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The annual cycle of oogenesis in the shanny, *Lipophrys pholis* (Pisces: Blenniidae)

FILIPA FERREIRA [1], MIGUEL M. SANTOS [1,2], MARIA ARMANDA REIS-HENRIQUES [1], NATIVIDADE M. VIEIRA [1,2] and NUNO M. MONTEIRO [3,4]

[1] CIMAR/CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 177, 4050-123 Porto, Portugal.
[2] Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal.
[3] CIBIO, Research Centre in Biodiversity and Genetic Resources, Campus Agrário de Vairão, R. Padre Armando Quintas, 4485-661 Vairão, Portugal.
[4] CEBIMED, Faculdade de Ciências da Saúde da Universidade Fernando Pessoa, Rua Carlos da Maia, 296, 4200-150 Porto, Portugal. E-mail: nmonteir@fc.up.pt

SUMMARY: *Lipophrys pholis* has been shown to be responsive to a variety of environmental contaminants, some of them able to impair reproduction. Description of the normal cycle of oogenesis of this newly proposed sentinel species is important since this data may function as a baseline for comparison in ecotoxicological studies, among other applications. Based on histological observations, *L. pholis* ovarian development in adult is asynchronous, and 7 ovarian germ cells can be described (oogonia, early and late perinuclear oocytes, cortical-alveolar oocytes, early vitellogenic oocytes, vitellogenic oocytes and spawning oocytes). Using a stereological approach together with the morphologic characteristics of ovarian cells, the ovarian cycle of *L. pholis* was divided into 3 maturation stages: early oogenesis (May); mid-oogenesis (September), and spawning (November to January). Ovarian cell proportions and gonadosomatic index confirmed that the reproductive period of *L. pholis* near the southern limit of distribution of the species occurs during cold-water periods, between November and May. The collected data will help to fill some of the gaps in information that still exist on *L. pholis* oogenesis, thus allowing a better integration of this species as a sentinel for the detection of contaminants in European coastal waters.

Keywords: *Lipophrys pholis*, sentinel species, intertidal, oogenesis, gonadal development.

RESUMEN: Ciclo anual de oogénesis en *Lipophrys pholis* (Pisces: Blenniidae). – En trabajos recientes, se ha visto que *Lipophrys polis* es sensible a diversos contaminantes medioambientales, algunos de los cuales pueden afectar a la reproducción. La descripción del ciclo normal de oogénesis de esta nueva propuesta de especie centinela es importante, ya que serviría como referencia para futuros estudios toxicológicos, entre otras aplicaciones. En base a observaciones histológicas, se pueden describir siete estados de los oocitos (oogonia, estado perinuclear temprano y tardío, estado cortico-alveolar, vitelogénesis temprana, vitelogénesis y oocitos desovados). Desde un enfoque estereológico, junto con las características morfológicas de las células ováricas, el ciclo ovárico de *L. polis* se ha dividido en tres estados de maduración: oogénesis temprana (mayo), oogénesis media (septiembre) y desove (de noviembre a enero). Las proporciones de las células ováricas y el índice gonadosomático, confirmaron que el periodo reproductivo de *L. polis*, en el límite meridional de distribución de la especie, se produce durante los periodos de agua fría, entre noviembre y mayo. Los datos obtenidos ayudarán a cubrir algunas de las lagunas que todavía existen en el conocimiento de la ovogénesis de *L. pholis*, permitiendo, por tanto, una mejor integración de esta especie como centinela para la detección de contaminantes en las aguas costeras europeas.

Palabras clave: *Lipophrys pholis*, especies centinela, intermareal, oogénesis, desarrollo gonadal.

