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ORIGINAL ARTICLE

Reproductive biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): Individual traits related to sexual reproduction

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Abstract

We studied individual traits related to the sexual reproduction of *Lychnorhiza lucerna* and reviewed earlier studies of sexual maturation in scyphomedusae, focusing on non-brooding species. *Lychnorhiza lucerna* is a gonochoric species and sexual dimorphism was noted in the gonadal colour. There were no brooding structures or any other distinguishable features enabling macroscopic determination of sex. Gametogenesis resembled descriptions available for other rhizostomes and semaeostomes. Both processes are asynchronous, with gametes at all stages of development occurring together. Oocytes arose from the gastrodermis and maintained contact with it via trophocytes throughout vitellogenesis. Spermatogenesis occurred within spermatic follicles arising from nested primary spermatogonia. Population features of sexual reproduction were defined by characterizing and quantifying individuals at different stages of sexual maturity. In Scyphozoa, sexually mature medusae can be detected by (1) the presence of fully developed gametes in the gonads indicating incipient spawning, or (2) the presence of spent follicles as evidence of ongoing or recent spawning. Whereas the former allows more detailed study of sexual reproductive patterns of any species, the latter constitutes an option for non-brooders (as in *L. lucerna*) equivalent to the search of fertilized eggs or planulae for brooder species.

Key words: *Non-brooder species, oogenesis, sexual maturity, South-western Atlantic, spermatogenesis*

Introduction

The study of reproductive strategies of jellyfishes gained importance in recent years given the presumed increases in their biomass and the socioeconomic and environmental problems caused by mass occurrences of these cnidarians (see Purcell et al. 2007; Uye 2008; Richardson et al. 2009). When population traits of sexual reproduction are studied, an essential objective is to identify those specimens that have reached sexual maturity and their spatial and temporal patterns of occurrence and abundance. In scyphomedusae, this phenomenon has been described thus far with different levels of detail and accuracy.

In brooder species the presence of fertilized eggs, embryos or planulae on brooding structures of females constitutes an evidence of sexual maturity (e.g. Kikinger 1992; Rippingale & Kelly 1995; Lucas & Lawes 1998), indicating ongoing or recent sexual reproduction events in the population. Different approaches are needed for the study of non-brooder species since the products of sexual reproduction are not retained by female medusae. One possible solution to this is examination of gonadal tissue through histological preparations. Such an approach has been used to investigate temporal patterns of sexual reproduction (Kon & Honma 1972; Rottini Sandrini & Avian 1991; Lucas & Lawes 1998; Pitt & Kingsford 2000) and for determining scales of sexual maturation (Pitt & Kingsford 2000; Toyokawa et al. 2010; Iguchi et al. 2010).

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Reliable identification of sexually mature medusae begins with studies of gametogenesis and gonadal development. Gametogenesis is basically the same among semaeostome and rhizostome Scyphozoa (Eckelbarger & Larson 1992; Eckelbarger 1994a). The few available criteria utilized in defining sexual maturation stages are similar (Pitt & Kingsford 2000; Schiariti 2008; Toyokawa et al. 2010; Iguchi et al. 2010). Although all of the studies have classified medusae on the basis of their gametogenic status (proportion of gametes at different stages of development), they differ mainly in the way sexually mature medusae have been defined. The differing definitions influence final results at a point such that contrasting reproduction patterns can be obtained depending on the applied criteria (Schiariti 2008).

Increasing awareness of jellyfish abundances, studies of their role in marine ecosystems, and economic losses from their negative impacts on human activities highlight the importance of knowledge about scyphozoan life histories and population dynamics. *Lychnorhiza lucerna* Haeckel, 1880 is one of the most frequently occurring medusae inhabiting coastal waters from southeastern Brazil (22–23°S) to northern Argentina (36–38°S) (Mianzan & Cornelius 1999); indeed, its economical exploitation is currently under consideration (Schiariti 2008). Furthermore, common mass occurrences of the species can generate serious problems for local fishermen by damaging their catches and even fishing gears and boats (Schiariti 2008; Nagata et al. 2009). The life cycle, planulae settlement preferences and asexual reproduction of *L. lucerna* have been described (Schiariti et al. 2008). Here we examine the individual traits related to sexual reproduction of *L. lucerna*, including a description of gonadal structure and gametogenesis. In doing so, we review how sexual maturation stages have been defined for scyphomedusae. Moreover, we discuss non-brooder species in particular, with the aim of finding a simple and uniform means of determining sexual maturity in scyphozoan medusae.

