Reproductive strategy of the Pacific cownose ray *Rhinoptera steindachneri* in the southern Gulf of California

María I. Burgos-Vázquez[A,E], *Valeria E. Chávez-García*[A,B], *Víctor H. Cruz-Escalona*[A], *Andrés F. Navia*[C] *and Paola A. Mejía-Falla*[C,D]

[A]Instituto Politécnico Nacional, Centro Interdisciplinario de Ciencias Marinas, Av. Instituto Politécnico Nacional s/n Col, Playa Palo de Santa Rita Apdo, Postal 592, Código Postal 23096, La Paz, BCS, México.

[B]Universidad del Mar, Ciudad Universitaria, Puerto Ángel, Distrito de San Pedro Pochutla, Código Postal 70902, Oaxaca, México.

[C]Fundación colombiana para la investigación y conservación de tiburones y rayas, SQUALUS, Calle 10A #72-35, Cali, Colombia.

[D]Wildlife Conservation Society, Avenida 5N #22N-11, Cali, Colombia.

[E]Corresponding author. Email: itzigueri@gmail.com

Abstract. *Rhinoptera steindachneri* is one of the most common batoid species in the artisanal gill net fishery of the Gulf of California. In this study we investigated its reproductive biology based on 317 specimens caught in Bahía de la Paz, Mexico. Females measured up to 94.2-cm disc width (DW) and males reached 82.5 cm DW; there were no significant differences in size or weight between sexes. The median size at maturity was estimated at 68.5 cm DW for males and 71.8 cm DW for females, and the median size at pregnancy was 84.3 cm DW. Only the left ovary and uterus were functional; a maximum of six preovulatory vitellogenic follicles per female was recorded, although uterine fecundity was one embryo per female. Ovulation and birth occurred in May, June and July, with birth sizes ranging from 38.1 to 42 cm DW. *R. steindachneri* in Bahía de la Paz exhibited low fecundity, large size at maturity and birth and a continuous and synchronous annual reproductive cycle.

Additional keywords: batoids, birth size, fecundity, life history, Myliobatiformes, size at maturity.

Introduction

It has been historically assumed that elasmobranchs as a group present a K-selected life history strategy, with low fecundity, late maturity and slow growth (Hoening and Gruber 1990; King and McFarlane 2003). Knowledge of the life history of a species, and in particular of its reproductive strategy, is one of the most important factors in evaluating populations, providing effective tools for decision makers to establish capture limits and prevent overfishing of species (Walker 2005). Some studies show that anthropogenic pressures on elasmobranch species can affect these life history strategies (Smith *et al.* 1998; Cortés 2000; Frisk *et al.* 2002).

Viviparous elasmobranchs exhibit a wide array of reproductive modes, which is reflected in the number of ways in which the mother contributes to the development of embryos (Wourms 1977; Conrath and Musick 2012). For example, lipid histotrophy occurs only in rays from the order Myliobatiformes. Mothers secrete a protein- and lipid-rich histotroph from highly developed secretory structures within the uterine lining called trophonemata (Wourms 1977; Hamlett *et al.* 2005). This mechanism of energy transfer seems to be more efficient, causing

Myliobatiformes to gain more weight during embryonic development than species that have other reproductive modes (Conrath and Musick 2012). In addition, lipid histotrophy could be related to low fecundity and large size at birth (Villavicencio-Garáyzar *et al.* 1994; Neer and Thompson 2005; Jacobsen *et al.* 2009).

The Pacific cownose ray *Rhinoptera steindachneri* (Evermann & Jenkins, 1982) is a batoid from the order Myliobatiformes and the only representative of the Rhinopteridae family, distributed in the Eastern Pacific (Robertson and Allen 2015). It inhabits shallow waters, especially over soft bottoms, and performs seasonal migrations related to water temperature (Bizzarro *et al.* 2007). Few studies on the reproductive biology of this species have been published. A study performed in the northern Gulf of California estimated a median size at maturity of 70-cm disc width (DW), fecundity of a single pup and a gestation period of 10–12 months (Bizzarro *et al.* 2007).

R. steindachneri is one of the most common batoid species in the artisanal gill net fishery of the northern (Bizzarro *et al.* 2007) and southern (González-González 2018) Gulf of California. It is also caught as bycatch in the shrimp fishery of the southern Pacific region of Mexico (Navarro-González *et al.* 2012).

Table 1.   Maturity stages of *Rhinoptera steindachneri* males, indicating the characteristics and indices of each reproductive organ

Maturity index	Maturity stage	Testes index	Testes condition	Seminal vesicle index	Seminal vesicle condition	Clasper condition
1	Immature – not developed	1	No testicular lobes, large amounts of testicular stroma, primary spermatogonia present	1	Undifferentiated	Not calcified, no semen
2	Immature – developing	2	Some testicular lobes, moderate testicular stroma; secondary spermatogonia and primary spermatocytes present	2	Differentiated and thick, no seminal fluid	
3	Mature – mating capable	3	Testicular lobes present throughout the organ, seminiferous ampullas present throughout the periphery, mature sperm cells present	3	Differentiated and coiled, no seminal fluid	Partially or completely calcified, without semen
4	Mature – actively mating	3		4	Differentiated and coiled, with seminal fluid	Calcified, with semen

Owing to the scarcity of information and the threats identified in its distribution range, the species is listed as Near Threatened in the IUCN Red List (Smith and Bizzarro 2006).

It has been found that reproductive characteristics can vary between populations of elasmobranchs, even at small spatial scales (Yamaguchi *et al.* 2000; Lombardi-Carlson *et al.* 2003; Walker 2007; Mejía-Falla 2012). However, others suggest further investigation to define whether those spatial differences are real or apparent (Trinnie *et al.* 2015). This highlights the importance of obtaining local data to avoid incorporating bias into demographic models and management strategies based on reproductive parameters defined for other locations. Therefore, the aim of the present study was to quantify the reproductive variables of *R. steindachneri* for the southern area of the Gulf of California, including sex ratio, size at birth, size at maturity and pregnancy, fecundity, gestation period and ovarian cycle.

Materials and methods

Study area, sample collection and laboratory analysis

Monthly samplings were performed from January 2014 to March 2017 in southern Bahía de La Paz, located in the southern Gulf of California (24°25′17.55″N, 110°18′31.64″W). Specimens were captured by artisanal fishermen using monofilament gill nets (100 m long × 1.5 m high, 8–10-inches or ∼20–25-cm mesh size) traditionally called '*chinchorros*', which are set in the afternoon at depths between 10 and 30 m over sandy bottoms and recovered the next morning. DW (cm) was measured and the sex determined by the presence of copulatory organs in males (claspers). The inner length (CL; cm) of one clasper from each male was measured, and the degree of calcification (calcified, partially calcified, not calcified) and presence or absence of semen were recorded. The gonads were weighed (GM; to the nearest 0.001 g) and fixed in 10% buffered formalin.

The biometry of the testes, seminal vesicles and claspers of males was evaluated. Testes length (±0.001 cm), width (±0.001 cm) and weight (±0.001 g), as well as seminal vesicle length (±0.001 cm) and width (±0.001 cm) were measured. The length, width (±0.1 cm) and weight (±0.01 g) of the ovaries,

uterus and oviductal glands of females were also measured. Visible ovarian follicles were extracted, quantified, measured (diameter to an accuracy of ±0.1 cm) and collected. The length of the longest trophonemata in the uterus was measured. Embryos were sexed and measured (DW), weighed and classified ontogenetically based on morphological characteristics, following criteria proposed by Hamlett *et al.* (1985) and Colonello *et al.* (2013).

