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A novel pathway of nutrient absorption in crustaceans: branchial amino acid uptake in the green shore crab (*Carcinus maenas*)

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Estuaries are environments enriched with dissolved nutrients such as amino acids. To date, marine arthropods are the only invertebrate group that have not been demonstrated to access this potentially important nutrient resource. Using *in vitro* gill perfusion techniques, we sought to investigate the ability of the green shore crab (*Carcinus maenas*) to take up the amino acid L-leucine directly from the water. Investigation of the concentration-dependent transport kinetics of radiolabelled L-leucine showed that there are two specific transport pathways across *Carcinus* gills, one with high affinity and low capacity, and the other with high capacity and low affinity. Using putative competitive substrates and reduced sodium preparations, we were able to identify the putative amino acid transport system associated with high-affinity uptake. This is the first study to demonstrate the absorption of dissolved organic nutrients across the gill epithelium of a marine arthropod.

1. Introduction

Marine arthropod species, such as the green shore crab (*Carcinus maenas*), are well adapted to the harsh environmental conditions that may prevail in estuaries. Although this species is an osmoconformer in full-strength seawater (internal osmolarity of 1050 mOsm), it is an excellent osmoregulator in dilute waters where it maintains an internal osmolarity of 650 mOsm [1]. This allows occupation of estuaries that fluctuate in salinity as a result of freshwater inputs and tidal flows. Estuaries are also key locations for the concentration of aquatic contaminants such as dissolved metals, and *Carcinus* has been shown to be relatively tolerant of common estuarine toxicants [1–3]. Environmental concentrations of dissolved amino acids in seawater are in the range 0.1–1 µM, while in estuaries measured concentrations of amino acids can be as high as 1–2 µM [4,5]. These relatively high levels arise from phenomena such as riverine inputs, tidal action on phytoplankton and hydrological processes such as entrainment [6,7]. Previous reports have shown that many soft-bodied marine invertebrates [8–10], and a single primitive vertebrate [11], are able to take up nutrients via the skin, while hard-bodied animals such as mussels can use their gills for this purpose. By taking advantage of localized elevations in dissolved organic nutrients, these species have been able to maximize energy resources in highly competitive feeding environments, such as estuaries.

Currently, 13 phyla and 18 classes of aquatic biota have displayed the ability to uptake amino acids across the integument. Arthropods, owing to their calcified exoskeleton and relative impermeability to solute and water flux [12], are not represented among groups with this ability. However, the gills of marine arthropods are an important transport surface, performing vital roles in physiological processes such as gas and ionic exchange [1,13,14]. Given the fine diffusive distance, the significant surface area and the high perfusion of the branchial surfaces with haemolymph, the gills are ideally suited as a site of dissolved nutrient uptake. Indeed, in a study where isolated gills from the crab species *Neohelice granulatus* were bathed in a medium containing radiolabelled amino acids,

tissue accumulation was measured. However, due to limitations in the methodological approach used, it was unable to be determined if transport was occurring or if the accumulation was non-specific and resulted from isotope trapped in the tissue after being bathed in isotope solution [15]. Therefore, the hypothesis that the gills of crabs transport amino acids has not been adequately tested.

The current study sought to investigate the ability of the green shore crab to take up the amino acid L-leucine directly from the water. This amino acid is one of the most enriched in estuarine settings [4]. A perfused gill technique was used to characterize transport kinetics of L-leucine (maximal rate of transport, J_{max}; transport affinity, K_m), and how transport is impacted by sodium (Na^+) gradient and putative transport competitors. Integumentary transport of amino acids is tied to external Na^+ concentrations [6], and as characteristics of the integumentary transport systems in invertebrates are generally similar to those of mammalian tissues [6], putative competitive substrates were based on known affinities of mammalian leucine transport systems [16].

Furthermore, the gills of *Carcinus* are known to be functionally heterogeneous, with anterior gills (pairs 2–5) being mainly respiratory in function, while posterior gills (pairs 6–9) are primarily ionoregulatory in function (e.g. [14]). Thus, the ability of gills to take up waterborne L-leucine was examined in both an anterior and a posterior gill, with posterior gills hypothesized to be more involved in amino acid transport owing to their greater importance in ion transport, a putative driver of amino acid uptake. This study provides the first evidence for the absorption of organic nutrients directly across the gills of a marine arthropod and also provides the first evidence for organic nutrient uptake by a surface other than the digestive system in this group.