Material and methods

Medusae of *Lychnorhiza lucerna* were collected at the Río de la Plata estuary (RLP; Argentina, 35°S) (n_{RLP} = 318; December 2006 to May 2007) and Cananéia region (CR; Brazil, 25°S) (n_{CR} = 404; November 1999 to January 2002) using several different methods: (i) demersal trawls; (ii) paired demersal trawling; (iii) gill-nets; (iv) hand nets; and (v) manual collecting of specimens stranded on beaches. Only those specimens showing no deterioration or damage were considered in this study. Additionally, ephyrae, metephyrae and young medusae cultured in aquaria were examined.

Bell diameter (BD, cm $\pm$ 0.1) of all medusae were measured within 2 h of collection. Gonads were exposed by cutting away the oral arms in medusae orientated with the oral surface facing upwards. Gonads were then examined macroscopically for sex and state of maturity based on colour. A piece of gonad (approx. 5 g) from each specimen was excised and preserved for 24 h in Bouin's solution prepared with seawater (specimens from Argentina) or directly in 4% formaldehyde solution in seawater (specimens from Brazil). After fixation, gonads were preserved in 70% ethanol prior to histological examination.

Fourteen mature females from RLP were carefully captured with hand nets, and their subgenital and gastric cavities and oral arms were examined under a dissecting microscope in a search for fully developed or fertilized eggs, embryonic stages or planulae.

Histological slides (n_{RLP} = 295; n_{CR} = 53; $n_{aquarium}$ = 2) were prepared for studies of gametogenesis and for establishing maturity stages. Gonadal tissues were prepared using standard ethanol dehydration and paraffin wax-embedding procedures. Samples used for maturity stages determination were sectioned into 5 μm ribbons and stained with Harris's haematoxylin followed by eosin counterstaining. Samples utilized for the description of gametogenesis were sectioned into 1 μm ribbons and stained the same way.

Interpretation of histological sections and gametogenesis stages were related to known gametogenic cycles stated for Cnidaria following Campbell (1974), Eckelbarger & Larson (1988) and Eckelbarger (1994a, b).

Results

Gonad colour

Differences in colour frequency were noted between males and females. Although whitish, greenish and brownish gonads were observed in both sexes, the relative abundance of each colour differed between males and females (Figure 1). Whitish and greenish gonads were more frequent in males (88%). In contrast, 72% of medusae with light or dark brown gonads were females (Figure 1). In a test, assuming whitish and greenish gonads were testes and brownish gonads were ovaries, an accuracy of 78.4% (n = 192) in a macroscopic determination of sex was achieved. Of the rest, 17.6% (n = 43) corresponded to males bearing brownish testes and the remaining 4.1% (n = 10) to females bearing whitish or greenish ovaries (Figure 1).

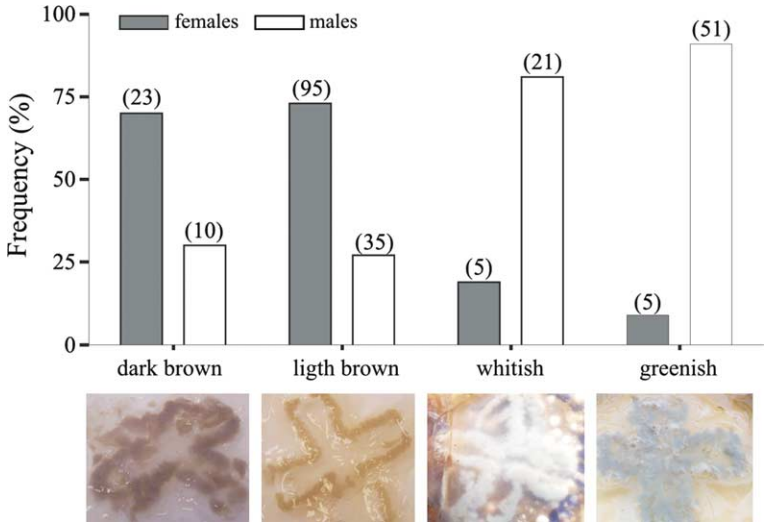

Figure 1. Different gonad colorations observed in adult *Lychnorhiza lucerna* and their frequency distribution (%) by sex. Number of specimens is indicated between brackets.

