Aquaculture 491 (2018) 368–374

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Sperm count of *Macrobrachium amazonicum* (Heller, 1862) populations with distinct life histories, with introduction of a simple counting method

Lucas Rezende Penido Paschoal[a,*], Fernando José Zara[a,b]

[a] Univ. Estadual Paulista (UNESP), 13506-900, Rio Claro, SP, Brazil
[b] Invertebrate Morphology Laboratory (IML), Departamento de Biologia Aplicada, CAUNESP and IEAMar, Univ. Estadual Paulista (UNESP), 14884-900 Jaboticabal, SP, Brazil

ARTICLE INFO

Keywords:
Spermatozoa
Sperm count
Morphotypes
Animals preserved in ethanol
Caridea

ABSTRACT

Sperm count is an important quality assessment tool in farming programs and stock improvement in crustaceans. However, this procedure is still little used in caridean shrimps and standardization of appropriate techniques for the group is lacking. In this study, we propose a simple protocol adapted to determine sperm count in carideans using as model the Amazon River prawn *Macrobrachium amazonicum*. Males of dominant morphotypes (GC1 and GC2) of this species with amphidromous and hololimnetic life cycles were collected and carefully dissected. The ejaculatory duct was removed from the vas deferens and dissociated in a solution of distilled water (9 μl) and methylene blue (1 μl). Subsequently, 1 μl of this new solution was added to distilled water (9 μl), and then 1 μl was pipetted and quantified in a Neubauer chamber. The feasibility of this technique was also evaluated in animals preserved (5–480 days) in 70% ethanol from collections and the structural morphology of spermatozoa (spz) was examined. Despite morphometric differences observed between different types of males, the mean sperm count was similar for the species. In amphidromous animals, 60,258 spz/μl were registered for GC1 and 65,308 spz/μl for GC2, while in hololimnetic prawns, 48,950 spz/μl were registered in GC1 and 53,850 spz/μl in GC2. The variation in sperm count among animals preserved for different periods of time was small and very similar to those of fresh animals. Also, no microscopic changes in the structures of spz were observed. This technique can be applied to obtain a spermiogram in fresh as well as preserved animals, being especially important in studies with animals examined in population studies and deposited in collections or laboratories. This protocol can be used as a general model for spermiogram in caridean shrimps due to the great similarity of male reproductive systems within the group.

1. Introduction

The vast majority of caridean shrimps are gonochoric and clearly sexually dimorphic. However, in this Infraorder, sex change during lifetime (i.e. hermaphroditism) has been reported for several species. Even with many types of sexual systems, the processes of spermatogenesis and spermiogenesis are similar in gonochoric or hermaphrodite carideans (Braga et al., 2009; Nunes et al., 2010; Sagi et al., 1988; Silva et al., 2009). Spermatogenesis in carideans occurs in the seminiferous tubules of the testicles and their spermatogonia and spermatocytes have similar characteristics to those of other invertebrates (Braga et al., 2009; Poljaroen et al., 2010). Nevertheless, the latter authors demonstrated that upon entering spermiogenesis, spermatids undergo a differentiation process with unique traits to the group. At the end of this process, tailless and immotile spermatozoa resembling an inverted umbrella are moved to the vas deferens.

Knowledge of sperm morphology and production, as well as preservation, quality, density and possible effects of nutrition are key tools in any shellfish farming program with emphasis on carideans (Chow et al., 1985; Daniels et al., 2010; Moraes-Valenti and Valenti, 2010; Samuel et al., 1999). The results obtained with the use of these practices can be easily seen with increase in production of the giant river prawn *Macrobrachium rosenbergii* (De Man, 1879) as reported by New et al. (2010). With an annual global production of over 215,000 tons in 2014, this is the most farmed freshwater prawn species worldwide (FAO, 2016). Despite that, approximately 250 species have been described for the genus *Macrobrachium* Spence Bate, 1868 (for revision, see De Grave and Fransen, 2011). Kutty and Valenti (2010) evaluated these species based on their potential for farming, ease of rearing and maintenance, having 30 species, including the Amazon River prawn *Macrobrachium amazonicum* (Heller, 1862) as the main species for South America.

* Corresponding author.
E-mail addresses: lucasrezende20@gmail.com (L.R.P. Paschoal), fjzara@fcav.unesp.br (F.J. Zara).

https://doi.org/10.1016/j.aquaculture.2017.12.046
Received 20 November 2016; Received in revised form 19 December 2017; Accepted 27 December 2017
Available online 28 December 2017
0044-8486/ © 2017 Elsevier B.V. All rights reserved.

Macrobrachium amazonicum has a wide geographic distribution, occurring in aquatic environments from Costa Rica to Argentina (Vergamini et al., 2011). This species is economically important in the Brazilian aquaculture and is highly consumed in northern and northeastern Brazil (Maciel and Valenti, 2009; Odinetz-Collart and Moreira, 1993). In addition, *M. amazonicum* is highly variable regarding its morphology, reproduction and phenotypic plasticity, allowing it to colonize several aquatic environments (Augusto and Valenti, 2016; Maciel and Valenti, 2009; Odinetz-Collart and Rabelo, 1996; Vergamini et al., 2011). In estuaries, this species has an amphidromous life history (Bauer, 2013; Meireles et al., 2013), but populations of *M. amazonicum* that recently colonized lentic environments became hololimnic (Pantaleão et al., 2014; Paschoal and Zara, 2017). Despite its economic importance and wide distribution in Brazilian aquatic environments, information on aspects of the male reproductive system of *M. amazonicum* is scarce. Currently, only Silva et al. (2009) have briefly discussed the histological pattern of the testicles of this species, but no information was provided regarding sperm production or sperm count.

Sperm concentration is one of the main parameters used to assess sperm quality in aquatic organisms such as mollusks (Naud and Havenhand, 2006), fishes (Billard et al., 1995; Felizardo et al., 2010) and amphibians (Dziminski et al., 2009; Olmstead et al., 2009). In these studies, the authors used spermatophores (mollusks) or sperm mass (fishes), as well as macerated testicles (amphibians), for sperm count with a hemocytometer. These techniques have already been used in penaeid shrimps (Ceballos Vázquez et al., 2003; Díaz et al., 2001; Leung-Trujillo and Lawrence, 1987), as well as in species of the genus *Macrobrachium* (Samuel et al., 1999). However, in all these studies, spermatophores or sperm masses of shrimps/prawns were obtained by electroejaculation or manual pressure, possibly increasing sample error due to variability of the ejaculate (Sandifer and Lynn, 1980; Sandifer et al., 1984). In this context, we describe a simple and practical technique for sperm count (spermiogram) in carideans that avoids sampling bias/errors, using *M. amazonicum* as model. We also test the feasibility of this procedure with animals from zoological collections, preserved for different periods of time and fixatives, and compare animals from populations with different types of life history.

