The importance of dietary calcium consumption in two species of semi-terrestrial grapsoid crabs

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ABSTRACT. Calcium (Ca) is essential for crustaceans, due to calcium carbonate ($CaCO_3$) deposition in the new exoskeleton to harden it. The purpose of this work was to study short term Ca balance in terms of dietary Ca ingestion in two phylogenetically related crabs (Superfamily Grapsoidea) showing different degrees of terrestrial adaptations: *Sesarma rectum* Randall, 1840 and *Neohelice granulata* (Dana, 1851). Dietary Ca ingestion was studied using purified diets with different Ca concentrations (0, 2.2 and 6.66 % Ca), together with measurements of Ca excretion and Ca hemolymph levels. The results showed that both crabs had the same response to foods containing different levels of Ca, with both species eating more of the high Ca diet. However, *S. rectum* consumed more per mg body mass at all Ca concentrations (6 mg.g^{-1} for *S. rectum* against 3 mg.g^{-1} for *N. granulata*). Both species excreted/egested Ca differently: *S. rectum* excreted Ca proportionally to ingestion, whereas *N. granulata* maintained constant faecal Ca output at all dietary Ca levels. Moreover, Ca hemolymph levels for crabs fed the different diets were independent of dietary Ca. In conclusion, both *S. rectum* and *N. granulata* seem to regulate the consumption of diets containing more Ca, which suggests a fine balance for Ca intake.

KEYWORDS. Diet, semi-terrestrial crabs, feeding behavior, *Sesarma rectum*, *Neohelice granulata*.

RESUMO. A importância do consumo de cálcio na dieta de duas espécies de caranguejos grapsóides semi-terrestres. O cálcio (Ca) é essencial para os crustáceos porque cristais de carbonato de cálcio ($CaCO_3$) são depositados no novo exoesqueleto para endurecê-lo. O objetivo do presente trabalho foi estudar o balanço do Ca em relação à sua ingestão em dois caranguejos filogenéticamente relacionados (Superfamília Grapsoidea), que apresentam diferentes graus de terrestrialidade: *Sesarma rectum* Randall, 1840 e *Neohelice granulata* (Dana, 1851). A ingestão de Ca foi estudada através do uso de dietas purificadas com diferentes quantidades de Ca (0, 2,2 e 6,66 % Ca), juntamente com a excreção de Ca nas fezes e níveis de Ca na hemolinfa. Os resultados mostraram que ambos apresentam a mesma resposta em relação aos níveis de Ca na dieta, ingerindo mais da dieta com maior quantidade de Ca. *Sesarma rectum*, porem, consumiu mais dieta por mg de peso para todas as concentrações de Ca utilizadas (6 mg.g^{-1} para *S. rectum* contra 3 mg.g^{-1} para *N. granulata*). As duas espécies excretaram Ca de maneira diferente: *S. rectum* excretou em proporção direta à quantidade ingerida, enquanto *N. granulata* manteve constante a excreção de Ca independente do Ca ingerido. Níveis de Ca na hemolinfa, por outro lado, foram iguais e independentes da quantidade de Ca ingerido para os dois caranguejos. Desse modo, tanto *S. rectum* como *N. granulata* parecem discriminar e ingerir dietas com maior quantidade de Ca, sugerindo uma regulação fina na ingestão do Ca.

PALAVRAS-CHAVE. Dieta, caranguejo semi-terrestre, comportamento alimentar, *Sesarma rectum*, *Neohelice granulata*.

Calcium (Ca) is essential for crustaceans, because calcium carbonate crystals (CaCO3) are deposited in the exoskeleton to harden it after the old exoskeleton has been shed. Critical periods for the intake of this mineral occur during molting-related events, when crustaceans shed their old exoskeleton and calcify the new one during the post-molt period. Whole body Ca uptake in these animals occurs mainly through the gills for aquatic species and transitions from intermolt (zero net flux) to premolt (net efflux) and postmolt (net influx at the rate of 2 mmol/kg/h; Zanotto & Wheatly, 2002). As such, crabs appear to have evolved mechanisms for calcium regulation through the gills and it is expected that regulation through dietary intake would also be important for the animals in terms of mineral regulation. Dietary intake of calcium, in particular, should be especially important for semi- and terrestrial crabs, the focus of this work, and it is already known that terrestrial crabs display different strategies to conserve Ca such as storage in the hemolymph and in the digestive system during premolt (Greenaway, 1988).

