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Do marine benthos breathe what they eat?

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Abstract

Intertidal benthos link tertiary predators and primary producers in marine food webs as well as directly contribute to sediment CO_2 emission. However, current methods for studying food sources of marine benthos are time-consuming and does not allow direct estimates on feeding regime-related CO_2 production. We examined the foods of mangrove crabs and gastropods as well as their corresponding CO_2 production by using cavity-ring down spectroscopy to measure the $\delta^{13}C$ of consumer-respired CO_2, considering the effects of feeding regime, benthos taxa, and dominant feeding habit. Benthos taxa and feeding habit have significant impact on $\delta^{13}C$ of respired CO_2. Particularly, the $\delta^{13}C$ of crab (-23.9±0.4‰) respired CO_2 was significantly lower than that from gastropod (-17.5±1.3‰) respiration. The $\delta^{13}C$ of deposit-feeder respired CO_2 was significantly higher than that from detritivores. There are significant differences in the amount of CO_2 emitted and $\delta^{13}C$ of crab respired CO_2 under different feeding regimes. The differences reflect diet-switching and fuel-switching in the crabs, i.e. 'you breathe what you eat'. Significant differences in CO_2 production of crabs also exist between those feeding on microphytobenthos (0.13±0.02 mmol g^{-1} day^{-1}) and on field collection (0.31±0.03 mmol g^{-1} day^{-1}). CO_2 production of crabs is strongly related to carapace width and length. The $\delta^{13}C$ of respired CO_2 from mangrove crabs reflects their diet while crab-respired CO_2 flux is related to crab size. These relationships enable partitioning the feeding habit and food sources of key benthos, and help incorporate their contribution into the overall sediment-atmosphere CO_2 fluxes in mangroves.

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1 Introduction

Intertidal benthos are well known to play different roles in processing nutrients in the intertidal food webs depending on their taxonomic affiliation and feeding habit. In mangroves, sesarmid crabs are reported to use mangrove leaf litter as the most important carbon source, with diatoms or live/dead animal prey as the dominant nitrogen source, while ocypodid crabs and gastropods are mainly deposit feeders, preferring the microphytobenthos (Kristensen et al., 2017; Lee, 2008). The different feeding habits (e.g. detritivorous or deposit-feeding) of the mangrove benthos may result in differences in their metabolic processes. Past mangrove studies revealing the food sources of the mangrove benthos have advanced from earlier gut analysis to stable isotope analyses (Lee et al., 2014). Stable isotope analysis unravels benthic food sources by extracting benthos muscle tissues and analyzing tissue and food stable isotope values of carbon, nitrogen and occasionally sulfur (Bui & Lee, 2014; Chong et al., 2001). However, dietary analysis is a time-consuming process that often requires close monitoring of individual predators or collection and analyses of prey remains in the gut or faeces of the predator (Caro, 1994; Wachter et al., 2012).

In contrast to traditional stable isotope analyses of predator tissues, $\delta^{13}C$ of predator-respired CO_2 have increasingly been applied to study the food sources and feeding habit of predators. The latter approach has advantages over isotope analyses of predator tissues because it can provide information about both the most recently consumed diet and the integrated diet over longer periods (Engel et al., 2009). While some adults of aerial and aquatic migratory species occupy intertidal habitats during specific seasons of the year, others are permanent inhabitants (Vernberg, 1993). The variable feeding habits of marine migratory species make it useful to study both their immediate diet and integrated diets. In contrast, some tissues (e.g. muscles) have

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slower turnover rates and their isotopes can only reflect the integrated diet over longer periods (Carleton et al., 2004). Further, breath $\delta^{13}C$ can be repeatedly measured non-destructively for the same predator and thus can track the changes in its food sources. Carleton et al. (2004) explored the use of $\delta^{13}C$ of hummingbirds exhaled CO_2 to demonstrate their shift from a C3 to C4 diet. Voigt et al. (2008) found free-ranging vampire bats prefer cattle blood by analyzing $\delta^{13}C$ of their respired CO_2. Its application was also tested in other animals such as grasshoppers (Engel et al., 2009). $\delta^{13}C$ of predator-respired CO_2 is sufficient for quickly unravelling the prey consumed without the involvement of diet and predator tissue $\delta^{13}C$ values. However, this approach still remains uncommon in marine studies for examining the food of marine benthos.

Moreover, the measurement of benthos-respired CO_2 and their isotope signatures does not only reveals their food sources but also helps assess their contribution to sediment respiration after adjusting for the effects of feeding habit and regime (e.g. active vs. dormant status, and feeding on microphytobenthos vs. yellow leaves). CO_2 emission rates from the sediment surface of mangroves are more than doubled if the contribution of marine benthos and their burrows is included (Kristensen et al., 2008; Ouyang et al., 2017; Penha-Lopes et al., 2010). Some crabs may remain dormant most of the time, with a short active period (e.g. 90 days, Katz (1980)) during the year. Should the starved condition during dormant periods be unaccounted for, it may lead to erroneous estimations on the animals' contribution to CO_2 emission from the sediment surface. Further, it is well established that C3 and C4 plants have distinct isotope signatures (Fry, 2006), which may result in differences in $\delta^{13}C$ of benthos-respired CO_2. Nonetheless, few studies explore whether the increase in CO_2 emission rates due to marine benthos is related to feeding regime or their feeding habit.