There were no brooding structures or other distinguishable features that enabled macroscopic determination of sex besides gonad coloration. *Lychnorhiza lucerna* had translucently whitish bodies with whitish or purplish-blue marginal lobes, but this colour variation was not related to sex (χ^2-test $p < 0.01$). Oocytes, embryonic stages or planulae were not found within the ovaries, the subumbrellar or gastric cavities or on the oral arms of females.

Structure of gonads

The so-called gonads of *Lychnorhiza lucerna* lie in the floor of the interradial gastric pouches, being clearly visible in the form of a Maltese cross from the exumbrelar side (see Figure 1). Each arm of the cross is a band-like evagination of the gastrodermis within the gastric pouch, forming a fold. On the inner side of the fold are digitate prolongations of the epithelium (gastric cirri), while the outer side bears the reproductive cells. The subgenital cavity is located between the subumbrella and the oral disc and opens to the exterior by four subgenital ostia between the oral pillars. As gastric pouches grow, early development begins by migration of cells from the gastric endoderm into the mesoglea. Developed gonads lie above the gastric cirri in the floor of interradial gastric pouches and appear clearly through the body wall as the previously described cross-shaped structure.

Gonads are two-layered in cross-section. Lining the gastrovascular cavity is the gastroderm, with gonadal mesoglea lying beneath. Interior to the mesoglea on its subumbrellar side, and separating it from the genital sinus, is the genital epithelium.

Gametogenesis

Gametogenesis (oogenesis and spermatogenesis) in *Lychnorhiza lucerna* is asynchronous, as gametes at all stages of development were observed simultaneously in almost all specimens examined.

Oogenesis

Female germ cells arise from the gonadal gastrodermis, indicating that they have an endodermal origin. Oogenesis begins with mitotic division of the oogonia (OG). Although OG were observed within the gastrodermis, no distinct mitotically dividing OG was distinguished. After an unknown number of mitotic divisions, OG develop into the first stage of development of the oocytes. Four stages of oocyte development were identified: previtellogenic oocytes (*pre*), early- (O*i*), mid- (O*ii*), and late-vitellogenic oocytes (O*iii*). Oocytes increased in size during development mainly due to the accumulation of yolk in their ooplasm (Table I). Previtellogenic oocytes are the most abundant stage in all ovaries and become visible when still embedded in the gastrodermis (Figure 2A). These cells are easily identifiable by their round shape and prominent nucleus with a single nucleolus. Their ooplasm appears darker in histological sections because of its strong basophilia due to the presence of free ribosomes. Upon growth they become larger than the surrounding cells and protrude into the gonadal mesoglea, but retaining contact with the gastrodermis through the trophocytes (Figure 2B). Early- to mid-vitellogenic oocytes are characterized by the presence of some yolk granules, intermediate levels of basophilia, and a conspicuous nucleus containing

Table I. Diameter (μm) of oocytes and their nuclei throughout oogenesis.

	Previtellogenic	Early-vitellogenic	Mid-vitellogenic	Late-vitellogenic
Min	3.1	9.6	20.6	46.1
Max	11.5	22.0	47.3	98.4
Mean	5.4	12.8	34.7	71.1
SD	1.8	5.2	7.4	5.2
N	125	100	100	168

Data from 50 gonads pooled.

a single spherical nucleolus. The nucleus appears to face the gastrodermis at the place where the oocyte contacts the trophocytes (Figure 2A). By the time oocytes have reached 59.8 ± 7.0 μm in diameter ($n = 100$), they have moved almost entirely to the mesoglea, but they still keep contact with the trophocytes (Figure 2A,B). At this stage, yolk granules increase in abundance with development and the ooplasm appears lighter in histological preparations, although some degree of basophilia may be maintained. The late-vitellogenic stage, the lightest stage in histological preparations (non-basophilic cells) (Figure 2), is reached when oocytes become filled with yolk granules. Late-vitellogenic oocytes have large nuclei, indicating that development has been arrested, probably at prophase I. They still maintain contact with the trophocytes. Oocytes are released from the ovary through a pit generated at the place where they were attached to the trophocytes (Figure 2C).

Differences between all stages of oocyte development were clearly visible in most cases under light microscopy. In the few cases where differences between stages were not clear, oocyte diameter was used in characterizations as shown in Table I.

