





## Article

# Histological Investigation of the Female Gonads of *Chiropsalmus quadrumanus* (Cubozoa: Cnidaria) Suggests Iteroparous Reproduction

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**Abstract:** The box jellyfish *Chiropsalmus quadrumanus* (Chirodromida: Cubozoa: Cnidaria) is common in warm waters. Although it is assumed that external fertilization is a characteristic of Chirodromida, the life history of *C. quadrumanus* is not yet known since its reproductive behavior has never been described, nor has the polyp has been found in nature. As a result, in the absence of documentation of reproductive behavior, we sought to test the hypothesis of external fertilization through a histological analysis of the female gonads. Herein, we analyze ten females collected in São Paulo and Rio de Janeiro (Brazil) and describe the gonadal organization and pattern of oocyte development. The discovery of four distinct stages of oocyte differentiation augments the scant existing reports of the structural and functional maturation of sex cells in Cubozoa species. Furthermore, the gonads of mature females comprise both mature (average diameter of 122  $\mu$ m) and immature oocytes, suggesting that *C. quadrumanus* is iteroparous and exhibits multiple reproductive cycles during its life. Medusa bell size was not found to correlate with maturity state as even small females possessed a high percentage of oocytes in late vitellogenesis, suggesting that sexual maturation occurs rapidly in *C. quadrumanus* females.

**Keywords:** oocyte; medusa; box jellyfish; fertilization; oogenesis; differentiation gradient; seasonality



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## 1. Introduction

Reproductive studies in Cubozoa (box jellyfish) are hampered because reports of their jellyfish (cubomedusae) or medusoid form often refer to sightings of a single individual [1] or a limited (seasonal) reproductive period [2,3], and many species are notoriously venomous to humans, making cubomedusae challenging to collect [4]. Cubozoans, like many medusozoans (jellyfishes), have complex metagenetic life cycles, metamorphosing from a sessile polyp stage (asexual larval stage) into the characteristic, free-swimming medusa (sexual adult stage) [5–7]. Asexual polypoid reproduction is favorable under certain environmental conditions because it allows for a rapid increase in the number of clonal medusozoan individuals; this is also the case for other bi- or multiphasic marine invertebrates [8,9]. Meanwhile, in marine invertebrates, sexual reproduction, which generates genetic novelties via mixing genotypes, is triggered by specific environmental

and genetic factors [8], many of which are undetermined. Unfortunately, female gonadal maturation (oogenesis), reproductive strategies and dynamics, and fertilization modes are particularly understudied in cubozoans [10]. This lack of knowledge limits actions to monitor cubozoan populations with the aim of managing the influence of cubomedusae on tourism and recreation while at the same time considering biodiversity conservation and public safety.

The accumulated knowledge on the sexual reproduction of cubomedusae is derived from seminal lab studies on five species of Carybdeida (*Alatina alata* (Reynaud, 1830) [3,11,12]; *Carybdea marsupialis* (Linnaeus, 1758) [13]; *Copula sivickisi* (Stiasny, 1926) [12,14–17]; *Morbakka virulenta* (Kishinouye, 1910) [18]; *Tamoya haplonema* F. Müller, 1859 [19,20]) and two species of Chirodropida (*Chironex fleckeri* Southcott, 1956 [21,22], and *Chiropsalmus quadrumanus* (F. Müller, 1859) [19]). The sexual behaviors of a few species have been described from the field (e.g., [3,23,24]) or from tank observations (e.g., [15,25]), proving the difficulty of performing in situ observations as cubomedusae are active swimmers [26,27] with long tentacles and the ability to avoid obstacles [27] using their complex eyes [28,29]. Accordingly, only *Tripedalia cystophora* Conant, 1897, a mild stinger and the second smallest cubomedusan species, has been reared to sexual maturity in vitro (cf. [30]). Therefore, mechanisms of gametogenesis and reproductive behaviors in this small class are often inferred via methods of histomorphology rather than relying exclusively on in situ observations.

The mode of fertilization in cubomedusae can be external or internal depending on the species, but external fertilization is considered plesiomorphic in marine invertebrates (cf. [31]). External fertilization is considered typical for Chirodropida based on scant reports for just two species, *C. fleckeri* [21] and *C. quadrumanus* [19]. In contrast, internal fertilization is considered a synapomorphy of Carybdeida [2] and is well documented for two species of Tripedaliidae (*C. sivickisi* and *T. cystophora*) that exhibit complex reproductive behaviors involving spermatophore transfer [14–16,23,32,33]. However, reports of external fertilization for *M. virulenta* (Carukiidae) [18] cast doubts on the universality of internal fertilization in Carybdeida. Regarding periodicity, most cubomedusae are considered seasonal spawners based on the few reports of reproductive events witnessed in the field (e.g., *C. marsupialis* [34]; *C. fleckeri* [35]; *C. sivickisi* [15]). However, the scattered knowledge about carybdeid species makes it difficult to establish either semelparity or iteroparity as a universal pattern at the level of class or even order. For example, *A. alata* medusae reproduce during monthly spermcasting aggregations in which the entire gonad tissue ruptures and the reproductive cells are released [36]; it may be unique among cubozoans as a semelparous species (for references on iteroparous cubomedusae, see [19,20]).

