Leucine transport across plasmamembranes from the scud *Echinogammarus stammeri* (Amphipoda: Gammaridae)

E. Berra, M. Forcella, R. Giacchini, P. Parenti*

Department of Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1 - 20126 Milano, Italy

The freshwater scud *Echinogammarus stammeri* is a very abundant benthic crustacean in riverine systems of northern Italy. The molecular physiology of L-[3H]leucine transport across cell membranes of *E. stammeri* was investigated. At pH 7.4, the presence of a NaCl gradient stimulated the leucine transport rate, when compared with KCl gradient. By contrast, alkaline pH strongly inhibited transport activity. In NaCl medium at acidic pH, leucine uptake displayed a specific activity 2.1-fold lower than that measured at neutral pH. Analysis of the uptake as a function of external leucine concentration revealed saturation kinetics. Leucine uptake appeared broadly unspecific and it was much strongly inhibited by the non-polar amino acids phenylalanine, methionine, and BCH, as well as by hydrophobic hydroxyacids. These results revealed important differences with respect to amino acid transport systems described in marine crustaceans. Their relevance in the feeding adaptation of the scud is briefly discussed.

Keywords : amino acid transport, brush border membrane vesicles, *Echinogammarus stammeri,* Gammaridae

Introduction

The crustacean digestive system consists of three main divisions: foregut, midgut, and hindgut. Early studies with radioactively labeled substances have demonstrated their specific role in overall nutrient absorption (Speck & Urich 1970). During the last two decades several studies have been performed on the crustacean gastrointestinal tract to examine the properties of nutrients transport and the physiology of the absorption of sugar, amino acids, and inorganic ions (Ahearn 1974, 1982, 1987). The hepatopancreas, a large multilobate diverticulum of the midgut with a single layer epithelium, has been proved to be responsible for the bulk of nutrient absorption in crustaceans (van Weel 1955, Wright & Ahearn 1997). Application of membrane vesicle technique to lobster hepatopancreas has resulted in purified brush border membrane fractions able to reproduce transport activities of sugars and amino acids (Ahearn et al 1985, 1986, Ahearn &

* Corresponding author :
E-mail: paolo.parenti@unimib.it

Clay 1988). These investigations, while confirming previous studies on perfused intestinal preparations (Maginnis 1977), added new data on the molecular physiology of transport mechanisms in crustaceans. In particular, they revealed the presence of several Na^+-dependent and Na^+-independent amino acids transport processes, which may or may not include chlorine ions as cosubstrates and all are strongly stimulated by acidic pH (Ahearn 1987). Thus, a model for nutrient transport, which differs from the vertebrate model has been proposed: the acidic luminal pH of crustaceans substantially modifies substrate charge resulting in substrate specificities which are considerably different between crustaceans and vertebrates. This model was mainly based on studies performed on the Atlantic lobster *Homarus americanus* and few additional decapod species (Wright & Ahearn 1997). No information is currently available from other crustacean taxa.

Amphipods are epibenthic peracarid crustaceans widespread on earth and very common in marine, brackish and fresh waters, and in a wide range of groundwater environments. In freshwaters they represent an important source of food for larger species, mostly fishes, and include several species that are able to tolerate or-

ganic contaminants, so that gammarids have been considered for development of specific biochemical biomarkers (McLoughlin et al. 2000). The gammarid *Echinogammarus stammeri* (Karaman 1931) is widely distributed in the Alpine region of northern Italy, slightly extending into the Apennine peninsula and crossing the border into France, Switzerland, and Slovenia. As most other scuds this species up benthic habits, it feeds on coarse particulate material of different origin and, consequently has an important role in detritus processing and nutrient cycling in freshwater ecosystems (Karaman 1993). While data about nutrient absorption in other macrobenthic invertebrates are available (Parenti et al. 2001, Forcella et al. 2004), nothing is known on freshwater crustaceans. Cell membranes purified in form of closed vesicles are a useful tool in the characterization of molecular features of transport systems (Lucke et al. 1978, Ahearn, 1987, Parenti et al. 2000). However, species of relatively small size, such as *E. stammeri,* have been excluded from these studies. Therefore, in order to add new data on comparative physiology of nutrient absorption in freshwater Crustacea, an investigation on the properties of leucine transport across purified plasmamembranes from *E. stammeri* was undertaken.

Material and methods

Animals

Specimens of *E. stammeri* (length > 5 mm) were collected at several localities along the Ticino River, northern Italy, maintained in large vessels at 6 ± 1 °C, and fed *ad libitum* with Tetramin® for 1-2 weeks before use for experiments.