2. Material and methods

(a) Animal collection

Green shore crabs (*C. maenas*) were collected by baited traps from Pipestem Inlet in Barkley Sound (Vancouver Island, BC N49°02.274–W125°20.710 and N49°01.749–W125°21.515) under a licence (XR202-2016) from Fisheries and Oceans Canada. They were transported to flow-through seawater holding facilities at Bamfield Marine Sciences Centre (BMSC). Animals were held in large 2000 l outdoor tarpaulin-covered tanks, subjected to natural summer lighting and water temperature (13°C). Crabs (mean mass (±s.e.m.): 70 (± 2.3) g; mean carapace length: 5.6 (± 0.4) cm) were fed every 3 days with salmon heads. Before experimentation, animals were removed from the outdoor tanks and placed in a carbon-filtered recirculating aerated seawater tank (60 l) at 18°C and allowed to acclimatize for 1 week. Water chemistry was as follows: salinity 32 ppt; pH 8.1; ion composition (in mM): Na^+, 475; K^+, 11; Ca^{2+}, 10; Mg^{2+}, 47; Cl^-, 515 [2]).

(b) Gill perfusion

Two gills were used to examine amino acid uptake. Gill 8 (a true posterior gill) was used for inulin trials, kinetic characterization, sodium dependence and amino acid blocker experiments. Gill 5 (an anterior gill) was used for uptake at 50 µM only. To avoid pseudo-replication, only a single gill 8 or 5 was used from an individual crab for each test solution. Crabs were fasted for 48 h before experimentation and then anaesthetized for 15 min on ice, followed by a rapid euthanization by a single spike to the ventral ganglion through the ventral wall of the carapace. The carapace was then removed and gills were dissected and placed in a Petri dish containing Bamfield seawater. All perfusion experiments were then performed using a method described by Siebers *et al.* [17] and Blewett *et al.* [2], using a peristaltic pump (Sci 323 Watson-Marlow Bredel Pump, Falmouth, England). Afferent and efferent haemolymph ducts were cannulated with a PE90 cannula (Intramedic, Clay Adams) and secured in place with a gill clip (electronic supplementary material, figure S1). Filtered seawater (see composition above) was used as the radiolabelled external/bathing medium. The artificial gill perfusion solution was made to resemble *Carcinus* haemolymph (see [2]) (in mM): 470 NaCl, 12 $CaCl_2$, 12 $MgCl_2$, 11 KCl, 9 $NaHCO_3$, 0.1 NH_4Cl, 0.3 glucose, 0.1 glutathione, 0.5 glutamine; pH 7.9. Gills were perfused at a rate of 130 ± 1.5 µl min^{-1} (based on the weight of the efferent perfusate) for 1 h. For each experiment the perfusate was collected in approximately 1 ml fractions, with the first fraction discarded, owing to the presence of the non-labelled perfusate, which would lead to dilution of the isotope signal.

Initial trials used the permeability marker ^{3}H-inulin (added to the bathing medium at 0.5 µCi/50 ml; PerkinElmer, Boston, MA, USA) to test the integrity of the preparation. Gills (gill 8) were suspended in 50 mL of inulin bathing solution and perfused for 1 h. ^{3}H-inulin levels collected from the efferent perfusate remained below background levels in all ($n = 4$) preparations examined (data not shown).

Thereafter, the concentration dependence of leucine uptake in gill 8 was examined at concentrations of 0, 0.5, 1, 2, 5, 10, 50, 100 and 500 µM L-leucine. Radiolabel (PerkinElmer, Boston, MA, USA) was added to 50 ml of bathing solution at a level of 1 µCi ml^{-1} for each L-leucine concentration. Uptake in each gill was tested at a single concentration, with 4–6 replicate gills (from different animals). Radiolabel appearance in perfusates was then quantified as described below.

Following determination of concentration-dependent L-leucine uptake kinetics, an assessment of the putative transport pathways for L-leucine was investigated. The putative transport competitors were other amino acids known to be substrates of transporters used by L-leucine in mammalian tissues [14]. In all studies, L-leucine at 2 µM was added to 50 ml of bathing solution with ^{3}H L-leucine (1 µCi ml^{-1}; PerkinElmer, Boston, MA, USA). Then, excess of D-leucine, L-threonine or L-lysine (to give a final concentration of 20 µM, Sigma-Aldrich) were added to the bathing medium, and perfusions were conducted, as described above, in a posterior gill (gill 8). To test the sodium dependence of L-leucine transport, a low-sodium artificial haemolymph was made by replacing sodium chloride with choline chloride (Sigma-Aldrich) at a concentration of 470 mM to maintain osmolality. Again, each replicate ($n = 4$–6) represents an individual gill tested under one exposure scenario.