In many gonochoristic marine organisms, sexual dimorphism can be exhibited in the form of marked phenotypic differences between males and females (e.g., in body size, color, and shape), but a disproportionate focus on a handful of “model” species has led to a skewed representation of the underlying mechanisms of the evolution of different modes of reproduction (reviewed by [8]). In cubozoans, although all species are gonochoristic, only species of Tripedaliidae exhibit sexual dimorphism with respect to gonadal shape, development, and/or color (e.g., *C. sivickisi* [15–17,37,38] and *T. cystophora* [23,32,37]). During gametogenesis, male and female cubomedusae develop their gonads (defined as the “area where gametes are formed” by [39], p. 142) from endodermal tissue in the bell [39–43]. In most cubomedusae, subtle phenotypic variation between males and females typically occurs at the level of reproductive tissues and in germ cell morphology [19,37,44]. However, reports of sexually dimorphic gonadal morphologies and patterns of gametogenesis are limited to cubomedusae that exhibit internal fertilization and exhibit sperm “packet” transfer [12], suggesting the potential for gonad morphology to infer reproductive modes in Cubozoa.

Recent histomorphological studies have elucidated spermatogenesis in several species of cubomedusae [19,20], but studies concerning oogenesis are limited to *C. marsupialis* [13] and *C. sivickisi* (as *Carybdea sivickisi* [15,17]). Most recently, oogenesis was reported in *Carybdea murrayana* Haeckel, 1880 (as *Carybdea branchi* [45]) from southern African waters,

and a “maturation scale” for female sex cells was established for the first time in the class. During the process of vitellogenesis, oocytes accumulate yolk protein granules and subsequently increase in diameter. Oocyte maturation patterns corresponded to significant egg size differences documented during the oogenesis of a single cubozoan species, *viz.* *C. murrayana* [45]. Nevertheless, scarce information on egg size and its relation to cubomedusae maturity precludes the ability to establish a baseline of sexual maturation related to reproductive season for the 50 estimated species.

Herein, we report on the hitherto obscure reproductive strategy of the chirodropid species *C. quadrumanus* (Chirodropida, Chiropsalmidae), a relatively common species in the western tropical Atlantic from Brazil to the USA [46–50]. *Chiropsalmus quadrumanus* is represented by conspicuous cubomedusae (with a bell height of 10 cm and a width of 12 cm on average) which are poorly studied; for instance, a mere three sightings have been reported in the literature in the past decade [19,20,51]. This species’ mode of fertilization was previously suggested to be external [19,20], but no evidence supports this claim as the life cycle and cubopolyp location in nature remain undetermined. Herein, we carry out a histomorphological analysis to elucidate an oocyte structural maturation “scale” in a chirodropid species for the first time while aiming to infer the sexual reproductive strategy and contemplate previous reports speculating on the fertilization mode of this species. We also refute the hypothesis that sexual maturity in all cubomedusae can be inferred accurately by bell size [15,16], as our outcomes for *C. quadrumanus* fail to align with previous findings on carybdeid species.

## 2. Materials and Methods

### 2.1. Material Samples

Medusae of *C. quadrumanus* have been collected in the field at typical marine ecosystem salinities (20–30‰) and at shallow depths ranging from 5 m to 10 m. In the northern hemisphere, specimens have been found during the summer (May–August) [52,53] and sometimes in the fall (September) (e.g., Matagorda Bay, Texas [54]). Conversely, in the southern hemisphere (e.g., Brazil), specimens have been collected during the winter, in the dry season (July–August) [20], and sometimes during spring (March–April) [19].

In this study, we analyzed 10 females (Table 1) collected via trawling from a depth of 5 m to 40 m during the dry season (April to September) in 2008, 2010, and 2014. The specimens were collected from the São Sebastião Channel (São Paulo State, *n* = 2), Santos Bay and São Vicente (São Paulo State, *n* = 2), and Macaé (Rio de Janeiro State, *n* = 6) (Table 1). The medusae were fixed in 10% formaldehyde solution in seawater. The bell height (BH, from the apex of the umbrella to the margin) and interpedalial distance (IPD, distance along the bell margin between alternate pedalia) were measured for all specimens (cf. [45,55]) (Figure 1A) (Table 1). All necessary approvals for the sampling of specimens were obtained (sampling permit 16802 SISBIO/ICMBIO—Instituto Chico Mendes de Conservação da Biodiversidade). The specimens from Macaé were deposited in the collection of the Museum of Zoology (MZ), and the remaining specimens were deposited in the Marine Evolution Lab (MEL), both belonging to the University of São Paulo (Table 1).

