Influence of mating strategies on seminal material investment in crabs

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Reproduction involves high energetic costs which are related to behaviour and gamete production. In females energy allocation to gamete production has been well documented. However, estimations of male investment in seminal material are scarce. The present study aims to assess and compare male investment in four brachyuran species by determining biochemical substrates present in the vasa deferentia to subsequently estimate energetic investment during the reproductive cycle. We identified two groups with contrasting energy investments. Two species, Homalaspis plana and Romaleon setosum, showed high investment due to significant quantities of proteins and lipids. Both species are characterised by large and complex vasa deferentia, and the formation of a remarkably large sperm plug deposited to the female after copulation as a sperm competition avoidance strategy. In contrast, Metacarcinus edwardsii and Taliepus dentatus invested little energy in their smaller-sized and simpler vasa deferentia. Morpho-functional traits may play a key role in determining the investment, which may also be influenced by mechanisms (i.e. mating tactics) to prevent sperm competition and the intensity of polygyny. This study emphasises the high amount of energy males invest in seminal material and highlights the diversity of mating strategies in Brachyura, which are reflected even on the physiological level.
numerous roles, the quantitative determination of the biochemical composition of the vas deferens is limited to very few species22,23,26.

Brachyuran crabs have highly variable mating strategies which are associated with different levels of polygyny. Intensity of sperm competition, which takes place inside of the seminal receptacle of females, may be a strong driver for reproductive effort in males27. For example, species with a high level of polyandry have a heavier reproductive system30 which has been detected in fishes, sharks and crickets29,30 and, in contrast, monogamous spiders have developed permanent sperm depletion after adulthood31. In the case of crabs, in species with fertilization given for last-male precedence32, male-male competition is increased, as well as male strategies to assure paternity. Male competitive mating tactics (i.e. morphological, physiological, and behavioural adaptations to avoid sperm competition), such as mate guarding, multiple copulations and sperm plugs, frequently appear when sperm competition is intense33. It has also been demonstrated that the intensity of sperm competition depends on the period of receptivity of the female, morphological traits of the female seminal receptacle and male-male agonistic competition abilities34–36. The coevolution of the female-male reproductive system in crabs32 suggests that the composition and quantity of biochemical components transferred during copulation may be closely linked to the morphology and complexity of the female sperm storage organ and the major functional roles they fulfill after mating in the female. In turn, male reproductive systems should also be complementary to female necessities of amount and features of seminal material.

Therefore, we expected that species that undergo a high level of polygyny and intense sperm competition (i.e. short period of female receptivity) would exhibit a large vas deferens and a large investment of energy in seminal material as these adaptations may help males compete when sperm competition occurs. In temperate regions there is an expected variation in energy investment in reproductive structures of marine invertebrates in order to match the offspring release with cycles of environmental factors like temperature and productivity, therefore seasonal variation in reproduction is very common37. Brachyura are ideal model organisms to assess male energetic investment in seminal material, because they exhibit a broad spectrum of reproductive traits and mating strategies which necessarily have an association with the energy involved to develop these strategies. The comparison of various crab species may allow determining patterns among species related to energetic investment in males.

Four brachyuran species, the purple crab *Homalaspis plana*, the hairy crab *Romaleon setosum* (synonymous *Cancer setosus*, *C. polyodon* and *R. polyodon*), the marble crab *Metacarcinus edwardsii* and the kelp crab *Taliepus dentatus*, are among the most important artisanal fishery resources along the Chilean coast. Studies concerning males are almost absent and principally descriptive22,23,26 although they are the main target of fishery. The crab species *R. setosum* and *M. edwardsii* belong to the same family (*Cancridae*). Our model species from other families, *Epialtidae* (*T. dentatus*) and *Platyxanthidae* (*H. plana*), are phylogenetically distant from the Cancridae, as shown in the phylogenetic tree for brachyuran families40. These four species were chosen as model species because they exhibit contrasting mating strategies, representing a gradient between a continuous mating period and a brief mating season as well as different levels of pre- and/or post-copulatory mate guarding, or the forma-
tion of a sperm plug during copulation (comparison of information related to reproduction see Table 1). Also, synchronization of cycles in males and females23 is not obvious because Brachyura have a seminal receptacle where after mating females can store sperm for a long period, but this could depend on the mating strategy (continuous or seasonal mating). The present study aims to assess seasonal patterns of main energetic substrates and energy investment per vasa deferentia during the reproductive cycle in four brachyuran species and associate them to their mating strategies.