Terrestrial crabs and hermit crabs show a strong capacity to control salt and water balance, through control of drinking and excretion of salts (Wolcott & Wolcott, 1988; Taylor et al., 1993; Greenaway, 1994). They are able to reabsorb ions from the urine through drinking after it has been voided and through recycling in the branchial chamber (Wolcott & Wolcott, 1988; Greenaway & Nakamura, 1991; Wolcott & Wolcott, 1991). Calcium, for example, is regulated in the final excretory fluid after post-renal modification of the urine, so that it is found in higher or lower concentration in this fluid, depending whether the crab is drinking diluted or concentrated seawater (Taylor et al., 1993). They also show behavioral osmoregulation, choosing between drinking water sources of appropriate salinity and adjusting the frequency and duration of drinking (Gross, 1955). Therefore, salt control through behavioral and physiological regulation are known in crabs and Ca, in particular, is an important ion for studies on physiological regulation of ingestion and excretion.

In spite of the great importance of nutrition, complete nutritional requirements have only been identified for a remarkably small number of living organisms. These include a variety of plants, some

bacteria, fungi, the laboratory mouse and rat, domestic animals and some few insects of economic importance (see review by Louw, 1993). This occurs because the analysis of individual nutritional elements is an extremely tedious and labor intensive work and involves a deep understanding of the organism's general biology, together with optimum laboratory holding conditions for the organism under study. Moreover, specific work in the literature on crustacean nutrition using purified diets has been focused mainly on shrimps, due to their commercial economic importance as human food (Chen *et al.*, 1991; Shiau & Liu, 1994; Chen & Chang, 1994; Shiau & Chin, 1998; Shiau & Hsu, 1999). Work on nutritional requirements of brachyuran crabs have been neglected (Zanotto, 2000). Based on that, minimal knowledge of nutritional aspects of brachyuran crabs have been developed through the use of purified diets. Moreover, focus on nutritional requirements of omnivores, such as the crabs to be studied here, have the potential to form conceptual links between dietary choice by herbivores, frugivores, detritivores and predators together (Buck *et al.*, 2003).

Therefore, the purpose of this work was to study short term calcium balance in two phylogenetically related crabs (*Sesarma rectum* Randall, 1840 and *Neohelice granulata* (Dana, 1851), Superfamily Grapsoidea) showing different degrees of terrestrial adaptations, according to the classification of crab terrestriality found in Hartnoll (1988). The hypothesis for the present work is that the more terrestrial crab species, *S. rectum*, will regulate calcium intake and excretion more tightly than the less terrestrial species, *N. granulata*, since the former will have a greater likelihood of finding itself without an opportunity for Ca uptake via the gills from the surrounding water. The diets offered to the animals were formulated according to a literature review on insect diets (Dadd, 1985) and a few references on crab diets (Sheen *et al.*, 1994; Ponat & Adelung, 1983; Sheen & Wu, 2002; Zanotto, 2000). The diets tested were tightly controlled for dietary calcium content.

MATERIAL AND METHODS

Sesarma rectum was collected from the Itaguaré River estuary near Bertioga (23° 47.716' S, 46° 03.327' W), in São Paulo State, Brazil. *Neohelice granulata* specimens were obtained from Laguna dos Patos, State of Rio Grande do Sul, Brazil. In the laboratory, the crabs were held in tanks containing aerated seawater at 16 ‰ (*S. rectum*) where they had free access to a dry surface. The animals were fed three times a week with mangrove leaves (*S. rectum*) and minced meat (*N. granulata*). The temperature was maintained at 22° C and the photoperiod at 13:11 L/D. *Neohelice granulata* was also maintained at 16 ‰.