Spermatogenesis

Spermatogenesis begins within the gonad gastroderm wall (subumbrellar side) with the mitosis of relatively large cells known as primary spermatogonia (SG) (Figure 3A). The SG are diploid and isodiametric, with a large and central nucleus containing chromatin in a dusty dispersion with one or two nucleoli. The successive mitosis of SG give rise to the formation of spermatogenic compartments named spermatic follicles where spermatogenesis takes place (Figure 3). Development of male gametes occurs, therefore, separated from the rest of the somatic tissues. After an unknown number of mitotic divisions, the SG developed into primary spermatocytes (S1) which migrate inwards from the follicle wall (Figure 3A–C). These cells undergo the first stage of meiosis (meiosis I) and prophase I is evidenced by dense and compact nuclei. The result is the formation of two diploid cells, known as secondary spermatocytes (S2) characterized by their small size and dense chromatin (Figure 3B). The S2 undergo meiosis II giving rise to spermatids (ST) (Figure 3B) with *n* chromosomes. The resulting ST differentiate into spermatozoa (SZ) by a process known as spermiogenesis or spermatotelosis. Spermiogenesis consists mainly of the transformation of ST into SZ without cellular division through cytoplasmic elimination of the ST and the gradual growth of the spermatozoan tail (Figure 3C). Reaching the final stages of the spermatogenesis, the oral half of the follicles became filled with S1, S2 and ST and the aboral half with SZ, which are grouped around many central masses with the heads attached to them and the tails radiating outwards until they are released through a pore on the distal wall of the spermatic follicle (Figure 3D). Spermatozoa are released into the genital sinus and probably reach

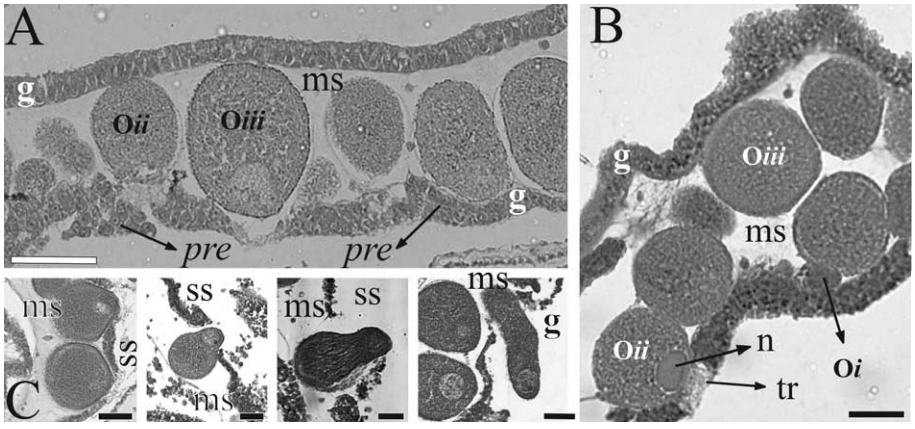

Figure 2. Oogenesis in *Lychnorhiza lucerna*. (A,B) Stages of oocyte development in gonadal tissue sections; (C) sequence of images showing fully developed oocytes during spawning. See details in text. g, gastroderm; ms, mesoglea; n, nucleus; ss, subgenital sinus; tr, trophocytes; *pre*, previtellogenic oocytes; O*i*, early-vitellogenic; O*ii*, mid-vitellogenic; O*iii*, late-vitellogenic. Scale bars = 35 μm.