2. Materials and methods

2.1. Sampling, morphotyping and measurement

Males of *M. amazonicum* with hololimnetic development were collected from the Grande River (20° 30′ 53,6″ S; 46° 50′ 16,4″ W), located in the reservoir of the Hydroelectric Power Station of Marechal Mascarenhas de Morais, municipality of Cássia, state of Minas Gerais, southeastern Brazil. From October 2014 to December 2015, two methods were used to collect animals: active sampling - one sieve (60 cm in diameter and 1.25 mm mesh) was moved several times for 30 min manually (one collector) along the marginal vegetation and macrophyte banks, and passive collection - six traps (85 cm in length × 35 cm in width/height) were placed near the margin (0.5–2 m), baited with small pieces of beef liver and removed after 4 h. Additionally, amphidromous males were obtained from the Crustacean sector of the Aquaculture Center of UNESP/Jaboticabal (CAUNESP). This population was collected in the municipality of Santa Bárbara do Pará, state of Pará, northern Brazil, where it inhabits estuarine areas connected to the ocean (Meireles et al., 2013). Animals from this population were collected from August 2015 to April 2016. After capture, live specimens were transported in thermal insulation boxes with aeration to the laboratory. The animals were kept in aquariums with dark bottoms, with basalt gravel as substrate and macrophytes and fragments of rocks for shelter. Prawns were acclimated and conditioned to a 12 h photoperiod and 26 °C (± 0.5).

Regardless of their life history (amphidromous or hololimnetic), four morphotypes have been described for male prawns of *M. amazonicum*: translucent claw (TC), cinnamon claw (CC), green claw 1 (GC1) and green claw 2 (GC2) (Moraes-Riodades and Valenti, 2004; Pantaleão et al., 2014). Dominant males (GC1 and GC2) have selective advantages over submissive individuals (TC and CC) such as obtaining more food resources and territories, and possibly more matings (Augusto and Valenti, 2016; Ibrahim, 2011). Specimens were separated into morphotypes based on macroscopic visual characteristics (body size, cheliped color, angles of the spines on the carpus and propodus, and the pubescence on dactyls) and then measured using an analog caliper (0.02 mm) following the anatomical portions established by Moraes-Riodades and Valenti (2004) and Pantaleão et al. (2014). The dimensions measured were: carapace length (CL), total length (TL), major cheliped length (MCL), propodus length (PPL) and dactyl length (DCL). The fresh weight (W) of each individual was obtained with an analytical balance (± 0.0001 g).

2.2. Sperm count and viability of the technique

In the present study, only GC1 and GC2 morphotypes of both populations of *M. amazonicum* were used to determine sperm count. After morphotyping and measurement, 15 males of each morphotype and population (N: 60) were anesthetized by chilling (− 20 °C/5 min) and had their reproductive systems carefully removed. Subsequently, testes were separated from vasa deferentia and maintained in distilled water. Each animal had one vas deferens randomly selected and the ejaculatory duct (ED) was measured and sectioned at the beginning of the dilation that formed this portion of the vas deferens (Fig. 1A) and momentarily maintained in distilled water. In this study, we considered this region of the vas deferens as the fixed area for sperm count in *M. amazonicum*, i.e. an anatomically standardized area (see Bauer, 1976). The entire ED was dissociated in a solution containing 9 µl of distilled water and 1 µl of methylene blue. After this step, 1 µl of this solution was removed and diluted in 9 µl distilled water, resulting in an 1:10 dilution. Later, 1 µl of the new solution was pipetted and carefully placed on the central portion of a Neubauer chamber for sperm count under light microscope (20 × magnification). The preparation, randomization and estimation of the sperm count followed the procedures described by Bastidas (2013) and Rouge (2002). In our study, sperm count was reported as: number of spermatozoa/µl (spz/µl). Since no statistical difference in sperm count was found between EDs of the same individual (Student's *t*-test - N: 12; $t = 0.147$; df = 22; $p = 0.88$), only one duct was used for counting per animal. After the sperm count, the macerated portion of ED was added to the rest of the vasa deferentia and dehydrated in an oven at 70 °C for 48 h and weighed with an analytical balance (± 0.0001 g). The same procedure was conducted for the testicles, in order to obtain the dry weight of the reproductive system of prawns from amphidromous and hololimnetic populations.

Ten prawns recently-preserved (one to five days) in 70% ethanol and other ten prawns recently-preserved in 4% paraformaldehyde in 0.2 M sodium phosphate buffer underwent the same procedure used for the dissection and sperm count. These animals had their sperm count compared to the fresh animal sperm count values. After that, the feasibility of this procedure was tested in animals from zoological collections, preserved in 70% ethanol for different periods of time. Prawns were preserved for different time intervals from 5 to 480 days and belonged to the collection of the Invertebrate Morphology Laboratory (IML) of DBAA (FCAV/Campus de Jaboticabal). Six time intervals were used: animals preserved for 5 to 15 days (0), one month (1), two months (2), between three and six months (3–6), between seven and twelve months (7–12) and between thirteen and sixteen months (13–16). For each time interval, regardless of morphotype and life history, six individuals (N: 36) were randomly sampled and later analyzed.

