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The reproductive pattern of the white barrel jellyfish *Rhizostoma pulmo* (Cnidaria, Scyphozoa) in the gulf of Taranto (Ionian Sea)

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Abstract

Understanding the mechanisms that lead to the proliferation of large jellyfish populations over time is crucial to prevent and mitigate any possible negative impacts on human activities and ecosystem functioning. *Rhizostoma pulmo* (Macri, 1778) is an outbreak-forming species of the Mediterranean Sea that has been observed in anthropized areas including the Gulf of Taranto. To describe seasonal changes in the reproductive patterns of *R. pulmo* from May to December 2016, we measured jellyfish diameter, the number and diameter of oocytes using histological and biochemical analyses (lipids, carbohydrates, proteins, and organic matter), as well as the gonadal somatic index (GSI). Jellyfish size did not fluctuate considerably during the collection period, with specimens measuring between 19.5 and 38.2 cm in diameter. Our findings revealed a strong gonadal activity over the summer and a spawning event between September and October. Male and female gonad morphology was similar to other previously reported Rhizostomeae. Oocytes were categorized into three developmental classes, recognizable by their diameter and shape: <15 µm for pre-vitellogenesis, 15-30 µm for vitellogenesis, and >30 µm mature oocytes. The number of mature oocytes peaked in August, with smaller eggs recorded in May and October and larger eggs recorded near the end of the summer. The GSI fluctuated greatly between sampling dates, peaking in August. Lipid levels varied significantly throughout the sampling period, with the greatest value in May (277.26±98 µg lipid mg AFDW⁻¹) and the lowest in September (70.36±41.5 µg lipid mg AFDW⁻¹). Carbohydrates, proteins, and organic matter (62 ± 6 %) remained stable during the sampling period. The biochemical analyses of jellyfish sexual reproduction we report on here can be used as a tool for identifying jellyfish spawning periods and better understanding the reproductive process that control jellyfish population dynamics.

Keywords: Reproductive cycle; Mediterranean Sea; Outbreaks; Biochemical composition; Lipids; Rhizostomeae.

Introduction

Jellyfish have rapid and seasonal population variations, which frequently result in blooming events when environmental conditions are favorable (reviewed by Bayha & Graham, 2014). However, in the last decade, climate change and human activities including overfishing, shipping, and habitat transformation could have boosted jellyfish natural proliferation (Purcell *et al.*, 2007; 2012).

Jellyfish influence nutrient cycling in ecosystems through their population dynamics (boom and bust), serving as carbon sinks during bloom/outbreaks periods (Condon *et al.*, 2011). Because of their high predatory pressure (e.g., fish eggs and larvae), when they are abundant, they cause a cascade effect on lower trophic level animals. They also have an impact on the pelagic food web by

overlapping diets and competing with zooplanktivorous fish, limiting resources for higher trophic levels (Purcell & Arai, 2001). Large jellyfish populations can also have an impact on coastal human activities like fishing and tourism, as well as pose a health risk (De Donno *et al.*, 2014; Bosch-Belmar *et al.*, 2017). This becomes particularly relevant when outbreaks appear. *Rhizostoma pulmo* (Macri, 1778), often known as “white barrel” or “sea lung,” is an outbreak-forming species that has increased in abundance throughout the Mediterranean basin in recent years (Leoni *et al.* 2021a). It is the largest endemic Mediterranean jellyfish, with outbreaks throughout the Mediterranean Sea, including the Adriatic, Ionian Sea, and the Tunisian shelf, Western and Eastern basin (reviewed by Mariottini & Pane, 2010; Leoni *et al.*, 2021a). This species has been seen in coastal marine environments with substantial an-

thropogenic impact, such as the Mar Menor coastal lagoon (Fuentes *et al.*, 2011), the Gulf of Trieste, and the Gulf of Taranto (Boero *et al.*, 2016). In Apulia, in the southwestern part of the Gulf of Taranto, the species was known to occur en masse already in 2005, when tourist resorts first adopted anti-jellyfish nets to protect bathers (Piraino, unpublished observation) and with swarms occurring from April to December, with the highest concentration during summer-autumn (from July to October) (Basso *et al.*, 2019; Stabili *et al.*, 2020), reaching up to 48,000 ind/km² during outbreaks (Leone *et al.*, 2015). These huge outbreaks have a severe influence on both tourists and fisheries, obstructing nets (Fuentes *et al.*, 2011). *R. pulmo* has also well-established positive ecological impacts. In addition to being a food source for marine species, it provides a safe shelter for juvenile stages of commercial fish such as *Trachurus mediterraneus* (Steindachner, 1868) (Hays *et al.*, 2018; Mir-Arguimbau *et al.*, 2019).

Rhizostoma pulmo has a life cycle similar to other Scyphomedusae, alternating between sexual and asexual generations. Adult jellyfish (pelagic) reproduce sexually by releasing gametes into the water column. Following fertilization, the newly produced larvae (planulae) settle and metamorphose into polyps, which reproduce asexually by strobilation, budding, and podocysts formation (Fuentes *et al.*, 2011). Long-term data reveal that *R. pulmo*'s recent increase in outbreaks frequencies is linked to positive temperature anomalies, implying that the species prefers warm water (Leoni *et al.*, 2021a). Furthermore, the white barrel jellyfish is very resistant to salinity and temperature fluctuations (Fuentes *et al.*, 2011). Temperature also regulates its life cycle, promoting budding and strobilation (Purcell *et al.*, 2012), as has been shown in other Rhizostomeae (Schiariti *et al.*, 2014). In addition to its increasing abundance, *R. pulmo* medusae season has increased from 5 to 7 months, most likely due to factors affecting early life stages and a longer adult survival period (Leoni *et al.*, 2021a).