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77 Cavity ring-down spectroscopy (CRDS) is one of the most recent advances in measuring the
78 concentration as well as isotopic values of many biogenic gases, including CO_2, CH_4, and other
79 gases that have unique near-infrared absorption spectra. CRDS works by quantifying the effect
80 of this absorption (Welch Jr et al., 2016). It has been used in marine studies to partition sources
81 of ecosystem and sediment respiration of mangrove seedlings (Ouyang et al., 2018), and the
82 variation of $\delta^{13}C$ of greenhouse gases emitted from estuaries and other ecosystems (Jacotot et al.,
83 2018; Munksgaard et al., 2014; Rosentreter et al., 2018; Sea et al. 2018; Ouyang et al., 2020).
84 We conducted the first study using CRDS to relate the feeding regimes of different intertidal
85 benthos with $\delta^{13}C$ of their respired CO_2. We also measured the different isotope enrichment
86 between marine benthos-respired CO_2 and their diets for different benthos taxa and feeding
87 habits. The relationship between benthos size and feeding regime to their CO_2 production was
88 also assessed. Intertidal benthos are hypothesised to "breathe what they eat" in terms of both
89 breath $\delta^{13}C$ and CO_2 production. We explored the influence of benthic taxa, feeding regime and
90 feeding habit on the $\delta^{13}C$ of benthos-respired CO_2 and/or benthos CO_2 production (i.e. CO_2
91 respired per unit mass per day), and the relationship between benthos CO_2 production and their
92 body sizes through laboratory experiments. We put forward the novel paradigm 'You Breathe
93 What You Eat', which adds to the well-known paradigm of 'You Are What You Eat' in food
94 web studies. This study will inform future efforts to estimate the contribution of different food
95 sources to the diet of mangrove benthos over both short and long periods.
96
97 **2 Materials and Methods**
98 **2.1 Sample collection**

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We collected small (2-4 cm carapace width) but numerically dominant brachyuran crabs (including sesarmids, varunids and ocypodids) and gastropods from the mangrove forests in Mai Po Nature Reserve (22°30'N,114°02'E) and Ting Kok (22°28'N,114°13'E), Hong Kong. Senescent leaves of the mangrove *Kandelia obovata* were hand-picked from the trees. The leaves can be identified by their yellow colour and easily detachable from the branches. Surface sediments (down to 1cm) were collected by a syringe with the needle end removed. The samples were kept on ice before transportation to the laboratory.

2.2 Sample pre-treatment and separation

Each animal was kept in a small container with seawater covering the bottom to avoid desiccation. The animal food items were treated and/or purified before the consumption experiments. Upon return to the laboratory, the leaves were immersed in seawater for around 24 hours to allow leaching of deterrent chemicals such as tannins, which may deter crabs from feeding. Microphytobenthos (MPB), mainly in the form of diatoms, were separated from the surface sediments by the 'sieve and spin' method (Bui & Lee 2014). Specifically, the sediments were suspended in seawater and sieved through a 45μm mesh. The filtrate was further passed through a 5 μm filter, and the residue was resuspended in Ludox colloidal silica (Sigma, density 1.34 g ml^{-1}). Then the mixture was vortexed and centrifuged at 4000 rpm for 10 minutes. The microphytobenthos concentrated in a distinct layer at the top of the colloidal silica were then separated via a pipette, before confirming to be predominantly microphytobenthos using a microscope. The MPB was washed in Milli Q water to remove the remnant colloidal silica and washed again when the microphytobenthos had settled down. This process was repeated several

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121 times until the water was clear. The MPB were then collected on pre-combusted GF/F filters
122 (Whatman).
123
124 **2.3 Experimental design**
125 We conducted a series of experiments to examine factors influencing C isotopic signatures
126 and/or CO_2 production of the mangrove benthos: (1) the effect of benthic taxa (i.e. crabs vs.
127 gastropods); (2) the effect of feeding regime (on collection vs. fasted, feeding on yellow
128 mangrove leaves vs. diatoms). Mangrove benthos "on collection" and "fasted" correspond to the
129 active and dormant status of benthos, respectively; (3) the effect of feeding habit (i.e. deposit
130 feeders vs. detritivores); and (4) the relationship between CO_2 production and animal body size.
131 Thirty animals were used each in experiments (1) and (3), while 10 sesarmid crabs were used in
132 experiment (2), and 25 animals (sesarmid crabs and gastropods) in experiment 4.
133 In experiment (1), the animals were put in plastic 0.8 l containers covered by aluminium foil to
134 minimise disturbance. There is a small hole on the lid of each container to keep the pressure
135 balance between the inside and outside of the containers. A soft plastic hose was connected to
136 the lid of each container with the other end closed by a stop-cock. Twenty minutes after closing
137 each container, a syringe was connected to the other outlet of the stop-cock, and 30 ml of gas
138 was collected from the container. The needle end of the syringe was also closed by a stop-cock
139 until analysis. Gases were collected over a period of 50 minutes (five times, every 10 minutes).
140 In experiment (2), gas samples were collected similarly collected upon arrival at the laboratory
141 as well as under starving conditions each day for three days to simulate the dormant status.
142 Afterwards, they were fed with mangrove leaves and then, after fasting for 3 days, fed with
143 MPB. Gas samples were collected separately when the crabs were fed on different foods. In