### Results

**Weight, biochemical components, and energy content of vasa deferentia.** The vasa deferentia of *H. plana* (Fig. 1a and Table 1) and *R. setosum* (Table 1) were extended and highly convoluted (i.e. complex). The vasa deferentia of *M. edwardsii* (Fig. 1b and Table 1) and in particular of *T. dentatus* (Table 1) were of simpler morphology. Annual median vasa deferentia of *H. plana* and *R. setosum* were larger, in comparison to those of *M. edwardsii* and *T. dentatus*, which were relatively small (Fig. 1c).

In *H. plana* significant seasonal changes in the quantity of proteins and lipids revealed a similar pattern: decreased values during winter and spring, followed by a continuous increase in summer and autumn (Table 2 and Fig. 2a). Quantity of proteins was generally at least twice the amount of lipids in each season; apart from in autumn quantity of both components was similar. In *R. setosum* no significant fluctuations in the quantity of

| Species (family) | Mating period | Mate guarding | Sperm plug | Sperm storage pattern | Vas deferens | Seminal receptacle | References |
|-----------------|---------------|---------------|------------|------------------------|--------------|-------------------|------------|
| *Homalaspis plana* (Platystomatidae) | Autumn | Not present | Present | Large sperm plug, without stratification | Large, complex | Large, very extensible | 22, LMP (pers. observation) |
| *Romaleon setosum* (Cancridae) | Spring and summer | Present | Present | * | Large, complex | Large | 23, LMP (pers. observation) |
| *Metacarcinus edwardsii* (Cancridae) | Spring and summer | Present, prolonged | Present | Clear stratification (sperm gel) | Small, simple | Small, simple | 44, 50, 57 |
| *Taliepus dentatus* (Epi-altidae) | Year-round | Present, brief | Absent | * | Small, simple | Medium, complex | 52, 56, 58 |

Table 1. Summary of mating tactics and size and complexity of male and female reproductive systems of the four brachyuran species studied. *Refers to not investigated yet.
The biochemical composition of the sperm plug itself is known in a few decapod species. For example, in part, the biochemical components of seminal material delivered by males may be involved in its formation. That can lead to genetic monogamy even in the presence of polyandry. The seminal receptacle of post-copulated females in most species (Fig. 3). The relatively scattered cluster of points corresponded to T. dentatus probably due to smaller seasonal variations. The two species, T. dentatus and M. edwardsii, were ably attributed to the great seasonal fluctuation of values. The species, T. dentatus, H. plana, R. setosum, and M. edwardsii, are characterized by the presence of a large sperm plug and a significant glycoprotein. Another example is the sperm plug of the portunid crab, Arenaeus cribrarius, whose sperm plug inside of the seminal receptacle of mated females consists of a layered strata of three types.

Discussion

Comparison among species. In the nMDS, H. plana was displayed as a group relatively separated from the other species (Fig. 3). The relatively scattered cluster of points corresponded to R. setosum and was probably attributed to the great seasonal fluctuation of values. The species, M. edwardsii and T. dentatus, were depicted aggregated together probably due to smaller seasonal variations.

