

Dynamics of energy reserves and the cost of reproduction in female and male fiddler crabs



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ABSTRACT

The physiological costs of reproduction can be measured as the energy allocated to reproductive activities. In fiddler crabs, females allocate energy to vitellogenesis and brooding, whereas males perform expensive courtship behaviors. We evaluated in a large-scale study the reproduction cost of females and males of *Leptuca uruguayensis* in a temperate estuary, where their reproductive efforts are synchronized in a short reproductive season. The reproductive investments (vitellogenesis, spermatophore production, and male reproductive behaviors) were measured and related to the dynamics of storage and expenditure of energy reserves (glycogen, total lipids, and total protein) in the hepatopancreas, ovary, and muscle of the enlarged cheliped, throughout one annual cycle. Maximum energy storage occurred in winter, a period of low activity, whereas minimum energy storage occurred during the reproductive period, characterized by expensive activities. The glycogen reserves of the hepatopancreas decreased about 66% in females and 61% in males, suggesting high and similar physiological costs of reproduction between sexes, despite their different reproductive strategies to maximize their fitness.

1. Introduction

The resources destined to growth, maintenance, and reproduction depend on a balance between the availability and demand of these resources, which can vary according to the life stage, life strategy, and environmental conditions (Reznick, 1985; Barnes and Partridge, 2003). Reproduction is an expensive process, and the physiological costs of reproduction can cause conflicting demands for resources with other life history traits (trade-offs between reproduction, growth, and maintenance) (Reznick et al., 2000; Kotiaho, 2001; Ramirez-Llodra, 2002; Reedy et al., 2016). Reproduction also has ecological costs, in which interactions with the environment during reproductive activities expose the organism to risks as disease, injury or predation (Reznick, 1992). Assessing the trade-offs between costs of reproduction and costs of other life variables, as well as their consequences on parental survival and future reproductive performance can contribute to a better understanding of the life history evolution of species (Calow, 1979; Reznick, 1985; Ellers and van Alphen, 1997; Candolin, 1998; Ramirez-Llodra, 2002; Hoy et al., 2016; Kindsvater et al., 2016).

The physiological costs of reproduction can be measured as energy allocated to reproductive activities (Calow, 1979; Kotiaho, 2001; Scharf et al., 2013). Usually, females allocate resources mainly to ensure

embryo development and many species show some level of maternal care, whereas males attempt to increase their mating opportunities. Old concepts supposed that female reproductive costs were greater than those of males, since males show an inexpensive and considered unlimited sperm production (Scharf et al., 2013). However, males of many species show sexually selected ornaments and complex behaviors (mate searching, courtship, combat, and territory defense) that maximize their reproductive success, but also increase the costs of reproduction (Matsumasa and Murai, 2005; Mowles, 2014; Greenspan et al., 2016). In these cases, although females and males have different strategies to maximize their fitness, the reproductive costs can be similar between sexes (Maklakov and Lummaa, 2013; Reedy et al., 2016).

In crabs, most of the knowledge about reproductive investment is based on female effort, probably because the reproductive traits ordinarily measured are those usually used as indicators of costs of reproduction (e.g. gamete production, fecundity, brooding time) (Hartnoll, 2006; Baeza et al., 2015; Bert et al., 2016). Reproductive female crabs load oocytes with yolk proteins (vitellogenesis) and, after fecundation, they brood the egg mass up to eclosion (maternal care). In female crabs, both vitellogenesis and brooding demand high energy expenditure (Eastman-Reks and Fingerman, 1985; Harrison, 1990; Fernández et al., 2000; Tsukimura, 2001; Brante et al., 2003;

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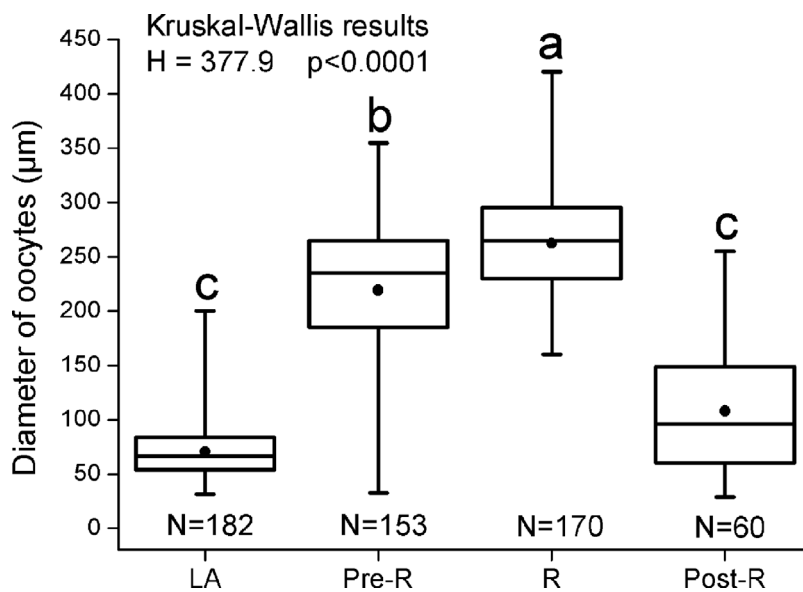


Fig. 1. Comparison of oocyte size (point = mean, line = median, box = 25–75%, whiskers = min–max) found in female gonads of *Leptuca uruguayensis* in each period of the reproductive cycle (LA, low-activity period; Pre-R, pre-reproductive period; R, reproductive period; Post-R, post-reproductive period). N, number of oocytes measured in each period. Different letters indicate statistically significant differences between medians ($p < 0.05$).

Guadagnoli et al., 2005; Zmora et al., 2007; Jimenez and Kinsey, 2015). In crabs, the hepatopancreas is the principal energy storage organ and plays an important role in reproductive activities, since it supplies energy and synthesizes yolk components (Harrison, 1990; Zmora et al., 2007; Girish et al., 2014; Thongda et al., 2015; Sacristán et al., 2017). Unlike females, male crabs allocate a low amount of energy to gamete production (Wu et al., 2010), but show several mating behaviors (Christy, 1987; McLay and Becker, 2015). Besides this behavioral diversity, little is known on the energetic requirements of males for reproduction. Only the glucose and lactate concentrations in the blood of male fiddler crabs have been evaluated as indicators of the metabolic demands of their reproductive behaviors (Matsumasa and Murai, 2005; Matsumasa et al., 2013).