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144 experiment (3), the benthos were fed treated yellow leaves or MPB, depending on their main
145 feeding habits. Deposit feeders, including gastropods (e.g. *Terebralia sulcata*), ocypodid crabs
146 (e.g. *Uca arcuata*) and varunid crabs (e.g. *Metaplax longipes*), were fed MPB. Detritivores,
147 including sesarmid crabs, e.g. *Parasesarma bidens* and *P. pictum*, were fed yellow mangrove
148 leaves.
149
150 **2.4 Sample analysis and measurement**
151 In experiment (4), the carapace width (CW) and length (CL) of crabs and shell length of
152 gastropods were measure by Vernier callipers. After measuring CO_2 production as described
153 above, the crabs were sacrificed and dried at 60°C until constant weight. Subsamples of dried
154 leaf and MPB samples (around 5 mg) were weighed into tin capsules for stable isotope analysis.
155 Another group of crabs were dissected and muscle tissues extracted, dried and prepared in the
156 same way for stable isotope analysis. Their elemental contents (carbon and nitrogen), $\delta^{13}C$ and
157 $\delta^{15}N$ values were analysed by a EuroVector Elemental Analyser - Nu Perspective Isotope Ratio
158 Mass Spectrometer (IRMS) at The University Hong Kong, with iACET standards used for
159 quality control check.
160 CO_2 and CH_4 concentrations and $\delta^{13}C$ values of the gas samples were measured by a Picarro
161 G 2201-i CRDS analyser (Picarro Inc., USA). The syringes were connected to the inlet of CRDS
162 analyser to allow gas to be sucked into the analyser by a vacuum pump. The analyser measured
163 $\delta^{13}CO_2$, CO_2 and CH_4 concentrations at an interval of five seconds with guaranteed precision of
164 <0.012‰, 200ppb + 0.005% and 50ppb + 0.05%, respectively. Standard gases of mixed CO_2
165 (1008 ppm) and CH_4 (10.2 ppm) (ARK NIC, West Indices) were used to check the accuracy of
166 the analyser.

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Keeling plots (Keeling, 1961) were used to estimate $\delta^{13}C$ of crab-respired CO_2. Keeling plots assume that $\delta^{13}C$ of CO_2 within a space reflects the mixture of some amount of CO_2 contributed by sources in the system and some background amount of CO_2 that is already present in the same space (Dawson et al., 2002). It is described by the isotope mixing model and mass balance model as below:

$$\delta^{13}C_{system}[CO_2]_{system}=\delta^{13}C_{atm}[CO_2]_{sample} + \delta^{13}C_{source}\,[CO_2]_{source} \quad (1)$$

$$[CO_2]_{system}=[CO_2]_{sample}+[CO_2]_{source} \quad (2)$$

where $\delta^{13}C_{system}$, $\delta^{13}C_{source}$, $\delta^{13}C_{atm}$ are the $\delta^{13}C$ values for the system, the source and sample space, respectively. $[CO_2]_{system}$, $[CO_2]_{source}$ and $[CO_2]_{sample}$ are the concentrations for the system, the source and sample space, respectively. The equations (1) and (2) can be combined and rearranged as below

$$\delta^{13}C_{system}=\delta^{13}C_{source}+[CO_2]_{sample}(\delta^{13}C_{sample} -\delta^{13}C_{source})\frac{1}{[CO_2]_{system}} \quad (3)$$

The same principle can be applied to measure $\delta^{13}C$ of benthos- and other animal-respired CO_2, as discussed elsewhere (Carleton et al., 2004). As indicated in equation (3), the y-intercept of a linear regression of $\delta^{13}C_{system}$ against $\frac{1}{[CO_2]_{system}}$ provides an estimate $\delta^{13}C$ of benthos-respired CO_2. $[CO_2]_{source}$ was estimated as the slope of the regression relationship between $[CO_2]_{system}$ (t=0, 10, 20, 30 and 40 minutes) and t. A conceptual model was used to describe the process of measuring benthos CO_2 production and $\delta^{13}C$ of respired CO_2 by CRDS (Fig. 1)

The containers used to collect greenhouse gases respired by the animals are similar to a static chamber, for which the respired greenhouse gas flux (mmol day^{-1}) was standardized across the diet treatments and estimated as below

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$$F = V\left(\frac{d[CO2]source}{dt}\right)\frac{1}{V_0}\frac{P}{P_0}\frac{T_0}{T} \quad (4)$$

Where V is the chamber volume subtracting the volume of the crab. $\frac{d[CO2]source}{dt}$ is usually taken to be the slope of the linear regression of $[CO_2]_{source}$ on t (Rolston, 1986). Here t is the series of gas collection time from the start to end, i.e. every 10 minutes for five times. V_0 is the gas molar volume under standard conditions. P_0 and T_0 is the standard pressure and temperature. P and T is the pressure and temperature in the container.