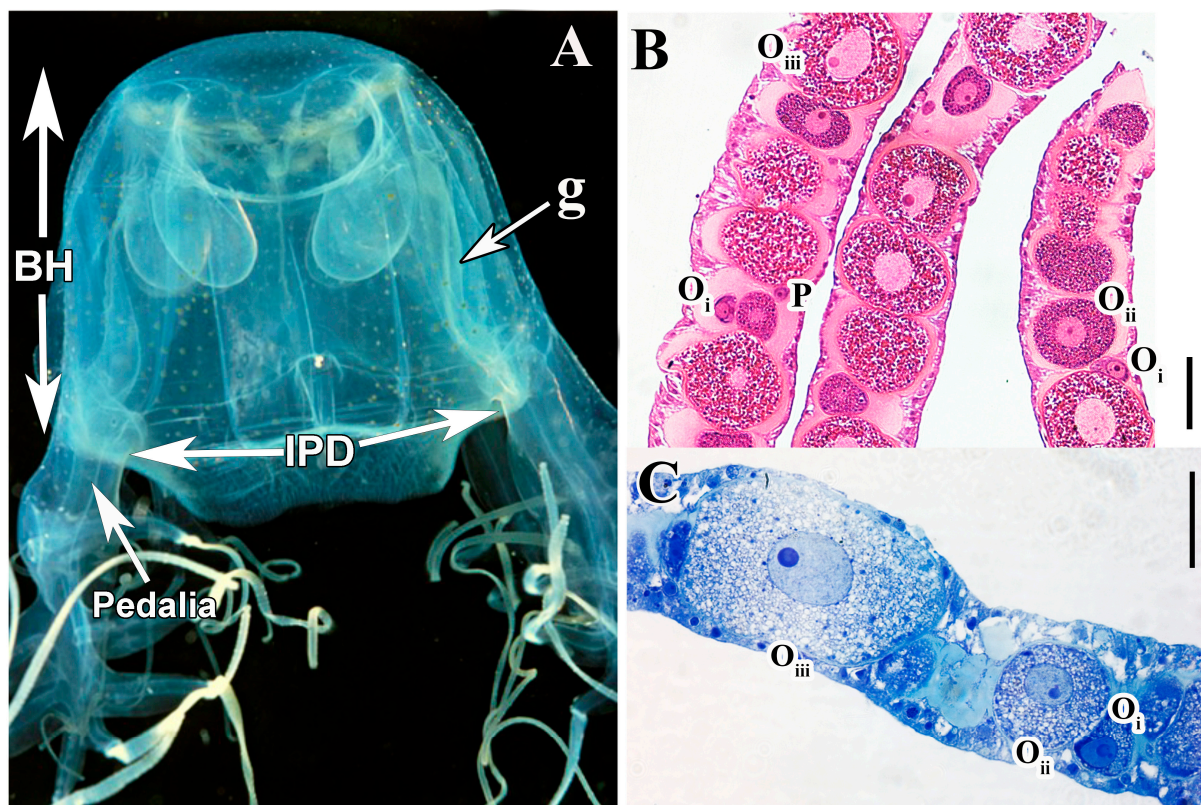
**Table 1.** Material data for *Chiropsalmus quadrumanus* females examined in this study. Abbreviation: MEL: Marine Evolution Lab; MZUSP: Museum of Zoology of the University of São Paulo; RJ: Rio de Janeiro; SP: São Paulo.

Specimen Number	Museum Number (MZUSP) and MEL Number	Bell Height (mm)	Interpedalial Distance (mm)	Locality	Depth (m)	Date (D/M/Y)	Collector
CH2	MEL-CH2	4.5	3.8	São Sebastião Channel (SP)	10	10 June 2014	JGR
CH6	MEL-CH6	6.5	4.7	São Sebastião Channel (SP)	10	19 August 2014	JGR



Table 1. Cont.

Specimen Number	Museum Number (MZUSP) and MEL Number	Bell Height (mm)	Interpedalial Distance (mm)	Locality	Depth (m)	Date (D/M/Y)	Collector
MA49	MZUSP-1920	6.5	4.5	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA50	MZUSP-1921	7.5	5	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA52	MZUSP-1923	6.5	4.4	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA53	MZUSP-1924	7	4.7	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA56	MZUSP-1927	7	5	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
MA57	MZUSP-1928	5.4	4	Macaé (RJ)	5–10	13 September 2008	F.P.L. Marques
53A	MEL-53A	6.7	10.54	Baía de Santos e São Vicente (SP)	10–40	April 2010	Tiseo, G. R.; Zara, F. J.
54C	MEL-54C	5.6	8	Baía de Santos e São Vicente (SP)	10–40	April 2010	Tiseo, G. R.; Zara, F. J.



**Figure 1.** (A). *Chiropsalmus quadrumanus* live specimen [20]. (B,C). Gonadal transversal sections of females of *C. quadrumanus* from São Sebastião Channel bearing oocytes in the four different stages of development. (B). Specimen (CH6, Table 1) stained with hematoxylin–eosin (HE). (C). Specimen (CH2, Table 1) stained with toluidine blue (TB). Scale bars 50  $\mu$ m. BH—bell height; IPD—interpedalial distance; g—gonads; O<sub>iii</sub>—late-vitellogenic oocyte; O<sub>ii</sub>—mid-vitellogenic oocyte; O<sub>i</sub>—early-vitellogenic oocyte; P—pre-vitellogenic oocyte.

## 2.2. Maturity of Specimens

*Chiropsalmus quadrumanus* has eight hemi-gonads arranged in four pairs, with each pair located at interradial septa [50]. Mature female medusae were distinguished based on the microscopic observation of oocytes, characterized by white spheres in the female gonads. Sexual maturity was inferred by the presence of more than 15% of gonadal oocytes in the late vitellogenic stage (O<sub>iii</sub>) (cf. [45,56]). We propose a maturity scale for the different oocyte developmental states observed in our samples, following the scale proposed for *Carybdea murrayana* (as *Carybdea branchi* [45]), based on oocyte diameter and quantity of yolk.