The fiddler crabs are interesting models to evaluate the cost of reproduction, because males have one of the chelipeds enlarged. This large cheliped is a sexually selected ornament, which is displayed vigorously to attract females, and used as a weapon in fights with other males to protect the mating burrow (Crane, 1975). This ornament increases the reproductive success of males, but the use of this enlarged appendage impacts on physiological costs, since it demands high energy expenditure (Matsumasa and Murai, 2005; Allen and Levinton, 2007; Gerald and Thiesen, 2014), and ecological costs, because it increases male vulnerability to predation (Bildstein et al., 1989; Koga et al., 2001; Cummings et al., 2008).

Among fiddler crabs, *Leptuca uruguayensis* is a eurythermal species that inhabits the temperate estuaries of the southwestern Atlantic coast (Argentina, 37°S). In these areas, the low winter temperatures prevent their activities and the gonad development in females, limiting the reproductive period to the warmer months (Colpo and López-Greco, 2017). Therefore, in temperate populations of *L. uruguayensis*, the reproductive efforts of females (vitellogenesis and brooding) and males (courtship behaviors) are synchronized in a short reproductive season. This feature makes the Argentine population of *L. uruguayensis* a suitable model to analyze the physiological costs of reproduction in females and males separately, considering their different strategies to maximize fitness. Throughout one annual cycle, we evaluated in this study the investment in reproduction and the dynamics of energy reserves in females and males of a temperate population of *L. uruguayensis*. For this, we measured throughout the year, the energy invested in vitellogenesis (female direct/gamete investment in reproduction), spermatophore production and accumulation in the vas deferens (male direct/gamete investment in reproduction), and the time spent in reproductive behaviors (male indirect/behavioral

investment in reproduction). We also assessed the physiological costs of these reproductive investments by surveying the dynamics of storage and expenditure of energy reserves (glycogen, total lipids, and total protein) in the hepatopancreas, ovary, and muscle, throughout the year. This way, we can estimate in a large temporal scale the costs of reproduction in a temperate fiddler crab. Moreover, knowledge on the energy invested in reproduction allows understanding the trade-offs among other life history traits throughout the year.

2. Material and methods

The study was performed in the Samborombón Bay, Río de la Plata estuary, Argentina. The field works were performed during low tide, from February 2014 to January 2015, in a muddy sand area of approximately 3,500 m² in the intertidal zone of the Saladero canal (36°25'0.3"S–65°57'11"W), in the locality of General Lavalle, Buenos Aires province. *Leptuca uruguayensis* is distributed from tropical estuaries in Rio de Janeiro, Brazil (22°S) to temperate salt marshes in Buenos Aires, Argentina (37°S) (Melo, 1996; Bezerra, 2012). In temperate estuaries, *L. uruguayensis* shows a conspicuous annual reproductive cycle affected by temperature, involving four periods: the low-activity period (LA, May–July), the pre-reproductive period (Pre-R, August–October), the reproductive period (R, November–January), and the post-reproductive period (Post-R, February–March) (more details about definition of the periods in Colpo and López-Greco, 2017). In each period, the fiddler crabs were sampled at three different time spans, to ensure appropriate temporal replication based on Underwood and Chapman (2013).

2.1. Cost of female and male gametes production

During vitellogenesis, the size of oocytes increases due to yolk accumulation (Castiglioni et al., 2007; Antunes et al., 2010). Therefore, to determine the temporal variations in energy expenditure in gamete production in *L. uruguayensis* females, the size of oocytes was measured in each period of their annual reproductive cycle. For this, the gonads of six random adult non ovigerous females (> 10 mm of maximum carapace width) by period were histologically examined. Ovaries were dissected and fixed in Bouin's solution for 4 h at 20 °C. Then, the gonads were dehydrated in ascending ethanol series, cleared in n-Butanol, embedded in Paraplast® and sectioned (6 µm thick) with a microtome. Slides were stained with Masson's trichrome (Subarna et al., 2012). Under a microscope, all oocytes with visible nucleus were measured