Maturity was defined based on the macroscopic observation of the reproductive organs of both sexes following proposals by Smith and Merriner (1986) and Poulakis and Grier (2014), and adapted for *R. steindachneri* males (Table 1) and females (Table 2). The characteristics used to define maturity in males were the presence or absence and degree of development of the testicular lobes in the testes, the degree of development of the epigonal organ (when present), the presence of seminal fluid and the degree of winding in the seminal vesicles, as well as absence or presence of the alkaline gland and the absence or presence of fluid in this structure. The characteristics used to define maturity in females were the presence of ovarian follicles and degree of vitellogenesis, the absence or presence and development of uterine villi and embryos and the thickness and weight of the muscular wall of the uterus. The total number of ovarian follicles (OF) per female was counted, but only the preovulatory vitellogenic ovarian follicles (VOF; diameter ≥0.8 cm) were used to evaluate ovarian fecundity.

Sex ratio, DW and individual weight

The sex ratio of adults, juveniles, neonates, embryos and all individuals together was evaluated using a Chi-Square test to determine whether the ratio differed from 1 : 1 (Sokal and Rohlf 1998). The significance of differences between males and females in DW and weight (excluding the weight of pregnant females) was evaluated using a Mann–Whitney *U*-test. Data were tested for normality and homogeneity of variances with Kolmogorov–Smirnov and Lilliefors tests respectively before analysis. All differences were considered significant at $P < 0.05$.

Table 2. Maturity stages of *Rhinoptera steindachneri* females, indicating the characteristics and indices of each reproductive organ

The 4a uterine index was not found in this study

Maturity Index	Maturity stage	Ovarian index	Ovarian condition	Oviductal gland index	Oviductal gland condition	Uterine index	Uterine condition
1	Immature – not developed	1	No follicles, large amount of ovarian stroma	1	Not visible or slightly differentiated from the anterior oviducts	1	Undifferentiated, no uterine villi, weight ≤0.6 g
2	Immature – developing	2	Follicles visible, small (diameter ≤0.79 cm) and previtellogenic	2	Slightly differentiated from the anterior oviducts but not completely developed	2	Slightly differentiated, tubular form, short uterine villi (≤0 cm), no histotroph present
3	Mature – virgin	2		3	Completely developed, widened and well differentiated from the oviducts	3	Completely differentiated, long and thick uterine villi (0.05–0.8 cm), no histotroph
4	Mature – pregnant	3	Follicles visible, large (diameter ≥0.8 cm) and vitellogenic; small amount of ovarian stroma	3		4a	Uterus with eggs
						4b	Uterus with villi (0.7–1 cm), differentiated and widened, with histotroph and embryos in early development
						4c	Uterus with villi (0.7–1 cm), differentiated and widened, with histotroph and embryos in mid development
						4d	Uterus with long villi (up to 1.8 cm), differentiated and widened, with histotroph and embryos in late development
5	Mature – postpartum	4	Follicles visible, large (diameter ≥0.8 cm) and vitellogenic, postovulatory follicles present; large amounts of ovarian stroma	3		5	Completely differentiated and flaccid, long and thick uterine villi (0.9–1 cm), with waste from histotroph; no embryos
6	Mature – resting	4	Follicles visible, large (diameter ≥0.8 cm) and vitellogenic	3		6	Completely differentiated, long and thick uterine villi (0.9 cm), no histotroph and no embryos

Reproductive structures and maturity

The logistic equation modified by Piner *et al.* (2005) was used to evaluate the relationship between DW and CL, using the following equation:

$$CL = \min CL + \frac{\max CL - \min CL}{1 + e^{b(a-DW)}}$$

where *a* is the inflection point of the curve, *b* is another parameter of the model, and min*CL* and max*CL* are minimum and maximum CL values respectively (Mejía-Falla *et al.* 2012).

The significance of differences in length, width and weight between the right and left reproductive structures (testes, seminal vesicle, ovary and uterus) was assessed using a Wilcoxon paired test; whereas the significance of differences in the length and width of the rigth and left oviducal glands was evaluated using Student's *t*-test.

The significance of differences in the weight of reproductive structures on the left (dorsal position) of the body (testes, ovaries, oviducal glands, and uterus) according to maturity stage, were evaluated using a Kruskal–Wallis test for independent samples; in the case of seminal vesicles, width was evaluated.

Median size at maturity and pregnancy

The median size at maturity ($DW_{50\%}$) was calculated for males and females using a logistic regression model with binomial data (0, immature; 1, mature) and the following equation:

$$P_i = (1 + e^{-(a+b \times DW_i)})^{-1}$$

where P_i is the fraction of mature individuals at DW_i (the size at class *i*), *a* and *b* are model parameters, with median size at maturity given by –*a*/*b* (Mollet *et al.* 2000). Males were considered mature if the claspers were partially calcified or calcified and semen or testicular lobes were present in the testes (Maturity Indices 3 and 4; Table 1). Females were considered mature if they had vitellogenic ovarian follicles or embryos (Maturity Indices 3–5; Table 2). Median size at maturity was also calculated using binomial data of the left ovary (DW_O; the only one exhibiting follicular development). Immature ovaries (Indices 1 and 2) were assigned a value of 0 and mature ovaries (Indices 3 and 4) were assigned a value of 1. Median size at maturity was calculated using binomial data of the left uterus (DW_U; the only functional one); immature uteri (Indices 1 and 2) were assigned a value of 0 and mature uteri (Indices 3, 4b, 4c, 4d and 5) were assigned a value of 1. The median size at pregnancy (DW_{P50}) was also calculated using binomial data, where a value of 1 corresponded to females at Maturity Indices 4, 5 and 6; other females were designated as 0 (Maturity Indices 1–3).

Fecundity and reproductive cycle

The range, mean and mode of the number of vitellogenic ovarian follicles in the ovary and the embryos in the uterus were estimated to evaluate ovarian and uterine fecundity (Pratt 1979). A linear regression was used to analyse the relationship between ovarian fecundity and uterine fecundity with DW. The significance of differences in OF and VOF and uterine fecundity across maturity stages was evaluated using a Kruskal–Wallis test.

The reproductive cycle was defined by ovulation (using only VOF) and gestation period. Ovulation was evaluated using the diameter of the largest ovarian follicle (preovulatory vitellogenic) of each female through the months, and the significance of differences between months were evaluated with a Kruskal–Wallis test. Gestation period was defined based on monthly embryo DW and weight; information for neonates was also analysed to infer birth months (DW and weight). Birth size was evaluated considering the DW and weight of the largest and heaviest embryo and the smallest and lightest neonate. Additional information from growth bands on neonate vertebrae (Pabón-Aldana 2016) was used to define birth months and birth sizes. The percentage of females and males by maturity stage by month was examined using a histogram. Where appropriate, data are presented as the mean ± s.d.

Results

Sex ratio, DW and weight

In all, 317 individuals (150 females, 163 males, 4 undifferentiated) were recorded, resulting in a sex ratio equal to the expected 1 : 1 proportion ($\chi_1^2 = 0.269$, $P = 0.539$). The sex ratio evaluated for each developmental stage was also 1 : 1 ($P > 0.05$ for all cases). Females were present during all months of the year except November and December, whereas males were not present in December. Females ranged in size from 40.1 to 94.2 cm DW (65.4 ± 13.8 cm DW), whereas weight ranged from 740.2 to 14 900.2 g (4301.1 ± 2793.3 g). Males ranged in size from 41.8 to 82.5 cm DW (62.7 ± 10.0 cm DW), whereas weight ranged from 850 to 8300 g (3754.3 ± 1764.6 g). There was no significant differences between the sexes in size (Z = 1.5, $P = 0.1$) or weight (Z = 0.5, $P = 0.6$).