In order to evaluate if the protocol applied in animals preserved in 70% ethanol altered the structure of spermatozoa under light microscope, histological analyzes of the testicle and ED of eight recently-

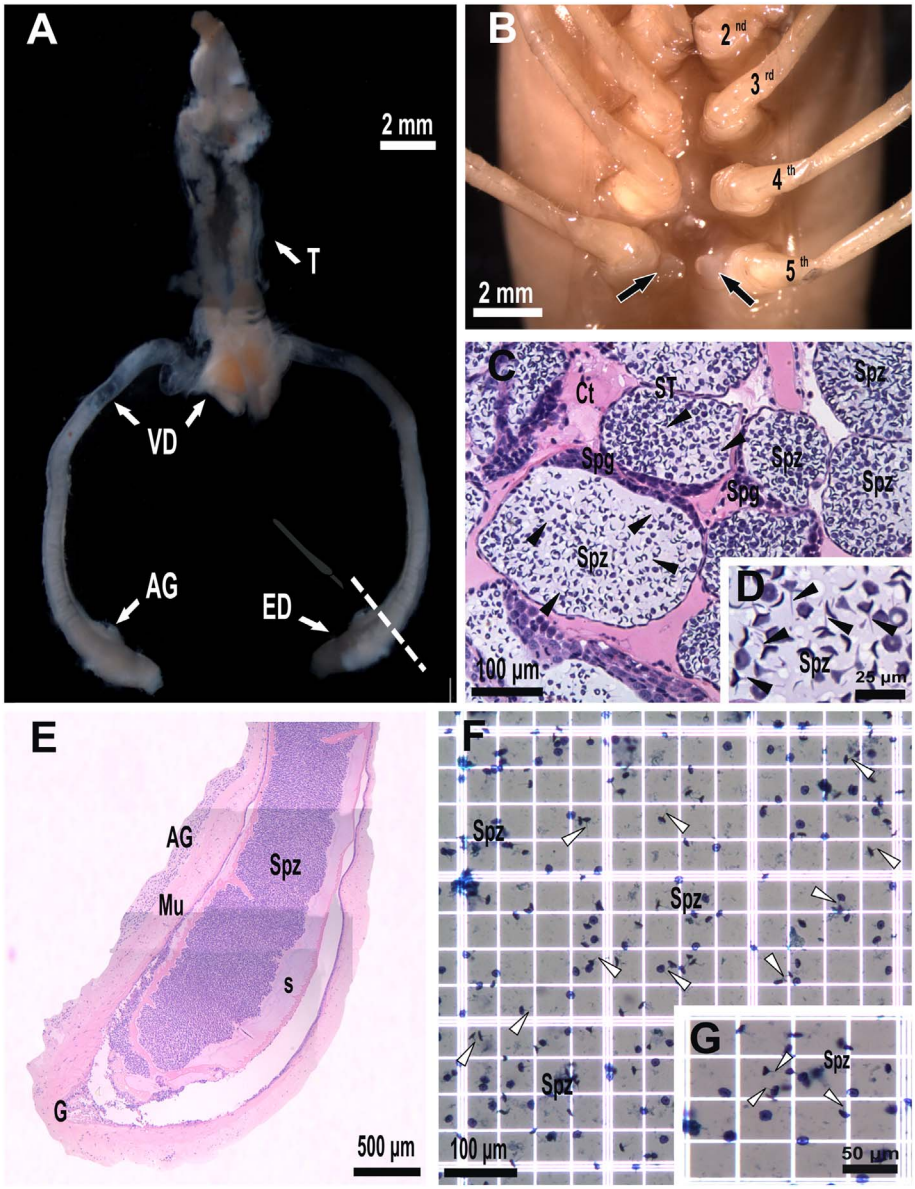

Fig. 1. A. Overview of the male reproductive system in *Macrobrachium amazonicum*. The scalpel shows the region where the ejaculatory duct was sectioned to determine the sperm count in an amphidromous prawn of morphotype green claw 2 (GC2). B. Ventral view of the abdominal sternum of a hololimnetic male of morphotype green claw 1 (GC1), with gonopore openings in the fifth pair of pereiopods covered by flaps (black arrows). C–D. Seminiferous tubules of the testicle of a hololimnetic GC2 male nearly completely filled with a large quantity of spermatozoa. Note the spike in the distal portion of the spermatozoon (black arrowheads) [H&E]. E. Longitudinal section of the ejaculatory duct of a hololimnetic GC1 male filled with spermatozoa surrounded by secretion [H&E]. F. Central view of the counting area of the Neubauer chamber with spermatozoa with a spike in their distal portion (white arrowheads) [methylene blue]. G. Detail in higher magnification of F, showing a grid used to determine the sperm count in an amphidromous GC2 male [methylene blue]. AG = androgenic gland. Ct = connective tissue. ED = ejaculatory duct. G = genital pore. mu = muscle. s = secretion. Spg = spermatogonia. Spz = spermatozoa. ST = seminiferous tubules. T = testicles. VD = vasa deferentia.

collected animals (two animals of each morphotype per population) were conducted according to Zara et al. (2012). After the dissection, fresh samples were fixed in 4% paraformaldehyde (24 h), washed twice in 0.2 M sodium phosphate buffer (pH 7.2), dehydrated in an increasing series of ethanol (70 to 95%) and embedded in glycol-methacrylate historesin Leica®. Blocks were cut in 4–5 µm sections with a rotary microtome and slides were stained with hematoxylin and eosin (H&E).

2.3. Data analysis

The morphometric variables of the farmed amphidromous population were compared to the variables of the wild hololimnetic population with the Hotelling's T^2 test. The dry weight of the reproductive systems of the amphidromous and hololimnetic populations was compared with the non-parametric Mann-Whitney test (normality assumption was not satisfied - Shapiro-Wilk: W-Statistic = 0.86, $p < 0.001$).

A two-way analysis of variance (ANOVA) was used to examine if sperm counts differed between morphotypes (GC1 and GC2) and different life histories (amphidromous and hololimnetic) in *M. amazonicum*. An interaction between these two factors (morphotypes X life histories) was also analyzed. The relationship between sperm count and CL of these animals was analyzed with a linear regression (best fitted regression). The coefficient of determination (r^2) and the degree of allometry (*b* - verified with the Student's *t*-test) of the regression lines were calculated in order to examine if the pattern of body growth influenced sperm density of animals.

The Student's *t*-test was used to examine if the values obtained for the fresh sperm count differed from those recorded for animals recently-preserved directly in 4% paraformaldehyde and 70% ethanol. An ANOVA was used to examine if the sperm counts of preserved individuals varied throughout time of preservation in 70% ethanol. All calculations and statistical analyzes were performed with the software R version 3.3.1. (R CORE TEAM, 2016).