During the jellyfish outbreak (July), lipidic extracts of female gonads of *R. pulmo* exhibit high contents of fatty acids, amino acid and osmolytes, known to perform important roles in marine biota (Angilè *et al.*, 2020). Understanding *R. pulmo*'s life cycle and reproduction strategies is thus a priority in order to predict abrupt outbreaks, devise mitigation and management techniques to prevent the potential detrimental effects of its outbreaks on human activities and the marine ecosystem.

The biochemical composition of jellyfish gonads can be used to understand seasonal energy inputs (Rossi *et al.*, 2017), and determine a species' nutritional status. More specifically, macromolecules such as carbohydrates or lipids can be used as an indirect measure of a species' ability to survive the reproductive phase (Rossi *et al.*, 2017). In this paper, we examine the reproductive cycle of *R. pulmo* in the Gulf of Taranto (Ionian Sea), a highly anthropized site in southern Italy. Through frequent sampling, histological analyses of female gonads, an assessment of number and diameter of oocytes, measurement of the gonad somatic index and biochemical analyses, we characterize *R. pulmo*'s spawning periods and investigate the energetic potential invested into reproduction.

Material and Methods

Sample collection

Sampling was conducted in the Gulf of Taranto (northwestern Ionian Sea, Southern Italy). Jellyfish were randomly collected. During the field sampling, *R. pulmo* specimens appeared homogeneous in size, seemingly belonging to the same cohort. Between May and December 2016 in six sampling times, 18 adult female jellyfish (based on gonad color and laboratory testing) were captured at depths ranging from 1 to 3 meters. In addition, a few adult males were harvested to study the male gonad morphology. During each sampling, at least three females and few male jellyfish were captured, rapidly rinsed with 0.2 µm-filtered seawater (FSW) and stored in a chilled box at 5°C during transfer to the laboratory.

Sample processing

In the laboratory, female jellyfish were washed in sterile FSW, examined under a stereomicroscope, and their diameters were measured. The gonads were carefully surgically removed from the umbrella using forceps and a scalpel. For each jellyfish, one piece of gonads was preserved for histology, while two pieces of gonads were washed with double-distilled water, frozen in liquid nitrogen, and stored at -80 °C before freeze-drying for biochemical analysis (lipids, carbohydrates, organic matter, and proteins).

Histological analysis

A piece of gonad from each *R. pulmo* specimen (n=18 female gonad parts from 18 individual medusae) was removed and dehydrated with a series of increasing ethanol concentrations (80% to 100%), then cleared in xylene (histological grade) and impregnated in paraffin (56-58 °C). The tissue was embedded in paraffin, sectioned at 0.7 µm using a Leica microtome (RM 2155), and stained with toluidine blue.

Oocytes count and measurement

Histological sections were studied using a light microscope (Nikon Eclipse 50i, Tokyo, Japan) outfitted with a camera and image processing software. The oocyte count was performed on ten sections per specimen (n=18 female jellyfish). All visible oocytes were counted using a manual counter in a 3 mm² region of the ovary. Each slice had 10 oocytes measured along a line (100 oocytes per specimen) from a random starting point. The mean diameter (d) was calculated according to Szafranski *et al.*, 2014 by using the formula:

$$d = \sqrt{l \cdot w}$$

where "l" is the length of oocytes (i.e., the major diame-

ter) measured in μm , and “w” is the width of oocytes (i.e., minor diameter) measured in μm .

Gonad somatic index

The gonad somatic index (GSI) is commonly utilized to assess the seasonality of the reproductive cycle (Byrne, 1990). Here, it was calculated for 3 jellyfish for each sampling date. Jellyfish were dissected under a stereomicroscope and gonads were frozen in liquid nitrogen and stored at -80°C . After freeze-drying, gonads were weighed and the GSI was computed as follows:

$$\text{GSI} = \frac{\text{Gonad dry weight}}{\text{Whole body dry weight}} \times 100$$

Biochemical analysis of the gonads

Total lipid content

Total lipids were quantified following the protocol reported in Milisenda *et al.* (2018) for 18 female jellyfish. Approximately 10 mg of gonads were grinded and the powder was resuspended in 3 mL of chloroform: methanol (2:1) following Barnes & Blackstock procedure (1973) (colorimetry). A calibration line was made using cholesterol as a standard and the final reaction was performed using vanillin. The measurements were taken using a spectrophotometer (UV mini1240, Shimadzu) at $\lambda = 520\text{nm}$. The lipid content is expressed in $\mu\text{g mg}^{-1}$ AFDW (i.e., ash free dry weight).

Carbohydrate content

Total carbohydrates were determined in gonads in each sampling time. Approximately 10 mg of samples dry weight (DW) were homogenized in 3 mL of distilled water following Dubois *et al.* (1956), modified by Rossi *et al.* (2006b) for cnidarians. A calibration line was made prior the samples measurements using glucose as a standard. Measurements were read using a spectrophotometer (UV mini1240, Shimadzu) using $\lambda = 480\text{nm}$. Carbohydrates are expressed in $\mu\text{g carbohydrates mg}^{-1}$ AFDW.

Proteins

Protein content was estimated by colorimetric determination ($\lambda = 750\text{nm}$) using approximately 10 mg of samples DW, homogenized in 2 mL of 1N NaOH following Lowry *et al.* (1951). Albumin was used as a standard. The protein content is expressed as $\mu\text{g mg}^{-1}$ of AFDW.