INTRODUCTION

Over the last few decades, many studies have emphasized the ability of certain chemicals to interfere with the endocrine system, causing reproductive impairment and threatening the survival of wild populations of invertebrates, fish, birds, reptiles, and wildlife in general (Sumpter 2005). The widespread distribution of these chemicals in aquatic ecosystems is an increasing factor of concern (Colborn *et al*. 1993, Jobling *et al*. 1998, Holbech *et al*. 2006, Scholz 2009). In aquatic environments, fish are commonly used as biological indicators of the ecosystem's health and integrity. While several studies have been conducted using species as models to assess the negative impacts of endocrine disrupting chemicals, many of the proposed sentinel species have important drawbacks that limit their use or reliability (Schladot *et al*. 1997, Frenzilli *et al*. 2004, Gercken *et al*. 2006), such as a narrow geographical distribution and migratory behaviour. Recently, the intertidal Blenniidae *Lipophrys pholis* (Linnaeus, 1758) has emerged as a promising sentinel species for monitoring pollution in the northeastern Atlantic and has already proven to be responsive to organic contaminants such as polycyclic hydrocarbons (Lima *et al*. 2008), neurotoxic compounds (Solé *et al.* 2008), oil spills (Santos *et al*. 2010, Lyons *et al*. 1997, Harvey *et al*. 1999), oil shale extracts (Lewis *et al*. 1986) and estrogenic chemicals (Ferreira *et al*. 2009).

Although the morphology (Ford 1922, Bath 1976, Arruda 1979, Laming *et al*. 1982), ecology and behaviour (Lebour 1927, Qasim 1956, 1957, Gibson 1967a,b, 1982, 1999, Dunne 1977, Shackley and King 1977, Milton 1983, Zander 1986, 1999, Almada *et al*. 1990a,b, 1992, Faria *et al*. 1996, 1998, 2002, Faria and Almada 1999, 2006) and diet (Gibson 1972, Mazé *et al*. 1999, Monteiro *et al*. 2005) of *L. pholis* are well characterized, only scattered and incomplete information exists on female gonadal development and maturation (Lebour 1927, Qasim 1956, 1957, Shackley and King 1977, Fives 1986). Data on gonad development is of prime importance because many contaminants interfere with reproductive pathways, affecting fertility parameters, causing dysfunction of sexual development (intersex), and altering sex ratio (Jobling *et al.* 1998, Schmitt *et al*. 2005). Although there are several methods for staging gonadal development, some are based on the external visual examination of the ovary (Qasim 1956, Shackley and King 1977), which, though simple and rapid, involves a high level of subjectivity that can lead to inaccurate results. Staging based on the appearance of whole oocytes can also be useful, though oocytes in transitional stages of development are a potential source of uncertainty (Qasim 1957). Sizing oocytes may also be used to measure development (Shackley and King 1977) but little information is given on the physiological status of the ovaries. Therefore, histological studies, although time consuming, appear to be one of the most reliable and objective sources of information on the determination of spawning cycles. In this study, a thorough description of the seasonal ovary maturation cycle was performed, clustering and comparing available information from previous studies in an attempt to provide a framework that can potentially be used in assessments of alterations caused by exposure to contaminants or other environmental insults, using *L. pholis* as a sentinel species.