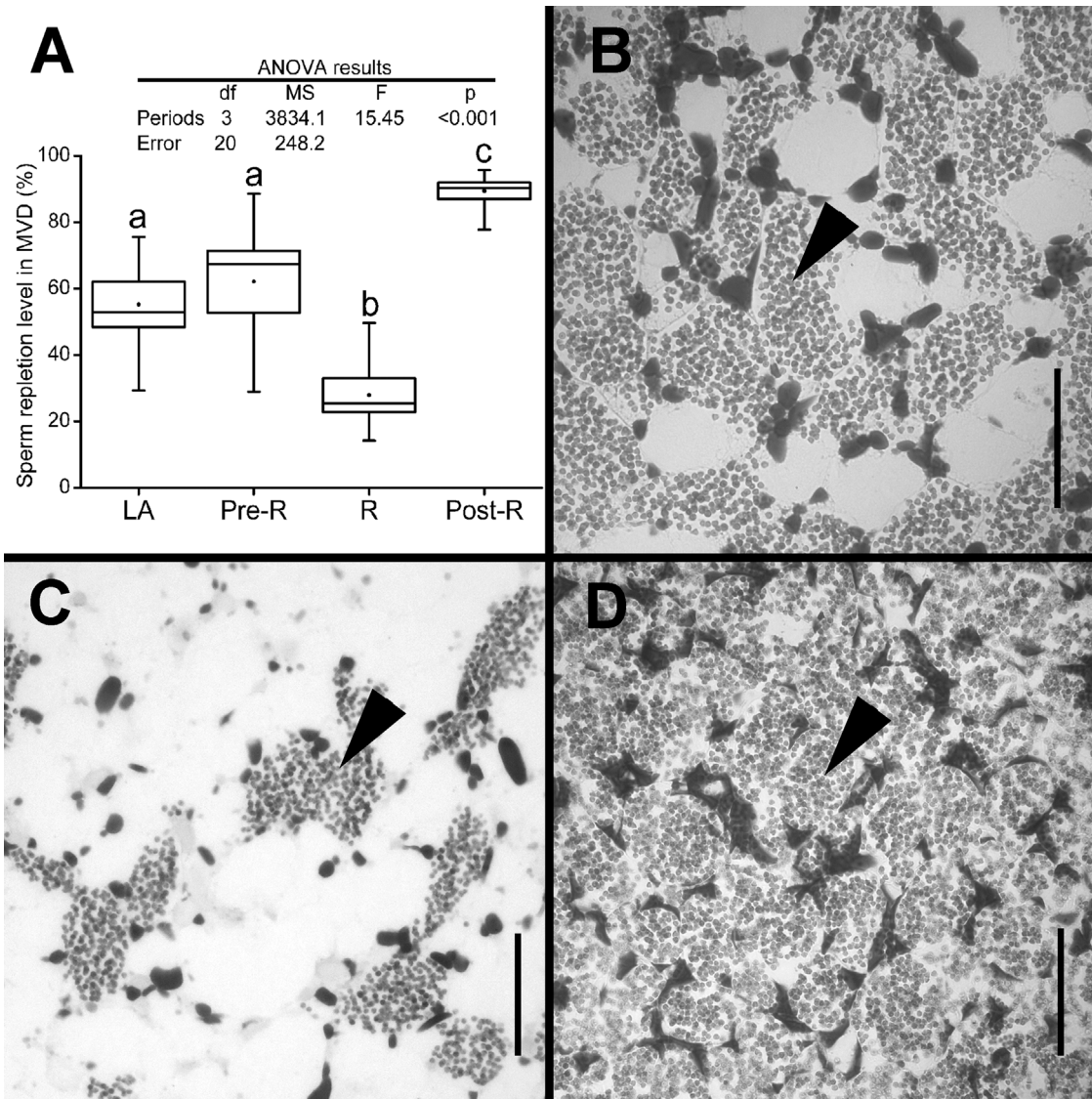


Fig. 2. Sperm repletion level in the middle vas deferens (MVD) of *Leptuca uruguayensis* throughout the reproductive cycle. (a) Comparison of sperm repletion level (point = mean, line = median, box = 25–75%, whiskers = min–max) between the periods of the reproductive cycle (LA, low-activity period; Pre-R, pre-reproductive period; R, reproductive period; Post-R, post-reproductive period). Different letters indicate statistically significant differences between means ($p < 0.05$). (b) Section of MVD showing the typical sperm repletion level during the LA and Pre-R periods. (c) Section of MVD showing the typical sperm repletion level during the R period. (d) Section of MVD showing the typical sperm repletion level during the Post-R period. Arrows indicate the spermatophores. Scale bar: 50 μ m.

Table 1
Description of behaviors performed by males of *Leptuca uruguayensis*, and their respective behavioral category.

| Behavioral category | Behaviors | Behavior descriptions |
|---------------------|-------------------------------------|--|
| Feeding | Feeding | Take portions of sediment with the minor cheliped and carry them to the mouth. |
| Reproductive | Waving display | Raise and lower the enlarged cheliped rhythmically to attract females for mating. |
| | Agonistic interaction between males | Combat or threatening postures involving two males, which end up with the separation of the individuals. |
| | Burrow maintenance | Preserve the burrow structure and reshape the burrow edge, using the pereiopods and chelipeds. |
| Other | Remaining buried | Remain totally or partially inside the burrow. |
| | Immobility | Remain still, with absence of perceptible motion. |
| | Walking | Slow or fast walking around, without apparent direction. |
| | Self-cleaning | Movement of minor cheliped over pereiopods, maxillipeds, and enlarged cheliped. |

(size of each oocyte = largest diameter + smallest diameter/2).

To estimate the variation in the sperm repletion level (indicative of mating activity) during the annual reproductive cycle, the male reproductive systems were histologically evaluated. The reproductive systems of six random adults by period were fixed and prepared using the same histological procedure described for the ovaries. Cross-

sections of the vas deferens of each male were photographed under a microscope and the images analyzed using the image processing program Image-J. The percentage area of the vas deferens occupied by spermatophores was recorded as indicative of sperm repletion.

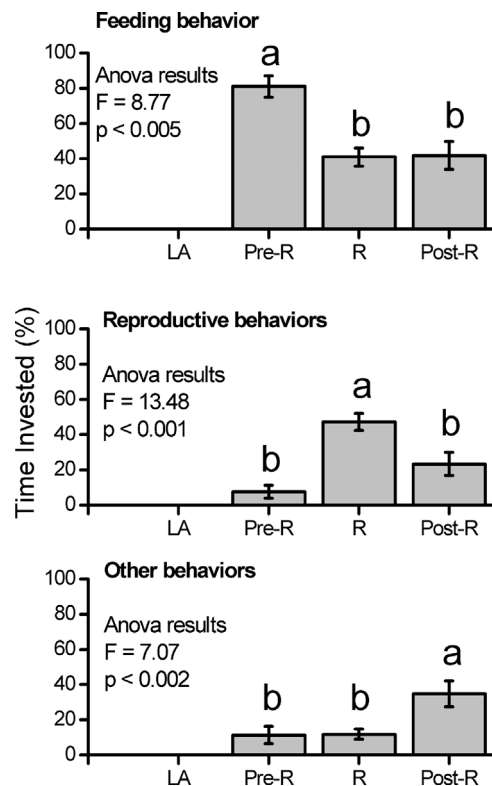


Fig. 3. Percentage (mean and standard error) of the time invested by males of *Leptuca uruguayensis* to perform each behavioral category (feeding, reproductive, and other behaviors) during the pre-reproductive (Pre-R), reproductive (R), and post-reproductive (Post-R) periods, respectively. Different letters indicate statistically significant differences between means ($p < 0.05$).

