The diet of the Harlequin crab *Lissocarcinus orbicularis*, an obligate symbiont of sea cucumbers (holothuroids) belonging to the genera *Thelenota*, *Bohadschia* and *Holothuria*

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Abstract The present paper characterizes, for the first time, the diet of the Harlequin crab *Lissocarcinus orbicularis*, an obligate symbiotic crab that associates with sea cucumbers (holothuroids) belonging to the genera *Thelenota*, *Bohadschia* and *Holothuria*. *These* tropical holothuroids host a rich symbiotic community in the Indo-West Pacific Ocean of which the Harlequin crab is the best known. The diet of *L. orbicularis* was characterized by analyzing the microscopic, molecular and isotopic signatures obtained from its gastric content. The presence of sea cucumber ossicles in the gastric mills of the crabs suggests that symbionts eat the superficial integument of their host and this was supported by the fact that Holothuroid DNA was detected in the stomach of *L. orbicularis* after DGGE and sequencing of the 18S rDNA gene. The stable isotopic δ^{13}C and δ^{15}N values of crab tissues were compared with diverse potential food sources including three holothuroids, three algae, one sea grass as well as the organic matter contained in the water column, in the sediment, and the second most abundant symbiont, the polychaete *Gastrolepidia clavigera*. The low δ^{15}N values of crabs suggests that the crabs do not exclusively feed on sea cucumber tissue but assimilate diverse food sources such as sea grasses and organic matter contained in sediment that have similar δ^{13}C values. There were no differences between the feeding of males and females but there was a positive correlation between the carapace length and the stable isotopic values indicating a shift of the food source as crabs grow larger.

Keywords Symbiotic crab · Crab diet · Holothuroid · Harlequin crab · Stable isotopes

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1 Introduction

Organisms belonging to different species which are closely and durably associated together form what is termed a symbiotic association that involves a host and one or several symbionts. Depending on the relative duration of the interaction and the impact on host fitness, the following categories of symbiotic association have been distinguished: parasitism, commensalism and mutualism (Parmentier and Michel 2013). With respect to sea cucumbers (holothuroids or holothurians), many symbionts have been discovered that live either as commensals or parasites (see Jangoux (1984) and Eeckhaut et al. (2004) for reviews). To date, most of the studies about holothuroid symbionts have focused on taxonomic descriptions but only a very few have concerned their feeding preferences. Five feeding guilds in holothuroid symbionts have been discovered as a result of past researches: (*i*) mucus feeding, (*ii*) suspension feeding, (*iii*) predation on other symbionts, (*iv*) parasitism on host tissues and (*v*) outside-feeding with symbionts that eat external food sources. Britaev and Lyskin (2002) demonstrated, by dissecting the gut of the polychaetes *Gastrolepidia clavigera*, that they feed on holothuroid tissues. Parmentier and Das (2004) used isotopic analyses on the obligate endosymbiotic pearlfishes and

found that *Encheliophis* species eat their holothuroid hosts while *Carapus* species probably leave their hosts to feed on unknown external sources. Gotto (1979) suggested that the copepod *Synaptiphilus tridens* feeds on the mucus produced by their hosts and that the crab *Hapalonotus reticulatus*, which lives in the respiratory tree of holothuroids, is a suspension feeder (Vandenspiegel et al. 1992). Finally, several symbionts are predators of other symbionts like the intradigestive turbellarian *Meara stichopi* (Westblad 1949).

Lissocarcinus orbicularis (Dana, 1852), also called the Harlequin crab, is the most well known symbiont associated with sea cucumbers inhabiting the shallow waters of the Indo-West Pacific (Fig. 1). It is a small portunid generally considered to be a commensal (Vannini and Innocenti 2000). The Harlequin crab is commonly observed as a single individual or as a heterosexual pair on the body wall or in the buccal and the cloacal cavities of its hosts (Ng and Jeng 1999; Caulier et al. 2012). The symbiosis is obligatory and no crab has ever been encountered in the absence of its sea cucumber host. Harlequin crabs mostly shelter on large holothuroids belonging to the genera *Thelenota*, *Bohadschia* and *Holothuria* (Caulier et al. 2012). The crabs could benefit from the chemical defense (*i.e.* saponins) produced by sea cucumbers that enable them to escape predators and they are so specifically adapted to their holothuroid hosts that saponins act as kairomones attracting the symbiotic crabs (Caulier et al. 2013).

There is no information on the feeding of the Harlequin crab and the goal of the present study was to characterize its diet to define its trophic niche and the nature of its symbiotic association. We used three complementary approaches: microscopic observations, genetic analyses and measurements of the stable isotopic composition. Gut content observations and genetic signatures in the food found in the gastric mills

allowed an initial assessment of the feeding habits but only provide "snapshots" of the crab's diet. Feeding ecology was also investigated using stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios in the Harlequin crab to provide further information on the food that crabs assimilate in their tissues over a long period.

