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CALCIUM LEVELS IN THE VULVAR OPERCULA OF GRAPSOID AND OCYPODOID CRABS (DECAPODA: BRACHYURA)

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ABSTRACT

The derived condition in Brachyura is exhibited when mating is not linked to moulting (i.e. hard-shell mating). Grapsoid and ocypodid crabs have this type of mating wherein females become receptive and have mobile vulvae covered by operculae without moulting. The mobility of the operculae has been attributed to a decalcification process although it has never been demonstrated experimentally. We described the morphology of the vulvae (operculum and hinge) and measure calcium levels employing X-ray Energy Dispersive Spectroscopy of the intermoult immature and mature female of several different species of grapsoid crabs: *Austrohelice crassa*, *Cyclograpsus lavauxi*, *Hemigrapsus crenulatus*, *Hemigrapsus sexdentatus* (Varunidae), *Guinusia chabrus* (Plagusiidae), *Leptograpsus variegatus* (Grapsidae), and the ocypodid *Hemiplax hirtipes* (Macrophthalmidae). We measured calcium levels during a reproductive cycle in the case of *H. sexdentatus*. In all the studied species the opercula and hinge had lower calcium levels compared to the adjacent exoskeleton, and the hinge showed the lowest values of calcium (9.7 and 40.8 weight%, respectively). Immature and adult crabs showed that the distribution of calcium remains the same after the pubertal moult as it was before. Furthermore, in *H. sexdentatus*, hinge calcium levels do not change during the female reproductive cycle when females become receptive. It is suggested that an alternative mechanism, not related to decalcification, could be involved in promoting mobility of the hinge in receptive females of these species. We need to modify our ideas of the processes involved in female receptivity in all these crabs with an operculate vulva and hence mate attraction. Answers to these questions are the key to understanding how the linkage of mating to moulting was broken.

KEY WORDS: *Austrohelice*, copulation, *Cyclograpsus*, decalcification, *Guinusia*, hard-shell mating, *Hemigrapsus*, *Hemiplax*, hinge line mobility, *Leptograpsus*, Macrophthalmidae, mate attraction, moulting, Varunidae

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INTRODUCTION

Mating in Brachyura is either linked to female molting soft-shell mating (ancestral condition) or it occurs during the intermoult when both sexes have hard exoskeletons (derived condition) (McLay and López Greco, 2011). Crabs belonging to the Cancridae and most Portunidae mate when the female is soft immediately after molting, whereas those belonging to Majidae (the first mating can be with soft females), Hymenosomatidae, Varunidae, and Ocypodidae mate when both sexes are hard shelled (Hines, 1991; Yamaguchi, 2001; Turner et al., 2003; Brockerhoff and McLay, 2005a; Sainte-Marie et al., 2008; van Den Brink and McLay, 2009). Regarding the timing and duration of female receptivity, mating in Brachyura can be restricted to a short period of time when females are morphometrically able to mate (as in the post-molt mating of portunoids and cancroids), or when the opercula of females, a soft or hard cover of the vulvae of many brachyurans (see Guinot et al., 2013), becomes temporarily mobile during the intermoult period (as in some grapsoids and ocypodids) (McLay and López Greco, 2011; McLay and Becker, 2015).

It is not surprising that the idea of "decalcification" became established as a necessary precursor to mating in crabs because this is the process whereby calcium was withdrawn from the old exoskeleton, allowing it to be shed, and for growth of the new exoskeleton to begin. Mating could, therefore, occur because the main structural obstacle, the operculum, could be displaced by insertion of the male gonopod. Consequently, in crabs that have hard-shell mating it was assumed that the same softening process was used. There remain the group of non-operculate, hard-shell females that do not need to decalcify any structures because their vulvae are not blocked. The primary aim of females is to gather sperm enabling them to fertilize their next brood, but combining this with growth could mean that there is more than one way of attracting males (McLay and López Greco, 2011).

The form of the vulva has important implications for the type of mating and in the length of the receptive period when males are attracted to females. We follow Guinot et al. (2013) and use the term "vulva" for eubrachyuran crabs because the aperture is used for both intromission and egg-laying. There are three possible forms of vulva closure

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in brachyuran females: 1) simple muscle action (opening of vulva, not restricted), 2) a muscle-operated non-hinged cover of the vulva, or 3) a calcified hinged operculum covering the vulva (Hartnoll, 1968, 2006; Thompson and McLay, 2005). It has been suggested in the last case that the female operculum becomes mobile due to a decalcification process that occurs during the intermoult (Hartnoll, 1968; Henmi and Murai, 1999), although this has never been demonstrated experimentally. A hard, immobile operculum is an obstacle to copulation, restricting the duration of receptivity and egg laying (Hartnoll, 1965; Brockerhoff and McLay, 2005a, b; Guinot et al., 2013).

The female vulva is composed of chitin, the same material as the integument of the exoskeleton. In the case of crustaceans, there is a high degree of mineralization, typically calcium carbonate, which gives mechanical rigidity (Chen et al., 2008). Crustaceans demonstrate drastic temporal differences within the same tissue with regard to the extent of and capacity for mineralization (Roer, 1980; Roer and Dillaman, 1984). Several studies have used scanning electron microscopy (SEM) and X-ray Energy Dispersive Spectroscopy (EDS) to measure calcium levels at different areas of the exoskeleton (e.g., Dillaman et al., 2005; Chen et al., 2008; Cribb et al., 2010). Females included in the superfamily Grapsoidea have the derived condition of mating not linked to moulting, thus, it has been suggested that operculae mobility during the intermoult could be due to a decalcification of the vulvae (Hartnoll, 1969; Henmi and Murai, 1999). The aims of our study were to describe the morphology of the vulvae and evaluate differences in calcium levels in the vulvae of different species of female grapsoid crabs such as *Austrohelice crassa* (Dana, 1851), *Cyclograpsus lavauxi* H. Milne Edwards, 1853, *Hemigrapsus crenulatus* (H. Milne Edwards, 1837), *Hemigrapsus sexdentatus* (H. Milne Edwards, 1837), *Guinusia chabrus* (Linnaeus, 1758), *Leptograpsus variegatus* (Fabricius, 1793), and in *Hemiplax hirtipes* Heller, 1865 (superfamily Ocypodoidea). For *H. sexdentatus* we also measured calcium levels in the vulva during a single reproductive cycle (when the female became receptive until she became unreceptive and laid eggs). In this species females produce a single brood of eggs each year and breeding is highly synchronized, limited to a short period from late March to mid-April. Females are receptive only when they have mobile opercula that lasts for approximately seven days (Brockerhoff and McLay, 2005b, c).