2.2. Cost of male reproductive performance

To determine the temporal variations in the energetic cost of reproductive performances in males, we measured the time invested by adult males in reproductive behaviors and compared them throughout the annual reproductive cycle. In temperate estuaries, during low temperature months, *L. uruguayensis* males are not active outside the burrows, whereas, in the remaining months, they are active for approximately 5 h, during the low-tide periods (Ribeiro et al., 2016; Colpo and López-Greco, 2017). Therefore, no behavior records were accounted during LA period, because fiddler crabs were not active on the surface. In each period with active crabs (Pre-R, R, and Post-R), 45 adult males were filmed, for 2 min. The recordings were made between 1 h before and 1 h after the low tide, from a minimum distance of 6 m to prevent the interference of the observer in the behavior of crabs. During image analysis, all kinds of behaviors performed by males were described, and the percentage of time spent in each behavior was estimated based on Matsumasa and Murai (2005). All behaviors recorded were grouped into three categories according to its purpose: feeding behavior, reproductive behaviors, and other behaviors.

2.3. Biochemical analysis of the energy reserves

To evaluate the dynamics of storage, mobility, and expenditure of energy reserves of *L. uruguayensis* throughout their annual reproductive cycle, the contents of glycogen, total lipids and total protein were evaluated in the hepatopancreas, ovary and muscle of the enlarged cheliped of males, which demand and mobilize great energy during the reproductive process of fiddler crabs (Mourente et al., 1994; Matsumasa and Murai, 2005; Hayes et al., 2013; Jimenez and Kinsey, 2015). Adult intermolt crabs of both sexes were transported to the laboratory, anesthetized and sacrificed in cold water, and dried with paper towels.

The maximum carapace width was measured, and the hepatopancreas (both sexes), the ovary (females), and the muscle of the enlarged cheliped (males) (essentially the propodus) were removed. Because the small size of *L. uruguayensis*, six crabs of the same sex were pooled to ensure enough mass for biochemical determinations. Twelve pools for each sex, in each period (LA, Pre-R, R, and Post-R) were analyzed to determine the contents of glycogen, total lipids, and total protein.

For glycogen determination, a modified protocol from Van Handel (1965) was used. Tissues were digested by boiling with alkaline solution (KOH 30%). Saturated SO_4Na_2 , absolute alcohol, and centrifugation were used to achieve glycogen precipitation. The resultant pellet was dissolved with 300 μl of distilled water. Afterward, the Anthrone reagent was used for colorimetric measurement of glycogen, in a spectrophotometer at 620 nm. Rabbit liver (Fluka®) was used to build the standard curve. For total lipid determination, a modified protocol from Folch et al. (1957) was used. Tissues were homogenized with a chloroform-methanol mixture (2:1 V/V volume). After 24 h, this homogenate was filtered, washed with NaCl (0.9%), and centrifuged to obtain a lipid phase. The sulfo-phospho-vanillin method was used to measure total lipids, in a spectrophotometer at 530 nm (procedures modified from Frings et al., 1972). Olive oil diluted with absolute ethanol was used to build the standard curve. For total protein determination, a homogenate of each tissue was prepared with 50 mM Tris-HCl buffer, pH 7.5, and centrifuged at 10,000 rpm, for 30 min, at 4 °C. The protein content of the supernatant was measured by the Coomassie blue dye method, in a spectrophotometer at 595 nm (Bradford, 1976). Serum bovine albumin (Fracc.V, Standard®) was used to build the standard curve. All concentrations of biochemical determinations were expressed as mg g^{-1} of tissue. All determinations were done on wet basis and performed in triplicate (three sub-samples for each sample) using spectrophotometric methods (Jasco – UV/VIS spectrophotometer, Model 7850).

2.4. Statistical analysis

The sizes of oocytes were compared between periods (LA, Pre-R, R, and Post-R) using a Kruskal-Wallis test, followed by Dunn test for multiple comparisons. The sperm repletion level was compared between periods (LA, Pre-R, R, and Post-R) using one-way ANOVA, followed by Tukey test for multiple comparisons.

The percentage of time used by male crabs to perform each behavioral category was compared between periods (Pre-R, R, and Post-R) using one-way ANOVA. Post-hoc Tukey tests were applied for multiple comparisons. Arcsine square root transformation was used to fulfill the requirements of the ANOVA. Additionally, the relationship between time spend feeding and reproducing was tested using a Pearson's Correlation.

The energy reserves (glycogen, total lipids, and total protein) recorded for each tissue and sex were compared between the periods of the annual reproductive cycle using the non-parametric Mann-Whitney or Kruskal-Wallis tests (multiple comparisons were performed by Dunn tests). Comparisons between sexes were performed using the *t*-Student test. For all statistical analysis, $p < 0.05$ was considered significant.

3. Results

The size of oocytes of *L. uruguayensis* varied throughout the annual reproductive cycle. Oocytes reached the largest diameters in the R period, intermediate sizes in the Pre-R period, and the smallest diameters in the Post-R and LA periods (Fig. 1). The sperm repletion level also showed temporal variation in the middle vas deferens (MVD), being lower in the R period, intermediate in the LA and Pre-R periods, and higher in the Post-R period (Fig. 2). The distal portion of the vas deferens (DVD) was empty in the R period and showed some sperm in the LA, Pre-R, and Post-R periods.