Reproductive structures and maturity

Males had paired oval testes fused at the lower end, just above the rectal gland. Both testes had epigonal organs next to the vertebral column; the right epigonal organ was more developed than the left and was observed only at Maturity Indices 3–4. Highly vascularised filamentous tissue was observed at Maturity Indices 2 and 3, and there was a tendency for this tissue to be reduced by Maturity Index 4 because of the increase in the size of the testicular lobes. Alkaline glands were identified on the side of the seminal vesicles above the kidneys; these were only present at Maturity Indices 3 and 4. The left testis was longer and heavier than the right one and although there was no significant difference in width between the left and right testes, there was a significant difference in weight (Table 3).

At Maturity Index 1, testes had abundant testicular stroma without testicular lobes. At Maturity Index 2 (Testes Index 2) some testicular lobes were visible in the ventral part of each testis. At Maturity Indices 3 and 4 (Testes Index 3), males had well-developed testicular lobes with seminiferous ampullas throughout the periphery of the testes. There were significant differences among maturity stages in the weight of the left testicle (Kruskal–Wallis $H_{3,61} = 44.1$, $P < 0.0001$). The heaviest male was at the actively mating stage (78.3 cm DW, 82.1 g), whereas the lightest male was at the immature – not developed stage (52.3 cm DW, 0.8 g; Fig. 1*a*).

Table 3. Mean (±s.d.) values of the right and left reproductive structures (dorsal position) in males and females of *Rhinoptera steindachneri*, and statistical results of Wilcoxon tests

Note, Student's *t*-test was used only for the length of the oviductal gland

	Right	Left	Z or t	d.f.	P-value
Males					
Testes					
Length (cm	7.5 ± 2.6	8.3 ± 2.2	2.5	57	0.01
Width (cm)	2.5 ± 1.3	2.5 ± 1.1	0.6	72	0.5
Weight (cm)	18.1 ± 19.7	21.6 ± 20.8	4.2	59	<0.0001
Seminal vesicle					
Length (cm)	5.4 ± 1.3	5.4 ± 1.2	0.6	65	0.57
Width (cm)	0.8 ± 0.6	0.9 ± 0.8	1.5	70	0.14
Females					
Ovaries					
Length (cm)	6.3 ± 2.7	6.4 ± 2.1	0.4	32	0.7
Width (cm)	2.4 ± 1.1	2.6 ± 1.2	0.9	32	0.4
Weight (cm)	8.4 ± 6.5	14.3 ± 12.5	3.9	30	<0.0001
Uterus					
Length (cm)	4.4 ± 0.8	4.9 ± 1.5	2.6	39	0.009
Width (cm)	1.3 ± 0.9	1.6 ± 1.4	3.3	54	0.009
Weight (cm)	12.5 ± 10.4	20.3 ± 22.4	2.4	36	0.02
Oviductal gland					
Length (cm)	1.8 ± 0.3	1.8 ± 0.4	−0.1	29	0.9
Width (cm)	1.1 ± 0.2	1.1 ± 0.3	1.4	23	0.2
Weight (cm)	1.5 ± 0.8	1.7 ± 0.8	1.7	25	0.09

No significant differences in length and width were found between the right and left seminal vesicles (Table 3). Seminal vesicles at Maturity Index 1 (Seminal Vesicle Index 1) were elongated, tubular and vascularised with thin walls, uncoiled, undifferentiated from extratesticular ducts and without seminal fluid. At Maturity Index 2, the seminal vesicles started to thicken and were also irrigated and without seminal fluid (Seminal Vesicle Index 2). At Maturity Index 3, irrigated, thickened and coiling seminal vesicles were evident, without seminal fluid (Seminal Vesicle Index 3). At Maturity Index 4, the seminal vesicles were morphologically equivalent to those seen at Maturity Index 3, but with the presence of seminal fluid (Seminal Vesicle Index 4). The width of the seminal vesicle differed significantly throughout the maturity stages (Kruskal–Wallis $H_{3,70} = 54.4$, $P < 0.0001$). Males with the largest seminal vesicles (≥1.5 cm) were at the actively mating stage (Fig. 1*b*).

Females had paired ovaries, elongated and fused at the lower end, just above the rectal gland. The epigonal organ was positioned on the lateral side of each ovary; it was visible starting at Maturity Index 2 and was slightly more protruding in the left ovary. However, only the left ovary showed evidence of oogenesis (follicular development), with the right ovary being rudimentary. The right and left ovaries were similar in size (length and width), but the left ovary was heavier than the right ovary (Table 3).

Ovaries showed no visible ovarian follicles at Maturity Index 1 (Ovarian Index 1). At Maturity Index 2 (Ovarian Index 2), the quantity of ovarian stroma in the right ovary was decreased and the first ovarian follicles started to be observed (diameter 0.05–0.79 cm); however, it was not possible to observe them externally. Three different follicular diameter cohorts were observed at the mature reproductive stages: (1) ≥0.05–0.79 cm; (2) 0.8–2.2 cm, previtellogenic; and (3) 3.0–3.15 cm, preovulatory yellowish, completely vitellogenic. The ovarian follicles could be observed externally (only those ≥0.8 cm, protruding from the ovarian covering tissue) at Maturity Indices 3–5 and 6 (Ovarian Index 3; only on the left ovary). The ovarian stroma of the right ovary increased in size and the epigonal organ widened throughout the maturity stages. There were significant differences in ovary weight throughout the reproductive stages (Kruskal–Wallis $H_{5,36} = 24.7$, $P = 0.0002$), with postpartum and resting females ≥84.4 cm DW having the greatest ovary weights (21.8–51.8 g; Fig. 1*c*).