To compare transport between an anterior and posterior gill, the transport of 50 µM L-leucine radiolabelled with ^{3}H-L-leucine (1 µCi ml^{-1}) was examined in an anterior gill (gill 5; $n = 4$–6) and compared to perfusion conducted at the same concentration in gill 8. Perfusions were conducted as described above.

(c) Determination of uptake

Collected perfusates (total volume minus the first 1 ml) were taken and a 1 ml subsample was added to 4 ml of ACS liquid scintillant (GE Healthcare, Amersham, UK). All samples were held in the dark for 12 h before counting (LS6500: Beckman Counter, Fullerton, CA, USA). Quench correction was performed based on the external standards ratio.

For calculation of uptake, the appearance of the isotope in the perfusate (counts per minute (CPM)) was converted into an amino acid concentration based on specific activity (SA), determined based on the recorded stock solution concentrations. This concentration was then converted into an amount of amino acid perfused over time by accounting for the total volume of perfusate collected

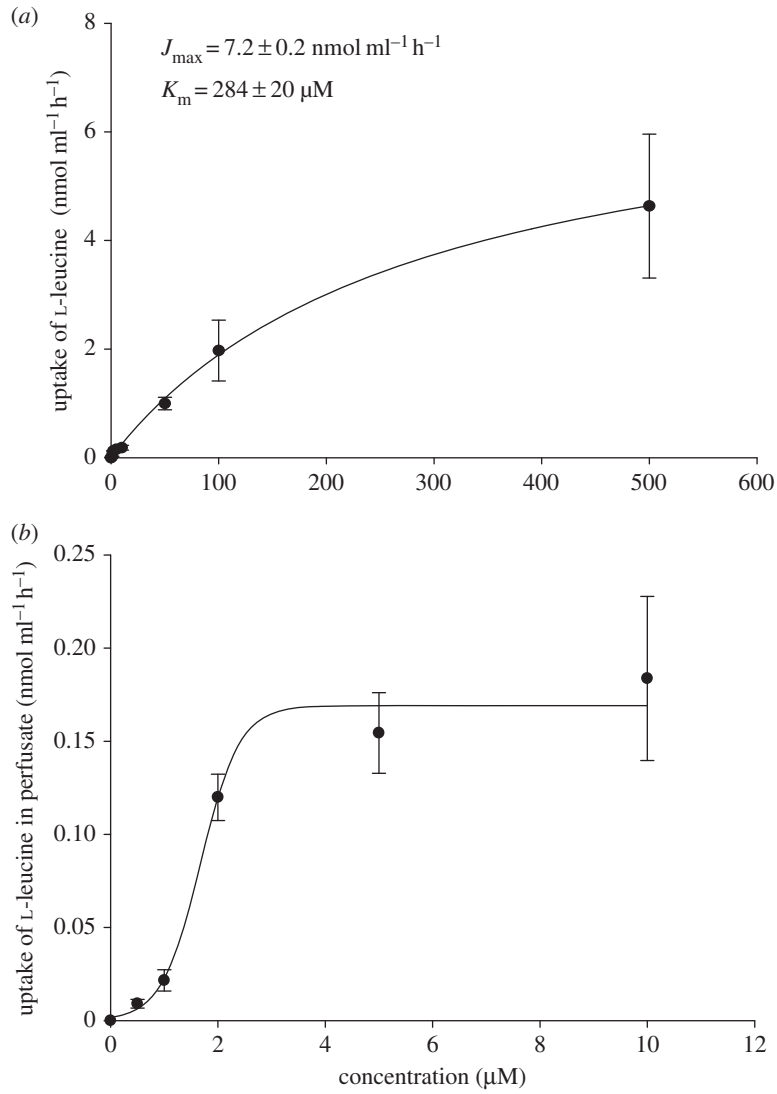

Figure 1. Concentration-dependent ^{3}H-L-leucine uptake in the posterior gill (8) of *Carcinus maenas* across (*a*) the entire substrate concentration range (0.5 to 500 µM) and (*b*) substrate concentrations from 0.5 to 10 µM, as determined via a gill perfusion technique. Values represent the mean ± s.e.m. of four to six replicates. Fitted lines were modelled on raw data using SigmaPlot (11.0).