The diets offered to the crabs were composed as detailed in table I. Usually, the amount of Ca in the diet of *S. rectum* is around 0.4-1.0 %, amount encountered in *Avicennia* sp. (Verbenaceae) leaves. For *N. granulata*, these animals were fed minced meat before experiments started, which contains Ca at around 0.02 %. The choice of dietary Ca used here was also based on Greenaway & Linton (1995), studying a terrestrial crab that consumes

leaves containing 4 % of Ca. Therefore, we used diets varying from 0 to 6.6 % of Ca.

All animals used were in the intermolt period. The animals were held without food for 48 h prior to experimentation. Then, the diets were offered to the animals for the next 4 days, every other day, before experiments started, to allow the animals to get used to the new purified diet (pre-treatment). After that, the animals were weighed. The experimental boxes used for feeding were inclined to separate a dry part, for the diets, and a wet part, where the animals could submerge. The dry part was separated from the wet part using a plastic square. The wet part of the box contained artificial seawater (Pantin, 1948; Tab. II). Therefore, the diets were offered for 2 h in the dry part of the experimental box individually (considered day 1). To facilitate consumption of the dry diets, these were moistened with 0.5 ml of distilled water. Next, the diets were offered for the next 15 days, on days 1, 3, 5, 7, 9 and 11, for 1 h again, and removed after that. Faeces, however, were collected every day and frozen for later measurement of total Ca. Hemolymph samples were collected on the last day of the experiment, for total hemolymph Ca measurements.

The uneaten diets were dried for 48 h at 35° C and weighed to calculate amounts eaten. Faeces were oven-dried at 70° C for 72 h, to get dry weight and ashed at 500° C for 2 h, to measure total Ca in a flame photometer. Haemolymph samples were also measured for total Ca.

The data was checked for normality and homogeneity of variance using a SPSS software. Repeated measures ANOVA was used to see the effects of time in the amount of diet consumed and to compare both species and different feeding stimulants added to the diets. Hemolymph Ca and faecal Ca excretion was compared between species and diets using two-way ANOVA.

RESULTS

Sesarma rectum fed diets containing betaine as a feeding stimulant, consumed more of the diet containing Ca at 6.66 % compared to the other diets (Fig. 1; repeated measures ANOVA, P < 0.01). The overall consumption was around 6 mg.g^{-1} for the latter diet and around
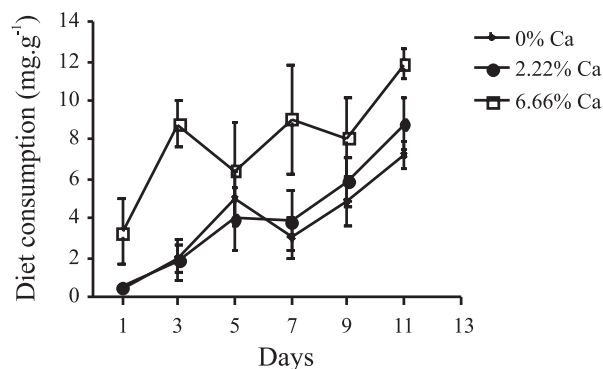

Figure 1. Mean diet consumption (mg.g^{-1}) for *Sesarma rectum* Randall, 1840 for every 2 days (total of 11 days), fed purified diets which contained 3 different Ca concentrations (0 %, 2.22 % and 6.66 %). There was a higher consumption for diets containing 6.66 % of Ca compared to the other diets (repeated measures ANOVA, P < 0.01; N= 10).

3-4 mg.g^{-1} for the other diets containing Ca at 0 % and 2.22 %. There was also an effect of day (repeated measures ANOVA, P < 0.01) showing that the overall consumption of diet varied over 11 days for all diets.

In the case of *N. granulata* fed diets with betaine at different Ca concentrations, the same pattern of consumption was found compared to *S. rectum* (Fig. 1, 2). *Neohelice granulata* ate more of the diet containing Ca at 6.66 % Ca, at around 3-6 mg.g^{-1} (repeated measures ANOVA, P < 0.01). There was also a strong effect of days, the consumption over the days showing a large variation for all diets (repeated measures ANOVA, P < 0.001). Again, the consumption for diets containing Ca at 0 and 2.22 % was lower and around 2-2.5 mg.g^{-1} (Fig. 2). Total Ca consumption was higher for diets containing 6.66 % Ca for both crabs, values around 4x higher compared to *S. rectum* fed diets containing 2.22 % of Ca (Tab. III) and 2.2x higher Ca ingestion was seen for *N. granulata* compared to the same animals fed 2.22 % of Ca.