## 2.3. Histological Analysis

Several fragments over the entire length of each gonad were dissected individually using forceps. The samples were fixed with formalin 4%, dehydrated in an ethanol series, and embedded in glycol methacrylate using the Leica Historesin Embedding Kit (Leica Microsystems Nussloch GmbH, Germany), following the manufacturer's protocol. The embedded material was cut into 5 µm transversal sections with a Leica RM2255 microtome, with ca. 12 sections set per slide (ca. 60 µm of gonad tissue); each gonad subsample was accommodated on 6–8 slides (i.e., ca. 480 µm of gonadal tissue). The slides were stained with hematoxylin–eosin (HE), toluidine blue (TB) and Gomori's trichrome (GT) (according to [57–62]) and cover-slipped using Entellan. The slides were subsequently analyzed using a Zeiss Axio Imager.M2 microscope, images enhanced with Adobe Photoshop CC 2017 version 24.0.1, and the oocytes were measured using the software ImageJ 1.53s [63].

This study is the first reported attempt to document oogenesis in jellyfish using a glycol methacrylate (historesin) method. This methodology has been successfully used in previous studies on other taxonomic groups [62,64] to obtain high-quality results.

## 2.4. Statistical Analysis

Several slides per specimen (ca. one third) were selected to measure the oocytes with a 4x objective lens on a compound microscope, and 12 oocytes (per tissue sample) were measured for each of the four established oogenesis stages. The maximum and minimum oocyte diameters of each specimen were averaged, and the standard deviations were calculated (Table 2). Average diameters for each stage were compared using the R package dplyr (v.1.0.7, function “summarise”) to discriminate between the different maturation stages based on size, and the results were visualized using R package ggplot2 (v.3.3.3). One slide per specimen was selected to calculate the percentage of oocytes present at each stage of oogenesis. Furthermore, evidence for a correlation between cubomedusa size (interpedalial distance) and maturity (percentage of oocyte stage) was tested using the Spearman correlation method with the R package ggpubr (v.0.4.0, function “ggscatter”). The code is available as Supplementary Material.

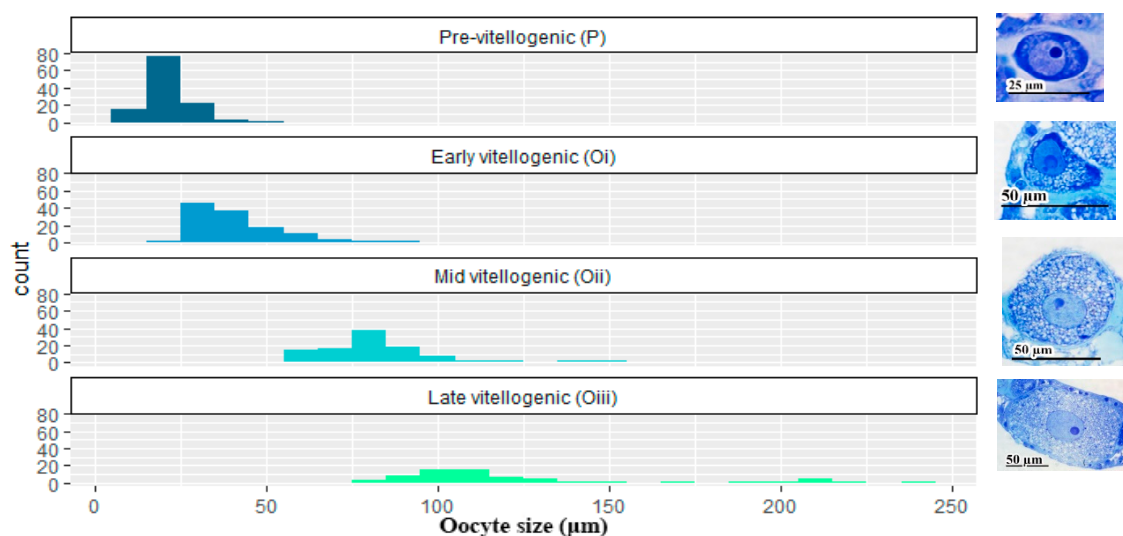
**Table 2.** Measurements of oocyte diameter (maximum, minimum, average, and standard deviation) in each stage of gonadal development of *Chiropsalmus quadrumanus*. Blank cells indicate no oocytes of that specific size category were observed in the sample. Oiii—late-vitellogenic oocyte; Oii—mid-vitellogenic oocyte; Oi—early vitellogenic oocyte; P—pre-vitellogenic oocyte.