The behaviors of 135 males of *L. uruguayensis* were recorded and

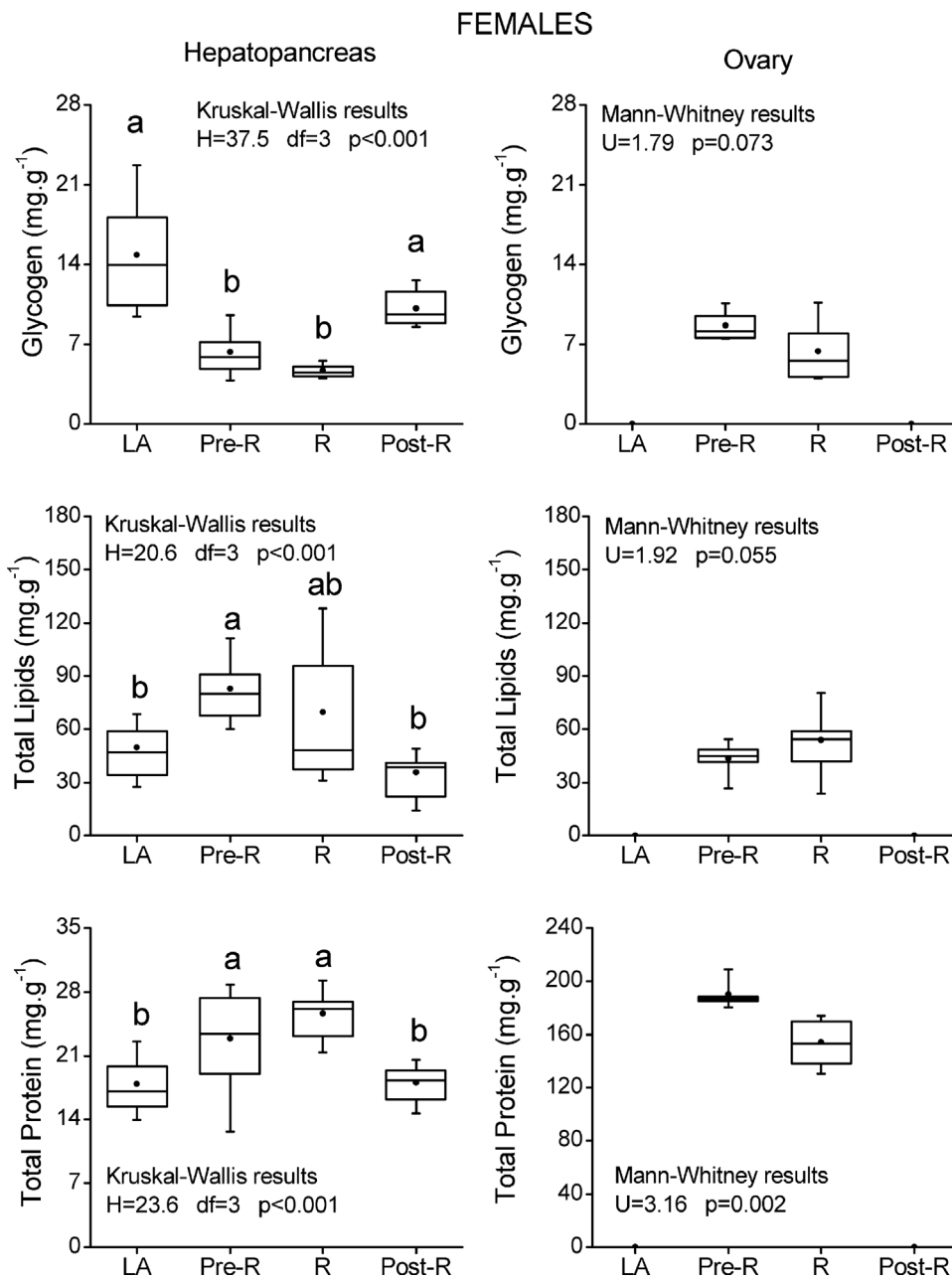


Fig. 4. Determinations of energy reserves (glycogen, total lipids, and total protein) in the hepatopancreas and ovary of females of *Leptuca uruguayensis* in each period of the reproductive cycle (LA, low-activity period; Pre-R, pre-reproductive period; R, reproductive period; Post-R, post-reproductive period). Point = mean, line = median, box = 25–75%, whiskers = min–max. Different letters indicate statistically significant differences between medians ($p < 0.05$).

evaluated during the Pre-R, R, and Post-R periods. The LA period was not included in the statistical analyses, because no fiddler crab was recorded on the substrate surface performing their activities. Eight behaviors were discriminated and grouped in the three behavioral categories: feeding behavior, reproductive behaviors, and other behaviors (Table 1). Their feeding behavior was more intense during the Pre-R period, their reproductive behaviors were more intense during the R period, and the other behaviors were more frequent during the Post-R period (Fig. 3). When males of *L. uruguayensis* spent more time performing reproductive behaviors, their feeding time decreased, in an inverse relationship (Pearson Correlation: $r_p = -0.896$; $t = -5.351$; $p = 0.001$).

The adult fiddler crabs analyzed for the determinations of energy reserves showed a mean size of 11.5 ± 4.0 mm of carapace width (N females = 288; N males = 288). In the hepatopancreas of females, the glycogen reserves were lower in the Pre-R and R periods, while lipid and protein content were about 33% and 77% higher, respectively, in these periods (Fig. 4). During most of the year, females showed no

developed gonads (Colpo and López-Greco, 2017); therefore, determinations of energy reserves in the ovary were only possible in October (Pre-R period), November, December, and January (R period). In the ovary, the content of glycogen and total lipids did not differ between the Pre-R and R periods, and was similar to that found in the hepatopancreas (Fig. 4). The amount of protein in the ovary during the Pre-R period was greater than that in the R period. Besides, in females, the protein content reached higher values in the ovary than in the hepatopancreas (approximately eight times) (Fig. 4). In the hepatopancreas of males, the glycogen content was lower in the R period, the total lipids decreased about 61% in the Post-R period, and the protein content was similar throughout the year (Fig. 5). In the muscle of the enlarged cheliped, the glycogen content was about 66% greater in the LA and Pre-R periods, the lipids did not differ between the periods, and the protein content increased about 70% during the Pre-R and R periods (Fig. 5). The maximum energy storage (high glycogen contents in the hepatopancreas) was observed in the LA period, and the maximum energy expenditure (low glycogen contents in the hepatopancreas) was