MATERIALS AND METHODS

Four sampling campaigns were conducted in January, May, September and November (2006), in an attempt to encompass time points characterized by distinct temperature and hydrological regimes. A total of 214 adult females, whose sex was determined in the field using genital papilla morphology (Ferreira *et al*. 2010), were collected from 7 rocky shores along the Portuguese coast [from North to South; Vila Praia de Âncora: 41°48'47''N, 8°51'55''W (N=31); Viana do Castelo: 41°41'61''N, 8°51'02''W (N=32); São Bartolomeu do Mar; 41°34'25''N, 8°47'54''W (N=33); Cabo do Mundo: 41°12'48''N, 8°42'50''W (N=26); Praia da Boa Nova: 41°11'56''N, 8°42'41''W (N=32); Foz: 41°09'29''N, 8°40'56''W (N=35); and Castelejo: 37°5'59''N, 37°5'59''W (N=25)]. Fishes were collected with hand-nets in rocky pools, during ebb tides. Only mature individuals, larger than 8 cm (see Faria *et al*. 1996; Monteiro *et al*. 2005) were collected. Captured fish were immediately transported to the laboratory and immersed in cold seawater to ensure rapid immobilization. All fish were measured (L_T), and weighed (W_T). After the initial measuring procedures, fish were quickly sacrificed by spinal transection. The gonads were excised and weighed (W_G) in order to determine the gonadosomatic index [GSI: 100 $W_G(W_T)^{-1}$]. Two locations were selected for posterior histological analysis, using a total of 35 mature *L. pholis* females (Cabo do Mundo, N=18; Castelejo, N=17). The whole ovarian tissue was preserved in Bouin's solution (Panreac) for 8-12 h and then transferred into 70º ethanol. Paraffin sections (3-5 μm thick) were stained with haematoxylin-eosin (H and E) and mounted with Entellan® (Merck). The stereological approach was designed based on point counting (Freere and Weibel 1967), using a Nikon microscope (Elipse 80i) equipped with a charge-coupled device camera (DS cooled camera Head DS-5Mc) able to record 4.9 megapixel digital images. The methodology was similar to the one proposed by Matta *et al*. (2009), with minor alterations. Briefly, in each female, 5 images were taken in each of 2 different areas of the ovary (10 fields per individual). A grid formed by 8x6 lines, creating a total of 48 interceptions, was overlaid on each image (recorded at 100x magnification) and the cell types encountered below the intersection points were registered (if no cell was present bellow the intersection, a zero was registered). The percentage values for each observed ovarian germ cell type [oogonia (Oog), perinuclear oocytes (PnO),

cortical-alveolar oocytes (CaO), early vitellogenic oocytes (pVtgO), vitellogenic oocytes (VtgO) and spawning oocytes (SpwO)], were calculated for the 4 sampling events. Microscopic developmental stages of oocytes were categorized based on Weber *et al*. (2003), the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (2009) and Lubzens *et al*. (2010).

A one-way ANOVA was conducted on the average monthly GSI values for all the sampled locations. The homoscedasticity assumption was met (Cochran's C=0.44). Furthermore, using image analysis software (UTHSCSA Image Tool Software 2.0), for each female that had mature gonads (N=22, out of the 35 that were used in the histological observations), the diameter of the 15 largest SpwO was measured in order to determine the average SpwO diameter. Regression analysis was conducted on the female size and i) female weight, ii) gonad weight and iii) SpwO diameter. Statistical analyses were performed using STATISTICA software, version 7.

RESULTS

The highest GSI value was observed in January (4.24±0.28%; average ± standard error), while the lowest was observed in May (1.19±0.29%; Fig. 1a). A trend was observed with a steady increase in the average GSI recorded from May to January (Fig. 1a). A one-way ANOVA revealed significant GSI differences between the sampled months [$F(3,27)=13.77$; $P<0.001$], with January GSI being higher than those of the other sampled months (NSK; data not shown).

The seasonal changes in ovaries were initially defined on the basis of major morphological characteristics and on the relative abundance of developing oocytes. Macroscopically, the ovaries of *L. pholis* are paired, bilobate organs located in the celomic cavity. During maturation, ovaries increase in mass and broadness and colour becomes more vivid. Covered by a thin, highly vascularized membrane (Fig. 1b,c,d), different stages of gonadal development oocytes can be observed, varying from bright orange to spherical golden-brown oocytes, with the adhesive disc in one extremity that indicates the end of maturation (Faria *et al*. 2002). Microscopically, large differences in cell type, size and arrangement can also be observed as maturation progresses (see Fig. 1e,f,g and, for more detail, Fig. 1h,i,j). Seven types of ovarian germ cells were identified during oogenesis: oogonia (Oog), early perinuclear oocytes ($_{e}$PnO); late perinuclear oocytes ($_{l}$PnO); cortical-alveolar oocytes (CaO); early vitellogenic oocytes (pVtgO); vitellogenic oocytes (VtgO); and spawning oocytes (SpwO) (Fig. 2c-h). Thus, according to both the macroscopic characteristics and the frequency of ovarian components, 3 stages of maturation were defined: early oogenesis (May; Fig. 1b,e,h); mid-oogenesis (September; Fig. 1c,f,i) and spawning (November to January; Fig. 1d,g,j).