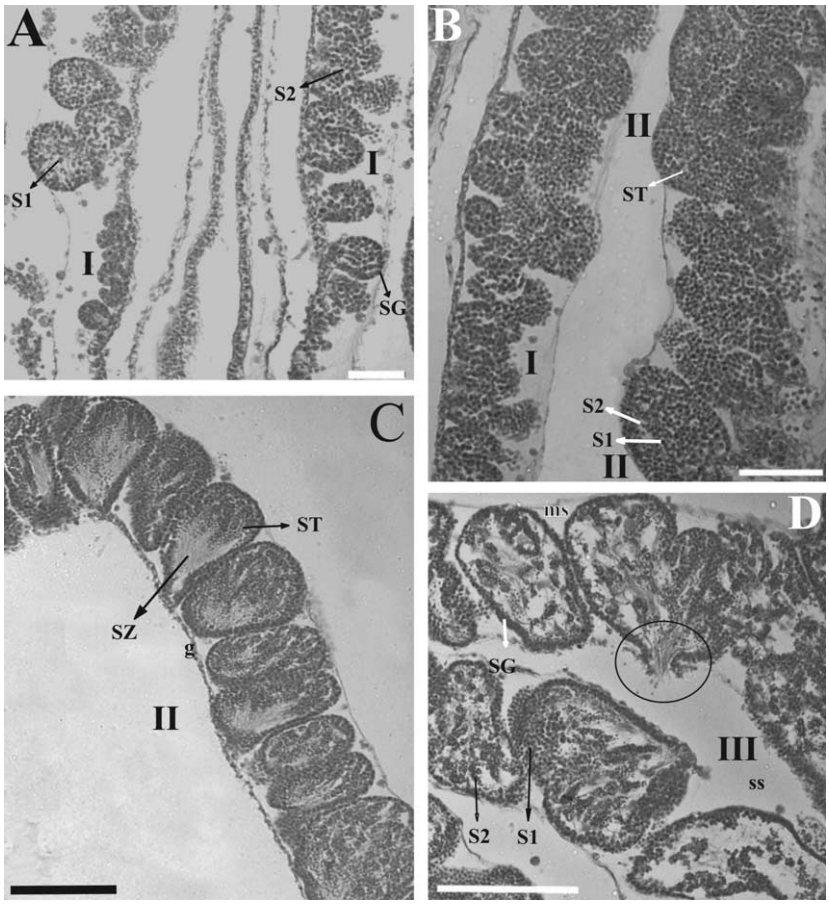

Figure 3. Spermatogenesis in *Lychnorhiza lucerna*. (A–D) Gonadal tissue sections showing development of follicles and spawning. SG, spermatogonia; S1, primary spermatocytes; S2, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; g, gastroderm; ss, subgenital sinus; I, II, III represents developmental stages of spermatic follicles. See details in text. In D, circle shows spawning. Scale bars = 100 μm.

the open water through the gastric canals via mouth-arms. Once the spermatozoa have been released, the spermatic follicles enter into a post-evacuation state (i.e. spent) where follicular recovery is evidenced by the presence of amoeboid cells and free spaces in the lumen (Figure 3D). Simultaneously, this follicular reversion, and the proliferative processes of spermatogenesis, recommences, as manifested by the mitotic divisions of the SG and further development of S1 and S2 (Figure 3D).

Maturity state

The following stages of sexual maturation were distinguished based on the state of differentiation of gonadal tissue: (1) sexually undifferentiated (no visible gonads, sexes indistinguishable); (2) sexually immature (absence of fully developed gametes; unspent gonads); (3) sexually mature (ripe) (fully developed gametes clearly visible; evidences of spawning may be present); (4) spent (medusae dying or already dead; gonads in advanced state of deterioration; oral arms usually lost or heavily damaged, umbrellar tissue opaque and hardened).

All sexually mature males possessed spent follicles (Figure 3D). After spawning of spermatozoa the spermatic follicle remained empty for an unknown period of time until new gametes develop, restarting the cycle. Spent follicles represented 'spawning prints' and confirmed the ripe stage of males despite the absence of spermatozoa.

Spawning in females consists of the expulsion of O*iii* through a pit formed in the gastrodermis. We observed O*iii* squeezing out the ovary (Figure 2C), although it was not possible to discern if oocytes were naturally expelled or if this was an artefact of histological slide preparation. Although this implies a disruption in the membrane, no spawning prints were found as evidence of spent ovaries.

Discussion

Gametogenesis

Oogenesis in *Lychnorhiza lucerna* resembled descriptions available from studies of other rhizostomes and semaeostomes (Uchida 1926; Widersten 1965; Eckelbarger & Larson 1988; Lesh-Laurie & Suchy

Table II. Sexual reproductive strategies in some species of Rhizostomeae.