Several tactics have been developed in male crabs to avoid sperm competition, such as sperm plugs and stratification of ejaculates, both with great use of non-sperm material. Sperm plugs serve as a paternity assurance device that prevents subsequent inseminations by other males. This appears as a highly effective strategy that can lead to genetic monogamy even in the presence of polyandry. The seminal receptacle of post-copulated females in H. plana and R. setosum is characterized by the presence of a large sperm plug and a significant part of the biochemical components of seminal material delivered by males may be involved in its formation. The biochemical composition of the sperm plug itself is known in a few decapod species. For example, in the portunid Arenaeus cribrarius, the sperm plug inside of the seminal receptacle of mated females consists of glycoprotein. Another example is the sperm plug of C. danae which is composed of a layered strata of three types.

**Figure 1.** Differences in size and complexity of the vasa deferentia of the brachyuran species studied. (a) Large and complex in Homalaspis plana. (b) Small and simple in Metacarcinus edwardsii. (c) Box plot of annual dry weight of paired vasa deferentia. HP, RS, ME, and TD refer to H. plana, Romaleon setosum, M. edwardsii and Taliepus dentatus, respectively. Boxes: interquartile range of the data (first quartile, median and third quartile). Whiskers: the values that extend to 1.5 times the interquartile range. Circles: outliers.
of secretions, each of a chemically distinct composition of proteins and polysaccharides, which are produced by males through the combined secretions from the three vas deferens regions47. In the sperm plug of distinct insect

| Factor          | df | MS              | No. of iterations | P       |
|-----------------|----|-----------------|-------------------|---------|
| Homalaspis plana|    |                 |                   |         |
| Quantity proteins |  | 1.23304×10^7 | 5000              | <0.001  |
| Residuals       | 15 | 9.056×10^7     |                   |         |
| Quantity lipids |    | 2.83779×10^7 | 5000              | <0.001  |
| Residuals       | 15 | 1.088×10^7     |                   |         |
| Energy          |    | 8.30444528×10^7| 5000              | <0.001  |
| Residuals       | 15 | 3.0645819×10^7|                   |         |
| Romaleon setosum|    |                 |                   |         |
| Quantity proteins |  | 4.99166×10^7 | 5000              | <0.001  |
| Season          | 3  | 7.5455×10^4  | 1259              | 0.105   |
| Residuals       | 13 | 3.1481×10^4  |                   |         |
| Quantity lipids |    | 2.108463×10^7| 5000              | 0.006   |
| Season          | 3  | 2.18988×10^4 | 233               | 0.613   |
| Residuals       | 13 | 2.37098×10^7 |                   |         |
| Energy          |    | 5.493224945×10^7| 5000              | 0.007   |
| Season          | 3  | 5.68766463×10^7| 292               | 0.387   |
| Residuals       | 13 | 5.32254899×10^7|                   |         |
| Metacarcinus edwardsii | |                 |                   |         |
| Quantity proteins |  | 6.71×10^2   | 5000              | 0.004   |
| Season          | 3  | 1.0654×10^2 | 473               | 0.289   |
| Residuals       | 14 | 7.89×10^1   |                   |         |
| Quantity lipids |    | 512.98×10^2 | 2792              | 0.034   |
| Season          | 3  | 2.66510×10^5 | 360               | 0.611   |
| Residuals       | 14 | 4.00560×10^5|                   |         |
| Energy          |    | 1.874968×10^6| 3422              | 0.028   |
| Season          | 3  | 2.66510×10^5 | 360               | 0.611   |
| Residuals       | 14 | 4.00560×10^5|                   |         |
| Taliepus dentatus|   |                 |                   |         |
| Quantity proteins |  | 1.85274×10^3 | 5000              | 0.006   |
| Season          | 3  | 9.9857×10^2 | 5000              | 0.008   |
| Residuals       | 15 | 1.9207×10^2 |                   |         |
| Quantity lipids |    | 1.71827×10^3 | 5000              | 0.001   |
| Season          | 3  | 5.9521×10^2 | 5000              | 0.04    |
| Residuals       | 15 | 1.7943×10^2 |                   |         |
| Energy          |    | 7.057318×10^6| 5000              | 0.001   |
| Season          | 3  | 2.215233×10^6| 5000              | 0.007   |
| Residuals       | 15 | 5.15481×10^5|                   |         |