Early oogenesis

In this stage, the oogonia, isolated or in small groups, are visible (0.71±0.59%; Fig. 2a,b). The low oogonia percentages were due to the cell's small size (31±0.002 μm; Fig. 2c), which reduced their chance of being spotted by the selected sampling technique used. These cells were characterized by a vesicular nucleus, a central nucleolus and scarce cytoplasm that stained weakly. Perinuclear oocytes were especially abundant during early oogenesis (43.97±12.89%; Fig. 2a,b) and were characterized by a highly basophilic cytoplasm with the presence of a nucleus containing several nucleoli and a thin follicular layer surrounding the oocytes (Fig. 2d). This stage can be further subdivided into early and late perinuclear oocytes. The early oocytes, larger than oogonia (78±0.003 μm), were surrounded by a thin layer of follicular epithelium. The bubble-shaped nucleus was located in the middle of the cell and numerous relatively large, basophilic nucleoli appeared at the periphery of the nucleus. The nucleoli increased in number and volume and tended to migrate to the periphery of the nucleus. In H and E stained sections, the cytoplasm was strongly basophilic (Fig. 2d).

The late perinuclear oocytes were approximately twice as large (126±0.005 μm). In H&E stained material, these 2 cell types are easily distinguished because the late perinuclear oocyte cytoplasm appears much lighter (Fig. 2d). The chromatin was dispersed throughout the nucleus, causing the nucleoplasm to appear granular. The inner layer, dense and deeply basophilic, and the outer layer, less dense and only slightly basophilic, began to differentiate. Numerous small round nucleoli were found in the periphery of the nucleus, quite close to the nuclear membrane. These oocytes, initially round-shaped but becoming more irregular due to lateral compression, were also present in the other sampling events. The average GSI value calculated during early oogenesis was 1.97% (±0.17).

Mid-oogenesis

This stage is mainly characterized by the presence of cortical-alveolar oocytes (22.46±3.53%) and early vitellogenic oocytes (24.31±6.88%) (Fig. 2a,b). The cortical-alveolar oocytes are characterized by the appearance of a typical vacuolization pattern (cortical-alveoli) in the cytoplasm. These spherical structures appear empty and they are not yet yolk (Fig. 2e). The cytoplasm loses some of its basophilic properties, with the number and size of the vesicles progressively increasing with the development of the oocytes. The nucleus now contains numerous nucleoli close to the nuclear membrane. These oocytes (233±0.007 μm) enlarge up to 50-100% relatively to the perinuclear oocytes. The zona radiata and the theca become perfectly visible.

The early vitellogenic oocytes (410±0.012 μm) are characterized by an increased centrifugal accumulation