Comparing the two crabs fed diets with Ca at 2.22% and added betaine or cadaverine as feeding stimulants, there were differences in consumption between both feeding stimulants (Fig. 3). *Sesarma rectum*, however, ate more diet than *N. granulata*, at around 6-8 mg.g^{-1}
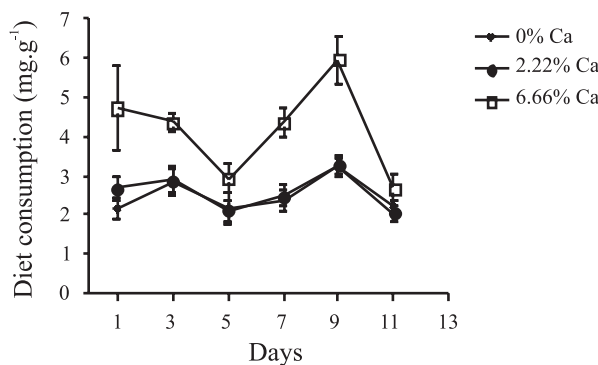
compared to 1-2 mg.g^{-1} for *N. granulata* (repeated measures ANOVA, P < 0.01). There was no difference in consumption for *N. granulata* in relation to the type of diet consumed, whether the diet had betaine or cadaverine (repeated measures ANOVA, P > 0.05; Fig. 3), differently to what was seen for *S. rectum*, consuming more of the diet that contained betaine (repeated measures ANOVA, P < 0.05; Fig. 3).

In relation to the egestion/excretion of Ca in the faeces (Fig. 4), very different regulatory mechanisms were seen between the two species. *Sesarma rectum* excreted Ca proportional to the intake of Ca in the diets, 1.5x more for diet 2.22 % Ca compared to 0 % Ca and 1.5x more for diet 6.66 % Ca compared to 2.22 % Ca (one-way ANOVA, P < 0.05). For *N. granulata*, the egestion/excretion of Ca was very similar on all diets, although the consumption of Ca between diets was different (Tab. III).

Total amounts of Ca in the hemolymph of the animals at the last day of the experiment are shown in figure 5. Although they were fed diets with different levels of Ca, hemolymph Ca was kept constant for each crab (one-way ANOVA, P > 0.05 for both crabs), showing that there was no effect of diet in relation to Ca levels in the hemolymph.

Figure 2. Mean diet consumption (mg.g^{-1}) for *Neohelice granulata* (Dana, 1851) for every 2 days (total of 11 days), fed purified diets which contained 3 different Ca concentrations (0 %, 2.22 % and 6.66 %). There was a higher consumption for diets containing 6.66 % of Ca compared to the other diets (repeated measures ANOVA, P < 0.01; N= 5 for diet with 0% Ca; N= 6 for the other diets).
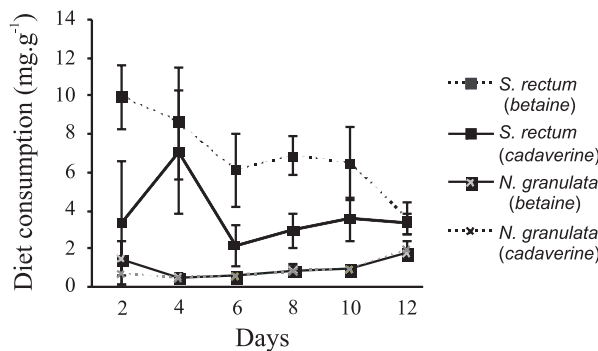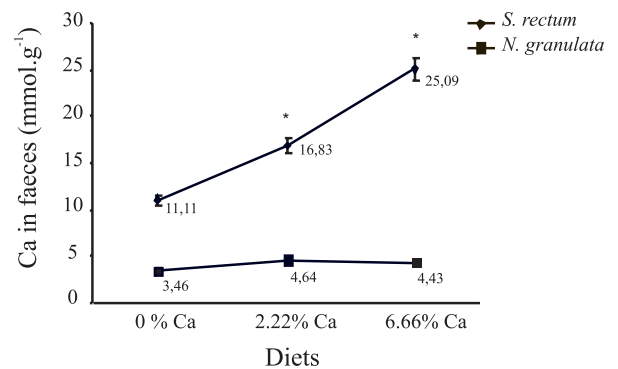