MATERIALS AND METHODS

Species and Areas Studied

Leptograpsus variegatus (Grapsidae) and *Guinusia chabrus* (Plagusiidae) were collected on the rocky shore of Matheson Bay and Leigh Harbor, North Island, New Zealand. The other species studied, *Austrohelice crassa*, *Cyclograpsus lavauxi*, *Hemigrapsus sexdentatus*, and *Hemigrapsus crenulatus* (Varunidae) were captured near Christchurch, South Island, New Zealand. For comparison, we also measured calcium values for intermoult *Hemiplax hirtipes* captured in the Avon-Heathcote Estuary near Christchurch. Crabs were transported to the Leigh Marine Laboratory, University of Auckland or to the School of Biological Sciences, Canterbury University for processing.

To evaluate differences in the calcium levels of vulvae during the reproductive cycle of *H. sexdentatus*, captive females were kept in 100 × 50 × 15 cm plastic tanks, approximately 20 female crabs per tank in a recycling seawater circulation system at 12°C and diurnal lighting. Rocks were provided for refuge. They were monitored twice weekly to check hinge line mobility and egg laying. Mobility of the hinge of receptive females was detected after it could be stretched by gentle pressure using a needle, allowing the operculum to be displaced inwards. Degrees of mobility were not measured, only its presence or absence. Crabs were fed twice weekly with seaweeds (*Codium fragile* (Suringa) Hariot, 1889; *Enteromorpha intestinalis* (Linnaeus) Nees, 1820; and *Ulva lactuca* Linnaeus, 1753) and crushed mussels (*Mytilus galloprovincialis* Lamarck, 1819 and *Perna canaliclus* Gmelin, 1791)) collected from the shoreline.

General Procedures

Individuals were killed in the laboratory by placing them in a freezer at 15°C for approximately 30 min. For the description of the external morphology, the area surrounding the vulva of five females of *L. variegatus* (ranging from 36 to 58.4 mm carapace width (CW)), *G. chabrus* (31.4 to 44 mm CW), *A. crassa* (8.9 to 11.2 mm CW), *C. lavauxi* (9.7 to 15.0 mm CW), *H. crenulatus* (12.4 to 15.9 mm CW), *H. sexdentatus* (13.8 to 28.0 mm CW), and *H. hirtipes* (8.9 to 15.5 mm CW) were dissected and examined by SEM. Mobility of the hinge and the opercula was checked in order to recognize if females where receptive or not. For the experiment comparing changes in vulva calcium levels over a reproductive cycle of *H. sexdentatus*, mature females ranged from 25.5 to 40.5 mm CW and 4 females were selected and processed by the same way.

Samples of vulvae were dried at ambient temperature (23 to 25°C), and coated with gold-palladium and viewed with a Jeol JSM-6460LV scanning electron microscope using an EDAX Genesis XM4-Sys 60, equipped with multichannel analyzer EDAX mod EDAM IV, sapphire Si (Li) detector, or 20 nm of carbon using an Emitech K975X coater and then examined using an Oxford Aztec EDS with a 50 mm X-Max silicon drift detector (SDD). Samples of the same species were analyzed by both methodologies and no differences in calcium levels were found, thus, we used both methods when necessary. Quantitative data were analyzed and displayed using software EDAX Genesis 5.11. The level of calcium was measured as its weight (%) using energy dispersive spectroscopy X-ray analysis (EDXA).

Following Hartnoll (1968) descriptions, three areas of the vulvae were differentiated to examine calcium levels: the operculum and hinge (both forming the vulva) and the adjacent exoskeleton (see below). Calcium levels in each area were measured using X-ray EDS.

The statistical analysis consisted of evaluating differences in the calcium levels (dependent variable) between the three vulvae areas, opercula, hinge and adjacent exoskeleton (independent variable) in both reproductive status, immature and mature (independent variable) of females of all species ($n = 4$ for each species) using a two-way ANOVA (Zar, 1999). In case of finding differences, a posterior Tukey-test was conducted to recognize these differences between the areas. For *H. sexdentatus*, differences between the calcium levels (dependent variable) in the same three vulvae areas measured (independent variable) over the reproductive cycle, approximately seven days, (independent variable) were analyzed using a two-way ANOVA.

RESULTS

The vulvae of all the species studied consisted of an operculum and a hinge (Fig. 1). The hinge was always recognized as the area where the cuticle was depressed and/or marked as a wrinkled area of the cuticle. These characteristics could allow the cuticle to be flexible during female receptivity, enabling the vulva to become mobile and facilitating insertion of the male gonopod during copulation. All mature females were unreceptive because of the immobility of the hinges and opercula.

Hemigrapsus sexdentatus

This species has vulvae closed by an operculum, with the hinge showing signs of distortions, which could be the result of compression and stretching of the cuticle during the previous copulation (Fig. 1A). The female from which the vulva is shown in Fig. 1 was mature and so would have mated and laid eggs at least once.