Specimen Number	Oiii Diameter (μm)				Oii Diameter (μm)				Oi Diameter (μm)				P Diameter (μm)			
	Max	Min	Average	Standard Deviation	Max	Min	Average	Standard Deviation	Max	Min	Average	Standard Deviation	Max	Min	Average	Standard Deviation
CH2	140.014	76.292	110.796	22.54687	75.617	52.308	62.1977	6.2079	48.703	16.492	28.484	7.81417	31.68	11.544	20.5662	5.98748
CH6	235.397	146.857	197.781	23.70315	170.486	99.398	132.068	22.297	92.352	49.656	69.34	12.3727	53.582	13.919	28.242	13.5341
MA49					83.02	58.667	74.9411	7.3262	61.273	34.359	46.186	8.19608	26.104	16.271	20.8476	3.6763
MA50					98.593	70.107	85.8052	8.959	58.029	31.074	42.923	8.00621	37.838	14.411	24.7551	7.08516
MA52	131.602	96.736	114.187	10.50545	97.173	65.079	81.6818	11.168	59.958	26.203	38.339	10.4808	27.778	13.889	20.9081	4.47119
MA53	113.525	95.89	103.493	5.407642	87.199	60.336	76.8158	8.5688	41.82	27.397	33.926	4.1092	34.013	13.784	20.6236	5.4142
MA56					119.398	75.18	90.0476	13.532	63.774	26.698	43.078	10.8529	27.68	10.99	20.4653	5.60585
MA57									47.507	33.333	38.761	4.44204	23.292	10.623	16.8126	4.18917
53A	122.144	89.443	101.199	10.10663	91.952	56.063	79.3338	8.9946	57.007	25.298	37.208	10.46	24.828	14.717	19.9075	3.43497
54C	131.966	82.262	99.711	15.20876	88.511	62.639	74.9315	8.3674	61.837	27.402	39.457	9.03138	29.829	14.583	20.7193	5.1459

### 3. Results

In *Chiropsalmus quadrumanus*, oogenesis presented a pattern of increased size and yolk density during the process of sexual maturation. Though late-vitellogenic oocytes were large (on average, 122  $\mu\text{m}$  in diameter), all gonads were intact with no sign of ovulation underway in the cubomedusae examined.

Four different oocyte states (Figures 2 and 3) are defined for *C. quadrumanus* females according to their diameter and vitellogenic content, following the scale previously established by [45]: (1) pre-vitellogenic oocytes (p), round-shaped, average diameter of  $21 \pm 6.88 \mu\text{m}$ , without vitellogenic content and basophil cytoplasm; (2) early-vitellogenic oocytes ( $O_i$ ) with some yolk granules, average diameter of  $42 \pm 13.5 \mu\text{m}$ ; (3) mid-vitellogenic oocytes ( $O_{ii}$ ), average diameter of  $84 \pm 21.6 \mu\text{m}$ ; (4) late-vitellogenic oocytes ( $O_{iii}$ ), rich in yolk granules, average diameter of  $122 \pm 39.1 \mu\text{m}$ . The average oocyte size differs significantly among the stages ( $\chi^2 = 355.82$ ;  $df = 3$ ;  $p < 2.2 \times 10^{-16}$ ), although their ranges overlap (Figure 2).

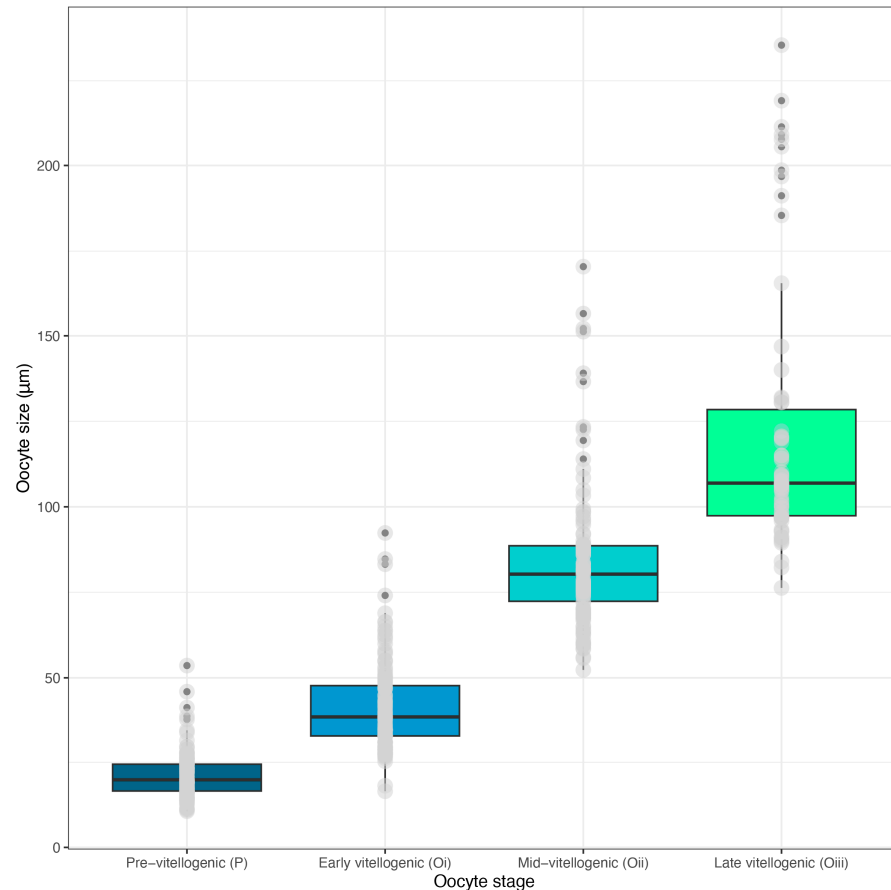


**Figure 2.** Distribution of oocyte diameter for each of the four stages of development in *C. quadrumanus*. Images on the right reveal morphological variations in oocyte features, including vitellogenic content.