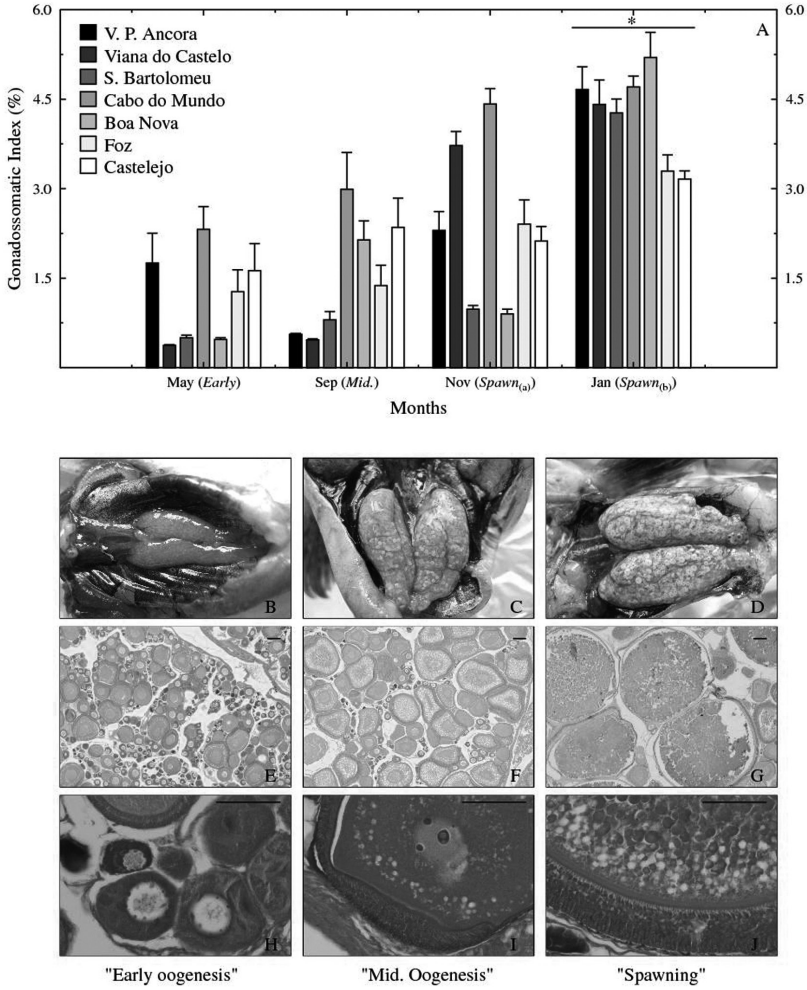

FIG. 1. – (a) GSI variation in the sampled rocky beaches (*vertical bars* depict standard errors and asterisk indicates the month in which the GSI was significantly higher) and macroscopic photos of ovarian gonadal development [(b) early oogenesis; (c) mid-oogenesis; (d) spawning]. Additionally, the histological architecture and photomicrographs of transverse sections of *L. pholis* ovaries within the three considered maturation stages are also depicted [e, h, early oogenesis; f, i, mid oogenesis; g, j, spawning] (e, f, g scale = 500 μm; h, i, j scale = 50 μm).

of little spherical, eosinophilic, vitellogenic yolk granules that tend to dislodge the cortical-alveolar material to the periphery of the cytoplasm (Fig. 2f). The nucleus tends to occupy a central or slightly eccentric position, with few nucleoli. The follicular layers are now well developed and can be easily distinguished. While the presence of perinuclear oocytes (20.66±3.57%) decreased, the GSI rose to 2.67% (±0.15).

Spawning

In this stage, the ovaries are mainly occupied by vitellogenic oocytes (22.17±1.82%) and spawning oocytes (23.35±3.76%) (Fig 2a,b). The process of final maturation approaches completion with the rise in abundance of vitellogenic oocytes (604±0.012 μm). The lipid droplets enlarge and occur scattered between the yolk spheres, granules or globules that occupy the whole ooplasm (Fig. 2g). As vitellogenesis proceeds, the cytoplasm becomes less basophilic. The envelope layers, including the follicle layer and zona radiata (which now assume a fine, acellular, striated appearance, homogeneously staining by eosin), become prominent. In these vitellogenic oocytes, the nucleus tends to be located approximately in the middle of the cell, from where it will initiate a migration towards the periphery. The nucleoli are placed right at the edge of the nucleus. Because of the lipophilic nature of these cells, this stage is sometimes difficult to process due to shrinkage and distortion.

In spawning oocytes (810±0.01 μm), a peripheral migration of the nucleus together with the membrane dissolution is visible. It seems important to stress that, given the cell dimensions, the nucleus is sometimes difficult to observe (Fig. 2h). The cytoplasm is completely filled with large yolk platelets, and a marked zona radiata can be seen. The protein yolk granules and lipid droplets start to coalesce and the oocytes rapidly increase in volume due to a hydration process. This is probably the most sensitive stage, in terms of histologi-