Organic matter

A fraction of the dry weight of each sample was heated for 4 h at 450°C . Ashes were re-weighted in order to

calculate the ash free dry weight as: $\text{AFDW} = \text{DW} - \text{AW}$ (ash weight).

Statistical analysis

One-way permutation univariate analysis of variance (PERMANOVA) was used to test monthly differences in jellyfish size, egg abundance and size and Gonadal Somatic Index (GSI). In addition, differences in organic matter and protein, carbohydrate and lipid contents of jellyfish gonads were investigated by using univariate and multivariate PERMANOVAs (Anderson, 2001). The analyses were based on Euclidean distances on untransformed data, using 9999 random permutations of the appropriate units (Anderson & Braak, 2003). The analyses were performed using PRIMER v. 6 software (Clarke & Gorley, 2006) including the PERMANOVA + add-on package (Clarke & Gorley, 2006; Anderson, 2008). All data are expressed as mean \pm SD.

Results

Size distribution

Jellyfish sizes (bell diameter) did not vary significantly during the sample period (Table 1, Fig. 1). The biggest individuals sampled seemed to appear in late summer reaching 39 cm, while the smallest were observed during spring with the smallest diameter of 16.9 cm.

Male and female gonads

Our histological analysis shows that male gonads contain sperm follicles, where spermatogenesis takes place. Some specimens were immature male while others were mature and spermatozoa with flagellum were visible inside the follicles (Fig. 2A, B). In the final stage of spermatogenesis, the aboral part of the follicle (facing gastrodermis) is filled by mature sperms (Fig. 2B) that are then released, through a pore, in the genital sinus (Fig. 2C).

Female gonads (Fig. 2D, E, F) have a convoluted shape. Pre-vitellogenic oocytes are embedded in the gastrodermis and are characterized by a round shape (Fig. 2F). As oogenesis proceeds, oocytes increase their size; vitellogenic oocytes are round shaped and contain yolk granules and a germinal vesicle facing the gastrodermis, containing a nucleolus (Fig. 2F). Late-vitellogenic /mature oocytes are lighter in color due to the increased yolk content and are characterized by a large germinal vesicle (Fig. 2D, E, F).

According to the description above, the oocytes were grouped into 3 size classes based on their diameter: <15 μm = oocytes in pre-vitellogenesis; 2) 15-30 μm = oocytes in vitellogenesis; 3) >30 μm mature oocytes

Table 1. Results of One-way PERMANOVA comparing the jellyfish size, egg number, egg size, gonad index (GI), together with the biochemical composition and organic matter of *R. pulmo* by month in the Gulf of Taranto across the sampling times. df - degrees of freedom; MS - mean squares; Pseudo-F - F critic; P(perm) - permutational level of probability; Perms - number of unique permutations; TI - sampling time; Res - residual. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns - not significant.

| Jellyfish Size | | | | | | OM | | | | |
|----------------|----|-------|----------|----------|-------|----|-------|----------|----------|-------|
| Source | df | MS | Pseudo-F | P (perm) | Perms | df | MS | Pseudo-F | P (perm) | Perms |
| TI | 5 | 32.84 | 1.32 | ns | 7089 | 5 | 39.43 | 0.98 | ns | 9947 |
| Res | 12 | 24.89 | | | | 12 | 40.41 | | | |
| Total | 17 | | | | | 17 | | | | |

| Egg number | | | | | | Carbohydrate | | | | |
|------------|-----|----------|----------|----------|-------|--------------|--------|----------|----------|-------|
| Source | df | MS | Pseudo-F | P (perm) | Perms | df | MS | Pseudo-F | P (perm) | Perms |
| TI | 5 | 2.69E+05 | 172.76 | *** | 9953 | 5 | 631.44 | 2.02 | ns | 9952 |
| Res | 174 | 1555.3 | | | | 12 | 311.96 | | | |
| Total | 179 | | | | | 17 | | | | |

| Egg size | | | | | | Lipid | | | | |
|----------|------|--------|----------|----------|-------|-------|--------|----------|---------|-------|
| Source | df | MS | Pseudo-F | P (perm) | Perms | df | MS | Pseudo-F | P(perm) | Perms |
| TI | 5 | 5698.4 | 38.71 | *** | 9950 | 5 | 17820 | 5.70 | ** | 9957 |
| Res | 1794 | 147.19 | | | | 12 | 3124.1 | | | |
| Total | 1799 | | | | | 17 | | | | |

| GSI | | | | | | Protein | | | | |
|--------|----|-------|----------|----------|-------|---------|-------|----------|----------|-------|
| Source | df | MS | Pseudo-F | P (perm) | Perms | df | MS | Pseudo-F | P (perm) | Perms |
| TI | 5 | 26.26 | 5.87 | * | 9947 | 5 | 46035 | 2.95 | ns | 9952 |
| Res | 12 | 4.472 | | | | 12 | 15583 | | | |
| Total | 17 | | | | | 17 | | | | |