The paired, bell-shaped oviducal glands were positioned in the anterior part of the uteri and were similar in size (length and width) and weight (Table 3). The oviductal glands were not visible macroscopically at Maturity Index 1 (Oviducal Gland Index 1). They could be slightly differentiated from the anterior oviducts and uteri at Maturity Index 2 (Oviducal Gland Index 2), but they were not yet completely developed. They were wider and well differentiated from the oviducts at Maturity Indices 3–5 and 6 (Oviducal Gland Index 3). There were significant differences in oviducal gland weight throughout the reproductive stages (Kruskal–Wallis $H_{4,26} = 18.5$, $P = 0.001$). The development of the oviductal glands was notable in mature females at the not pregnant stage (≥1.3 g), and the heaviest oviductal gland (3.2 g) occurred in a mature postpartum female (88.4 cm DW; Fig. 1*d*). However, this stage was only significantly different from the developing stage ($P = 0.006$). Both uteri had uterine villi, but the left uterus was functional, wider, longer and heavier than the right one (Table 3), which was rudimentary.
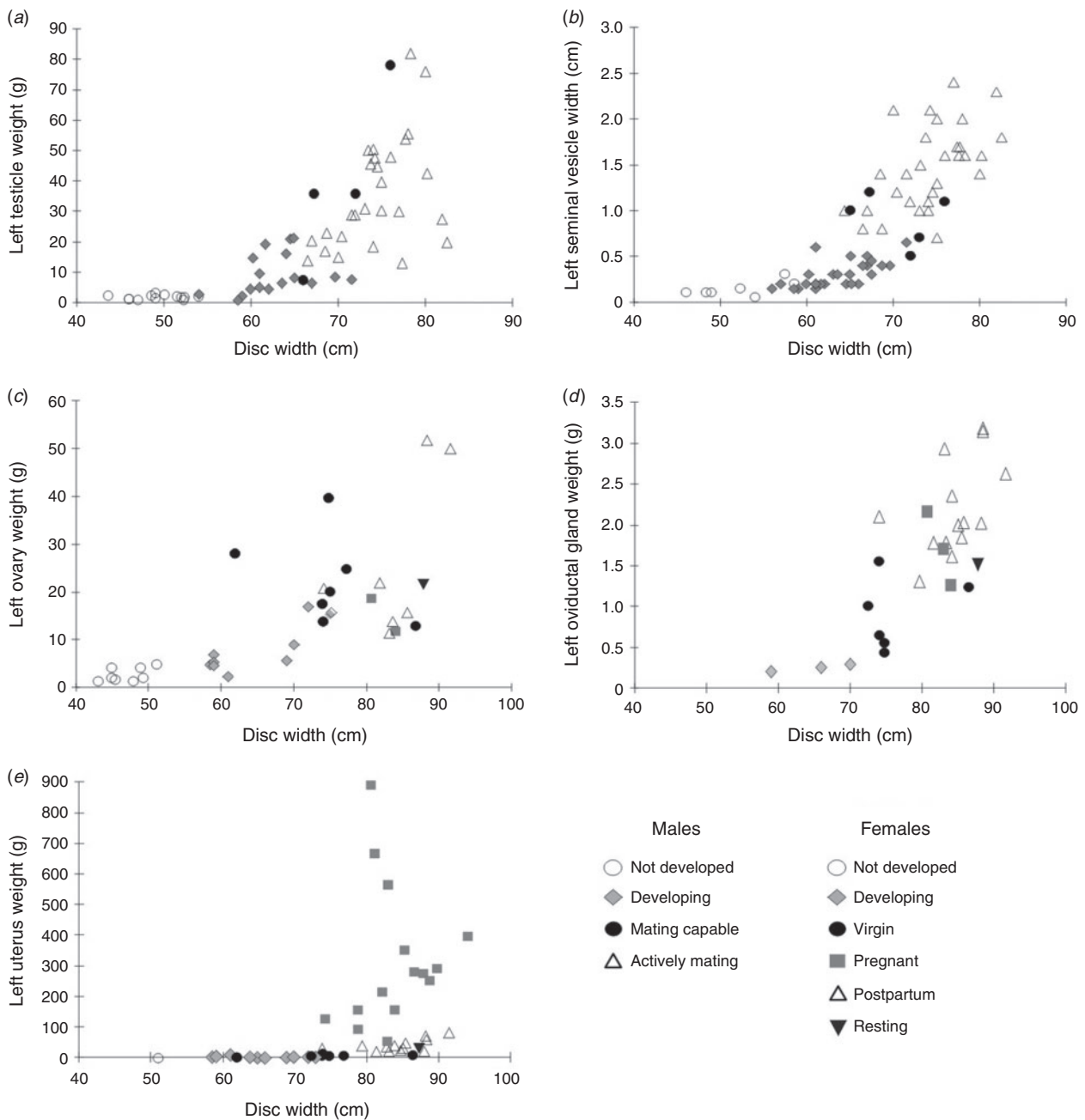
Fig. 1. Relationships in *Rhinoptera steindachneri* between disc width and (*a*) left testicle weight and (*b*) left seminal vesicle width for males, and the weight of the (*c*) left ovary, (*d*) left oviductal gland and (*e*) left uterus for females.

At Maturity Index 1 (Uterine Index 1), both uteri were tubular, undifferentiated from the oviductal gland and without uterine villi (trophonemata), and the cervix was not differentiated (Fig. 2*a*). At Maturity Index 2 (Uterine Index 2), the uteri were thin and flaccid, partially fused at the posterior end, and the cervix started to be distinguishable and the villi had started to develop (0–0.2 mm) in both uteri (Fig. 2*b*). At Maturity Index 3 (Uterine Index 3), the left uterus started to thicken, the muscular layer was thicker and the uterine villi were longer (0.5–8 mm) and homogeneous throughout the endometrium, whereas the right uterus became thicker (Fig. 2*c*). At Maturity

Index 4 (Uterine Indices 4b, 4c and 4d), only the left uterus contained embryos, the uterine villi were well irrigated and longer (7–18 mm) and secreted histotroph (uterine milk; Fig. 2*d*). The right uterus remained the same as seen at Uterine Index 3. At Maturity Indices 5 and 6 (Uterine Indices 5 and 6), the left uterus was similar to that at Maturity Index 4, but lacked embryos, had a flaccid structure and had histotroph residues. The uterine weight varied significantly by maturity stage (Kruskal–Wallis $H_{5,52} = 45.7$, $P < 0.0001$). Postpartum and pregnant females had the greatest uterine weights (≥ 56.3 g). (Fig. 1*e*).
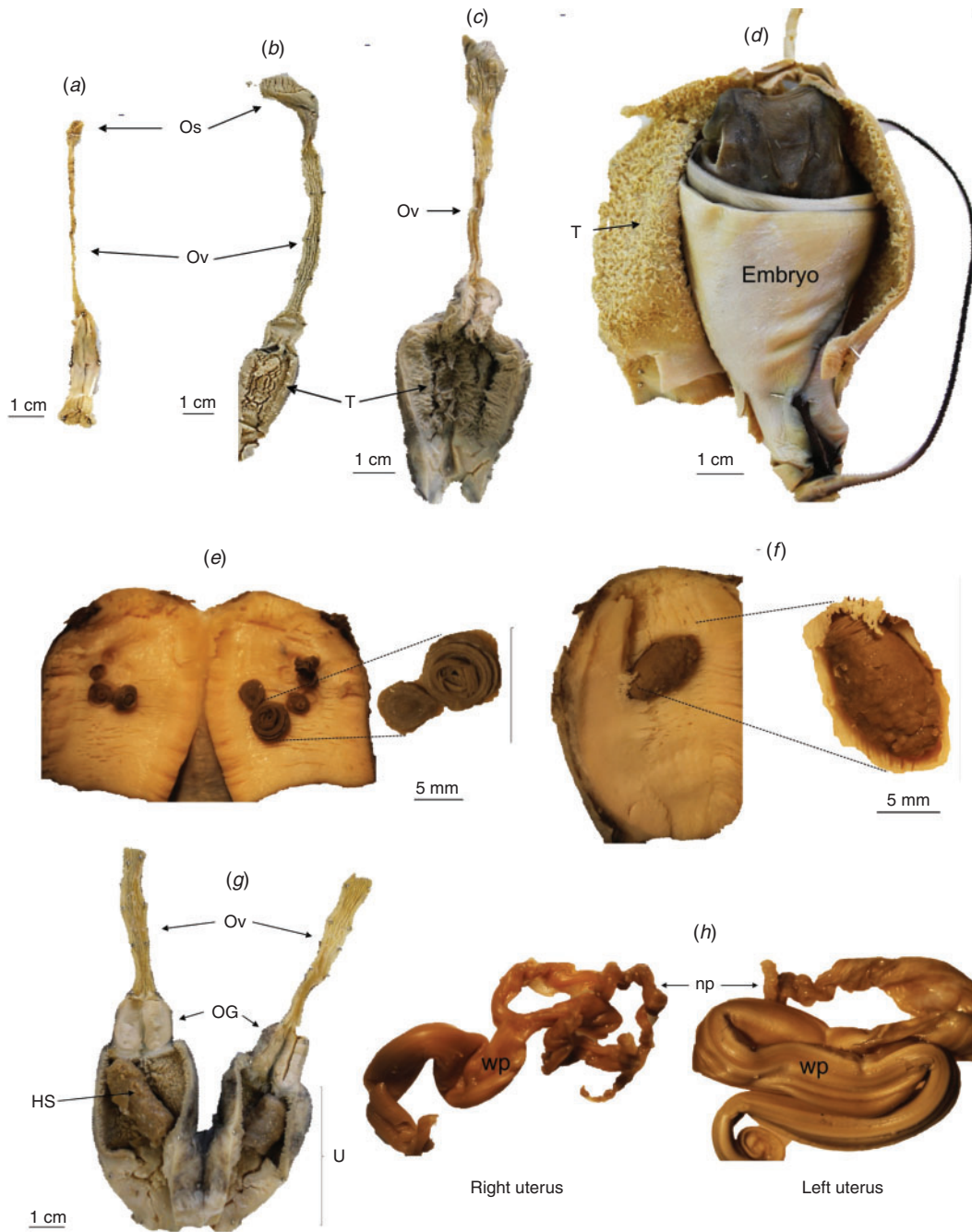
Fig. 2. Macrostructures of the reproductive system of *Rhinoptera steindachneri* females. Longitudinal sections of the left ostium, oviduct and uterus in (*a*) Stage 1 (immature), (*b*) Stage 2 (developing), (*c*) Stage 3 (mature–virgin) and (*d*) Stage 4 (mature–pregnant) females. Hard structures were found in the (*e*) left oviductal gland, (*f*) right oviductal gland, (*g*) both uteri (inside) and (*h*) those extracted organs. Structures are considered in the text in the dorsal position, but all photographs were taken in the ventral position. Os, ostium; Ov, oviduct; T, trophonemata; HS, hard structure; OG, oviducal gland; U, uterus; np, narrowest part; wp, widest part.