2.5 Statistical analysis

We conducted a two-way analysis of variance (ANOVA) to examine the impact of benthos taxa and feeding habit on $\delta^{13}C$ of benthos-respired CO_2. When significant treatment effects were found, Tukey's HSD test was used to detect difference among groups. We also conducted a one-way ANOVA to examine the impact of feeding regime on $\delta^{13}C$ of benthos-respired CO_2 and benthic CO_2 production. Before ANOVA, the assumptions of normality and homoscedasticity were tested (α=0.05). Normality was tested using the Shapiro-Wilk normality test. Homoscedasticity was tested using the Levene test. Linear regression was used to examine the relationships between crab CO_2 production and carapace width/length/ weight, as well as between gastropod CO_2 production and shell length/weight. The assumption of normality was tested as described above. Given only significant relationships were found for sesarmid crabs, student's t test was performed to test the difference in the ratios of carapace length and width between ocypodid crabs and sesarmid crabs, to show if the relationship between CO_2 production of sesarmid crabs and crab size applies to ocypodid crabs. Student's t test was also used to compare (1) the difference in C/N ratios; (2) $\delta^{13}C$ and $\delta^{15}N$ values between mangrove yellow leaves and the microphytobenthos; and (3) $\delta^{13}C$ of CO_2 respired by crabs on collection and that

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of muscle tissues. Data are presented as mean±standard error (SE). All the data analyses were conducted using R programming language (R Core Team, 2014). The R package 'car' was used to conduct ANOVA (Fox & Weisberg, 2011).

3 Results

3.1 The sources of variations of $\delta^{13}C$ of intertidal benthos respired CO_2

Our results show that $\delta^{13}C$ of intertidal benthos respired CO_2 varied significantly with benthos taxa, dominant feeding habit and/or feeding regime. Benthos taxa (1) and dominant feeding habit (2) had a significant influence on $\delta^{13}C$ of benthos-respired CO_2 (ANOVA, F_1=48.4, P<<0.001; F_2=12.9, P<<0.001). Further post-hoc analyses showed that the $\delta^{13}C$ of crab (-23.9±0.4‰) respired CO_2 was significantly lower than that of gastropod (-17.5±1.3‰) respired CO_2 (Tukey's HSD test, P<<0.001, Fig. 2a). The $\delta^{13}C$ of deposit-feeders (e.g. *Terebralia sulcata* and *Uca arcuata*) respired CO_2 (-19.8±0.8‰) was significantly higher than those of detritivores (e.g. *Parasesarma bidens*) respired CO_2 (-24.7±0.3‰, Tukey's HSD test, P=0.004, Fig. 2b).

There were also significant differences in $\delta^{13}C$ of crab-respired CO_2 among different feeding regimes (ANOVA, F=5.4, P<0.001, Fig. 3). In particular, $\delta^{13}C$ of crab-respired CO_2 was significantly lower when crabs were fed on leaves (-26.6±0.3‰) than on MPB (-23.8±0.4‰, P<0.001), and on collection (-24.8±0.6‰, P<0.05). This is consistent with the diet $\delta^{13}C$ values. $\delta^{13}C$ values of mangrove yellow leaves were significantly lower (-27.8±0.2‰) than those of the MPB (-27.1±0.05‰) (t = -3.8, p<0.01, Fig. 2c). In contrast, the ratio of C/N for mangrove yellow leaves (95±1.8) was significantly higher than that for the MPB (16.3±0.3) (t = 44.307, p<<0.001, Fig. 2d). $\delta^{13}C$ of benthos-respired CO_2 was also significantly higher when crabs fed

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on MPB than when they were fasted for 2 days (-25.7±0.4‰, $P<0.05$) and 3 days (-25.8±0.4‰, $P<0.05$). Further, $\delta^{13}C$ of CO_2 respired by detritivorous crabs on collection (-24.0±0.6‰) was not significantly different from that of crab muscles (-22.6±0.6‰, $P>0.05$). Also no significant differences were found between other comparisons.

3.2 Variation of CO_2 production with benthos size and/or feeding regimes

CO_2 production was related to consumer size and/or feeding regimes. There was a significant difference in CO_2 production of crabs among different feeding regimes (ANOVA, $F=2.9$, $P<0.05$, Fig. 4). In particular, CO_2 production of crabs was significantly lower when they fed on MPB (0.13±0.02 mmol g^{-1} day^{-1}) than on collection (0.31±0.03 mmol g^{-1} day^{-1}, $P<0.05$) or fasted for one day (0.3±0.05 mmol g^{-1} day^{-1}, $P<0.05$). No significant differences were found for other comparisons. There were significant relationships between CO_2 production of crabs and carapace width ($R^2=0.73$, $P<0.001$, Fig. 5a), and between CO_2 production of crabs and carapace length ($R^2=0.61$, $P<0.01$, Fig. 5b). Significant differences were found in the ratios of carapace length and width between ocypodid crabs (0.59±0.002) and sesarmid crabs (0.79±0.025) (t test, $t=-7.9$, $P<0.01$), which application of the above relationships for intraspecific comparison may depend on the size measurement used. The CO_2 production of the crabs was 0.45±0.05 mmol g^{-1} day^{-1} at the average dry weight of 0.95g (0.12±0.01 mmol g wet wt^{-1} day^{-1}). Similarly, there was a significant relationship between CO_2 production of gastropods and shell length ($R^2=0.58$, $P<0.05$, Fig. 5c). The CO_2 production of the gastropods was 0.014±0.003 mmol g^{-1} day^{-1} at the average dry weight of 2.09g (0.045g without shell). No significant relationships were found between CO_2 production and crab or gastropod weight ($P>0.05$).