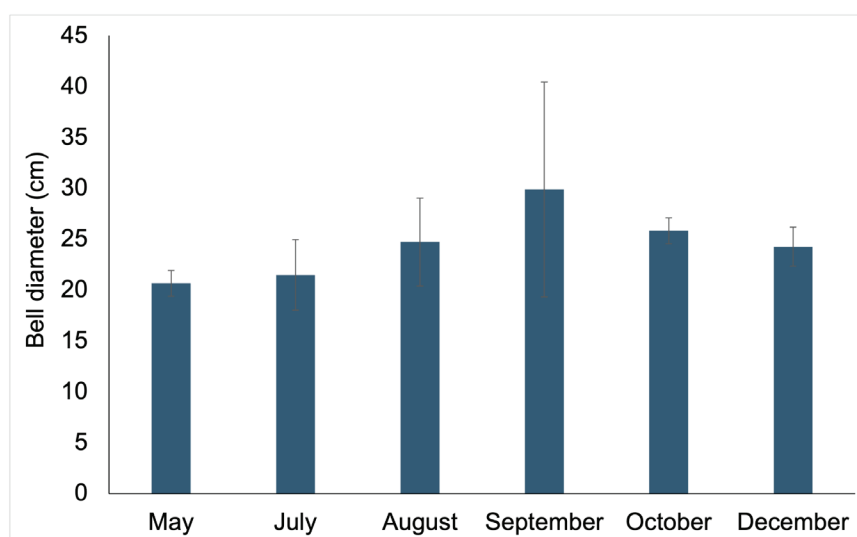


Fig. 1: Jellyfish diameter during the sampling period. Data represent mean and SD.

Histological analysis (number and size)

The number of oocytes fluctuated during the sampling interval (Table 1). In particular, lower quantities of oocytes were observed in May and October while the peak was reached during August (Fig. 3). The maximum number of oocytes found was 101/mm², while the minimum was 15.81/mm².

Oocytes at different stages of maturation were ob-

served during the sampling period and within each sampling month, as shown by temporal variations of jellyfish oocyte diameter (Table 1). The largest eggs (i.e., diameter length) were observed at the end of summer (35±14 μm) reaching a maximum value of 67.33 μm. Conversely, May and October were characterized by the smallest average egg sizes, 23±10 μm and 26±14 μm respectively, with a minimum value of 5.37 μm (Fig. 4A).

Size class distribution followed a similar trend to that

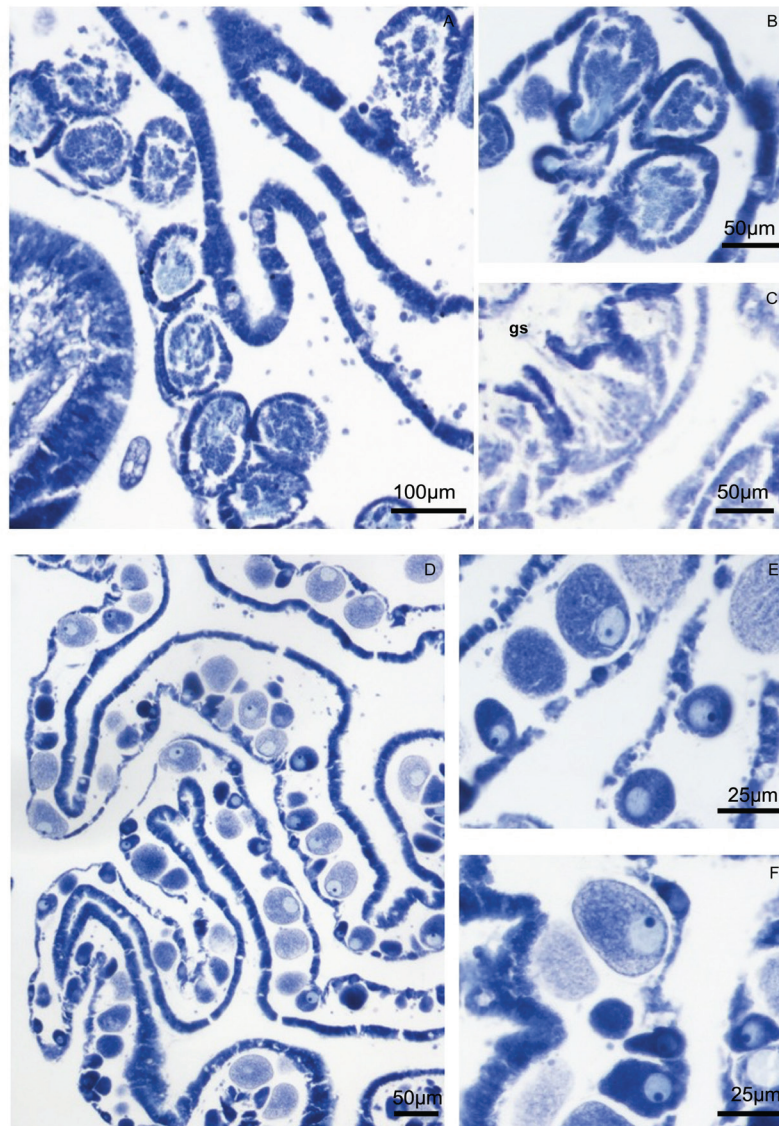


Fig. 2: Histological section of *R. pulmo* gonads. **A** Shows a view of the male gonad. **B** Sperm follicles with mature spermatozoa. **C** Follicle during a spawning event. **D** General view of a female gonad. **E, F** Oocytes at a different stage of maturation; gs: genital sinus.