3. Results

The male reproductive system of *M. amazonicum* consists of a pair of testicles and vasa deferentia with ejaculatory ducts (ED) connected to gonopores. The testicles are united at the anterior and posterior regions of the organ and are positioned above the hepatopancreas of the animal. Vasa deferentia are arranged laterally to the testis, directly connected to this organ (Fig. 1A). These long ducts open into the gonopores

Table 1
Comparison of morphometric variables and other variables (± standard deviation) analyzed in *Macrobrachium amazonicum* male prawns of morphotypes green claw 1 and green claw 2 with amphidromous and hololimnetic life histories.

Morphotype	Life history	CL (mm)	TL (mm)	MCL (mm)	PPL (mm)	DCL (mm)	W (g)	RS (g)	ED (mm)
GC1	A	22.86 (± 2.40)	70.50 (± 4.87)	72.01 (± 13.50)	23.50 (± 5.30)	10.43 (± 2.34)	6.9015 (± 1.3539)	0.0044 (± 0.0022)	3.46 (± 0.59)
GC2	A	24.58 (± 2.58)	74.85 (± 6.33)	97.35 (± 10.97)	32.11 (± 3.83)	13.46 (± 1.51)	8.8926 (± 2.5100)	0.0067 (± 0.0026)	3.78 (± 0.62)
GC1	H	19.04 (± 1.68)	59.34 (± 4.03)	55.15 (± 9.00)	15.79 (± 3.77)	7.61 (± 1.47)	3.7112 (± 0.8590)	0.0021 (± 0.0011)	3.09 (± 0.57)
GC2	H	21.55 (± 2.13)	65.44 (± 4.94)	80.21 (± 11.97)	26.44 (± 4.79)	12.28 (± 2.16)	5.5246 (± 1.3165)	0.0020 (± 0.0008)	3.16 (± 0.47)

A = amphidromous.
CL = carapace length.
DCL = dactyl length.
ED = ejaculatory duct length.
GC1 = green claw 1 morphotype.
GC2 = green claw 2 morphotype.
H = hololimnetic.
MCL = major cheliped length.
PPL = propodus length.
RS = reproductive system.
TL = total length.
W = fresh weight.

of the coxopodites of the fifth pair of pereiopods. In males, the gonopores are covered by flaps and their openings are oriented towards one another for the formation of spermatophoric mass (Fig. 1B). Microscopically, the testicles are formed by seminiferous tubules grouped in lobules, where spermatogenesis and spermiogenesis take place. The tubules are surrounded by accessory or Sertoli-cells lying on a thin layer of connective tissue. Spermatozoa with a spike in their distal portion were abundant enough to fill nearly entirely the tubules (Fig. 1C–D). The EDs have a large quantity of centrally compacted spermatozoa surrounded by secretion and open into the gonopores through a genital pore. The androgenic gland lies perpendicularly on the muscle layer (Fig. 1E). No anatomical or histological differences were observed in the male reproductive system between the studied populations or morphotypes.

The populations analyzed in our study had distinct life histories and morphological characteristics (Table 1). The body size and morphometric variables of males of the amphidromous population were larger when compared to individuals of the hololimnetic population (Hotelling's T^2 - $F = 11.88$; $p < 0.0001$). In addition, the male reproductive system was heavier than those of the wild hololimnic population ($U = 129.50$; $p < 0.001$). Despite these differences in morphometric parameters and weight between populations, the mean values of sperm count were similar. Also, morphological abnormalities such bent or missing spikes were not observed in spermatozoa of *M. amazonicum*. In amphidromous GC1 males, the mean sperm count was $60.3 \pm 23.6 \times 10^3$ spz/μl, while for the GC2 morphotype, $65.3 \pm 25.0 \times 10^3$ spz/μl. The mean density obtained for hololimnetic animals was slightly lower than that recorded for amphidromous prawns, with $48.9 \pm 13.9 \times 10^3$ and $53.8 \pm 21.2 \times 10^3$ spz/μl for GC1 and GC2 individuals, respectively (Table 2). No differences in sperm count were found between the amphidromous and hololimnetic populations ($F = 3.37$; $p = 0.07$) or between morphotypes ($F = 0.48$; $p = 0.49$). Also, no interaction between the two factors analyzed was observed ($F = 0.01$; $p = 0.97$), and sperm density was not correlated to the size of the animals (Fig. 2 and Table 3).

The values of sperm counts did not differ between animals preserved directly in 4% paraformaldehyde ($\overline{X}$ ± SD: $44.8 \pm 11.7 \times 10^3$ - Student's *t*-test: $t = 0.20$; df = 18; $p = 0.83$) or 70% ethanol ($\overline{X}$ ± SD: $47.5 \pm 19.1 \times 10^3$ - Student's *t*-test: $t = -0.09$; df = 18; $p = 0.93$) when compared to the values obtained in fresh animals ($\overline{X}$ ± SD: $46.6 \pm 24.1 \times 10^3$) (Table 4). The absence of statistical differences in sperm count of fresh or preserved animals allowed the randomization of specimens in collection samples. Using the same procedures for sperm count, no changes were observed in spermatozoon structure in individuals preserved in 70% ethanol under light microscope, characterized by its tack-like shape, with a concave basal portion and a spike in its distal portion (Fig. 1F–G). The analysis of time intervals for the preservation of specimens in 70% ethanol did

Table 2
Mean (± standard deviation), minimum and maximum values for fresh sperm count (number of spermatozoa/μl) in *Macrobrachium amazonicum* male prawns of morphotypes green claw 1 and green claw 2 with amphidromous and hololimnetic life histories.

Morphotype	Life history	Mean (± SD)	Minimum	Maximum
GC1	A	60,258 (± 23,611)	32,750	100,500
GC2	A	65,308 (± 25,004)	19,250	115,750
GC1	H	48,950 (± 13,860)	20,750	83,000
GC2	H	53,850 (± 21,211)	32,000	97,500

A = amphidromous.
GC1 = green claw 1 morphotype.
GC2 = green claw 2 morphotype.
H = hololimnetic.