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4 Discussion

4.1 Differences in $\delta^{13}C$ of intertidal benthos due to different food categories

Our data suggest that $\delta^{13}C$ of benthos-respired CO_2 can be used to infer the categories of benthos foods being used. The $\delta^{13}C$ of benthos-respired CO_2 in the gastropod group was higher than that of the crab group, and that of the deposit-feeding group was higher than the detritivorous group. These different patterns may reflect different food categories of the mangrove benthos. In our laboratory experiment, the gastropods investigated mainly forage on the MPB, while the crabs may use both the MPB and mangrove leaf litter depending on their feeding habit. Yellow leaves of mangrove species in Ting Kok (where leaves and sediments were collected) have $\delta^{13}C$ at -27.8±0.16‰ and C/N at 95±1.8, while the corresponding values for MPB were -27.1±0.05‰ and 16.3±0.3. A C/N ratio of < 20 is generally required for sustainable animal nutrition (Russell-Hunter, 1970). Thus, crabs may need to use the MPB or other N-rich sources to meet their nutritional requirement to supplement the low-N leaf diets. The isotopic fractionation ($\Delta^{13}C$) is 3.2‰ and 3.9‰ between $\delta^{13}C$ of crab (-23.9±0.4‰) respired CO_2 and $\delta^{13}C$ of MPB and mangrove yellow leaves respectively, while it is 9.6‰ between $\delta^{13}C$ of gastropod (-17.5±1.3‰) respired CO_2 and $\delta^{13}C$ of the MPB (Table 1). Similarly, $\Delta^{13}C$ is 7.3‰ between $\delta^{13}C$ of deposit-feeder (including ocypodid and varunid crabs and gastropods) respired CO_2 (-19.8±0.8‰) and $\delta^{13}C$ of the MPB and 3.1‰ between $\delta^{13}C$ of detritivore (including sesarmid crabs) respired CO_2 (-24.7±0.3‰) and $\delta^{13}C$ of yellow mangrove leaves. The lack of significant difference between $\delta^{13}C$ of CO_2 respired by detritivorous crabs on collection and that of crab muscles is consistent with findings on terrestrial herbivores (Engel et al. 2009).

4.2 Differences in $\delta^{13}C$ of intertidal benthos due to the feeding regime

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280 The significant differences in $\delta^{13}C$ of crab-respired CO_2 among different feeding regimes may be
281 attributed to fuel-switching and also to food categories consumed. Proteins, lipids and
282 carbohydrates are the three major classes of metabolic fuels (McCue & Welch, 2016). The long-
283 standing paradigm lies in that fasting animals pass through three sequential physiological phases
284 whereby they predominantly oxidize endogenous fuels in the sequence of carbohydrates,
285 followed by lipids, and then proteins (Caloin, 2004; Castellini & Rea, 1992). $\delta^{13}C$ of crab-
286 respired CO_2 was not significantly different among the MPB diet, on collection or starved for
287 one day, but was significantly higher compared to that of crabs starved for two or three days. In
288 our experiment, crabs may metabolise carbohydrates and/or mixed with lipids when they were
289 just collected or fasted for one day, similar to the small animals in another study (Carleton et al.,
290 2004). After fasting for two or three days, crabs started to consume lipids, which have $\delta^{13}C$
291 values 0.5-8‰ lower than those of carbohydrates (DeNiro & Epstein, 1977; McCue & Welch,
292 2016; Stott et al., 1997). This can explain the decline (but non-significant) in $\delta^{13}C$ of crab-
293 respired CO_2 during the fasting periods. After fasted for three days, $\delta^{13}C$ of crab-respired CO_2 (-
294 26.6±0.3‰) was about 1.2‰ higher than when they fed on yellow *Kandelia obovata* leaves.
295 Similarly, $\delta^{13}C$ of crab-respired CO_2 (-23.8±0.4‰) was about 3.3‰ higher than the diet when
296 they fed on MPB after starvation. These results are supported by the higher $\delta^{13}C_{breath}$ values than
297 $\delta^{13}C_{diet}$ (0.8-3.1‰) for different animals including steers, pigs and rabbit (Passey et al., 2005).
298

299 **4.3 Differences in benthos CO_2 production due to body size and feeding regime**

300 The significant relationships between benthos CO_2 production and body size suggest that animal
301 size distribution should be considered when estimating the contribution of the benthos to CO_2
302 emission rates from the sediment surface. Crab CO_2 production (0.12 mmol g^{-1} wet wt day^{-1}) for

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303 the sesarmid crabs in our study falls within the reported range of 0.05-0.15 mmol g^{-1} wet wt day^{-1} for ocypodid crabs (Kristensen et al., 2008; Penha-Lopes et al., 2010). Average gastropod CO_2 production in Penha-Lopes et al. (2010) (0.011 mmol g^{-1} day^{-1}) was similar to that in our study (0.014 mmol g^{-1} day^{-1}).

We established the relationships between benthos CO_2 production and body size (carapace width or length). They can be used for different purposes. Particularly, when estimating crab CO_2 production related to crab burrow size (i.e. the diameter of burrow openings) (e.g. Cameron et al. (2019)), the relationship between CO_2 production and carapace length rather than carapace width should be used as crabs walk side-ways. However, carapace length is not as good a measurement of animal body size as carapace width when different crab families are involved. For example, our data suggest that ocypodid crabs had significantly smaller carapace length/width ratios (0.59±0.002) than those of sesarmid crabs (0.79±0.025).