**Table 2.** Permutation tests of the linear model comparing the effects of size (covariate) and season (fixed factor) on quantity of each biochemical component and energy in crab species. Bold indicates significant differences (**P** < 0.05). Note that analyses of *Homalaspis plana* and quantity of lipids of *Metacarcinus edwardsii* do not include size as a covariate.
species, proteins and lipids have been detected\textsuperscript{14,48,49}. For example, in Drosophila protein contributes to forming of the plug\textsuperscript{49}, but in the bumblebee B. terrestris the active substances are fatty acids\textsuperscript{14,48}. The presence of a sperm plug may increase the probability of paternity\textsuperscript{32}, and males may benefit by increasing investment in a single female. Though, investment in a sperm plug and mating with only one female could lead to ejaculate depletion. Recently, it has been suggested that males of H. plana are likely able to mate only once during their reproductive period due to the great amount of ejaculate received by the female seminal receptacle\textsuperscript{26}. In M. edwardsii seminal fluids transferred by the male form a sperm plug which is very discrete and only blocks the narrow vaginal lumen to avoid further matings with rival males\textsuperscript{50}. In contrast, males of T. dentatus do not produce a plug\textsuperscript{51}. Seminal fluids may play an important role in sperm stratification, which is another mechanism with which males can influence fertilization in their favour through clearly separating ejaculates transferred by different mates and reducing mixing of sperm\textsuperscript{52,53}. The exact biochemical composition of sperm gel in crabs has been poorly studied. For example, in the seminal receptacle of the majoid Stenorhynchus seticornis stratified sperm packets are surrounded by secretions that consist mainly of proteins, combined with acid and neutral polysaccharides\textsuperscript{54}. In M. edwardsii, proteins and lipids in the vas deferens may play an important role in avoiding sperm competition in the form of sperm gel, which separates sperm received by different males, thereby allowing a clear stratification of sperm in the receptacle\textsuperscript{50}. In the ventral-type seminal receptacle of M. edwardsii new ejaculates displace old ones toward the blind end of the receptacle and are deposited closer to the oviduct connection promoting last-male precedence\textsuperscript{32}. In R. setosum the storage pattern of ejaculates in the seminal receptacle has not been investigated yet, but in the family Cancridae in which females may mate with various males, sperm stratification has been reported in several species and seems to be widespread\textsuperscript{50,55}. In T. dentatus

Figure 2. Seasonal pattern of biochemical components and energy present in the paired vasa deferentia of the four brachyuran species studied. (a) Quantity of proteins and lipids. Note different scale for each species. Values correspond to adjusted means + SE in all species, except for quantity of proteins and lipids in Homalaspis plana and lipids quantity in Metacarcinus edwardsii which correspond to means + SE. Seasons correspond to austral seasons. (b) Energy content. Note different scales of energy investment of H. plana and R. setosum, and M. edwardsii and Taliepus dentatus. Values correspond to adjusted means + SE in all species, except for in H. plana which correspond to means + SE. Energy content was estimated by converting amounts of lipids and proteins to their caloric equivalents. Seasons correspond to austral seasons.
post-copula. Especially for males of *R. setosum*. Males of *R. setosum* mate multiply under a female-biased sex ratio (58). Therefore, in *T. dentatus* a continuum of sperm gel and spermatophores has been observed in the blind end of the sac, which suggests no stratification of ejaculates (LMP, pers. observation).

During this vulnerable state, females may also benefit from protection by males from predators, cannibalism or other males (59). Mate guarding may also be associated with the necessity of protecting the valuable energy invested. In *T. dentatus*, males transfer on average 37% of their initial seminal material stock during one mating (60). The imminent high risk of sperm competition and uncertainty of paternity may explain the strategy of allocating relatively little energy and small percentage of the initial seminal material stock in each female compared to the reproductive season (58,61) and increasing chances of paternity through partitioning seminal material among various females (62). Therefore, in *T. dentatus* the decrease of proteins and lipids probably corresponds to multiple matings indicating increased reproductive activities especially in spring. This suggests that a high level of polygyny (*M. edwardsii* and *T. dentatus*) may be associated with investing little energy in each female but engaging in frequent copulations. The frequency of mating of males of *R. setosum* has not been investigated yet.