Rare and hard structures in females

Sixteen females had hard structures of unknown material in the left and right oviducal glands and uteri. Only two females (Maturity Index 3) had these structures in the anterior part of the oviducal glands. The first female (74.1 cm DW) had four hard structures in the left oviducal gland shaped like flat capsules with ringed edges (like roses; Fig. 2*e*). The second female (85 cm DW) had a greyish single structure in the left oviducal gland in the form of a capsule such as a seed or grenade, with both extremes ringed (Fig. 2*f*). The other
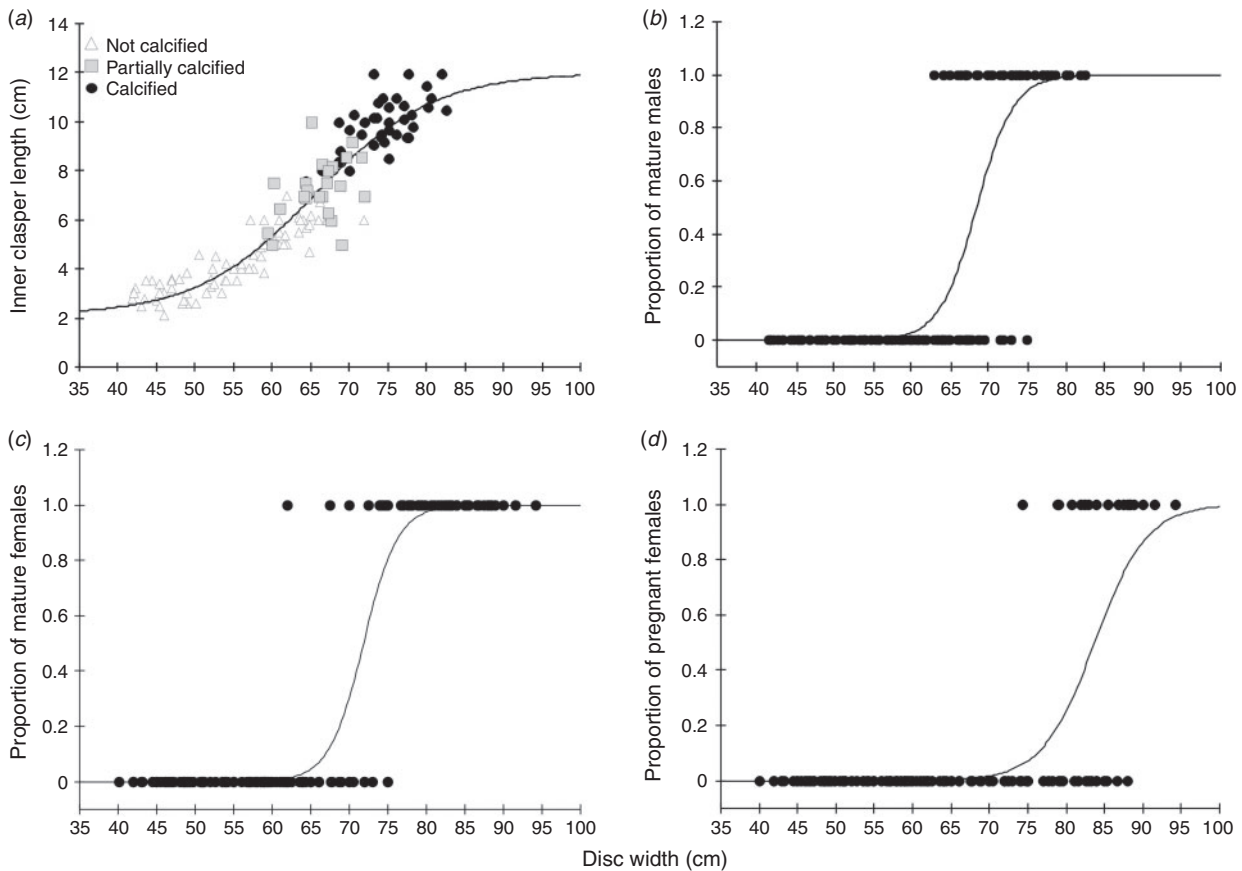
Fig. 3. (*a*) Relationship between disc width and inner clasper length in male *Rhinoptera steindachneri*. (*b*, *c*) Maturity ogives in relation to the maturity stage of males (*b*) and females (*c*). (*d*) Pregnancy ogive in female *R. steindachneri*.

females that had hard structures in both uteri ($n = 14$; 74.1–91.6 cm DW) were also mature (Maturity Indices 3–5). Each female contained a single brown and translucent structure per uterus (two per female; Fig. 2*g*) in the form of an elongated capsule (at its widest part) with wrinkled ends similar to tendrils (at its narrowest part) and empty inside (Fig. 2*h*). Those structures were found in February, May and July, but it was not possible to determine how long they lasted in the oviducal gland or in the uterus.

Size at maturity and pregnancy

Males with uncalcified claspers ranged in size between 41.8 and 69.6 cm DW (59% of all recorded males) and were categorised as Maturity Indices 1 and 2. Males with partially calcified claspers ranged in size between 59.5 and 72 cm DW (13%) and were categorised as Maturity Indices 2 and 3. Males with calcified claspers ranged in size between 64.3 and 82.5 cm DW (28%), and were classified as Maturity Indices 3 and 4. The smallest clasper measured 2.1 cm CL (belonging to a neonate of 46 cm DW) and the largest measured 12 cm CL (recorded for three reproductively active males measuring 73, 77.6 and 81.9 cm DW). The inflection point found in the logistic relationship was 65.6 cm DW, with a CL of 7 cm (Fig. 3*a*). Therefore, males with claspers $\geq$7 cm CL were considered mature. Immature males (68.1% of all males sampled) measured

between 41.8 and 75.0 cm DW, whereas mature males (31.3%) measured between 63.0 and 82.5 cm DW. The median size at maturity was estimated to be 68.5 cm DW (95% confidence interval (CI) 58.9–78.1; Fig. 3*b*).