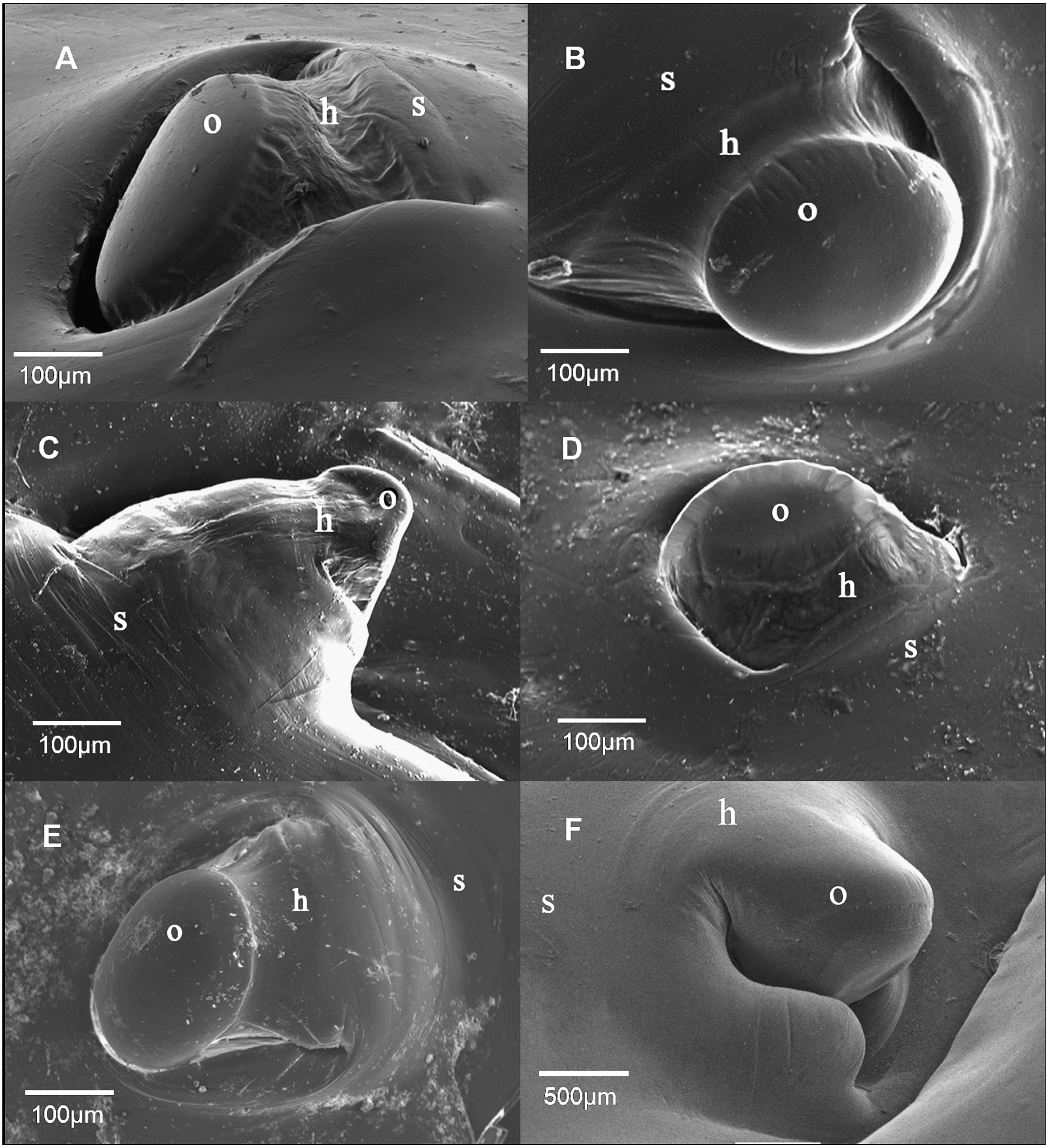


Fig. 1. Vulvae of the females of the grapsoid and ocypodoid crabs studied. A, *Hemigrapsus sexdentatus*; B, *Hemigrapsus crenulatus*; C, *Cyclograpsus lavauxi*; D, *Austrohelice crassa*; E, *Hemiplax hirtipes*; F, *Guinusia chabrus*. Abbreviations: s, adjacent skeleton; h, hinge; o, operculum showing where calcium levels were measured.

Levels of calcium showed differences between the three areas measured (operculum, hinge, and adjacent exoskeleton) but not between immature and mature females (Table 1, Fig. 2A). A posterior Tukey-test showed differences between the three areas, with the hinge having the lowest values and the adjacent exoskeleton the highest (Table 2). The hinge area is primarily composed of chitin, which has to be continuous over the entire surface of the crab, but the calcified operculum is a patch of calcified tissue isolated from the surrounding calcified sternum.

Hemigrapsus crenulatus

The vulva is closed by an elevated hinged operculum similar to *Hemigrapsus sexdentatus* (Fig. 1B). The operculae of both

Table 1. Results of the two-way ANOVA comparing calcium levels in the vulvae area (adjacent skeleton, hinge, and operculum) and immature and mature (reproductive status) females of seven species of grapsoid and ocypodoid crabs. There are highly significant differences for vulvae area (***$p < 0.001$) but no significant differences for reproductive status ($p < 0.05$).

Species	*F*	df	*p*
Hemigrapsus sexdentatus			***
Model	79.56	5	***
Vulvae area	198.12	2	
Reproductive status	0.74	1	0.4
Hemigrapsus crenulatus			
Model	22.59	3	***
Vulvae area	33.42	2	***
Reproductive status	0.93	1	0.35
Cyclograpsus lavauxi			
Model	260.69	3	***
Vulvae area	389.61	1	***
Reproductive status	2.84	1	0.12
Austrohelice crassa			
Model	204.65	3	***
Vulvae area	308.80	2	***
Reproductive status	2.35	1	0.14
Hemiplax hirtipes			
Model	54.33	3	***
Vulvae area	81.23	2	***
Reproductive status	0.52	1	0.48
Guinusia chabrus			
Model	34.84	3	***
Vulvae area	51.94	2	***
Reproductive status	0.65	1	0.43
Leptograpsus variegatus			
Model	108.74	3	***
Vulvae area	162.52	2	***
Reproductive status	1.17	1	0.29

Table 2. Results of Tukey tests after a significant two-way ANOVA in immature and mature specimens of the species studied. Abbreviations: S, skeleton adjacent to vulva; H, hinge; O, operculum; $p > 0.05$.