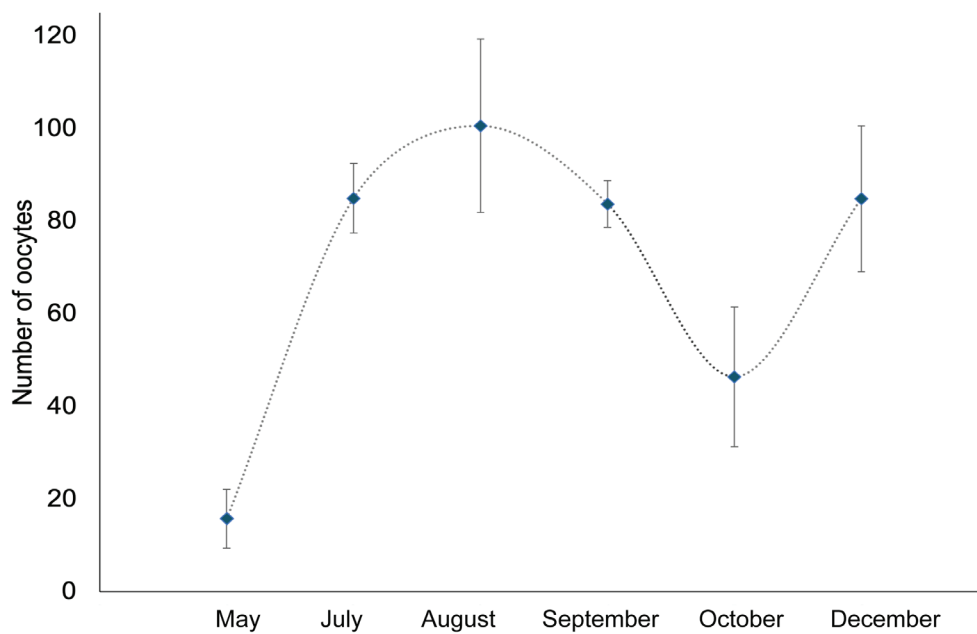


Fig. 3: Number of oocytes in female specimens along the sampling period. The number of oocytes refers to an area of 1 mm².

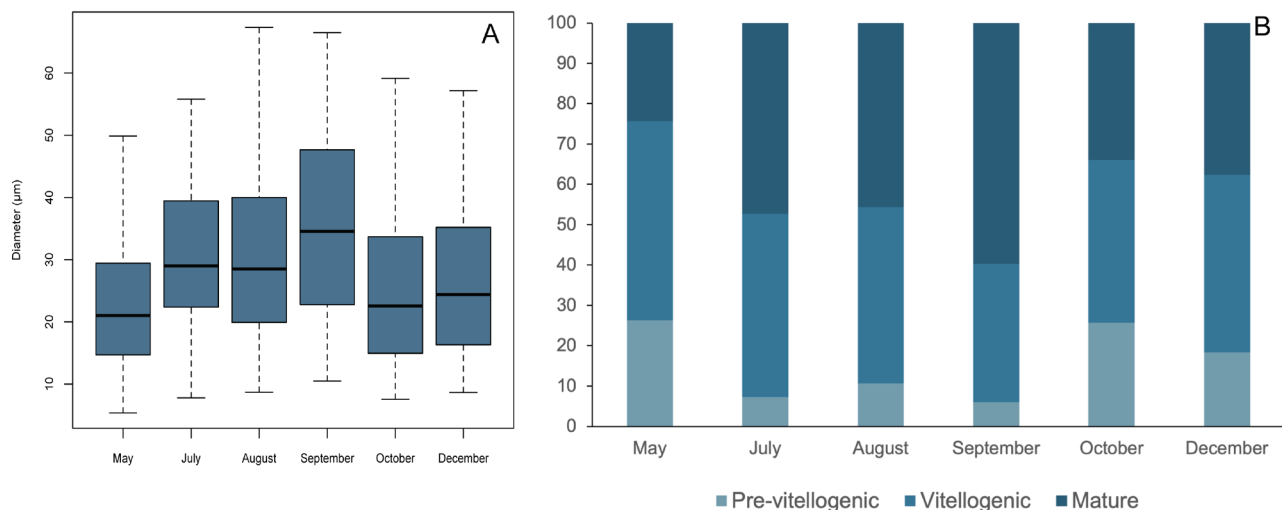


Fig. 4: Oocytes diameter distribution. **A** Oocytes diameter during the sampling period. **B** Percentage of each size class during the sampling period.

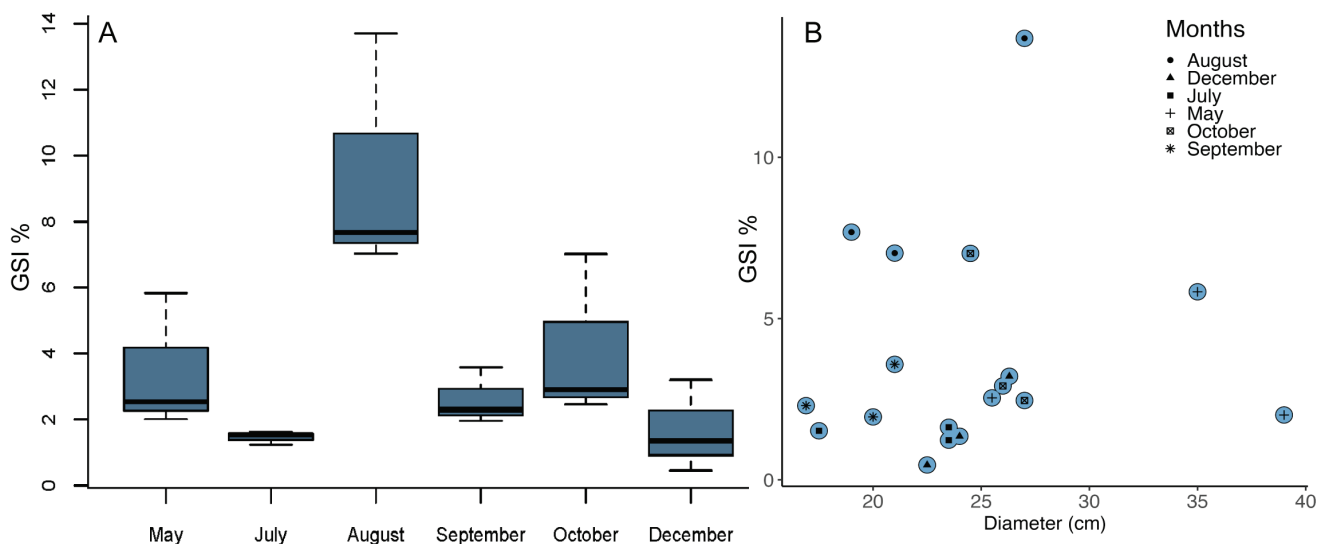


Fig. 5: Gonad somatic index. **A** Percentage of the gonad somatic index during the sampling period. **B** Relationship between gonad somatic index (GSI) and jellyfish bell diameter.