Figure 4. Mean Ca concentration in faeces of *Sesarma rectum* Randall, 1840 and *Neohelice granulata* (Dana, 1851) (mmol. g^{-1}) for 3 different diets (0 % Ca, 2.22 % Ca and 6.66 % Ca; N = 4).

Figure 3. Total diet consumption (mg.g^{-1}), with added betaine or cadaverine, for *Neohelice granulata* (Dana, 1851) and *Sesarma rectum* Randall, 1840 fed diet containing 2.22 % Ca. There was a significant higher consumption of diet by *S. rectum* compared to *N. granulata* for both feeding stimulants (repeated measures ANOVA, P < 0.01).
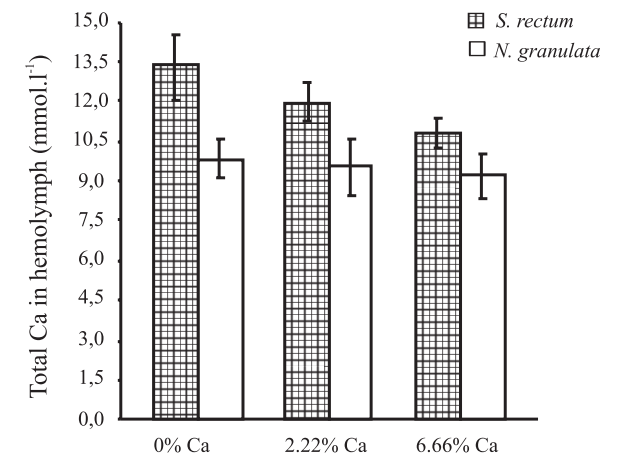

Figure 5. Mean hemolymph Ca concentration (mmol.l-1) in *Sesarma rectum* Randall, 1840 and *Neohelice granulata* (Dana, 1851) fed purified diets which contained 3 different Ca concentrations (0 %, 2.22 % and 6.66 %). There was no difference in total hemolymph Ca for both crabs (one-way ANOVA, P > 0.05; N=10).

Table I. Diet composition offered to the crabs *Sesarma rectum* Randall, 1840 and *Neohelice granulata* (Dana, 1851) for 15 days. Diet A, B and C differed only in relation to Ca content (*calcium free salt mixture (ICN): KPO_4: 52.81 %; $NaPO_4$: 10.31 %; $MgSO_4.7H_2O$: 8.19 %; NaCl: 23.13 %; iron citrate: 4.5 %; KI: 0.13 %; $MgSO_4.1H_2O$: 0.74 %; ZnCl: 0.08 %; $CuSO_4.5H_2O$: 0.05 %; sodium selenite: 0.001 %; chromium potassium sulfate: 0.06 %; **AIN Vitamin Mixture 76 (ICN) – kg of mixture: thiamine hydrochloride: 600 mg; riboflavin: 600 mg; pyridoxine hydochloride: 700 mg; nicotinic acid: 3 g; D-calcium pantothenate: 1.6 g; folic acid: 200 mg; D-biotin: 20 mg; vitamin B12: 1 mg; vitamin A: 1.6 g (250.000 UI/g); DL-tocopherol acetate: 20 g (250 UI/g); vitamin D3: 250 mg (400.000 UI/g); vitamin K2: 5 mg; sucrose 972.9 g).