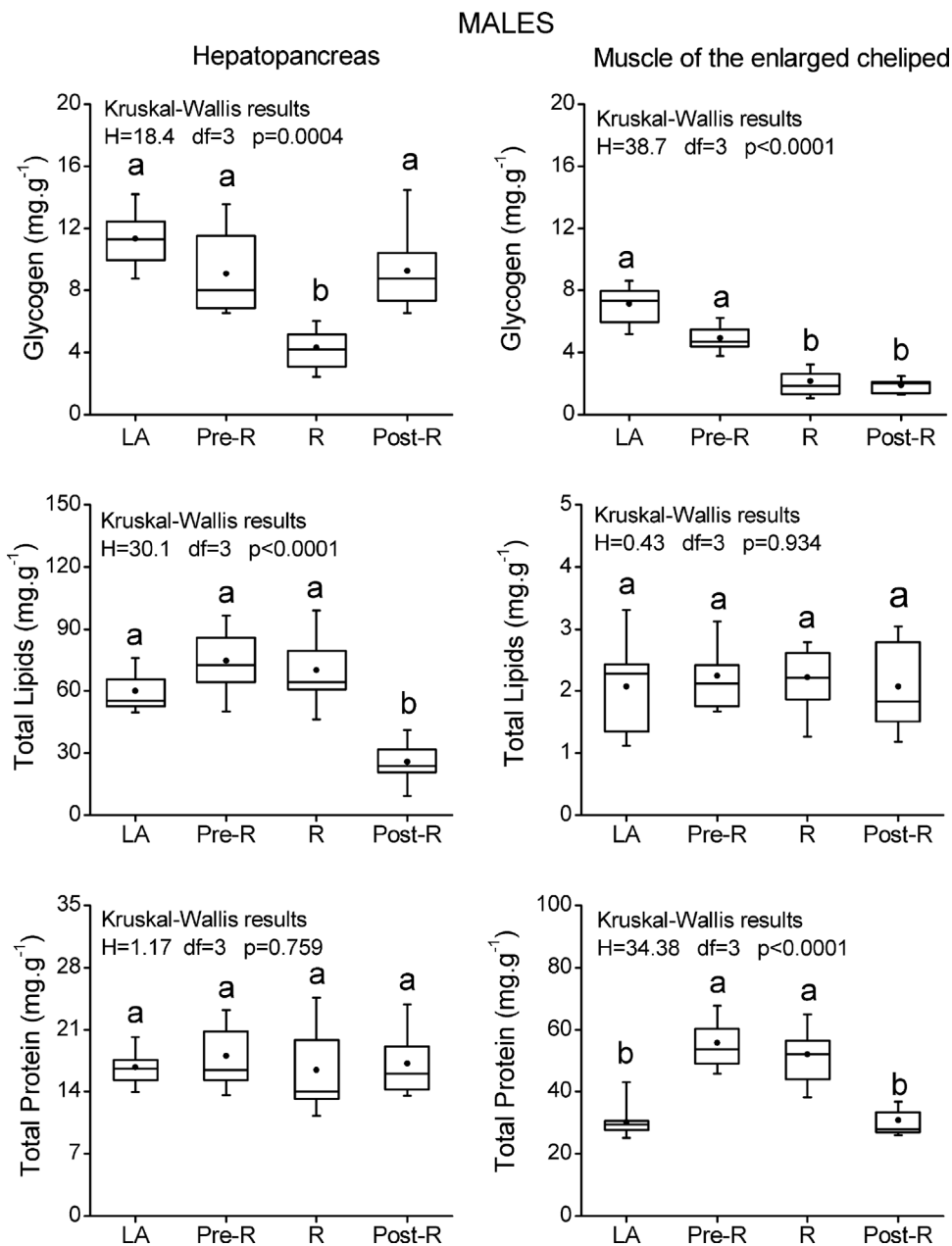


Fig. 5. Determinations of energy reserves (glycogen, total lipids, and total protein) in the hepatopancreas and muscle of the enlarged cheliped of males of *Leptuca uruguayensis* in each period of the reproductive cycle (LA, low-activity period; Pre-R, pre-reproductive period; R, reproductive period; Post-R, post-reproductive period). Point = mean, line = median, box = 25–75%, whiskers = min–max. Different letters indicate statistically significant differences between medians ($p < 0.05$).

observed in the R period. The consumption of glycogen reserves from the LA to the R period was calculated for females and males as a simple indicator of the physiological costs of reproduction. The glycogen reserves of the hepatopancreas decreased about 66% in females and 61% in males, and no differences were found in glycogen consumption between sexes (t-Student test: $t = 1.12$, $df = 22$, $p = 0.273$).

4. Discussion

In temperate estuaries, the fiddler crab *L. uruguayensis* shows a conspicuous annual cycle, in which the reproductive period is marked by the maturation of oocytes in females, and intense sperm release (indicative of mating) and courtship behaviors in males. Our results showed that for both sexes, the reproductive experience was expensive, since the levels of energy reserves (especially those of the glycogen of the hepatopancreas) remained low during the reproductive period compared to other periods of the year. The results about the effects of reproductive investments on the dynamic of energy reserves are at a large temporal scale, because the year was divided into four periods

(LA, Pre-R, R, and Post-R), and the general aspects of the population for each period were recorded. Fine scale details or individual variations were not discussed, but we recognize disparity among crabs within periods, which can be observed in the dispersion of data. For instance, during the Pre-R period, the gonad maturation can start earlier in some females than others; or in the R period, some females can be ovigerous inside the burrows, while others are searching to mate on surface. Some males can be mating, while others are waving to attract females. Despite these and other individual variations, the activities and therefore the energy allocation of most fiddler crabs in this population aim the reproduction during the R period.