Species	Vulvae area	Mean
Hemigrapsus sexdentatus	S	44.01
	H	3.05
	O	25.14
Hemigrapsus crenulatus	S	42.99
	H	5.98
	O	35.63
Cyclograpsus lavauxi	S	40.31
	H	3.76
	O	11.94
Austrohelice crassa	S	41.73
	H	5.56
	O	32.31
Hemiplax hirtipes	S	35.38
	H	3.70
	O	22.75
Guinusia chabrus	S	29.88
	H	4.13
	O	14.70
Leptograpsus variegatus	S	23.08
	H	2.33
	O	13.03

species are at the same level as the sternum, but they do not close tightly, leaving a small gap around the operculum edge. Presumably vaginal musculature provides a tighter seal.

There were differences between the operculum, hinge, and adjacent exoskeleton but not between immature and mature females (Table 1, Fig. 2B). A posterior Tukey-test showed differences between the three areas, the hinge having the lowest values and the adjacent exoskeleton the highest (Table 2).

Cyclograpsus lavauxi

The vulva is characterized by the presence of a simple, elevated, and hinged operculum covering the female aperture (Fig. 1C). The hinge area is wide, with the operculum almost at right angles to the hinge, and surrounded by an elevated rim (Fig. 1C).

Levels of calcium showed differences between the operculum, hinge, and adjacent exoskeleton but not between immature and mature females (Table 1, Fig. 2C). A posterior Tukey-test showed differences between the three areas, the hinge having the lowest values and the adjacent exoskeleton the highest (Table 2).

Austrohelice crassa

The vulva is covered by a convex, hinged operculum surrounded by an elevated rim (Fig. 1D). Although belonging to a different genus, the vulva of this species is very similar to the two species of *Hemigrapsus* described above. In all of the species examined here the operculae are oriented medially, with the hinge line on the lateral side to accommodate the orientation of the male gonopod.

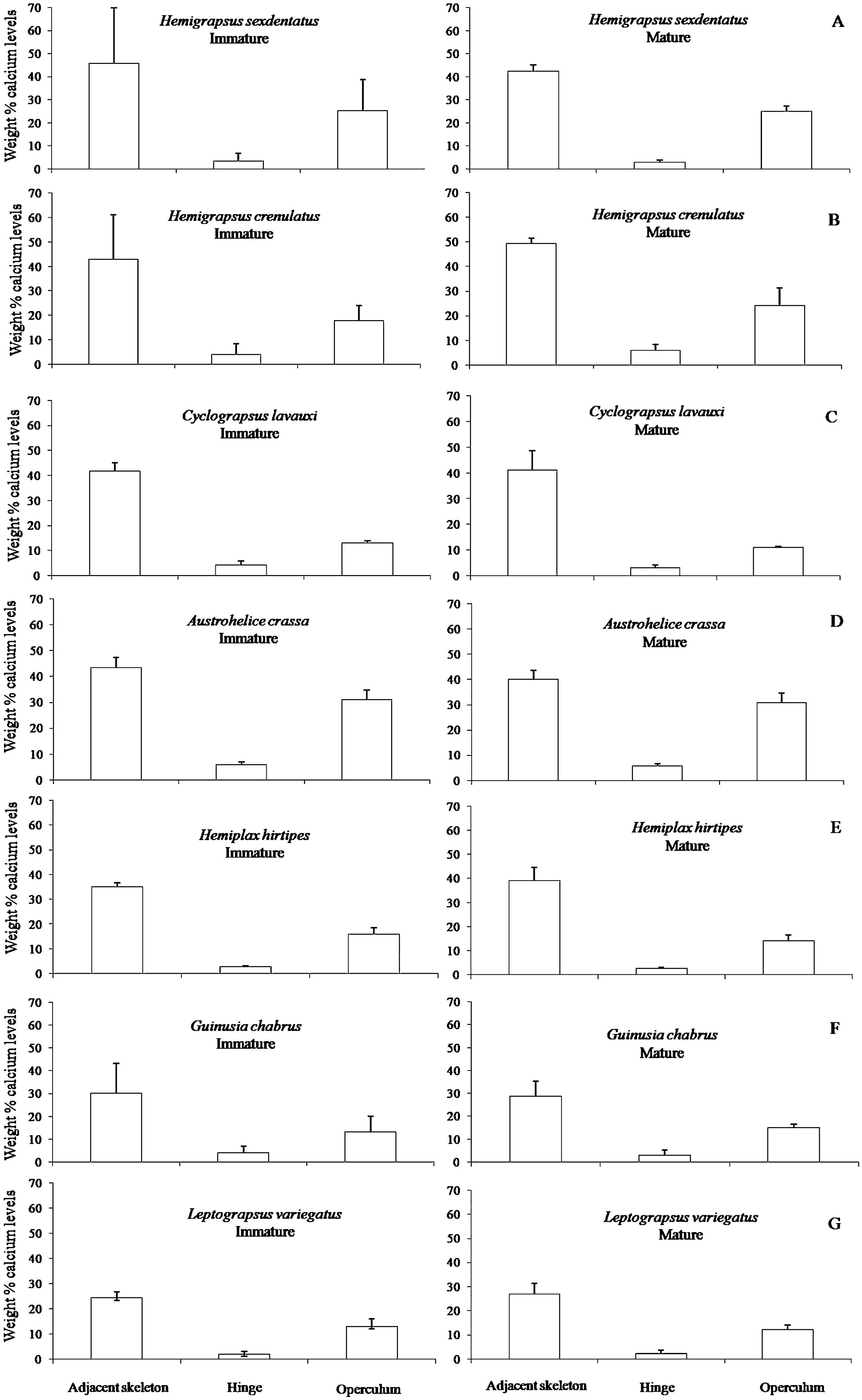
Levels of calcium showed differences between operculum, hinge, and adjacent exoskeleton but not between immature and mature females (Table 1, Fig. 2D). A posterior Tukey-test showed differences between the three areas, having the hinge the lowest values and the adjacent exoskeleton the highest (Table 2).

Hemiplax hirtipes

Although from a different family, Macrophthalmidae, *Hemiplax hirtipes* has a vulva very similar in structure to that of varunids, being characterized by the presence of an elevated operculum followed by the hinge (Fig. 1E).