Oogenesis in *C. quadrumanus* was found to be asynchronous, and oocytes of all four stages of development (p– $O_{iii}$ ) were observed in almost all specimens analyzed (Figure 1B,C and Table 2). All the specimens possessed pre-vitellogenic oocytes, but late-vitellogenic oocytes ( $O_{iii}$ ) were not present in four specimens from Macaé, suggesting that they were immature despite their relatively large bells (IPD 4–5 mm). Conversely, a small specimen (IPD 3.8 mm) from the São Sebastião Channel had late-vitellogenic oocytes ( $O_{iii}$ ), suggesting a non-linear relationship between sexual maturity and bell size. Although all females examined possessed gonads, the maximum oocyte diameter observed for each of the four stages varied between specimens collected at different geographic localities, with maximum diameters of 235  $\mu\text{m}$  for the São Sebastião Channel and 113  $\mu\text{m}$  for Macaé, despite the similarity of the bell sizes (IPD 4.7 mm) (Table 2).

Female gonads are composed of an epithelial bilayer corresponding to the external gastrodermal epithelium and an internal gonadal layer within the mesoglea composed of oocytes (Figure 4A). Epithelial gonadal cells are columnar with basal vacuoles (Figure 4B). While there is no developmental gradient seen along the length of the gonad, oocytes increase in size due to the accumulation of yolk (vitellogenesis) during maturation (Figures 1 and 2). Histological cross-sections revealed that late-vitellogenic oocytes ( $O_{iii}$ ) were found across the entire length of the gonad within the gonadal epithelium (Figure 1C). Neither trophocytes (specialized nutritive gastrodermal cells) nor nurse cells were found in contact with the oocytes and gastrodermal epithelium (Figures 1 and 4), suggesting

that mature oocytes develop freely in the mesoglea (in direct contact with the gastrodermis) (cf. [65]). However, we also report here that some specimens did show inclusions of unknown natures within the oocytes (Figure 5).



**Figure 3.** Boxplot representing the four stages of development based on the oocyte diameter of *Chiropsalmus quadrumanus*.

We defined mature females by the presence of gonads with >15% of oocytes in the late-vitellogenic stage [45]. Accordingly, there were four females with this pattern; therefore, they were considered sexually mature (Table 3). Although the ten female specimens had IPD values  $\geq 4.4$  mm, not all were found to be mature. Two specimens from Macaé (IPDs = 4.5 and 5 mm) had no late-vitellogenic oocytes. One specimen from Macaé (MA57, IPD = 4 mm) had pre-vitellogenic (14%) and early-vitellogenic (86%) oocytes, representing the most immature female studied herein (Figure 4). The Spearman correlation test indicates that the bell size of *C. quadrumanus* is not correlated with the proportion of late-vitellogenic oocytes present ( $r = 0.019$ ;  $p = 0.96$ ).

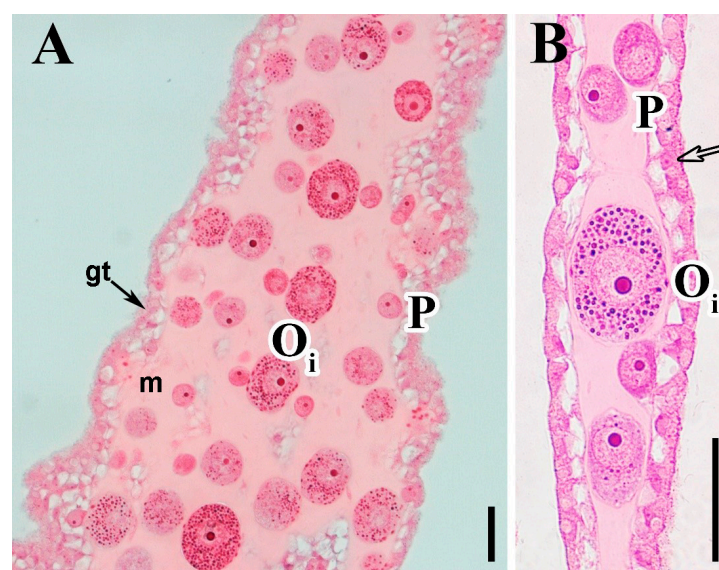
**Table 3.** Percentage of oocytes in the four different stages of the oogenesis of *Chiropsalmus quadrumanus*. \* Mature females; IPD—interpedalial distance (mm); Oiii—late-vitellogenic oocyte; Oii—mid-vitellogenic oocyte; Oi—early-vitellogenic oocyte; P—pre-vitellogenic oocyte.