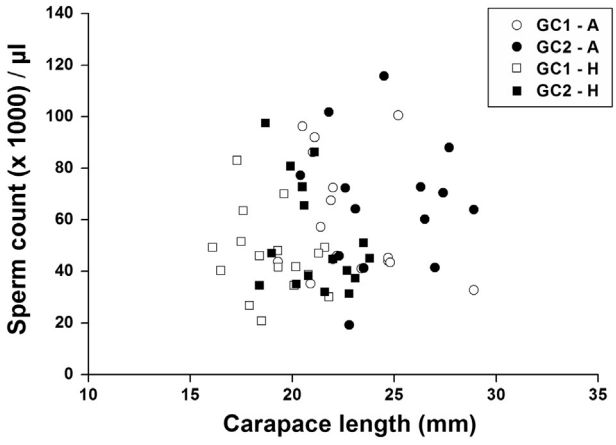

Fig. 2. Scatter plot of sperm count (number of spermatozoa/μl) vs. carapace length for males of *Macrobrachium amazonicum* with morphotypes green claw 1 and green claw 2 with amphidromous and hololimnetic life histories. A = amphidromous. GC1 = green claw 1 morphotype. GC2 = green claw 2 morphotype. H = hololimnetic.

Table 3
Relationship between sperm count and carapace length of *Macrobrachium amazonicum* males of dominant morphotypes with amphidromous and hololimnetic life histories.

Morphotype	Life history	Linear regression	r^2	*t*	Al
GC1	A	CL: - 3.01*SC + 128.80	0.09	1.59^{ns}	0
GC2	A	CL: 0.65*SC + 49.38	0.01	0.13^{ns}	0
GC1	H	CL: - 2.17*SC + 87.30	0.06	1.44^{ns}	0
GC2	H	CL: - 4.72*SC + 152.40	0.14	1.86^{ns}	0

A = amphidromous.
Al = allometry.
CL = carapace length.
GC1 = green claw 1 morphotype.
GC2 = green claw 2 morphotype.
H = hololimnetic.
ns = not significant.
r^2 = coefficient of determination.
SC = sperm count.

Table 4
Comparison of *Macrobrachium amazonicum* sperm count (number of spermatozoa/μl) values obtained in ten randomly selected fresh prawns and recently-preserved prawns in 70% ethanol and 4% paraformaldehyde.

Prawns	Fresh	Ethanol	Paraformaldehyde
1	88,000	86,250	26,750
2	60,250	46,125	32,750
3	70,500	57,250	44,750
4	41,000	43,750	38,750
5	19,250	72,500	49,250
6	14,500	33,750	33,250
7	67,500	26,750	48,000
8	37,250	40,250	55,750
9	42,000	31,250	60,250
10	26,000	37,250	59,000
Mean (± SD)	46,625 (± 24,155)	47,513 (± 19,087)	44,850 (± 11,697)

Table 5
Mean (± standard deviation), minimum and maximum values for sperm count (number of spermatozoa/μl) in *Macrobrachium amazonicum* preserved for different periods of time in 70% ethanol.

Time (months)	Mean (± SD)	Minimum	Maximum
0	57,750 (± 24,256)	19,250	88,000
1	61,688 (± 16,189)	43,750	86,250
2	45,875 (± 18,716)	26,750	72,750
3–6	51,750 (± 19,920)	30,000	80,750
7–12	52,375 (± 18,531)	32,000	83,000
13–16	65,208 (± 26,060)	26,000	97,500

not reveal significant differences in sperm counts ($F = 0.70$, $p = 0.63$) (Table 5).

4. Discussion

The populations of *Macrobrachium amazonicum* have very distinct life histories and morphological traits, but not statistically different sperm counts. Males of the farmed amphidromous population had larger body size and heavier reproductive system than individuals of the wild hololimnetic population. This difference is due to the additional nutritional food received during rearing (Moraes-Valenti and Valenti, 2010). The similarities in sperm count may be associated to highly conservative patterns of spermatogenesis, spermiogenesis and other sperm peculiarities at the family or subfamily levels in aquatic organisms (Jamieson, 1991; Poljaroen et al., 2010), which would explain the comparable values despite differences in morphology and life history. Unlike females, whose gametes are produced directly proportional to their body size, (Bauer, 2004; Meireles et al., 2013), sperm production in males of *M. amazonicum* was not correlated to somatic growth, occurring independently to body size increase. In *M. amazonicum*, males have a social hierarchy and during the mating period, they protect and guard females (Paschoal, 2017). Thus, a smaller portion of energy seems to be destined to the production of gametes and seminal fluid, while a greater portion may be directed to body size increase and production and maintenance of robust chelipeds (i.e. sexual weapons), increasing the fitness of males in intraspecific competitions (Correa and Thiel, 2003; Paschoal et al., 2016). *Macrobrachium amazonicum* has great adaptive and morphological plasticity that can be explained by the synergy of reproductive variability and physiological responses to the environments in which it occurs (Maciel and Valenti, 2009; Vergamini et al., 2011). Recently, individuals of this species with traits only seen in amphidromous populations, such as presence of four morphotypes in males, large body size and ovigerous females with large quantities of small eggs, were found in lentic environments, indicating that this species might be changing its life history pattern (i.e. hololimnia) (Pantaleão et al., 2014; Paschoal, 2017; Paschoal and Zara, 2017).

The anatomical and histological pattern of the male reproductive system of *M. amazonicum* is similar to those commonly described for carideans. In *M. amazonicum*, the testicles and vasa deferentia with ejaculatory ducts connect to gonopores in the coxopodites of the fifth pair of pereiopods. The testicles are joined in the anterior and posterior regions of the organ. The reproductive system of this species is very similar to those of other species of the genus *Macrobrachium* and *Palaemon* Weber, 1795 (see Bauer, 2004; Sandifer and Lynn, 1980), although some differences may be observed, such as those described for *M. rosenbergii*, where the testicle is connected only in the anterior region of the organ (Chow et al., 1982). Histologically, the male reproductive system of *M. amazonicum* is similar, nearly identical, to those of gonochoric and hermaphrodite caridean shrimps already studied (Braga et al., 2009; Chow et al., 1982; Nunes et al., 2010; Poljaroen et al., 2010; Sagi et al., 1988; Silva et al., 2009). The similarity of the male reproductive system in carideans allows this technique to be extended to other species of the group. The application of this protocol to determine sperm count in *Macrobrachium olfersii* (Wiegmann, 1836) revealed similar results (N: 3, $\overline{X}$ ± SD: 67,250 ± 17,430 spz/μl) to that obtained for *M. amazonicum* (Paschoal and Zara, personal data). In hermaphrodite species with a viable male reproductive system throughout their development and sex change, such as in *Exhippolysmata oplophoroides* (Holthuis, 1948) and *Lysmata wurdemanni* (Gibbs, 1850), this technique may be easily performed, since in these species, EDs are anatomically quickly identified and contain considerable quantities of spermatozoa in all sexual stages (Bortolini and Bauer, 2016; Braga et al., 2009).