of the oocyte diameter. Mature oocytes were most abundant the end of summer (less pre-vitellogenic oocytes), with the lowest abundance occurring in May and October, when higher pre-vitellogenic oocytes presence was observed (Fig. 4B).

Gonad-somatic index

Temporal fluctuations in GSI values were detected by the PERMANOVA analysis (Table 1). The GSI value fell in a range between 0.46 and 7.02 % during the duration of our investigation. In August, it reached the highest values between 7.03 and 13.69 % (Fig. 5). No significant correlation was found between GSI and jellyfish bell diameter ($R^2=0.0268$; $p<0.05$).

Biochemical analysis (lipids, carbo, proteins, and OM)

Significant fluctuations were found in gonad lipid composition throughout the sampling period. Lipids were higher in May ($277.26\pm98 \mu\text{g lipid mg AFDW}^{-1}$), while they reached the lowest value in September ($70.36\pm41.5 \mu\text{g lipid mg AFDW}^{-1}$) (Fig. 6).

The statistical analysis on carbohydrates and proteins did not show significant differences across the sampling times. Carbohydrates did not show large fluctuations during the investigated sampling period (Fig. 6); the highest carbohydrate concentration was found in May ($90.21\pm22.3 \mu\text{g carbohydrates mg AFDW}^{-1}$), the lowest in October ($53.21\pm10.4 \mu\text{g carbohydrates mg AFDW}^{-1}$). Protein content was high in September and October ($1000.66\pm133.2 \mu\text{g proteins mg AFDW}^{-1}$). In addition, organic matter did not vary significantly throughout the sampling period covering a range between $62 \pm 6 \%$.

Looking at the overall biochemical analysis, multivariate PERMANOVA showed significant changes in the

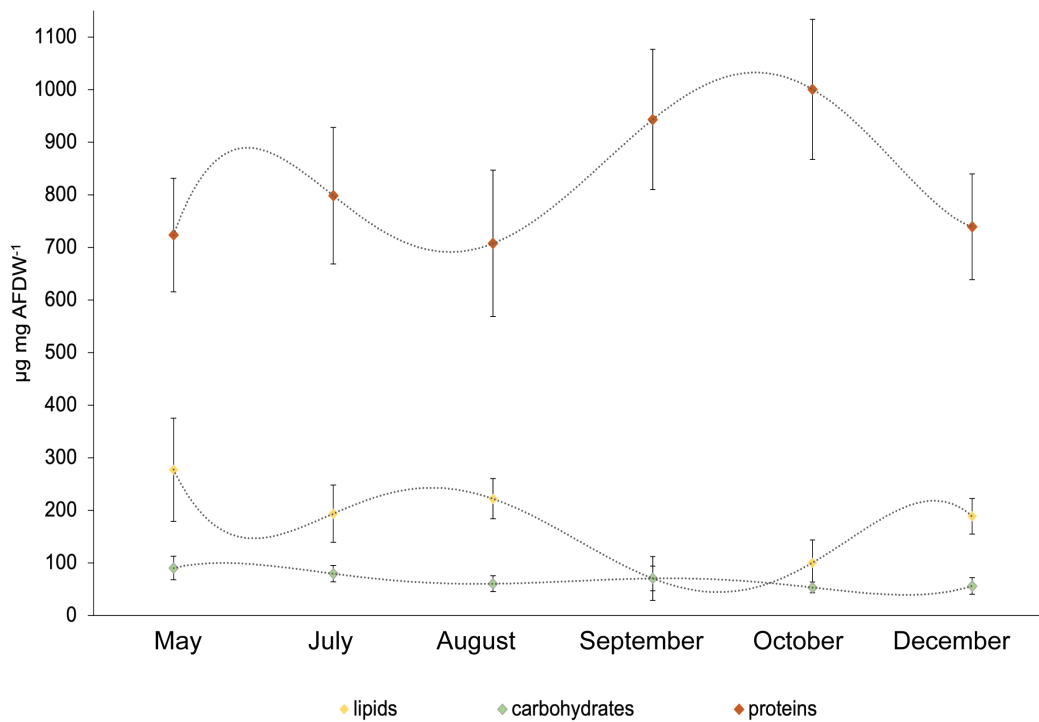


Fig. 6: Biochemical analysis of *R. pulmo* gonadal tissue showing the variation of lipids, carbohydrates, and proteins along time. Lipids, and carbohydrates are expressed in $\mu\text{g mg AFDW}^{-1}$. Data represents mean and SD.

Table 2. Multivariate PERMANOVA comparing the biochemical composition of jellyfish gonad in *R. pulmo* across the sampling times. df - degrees of freedom; MS - mean squares; Pseudo-F - F critic; P(perm) - permutational level of probability; TI - sampling time; Res - residual; Perms - number of unique permutations. * = $p < 0.05$.

| Source | df | MS | Pseudo-F | P (perm) | Perms |
|--------|----|-------|----------|----------|-------|
| TI | 5 | 64487 | 3.39 | * | 9939 |
| Res | 12 | 19019 | | | |
| Total | 17 | | | | |

biochemical composition of jellyfish gonad in *R. pulmo* across the sampling times (Table 2).