2 Materials and methods

Two hundred and forty-nine Harlequin crabs *Lissocarcinus orbicularis* were collected by scuba diving and with the collaboration of local fishermen on the Great Reef of Toliara (23°24′49″S; 43°38′59″E), South-West of Madagascar from December 2006 to February 2007. Potential food sources (see isotopic analyses for more details) were also collected in the same biotopes where holothuroids were found (*i.e.* near patch reefs in the lagoon, on sea grass beds and on the reef flat close to the external slope) in the corresponding period. Two other symbiotic associations collected by scuba diving (crinoids/polychaete) or from the intertidal flat reef (urchins/gastropod) in the same area were studied as controls, for a comparison of their isotopic compositions with that of the Harlequin crab/sea cucumber symbiosis.

During sampling, the sex of each crab was determined and the carapace length (CL) measured from the rear edge of the carapace to the tip of the rostrum. Crabs were then fixed appropriately according to the required analyses (*i.e.* microscopic, molecular or isotopic analysis). The gastric mills were removed from 100 crabs and studied using optical microscopy.

Genetic analyses were carried out on two occasions using samples collected in January and February 2007. Total DNA was extracted from pooled food found in the gastric mills of 5 *L. orbicularis* for the first extraction and of 55 individuals for the second. An 18 S rDNA gene fragment was specifically amplified by PCR using 1 μl of extraction product as a template DNA and the primers Euk-1A and Euk516r-GC-clamp (Diez et al. 2001). These primers amplify a 560 bp fragment of the 18S rDNA suitable for subsequent DGGE analysis and sequencing. PCR amplifications were performed with the kit Ready-To-Go PCR Beads (Amersham Pharmacia) in a Thermal iCycler (Bio-Rad). The PCR cycle conditions consisted of 30 s at 94 °C (denaturation), 45 s at 56 °C (annealing) and 130 s at 72 °C (elongation). This cycle was repeated 35 times; it was preceded by a step of 130 s at 94 °C (first denaturation) and was followed by a step of 420 s at 72 °C (last elongation). DGGEs were performed twice on the total DNA extracted with a Bio-Rad Protean II system using 15 μl of PCR product applied directly onto 8 % (w/v) polyacrylamide gels in a 0.5× TAE buffer (20 mM Tris-acetate [pH 7.4], 10 mM acetate, 0.5 mM disodium EDTA) with a denaturing gradient ranging from 25 to 75 % of
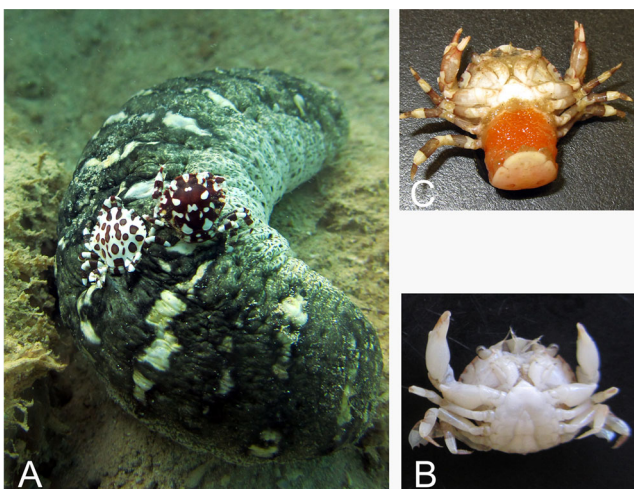

Fig. 1 a Harlequin crab *Lissocarcinus orbicularis* living on the body wall of its holothuroid host *Holothuria scabra*. **b** Ventral view of a (gravid) female Harlequin crab. **c** Ventral view of a male Harlequin crab

denaturant (100 % corresponded to 7 M urea and 40 % [v/v] formamide). The gradient was performed using a gradient-maker (Bio-Rad) and a Masterflex peristaltic pump (Cole-Parmer). Electrophoreses were performed for 16 h at a constant 75 V and a temperature of 60 °C. After electrophoresis, the gels were incubated for 30 min in 0.5× TAE buffer containing ethidium bromide (0.5 mg l^{-1}), photographed and analyzed with the Gel Doc System 1000/2000 of Bio Rad. The number of bands per lane was determined with the Quantity One 4.1 program and checked manually.

DGGE bands were excised from the gels for sequencing and identification. The acrylamide with the DNA was crushed in 1.5 ml microcentrifuge tubes containing 300 µl of Tris-EDTA. After one night at 4 °C, the tubes were centrifuged and the DNA, present in the supernatant, was precipitated with ethanol. DNA obtained after precipitation was used for a new PCR amplification using the same primers and the same protocol as previously. The amplified products were then purified with a QIAQuick Purification kit (Qiagen). Sequences were obtained with the BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) in a Prism 3100 Genetic Analyzer (ABI). The cycle sequencing reaction consisted of 25 cycles with a 10 s denaturing step at 96 °C, a 5 s annealing step at 50 °C and a 4 min elongation step at 60 °C. The primers used were the same as previously. Sequences were checked against the GenBank database (www.ncbi.nlm.nih.gov/GenBank) using the BLAST tool, in order to find related species (Altschul et al. 1990).