In Decapoda, vitellogenesis is a process in which maturing oocytes increase their size due to the accumulation of yolk granules in the cytoplasm (Castiglioni et al., 2007; Antunes et al., 2010). Vitellogenesis is energetically costly, since it involves the synthesis and mobilization of great amounts of lipids and proteins, which are the main components of yolk (Eastman-Reks and Fingerman, 1985; Harrison, 1990; Tsukimura, 2001; Zmora et al., 2007; Jimenez and Kinsey, 2015). However, glycogen, which is mainly stored at the hepatopancreas and muscle, and

showed low levels in the ovary of *L. uruguayensis*, is the principal fuel that supplies the variety of activities of crabs (Herreid and Full, 1988; Harrison, 1990; Sánchez-Paz et al., 2006; Antunes et al., 2010). In this study, during the Pre-R and R periods of the annual reproductive cycle, the ovary of *L. uruguayensis* showed large germinative cells (vitellogenic and mature oocytes), whereas in the LA and Post-R periods, it showed only a germinative area with predominance of smaller cells (oogonias and primary oocytes). The macroscopic appearance of the ovary in the LA and Post-R periods was hollow and transparent, resulting in low gonadosomatic indices (Colpo & López-Greco, 2017). This annual cycle of the ovary is followed by changes in the energy reserves of the hepatopancreas. In the Pre-R and R periods, the glycogen content of the hepatopancreas decreased significantly, suggesting that this reserve plays an important role as fuel of the energetic requirements of the intense biosynthesis and mobilization of nutrients for vitellogenesis (Harrison, 1990). Besides, more than 40% of females were ovigerous in the R period (Colpo and López-Greco, 2017), and the brooding process (carrying, cleaning, and providing oxygen for the embryos) increases the metabolic demand and represents an important cost to decapod females (Fernández et al., 2000; Brante et al., 2003; Guadagnoli et al., 2005). Females of *L. uruguayensis* remain underground during the brooding time (about 15 days), and can be easily found by excavation of 30–40 cm deep (Colpo, unpublished data). Because of these high costs of reproduction (vitellogenesis and brooding), glycogen is greatly used, and the ability of *L. uruguayensis* females to store it during the reproduction time decreased. However, in the Post-R period, the non-vitellogenic ovary probably reduced the energetic demand of females, favoring the accumulation of glycogen in the hepatopancreas. In the LA period, *L. uruguayensis* was not active on the substrate surface, and the ovary was not vitellogenic so no cost in reproduction would be involved. However, the highest level of glycogen content (present study) and hepatosomatic index (Colpo and López-Greco, 2017) during LA period, allow us to suppose that more than saving energy, this fiddler crab could feed on underground. Also, we can consider that inside of the plugged burrows the oxygen availability is low. Herreid and Full (1988) stated that under anoxic conditions glycogen is a more suitable reserve than lipids. Hence, reserving energy as in glycogen is a more adaptive strategy to such condition than in lipids, although lipids can reserve more energy than glycogen per gram basis (Herreid and Full, 1988). Therefore, remaining inside the burrow during the cold months or during the incubation period would be a good strategy, since this behavior can minimize the ecological costs (e.g. thermal stress, predation risk, eggs loss) without impairing the energy reserves.

During ovarian development, there is synthesis of the yolk precursor (vitellogenin) and the main yolk component (lipovitellin) (Harrison, 1990; Zmora et al., 2007). Vitellogenin and lipovitellin are serologically identical lipoproteins (Zmora et al., 2007), and different authors disagree about the tissue where they are synthesized (Lui and O'Connor, 1977; Eastman-Reks and Fingerman, 1985; Harrison, 1990; Tsukimura, 2001; Antunes et al., 2010). Recently, molecular studies have evidenced that the hepatopancreas, hemolymph, and ovary show a complex interaction in the synthesis, transport, and transformation of vitellogenin, as well as in the subsequent storage of lipovitellin in the oocytes (Puengyam et al., 2013; Girish et al., 2014; Thongda et al., 2015). Thongda et al. (2015) proved that 99% of the vitellogenin of *Callinectes sapidus* is produced in the hepatopancreas. Probably, the hepatopancreas of *L. uruguayensis* females also plays an important role in the synthesis of vitellogenin, since we recorded higher contents of lipids and proteins in the hepatopancreas of females during the vitellogenic periods (Pre-R and R). Therefore, it is probable that a great proportion of these lipids and proteins are more related to yolk synthesis than to energy supply for females.

The lipovitellins stored in the egg cover all the nutritional and energetic requirements for embryo development. The results of our biochemical determination in ovarian tissue during the vitellogenic phases suggest that the egg yolk of *L. uruguayensis* is richer in protein than in

lipids (about 3–4 times higher). The fiddler crab *Afruca tangeri* also shows greater content of protein than lipids in the ovary (Mourente et al., 1994). The protein content (and amino acid profiles) of lipovitellin can vary among crustacean species, probably to meet the requirements of each species (Harrison, 1990). A greater protein:lipid ratio is more usual in decapods with indirect development than in decapods with direct development (Tropea and López-Greco, 2015). The high protein proportion recorded in the ovary of *L. uruguayensis* suggests that embryo development needs high levels of this nutrient, following the trend for marine decapods with indirect development. Besides, the great protein content accumulated in the ovaries of *L. uruguayensis* indicates that the Pre-R and R periods show intense biosynthesis. The requirements for this high biosynthesis can be supplied by a greater protein intake, considering that females also became active and increased their time spent feeding during the Pre-R period.