The technique described here for *M. amazonicum* was very effective to determine the sperm count of fresh and preserved animals, in addition to maintain the structures that compose the spermatozoa preserved under light microscope. The small number of studies on sperm count in *Macrobrachium* limits the comparison of the results obtained in our study with those of other populations of the species and other congeners. Only Samuel et al. (1999) has reported the use of sperm count with a hemocytometer for the monsoon river prawn, *M. malcolmsonii* (Milne-Edwards, 1844). Comparing the results obtained here for *M. amazonicum* using EDs as standardized area (maximum count: $\sim 120 \times 10^3$ spz/μl) with those obtained by Samuel et al. (1999) that obtained spermatophoric masses by electroejaculation (maximum count: $\sim 500 \times 10^6$ spz/spermatophoric mass), a wide difference is observed. This might be due to the absence of complex spermatophores in male prawns of the genus *Macrobrachium*, which produce a simple spermatophoric mass instead that varies considerably in size when formed and deposited on the female sternum, added to the possible sampling error promoted by electroejaculation (for revision see Chow et al., 1982; Sandifer and Lynn, 1980; Sandifer et al., 1984).

Although few studies have determined the sperm count of *Macrobrachium* species, it has been reported for the brachyurans:

Callinectes sapidus Rathbun, 1896 by Hines et al. (2003) and Kendall et al. (2001), and the parastacids: *Cherax quadricarinatus* (von Martens, 1868) by Bugnot and López-Greco (2009a, 2009b). This protocol has been widely used in penaeid shrimps, such as *Farfantepenaeus paulensis* (Perez-Farfante, 1967) in Braga et al. (2010), *Litopenaeus setiferus* Linnaeus, 1767 in Diamond et al. (2008) and Leung-Trujillo and Lawrence (1987), *L. vannamei* (Boone, 1931) in Ceballos Vázquez et al. (2003) and *Pleoticus muelleri* (Bate, 1888) in Díaz et al. (2001). However, all previously mentioned studies involving penaeids shrimps did not evaluate a morphologically standardized sampling area, but rather spermatophores to determine sperm count. These studies might have overestimated sperm density in these species (values expressed between 10^6 and 10^9), since these structures have considerable variability in size and number of spermatozoa (Bugnot and López-Greco, 2009a). Also, they are usually modulated by the size and shape of the receptacles and sternum of females, where they are deposited after mating (Bauer, 1986). The wide variability of sperm-carrying structures (spermatophores and spermatophoric mass) would justify the use of the technique described herein, by reducing this type of sample bias, since only an anatomically standardized area (i.e. ejaculatory duct) seems to be more feasible.

The present study proposes a simple technique to determine sperm count in fresh and well-preserved caridean shrimps. In Amazon River prawn *M. amazonicum*, it can be used for the maintenance of breeders, improvement of stocks, assessment of temporal series of breeders, as well as to aid future assays aimed at cryopreservation of spermatophoric masses in this species and possible crosses between amphidromous and hololimnetic populations. Viability techniques can be performed together with sperm count protocol here proposed. Simple techniques such trypan blue or eosin-nigrosin smears can be used to evaluate the viability of spermatozoa in fresh animals using light or phase-contrast microscopy (see Supplementary material Fig. S1A, D). In *M. amazonicum*, the sperm viability varies from 90-95% (N: 5). Sperm viability could not be determined in animals preserved in any type of fixative (e.g. ethanol or paraformaldehyde). The preservation process promotes the death of the spermatozoa, i.e. 0% viability (see Supplementary material Fig. S1B-C, E-F). Also, the sperm count protocol was successfully performed in animals preserved in 70% ethanol for over 24 months as well as animals preserved in 10% formaldehyde (Paschoal and Zara, personal data). Thus this protocol may be a useful tool for researchers whose collections are preserved in ethanol or formaldehyde, providing opportunities for new studies on sperm production in different species of carideans.

5. Conclusions

The sperm count protocol proposed here is easy to perform, practical and reproducible, and can be applied successfully in both fresh and preserved animals in various types of fixatives (e.g. ethanol, paraformaldehyde and formaldehyde). The technique used in specimens preserved for long periods of time (> 1 year) did not interfere with sperm concentration. Also, no changes in the spermatozoa structure were observed applying this protocol. The choice of the Amazon River prawn *M. amazonicum* as the standard model for the sperm count protocol allows its extrapolation to other species of the Infraorder Caridea, since this species presents great reproductive and morphometric variability, added to different types of life history (amphidromy or hololimnia). The standardization of an anatomical area makes the results closer to each other, thus reducing sample bias and errors in total sperm concentration values.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2017.12.046.

Acknowledgements

The first author thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for granting a doctoral fellowship. We thank CAPES Ciências do Mar CIMAR II #1989/2014. FJZ also thanks Fapesp Biota (grants 2010/50188-8 and 2016/25344-2). We thank the Editor-in-Chief and the reviewers, for the constructive reviews. This study complied with Brazilian regulations (MMA-ICMbio, license 47653-1 to LRPP, and MMA-ICMbio, permanent license 34587-1 to FJZ).

References

Augusto, A., Valenti, W.C., 2016. Are there any physiological differences between the male morphotypes of the freshwater shrimp *Macrobrachium amazonicum* (Heller, 1862) (Caridea: Palaemonidae)? J. Crustac. Biol. 36, 716–723.

Bastidas, O., 2013. Cell counting with Neubauer chamber, basic hemocytometer usage. In: Technical Note - Neubauer Chamber Cell Counting, . http://www.celeromics.com/en/resources/Technical%20Notes/cell-article-chamber.php/, Accessed date: 21 October 2015.

Bauer, R.T., 1976. Mating behaviour and spermatophore transfer in the shrimp *Heptacarpus pictus* (Stimpson) (Decapoda: Caridea: Hippolytidae). J. Nat. Hist. 10, 415–440.