In addition to the anatomical adaptation to avoid sperm competition, mate guarding is an effective behavioural adaptation to ensure paternity. Post-copulatory guarding has often been observed in species in which receptivity of the female is restricted to a short time after its moult (i.e. female ‘soft-shelled’ during mating) (44,59). During this vulnerable state, females may also benefit from protection by males from predators, cannibalism or other males (59). Mate guarding may also be associated with the necessity of protecting the valuable energy invested. Guarding is particularly common in the family of the cancrids (44,55,59), such as in *R. setosum* and *M. edwardsii*. Males of *R. setosum* perform copulatory guarding approximately one week before the females’ moult and 2–3 days post-copula (60). Especially for males of *R. setosum*, which spent large quantities of energy on reproduction, it may be crucial to protect their investment through guarding. In *M. edwardsii* pre- and post-copulatory mate guarding is extended (44). In the presence of rival males the duration of guarding in males of *M. edwardsii* is increased; thus, it serves as a strategy against sperm competition and provides better protection for their energy invested. In the case of *H. plana*, only precopulatory guarding has been detected and probably the large sperm plug present in this species is enough to ensure paternity. In *T. dentatus* both pre- and post-copulatory mate guarding is brief (41). Likely in *T. dentatus*, relatively low energetic investment in reproduction does not require extended protection of the ejaculate after mating as well as females are receptive year-round which may relax male-male competition. Mating with ‘hard-shelled’ females, energy-conserving short mate-guarding and the absence of costly prevention of competition (e.g. no sperm plug) may facilitate males of *T. dentatus* to recover seminal reserves fast (within 15 days) (60) and to increase the mating frequency. Although males of *H. plana* inverted the greatest quantity of energy in reproduction of the four species studied, this species normally does not perform post-copulatory guarding (LMP, pers. observation). Probably the large internal sperm plug is sufficient to efficiently protect the ejaculate, prevent further pairings and ensure paternity.

Figure 3. Non-metric multidimensional scaling (nMDS) plot consisting of seasonal data of four brachyuran species. Non-metric multidimensional scaling is a tool to assess similarity between samples when considering multiple variables of interest. The value stress represents how well points fit within the specified number of dimensions (stress < 0.05 indicates good fit). The variables included in the nMDS were quantities of proteins and lipids, the vasosomatic index and the total energy content per paired vasa deferentia. Data were square root transformed for analysis.

The quantity of each biochemical component and energy stored in the vas deferens may be influenced by the amount of ejaculate transferred in one mating event and particularly the mating frequency. Males of *H. plana* likely mate only once during the reproductive season (58), thus inverting their entire reproductive energy in only one female. In *M. edwardsii*, lipids in the vasa deferentia decreased during the reproductive season; however, reductions were of small amount probably because males deliver on average only 15% of their vasa deferentia weight in one mating event (57) and portion their seminal material to various females (high level of polygyny (44)). In *T. dentatus*, males transfer on average 37% of their initial seminal material stock during one mating (60). The imminent high risk of sperm competition and uncertainty of paternity may explain the strategy of allocating relatively little energy and small percentage of the initial seminal material stock in each female compared to *H. plana* and increasing chances of paternity through partitioning seminal material among various females (high level of polygyny: 42% of males mate multiply under a female-biased sex ratio (58)). Therefore, in *T. dentatus* the decrease of proteins and lipids probably corresponds to multiple matings indicating increased reproductive activities especially in spring. This suggests that a high level of polygyny (*M. edwardsii* and *T. dentatus*) may be associated with investing little energy in each female but engaging in frequent copulations. The frequency of mating of males of *R. setosum* has not been investigated yet.