The significant impact of feeding regimes on CO_2 production of sesarmid crabs may be a result of the differences in carbon content of the diet and endogenic fuel. CO_2 is a waste product of oxidising reduced carbon compounds during crab metabolism. When sesarmid crabs were just collected from the field, their main carbon source is mangrove leaves, which were found to have a higher carbon content than the MPB (45.6% vs. 30.6%) (Bui & Lee, 2014). This could account for the significant differences in CO_2 production of crabs on collection and while feeding on the MPB. As small animals, when fasted for one day, our collected crabs may consume a mixture of recent diet (mangrove leaves) and stored carbohydrates, which are easier to decompose than the structural carbon-rich mangrove leaves and other organic compounds, e.g. lipids and proteins. However, when they were fasted for two or three days, their energy source may shift to lipids and proteins, which are more difficult to be metabolised to CO_2 than carbohydrates. This could

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account for the significantly higher CO_2 production of crabs when they were fasted for one day but not more days than they fed on MPB. CO_2 production of *Carollia perspicilata* was also reported to generally decline over time after feeding (Welch Jr et al., 2016), corroborating with our findings.

5 Conclusions and Implications

Our study tested the hypothesis that intertidal benthos breathe what they eat. This hypothesis is supported by the significantly higher $\delta^{13}C$ of deposit feeders-respired CO_2 than that of detritovore-respired CO_2, and the significant difference in CO_2 production under different feeding regimes due to different carbon content in diet or decomposability of carbon compounds in fuel.

Our study supports the notion that $\delta^{13}C$ of benthos-respired CO_2 can be used to differentiate food categories of the benthos, as has been demonstrated in mammals and other animals. It may be further applied to reveal the contribution of different food sources to their diet over short and long periods when combined with (compound-specific) stable isotope analyses of animal tissues and diets. For partitioning the food sources of intertidal animals, past studies combining stable isotope analyses of animal tissues and their diet have obvious limitations because they can only figure out their integrated food over long periods. However, intertidal benthos (e.g. crabs) may consume endogenous fuels such as lipids which are lower in ^{13}C (Stott et al., 1997) when they are inactive without feeding. There are also differences among endogenous fuels. For example, the difference in $\delta^{13}C$ of synthesised lipids and carbohydrates are assumed to be -3‰ (Carleton et al., 2004). Nevertheless, few studies can exclude the influence of endogenous fuels on the apparent food sources of intertidal benthos when using stable isotope analyses of animal tissues.

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Our study also reiterates the necessity of integrating the influence of benthos feeding regime and size into estimating the contribution of intertidal benthos to CO_2 emission rates from the sediment surface. To date, a few studies have established benthos CO_2 production related to crab mass but are limited to a few families, e.g. ocypodid crabs (Penha-Lopes et al., 2010) and sesarmid crabs in this study. The former study established a significant polynomial relationship between crab CO_2 production and body mass for ocypodid crabs, while we found a significant linear relationship between crab CO_2 production and size (i.e. crab carapace width/length and gastropod shell length) for sesarmid crabs. It highlights that the relationships between benthos CO_2 production and size indicators (i.e. weight or size) may be different depending on the benthos taxa or families. Therefore, caution should be exercised in estimating benthos CO_2 production from size indicators based on relationships developed for other benthos taxa. Moreover, our study demonstrates the importance of considering the activity status of the animals when estimating benthos CO_2 production; in particular, a significant consideration for up-scaling estimates on the contribution of benthos CO_2 production to system sediment CO_2 emission rates. The same benthic animals may forage alternatively on MPB and mangrove litter/detritus, as well as switch between active-feeding and fasting over long periods.

Code availability

Computer codes are available upon request sent to Xiaoguang Ouyang.

Authors' contributions

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370 XO and SYL designed the study. XO conducted field survey, laboratory experiment and data
371 analysis, and wrote the manuscript revised by SYL. CYL contributed to data acquisition and
372 analysis.
373
374 **Competing interests**
375 The authors declare that they have no conflict of interest.
376
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Figure legends

Fig.1 A conceptual model showing the process of measuring CO_2 production and $\delta^{13}C$ of respired CO_2 by CRDS: the closed container, a sesarmid crab, the syringe used to collect gas from the container, a stop-cock, and CRDS. CRDS denotes cavity ring-down spectroscopy.

Fig. 2 The variation of $\delta^{13}C$ of benthos-respired CO_2 with different benthos taxa (a), dominant feeding habits (b) and diets (c), as well as C/N of diets (d). Bars with different letters have significantly different values.

Fig. 3 The variation of $\delta^{13}C$ of crab-respired CO_2 with different feeding regimes. Bars with different letters are significantly different.

Fig. 4 The variation of the rate of crab CO_2 production with different feeding regimes. MPB denotes microphytobenthos. Bars with different letters have significantly different CO_2 production.

Fig. 5 The relationships between crab CO_2 production and carapace width (a), and carapace length (b), as well as between gastropod CO_2 production and shell length (c).