Measurements of isotopic compositions of crab tissues were performed on 89 entire *L. orbicularis*. Their potential food sources were also investigated for their isotopic composition: three sea cucumber (holothuroid) host species, three algae species, one sea grass, another common symbiont (the polychaete *Gastrolepidia clavigera*), the organic matter contained in sediment and the organic matter contained in suspension in the water column (see Table 2 for more details). We decided to study *G. clavigera* because it is the second most abundant symbiont found on the sea cucumbers investigated in this study. To test the efficiency of isotopic analyses on symbiotic organisms, we also measured the $\delta^{15}N$ and $\delta^{13}C$ values of two other symbiotic associations from the same area and which have been examined in our laboratories. These were the "*Myzostoma fissum* (Myzostomida) – *Lamprometra palmata* (Crinoidea)" association and that between "*Vexilla vexillum* (Gastropoda) and *Echinometra mathaei* (Echinoidea)". In the first, *M. fissum*, eats the food particles caught by its crinoid host as all ectocommensal myzostomids, (Eeckhaut and Jangoux 1993). It should thus have similar $\delta^{15}N$ and $\delta^{13}C$ values as its host. In the second association, *V. vexilum* is a parasitic gastropod that exclusively eats the tissues of its urchin host (Vaïtilingon et al. 2004). It should therefore have similar $\delta^{13}C$ values but a higher $\delta^{15}N$ values than its urchin host as a result of isotopic fractionation.

Samples were oven-dried at 60 °C for 48 h. They were crushed with a mortar and pestle to obtain a fine powder. All samples were acidified with 37 % fuming HCl in a bell jar for 48 h in order to remove skeleton carbonates. Isotopic ratios and elemental content measurements were performed with a mass spectrometer (VG Optima, Isoprime, UK) coupled to a C-N-S elemental analyzer (Carlo Erba, Italy) for combustion and automated analysis. Nitrogen and carbon contents are expressed in percent relative to dry weight (% DW). Isotopic ratios are presented as δ values (‰), expressed relative to the VPDB (Vienna Peedee Belemnite) standard and to atmospheric N_2 for carbon and nitrogen, respectively. Reference materials were IAEA-N1 ($\delta^{15}N=+0.4\pm0.2‰$) and IAEA CH-6 (sucrose) ($\delta^{13}C=-10.4\pm0.2‰$). The standard deviation on replicated measurements of our sample is 0.3‰ for both $\delta^{15}N$ and $\delta^{13}C$ values.

Spearman's correlation coefficient was used to study a potential correlation between CL, $\delta^{15}N$ and $\delta^{13}C$. Non parametric statistical Mann–Whitney and Kruskal–Wallis tests were used to compare the isotopic signatures of (*i*) myzostomids, gastropods and their respective hosts and (*ii*) Harlequin crabs and their potential food sources.

3 Results

3.1 Microscopic and genetic characterizations

Microscopic analyses of 100 gastric mills of the Harlequin crab revealed that only 52 contained food, the others were totally empty. Sediment and non-identified fractions were regularly observed in the stomach contents (90 % of the non-empty gastric mills). Thirty-five percents of the 52 gastric contents proved to contain sea cucumber (holothuroid) ossicles (Fig. 2). Studies on the ossicles indicated that these originated from the terminal plates of ventral podia, from dorsal papilla and from the cloacum. Finally, non-identified fragments of algae and sea grasses were found in 5 % of the non-empty gastric mills. We did not find any difference between males and females or between juveniles and adults.