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Homalaspis plana and R. setosum invested remarkably high energy in their vasa deferentia, reaching maxima of nearly 40,000 J (autumn and summer, respectively). The energy peak in each of the two species was followed by a sharp decline in both. The pattern of seasonal energy fluctuations may be indicative of the reproductive period of a species. An abrupt decrease of energy may coincide with the mating period or the end of it. In H. plana the decrease of ~80% of energy in winter compared with autumn probably indicates that matings have occurred shortly before this period. Energy remained low in spring and successively increased during summer and autumn. In R. setosum the abrupt decrease of two-thirds of the energy in autumn compared with summer likely indicates that males transferred seminal material to females just before this period and recovering values from winter until summer. The presence of ovigerous females of R. setosum was observed throughout the austral winter until larvae hatching in spring in a similar latitude60, whereby in both species, mating occurs when the cycle of the ovary development begins (as has been detected in other crab species46,50,62) and a high quantity of proteins in the seminal fluid delivered to the female seminal receptacle could function as a cue for gonadal development. Seminal fluid proteins (SFPs) produced in the male reproductive system are well recognized to induce numerous physiological and behavioural post-mating changes in female insects, such as egg production31. However, SFPs have been poorly studied in decapods63. Other key functional properties associated with the great quantity of biochemical components in the vas deferens may be related to mechanisms that increase the reproductive return. In R. setosum male seminal fluids may be involved in enhancing sperm viability during prolonged storage within the female seminal receptacle as this species is able to produce several viable clutches without re-mating62. There is evidence from females of M. magister, which store viable sperm for at least 2½ years in their seminal receptacle, which can be used to inseminate more than one egg mass64. Especially proteins have been formerly associated with this function in insects37; however, lipids may also be valuable in prolonged sperm storage due to their high energetic value. Beyond that, lipids may serve as a contribution of energy to the female in H. plana and R. setosum due to the great amount of this highly energetic component. In insects, males commonly transfer gifts that contain many different non-gametic materials to females during courtship and mating65. In this way, the provision of nutrients deposited in the female’s reproductive tract can occur.

While H. plana and R. setosum assigned similarly high amounts of energy, the energy invested by H. plana was linked to the production of large amounts of proteins and lipids, in contrast to R. setosum, which produced highly energetic lipid-rich vasa deferentia. In H. plana the vasa deferentia dry weight as a proportion of the crab’s whole body dry weight accounts for up to nearly 6% displaying one of the largest VSI recorded so far in crabs26 (Fig. 4). This emphasizes the large energy expenditure, which may result from the capacity of the vas deferens to produce and store large quantities of proteins and lipids. In this species, especially the voluminous posterior region of the vas deferens has been identified as the main site of seminal fluid production with a very high energy content26. The main energetic substrates in H. plana were proteins and lipids, and in winter, quantities of lipids and proteins dropped nearly 90% and two-thirds, respectively, compared with autumn. These seasonal fluctuations indicate that males delivered large quantities of both components during mating to the female. Especially in H. plana quantities of proteins and lipids remained relatively low during winter and spring. Recovery may require a long period after mating suggesting that the elaboration of these large amounts of proteins and lipids may have elevated energetic costs. In this context, interesting future research questions may arise, such as whether the elaboration of specific components or the composition of the seminal reserves influences the recovery period after mating.

The crab species M. edwardsii and T. dentatus showed low energetic investment per vasa deferentia. They had similar patterns of investment with relatively constant energy values throughout the seasons, except for in austral spring the lowest energy values (around 900 J) were recorded in both species. In M. edwardsii the reduction of energy coincides with the well-known mating season from October to January (i.e. austral spring and summer)30. In T. dentatus the marked decline of energy content during spring corresponds to the formerly described continuous reproductive pattern but with a greater mating intensity in spring31. Both species with low male energy investment were characterized by smaller-sized vas deferens and intermediate VSI (Fig. 4). Annual mean vasa deferentia dry weight of M. edwardsii and T. dentatus only accounted for approximately 10% of those of H. plana and R. setosum. While the two crab species show a similar investment in terms of energy, this is likely related to the mating strategies.