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502 Fig. 1

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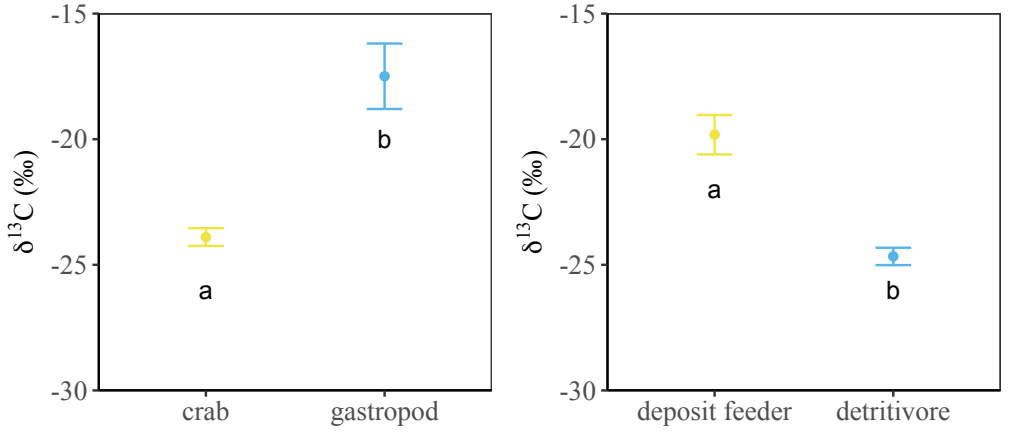
504 Fig. 2

505 a) b)



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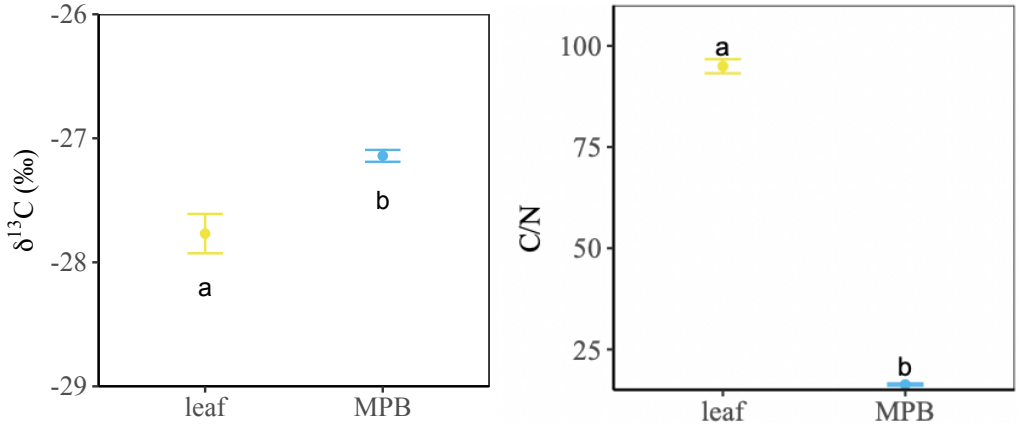
507 c) d)



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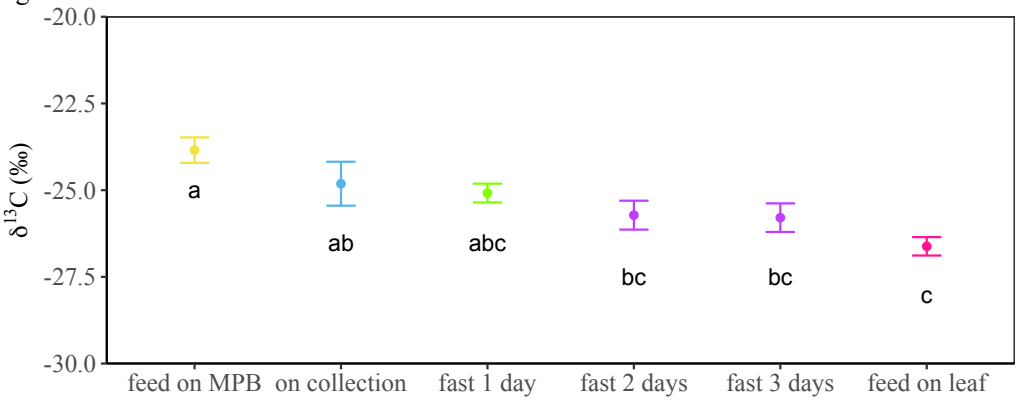
522 Fig. 3