Ingredients	Diet A (0 % Ca)		Diet B (2.22 % Ca)		Diet C (6.66 % Ca)	
Casein	20.5	g	20.5	g	20.5	g
Peptone	5.0	g	5.0	g	5.0	g
Sucrose	5.0	g	5.0	g	5.0	g
Starch	11.0	g	11.0	g	11.0	g
Salt mixture*	3.0	g	3.0	g	3.0	g
Vitamins**	0.1	g	0.1	g	0.1	g
Vitamin C	0.2	g	0.2	g	0.2	g
Betain or cadaverine	0.8	g	0.8	g	0.8	g
Corn oil	1.9	ml	1.9	ml	1.9	ml
Cod oil	1.9	ml	1.9	ml	1.9	ml
Cholesterol	0.5	g	0.5	g	0.5	g
$CaCl_2$	0.0	g	1.11	g	3.33	g
Total	49.9	g	51.01	g	54.34	g

Table II. Composition of artificial brackish water at 16 ‰ (adapted from PANTIN, 1948).

Salt	g/L
NaBr (anhydrous)	0.03
$NaHCO_3$	0.1
KCl	0.34
$CaCl_2$ (anhydrous)	1.12
Na_2SO_4 (anhydrous)	1.8
$MgCl_2 . 6 H_2O$	4.84
NaCl	10.7

Table III. Total Ca consumption (mg) over 13 days for *Sesarma rectum* Randall, 1840 and *Neohelice granulata* (Dana, 1851) fed 3 different diets containing betaine (mean ± SE; N= 10 *for S. rectum* and N=6 for *N. granulata*). Asterisks denote significant differences.

	Ca consumption (mg)	
Diet Ca	*Sesarma rectum*	*Neohelice granulata*
0 %	0	0
2.22 %	11.39 ± 2.15	12.78 ± 6.28
6.66 %	48.29 ± 5.52*	28.29 ± 1.19*

DISCUSSION

We found here that two phylogenetically related crabs with different feeding habits and different degrees of terrestriality show different patterns of regulation of ingested Ca. The results showed that both crabs had the same response to foods containing different levels of Ca, with both species eating more of the high Ca diet. However, *S. rectum* consumed more per body mass at all Ca concentrations and show a higher degree of terrestriality compared to *N. granulata*. Moreover, the

species excreted/egested Ca differently: *S. rectum* excreted Ca proportionally to ingestion, whereas *N. granulata* maintained constant faecal Ca output. *Sesarma rectum* usually feeds on a mixed diet but the diet is composed mainly of mangrove leaves (around 75 %, see STEINKE *et al.*, 1993) and *N. granulata* feeds on a diet composed of fewer plant matter, showing an opportunist behavior in relation to foods encountered (KUCHARSKI & SILVA, 1991). This study also demonstrated for the first time the effects of known feeding stimulants, such as betaine or cadaverine (see COMAN *et al.*, 1996) in the intake patterns of crabs, an understudied group of crustaceans in relation to non-natural food sources.

In general, animals have evolved mechanisms to balance the intake of nutrients they get from the food. These mechanisms involve nutrient balancing through behavioral means, such as food selection and regulation of amounts eaten (see WALDBAUER & FRIEDMAN, 1991; SIMPSON & SIMPSON, 1990). Other means of balancing nutrient intake involves physiological regulation, in contrast to behavioral regulation, and is achieved through change in the amount of digestive enzyme produced (SABAT & NOVOA, 1998), absorptive regulation through differential absorption in the digestive system (TITUS *et al.*, 1991; DIAMOND, 1991; CAVIEDES-VIDAL & KARASOV, 1996) and/or *post*-absorptive regulation, achieved mainly through differential excretion (ZANOTTO *et al.*, 1993, 1994).

Physiological regulation of food intake has been shown earlier in herbivorous land crabs, using natural food sources. This has involved changing food residence time in the gut and increasing assimilation efficiencies of minerals, nitrogen and dry matter content present in natural foods such as leaves (GREENAWAY & LINTON, 1995). Calcium assimilation from the leaves by *Gecarcoidea natalis* (Pocock, 1888) was 34-40 % of the Ca present in the diet, indicating that much of the Ca was lost in the faeces. In our work we saw different patterns of Ca egestion in two different crabs, one retaining Ca differently when subjected to diets with different levels of Ca (*S. rectum*), and the other excreting Ca at quantities which were independent of the levels in the diet (*N. granulata*). The excretion/egestion was independent of Ca levels in the hemolymph for both crabs, levels being maintained the same independent of dietary Ca consumed. Here, the assimilation of Ca from purified diets was around 47 % for *S. rectum* fed diet containing 2.22 % Ca and 78 % for crabs fed diet with 6.66 % Ca, showing an increase in Ca assimilation with increasing dietary levels. For isopods, for example, Ca assimilation is around 79-84 % of total Ca eaten, although these crustaceans have more terrestrial features than the crabs studied here (RADU *et al.*, 1971).