The two DGGE analyses of the food contained in the gastric mills of the Harlequin crabs revealed 7 and 3 bands that theoretically represent 7 and 3 different phylotypes (Fig. 3, Table 1). The sequences obtained were of very bad quality probably due to the digestion of the food. The primers used would normally have amplified sequences of 560 paired bases but the only retrieved sequences were readable for 48 to 172 pb, spaced by undetermined nucleotides. Blast searches on these fragmented sequences revealed that six bands originate from crustaceans (and most probably from the crab *L. orbicularis*), one corresponds to a holothuroid and 3 showed no similarity with anything in the database.
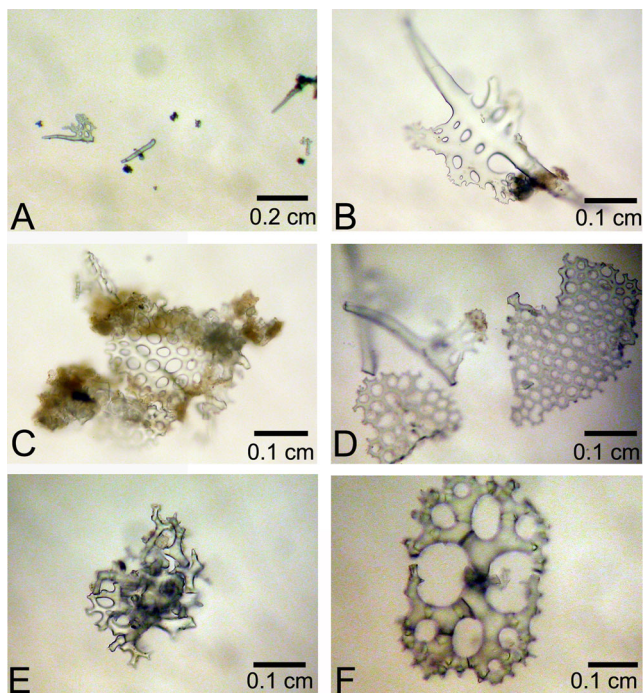
Fig. 2 Microscopic determination of holothurian ossicles found in the gastric mills of Harlequin crabs. Stick like ossicles are from ambulacraria appendage (*e.g.* podia or sensilla) (**a** and **b**) and plate shaped ossicles originate from cloacum membrane (**c**, **d**, **e**, **f**)

3.2 Isotopic characterization

When comparing the isotopic signatures of the control crinoid and urchin symbiotic associations (Table 2, Fig. 4), the Mann–Whitney statistical test revealed no differences between either the δ^{13}C ($U=1$, $N_1=3$, $N_2=3$, $P>0.05$) or the δ^{15}N ($U=1$, $N_1=3$, $N_2=3$, $P>0.05$) composition of myzostomids and crinoids, or between the δ^{13}C values of the gastropods and urchins ($U=6$, $N_1=6$, $N_2=5$, $P>0.05$). The δ^{15}N values of the gastropod, *V. vexillum*, were significantly higher than its Urchin host *E. mathaei* ($U=0$, $N_1=6$, $N_2=5$, $P<0.01$), with a mean enrichment of 2‰.
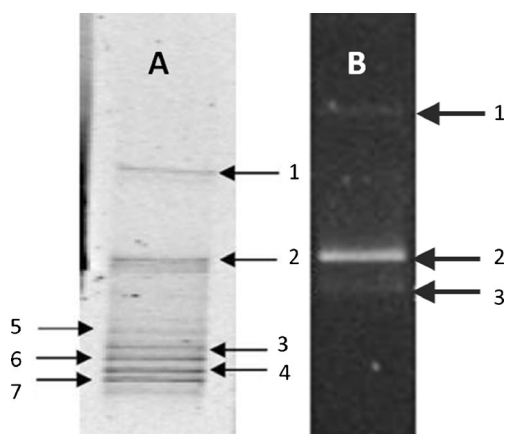

Fig. 3 Molecular analysis obtained by two DGGEs showing ten metazoans molecular signatures found in the gastric mills of Harlequin crabs

With respect to the sea cucumber symbionts, tests on δ^{15}N values highlighted a statistical difference between crabs and polychaetes. The crabs exhibited lower δ^{15}N values than the sea cucumber ($U=206$, $N_1=89$, $N_2=20$, $P<0.01$), while the polychaetes possessed a significantly higher δ^{15}N value than their holothurian hosts ($U=0$, $N_1=6$, $N_2=20$, $P<0.01$).

Algae (Table 2, Fig. 5), had δ^{15}N values that were low in comparison with those of animal tissues (2.7‰ to 3.11‰) but the δ^{13}C values were highly variable between species (from -17.2‰ to -10.2‰). Both δ^{15}N (2.1‰) and δ^{13}C values (-9.3‰) were low for the sea grass *Cymodocea rotundata*. Organic matter values were highly different depending on their origin; δ^{15}N was the lowest (1.4‰ compared to 4.8‰) for organic matters contained in sediment, δ^{13}C values were the lowest for the particulate organic matters in water (-19.3‰ compared to -10.4‰). There was no statistical difference between the δ^{13}C of organic matter contained in water, the suspensive feeder crinoids ($U=5$, $N_1=11$, $N_2=3$, $P>0.05$) and the myzostomids ($U=12$, $N_1=11$, $N_2=3$, $P>0.05$).