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524 Fig. 4
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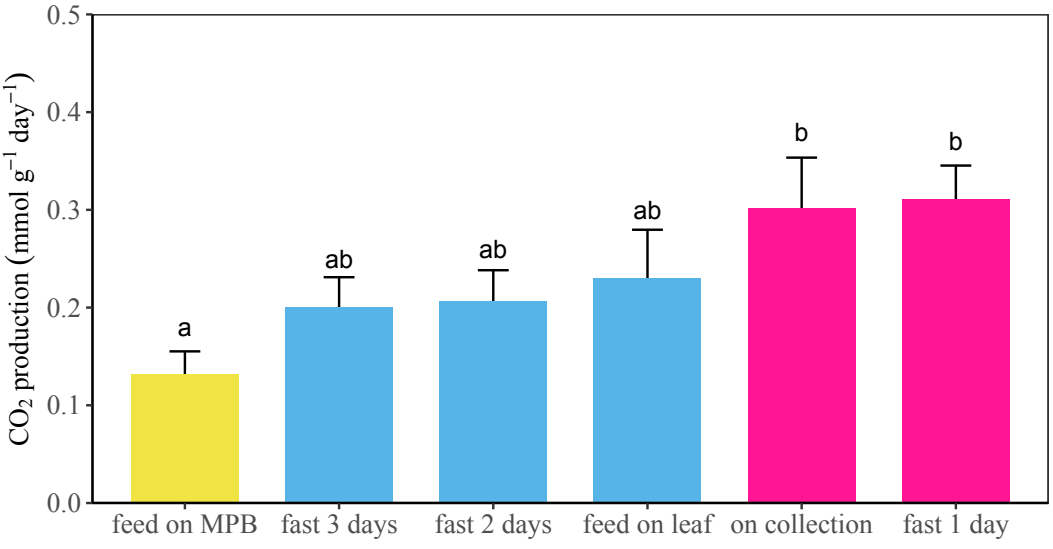

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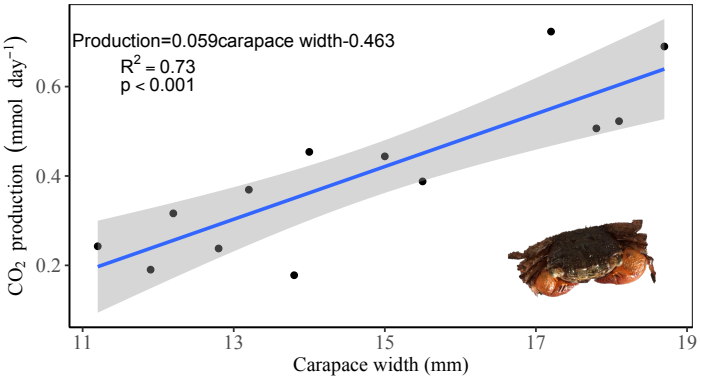
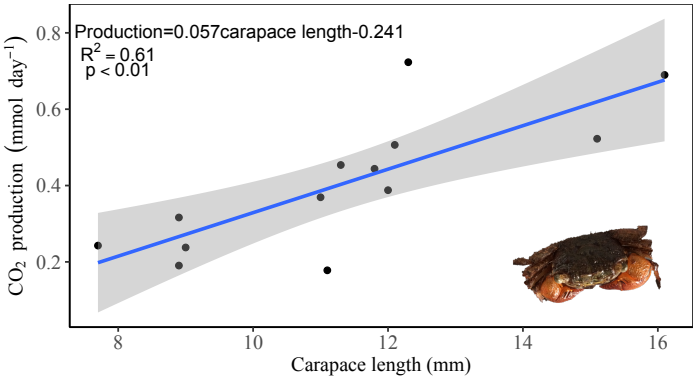
Fig. 5

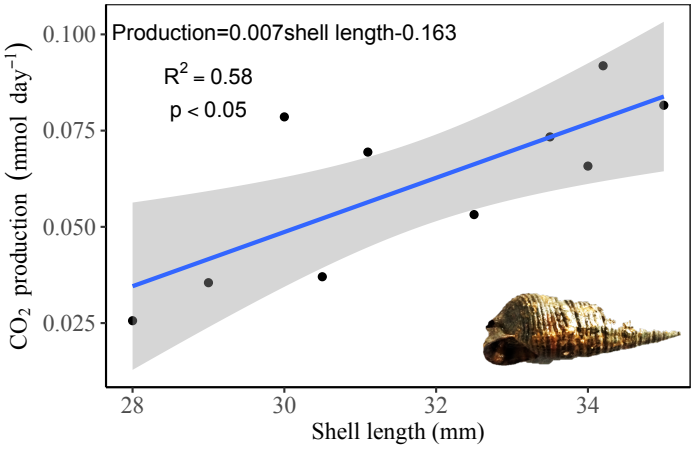
a)



b)



c)



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568 Table 1 Isotopic fractionation between mangrove animal respired CO_2 and their diets. MPB
569 denotes microphytobenthos.
570

Benthos taxa		Diet		Isotopic fractionation	
$\delta^{13}C$ of crab respired CO_2	$\delta^{13}C$ of gastropod respired CO_2	$\delta^{13}C$ of MPB	$\delta^{13}C$ of yellow leaves	$\Delta^{13}C_{benthos\text{-}MPB}$	$\Delta^{13}C_{benthos\text{-}leaves}$
-23.9±0.4‰	-17.5±1.3‰	-27.1±0.05‰	-27.8±0.2‰	3.2‰[a], 9.6‰[b]	3.9‰[a]
Feeding habit		Diet		Isotopic fractionation	
$\delta^{13}C$ of deposit-feeder respired CO_2	$\delta^{13}C$ of detritivore respired CO_2	$\delta^{13}C$ of MPB	$\delta^{13}C$ of yellow leaves	$\Delta^{13}C_{deposit\text{-}feeder\text{-}MPB}$	$\Delta^{13}C_{detritivore\text{-}leaves}$
-19.8±0.8‰	-24.7±0.3‰	-27.1±0.05‰	-27.8±0.2‰	7.3‰	3.1‰

571 [a] crab, [b] gastropod