Levels of calcium showed differences between the operculum, hinge, and adjacent exoskeleton but not between immature and mature females (Table 1, Fig. 2E). A posterior Tukey-test showed differences between the three areas,

Fig. 2. Calcium levels (% weight) of exoskeleton adjacent to vulva, hinge, and operculum of immature and mature females of some crab species. A, *Hemigrapsus sexdentatus* (Grapsidae); B, *Hemigrapsus crenulatus* (Grapsidae); C, *Cyclograpsus lavauxi* (Grapsidae); D, *Austrohelice crassa* (Varunidae); E, *Hemiplax hirtipes* (Macrophthalmidae); F, *Guinusia chabrus* (Plagusiidae); G, *Leptograpsus variegatus* (Grapsidae).


Weight % calcium levels
0
10
20
30
40
50
60
70
Hemigrapsus sexdentatus Immature
Hemigrapsus sexdentatus Mature
A
Hemigrapsus crenulatus Immature
Hemigrapsus crenulatus Mature
B
Cyclograpsus lavauxi Immature
Cyclograpsus lavauxi Mature
C
Austrohelice crassa Immature
Austrohelice crassa Mature
D
Hemiplax hirtipes Immature
Hemiplax hirtipes Mature
E
Guinusia chabrus Immature
Guinusia chabrus Mature
F
Leptograpsus variegatus Immature
Leptograpsus variegatus Mature
G
Adjacent skeleton
Hinge
Operculum

the hinge the lowest values and the adjacent exoskeleton the highest (Table 2).

Guinusia chabrus

The vulva has a cuticular projection that covers most of the aperture. The projection ends in the operculum (Fig. 1F). The hinge of the operculum is not immediately obvious, but it can be found at the base of the operculum where the cuticle is seen slightly depressed (Fig. 1F). Using macrophotography it is possible to see a difference in the colour of the cuticle marking the position of the hinge. The skeleton around the edge of the vulva is raised, forming a rounded border of cuticle (Fig. 1F).

Levels of calcium showed differences between the operculum, hinge, and adjacent exoskeleton but not between immature and mature females (Table 1, Fig. 2F). A posterior Tukey-test showed differences between the three areas, the hinge having the lowest values, the adjacent exoskeleton the highest (Table 2).

Leptograpsus variegatus

The vulva is very different from the rest of the studied species, being a complex structure characterized by an operculum, which shows signs of distortion, perhaps the result of compression and stretching of the cuticle during past copulations because of gonopod pressure (Fig. 3). The operculum is divided at its base into six portions looking like fingers. Each "finger" shows a depressed region of the cuticle, which could constitute the hinge (Fig. 3). The skeleton around the vulva edge is raised, thus the general operculum is elevated from the surface of the adjacent exoskeleton by a rounded border of cuticle (Fig. 3).

Levels of calcium showed differences between the operculum, hinge, and adjacent exoskeleton but not between immature and mature females (Table 1, Fig. 2G). A posterior Tukey-test showed differences between the three areas, having the hinge the lowest values and the adjacent exoskeleton the highest ones (Table 2).

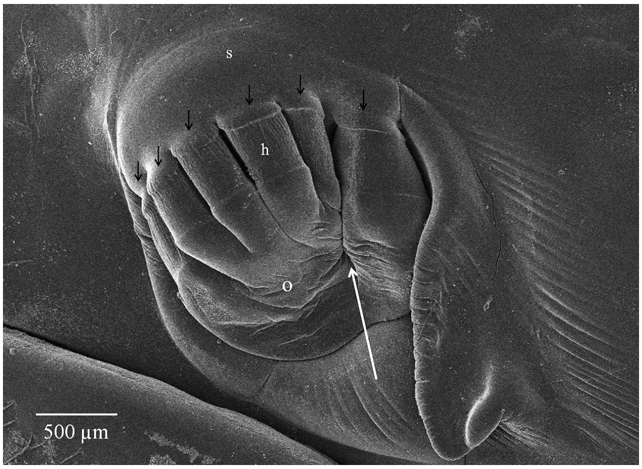


Fig. 3. Vulva of *Leptograpsus variegatus*. The operculum cuticle looks stretched perhaps as a result of a male gonopod pushing it aside during copulation. The white arrow indicates where the gonopod pushed the operculum into the vagina. The operculum is divided at its base into six finger-like portions, each showing a depressed region of the cuticle, which could constitute the hinge (black arrows). Abbreviations: s, adjacent skeleton; h, hinge; o, operculum showing where calcium levels were measured.

Changes in Calcium Levels in the Vulva During the Reproductive Cycle of *Hemigrapsus sexdentatus*

Four stages were recognized in a single reproductive cycle: 1) pre-breeding non-receptive females with an immobile hinge and not bearing eggs, 2) receptive females with mobile hinge and not bearing eggs, 3) receptive females with mobile hinge and bearing eggs, and 4) non-receptive females with immobile hinge and bearing eggs. The vulvae of female *H. sexdentatus* maintained similar calcium levels in each of the three vulvae areas measured during their reproductive cycle (two-way ANOVA, $F = 0.48$, df = 3, $p = 0.7$), whereas calcium levels differed between operculum, hinge, and adjacent exoskeleton in the different reproductive stages (two-way ANOVA, $F = 275.34$, df = 2, $p < 0.001$) (Fig. 4A-D). At all four stages the spatial pattern was hinge line (3.6% weight) $\ll$ operculum (24.3% weight) $<$ skeleton (40.9% weight). The calcium level showed no significant change when the hinge became mobile (ready to mate and lay eggs) nor when the hinge became immobile again (after egg laying). Hinge line calcium levels thus remain similar before, during, and after breeding. The same occurred in the adjacent skeleton and operculum.