With respect to sea cucumber associations, the polychaete symbiont *G. clavigera* had a high δ^{15}N value of 7.8‰. No significant differences were observed between δ^{13}C values of the Harlequin crab, *L. orbicularis,* the holothuroids, ($U=869$, $N_1=89$, $N_2=20$, $P>0.05$), the sea grass ($U=138$, $N_1=89$, $N_2=5$, $P>0.05$), the organic matter contained in sediment ($U=169$, $N_1=89$, $N_2=5$, $P>0.05$), and the symbiotic polychaete ($U=98$, $N_1=89$, $N_2=6$, $P>0.05$). In contrast, the δ^{13}C values of Harlequin crabs and the organic matter contained in water were significantly different ($U=0$, $N_1=89$, $N_2=11$, $P<0.01$). Harlequin crabs showed a mean δ^{15}N value of 3.9‰ (±0.6) and a δ^{13}C of -10‰ (±1.4) (Table 2). No significant difference was found between isotopic composition of male and female crabs analyzed ($U=800$, $N_1=45$, $N_2=144$, $P>0.05$). The δ^{15}N values varied from 1.9 to 5.6‰ and the δ^{13}C from -13.8 to -6.5‰. We also did not find any difference when comparing the individuals sampled in December, January and February ($K=147$, $N_1=31$, $N_2=33$, $N3=25$, $P>0.05$). The length of the Harlequin crabs that were measured varied from 0.2 to 1.4 cm from the tip of the rostrum to the end of the cephalothorax. A significant positive correlation was obtained when CL (Carapace Length) was compared to both stable isotopic ratios. Notably, the correlation is more significative for carbon than nitrogen (δ^{13}C: $r_s=0.67$, $N=89$, $P<0.01$) (δ^{15}N: $r_s=0.36$, $N=89$, $P<0.01$) (Fig. 6). The smaller individuals exhibited lower carbon and nitrogen isotopic values compared with the bigger ones.

4 Discussion

Microscopic analyses revealed that nearly 50 % of the crab gastric mills were empty which indicate that they do not feed

Table 1 Sequences and blast results obtained for each phylotype revealed by DGGE A and B

Phylotype	Retrieved sequences	Best BLAST match	MI
DGGE A			
1	TACCTGGNNNNNNNCTGCCAGTAGTCATATGCTTGTCTCGTTGTCTCGTTG CCTGCTGTCTAAGTACAAGCCGATTCAANNNNNNNAACCGCGAATGG CTCATTAAATCAGCTATGATTCATTGGATCTGTACCCACATTTACTTG GATAACTGTGGTAATTCTAGAGCTAATAC	*Pugettia quadridens* (Eubrachyura; Epialtidae)	96
	TTAGTTCNNNNNCGGTAACTTGGCCCCCTCCGTCACCCTGGTGTGTTG AATCTGAATAACTTTTCT	*Pugettia quadridens* (Eubrachyura; Epialtidae)	71
2	AGTCATATNNTTGTCTCGTTGTCTCGTTGCCTGCTGTCTAAGTACAAGCC GATTCAAGGCGAANNNNCGAATGGCTCATTNNNNNAGCTATGATTCAT TGGATCTGTACCCACATTTAC	*Pugettia quadridens* (Eubrachyura; Epialtidae)	100
3	AGCGCCGACCCTTCNNNNNGCGTGCATTTTTTAGGAACAAGGCC AGCCCGGTGTCTCGCTTCGGCGTGCCCGGTCTCCGCTGGTGA ACTCTAGATAAC	*Bohadschia vitiensis* (Holothuroidea)	96
4	TTACGGGTATTCAGNNNTTTATTCCGTATTCCCCCTTGGGAACGGGTA	*Pugettia quadridens* (Eubrachyura; Epialtidae)	86
5	AGGAAGGCCGGAGGGACGGCNNNNNCCAACCCGGCACGGGGAGG TAGTGAC	No significant similarity	/
6	TTCNNNNCGAAACCGCGAATGGCTCATTAAATCAGCTATGATTCATTG GATC	*Thenus unimaculatus* (Decapoda; Scyllaridae)	84
7	AGGGCCGCCTCTTTCAANNNNNTGCCTTATCAGCTTTCGATTGTAAGGT	*Pugettia quadridens* (Eubrachyura; Epialtidae)	72
DGGE B			
1	GGCGAAACCNNNNNNNGGCTCATAATTTCAGCTATTTAGCATTGGATCT GTACCCACAATTTC	No significant similarity	/
2	AGTCATATGCTNNNNNNGTTGTCTCGTTGCCTGCTGTCTAAGTACAAGC CGATTCAAGGCGGCCAAACGAATGGCTCATTAAATCAGC	*Pugettia quadridens* (Eubrachyura; Epialtidae)	99
	AGGGTTTTATTCCGNNNNNCCCCCCTTGGGAACGGGTACCA	*Pugettia quadridens* (Eubrachyura; Epialtidae)	52
3	GAAACTGCAGTTGGCTCATATGATCAGTTATGGAACATTGGAGCCAGTTC ACGANNNNAAATCTGTCCTAATTCTAGAG	No significant similarity found	/

Band 3 originates from an holothuroid species, 5 others correspond to crustaceans and 3 others were not recognized in the database

MI maximum identification (%)

regularly. But there is a possibility that part of the food was lost by digestion because of the delay between crab collection and dissection.