Complex and well-developed male reproductive systems appear to have evolved at different times in the evolution of crabs as is noted in a contrasting composition and size of the vas deferens within one family and similarities between phylogenetically not closely related species. Thus, the composition and size of the vas deferens is related to the mating strategy and level of polygyny of each species. To summarize, the morpho-functional traits of the reproductive system and competition avoidance strategies may interact to determine the optimal mating frequency and the quantity of biochemical components, which can also be reflected in the energy invested (Table 3). Energy allocation may also be associated with the intensity of polygyny of a species. We suggest that a gradient of the intensity of polygyny exists related to energetic investment: allocation of great energy amount and low mating frequency, in contrast to low energetic investment and multiple matings. Interestingly, our findings of energy investment in reproduction resemble the two distinct hypotheses concerning the evolution of large testes stated by Vahed and Parker28: the numerical sperm competition hypothesis may correspond to H. plana and R. setosum vs. the male mating rate hypothesis matches our findings of M. edwardsii and T. dentatus. Especially in the face of sperm limitation (number of sperm is insufficient to fertilize all oöcytes produced by females) as triggered by size-selective male fishery management66, it is crucial to evaluate the ejaculate as a whole because males may be depleted of specific biochemical components of the seminal material31. Depletion of distinct biochemical components may reduce the functionality of particular components and affect the reproductive success. Thus, ejaculate limitation rather than sperm limitation could affect fished species. Moreover, in the aquaculture industry, biochemical composition and the quantity of each component may be important indicators for the quality of seminal material. Our study is a first step towards identifying investment in the
Figure 4. Seasonal pattern of vasosomatic index of the four brachyuran species studied. Values correspond to means ± SE. Seasons correspond to austral seasons.

Table 3. Summary of energy investment in the four brachyuran species studied. The complexity of the reproductive system, intensity of mating, polyandry (based on observations in the laboratory) and existence of a strategy to prevent competition (i.e. sperm plug) are indicated for each species and may play key roles in energy investment. *Refers to not investigated yet.
male seminal storage structure in crabs which highlights the diversity of mating strategies in Brachyura which is reflected even on the physiological level.

Methods
Species sampling and experimental procedures. Males of the following four crab species, *H. plana*, *R. setosum*, *M. edwardsii*, and *T. dentatus* were collected seasonally (austral seasons) from Los Molinos Bay, Southern Chile (39° 51’ 16.7’’ S; 73° 23’ 40.3’’ W) from June 2016 to May 2017 and transported to the Laboratorio Costero de Recursos Acuáticos de Calfuco, Universidad Austral de Chile. Crabs were maintained in the laboratory and provided running seawater, air supply and ad libitum food (mussels). All crabs were measured along their greater body axis; carapace width (CW) in *H. plana*, *R. setosum* and *M. edwardsii* and carapace length (CL) in *T. dentatus*. Mean ± SE size of *H. plana*, *R. setosum*, *M. edwardsii* and *T. dentatus* were 94.3 ± 1.4 mm CW, 128.6 ± 3.1 mm CW, 133.9 ± 2.9 mm CW, and 102.7 ± 1.6 mm CL, respectively. No difference in size among seasons existed in each species except for *M. edwardsii* (one-way ANOVA, season: $F_{3,15} = 10.05, P < 0.001$; smaller sized crabs in summer). Each crab species and season comprised five replicates (except for $n = 4$ each in *M. edwardsii* in winter, *H. plana* in winter, and *R. setosum* in winter and autumn).