The temporal variation in the sperm repletion level in the middle vas deferens (MVD) and the presence of sperm in the distal vas deferens (DVD) of males also indicate seasonality in the reproduction of *L. uruguayensis*. The lower sperm level in MVD and the absence of sperm in DVD in the R period indicate intense sperm release due to the successive matings, which can be 5–6 per reproductive season (Ribeiro et al., 2016). This indicative of male mating coincides with greater fullness of female seminal receptacles in the R period (Colpo and López-Greco, 2017). The sperm repletion level in MVD during the LA, Pre-R, and Post-R periods suggests that the frequency of mating decreased, allowing crabs to store spermatophores in MVD. The lack of females with mature gonads (Colpo and López-Greco, 2017) and the reduction of courtship behaviors performed by males during these periods also corroborate the assumption of a decrease in the number of mating during the LA, Pre-R, and Post-R periods. Additionally, the sperm repletion level in MVD was lower in the LA and Pre-R periods than in the Post-R period. This difference can indicate a reduction of sperm production during the LA and Pre-R periods, as suggested by Cuartas and Petriella (2010). Although sperm production demands lower energy expenditure than vitellogenesis, its costs and time requirements cannot be neglected. Probably, the decrease in production of sperm and associated secretions for spermatophore transfer during the period with low mating opportunities is an efficient strategy to economize energy and sperm reserves to maximize the male reproductive success (Sato and Goshima, 2007). However, the periodicity in sperm production should not significantly affect their energy reserves, since the gonadal development of male crabs shows low energetic requirements (Wu et al., 2010). The great reproductive effort of male fiddler crabs is related to courtship and territory defense behaviors, in which the enlarged cheliped plays an essential role (Christy and Salmon, 1984; Hayes et al., 2013; Ribeiro et al., 2016; Rodrigues et al., 2016). The costs of carrying such an enlarged appendage, which represents between 25 and 35% of the male's body weight (34% in *L. uruguayensis* [Colpo, unpublished data]), are high (Allen and Levinton, 2007; Gerald and Thiesen, 2014). Therefore, when males use this enlarged cheliped in reproductive behaviors, the energetic costs increase and have an important impact on their energy budget (Matsumasa and Murai, 2005; Hayes et al., 2013; Matsumasa et al., 2013).

Most of the energy needed for fueling activities of crabs is provided by glycolysis, which uses glucose as substrate and/or promotes the breakdown of glycogen reserves (Full and Herreid, 1984; Herreid and Full, 1988; Matsumasa and Murai, 2005; Sánchez-Paz et al., 2006). In crustaceans, the main glycogen reserves are the hepatopancreas and the muscle (Jimenez and Kinsey, 2015; Sacristán et al., 2017). Like in females of *L. uruguayensis*, the reduction of activity in males and their possibility of feeding inside the burrows during the LA period, would ensure energy storage, increasing the glycogen reserves in the hepatopancreas and in the muscle of the enlarged cheliped. When the temperature rises, in the Pre-R period, fiddler crabs leave their burrows, showing a high activity level (Colpo and López-Greco, 2017) and spend about 80% of the time feeding. Despite this high food ingestion in Pre-

R, the glycogen reserves in the hepatopancreas and muscle did not increase, probably because glycogen storage in animals is limited and also because males display several activities in this period (e.g. burrowing, walking, self-cleaning) that consume part of energy obtained from food. However, although the statistical test showed no differences, we noted an increasing trend of the lipid content in the hepatopancreas, when fiddler crabs enhanced the food intake during the Pre-R period. In the R period, the social activities and the reproductive behaviors intensified. The presence of active crabs in the area is an important stimulus that increases and maintains the high blood glucose concentrations in male fiddler crabs (Matsumasa and Murai, 2005). The levels of glucose in the hemolymph can also be a measure of 'male quality', since it is the immediate fuel for the expensive courtship activities (Matsumasa and Murai, 2005; Matsumasa et al., 2013). Therefore, the decrease in glycogen content recorded in the hepatopancreas and in the cheliped muscle of males in the R period is probably related to an increased glucose demand, to supply the high activity of the enlarged cheliped during reproductive performances.

The greater values of protein measured in the muscle of the enlarged cheliped of males in the Pre-R and R periods can be associated with factors that favor protein synthesis, such as the increase in environmental temperatures and the greater intake of protein, probably promoted by the increased feeding behavior of *L. uruguayensis* in the Pre-R period (Carter and Mente, 2014). In crustaceans, the hepatopancreas is the main lipid storage tissue. Lipids are an important energy source for the growth and regeneration of tissues following molting; therefore, the lipid reserves in the hepatopancreas of crabs usually decrease during pre- and post molting (Herreid and Full, 1988; Sánchez-Paz et al., 2006). The proportion of molting in *L. uruguayensis* increases in the Post-R period (between 3 and 6% [Colpo, unpublished data]). Hence, the significant decrease in lipid reserves in the hepatopancreas of *L. uruguayensis* in Post-R could be related to the molting cost.

Performing reproductive activities at the expense of foraging can affect the reproductive success of males by limiting energetic supplies (Scharf et al., 2013). Male fiddler crabs often show an inverse relationship between reproductive and feeding behaviors (Matsumasa and Murai, 2005; Matsumasa et al., 2013). In some species, males can engage in short starvation periods during the breeding process (Christy and Salmon, 1984). Despite the decrease in feeding time during the R period, males of *L. uruguayensis* keep the food intake (Fig. 3). The female behaviors were not measured, but we observed several females feeding on the surface during the R period. Therefore, we assumed that they were also feeding between broodings. Although *L. uruguayensis* preserves some intake of nutrients and energy during the R period, the glycogen reserves of the hepatopancreas decreased significantly in females and males. This result suggests the high energetic expenditure for reproduction, since the energy from feed (glucose directly absorbed from diet) was not enough, and the breakdown of hepatopancreas glycogen had to be activated to supply the reproductive demands in both sexes. The similar consumption of glycogen reserves in females and males also allows us to infer that the physiological costs of reproduction are similar between sexes of fiddler crabs, despite the different reproductive strategies of females (vitellogenesis and brooding) and males (courtship behaviors) to maximize their fitness. To our knowledge, this is the first empirical study to evaluate the cost of reproduction in both sexes of fiddler crabs.

Limited resources usually affect the reproductive time and effort of species (Reznick et al., 2000). In the study area, the food availability for *L. uruguayensis* is constant throughout the year (Colpo and López-Greco, 2017), and does not seem to be a factor restricting a longer reproduction season. However, the low winter temperatures are stressful and probably demand allocation of resources for the maintenance of these fiddler crabs. Because the amount of energy devoted to reproduction is opposed to that devoted to maintenance (Calow, 1979; Reznick, 1992; Reedy et al., 2016), we suggest that the reproductive period of *L. uruguayensis* is not necessarily dependent on resource plenitude, but

would occur when the environmental conditions minimize the energetic costs of crabs with other traits. Therefore, a seasonal trade-off between reproduction and maintenance would ensure the optimal life history of *L. uruguayensis* in temperate estuaries.

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