Microscopic observations of the gastric mills indicated that Harlequin crabs can eat the epidermis and the upper part of the dermis of sea cucumbers. The ossicles in the crab gut contents originate from the papillae, the podia and from the cloacum of holothuroids which corresponds to the location where *L. orbicularis* is found (Caulier et al. 2012). This was confirmed by gut content genetic analysis as one band corresponds to holothuroid tissues.

The amplified crab sequences from the crab gastric content indicated that they were either coming from conspecific individuals or are parasite sequences originating from the gastric mill tissues. It was not easy to separate gastric mill tissues from the food and it is highly probable that these DGGE bands are artifacts. However, in the field, we have observed that when confined inside a small aquarium, adult crabs may eat conspecific juveniles (*unpub. obs.*) but this may not take place in nature. Many crustaceans, and perhaps Harlequin crabs eat their exuvia after molting (Bliss and Mantel 1985), to that the

presence of crab tissues in the crab gut may directly be related to the recent intake of its molt.

Most NCBI blast results indicated the presence of the genetic signature of the spider crab, *Pugettia quadridens* (de Haan 1850), in the gastric content of *L. orbicularis* (Table 1). This spider crab occurs in the cold waters of the Sea of Japan (Kornienko and Korn 2004) and it does not coexist in the same biotope as the Harlequin crab so that this result is due to the poor quality of the DNA sequences retrieved from the predigested samples from the gastric mills.

Even if Harlequin crabs eat tissues from their sea cucumber hosts, the colonized holothuroids never appeared unhealthy and there was no obvious injury induced by the crabs. This may be partly due to the fact that holothurians host only one or two adult crabs. Moreover, the morphology of chelipeds is usually related to the diet preference of brachyuran crabs (Lee 1995). The Harlequin crab is not adapted to be a sea cucumber parasite as its chelipeds are smooth and not designed to tear the holothuroid integument. Probably, the smooth chelipeds enable the crabs to attach firmly to their hosts without harming them (Caulier et al. 2012). Several parasitic crabs, for

Table 2 Mean values of δ^{15}N and δ^{13}C ($\pm$ SD) of the Harlequin crab *L.orbicularis*, its potential food sources, and control symbionts

Species	Number of tested individuals	Isotopic ratios measurements	
		δ^{15}N	δ^{13}C
Control symbioses			
Vexilla vexillum (Gmelin, 1971)	6	5.0 ($\pm$0.6)	-10.0 ($\pm$0.4)
Echinometra mathaei (Blainville, 1825)	5	3.0 ($\pm$0.3)	-10.3 ($\pm$0.8)
Myzostoma fissum (Graff, 1884)	3	5.8 ($\pm$0.8)	-21.3 ($\pm$2.4)
Lamprometra palmata (Muller, 1841)	3	5.2 ($\pm$0.8)	-17.7 ($\pm$2.7)
Brachyurian			
Lissocarcinus orbicularis (Dana, 1852)	89	3.9 ($\pm$0.7)	-10.1 ($\pm$1.5)
♀ *L. orbicularis*	45	3.8 ($\pm$0.6)	-9.9 ($\pm$1.5)
♂ *L. orbicularis*	44	4.1 ($\pm$0.7)	-10.1($\pm$1.4)
Holothuroids			
Bohadschia atra (Massin *et al.*, 1999)	5	6.7 ($\pm$0.6)	-11.6 ($\pm$1.6)
Holothuria atra (Jaeger, 1833)	7	6.1 ($\pm$0.6)	-9.5 ($\pm$1.1)
Holothuria scabra (Jaeger, 1833)	8	4.7 ($\pm$1.4)	-9.9 ($\pm$1.3)
Sea grass			
Cymodocea rotundata (Aschers et Schweint, 1968)	5	2.1 ($\pm$0.4)	-9.3 ($\pm$0.2)
Algae			
Ulva reticulata (Linnaeus, 1753)	3	3.1 ($\pm$1.7)	-17.2 ($\pm$0.2)
Padina boryana (Adanson, 1763)	4	2.7 ($\pm$0.6)	-10.2 ($\pm$0.6)
Sargassum densifolium (Zanardini, 1858)	3	2.7 ($\pm$0.3)	-14.2 ($\pm$0.6)
Organic matter			
In sediment	5	1.4 ($\pm$0.4)	-10.4 ($\pm$1.8)
In water	11	4.8 ($\pm$1.6)	-19.3 ($\pm$1.2)
Holothuroid symbiont			
Gastrolepidia clavigera (Schmarda, 1861)	6	7.8 ($\pm$0.6)	-12.3 ($\pm$1.7)