Discussion

In this paper, using an integrated approach that combines histological and biochemical analyses, we study the scyphozoan *Rhizostoma pulmo*'s intra-annual changes in gonad content and investigate its energetic investment in reproduction.

We show that the diameters of *R. pulmo* ranged from 16.9 to 39 cm, in accordance with prior observations in the same area (Leone *et al.*, 2015; De Domenico *et al.*, 2019; Basso *et al.*, 2021). *R. pulmo* individuals typically grow following a Von Bertalanffy Growth Function (Fernández-Alías *et al.*, 2020; Leoni *et al.*, 2021b), and the average size of the individuals within the population increase with time (Fernández-Alías *et al.*, 2023). Although jellyfish size did not change significantly throughout the sampling months, larger specimens of *R. pulmo* were de-

tected during the spawning period. Data collected from *R. pulmo* in the Black Sea, showed a seasonal change in jellyfish diameter ranging between 14.5 to 42.5 cm (corresponding to August and October peak) (Dönmez & Bat, 2019). Data from other outbreak-forming species, such as *Pelagia noctiluca* and *Aurelia coerulea* von Lendenfeld, also show seasonal variation in sizes. In particular, *A. coerulea*, endemic to the Mediterranean lagoons, has varied size classes throughout the year, with larger specimens found in September (Scorrano, 2014; Fernández-Alías *et al.*, 2023; Marques *et al.*, 2015). *Pelagia noctiluca* is characterized by a jellyfish cohort (6-8 cm diameter) present the whole year, and two more cohorts with smaller and bigger jellyfish that appear from February to May. This period (spring) is also distinguished by *Pelagia* spawning events, which are characterized by larger specimens (>120mm) (Milisenda *et al.*, 2018).

We also show that *R. pulmo* is a gonochoric species, with oocytes and sperm follicles evident in the gonads of several individuals. During spermatogenesis, spermatocytes develop near the follicle wall and mature within

the follicular cavity. Male spawning occurs once mature through the release of free spermatozoa in the genital sinus (Fig. 2C), as seen in other rhizostomeae (Schiariti *et al.*, 2012), although other species, such as *Cassiopea andromeda*, spawn via spermatozeugmata (i.e., packages of spermatozoans) (Hofmann, 1996; Mammone *et al.*, 2023). Once the sperms have been discharged, the follicle enters the spent phase (post-evacuation), which is distinguished by open spaces in the lumen (Schiariti *et al.*, 2012). All sexually mature males have spent follicles, which indicate a “spawning print”. However, it remains unclear whether spermatogenesis resumes and further investigations with male *R. pulmo* specimens should be conducted to gain a fuller picture of the species’ reproduction.

Histological slides show that in *R. pulmo*’s female gonads, oogonia can form anywhere in the endoderm, without a centrifugal maturation gradient (i.e., oogonia form only in the proximal portion of the endoderm) as previously observed by Avian & Sandrini (1991). This is also a characteristic of *A. aurita*, but unlike other outbreaks-forming species like *P. noctiluca*. *R. pulmo* spawning involves the gastrodermis to thin and fenestrate, allowing the release of oocytes, followed by external fertilization (Holst *et al.*, 2007; Hamner & Dawson, 2008).

Female specimens from the Gulf of Taranto displayed oocytes from pre-vitellogenesis to mature, reaching a maximum diameter (of 67.3 μm) during late summer, smaller (150-180 μm) than previously recorded by Avian & Sandrini (1991). Mature oocytes were smaller than those of other Mediterranean and outbreak-forming jellyfish, such as *P. noctiluca* (250-320 μm) (Ramondenc *et al.*, 2019), *A. aurita* (160-180 μm) (Avian & Sandrini, 1991), and *C. andromeda* (140 μm) (Mammone *et al.*, 2023).

In both male and female specimens, gametes were present at various stages of maturation, indicating an asynchronous mechanism similar to that observed in rhizostomes and semaeostomes (Avian & Sandrini, 1991; Schiariti *et al.*, 2012; Mammone *et al.*, 2023).

In female specimens, oocytes were detected throughout the sampling period, with increased gonadal activity throughout the summer. The number of oocytes is highly variable in August and October, with the lowest fluctuation in May. Other outbreaks-forming species, such as *P. noctiluca*, produce oocytes throughout the year, with two distinct peaks in the spring and late summer, just before spawning (Milisenda *et al.*, 2018). *R. pulmo* and *P. noctiluca* appear to use a semi-continuous reproductive strategy, with an increased gonadal activity as temperatures rise. Furthermore, our findings reveal an increase in egg numbers in late fall. Recent studies revealed the presence of mature specimens during the winter, supporting the hypothesis that rising temperatures affected not only abundance and ontogenic rate, but also the duration of the medusae season, which for *R. pulmo* increased from 5 to 7 months (2008-2018) (Fuentes *et al.*, 2011; Leoni *et al.*, 2021b).