Immature females (63.5%) measured between 40.1 and 75.0 cm DW, whereas mature females (36.5%) measured between 62.0 and 94.5 cm DW. Female median size at maturity was estimated to be 71.8 cm DW (95% CI 58–85.7; Fig. 3*c*). Pregnant females (10.1%) were between 74.4 and 94.5 cm DW in size, with an estimated median size at pregnancy of 84.3 cm DW (95% CI 73.7–95.02; Fig. 3*d*). The $DW_{50\%}$ based on ovarian development was estimated to be 74.4 cm DW (95% CI 60.3–88.4), whereas that based on uteri was estimated to be 72.5 cm DW (95% CI 64.5–73.4).

Ovarian and uterine fecundity

In all females, only the left ovary (dorsal position) exhibited follicular development. The OF per female varied between 1 and 44 (mean 21.7 ± 11.8; mode $= 22$). A significant but weak positive relationship was detected between OF and DW ($r^2 = 0.4$, $P < 0.0001$), but a greater number of OFs ($\geq$30) were present in females $\geq$74.1 cm DW (Fig. 4*a*). Significant differences were found in the total number of OF per maturity stage (Kruskal–Wallis $H_{4,36} = 13.7$, $P = 0.0082$). The developing stage had the least number of OF (4–21), whereas the most OF
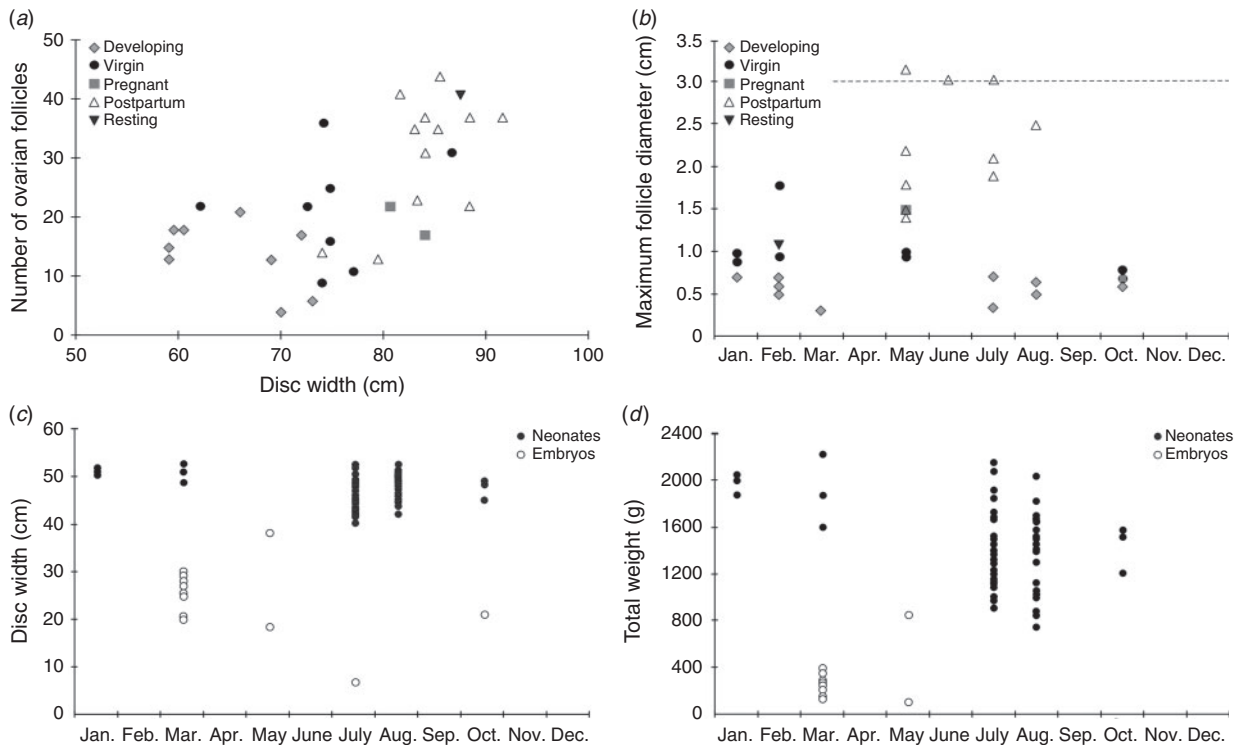
Fig. 4. (*a*) Relationship between the total number of ovarian follicles and disc width in *Rhinoptera steindachneri*. (*b*) Maximum follicle diameter by month (the dashed line indicates ovulation diameter). (*c*) Disc width and (*d*) total weight of embryos and neonates by month.

were seen in postpartum and resting females (37–41) and those with embryos in late development (44).

VOF was estimated to be between 1 and 6 (mean ± s.d. 3.0 ± 1.6; mode = 2). No clear significant relationship was found between VOF and DW ($r^2 = 0.03$, $P = 0.6402$) or between VOF and maturity stages (Kruskal–Wallis $H_{3,11} = 1.7$, $P = 0.6376$). However, the highest ovarian fecundity (based on VOF) was found in the largest female (91.6 cm DW) at the postpartum stage.

In all, 13 embryos (size 6.8–38.1 cm DW) were recorded in 13 females. There was uterine fecundity of one embryo per female, all in the left uteri (dorsal position). No evidence of abortions and no females with eggs in the uterus were observed. A single embryo at the early developmental stage was recorded (6.8 cm DW). This embryo had a yolk sac with no pigmentation, the cephalic lobes were not yet fused and it had the same body shape as the adult. Eleven embryos were recorded at the mid-development stage, with sizes ranging between 18.3 and 30.1 cm DW (mean 24.7 ± 4.1 cm DW), total mass between 85.4 and 386.8 g (mean 234.9 ± 96.0 g), little pigmentation and the same body shape as the adult. Only one embryo was found to be in the late development stage (size 38.1 cm DW, weight 841 g) and this embryo was characterised by an absent yolk sac, a completely pigmented body and the same body shape as the adult.

Reproductive cycle

There were significant differences in maximum follicle diameter throughout the months (Kruskal–Wallis $H_{7,37} = 14.5$, $P = 0.04$). Lowest values were obtained in October (0.7–0.8 cm), January

(0.7–1.0 cm) and February (1–1.8 cm), whereas the highest values were obtained in May (3.2 cm; postpartum stage), June and July (3.0 cm). These last 3 months correspond to the period of ovulation, considering only ovarian follicles ≥3.0 cm as those that can be soon ovulated, which corresponds to a follicle development period of 7–9 months (Fig. 4*b*).

The smallest embryo (6.8 cm DW) was found on 21 July 2015, whereas the largest (38.1 cm DW) and heaviest (841 g) was found on 21 May 2015. The smallest neonate (40.1 cm DW) was found on 4 July 2015 (Fig. 4*c*), and the lightest (740 g; 42 cm DW) was found on 2 August 2016 (Fig. 4*d*). Based on this information, the identification of postpartum females in the period 21 May–3 August and the high frequency ($n = 24$) of neonates before 4 August, it was proposed that May–July are the birthing months (Fig. 4*c*). Considering the ovulation peaks (May–July) and subsequent start of embryonic growth (June–August) with the defined birthing months, a gestation period of between 10 and 14 months is suggested for the species. Although birth sizes could be estimated at between 38 and 42 cm DW, based on traditional estimates (based on the largest and heaviest embryo and lightest neonate), the absence of growth bands in neonates, between 40.1 and 52.1 cm DW found in July and August (Pabón-Aldana 2016), suggests a wider range of birth sizes (38.1–52.1 cm DW; Fig. 4*c*).