Crabs were anaesthetised (thermal shock ~ 20 °C for 15 min) and paired vasa deferentia were extracted. Samples were immediately frozen and stored in pre-weighed vials at ~ 80 °C in an ultrafreezer (Thermo Scientific®, Forma Series 700) until further analyses. The left vas deferens was used for biochemical analyses and the right vas deferens was used to determine the dry weight to relate the quantity of each biochemical component to it. Normally both vasa deferentia have similar weight. Dry weight was determined by oven drying the right vas deferens for 4 days at 70 °C and weighing it to a precision of 0.00001 g. Total dry weight of paired vasa deferentia (i.e. doubling dry weight of right vas deferens) is referred to as VDW. The vasosomatic index (VSI, expressed as percentage) was calculated: VSI = (VDW/BDW) × 100, BDW being the dry weight of the body (oven-dried for 4 days at 70 °C and weighed to a precision of 0.01 g). To estimate the VSI, dry weight of crabs without legs and chelae were used to increase accuracy.

Biochemical analyses. Samples were analysed for total protein and lipid content at the Laboratorio de Ecología y Fisiología de Crustáceos, Universidad Austral de Chile, in Puerto Montt, Chile. Stored samples were lyophilised (Labconco, FeeZone 2.5) for 48 h, and dry weight was determined (precision of 0.00001 g). Samples were homogenised in a phosphate buffer and Milli-Q ultrapure water to achieve a concentration of ~ 10 mg mL$^{-1}$ by a motorised homogenizer (Ultra-Turrax); during this process, samples were kept on ice. Complementary ultrasound (Branson, Sonifier, Cell Disruptor B 15) was applied in pulses of 4 s and 4 s rest (cycle repeated four times maximum to avoid warming) on tissues difficult to homogenize. Each homogenate was subdivided into new cryovials (proteins: 10 µL (3 µL per duplicate in Lowry method) and lipids: 50 µL in sulfo-phospho-vanillin method) and stored frozen at − 80 °C until respective analysis.

Protein content was assessed in duplicate by the Lowry method using a commercial kit (DC Protein Assay Kit, Bio Rad; Standard BSA; 750 nm). Total lipid content was estimated in duplicate by the sulfo-phospho-vanillin method (Standard cholesterol; 530 nm) modified for microplate format by Torres et al. A PowerWave HT spectrophotometer (BioTek) was used for all colorimetric methods. The biochemical composition was calculated based on the calibration curves and values of absorbance. Quantities of biochemical components and dry weight of the right vas deferens were doubled to refer to the paired vasa deferentia. Results were expressed as the averaged quantity of each biochemical component per paired vasa deferentia. The energy content in vasa deferentia of crabs was estimated from the protein and lipid data and was converted to their caloric equivalents in Joule corresponding to 23.64 J/mg protein and 39.54 J/mg lipid. In preliminary analyses quantity of carbohydrates was very small therefore, they were not estimated.

Data analyses. To test seasonal variation in quantity of biochemical components and energy invested in the vasa deferentia in each species, one-way ANCOVAs with permutation were performed with season as a fixed factor and male size as a covariate. However, when the covariate was not significant, a one-way ANOVA with permutation was performed instead. Analyses were chosen because they are distribution-free and allow analysis with relatively small sample sizes. The `Aovp` function in the package `lmPerm` was used. To check for size similarities among seasons, a one-way ANOVA was performed for each species.

Non-metric multidimensional scaling (nMDS) was performed to visualize the pattern of energy investment in seminal material and to identify species with similar investment strategies. It is a tool to assess similarity between samples when considering multiple variables. Data comprised seasonal values of each of the four brachyuran species. The variables included in the nMDS were quantities of proteins and lipids, VSI and the total energy content per vasa deferentia. The `isoMDS` function in the package `MASS` was used. All statistical analyses were performed in R v. 3.5.3.

Data accessibility. The datasets generated during the current study are available in the Zenodo open science repository, https://doi.org/10.5281/zenodo.5654807.
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Author contributions

L.M.P., K.Pa. and K.Pr. conceived the ideas and designed methodology; K.Pa., K.Pr. and M.P.R. collected the data; K.Pa., K.Pr. and L.M.P. analysed the data; K.Pr. and L.M.P. led the writing of the manuscript. All authors reviewed the manuscript and gave final approval for publication.

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Competing interests

The authors declare no competing interests.