(v) and time of perfusion (t). Data are expressed as nmol ml^{-1} h^{-1}:

amino acid uptake: $\frac{\text{CPM}}{\text{SA}} \times \frac{1}{V} \times \frac{1}{t}$.

(d) Data analysis and statistics

Concentration-dependent L-leucine uptake was analysed via an iterative curve-fitting analysis using SigmaPlot version 11.0 with SigmaStat integration v. 3.5 (Systat Software, San Jose CA, USA). Curves were best fitted to either a sigmoidal or a hyperbolic (Michaelis–Menten) distribution according to the equations below:

sigmoidal distribution: $y = \frac{a}{1 + e^{\left(-x - \frac{x_0}{b}\right)}}$

hyperbolic distribution: $y = \frac{a \times x}{a + b}$

where x is the leucine concentration, a is the maximal rate of transport (J_{max}), and b (hyperbolic) or x_0 (sigmoidal) is the leucine concentration resulting in half the maximal rate of transport (K_m).

All other data were subjected to a one-way analysis of variance (ANOVA) followed by a Tukey *post hoc* test to determine significant differences between treatments. All data have been expressed as means ± s.e.m. (n values), and significance for all tests was accepted at $\alpha = 0.05$.

3. Results

Uptake across gill 8 was best characterized by two concentration-dependent curves (figure 1*a*,*b*). When including all L-leucine exposure concentrations, there was a gradual increase in uptake as substrate concentrations of L-leucine increased, eventually reaching a saturation whereby further increases in L-leucine resulted in only minimal further increases in L-leucine transport (figure 1*a*). This hyperbolic curve facilitated calculation of transport kinetic parameters, which resulted in a J_{max} of 7.2 ± 0.2 nmol ml^{-1} h^{-1} and a K_m of 284 ± 20 µM ($R^2 = 0.70$). However, when examining low exposure concentrations of leucine (up to 10 µM), a second concentration-dependent curve was obtained. The best-fit relationship between substrate concentration and uptake was sigmoidal ($R^2 = 0.80$ versus $R^2 = 0.76$ for a hyperbolic curve; figure 1*b*). This curve displayed a maximal uptake rate of 0.17 ± 0.01 nmol ml^{-1} h^{-1}, and a substrate concentration giving 50% of this maximal uptake at 1.7 ± 0.2 µM.

There was a significant effect of sodium on L-leucine transport. Reducing sodium in the perfusate led to a significant 79% decrease in L-leucine transport (figure 2*a*; $p < 0.05$).

The presence of putative transport competitors D-leucine, threonine and lysine all decreased transport of L-leucine

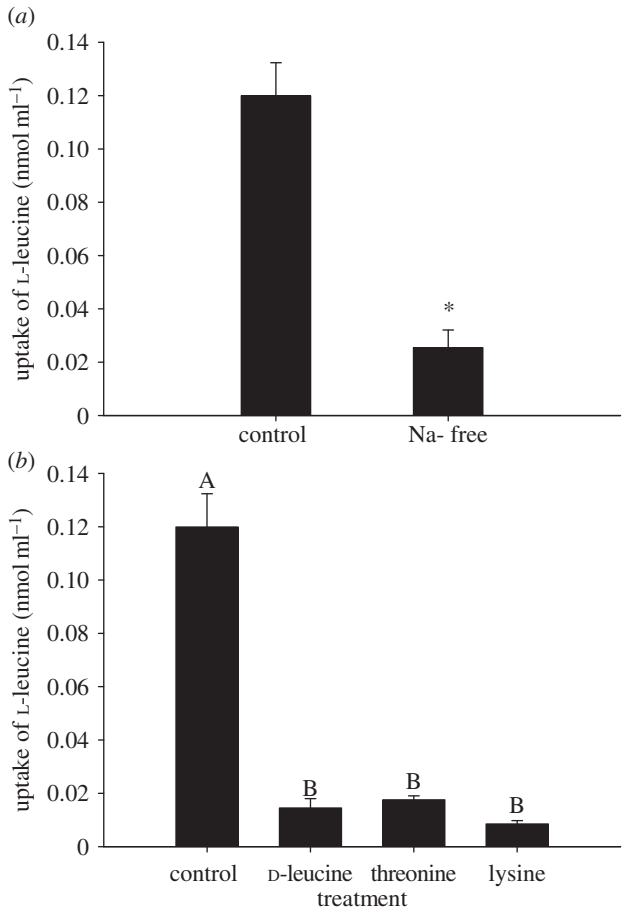

Figure 2. Effect of (*a*) replacing sodium chloride with choline chloride in the perfusate and (*b*) the presence of 10-fold higher concentration of putative competitive substrates (D-leucine, threonine and lysine) on branchial uptake (gill 8) of ^{3}H L-leucine in the green shore crab (*Carcinus maenas*). Bars represent mean ± s.e.m of four to six replicates. Bars sharing letters are not significantly different at $\alpha = 0.05$ using a one-way ANOVA followed by a Tukey *post hoc* assessment.