Specimen number	IPD	Oiii%	Oii%	Oi%	P%	Sampling Month
CH2	3.8	6.45	45.16	41.94	6.45	June/2014
CH6 *	4.7	37.68116	27.53623	24.63768	10.14493	August/2014
MA49	4.5	0	8.474576	83.89831	7.627119	September/2008

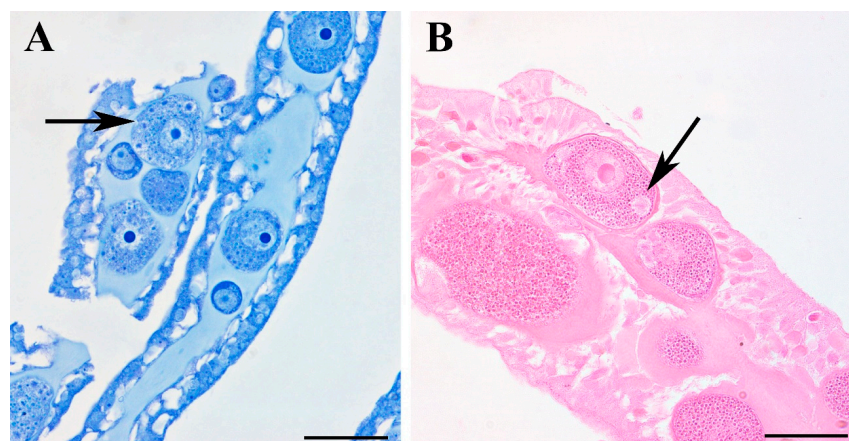


Table 3. Cont.

Specimen number	IPD	Oiii%	Oii%	Oi%	P%	Sampling Month
MA50	5	0	38.70968	38.70968	22.58065	September/2008
MA52 *	4.4	35.38462	47.69231	12.30769	4.615385	September/2008
MA53 *	4.7	26.05042	35.29412	24.36975	14.28571	September/2008
MA56	5	0	62.63736	32.96703	4.395604	September/2008
MA57	4	0	0	85.65022	14.34978	September/2008
53A *	10.54	32.91139	44.3038	14.76793	8.016878	April/2010
54C	8	4.659498	60.9319	28.67384	5.734767	April/2010



**Figure 4.** Gonadal section of the most immature studied female of *Chiropsalmus quadrumanus* (from Macaé, Rio de Janeiro). Stained with Gomori trichrome and hematoxylin (GT + H) and HE, respectively (A,B). Arrows show the columnar gonadal epithelium with basal vacuoles. Scale bars: 50 µm. gt—gastrodermal epithelium; m—mesoglea; Oi—early-vitellogenic oocyte; P = pre-vitellogenic oocyte.



**Figure 5.** Gonads section of two different specimens of *Chiropsalmus quadrumanus* (from Macaé, Rio de Janeiro) stained with toluidine blue (A) and HE (B), respectively. Arrows show inclusions of unknown natures within the oocytes. Scale bars 50 µm.

#### 4. Discussion

Patterns of female sex cell maturation (oogenesis) and the reproductive strategy of the cubomedusa *C. quadrumanus* are presented for the first time based on our histological approach. Females have four different stages of oocyte development, as determined via a comparative analysis of vitellogenic content, which was present in most specimens examined (Figures 2 and 3). The presence of pre-vitellogenic oocytes even in mature female gonads is interpreted as an indication of asynchronous oogenesis in which a mature female develops immature oocytes in order to reproduce more than once in her lifetime. This pattern diverges from the pattern reported for *C. murrayana* in which only late-vitellogenic oocytes were observed in mature females, suggesting a single spawning event occurring upon cubomedusae maturity [45]. Several reports of oogenesis in non-cubozoan jellyfishes (e.g., *Cassiopea andromeda*, cf. [66]) have mentioned trophocytes associated with mature oocytes during ovulation; the absence of these specialized nutritive cells in *C. quadrumanus* corroborates findings reported by [45] for the cubomedusa *C. murrayana*. The absence of embryos or planulae within the gastrovascular cavity in females, taken together with knowledge that sperm are released via the rupture of the follicle wall in males of this species [19], strongly indicates that *C. quadrumanus* exhibits external fertilization as a reproductive strategy.

##### 4.1. Seasonality

*Chiropsalmus quadrumanus* is a shallow-water species inhabiting the Atlantic coast of the Americas. Its seasonality is marked by the presence of mature medusae from April to September in the Southern Hemisphere (e.g., [19,20] and this study). To date, the most sexually mature female (>37% of O<sub>iii</sub>) was trawled in São Paulo in August (Tables 2 and 3), suggesting that the reproductive season occurs in the dry season in the Southern Hemisphere. Occurrence data for *C. quadrumanus* for the Northern Hemisphere recorded “blooms” in Georgia in July of 1971 [52], a “predominance” in the Mississippi Sound in August of 1968 [53], and floating dead specimens in the fall (September–November) after a Texas rainy season [54]. However, a broader understanding of the distribution and seasonality of this species requires phylogenetic studies to determine whether specimens in Brazil, the Gulf of Mexico, and North Carolina [46] belong to the same species or if a complex of cryptic species exists instead (cf. [1] for a similar western Atlantic cubomedusae). Elucidating the taxonomy of the species will provide a better understanding beyond seasonality, for example, in determining possible differences in relation to its venom, an important public safety area given the case of the death of a child in the Gulf of Mexico associated with *C. quadrumanus* [67].