δ^{15}N and δ^{13}C in ‰

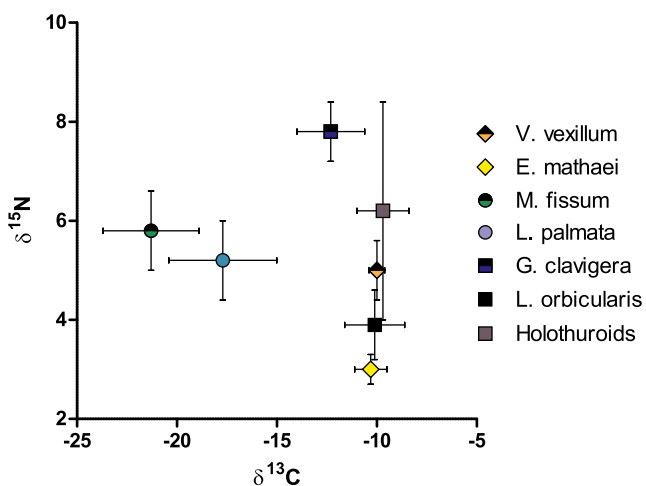**Fig. 4** Average values ($\pm$ S.D.) of δ^{15}N and δ^{13}C of different invertebrates with echinoderms symbioses: gastropod *V. vexillum* with urchin *E. mathaei* (*diamonds*) ; myzostomid *M. fissum* with crinoid *L. palmata* (*circles*); polychaete *G. clavigera* and crab *L. orbicularis* with holothuroids (*squares*)
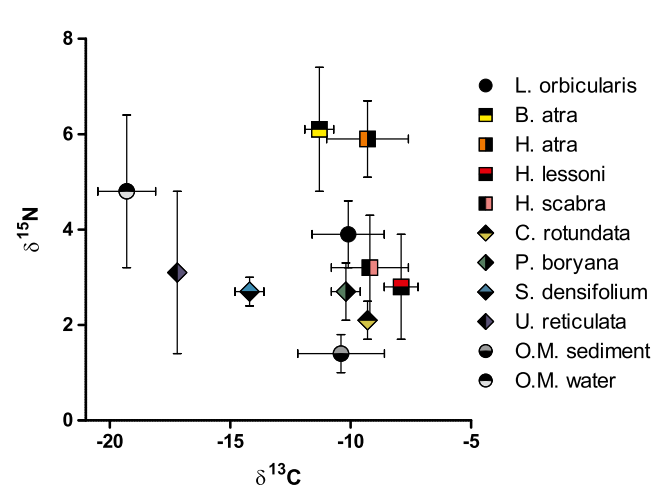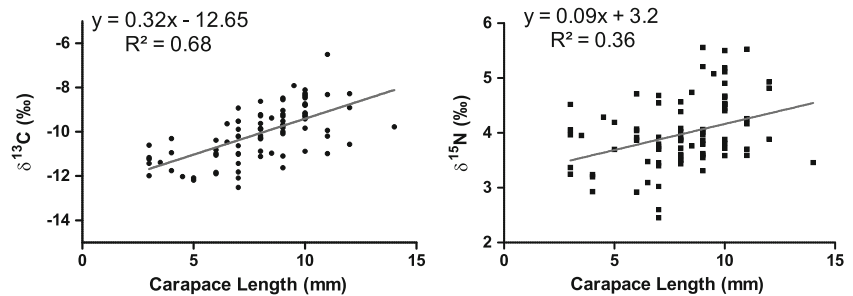
Fig. 5 Average values ($\pm$ S.D.) of δ^{15}N and δ^{13}C of Harlequin crabs and their potential food sources. Holothuroids are represented by *squares*, phanerogam and algae by *diamonds* and organic matter of sediment and water column by *circles*

Fig. 6 Carapace length (CL, mm) versus stable isotopic composition in Harlequin crabs

example, those belonging to the genus *Dissodactylus* directly eat their sea urchin host tissues, and their chelipeds morphology allows them to cut the urchin spines, causing serious damage to their host (Telford 1982, De Bruyn et al. 2009, Jossart et al. 2013). Therefore, even if holothuroid tissues contribute as a food source, they do not constitute the exclusive diet of the Harlequin crabs.

The isotopic approach relies upon the fact that stable isotopes composition of an animal is the weighted average of the stable isotopic compositions of its different foods, modified by the isotopic fractionation process (*i.e.* the isotopic composition is modified by metabolic activity) (DeNiro and Epstein 1981). Because $\delta^{15}N$ is selectively incorporated in tissues, nitrogen isotope ratios present higher level in consumer tissues as trophic level increases. Thus, nitrogen isotope analysis is helpful in establishing trophic level and predator–prey relationships (Kelly 2000). On the other hand, carbon isotope ratios of an organism define the contribution of its different potential food sources (DeNiro and Epstein, 1981).