Bauer, R.T., 1986. Phylogenetic trends in sperm transfer and storage complexity in decapod crustaceans. J. Crustac. Biol. 6, 313–325.

Bauer, R.T., 2004. Remarkable Shrimps. Adaptations and Natural History of the Carideans, first ed. University of Oklahoma Press, Oklahoma.

Bauer, R.T., 2013. Amphidromy in shrimps: a life cycle between rivers and the sea. Lat. Am. J. Aquat. Res. 41, 633–650.

Billard, R., Cosson, J., Crim, L.W., Suquet, M., 1995. Sperm physiology and quality. In: Bromage, N.R., Roberts, R.J. (Eds.), Brood Stock Management and Egg and Larval Quality. Blackwell Science Ltd, Oxford, England, pp. 25–52.

Bortolini, J.L., Bauer, R.T., 2016. Persistence of reduced androgenic glands after protandric sex change suggests a basis for simultaneous hermaphroditism in a caridean shrimp. Biol. Bull. 230, 110–119.

Braga, A.A., López-Greco, L.S., Santos, D.C., Fransozo, A., 2009. Morphological evidence for protandric simultaneous hermaphroditism in the caridean *Exhippolysmata oplophoroides*. J. Crustac. Biol. 29, 34–41.

Braga, A.L., Nakayama, C.L., Martins, J.G., Colares, E.P., Wasielesky, W., 2010. Spermatophore quality of the pink shrimp *Farfantepenaeus paulensis* (Decapoda, Dendrobranchiata) broodstock fed with different maturation diets. Aquaculture 307, 44–48.

Bugnot, A.B., López-Greco, L.S., 2009a. Sperm production in the red claw crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). Aquaculture 295, 292–299.

Bugnot, A.B., López-Greco, L.S., 2009b. Structural alterations in the male reproductive system of the freshwater crayfish, *Cherax quadricarinatus* (Decapoda, Parastacidae). J. Invertebr. Pathol. 102, 160–166.

Ceballos Vázquez, B.P., Rosas, C., Racotta, I.S., 2003. Sperm quality in relation to age a weight of white shrimp *Litopenaeus vannamei*. Aquaculture 228, 141–151.

Chow, S., Ogasawara, Y., Taki, Y., 1982. Male reproductive system and fertilization of the palaemonid shrimp *Macrobrachium rosenbergii*. Bull. Jpn. Soc. Sci. Fish. 48, 177–183.

Chow, S., Taki, Y., Ogasawara, Y., 1985. Cryopreservation of spermatophore of the fresh water shrimp, *Macrobrachium rosenbergii*. Biol. Bull. 168, 471–475.

Correa, C., Thiel, M., 2003. Mating systems in caridean shrimp (Decapoda: Caridea) and their evolutionary consequences for sexual dimorphism and reproductive biology. Rev. Chil. Hist. Nat. 76, 187–203.

Daniels, W.H., Cavalli, R.O., Smullen, R.P., 2010. Broodstock management. In: New, M.B., Valenti, W.C., Tidwell, J.H., D'abramo, L.R., Kutty, M.N. (Eds.), Freshwater Prawns: Biology and Farming. Wiley-Blackwell, Oxford, England, pp. 40–54.

De Grave, S., Fransen, C.H.J.M., 2011. Carideorum Catalogus: the recent species of the Dendrobranchiate, Stenopodidean, Procarididean and Caridean shrimps (Crustacea: Decapoda). Zool. Meded. 85, 195–589.

Diamond, S., Powell, A., Shields, R.J., Rowley, A.F., 2008. Is spermatophore melanisation in captive shrimp (*Litopenaeus vannamei*) a result of an auto-immune response? Aquaculture 285, 14–18.

Díaz, A.C., Fernández Gimenez, A.V., Harán, N., Fenucci, J.L., 2001. Reproductive performance of male red shrimp *Pleoticus muelleri* Bate (Decapoda, Penaeoidea) in culture conditions. J. World Aquacult. Soc. 32, 236–242.

Dziminski, M.A., Roberts, J.D., Beveridge, M., Simmons, L.W., 2009. Sperm competitiveness in frogs: slow and steady wins the race. Proc. R. Soc. Lond. B Biol. Sci. 276, 3955–3961.

FAO (Food and Agriculture Organization of the United Nations), 2016. *Macrobrachium rosenbergii* (De Man, 1879). Fishstat Plus version 2. pp. 32. http://www.fao.org/fishery/culturedspecies/Macrobrachium_rosenbergii/en/, Accessed date: 9 March 2016.

Felizardo, V.O., Mello, R.A., Murgas, L.D.S., Andrade, E.S., Drumond, M.M., Rosa, P.V., 2010. Effect of cryopreservant combinations on the motility and morphology of curimba (*Prochilodus lineatus*) sperm. Anim. Reprod. Sci. 122, 259–263.

Hines, A.H., Jivoff, P.R., Bushmann, P.J., Van Montfrans, J., Reed, S.A., Wolcott, D.L., Wolcott, T.G., 2003. Evidence for sperm limitation in the blue crab, *Callinectes sapidus*. Bull. Mar. Sci. 72, 287–310.

Ibrahim, A.N.A.F., 2011. Controle social do crescimento do camarão-da-amazônia *Macrobrachium amazonicum* (MSc. thesis). Universidade Estadual Paulista - UNESP Jaboticabal, São Paulo, Brazil (39 pp.).

Jamieson, B.G.M., 1991. Fish Evolution and Systematics: Evidence from Spermatozoa. Cambridge University Press, Cambridge, UK.