(figure 2*b*). The largest decrease of L-leucine occurred with lysine in the solution (a 93% decrease from control uptake values; $p < 0.05$). The presence of D-leucine and threonine significantly reduced uptake rates by 88 and 86%, respectively (figure 2*b*).

There was a significant difference in L-leucine uptake between the anterior gill (5) and the posterior gill (8). Gill 8 displayed a threefold higher rate of uptake than the anterior gill, at an L-leucine concentration of 50 µM (figure 3).

4. Discussion

The current study demonstrates that the crab gill is capable of absorbing amino acids (in this case L-leucine) directly from seawater. This is the first demonstration of direct waterborne nutrient acquisition across the gills in arthropod crustaceans.

(a) Branchial transport of amino acids

Branchial transport of L-leucine displayed saturable concentration-dependent kinetics indicative of a specific carrier-mediated pathway. The presence of saturation indicates that movement of this amino acid was not from simple diffusion, and that there are therefore specific mechanisms by which L-leucine is absorbed across the gills of *Carcinus*.

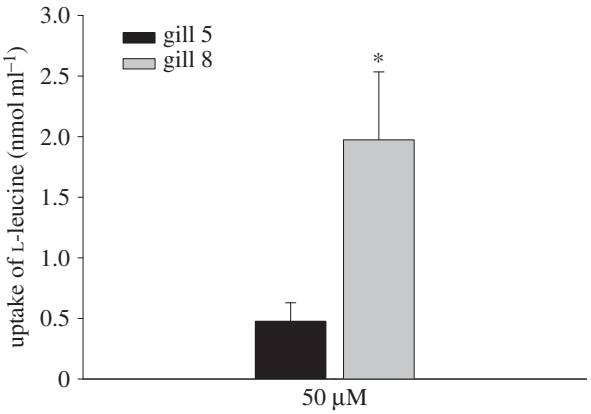

Figure 3. Uptake of ^{3}H L-leucine at a concentration of 50 µM in posterior (8) and anterior (5) gills of *Carcinus maenas* using an *in vitro* gill perfusion techniques. Significant differences (*) in uptake between the gills were assessed using a Student's two-tailed *t*-test with $\alpha = 0.05$. All data are represented as means ± s.e.m. of four to six replicates.

Curve-fitting analysis showed two transporter pathways: a high-affinity, low-capacity pathway over substrate concentrations less than 10 µM, and a high-capacity, low-affinity pathway, when all substrate concentrations were examined (0.5–500 µM). Given the relative concentrations of dissolved amino acids in the environment (see below), only the high-affinity pathway is likely to be of physiological significance. The substrate concentration at which transport is approximately half of the maximal rate of uptake through this high-affinity pathway in *Carcinus* gills (1.7 µM) is comparable with similar affinity values determined in other marine animals. The marine mollusc *Mytilus californianus*, which obtains 75% of its nutrients via the gills [17], displays a branchial affinity value of 1–4 µM for L-leucine [18]. Juvenile oysters (*Ostrea edulis*) have a lower affinity for amino acids with K_m values at 15–20 µM [19], but this is still within the scope of the value obtained for *Carcinus* in the current study.

(b) Delineating the amino acid transporter

The specific transport systems that achieve amino acid uptake in crustaceans are not well described. However, the relatively conserved mechanisms of uptake between invertebrate systems studied to date and mammalian amino acid transport [6,20,21] suggests that the transport specificities of mammalian amino acid transport systems could be used to identify the putative L-leucine transporters in *C. maenas*. In mammals, L-leucine is transported via one of four solute carrier systems [15]. System $y^{+}L$ is a Na^{+}-dependent antiporter, and in addition to L-leucine, also transports L-lysine. System L consists of antiporters that can transport threonine and L-leucine, while system $b^{0,+}$ transports threonine and lysine, in addition to L-leucine. Finally, system $B^{0,+}$ is a $2Na^{+}/1Cl^{-}$/AA co-transporter which transports L-leucine and weakly transports threonine and lysine. Our results showed that both L-threonine and L-lysine reduced uptake of L-leucine by 86%, when present in a 10-fold excess (figure 3). Based on the L-leucine concentration used in this study (2 µM; close to the K_m of the high-affinity pathway), and the fact that both putative competitors significantly inhibited L-leucine uptake, the most likely candidate for achieving L-leucine uptake via the crab gill is therefore an analogue of system $b^{0,+}$.