In the present study, the stable isotope data provide further insights into the food source of the Harlequin crab. Firstly, the isotopic characterization of the control symbiotic associations studied effectively matches with the diet preferences of the investigated symbionts. Our results indicate that the gastropod *V. vexillum* is a parasite of its urchin host, feeding almost exclusively on *Echinometra mathaei*. Indeed, the gastropods exhibit almost the same $\delta^{13}C$ values, but a significantly higher $\delta^{15}N$ value than their host. This fits the average isotopic fractionation (*i.e.* isotopic composition change) classically observed between a consumer and its prey (McCutchan et al. 2003). We found that the $\delta^{15}N$ values of the polychaetes *G. clavigera* were higher compared with their sea cucumber host (Fig. 4). Indeed, this symbiont is known to eat holothuroid tissues in a large part of its diet (Britaev and Lyskin 2002). In the second control symbiotic association, namely that between the myzostomid *M. fissum* and the crinoids *L. palmata,* both $\delta^{13}C$ and $\delta^{15}N$ were impossible to distinguish, indicating that the host and the symbionts share food source and therefore exhibit the same isotopic composition (Eeckhaut and Jangoux 1993). Interestingly, the $\delta^{13}C$ of this symbiotic association is similar to that of the organic matter contained in the water, which confirms that they are suspension-feeders. A $\delta^{15}N$ increase was not observed for

the Harlequin crabs. Their $\delta^{15}N$ values were generally lower to those of their holothuroid hosts, further evidence that sea cucumber tissues are not the main food item of this crab.

$\delta^{13}C$ values were not significantly different between the different benthic ecosystem components investigated (*i.e.* organisms and organic matter in sediment), but were well separated from values found for the organic matter contained in the water column (*i.e.* planktonic organism and detritic organic matter). Therefore, the carbon isotopic composition of the Harlequin crabs precludes a large contribution from the water column components to the crab diet. This is confirmed by the absence of filtering structure on mouth apparatus or the observation of filtering behavior in *L. orbicularis*. The more probable food source of Harlequin crabs considering low $\delta^{15}N$ values and their $\delta^{13}C$ is therefore the organic matter found in the sediment. This is consistent with the gut content study where most of the filled gastric mills comprised fine sediments. Nevertheless, small pieces of algae and sea grasses were also recorded. Our observations show that the Harlequin crab is never found outside its host and therefore direct ingestion of sediment outside the sea cucumber is unlikely. Most probably, crabs rely on the sediment and detritic organic matter ingested by their host or found on the dorsal/cloacal integument of their host but microscopic observations indicated a contribution from other food sources particularly host tissue and phytodetritus. Our results showed no evidence that the Harlequin crab, *L. orbicularis,* feeds on the symbiotic polychaete *G. clavigera* when the host is colonized by both animals, but field observations showed that in 90 % of the cases, there was no symbiotic polychaete present when sea cucumbers were found associated with a Harlequin crab (*unpubl. data*).

There was no isotopic difference in the diet of males and females and the diet seems similar whatever the period of the year investigated. On the other hand, we observed a positive correlation between stable nitrogen and carbon isotopic values and the size of crabs. This suggests that the diet of *L. orbicularis* varies with the growth of the individuals. On average, small crabs have a lower trophic level which could be explained by the fact that they do not eat sea cucumber tissues and that organic matter contributes more to the diet than for older individuals. Very few studies have investigated the variations in nitrogen and carbon stable isotopic signatures

in decapod crustaceans in relation to age. In two spider crab (*Maja brachydactyla*) populations inhabiting different biotopes of Western Europe (Bodin et al. 2007), $\delta^{13}C$ values increased significantly both with body size and age, revealing a shift towards "benthic-component" prey with spider crab growth. Trends in the carbon and nitrogen isotope signatures with size in the crayfish (*Nephrops norvegicus*) and the squat lobster (*Munida sarsi*) revealed that these two omnivorous crustaceans, like *Maja brachydactyla*, change their diet as they grow (Loc'h & Hily 2005). Harlequin crabs like other portunid crabs (Warner 1977), display opportunistic behavior and trophic changes to some extent as they grow larger.

Sea cucumbers (holothuroids) host a large community of symbionts from diverse taxa on/in their body. Studying the trophic relationships between the symbiotic entities, their hosts and the external food sources provide new insight into these micro-ecosystems. The case of the Harlequin crab reveals the difficulties of characterizing these complex ecological interactions. Indeed, just with respect to the trophic relationships, this crab could be considered as a parasite (*i.e.* by the presence of sea cucumber spicules in its stomach), a kleptoparasite (*i.e.* feeding on the same sediments as its host) or a commensal (*i.e.* using external food intakes). The symbiotic status of the Harlequin crab provides a good illustration of how difficult it is to categorize the symbiotic interactions found in oceans, a feature emphasized by Parmentier and Michel (2013).

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