Kendall, M.S., Wolcott, D.L., Wolcott, T.G., Hines, A.H., 2001. Reproductive potential of

individual male blue crabs, *Callinectes sapidus*, in a fished population: depletion and recovery of sperm number and seminal fluid. Can. J. Fish. Aquat. Sci. 58, 1168–1177.
Kutty, M.N., Valenti, W.C., 2010. Culture of other freshwater prawn species. In: New, M.B., Valenti, W.C., Tidwell, J.H., D'ABramo, L.R., Kutty, M.N. (Eds.), Freshwater Prawns: Biology and Farming. Wiley-Blackwell, Oxford, England, pp. 502–523.
Leung-Trujillo, J.R., Lawrence, A.L., 1987. Observations on the decline effect in sperm quality of *Penaeus setiferus* under laboratory conditions. Aquaculture 65, 363–370.
Maciel, C.R., Valenti, W.C., 2009. Biology, fisheries, and aquaculture of the Amazon River prawn *Macrobrachium amazonicum*: a review. Nauplius 17, 61–79.
Meireles, A.L., Valenti, W.C., Mantelatto, F.L., 2013. Reproductive variability of the Amazon River prawn, *Macrobrachium amazonicum* (Caridea, Palaemonidae): influence of life cycle on egg production. Lat. Am. J. Aquat. Res. 41, 718–731.
Moraes-Riodades, P.M.C., Valenti, W.C., 2004. Morphotypes in male Amazon river prawns, *Macrobrachium amazonicum*. Aquaculture 236, 297–307.
Moraes-Valenti, P.M., Valenti, W.C., 2010. Culture of the Amazon River prawn *Macrobrachium amazonicum*. In: New, M.B., Valenti, W.C., Tidwell, J.H., D'ABramo, L.R., Kutty, M.N. (Eds.), Freshwater Prawns: Biology and Farming. Wiley-Blackwell, Oxford, England, pp. 485–501.
Naud, M.J., Havenhand, J.N., 2006. Sperm motility and longevity in the giant cuttlefish, *Sepia apama* (Mollusca: Cephalopoda). Mar. Biol. 148, 559–566.
New, M.B., Valenti, W.C., Tidwell, J.H., D'Abramo, L.R., Kutty, M.N., 2010. Freshwater Prawns: Biology and Farming. Wiley-Blackwell, Oxford, UK.
Nunes, E.T., Braga, A.A., Dos Santos, D.C., Camargo-Mathias, M.I., 2010. Cytodifferentiation during the spermatogenesis of the hermaphrodite caridea *Exhippolysmata oplophoroides*. Micron 41, 585–591.
Odinetz-Collart, O., Moreira, L.C., 1993. Potencial pesqueiro de *Macrobrachium amazonicum* na Amazônia Central (Ilha do Careiro): variação da abundância e do comprimento. Amazoniana 12, 399–413.
Odinetz-Collart, O., Rabelo, H., 1996. Variation in egg size of the freshwater prawn *Macrobrachium amazonicum* (Decapoda, Palaemonidae). J. Crustac. Biol. 16, 684–688.
Olmstead, A.W., Korte, J.J., Woodis, K.K., Bennett, B.A., Ostazeski, S., Degitz, S.J., 2009. Reproductive maturation of the tropical clawed frog: *Xenopus tropicalis*. Gen. Comp. Endocrinol. 160, 117–123.
Pantaleão, J.A.F., Hirose, G.L., Costa, R.C., 2014. Ocurrence of male morphotypes of *Macrobrachium amazonicum* (Caridea, Palaemonidae) in a population with an entirely freshwater life cycle. Braz. J. Biol. 74, 223–232.
Paschoal, L.R.P., 2017. História natural de *Macrobrachium amazonicum* (Heller, 1862) (Decapoda: Palaemonidae) e sua importância em reservatórios neotropicais do sudeste brasileiro (PhD thesis). Universidade Estadual Paulista - UNESP Rio Claro, São Paulo, Brazil (320 pp.).
Paschoal, L.R.P., Zara, F.J., 2017. First record of intersexuality in the Amazon River shrimp *Macrobrachium amazonicum* (Heller, 1862) (Caridea: Palaemonidae). J. Crustac. Biol. 37, 507–511.
Paschoal, L.R.P., Guimarães, F.J., Couto, E.C.G., 2016. Growth and reproductive biology of the amphidromous shrimp *Palaemon pandaliformis* (Decapoda, Caridea) in a Neotropical river from northeastern Brazil. Zoologia (Curitiba) 33, 1–14 epub.
Poljaroen, J., Vanichviriyakit, R., Tinikul, Y., Phoungpetchara, I., Linthong, V., Weerachatyanukul, W., Sobhon, P., 2010. Spermatogenesis and distinctive mature sperm in the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879). Zool. Anz. 249, 81–94.
R CORE TEAM, 2016. R (The R Project for Statistical Computing). Software version 3.3.1. https://www.r-project.org/, Accessed date: 8 September 2016.
Rouge, M., 2002. Counting Cells with a Hemacytometer. http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/hemacytometer.html/, Accessed date: 21 October 2015.
Sagi, A., Milner, Y., Cohen, D., 1988. Spermatogenesis and sperm storage in the testes of the behaviorally distinctive male morphotypes of *Macrobrachium rosenbergii* (Decapoda, Palaemonidae). Biol. Bull. 174, 330–336.
Samuel, M.J., Kannupandi, T., Soundarapandian, P., 1999. Nutritional effects on male reproductive performance in the freshwater prawn *Macrobrachium malcolmsonii* (H. Milne Edwards). Aquaculture 172, 327–333.
Sandifer, P.A., Lynn, J.W., 1980. Artificial insemination of caridean shrimp. In: Clark Jr.W.H., Adams, H.S. (Eds.), Recent Advances in Invertebrate Reproduction. Elsevier, Amsterdam, pp. 271–288.
Sandifer, P.A., Lawrence, A.L., Harris, S.A., Chamberlain, G.W., Stokes, D.A., Bray, W.A., 1984. Electrical stimulation of spermatophore expulsion in marine shrimp, *Penaeus* spp. Aquaculture 41, 181–187.
Silva, G.M.F., Pantoja Ferreira, M.A., Von Ledebur, E.I.C.F., Da Rocha, R.M., 2009. Gonadal structure analysis of *Macrobrachium amazonicum* (Heller, 1862) from a wild population: a new insight into the morphotype characterization. Aquac. Res. 40, 798–803.
Vergamini, F.G., Pileggi, L.G., Mantelatto, F.L., 2011. Genetic variability of the Amazon River prawn *Macrobrachium amazonicum* (Decapoda, Caridea, Palaemonidae). Contrib. Zool. 80, 67–83.
Zara, F.J., Toyama, M.H., Caetano, F.H., López-Greco, L.S., 2012. Spermatogenesis, spermatophore and seminal fluid production in the adult blue crab *Callinectes danae* (Portunidae). J. Crustac. Biol. 32, 249–262.