Female gonad maturity can be estimated using egg

diameter as a proxy (Toyokawa *et al.*, 2009). Eggs in various stages of maturation were found throughout the sampling period, suggesting an iteroparous or seasonal iteroparous reproduction strategy, as seen in other rhizostomeae such as *C. andromeda* (Mammone *et al.*, 2023) and outbreaks -forming species such as *P. noctiluca* (Canepa *et al.*, 2014; Milisenda *et al.*, 2018). Our results for the percentage of each size class reveal a trend of more pre-vitellogenic oocytes in May and October. Furthermore, the mean egg diameter is reduced throughout these months, which coincides with a decline in oocyte abundance, possibly indicating either the commencement of a new gametogenic cycle for the examined population, or a cohort replacement (Fernández-Alías *et al.*, 2020, Leoni *et al.*, 2021b). Some reproduction phases can be identified in *R. pulmo*, just as they are in *P. noctiluca*. Indeed, moments with a higher ratio of vitellogenic eggs represent the moment of maximal gamete differentiation; lower egg number and higher pre-vitellogenic represent the spawning period/beginning of the gametogenic cycle, while the remainder of the sampling period represents an intermediate situation (Milisenda *et al.*, 2018). Comparing the phases of these two species, we note that, in addition to the occurrence of these distinct phases, *R. pulmo* appears to have a later spawning season than *P. noctiluca*, which is most likely due to the two species’ different life cycles and environmental conditions in the sampled region.

To assess the spawning period, also the morphometric index (GSI) was utilized. It indicates the energy investment during gonadal development, which is related to environmental parameters such as temperature and food resources. Our findings reveal that the highest value of *R. pulmo* GSI was seen in August, indicating an increase in energy directed toward gonadal growth rather than somatic tissue in preparation for a subsequent spawning episode (late summer). Our findings support Fernández-Alías *et al.* (2020) conclusion that gonadal development is seasonal and that is influenced by favorable environmental conditions rather than bell size, since no correlation between bell diameter and GSI is observed. *R. pulmo* GSI appears to be consistent with the peak of oocyte quantity but not with the fraction of higher developed eggs.

The GSI of the outbreaks-forming Mediterranean jellyfish *P. noctiluca* reaches the peak in May, however it is not observed to be associated to its peak in oocyte abundance recorded in April (Milisenda *et al.*, 2018).

Macromolecules such as lipids and carbohydrates are trackers of metabolic processes that occur over a short period of time, potentially affecting animal fitness and reflecting moments of food scarcity (Rossi *et al.*, 2006a). Furthermore, lipid-carbohydrate storage enables to better comprehend a species’ energetic investment in reproduction. Our data show a high proportion of *R. pulmo* gonads organic matter ($62 \pm 6\%$), similar to what was discovered by Stabili *et al.* (2018) where the organic matter percentage was around $60 \pm 10\%$, and in accordance with other outbreaks-forming species such as *P. noctiluca* (41-59%) (Milisenda *et al.*, 2018).

R. pulmo jellyfish has a greater dry weight percentage (29.5 ± 6.6) compared to outbreaks-forming species such as *Aurelia* sp.1 (23.9 ± 3.3) (Leone *et al.*, 2015). The biochemical composition was dominated by protein, followed by lipids and carbohydrates, which confirmed previous observations by Stabili *et al.* (2018) in other specimens of *R. pulmo* as well as in other outbreaks-forming jellyfish such as *P. noctiluca* (Milisenda *et al.*, 2018). Despite their huge size, the dry weight of most rhizostomeae is primarily constituted of proteins, with lipids and carbohydrates accounting for small components (Leone *et al.*, 2013; Lucas, 2009).

Protein concentrations were high ($> 600 \mu\text{g mg AFDW}^{-1}$), consistent with earlier data (761.76 ± 25.11 ; Stabili *et al.*, 2018), but significantly lower than in *P. noctiluca* (max: 187.83 ± 6.38 ; Milisenda *et al.*, 2018). Overall, our findings are consistent with biochemical composition changing with reproductive cycle time, diet, and seawater temperatures (Lucas & Lawes, 1998; Roff, 1992).

Lipids varied significantly during the sampling period, beginning in May and peaking in August, coinciding with the peak of oocyte quantity. Lower lipid content was observed between September and October, consistent with a decline in oocyte abundance and supporting the idea of a spawning event at this time of year. The peak of lipids observed in August is consistent with the reproductive peak, further testifying that lipids are the most effective macromolecules in terms of energy storage. The lipid content ($70\text{-}277 \mu\text{g mg AFDW}^{-1}$) was consistent with prior observations for this species (Stabili *et al.*, 2018), and greater than the lipid content of whole jellyfish reported by Leone *et al.* (2015).

Our results demonstrate a steady carbohydrate content (53.21 ± 10.43 to $90.21 \pm 22.33 \mu\text{g mg AFDW}^{-1}$) across the whole period, indicating a continuous energy source for the specimens. Similar values were obtained in other *R. pulmo* specimens (59.66 ± 2.72) sampled by Stabili *et al.* (2018). Our results highlight no substantial difference in organic matter content across the sampling period, as seen in other species such as *P. noctiluca* (Milisenda *et al.*, 2018).

Conclusion

Rhizostoma pulmo is the largest outbreaks-forming species, which can affect the environment and produce socioeconomic problems in various areas, such as on tourism (e.g., stings) or fishing (e.g., clogging nets). Moreover, *R. pulmo* appears to be benefiting from climate change, although the temperature is not the only factor influencing the population dynamics.

Thus, studying its inter-annual changes can provide vital insights for predicting potential outbreaks. Our findings revealed a strong gonadal activity over the summer and a spawning event between September and October. The number of oocytes peaked in August, and larger eggs were detected near the conclusion of the summer, whereas May and October had the smallest eggs on average.

These findings were backed by biochemical data, which showed significant variation in lipid levels during the sample period, with the greatest value occurring in May. Overall, our findings suggest that precise knowledge on the barrel jellyfish's life cycle should be prioritized in order to assist in predicting large outbreaks and devising potential ways to control their spread.

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