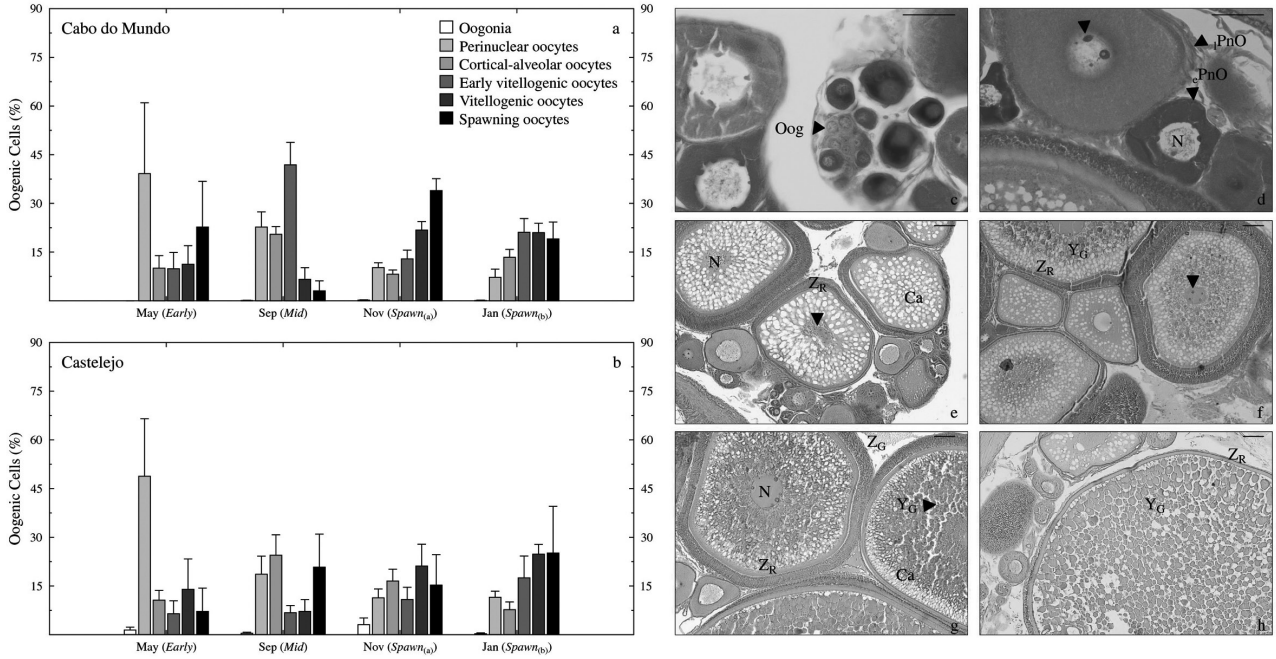

FIG. 2. – Percentage of oogenic cells during the gonadal development of *Lipophrys pholis,* at Cabo do Mundo (a) and Castelejo (b) (vertical bars represent standard errors). Microscopic morphology of *L. pholis* oocytes [c, Oogonia (Oog)], [d, perinuclear oocytes in an initial ($_e$PnO) and advanced stage ($_l$PnO). Early perinuclear stage showing the nucleus (N) and the late perinuclear stage with multiples nucleoli (arrowhead)], [e, cortical-alveolar oocytes. Lipid accumulation in the oocytes; cortical-alveoli (Ca) and the follicular envelope / zona radiata (Z_R). Several nucleoli (arrowhead) in a central nucleus (N)], [f, early vitellogenic oocytes. A central nucleus, with several nucleoli (arrowhead). Yolk granules (Y_G) and the follicular envelope (Z_R)], [g, vitellogenic oocytes. Cortical-alveoli (Ca) in the periphery and yolk granules (Y_G). The follicular envelope is well developed with the 2 visible layers: zona radiata (Z_R) and zona granulosa (Z_G)] [h, spawning oocytes. Fully hydrated oocytes with yolk platelets (Y_G) and the ooplasm is surrounded by a marked zona radiata (Z_R)] (c, d, scale = 50 μm; e, f, g, h, scale = 200 μm).

cal procedures, as cells easily rupture. During spawning, the GSI reached 3.6% (±0.11). Oogonia and perinuclear oocytes can also be observed, and may function as a repository that allows for several spawning events [*L. pholis* can have 3 to 8 spawnings per year, according to Qasim (1957) and Shackley and King (1977), respectively)]. Finally, atresic follicles (from non-ovulated mature oocytes) were also occasionally observed, mostly in January. These post-ovulatory follicles had a lumen and wall made up of follicular cells and conjunctive theca.