Because females exhibit continuous follicular development and ovulate immediately once they have given birth, the reproductive cycle is continuous. The synchrony of the reproductive cycle was based on two pieces of information: (1) the female with the largest embryo (38.1 cm DW), which contained

follicles 2.2 cm in diameter, close to the ovulation diameter (3 cm) recorded in May; and (2) females at the postpartum stage during May and June having follicles with the largest diameters (3.0–3.2 cm; Fig. 4*b*). Both pieces of information indicate that ovulation occurred in the same month or 1 month after birth (Fig. 4*b*, *c*).

According to percentages by developmental stage, adult males were more frequent in the summer months (from May to August; Fig. 5*a*), whereas adult females were only frequent in March and May (Fig. 5*b*). Neonates of both sexes were absent from April to June and were more frequent in July and August (Fig. 5*a*, *b*), which indicates that births occurred in May, June and July. This information, along with the synchronous and continuous annual reproductive cycle described for the species, indicates that the reproductive activity (ovulation, mating and births) was concentrated in the summer months. Juveniles of both sexes observed from July to March represent the recruits of each reproductive event (Fig. 5*a*, *b*).

Discussion

This study is the first to provide an anatomical description of the gonadal structures of male and female *R. steindachneri* and to propose a maturity scale for the species. *R. steindachneri* is a matrotrophic species, with trophonemata to nourish the embryo through the secretion of histotroph (uterine milk), and with a continuous and synchronous annual reproduction.

A higher frequency of *R. steindachneri* individuals in the summer has been reported for the Gulf of California (Bizzarro *et al.* 2007). The absence of individuals in November and December can be explained by migratory activities, as reported by Schwartz (1990) for the entire *Rhinoptera* genus.

Although no size differences by sex were identified in the present study, females reached greater sizes due to their viviparous condition and the advantages for survival (Wourms and Lombardi 1992), as has been reported previously for other viviparous ray species (Smith *et al.* 2007; Alkusairy *et al.* 2014; Romero-Caicedo and Carrera-Fernández 2015; Burgos-Vázquez *et al.* 2017). The average sizes found for the Bahía de La Paz population were similar to those reported in previous studies within the Gulf of California (Villavicencio-Garáyzar 1996; Bizzarro *et al.* 2007), but lower than those recorded for the west coast of Baja California Sur state (México) (Bizzarro *et al.* 2007). Because these latter two studies were conducted using similar fishing gear, it is likely that the differences in size were due to the environmental characteristics of the two areas, because it has been reported that batoids within the Gulf of California are smaller (Villavicencio-Garáyzar 1993; Bizzarro *et al.* 2007; Márquez-Farías 2007; Burgos-Vázquez *et al.* 2017).

Although the degree of clasper calcification has been used previously to evaluate maturity in males (Pratt 1979; Smith and Merriner 1986; Walker 2005), the results of the present study suggest that this measure could underestimate maturity in *R. steindachneri*. We identified six specimens (size 63–77 cm DW) with mature testicles but partially calcified claspers, leading to their initial (visual) classification as immature. This inconsistency was reported formerly by Walker (2005) for *Galeorhinus galeus* and by Poulakis (2013) for *Rhinoptera bonasus*. We suggest considering the presence of testicular lobes in the
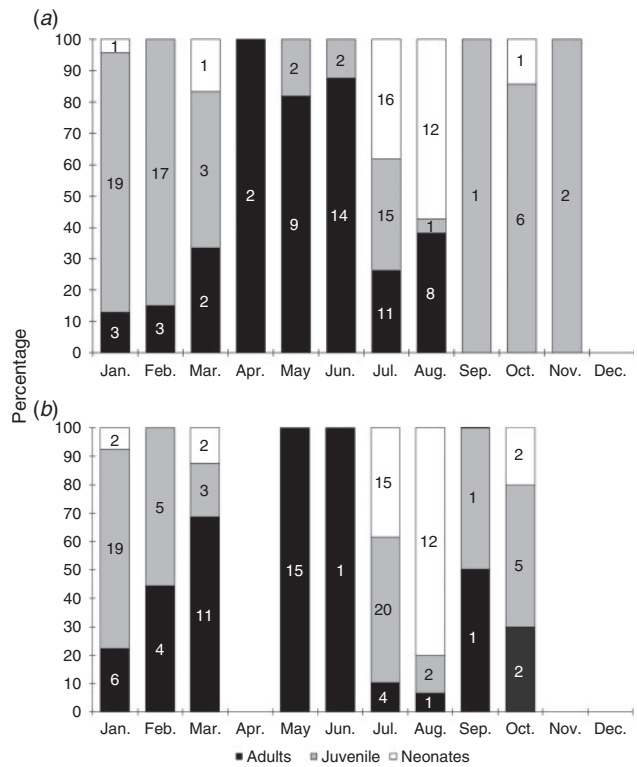
Fig. 5. Proportion of (*a*) male and (*b*) female *Rhinoptera steindachneri* individuals in each reproductive stage by month. Numbers within columns are the number of individuals in each given stage by month.

testicles, the thickening of the seminal vesicle and the presence of the alkaline gland as the most trustworthy and effective way to assess maturity in *R. steindachneri* males. The minimum size at maturity of males in Bahía de La Paz (63 cm DW) based on the degree of calcification and the CL was similar to that reported by Bizzarro *et al.* (2007) for *R. steindachneri* off the Sonora coast (65 cm DW). The wide range of sizes found in this study in clasper increase (60–70 cm DW, range which is the change between individuals with claspers not calcified and partialy calcified) was similar to that reported by Bizzarro *et al.* (2007; 65 cm DW). Martin and Cailliet (1988) found that the abrupt change in clasper length allowed identification of maturity in *Myliobatis californica*, and the inflection point of the logistic model corresponded to the middle of the range (65.6 cm DW); however, in the present study the median size at maturity was different (68.5 cm DW). This could be due to the fact that size at maturity was evaluated considering characteristics such as the condition of the testicles, seminal vesicles and the presence or absence of seminal fluid. The size at maturity in this study was similar to that determined by Bizzarro *et al.* (2007) for *R. steindachneri* off the Sonora coast (69.9 cm DW). It is therefore advisable to use qualitative characteristics to evaluate size at maturity, because the exclusive use of CL could lead to underestimation of the size at maturity.

Differences in weight (but not size) between the right and left ovary may be due to the fact that as females mature follicular development increases in the left ovary, whereas no follicular development is observed in the right ovary. This has already

been reported for other Myliobatiformes, such as *R. bonasus* (Smith and Merriner 1986; Pérez-Jiménez 2011; Poulakis 2013), *M. californica* (Martin and Cailliet 1988), *Gymnura micrura* (Yokota *et al.* 2012) and *Gymnura altavela* (Capapé *et al.* 1992), and was attributed to the fact that the non-functional structure is compensatory at a physiological level, as a hormone-secreting structure (Møller 1994). It is likely that because of the low fecundity of *R. steindachneri* (one embryo per female), the energy that would have been dedicated to follicular development of the rudimentary ovary is channelled towards other reproductive functions, such as hormone production.

As reported for *R. bonasus* (Smith and Merriner 1986; Pérez-Jiménez 2011), *Myliobatis goodei* (Colonello *et al.* 2013) and *R. steindachneri* in the Gulf of California (Villavicencio-Garáyzar 1996), the results of the present study showed that only the left uterus was functional. This is probably an ancestral condition, derived from a reproductive mode where both uteri were viable; however, because of the low fecundity, the right uterus ceased to be functional. Colonello *et al.* (2013) suggested that asymmetry is not a condition of Myliobatiformes and that it may be related to the fertility of the species, which has also been seen in *Urolophus paucimaculatus*, with only the left uterus functional and very low fecundity (one to two embryos; White and Potter 2005).