Suborder	Family	Species	Sexual dimorphism	Brooding behaviour	Male spawning	References
Kolpophorae	Cassiopeidae	*Cassiopea andromeda* Forskål, 1775	√	√	Spermatozeugmata	Gohar & Eisawy 1960a,b; Hofmann et al. 1996
		Cassiopea frondosa Pallas, 1774	√	√	Spermatozeugmata	Gohar & Eisawy 1960a,b; Hofmann et al., 1996
		Cassiopea xamachana Bigelow, 1892	√	n. d.	n.d.	Gohar & Eisawy, 1960a,b; Hofmann et al. 1996
	Mastigiidae	*Mastigias papua* Lesson, 1830	n. d.	√	Spermatozeugmata	Uchida 1926
		Phyllorhiza punctata von Lendenfeld, 1884	n. d.	√	Spermatozoa + spermatozeugmata	Rippingale & Kelly 1995; Rouse & Pitt 2000
	Cepheidae	*Cephea cephea* Forskål, 1775	n. d.	√	n.d.	Sugiura 1966
		Cotylorhiza tuberculata Macri, 1778	√	√	Spermatozeugmata	Avian 1986; Kikinger 1992
Daktyliophorae	Catostylidae	*Catostylus mosaicus* Quoy & Gaimard, 1824	×	√	Spermatozoa	Pitt 2000; Pitt & Kingsford 2000; Rouse and Pitt 2000
	Lychnorhizidae	*Lychnorhiza lucerna* Haeckel, 1880	√[1]	×	Spermatozoa	Present work
	Rhizostomatidae	*Rhizostoma octopus* Macri, 1778	√[1]	×	n.d.	Holst et al. 2007
		Rhizostoma pulmo Macri, 1778	n. d.	×	Spermatozoa	Paspaleff 1938; Widersten 1965
		Rhopilema esculentum Kishinouye, 1891	n. d.	×	n.d.	Ding & Chen 1981; Dong et al. 2006
		Rhopilema nomadica Galil et al., 1990	n. d.	×	n.d.	Lotan et al. 1992; Lotan et al. 1994
		Rhopilema verrilli Fewkes, 1887	n. d.	√	n.d.	Cargo 1971; Calder 1973
	Stomolophidae	*Nemopilema nomurai* Kishinouye, 1922	√[1]	×	Spermatozoa	Kawahara et al. 2006; Ohtsu et al. 2007; Iguchi et al. 2010; Toyokawa et al. 2010; Ikeda et al. 2011
		Stomolophus meleagris L. Agassiz, 1862	n. d.	×	n.d.	Calder 1982

[1]Only detectable in sexually mature specimens; n.d.: no available data in literature; √: present; X: absent. Classification from Mianzan & Cornelius (1999).

1991; Kikinger 1992; Eckelbarger 1994a, b). Oocytes originate within the gastrodermis, increase in size, and bulge into the mesoglea, retaining contact with the germinal epithelium through trophocytes; this connection is not found among coronates (Eckelbarger & Larson 1992; Eckelbarger 1994a; Morandini & Silveira 2001), except in the deep water species *Periphylla periphylla* Péron & Lesueur, 1810 (Tiemann & Jarms 2010). Oogenesis is stopped at prophase I after completion of vitellogenesis in the majority of species. Where known, oogenesis is basically similar in all Discomedusae and our description of *L. lucerna* follows the same pattern observed in other studied species (under light microscopy).

Widersten (1965) proposed that fertilization may occur within the ovary; however, it was always assumed that oocytes complete their development outside the gonad, either in the gastric cavity, canals of the mouth arms, or the surrounding water (Arai 1997). Kevin Eckelbarger (pers. comm.) is convinced that developed oocytes pass through the gastrodermis at the point they were attached. Moreover, Ohtsu et al. (2007), from in vivo examination of gonads, reported that oocytes are squeezed out through pits in the ovarian epithelium. Our observations agree with the conclusions of Ohtsu et al. (2007), and support Eckelbarger's assumption; we observed that oocytes of *L. lucerna* are squeezed out through a pit in the gastrodermis at the point they were in contact with trophocytes. The same observations were also reported by Toyokawa et al. (2010) and Ikeda et al. (2011) in studies of *Nemopilema nomurai* Kishinouye, 1922. Tiemann & Jarms (2010) found an aperture in the genital epithelium of *P. periphylla* which they defined as a gamete-releasing pore. Although our observation could have been an artefact caused by the standard paraffin histology method we used (Eckelbarger & Larson 1992), the ovary is a blind sac and there must be a naturally occurring break (mechanical or chemical) at some place of the ovarian epithelium allowing oocytes to be shed. Therefore, we assumed that the oocytes are released from the ovary through a pit opening naturally at the weakest point of the gastrodermis, just at the place where oocytes are in contact with trophocytes.