Positive correlations were observed between female size and weight (R^2=0.945; P<0.001) as well as with SpwO diameter (R^2=0.544; P<0.001) and gonad weight (R^2=0.717; P<0.001) (Fig. 3).

DISCUSSION

Although some degree of variance in the GSI exists among the 7 sampled locations, this index seems to provide a good indication of the reproductive status of female *L. pholis*, coinciding with the already described breeding season in Portuguese waters (Almada *et al*. 1990a, Faria *et al*. 1996). Interestingly, GSI also produced fairly good results for *L. pholis* males (Ferreira *et al*. 2011). The stereological approach, although highlighting several similarities between sites and sampling seasons (e.g. percentages of perinuclear oocytes or vitellogenic oocytes), also showed some site-specific differences (e.g. percentage of spawning oocytes). As Cabo do Mundo and Castelejo are two geographically

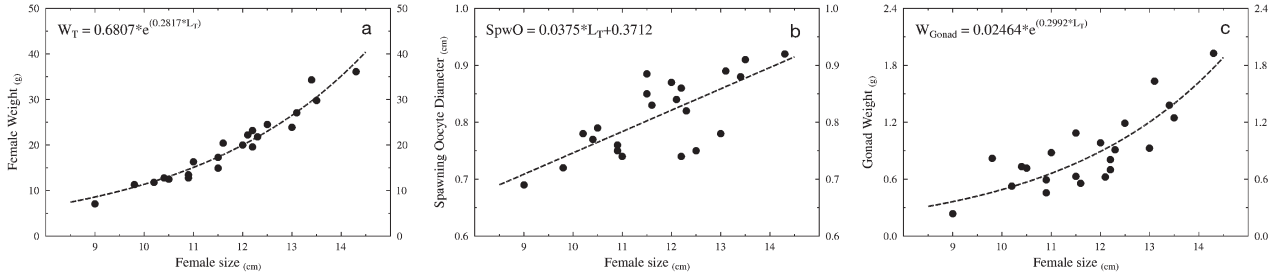

FIG. 3. – The relationship between *L. pholis* female size and a) female weight (W_T), b) spawning oocytes diameter (SpwO), and c) gonad weight (W_{Gonad}). Best-fit equations are shown in each graph.

distant locations, the differences observed might be due to differences in water temperature regimes, which greatly influence fish reproduction (Cushing 1975). If this is true, then a stereological approach might be much more sensitive than a traditional GSI-based approach for highlighting subtle changes in local abiotic conditions.

Overall, based on oocyte morphology and cell type prevalence, as observed under a light microscope, the ovarian cycle of *L. pholis* can be divided into 3 maturation stages: early oogenesis (May), mid-oogenesis (September) and spawning, which can be further subdivided into early (November) and late spawning (January). Although the number and characteristics of these stages may differ slightly in the literature (Qasim 1957, Shackley and King 1977), the differences might be attributed to the selected methodology because the previous descriptions of the oogenesis process were not based on histological procedures. The stereological analysis of the ovaries, in addition to the macroscopic characteristics and GSI indices, enabled a more accurate and thorough evaluation of the several developmental stages.

The basic pattern of oogenesis in *L. pholis* is similar to that already described for other blenniids (Qasim 1957, Dunne 1977, Shackley and King 1977, Fives 1980, Patzner 1983, Santos 1995, Carrassón and Bau 2003) or even other teleosts (Tayler and Sumpton 1996, Lubzens *et al*. 2010). Histological observations showed that all stages of development were represented in the ovaries during all 4 seasons studied, suggesting that *L. pholis* is an asynchronous spawner, with eggs being recruited in several batches during the breeding season. It is possible that the asynchronous production of multiple batches functions as a bet hedging strategy, allowing the eggs to be distributed among several males, thus reducing the risks of complete loss of progeny because of inadequate mate choice, environmental constraints and failure in larval recruitment, among other equally valid causes.