The female cubomedusae studied herein presented oocytes at different stages of development, but none of the females was undergoing ovulation. The presence of pre-vitellogenic oocytes in all specimens (4–22%, Tables 2 and 3) supports the existence of continuous oogenesis, making it likely that *C. quadrumanus* cubomedusae are iteroparous (shedding their gametes more than once during their lifetime), a pattern observed in a few cubomedusae (e.g., [68]) and the tripedaliid cubozoans [15]. Although the histological data are robust, in situ data on spawning females are needed to corroborate our findings of a maturation scale and its persistence within the population in order to fully validate *C. quadrumanus* as a truly iteroparous species. Large and predictable spermcasting aggregations of the semelparous *A. alata* demonstrated that the leaf-like hemi-gonads visible on radial septa break, becoming a thin line after spawning, and stranded medusae along the rocks on the beach have varying degrees of gonadal rupture (from partial to almost complete) (more details in [3]). However, as is the case for most cubozoan species due to a dearth of histological data on cubomedusa gonad maturation, it remains difficult to corroborate observations on cubomedusae sexual reproduction in situ.

#### 4.2. Fertilization Mode and Oocyte Nutrition

The challenges inherent in rearing cubomedusae for observing sexual reproduction in vitro hamper the experimental study of the modes of fertilization in this class. Even without visual confirmation of sexual reproduction, the presence of fertilized eggs or planulae in the gastric cavity of collected females has served as evidence of internal fertilization in some medusozoan species [15,69]. Additionally, external fertilization in Chirodropida is inferred for *C. fleckeri* [21] and *C. quadrumanus* based on observations of sperm release via the rupture of the follicle wall [19]. Our data corroborate the external fertilization theory for *C. quadrumanus* due to the absence of sperm or embryos within the female gastrovascular cavity, but as no samples had signs of insufficient spawning, gonadal maturity may have precluded the females from signaling males of an ensuing spawning event (for a review of such genetic signals, see [25]).

The presence of inclusions of unknown nature in some oocytes (Figure 5) may indicate that the yolk is supplied to the oocytes by phagocytic bodies, a mode of vitellogenesis previously reported in at least one medusozoan (viz., *Hydra*, [70,71]). However, we avoid further speculation on their origin or function prior to conducting further investigations.

#### 4.3. Individual Size and Maturity

Egg size can indicate the degree of sexual maturity [72–74], defined as the period in which a female is able to reproduce sexually [75]. The average oocyte diameter in *C. quadrumanus* (122 µm) is considerably larger than that reported for the cubomedusa *C. murrayana* (55 µm). This difference may be related to general medusa size, since the bell height of a mature *C. quadrumanus* specimen (>4.4 cm) is greater than that of a mature *C. murrayana* (3.68 cm, as *C. branchi* in [45]). Aside from indicating sexual maturity, egg size is often considered a proxy for understanding the reproductive and developmental traits of medusozoans, as large eggs are related to direct development [76–78] and are found in some deep-sea scyphomedusae [79].

Sexual maturity in medusozoans has previously been connected with bell size [15,80], but these studies have the caveat of defining the sexual maturity of a female cubomedusae simply through the presence of visible oocytes [3], during the act of ovulation [25], or stating that the gonads are full of “mature eggs” [81]. However, our maturation scale and statistical analysis thereof demonstrate that bell size is not necessarily correlated with gonadal maturity in *C. quadrumanus*. A possible explanation for the lack of correlation between bell size and sexual maturity could be that small individuals with high percentages of late-vitellogenic oocytes (e.g., specimen MA52, Table 3) might have undergone more rapid development under different environmental parameters such as temperature, food availability, or breeding period, even within the same population (cf. [3,56,80] for other medusozoan examples). Future in situ studies documenting the reproductive behavior of cubomedusae that are carried out in conjunction with histological studies of the gonads should further elucidate and complement the patterns revealed in this study.

### 5. Conclusions

In this study, we presented a female oocyte maturation scale based on histological data for the cubozoan *Chiropsalmus quadrumanus*, making it the first of its kind presented for a chirodropid. We demonstrated the existence of four distinct stages of oogenesis, congruent with an iteroparous mode of sexual reproduction with external fertilization. This framework shall serve as a standard for future investigations into sexual maturation and reproductive strategies in cubomedusae. Although large and unpredictable aggregations or “blooms” of *C. quadrumanus* have not been recorded on the Brazilian coast, it is important to be vigilant for potential events related to the emergence of these cubomedusae at the surface to understand the environmental conditions that favor both the sexual and asexual proliferation of this species. Thus, year-round local surveys along the Brazilian coast are needed to fully elucidate the reproductive season of *C. quadrumanus* and corroborate our hypotheses of its iteroparity. Furthermore, additional specimen collections directed

at molecular phylogenetic analyses will be important to validate whether the widely distributed *C. quadrumanus* lineage (Gulf of Mexico, North Carolina, Brazil) correspond, in fact, to the same species examined in this work. We expect our findings will serve as a baseline for understanding the structural and functional maturation of female sex cells and the evolution of sexual reproductive strategies within species of the order Chirodromida.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d15070816/s1>.

**Author Contributions:** A.C.M. (André Carrara Morandini) and A.C.M. (Antonio Carlos Marques): Conceptualization, Writing—review & editing. All authors contributed to the study conception and design. J.G.-R. and A.J.-B. conceived the ideas; Material preparation, data collection, and analysis were performed by J.G.-R., A.J.-B. and G.R.T. The first draft of the manuscript was written by J.G.-R. and C.L.A., and all authors reviewed and commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

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