There are two current views that try to explain the evolutionary lineage of rhizostome scyphozoan jellyfishes. The first presents the order Rhizostomeae as a non-monophyletic group, with two main divisions (Thiel 1970; Straehler-Pohl 2009). One group more related to Cubomedusae called Cepheida/Cassiopeida (also known as Kolpophorae) (Straehler-Pohl 2009, figure 84), in which members produce spermatozeugmata and brood larvae. The other group more related to part of the semaeostome family Ulmaridae (genus *Aurelia*) called Rhizostomida (also known as Daktyliophorae), in which members rarely produce packages of spermatozoa and brood larvae. When considering this view as a working hypothesis, there may be one inconsistency: members of the genus *Aurelia* are the only semaeostome group that brood larvae (Widersten 1965).

The second view is based on the traditional hypothesis (e.g. Thiel 1966) expressed here based on the most recent molecular phylogeny of the Scyphozoa (Bayha et al. 2010). The authors present a different topology for the families, and the main difference is that the order Rhizostomeae is considered a monophyletic group (except their figure 5B). Based on that hypothesis, we can look at the spermatozeugmata production and brooding capabilities as derived characters (except for the species *Rhopilema verrilli* Fewkes, 1887 and *Catostylus mosaicus* Quoy & Gaimard, 1824 regarding the brooding capacity).

In general there is a trend among semaeostome scyphozoan jellyfish to increase the complexity of the gastrovascular system from basal to derived clades (from Pelagiidae to Ulmaridae). The same can be observed regarding brooding capacities and internal fertilization in the Pelagiidae where species of the genus *Chrysaora* may release gametes and larvae into the water column or inside the female's body (e.g. Widersten 1965; Morandini et al. 2004; Widmer 2008). Cyaneidae, e.g. the genus *Cyanea*, release male gametes and fertilization occurs inside female body (Widersten 1965); larvae can be found on the oral arms, but no brooding structures exist. Although the life cycle of the monospecific Phacellophoridae (*Phacellophora camtschatica* Brandt, 1838) was recently described (Widmer 2006), no information about brooding and release of gametes is available. Ulmaridae species, e.g. the genus *Aurelia*, release only male gametes; fertilization occurs within the gastrovascular system of females which brood larvae in the oral arms (Widersten 1965; Russell 1970). In this article, we are not interested in presenting evidence supporting either view, but interestingly, our observations (summarized in Table II) can be used in favour of both hypotheses with the appropriate explanations.

Sexual maturation in Scyphozoa

Population features of sexual reproduction are defined by characterizing and quantifying individuals at different stages of sexual maturity. In Scyphozoa, the process of sexual maturation has been divided into a variable number of stages depending on criteria and goals adopted by various authors.

The simplest approach classifies medusae into immature (unripe) and mature (ripe) based on absence or presence of evidences of sexual reproduction as indicated by existence of fertilized eggs, embryos or planulae carried by females (e.g. Kikinger 1992; Rippingale & Kelly 1995; Lucas & Lawes 1998). Other than differences in terminology, Brewer (1989) also detected sexual reproduction by the presence of blastulae carried by females, but he included the stages 'maturation' (medusae bearing gonads, but lacking blastulae) and 'deterioration' (with spawning finished and medusae dying or already dead). Although simple and effective, this methodology can only be applied to brooding species only where proof of sexual reproduction can be found in females. Conversely, in non-brooding medusae, fully developed gametes are immediately shed into the sea after spawning (Ohtsu et al. 2007; Iguchi et al. 2010; Ikeda et al. 2011). Alternatively, the classification of maturation stages of scyphomedusae has been undertaken based on their gametogenic status (proportion of gametes at different stages of development). In this way, gametogenic stages are defined according to oocyte diameter or yolk density (Rottini Sandrini & Avian 1991; Pitt & Kingsford 2000; Jarms et al. 2002; Iguchi et al. 2010; Toyokawa et al. 2010) and by the presence of spermatozoa within the spermatic follicles (Pitt & Kingsford 2000; Iguchi et al. 2010; Toyokawa et al. 2010).