However, arguing against this is the fact that L-leucine transport exhibited sodium dependence. Replacing the NaCl in the

artificial haemolymph perfusate with choline chloride led to a 79% reduction in transport (figure 2). System $b^{0,+}$ in mammals is sodium-independent [16], and thus if this was the main transporter achieving branchial L-leucine uptake in *Carcinus*, a relative insensitivity to sodium should have been noted. It is notable, however, that system y^+L is a sodium-dependent antiporter, such that if this transporter was achieving L-leucine uptake in *Carcinus*, then the reduction in perfusate sodium would have been expected to reduce the absorption of the amino acid, similar to the pattern observed in the current study. However, in mammals, this system is not known to transport threonine [16]. It is therefore possible that one of these systems is responsible for high-affinity L-leucine uptake, but that the characteristics of the transport system do not correspond exactly to those described for mammalian epithelia.

The finding that D-leucine decreased the uptake of L-leucine confirmed the presence of a specific transport pathway. Previous studies have shown that D-leucine inhibits 95% of L-leucine flux through brush-border membrane vesicles prepared from whole *Chironomus* larvae [22]. This effect can be mediated by direct competition, with both D- and L-amino acids being substrates for a transport pathway, or through blockage of the transporter binding site by the stereoisomer [23]. Either mechanism is indicative of a specific transport pathway for the amino acid, and is consistent with the findings of sodium dependence and inhibition by other putative substrates in the current study for L-leucine uptake across the gills of *C. maenas*.

(c) Ionoregulatory versus respiratory gills

There are two functional types of epithelial cells in the gill—respiratory cells characterized by a thin epithelium, and ion-transporting cells characterized by a relatively thick epithelium [13]. In *C. maenas*, the gills are heterogeneous with the four posterior gills (numbers 6–9) displaying thicker cells and having primary roles in ionoregulation, while the anterior four sets of gills are primarily respiratory surfaces with thinner epithelia [14]. Considering that amino acid transport is strongly tied to electrochemical gradients and osmoregulation, it was hypothesized that the posterior gills would have higher uptake rates than the anterior gills. Uptake was evident in both gill types; however, the posterior gills transported L-leucine at a rate threefold greater than that of the anterior gills (figure 3). This pattern is in accordance with other ion-linked transport processes in crustaceans. For example, nickel transport is fourfold higher through posterior than anterior gills of *C. maenas* [2].

(d) Environmental context

Many invertebrate species have been shown to transport amino acids via their integument. In most species in which this phenomenon has been described, the integument is 'soft'; and therefore thought to be relatively amenable to environmental exchange. Crab species have a hardened exoskeleton that probably contributes to their relative impermeability to salts and water, and thus plays an important role in their ability to osmoregulate in the fluctuating salinities of estuarine waters. However, even with the thin cuticle covering the gills, these structures are ideal transport surfaces. Given their high flow of water, significant perfusion of haemolymph, large surface area and small diffusive distances, the gill surface is clearly able to extract low concentrations of amino acids from the water. Green shore crabs have a transport pathway that is half-saturated at 1.7 µM, a value within environmental levels (1–2 µM), suggesting that transport of this substrate across the gills is environmentally relevant. It is also worth noting that under conditions where the crab is actively osmoregulating (e.g. in dilute salinities), ion-uptake mechanisms that might facilitate amino acid uptake are likely to be more active. Thus the current study, conducted under osmoconforming conditions, may be a conservative estimate of dissolved amino acid uptake capacity in *C. maenas*.

Ethics. All procedures were approved by the Bamfield Animal Research Ethics Board and were in accordance with the Guidelines for the Canadian Council on Animal Care.

Data accessibility. The data used for this study have been deposited in Dryad as http://dx.doi.org/10.5061/dryad.h7kt0.

Authors' contributions. T.A.B conceived the study design, carried out all experimental as well as statistical analyses, and wrote the manuscript. G.G.G. coordinated the study and helped draft the manuscript. All the authors gave their approval for publication.

Competing interests. We have no competing interests.

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