Although all oocyte developmental stages were present throughout the sampled seasons, a predominance of certain ovarian cells was visible in each particular season. Averaging both sampled locations, vitellogenic and spawning oocytes were predominant in November and January, an observation that is in concordance with the already defined breeding season for the continental Portuguese coast (Almada *et al.* 1990a, Faria *et al.* 1996). In May, even though spawning oocytes were still found in a few females, most ovaries had perinuclear oocytes whereas in September, cortical-alveolar and early vitellogenic oocytes were predominant, indicating the upcoming onset of the breeding season.

In this study, it was not possible to observe a discrete 'rest' or 'spent' period, contrarily to what was observed by Qasim (1957) and by Shackley and King (1977). This fact could be due to (i) the sampling strategy, which did not cover time periods in which these stages might have occurred or, alternatively (ii) the fact that these stages simply do not occur at lower latitudes because the extended breeding season prevents a halt in the gonad development cycle, with each stage partially overlapping the following one (as can be observed in Fig. 2).

As in other teleosts, atresic follicles were occasionally observed, especially at the end of the spawning stage. The presence of these follicles is not only an indicator of reproductive maturity and recent spawning events, but can also indicate adverse conditions such as an increase in stress due to changes in environment (Agostinho 2007). Because the mechanism that initiates and regulates oocyte re-absorption in teleosts is still poorly understood (Santos *et al*. 2005), the phenomenon of atresia and its consequences in oocyte production is a major issue to be addressed in future studies.

Although the process of oogenesis in *L. pholis* shows several similarities with that in other Blenniidae, as stated above, there are some specific differences at the intraspecific level that deserve comment. As expected, positive correlations were observed (Fig. 3) between female size and the other 3 selected variables [female weight (a), spawning oocyte diameter (b) and gonad weight (c)], which suggest that larger females have the potential to produce larger broods. Similar trends were observed in the British Isles (Qasim 1957; Shackley and King 1977). Nevertheless, in these more northern populations, although fish size [Qasim (1956) reports a maximum female size of 15.7 cm] and gonad weight [Shackley and King (1977) report a weight of 1.8 g] can be considered similar to those observed in this study, the diameter of spawning oocytes seems to differ. While along the Portuguese coast the maximum spawning oocyte recorded was only 0.92 mm (Fig. 3b) (average of 0.81 mm), Shackley and King (1977) report a size of 1.35 mm for the British Isles. According to the regression equation obtained (Fig. 3a), considering fish size and spawning oocyte diameter, an oocyte this big would have been laid by an equally large female (26 cm). Alternatively, it seems more parsimonious to infer that more northern populations may have a distinct investment pattern in reproduction in comparison with those that live near the species southern limit of distribution. Given that gonad weight is similar but spawning oocytes are smaller, it also seems reasonable to infer that females of southern populations, through a broader breeding season (Faria *et al*. 2002), disperse their total reproductive investment over a longer time period. Alternatively, this difference could just be considered as an artefact resulting from different measurement techniques, because newly born larvae size is similar in Portugal and the UK [Faria *et al*. (2002) and Qasim (1956) report similar hatch sizes, slightly above 5 mm]. It should be stressed, however, that the extension of the embryonic development period differs according to temperature (Faria *et al.* 2002), which means that the energetic demands needed to produce a larvae of similar size might differ according to latitude.

The proposed evaluation of *L. pholis* oogenesis summarizes the available information and adds a more cell-centred description of the annual cycle of gonad maturation. It also clearly highlights that, in addition to minor differences among populations inhabiting different latitudes, there is a general consistent pattern that can now be further explored in pollution-monitoring studies. Because of the well-established responsiveness of the species to chemical pollution, its unique biological characteristics and the growing body of literature, data on the annual cycle of oogenesis can be viewed as an additional tool for the integration of *L. pholis* as a sentinel species that is especially suited to the evaluation of environmental contamination in European marine ecosystems.

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