In rhizostomes and semaeostomes, gametogenesis is an asynchronous process because gametes at all stages of development occur simultaneously in both sexes (Rottini Sandrini & Avian 1991; Lucas & Lawes 1998; Pitt & Kingsford 2000; Iguchi et al. 2010). In this regard, Pitt & Kingsford (2000) established a maturation scale for *Catostylus mosaicus* based on different percentages in the development of oocytes (previtellogenic, early–mid vitellogenic, and late vitellogenic oocytes) and follicles with and without spermatozoa. However, this scale is not suitable for other rhizostomes such as *Lychnorhiza lucerna* and *Nemopilema nomurai* (Schiariti 2008; Iguchi pers. comm.) and particularly for *L. lucerna*, as no sexually mature specimens were detected by applying the Pitt and Kingsford approach, leading to a misinterpretation of the sexual reproduction patterns of this species (Schiariti 2008).

Specifically, Pitt & Kingsford (2000) stipulated that a ripe medusa presents more than 15% of late-vitellogenic oocytes (O*iii*), or more than 70% of the spermatic follicles containing spermatozoa. For females, the estimation of the proportion of O*iii* included all gametogenic stages (*pre*, O*i*, O*ii*, O*iii*) (Pitt pers. comm.). However, Schiariti (2008) observed that the percentage of *pre* in *L. lucerna* exceeds other stages of oocyte development. Thus, if we include this early cellular stage in the estimation of O*iii*, none of the females attained the threshold established by Pitt & Kingsford (2000) as sexually mature. On the other hand, ripe males were distinguished by Pitt & Kingsford (2000) based on the percentage of follicles containing spermatozoa (SZ). This means that spent follicles, lacking spermatozoa (already shed), were not included in the estimation of the threshold percentage from where males are considered as ripe. Many of *L. lucerna* males examined by Schiariti (2008) bore a high percentage of spent follicles, so that the percentages of ripe males could have been strongly underestimated by excluding them as evidence of sexual maturity.

Later, Toyokawa et al. (2010) classified medusae of *N. nomurai* into maturity indexes defined only by the presence (not percentages) of gametes at different stages of development. This approach has the advantage of classifying medusae into different maturation stages avoiding quantification of gametes. However, Toyokawa et al. (2010) omitted spent follicles when classifying males into immature and mature stages. For females they established the most advanced maturation stage as 'ovulating' referring to females having oocytes being shed as by us for *L. lucerna* (Figure 2c). However, Toyokawa et al. (2010) used standard paraffin histological methods which physically disrupts the delicate oocyte–trophocyte junctions, and probably caused developing oocytes to break free from the gonad (Eckelbarger & Larson 1992). Therefore, the number of 'ovulating' females estimated from such samples is questionable.

Like Toyokawa et al. (2010), Iguchi et al. (2010) described maturation stages of *N. nomurai* considering the most advanced gametogenic stage for each individual as the maturation stage. They also observed fully developed gametes (oocytes and spermatozoa) already shed into the subgenital sinus. While these observations constitute clear evidences of spawning, gametes are thought to flow into the sea immediately and were not considered as proofs of sexual maturity (Iguchi et al. 2010). This reproductive process in all non-brooding species, together with problems related to standard paraffin histology techniques, complicates the study of sexual reproduction patterns in such medusae.

Whereas ripe brooders are recognized by the presence of their 'products of sexual reproduction', sexual reproduction in non-brooders must be detected indirectly by looking for evidence of spawning. Although proof of sexual maturity, the presence of spawned gametes within females or males is not sufficient for characterization of sexual reproductive

patterns. Indeed, observation of oocytes squeezing out of the gonads from examination of histological slides does not solve the problem either. However, spent follicles constitute a 'spawning print' that can be utilized as evidence of sexual maturity. On the other hand, we assume oocytes with onset of vitellogenesis will finish the process and become ready for fertilization within a relatively short period (see Ohtsu et al. 2007; Ikeda et al. 2011). Therefore, in agreement with Iguchi et al. (2010), we suggest that fully developed gametes can be used as evidence of sexual maturity, indicating ongoing or imminent spawning events.

In summary, a complete picture of sexual reproduction patterns in scyphozoan populations can be achieved by defining maturation stages of each medusa on the basis of gametogenic status. Within these stages, sexually mature medusae can be detected by (i) the presence of fully developed gametes in the gonads or (ii) by the presence of spent spermatic follicles. Whereas the former permits greater insights into sexual reproduction patterns of any species, the latter constitutes an option for non-brooders equivalent to the search for fertilized eggs or planulae for brooder species.

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