DISCUSSION

The operculae in the varunid crabs described above are similar to those found in other genera in this family (Sakai et al., 2006). The operculae can only be pushed downward into the vagina by the male gonopod when the hinge line is mobile. The operculae of grapsids like *Grapsus albolineatus* Latreille, in Milbert, 1812, *Metopograpsus messor* (Forskål, 1775), and *M. thukuhar* (Owen, 1839) reported by Naderloo (2011) resemble varunid operculae. The grapsid *Leptograpsus variegatus*, however, has a vulva that is remarkably different from other grapsids, having a hinge that is divided into finger-like folds (Fig. 3). The vulva and operculum of *Hemiplax hirtipes* are similar to those of other macrophthalmid crabs (Komai et al., 1995; Naderloo et al., 2011; Teng and Shih, 2015). Moreover, although from a different family, the vulva of *H. hirtipes* has a structure very similar to that found in varunids. Kitaura et al. (2010), using mitochondrial 16S ribosomal DNA found that *H. hirtipes* grouped with Australian and New Zealand varunids rather than Australian macrophthalmids, prompting Davie (2012) to omit *H. hirtipes* from this family. We nevertheless continue to treat *H. hirtipes* as a macrophthalmid until the two families are revised and rationalized.

Crabs with operculate vulvae normally have a vagina that is concave in cross section (Guinot et al., 2013). Crabs with hinged operculae also have indeterminate growth, ventral seminal receptacle and hard-shell mating (McLay and López-Greco, 2011). Most of these crabs are included in Thoracotremata (see Guinot, 1977). Many species live in burrows in soft sediment where an operculum could be advantageous. An operculum could be necessary to protect stored sperm in crabs that live in shallow water and the intertidal, where they could be exposed to air for long

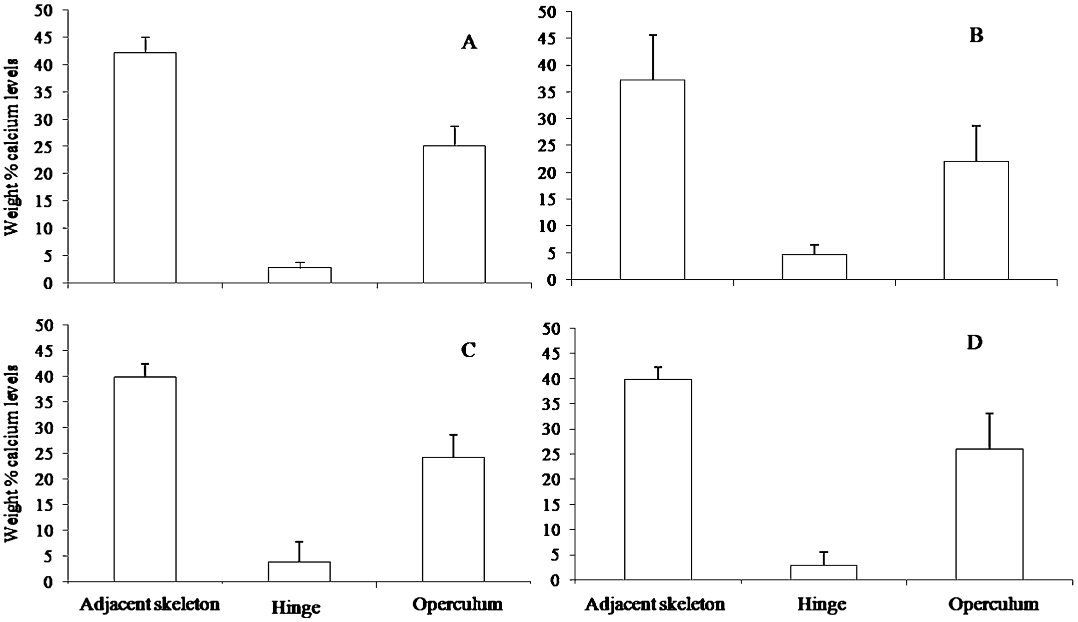


Fig. 4. Changes in calcium levels (% weight) in the exoskeleton adjacent to vulva, hinge, and operculum during the reproductive cycle of female *Hemigrapsus sexdentatus*. Abbreviations: A, pre-breeding females with immobile hinge not bearing eggs; B, receptive females with mobile hinge not bearing eggs; C, receptive females with mobile hinge bearing eggs; D, females with immobile hinge bearing eggs.

periods and become dehydrated. Operculae could also help avoid water-borne microbial infections.

It has been demonstrated that when grapsoid females are ready to mate their operculae become mobile and they attract males (Brockerhoff and McLay, 2005a, b, c). This has been assumed to be a result of decalcification of the hinge line (Hartnoll, 1968, 2006; Henmi and Murai, 1999; McLay and López Greco, 2011), and that this local decalcification during the intermoult employs the same mechanism used to recover calcium from the exoskeleton when the animal moults (Hartnoll, 1965, 1982; Guinot et al., 2013). How such a process might be targeted on the hinge line has never been explored. We found that females of some grapsoid (and ocypodoid) species showed lower calcium values in the operculum and hinge compared to the adjacent exoskeleton during the intermoult period. If "decalcification" is the cause of hinge-line mobility then the amount of calcium that needs to be removed would be much less than if it was the same as the nearby skeleton. The derived condition of mating not linked to moulting could have therefore evolved from vulvae with lower calcium levels, which could facilitate decalcification of only this area and not the rest of the animal during the intermoult period. The pattern of low calcium levels in the hinge and operculum in both immature and mature females indicates that this is not a consequence of becoming mature at the puberty moult (i.e. a secondary sexual character) but a character present in juveniles.

Our results show that in *H. sexdentatus* calcium levels of the hinge line did not vary during the reproductive cycle, while being receptive with mobile operculae, suggesting that there could be another mechanism, not related to decalcification, involved in mobilization of the hinge. The hinge line tissue is not a target for calcification after each moult, thus resembling a limb joint that must be freely movable so the limb can perform its role. Unlike the limb joint, however, the hinge does not remain flexible throughout life, being immobile most of the time. It is thus suggested that there must be another way of hardening chitinous tissue that does not necessarily involve calcium.