Earlier work has shown that terrestrial crabs also display behavioral regulation during food intake (GREENAWAY, 1993; GREENAWAY & RAGHAVEN, 1998). Two species of herbivorous land crabs, *G. natalis* and *Discoplax hirtipes* (Dana, 1852) displayed different feeding strategies when offered leaves found in their natural environment (GREENAWAY & RAGHAVEN, 1998) and as a result *G. natalis* had a higher intake of Ca in the field when compared to *D. hirtipes,* through selection of yellow leaves that already contained more Ca. Interestingly,

another study with a salt marsh crab *Armases cinereum* (Bosc, 1802), found that salt added as NaCl to the artificial diets stimulated feeding by *A. cinereum*, in concentrations even higher than found in their natural environment (PENNINGS & CAREFOOT, 1998). Overall, it seems that salts in general, as well as Ca, are upregulated for crabs living in salt marsh environments, where Ca can be limiting. This has been suggested before for terrestrial crabs that live in areas far from the sea and where the water available is of reduced salinity (WOLCOTT & WOLCOTT, 1984, 1988, 1991).

Crustaceans are known to use the antennules as distant chemoreceptors (HAZLETT, 1971; STEULLET *et al.*, 2002). Dactyl chemoreception is also involved in food detection (HAMILTON & CASE, 1983) and both seem to act together to increase food detection (HAZLETT, 1971). Land hermit crabs, with terrestrial features, are scavengers that also use olfaction to locate their food. They frequently chose foods that they had not experienced during the previous 24 h. As a consequence of this behavior, land hermit crabs consume a broader diet, which may result in obtaining a more nutritionally balanced diet (THACKER, 1996). Presently, our crabs consumed more of the high Ca diet but were not given a choice of diet. Interestingly, it has been found that hermit crab is able to behaviorally choose shells in the environment (MESCE, 1982) and detect calcium through chemoreceptors in the dactyls which are sensitive to calcium (MESCE, 1993). This suggested "ion sensitivity" has also been noted in terrestrial isopod antennae which showed sensitivity to calcium solutions in the range of 10-100mM (cited in MESCE, 1993).

In rats, diets containing elevated concentration of Ca are regulated so that the animals absorb less Ca compared to a low Ca diet (FERRARIS & DIAMOND, 1989; BRONNER, 1996). However, in flies, there is no absorptive regulation of dietary Ca when they are fed high Ca diets, presenting a regulatory system different to that seen in rats (TAYLOR, 1985). In the results presented here, Ca was in one case excreted/egested according to dietary levels and in another case was retained despite of a higher intake on high Ca diets. This retention could have happened through re-absorption in the hindgut, before excretion through the faeces. The amount of Ca in the diet of *S. rectum* is around 0.4-1.0g % in *Avicennia* leaves found in their natural habitat. *Neohelice granulata* are omnivores and were fed minced meat before experiments started, which contains Ca at around 0.02g % Ca. Probably the levels of Ca encountered by these animals in the field could also reflect the differences in Ca retention between both crabs.

The hypothesis of increased terrestriality, using two species of crabs related phylogenetically, and an increase in Ca ingestion in the more terrestrial crab, *S. rectum*, was observed here. Similar response was seen for dietary copper, a heavy metal (SÁ *et al.*, 2008). Interestingly, further exploring the subject of Ca intake in semi-terrestrial crabs should help discriminate strategies for Ca intake in a group of animals that represent a paradigm for the transition from aquatic to terrestrial habitats, representing the earlier stages of evolution to terrestriality (BURGGREN & MCMAHON, 1988) and also help to elucidate evolutionary mechanisms between levels of terrestriality and strategies to regulate dietary Ca levels. Further work should involve the use of postmolt crabs to see how dietary Ca intake is regulated during this critical period of high Ca intake for the purpose of hardening the new exoskeleton.

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