Herein we describe for the first time the presence of hard structures in the oviducal glands and uteri of *R. steindachneri*. It was not possible to identify the origin of the material making up these structures; however, the ringed patterns, the shape of the capsule and the rigidity of the material could be explained as a vestige of reproduction, because one of the functions of the oviducal gland is to produce the tertiary egg envelope or flexible candle that wraps the fertilised egg in species with a yolk sac (Hamlett *et al.* 1998, 2005; Hamlett and Koob 1999). In the case of the capsules found in the uterus of mature females, Smith and Merriner (1986) reported two *R. bonasus* females with capsules in their uterus that had similar morphological characteristics, but a female had one egg in one capsule and the other female had three ovules. In the specific case of *R. steindachneri*, the capsules found did not have any type of material. It is advisable that a histochemical analysis be performed to establish the origin of these hard structures.

The median size at maturity estimated for *R. steindachneri* males in this study (68.5 cm DW) represented 83.0% of the maximum size found, whereas for females (71.8 cm DW) it represented 76.2% of the maximum size found. This represents a high value for the species, suggesting late size at maturity. Off the Sonora coast, northern Gulf of California, Bizzarro *et al.* (2007) estimated a median size at maturity for males and females of 69.9 and 70.2 cm DW respectively, which is similar to the findings in this study. In Bahía Almejas, Mexico, Flores-Pineda *et al.* (2008) estimated size at maturity of *R. steindachneri* males and females of 79.2 and at 80.4 cm DW respectively. The differences observed in this parameter between populations of the Gulf of California and Bahía Almejas are attributed to temperature differences between the two areas, because Bahía Almejas has lower temperatures than the Gulf area (Salinas-González *et al.* 2003; Zaitsev *et al.* 2010). This could affect metabolic rate and reflect the effect of temperature on the maximum size that organisms can reach (Brown *et al.* 2007; Bernal *et al.* 2012). Bizzarro *et al.* (2007) commented that

differences between the *R. steindachneri* populations of Bahía Almejas and the northern Gulf of California could be due to limited genetic exchange, which is reflected in the life history traits of the two populations.

The evaluation of ovarian fecundity through the total count of OF allowed us to define three different groups or cohorts of follicular production, and although ovarian fecundity is not equal to uterine fecundity (1), it is likely that the number of ovarian follicles found was due solely to the result of the meiotic division in gametogenesis. In addition, the presence of only one embryo per female and of the absence of eggs in the uterus suggest that the other preovulatory vitellogenic follicles that are not ovulated are reabsorbed in the ovary (atretic follicles; V. E. Chávez-García, unpubl. data). It is well documented that in Myliobatiformes uterine fecundity is low (Musick and Ellis 2005) and it has been reported that *R. steindachneri* has one of the lowest fecundities within the order (one embryo per female; Villavicencio-Garáyzar 1996; Bizzarro *et al.* 2007), which coincides with the observations of this study. However, similar fecundity has been reported for *U. paucimaculatus*, with one embryo per female and rarely two (White and Potter 2005). Although we found no relationship between the DW of the mother and the DW of the embryo, Bizzarro *et al.* (2007) reported a relationship between these two values; however, Bizzarro *et al.* (2007) did not present statistical evidence to support their findings because of low sample numbers.

The annual reproductive cycle described in this study is similar to what has been described by others for the Mexican north-west (Villavicencio-Garáyzar 1996; Bizzarro *et al.* 2007), and even for *R. bonasus* in the Gulf of Mexico (Poulakis 2013). The results of the present study show that follicular development occurred during almost all months sampled (i.e. in 9 months). This allowed us to corroborate a continuous reproductive cycle. Once the larger follicles are ovulated, the next cohort begins to mature; this was also demonstrated by the presence of preovulatory vitellogenic follicles (VOF = 6) in a female at the postpartum maturity stage.

The greatest follicle diameters were observed in May, which also coincides with the presence of the greatest number of mature males in the area. May is probably the month when mating starts, ending in July, because this was when females with large vitellogenic follicles (diameter 3 cm) were recorded. Therefore, mating for *R. steindachneri* in Bahía de La Paz could last 3 months. Unlike other previously mentioned reproductive parameters, there were no differences in ovulation among the *R. steindachneri* populations studied in the Mexican Pacific, for which ovulation always occurs during the summer months (Bizzarro *et al.* 2007).

Synchrony of the reproductive cycle has also been reported by other authors for *R. steindachneri* (Flores-Pineda *et al.* 2008; Bizzarro *et al.* 2007) and *R. bonasus* along the coasts of North Carolina (Smith and Merriner 1986) and Florida (Poulakis 2013). This condition results from the continuous production of ovarian follicles in the ovary while gestation occurs. It is very likely that these species do not have a period of cessation of the reproductive cycle, with exception of the only report made by Pérez-Jiménez (2011) for the south-eastern Gulf of Mexico, where *R. bonasus* reproduce biennially without synchrony in the reproductive period, proposing that

females give birth every 2 years. This last condition could be also exhibited by *R. steindachneri* in the present study, because a resting female (87.5 cm DW) was registered in February. Although the uterus was not flaccid, weighed 34.4 g and the uterine villi measured 0.9 cm in length, it is probable that this female gave birth in July, was not fertilised and, given that the maximum diameter of its follicles was 1.1 cm, probably restarted its reproductive cycle again in May, when the follicles reach the ovulation diameter.

The range of birth sizes evaluated in this study is similar to that reported by other authors for the same species: 38–45 cm DW off the Sonora coast (Bizzarro *et al.* 2007), 40–44 cm DW off both coasts of the BCS peninsula, Mexico (Villavicencio-Garáyzar 1996), and 40 cm DW in the northern Gulf of California (Villavicencio-Garáyzar 2000). Although not enough information was collected to describe the entire embryonic development, we report the smallest embryo size for the species (6.8 cm DW), recorded in July. A 21-cm-DW embryo was recorded in October, suggesting rapid embryo growth. Embryos subsequently reach sizes between 19.9 and 30.1 cm DW in March; the largest embryo (38.1 cm DW) was recorded in May. This suggests that embryonic growth is rapid during the first months (summer), slows between autumn and winter, when the number of individuals of the species is reduced in Bahía de La Paz, and finally starts to increase in May. This same temporal behaviour was previously reported for *R. bonasus* in northern Carolina (Smith and Merriner 1986). In that study, the authors proposed that the migratory behaviour of the species results in mothers needing energy, leading to the cessation of embryo growth. It is also likely that the decrease in water temperature during the winter months leads to a decrease in the metabolic rate of embryos.

We propose a gestation period of between 10 and 14 months for *R. steindachneri* in Bahía de La Paz (females can be fertilised in May, June or July and give birth during the same period the following year). This period was defined taking into account all the pregnant females in March (with embryos in mid-development), the presence of females in the postpartum stage in May and the high frequency of neonates at the beginning of August, which confirmed recent births (July) in the population. A similar gestation period (11–12 months) has been proposed for this species off the Sonora coast and for Bahía Almejas (Bizzarro *et al.* 2007; Flores-Pineda *et al.* 2008).

Bahía de La Paz is occupied mainly by juvenile animals that enter the bay in January; these individuals probably represent those born the previous summer. Mature animals enter the bay in the summer to copulate and give birth and, once this activity is over, they begin to migrate in autumn–winter, leaving only new recruits in the bay, who leave the area in November. Because this bay is occupied mostly by neonates and juveniles, and because the species reaches maturity at large sizes, has low fecundity and only reproduces once per year, it is advisable that demographic studies are undertaken to assess the degree of vulnerability to overfishing of this ray species of commercial importance in the Gulf of California.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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