It has long been assumed that in species showing mating linked to moulting males are attracted to females ready to mate by the moulting hormone crustecdysone (Hardedge et al., 2002), but this could not apply to species with hard-shell mating. These crabs are only going to reproduce and not grow so they cannot use crustecdysone. An alternative explanation could invoke an ovarian hormone, released when the gonad was reaching maturity and the female needed sperm to fertilize her oocytes. Males could use such a signal to detect the reproductive status of females and also the signal could be involved in promoting mobilization of the hinge by an alternative mechanism, perhaps by polymerization or glycosylation of specific chitin glycoproteins of the hinge cuticle. It was demonstrated that there are changes in glycoprotein composition in the cuticle by glycosylation during the moulting cycle (Shafer et al., 1994), and that glycoproteins could act as contact pheromones as well (Caskey et al., 2009). Besides, characterization of protein features of the abdominal arthrodial membrane (intersegmental membrane) in the lobster *Homarus americanus* H. Milne Edwards, 1837 in comparison to proteins of the skeleton mineralized cuticle demonstrated that different proteins are found in each of them (Andersen, 1998). If such a new ovarian hormone is discovered "hardening" and "calcification" would no longer be synonymous.

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REFERENCES

Andersen, S. O. 1998. Characterization of proteins fromrthroial membranes of the lobster, *Homarus americanus*. Comparative Biochemistry and Physiology, Part A Molecular and Integrative Physiology 121: 375-383.

Brockerhoff, A. M., and C. L. McLay. 2005a. Comparative analysis of the mating strategies in grapsid crabs with special reference to two common intertidal crabs, *Cyclograpsus lavauxi* and *Helice crassa* from New Zealand. Journal of Crustacean Biology 25: 507-520.

———, and ———. 2005b. Mating behaviour, female receptivity and male-male competition in the intertidal crab *Hemigrapsus sexdentatus* (Brachyura: Grapsidae). Marine Ecology Progress Series 290: 179-191.

———, and ———. 2005c. Factors influencing the onset and duration of receptivity of female purple rock crabs, *Hemigrapsus sexdentatus* (H. Milne Edwards, 1837) (Brachyura: Grapsidae). Journal of Experimental Marine Biology and Ecology 314: 123-135.

Caskey, J. L., K. H. Hasenstein, and R. T. Bauer. 2009. Studies on contact sex pheromones of the caridean shrimp *Palaemonetes pugio*: I. Cuticular hydrocarbons associated with mate recognition. Invertebrate Reproduction and Development 53: 93-103.

Chen, P. Y., A. Y. M. Lin, J. McKittrick, and M. A. Meyers. 2008. Structure and mechanical properties of crab exoskeletons. Acta Biomaterialia 4: 587-596.

Cribb, B. W., C. L. Lin, L. Rintoul, R. Rasch, J. Hasenpusch, and H. Huang. 2010. Hardness in arthropod exoskeletons in the absence of transition metals. Acta Biomaterialia 6: 3152-3156.

Dana, J. D. 1851. Crustacea Grapsoidea (Cyclometopa, Edwardsii): Conspectus Crustaceorum quae in Orbis Terrarum Circumnavigatione, Carolo Wilkes e Classe Reipublicae Foederatae Duce. Proceedings of the Academy of Natural Sciences of Philadelphia 5: 247-254.

Davie, P. J. F. 2012. A review of *Macrophthalmus* sensu lato (Crustacea: Decapoda: Macrophthalmidae) from Australia, including two new species and new records. Memoirs of the Queensland Museum – Nature 56: 149-219.

Dillaman, R., S. Hequembourg, and M. Gay. 2005. Early pattern of calcification in the dorsal carapace of the blue crab, *Callinectes sapidus*. Journal of Morphology 263: 356-374.

Fabricius, J. C. 1793. Entomologia Systematica Emendata et Aucta. Secundum Classes, Ordines, Genera, Species, Adjectis Synonimis, Locis, Observationibus, Descriptionibus. Vol. 2. Proft and Storch, Hafniae [= Copenhagen].

Forskål, P. 1775. Descriptiones Animalium, Avium, Amphibiorum, Piscium, Insectorum, Vermium; quae in Itinere Orientali Observavit Petrus Forskål. Post Mortem Auctoris editit Carsten Niebuhr. Adjuncta est materia Medica Kahirina. Mölleri, Hafniae [= Copenhagen].

Guinot, D. 1977. Propositions pour une nouvelle classification des Crustacés Décapodes Brachyoures. Comptes Rendus hebdomadaires des séances de l'Academie des Sciences (Paris) (Séries D) 285: 1049-1052.

———, M. Tavares, and P. Castro. 2013. Significance of the sexual openings and supplementary structures on the phylogeny of brachyuran crabs (Crustacea, Decapoda, Brachyura), with new nomina for higher-ranked podotreme taxa. Zootaxa 3665: 1-414.

Hardedge, J. D., A. A. Jennings, D. Hayden, C. T. Muller, D. Pascoe, M. G. Bentley, and A. S. Clare. 2002. Novel behavioural assay and partial purification of a female-derived sex pheromone in *Carcinus maenas*. Marine Ecology Progress Series 244: 179-189.

Hartnoll, R. G. 1965. Notes on the marine grapsid crabs of Jamaica. Proceedings of the Linnean Society of London 176: 113-147.

———. 1968. Morphology of the genital ducts in female crabs. Journal of the Linnean Society of Zoology (London) 47: 279-300.

———. 1969. Mating in the Brachyura. Crustaceana 16: 161-181.

———. 1982. Growth, pp. 11-196. In, D. E. Bliss and L. G. Abele (eds.), The Biology of Crustacea. Vol. 2. Academic Press, New York, NY.

———. 2006. Reproductive investment in Brachyura. Hydrobiologia 557: 31-40.

Heller, C. 1865. Crustaceen. Reise der Österreichischen Fregatte Novarra um die Erdre, in den Jahren 1857, 1858, 1859 unter den Befehlen des Commodore B. von Wüllerstorf-Urbair. Zoologischer Theil 2(3): 1-280.

Henmi, Y., and M. Murai. 1999. Decalcification of vulvar operculum and mating in the ocypodid crab *Ilyoplax pusilla*. Journal of Zoology 247: 133-137.

Hines, A. H. 1991. Fecundity and reproductive output in nine species of *Cancer* crabs (Crustacea, Brachyura, Cancridae). Canadian Journal of Fisheries and Aquatic Science 48: 267-275.

