumulated during spring (from March to June), consumed during summer (from June to September) and accumulated again during the autumn (from September to December). However, in both males and females a relationship between the development status of the gonad and the lipid reserves of the digestive gland was not evident. For females, in March, the low amount of lipid reserves coincide with the maximum development of the ovary, while in September a low value of lipid reserves coincided with a low GSI value. For males, the substantial reduction of the GSI in September was not followed by any significant variation of lipid reserves in the digestive gland.

For *N. lapillus*, spring has been considered the main spawning season on the coast of England (Feare, 1970; Crothers, 1985). However, the laying of egg capsules can occur almost all year (Morton, 2009). Nevertheless, at a collection site on the south coast of England, the number of egg capsules laid each month from May 2004 to September 2008 attained a maximum in March and minimum numbers were recorded from August to October (Morton, 2009). These results match well with our own data. Mature females were collected only in March and the most mature males were also collected in March, suggesting that on the Portuguese coast, spring is also the main spawning season. The GSI of males remained high most of the year and a large number of spermatozoa were found in testes except in September, suggesting that they can fertilize females with the exception of late summer and early autumn. However, we did not find fertile females except in March, but if mature females are much less frequent during the rest of the year they could have been missed in the small samples of the population used in this study.

Among molluscs, seasonal variations of lipid, protein and glycogen reserves have been studied mainly in species with commercial value, like abalone and scallops. These reserves are accumulated when food intake exceeds metabolic requirements and can be consumed in variable amounts to allow energy demanding processes (Barber & Blake, 1991). In molluscs, energetic resources can be stored in the digestive gland, muscle and specialized connective tissue cells (Robinson *et al.*, 1981; Da Silva & Zancan, 1994; Lobo-da-Cunha *et al.*, 2006). Development of gonads consumes significant amount of resources, depending on recently ingested food and mobilization of stored reserves. In scallops, digestive gland lipids

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are consumed during gametogenesis, but glycogen stored in the adductor muscle has been considered the primary energy source in this case (Barber & Blake, 1991; Le Pennec *et al.*, 2001).

Organ indices can be useful to evaluate accumulation or consumption of reserves. In the scallop *Placopecten magellanicus*, for males and females, the digestive gland index and the lipid reserves of this organ are low in winter and both increase during spring due to food abundance. The digestive gland index and lipid reserves decrease towards late spring and summer as the gonads reach maturity. Following spawning, a slight recovery of digestive gland lipids and index was detected (Robinson *et al.*, 1981). In *N. lapillus* females, the digestive gland index is lower in early spring (March) coinciding with ovary maturation and increases subsequently to reach a peak at late spring/early summer (June). However, during the summer the digestive gland index falls reaching in September a value that is identical to March. This cannot be justified by a transfer of reserves to the ovary, because in late summer/early autumn oocytes are immature, laying of egg capsules is basically absent in this period (Morton, 2009) and even the male gonad is at a resting stage. Digestive gland index of the female increases again during autumn and only a decline in late winter/early spring could be justified by the transfer of lipids to the developing ovary. The fluctuation of the digestive gland index matches with the variation of the V_V of lipid droplets, suggesting that weight gains and losses in the digestive gland are, at least in part, due to the accumulation or consumption of lipid reserves, respectively. In June and December, females accumulate more fat in the digestive gland than males, pointing to a sex related difference in lipid metabolism.

In the abalone, *Haliotis rubra*, the GSI of females is inversely correlated to the digestive gland index, pointing to a translocation of energy from the digestive gland to the ovary. In this species the digestive gland was considered the main provider of reserves during ovary maturation, and these reserves are provided mainly in the form of lipids (Litaay & De Silva, 2003). Seasonal variations of the lipid content were also studied in the land pulmonate gastropod *Megalobulimus oblongus*. In this species, the variation pattern was very different; lipid content of the digestive gland reaches a maximum value during spring coinciding with oocyte maturation and the highest amounts of lipids in the gonad (Da Silva & Zancan, 1994). These discrepancies between species are probably related to habitat, food source and physiological differences. For bivalves feeding on phytoplankton, fluctuations in lipid content were correlated to food concentration and the reproductive cycle (Pazos *et al.*, 1996; Le Pennec *et al.*, 2001). For *N. lapillus*, feeding on mussels and barnacles (Crothers, 1985), prey are available all year and food shortage seems unlikely in normal conditions. Nevertheless, feeding activity is temperature dependent in *N. lapillus* and during winter a reduction in prey consumption is expected (Largen, 1967).

Starvation experiments revealed the usefulness of digestive gland lipids as an energetic reserve. As in *N. lapillus*, in the digestive gland of *Helix lucorum* the amount of lipid droplets decreases substantially after 40 days of starvation (Dimitriadis & Hondros, 1992). But, in the starvation experiment with *N. lapillus* it is interesting to analyse the variation pattern of the different parameters used to evaluate lipid consumption. Volume density alone did not show a reduction in lipid reserves during the first 30 days of starvation. However, the volume of the digestive gland itself was reduced during the first 30 days of starvation. These results imply an actual reduction of lipid droplets volume, but in proportion to the volume reduction of the whole organ. After 30 days of starvation both the volume density of lipid droplets and digestive gland volume decreased. In this phase lipid consumption was significantly accelerated, probably because other reserves like glycogen in muscle tissue were already depleted.

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