Kitaura, J., K. Wada, Y. Fukui, and C. L. McLay. 2010. Molecular phylogenetic position of the New Zealand sentinel crab, *Macrophthalmus* (*Hemiplax*) *hirtipes* (Jacquinot, in Hombron & Jacquinot, 1846) (Decapoda, Brachyura, Macrophthalmidae). Crustaceana 83: 1315-1326.

Komai, T., S. Goshima, and M. Murai. 1995. Crabs of the genus *Macrophthalmus* of Phuket, Thailand (Crustacea: Decapoda: Ocypodidae). Bulletin of Marine Science 56: 103-149.

Latreille, P. A. 1812. Crustacés et insects, pp. 270-280. In, J. Milbert, Voyage pittoresque à l'Île-de-France, au cap de Bonne-Espérance et à l'Ile de Ténériffe. Peintre embarqué sur la corvette le Géographe, et Directeur des gravures de la partie historique du Voyage aux Terres-Australes. Avec un Atlas, composé de trois cartes géographiques, et de quarante-cinq vues pittoresques déssineéessur les lieux, et gravées en partie par l'auteur. Vol. 2. A. Nepveu, Paris.

Linnaeus, C. 1758. Systema Naturae per Regna Tria Naturae, Secundum Classes, Ordines, Genera, Species, cum Characteribus, Differentiis, Synonymis, Locis. Vol. 1. 10th Edition. Laurentii Salvii, Holmiae [= Stockholm].

McLay, C. L., and C. Becker. 2015. Reproduction in Brachyura, pp. 185-243. In, P. Castro, P. J. F. Davie, D. Guinot, F. R. Schram, and J. C. von Vaupel Klein (eds.), Decapoda Brachyura, Treatise on Zoology – Anatomy, Taxonomy, Biology. Vol. 9C, Part 1. Brill, Leiden.

———, J. Kitaura, and K. Wada. 2010. Behavioural evidence for the systematic position of *Macrophthalmus* (*Hemiplax*) *hirtipes* Hombron and Jacquinot, 1846, with comments on macrophthalmine sub-genera. Crustaceana Monographs 14: 483-503.

———, and L. López Greco. 2011. A hypothesis about the origin of sperm storage in the Eubrachyura, the effects of seminal receptacle structure on mating strategies and the evolution of crab diversity: how did a race to be first become a race to be last? Zoologischer Anzeiger 250: 378-406.

Milne Edwards, H. 1837. Histoire naturelle des Crustacés, comprenant l'anatomie, la physiologie et la classification de ces animaux. Vol. 2. Librairie Encyclopédique de Roret, Paris.

———. 1853. Mémoire sur la famille des Ocypodides. Suite (1). Deuxiéme Tribu Principale. Annales des Sciences Naturelles (Séries 3, Zoologie) 20: 163-228.

Naderloo, R. 2011. Grapsoid crabs (Decapoda: Brachyura: Thoracotremata) of the Persian Gulf and the Gulf of Oman. Zootaxa 3048: 1-43.

———, M. Turkay, and M. Apel. 2011. Brachyuran crabs of the family Macrophthalmidae Dana, 1851 (Decapoda: Brachyura: Macrophthalmidae) of the Persian Gulf. Zootaxa 2911: 1-42.

Owen, R. 1839. Crustacea, pp. i-xii, 77-92. In, The Zoology of Captain Beechey's Voyage; Compiled From the Collections and Notes Made by Captain Beechey, the Officers and Naturalist of the Expedition, During a Voyage to the Pacific and Behring's Straits Performed in His Majesty's Ship Blossom, Under the Command of Captain F. W. Beechey, R.N., F.R.S. &c. &c. in the Years 1825, 26, 27, and 28. Henry G. Bohn, London.

Roer, R. 1980. Mechanisms of resorption and deposition of calcium in the carapace of the crab *Carcinus maenas*. Journal of Experimental Biology 88: 205-218.

———, and R. Dillaman. 1984. The structure and calcification of the crustacean cuticle. American Zoologist 24: 893-909.

Sainte-Marie, B., T. Gosselin, J. M. Sevigny, and M. Urbani. 2008. The snow crab mating system: opportunity for natural and unnatural selection. Bulletin of Marine Science 83: 131-161.

Sakai, K., M. Türkay, and S. L. Yang. 2006. Revision of the *Helice/Chasmagnathus complex* (Crustacea: Decapoda: Brachyura). Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft 565: 1-76.

Shafer, T. H., R. D. Roer, C. G. Miller, and R. M. Dillaman. 1994. Postecdysial changes in the protein and glycoprotein composition of the cuticle of the blue crab *Callinectes sapidus*. Journal of Crustacean Biology 14: 210-219.

Teng, S.-J., and H.-T. Shih. 2015. A new species of *Macrophthalmus* Desmarest, 1823 (Crustacea: Decapoda: Brachyura: Macrophthalmidae) from Taiwan, with notes on four new records. Zootaxa 4058: 451-470.

Thompson, G., and C. L. McLay. 2005. Mating behavior of *Heterozius rotundifrons* (Crustacea: Brachyura: Belliidae): is it a hard or soft shell mater? Marine Freshwater Research 56: 1107-1116.

Turner, H. V., D. L. Wolcott, T. G. Wolcott, and A. H. Hines. 2003. Post-mating behavior, intramolt growth, and onset of migration to Chesapeake Bay spawning grounds by adult female blue crabs, *Callinectes sapidus* Rathbun. Journal of Experimental Marine Biology and Ecology 295: 107-130.

van den Brink, A. M., and C. L. McLay. 2009. Use of the sterile male technique to investigate sperm competition, storage and use in a pill box crab, *Halicarcinus cookii* (Brachyura: Hymenosomatidae). Journal of Crustacean Biology 29: 62-69.

Yamaguchi, T. 2001. The mating system of the fiddler crab, *Uca lactea* (Decapoda, Brachyura, Ocypodidae). Crustaceana 74: 389-399.

Zar, J. H. 1999. Biostatistical Analysis. Prentice-Hall, NJ, USA.

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