Chemicals

L-[4,5-^{3}H]-leucine 152 Ci/mmol was purchased from Amersham Pharmacia Biotech. Leucine, HEPES (4-(2-hydroxyethyl)piperazine-1-propansulfonic acid), Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol), enzyme, substrates, and coenzymes were from Sigma-Aldrich.

Brush border membrane preparation

Membrane vesicles were prepared using the method described by Wolfersberger et al. (1987) with minor modifications. Briefly, animals (roughly 3 g per experiment) were suspended with 9 volumes of a hypotonic Tris buffer (Tris-HCl 17 mM, pH 7.4, mannitol 100 mM, EGTA 5 mM) and homogenized at 4°C in a glass and teflon Potter-Helvehjem, two 9-strokes at 3,000

rpm, separated by 2 min in ice. The suspension was then filtered through two layers of surgical gauze. An equal volume of 24 mM $MgCl_2$ was added to the recovered volume. The resulting mixture was thoroughly stirred for 15 min in an ice bath and then centrifuged at 2,500 g for 15 min at 4°C. The resulting pellet was discarded and the supernatant centrifuged at 48,000 g for 20 min at 4°C. The pellet was resuspended in 0.5 homogenate volume of cold Tris buffer and homogenized in a glass and teflon Potter-Helvehjem, two 6-strokes at 2,000 rpm, separated by 1 min in ice. After addition of an equal volume of 24 mM $MgCl_2$, the mixture was stirred for 15 min in an ice bath and centrifuged as above at low speed and then at high speed. The final pellet was resuspended in 1 vol of initial buffer, homogenized in Potter-Helvehjem as before, and centrifuged again at 48,000 g for 20 min at 4°C. The obtained pellet was resuspended in HEPES-Tris 10 mM, pH 7.4, mannitol 100 mM, and represented the membrane preparation used for transport experiments. The recovery was about 0.8 mg membrane proteins/g fresh individuals. For transport experiment, the suspension was made at a protein concentration of 2-3 mg/ml as assayed according to Bradford (1976) using bovine serum albumin as standard.

Transport experiments

Amino acid uptake studies were performed at 22°C by incubating 10 µl of freshly prepared vesicles and 40 µl of an appropriate radioactive cocktail, including L-^{3}[H]-Leucine (30-90 µCi/ml), as reported in the figure legends. Uptake studies were performed by a rapid filtration technique described elsewhere (Parenti et al. 1992). Incubation was stopped by the addition of 50 volumes of ice-cold stop solution (150 mM NaCl, 10 mM HEPES-Tris, pH 7.2) and the overall solution was filtered under vacuum through pre-wetted mixed-esters cellulose filter (0.45 µm pore size, Micro Filtration System, Dublin, California). Filters were quickly rinsed with 10 ml of ice-cold stop solution and filter-associated radioactivity was counted in a scintillation spectrometer Tri-Carb, Packard, model 300 C. Each experiment was performed in triplicate and repeated at least three times with different membrane preparations. Uptakes were expressed as nmol/mg of membrane proteins. Experimental data are given with ± SE and analyzed by computer using a multiparameter, iterative, non-linear regression program based on the Marquardt-Levenberg algorithm (Sigma Plot, Jandel, CA).

pH measurements in vivo

To estimate the lumen pH (Zhuang et al. 1999), individual gammarids were dissected alive on a microsco-

pic slide, the intestine was isolated and examined under a Leica stereomicroscope adding one of the following indicators (in powder to not dilute body fluids): phenolphthalein, thymol blue and cresol red. Segments were compared visually with standards prepared in step of approximately 0.5 pH units.

Results

Purity of membrane preparation from whole *E. stammeri* prepared by magnesium precipitation was assessed by comparing the activity of the membrane-bound enzyme leucine aminopeptidase (LAP) in whole homogenate and in final membrane suspension. LAP specific activity was enriched significantly over the specific activity in the whole animal homogenate. The values were 0.6 ± 0.1 mU/mg and 6.2 ± 0.2 mU/mg in crude extract and in membrane preparation, respectively. In contrast cytochrome c oxidase did not exhibit increased activity in the enriched membrane fraction. This result indicates that the preparation was mainly represented by brush border membranes of gut origin. The yield was 0.34 ± 0.04 mg of membrane proteins per gram of wet weight.

The incubation of the membrane preparation from *E. stammeri* in a medium containing tracer amount of labeled substrates demonstrated that these membranes are constituted by closed vesicles able to take up the labeled compounds as it has been demonstrated by similar preparations made from mammalian intestine (Lucke et al. 1978), crustacean hepatopacreas (Ahearn et al. 1986) or insect midgut (Parenti et al. 1992). The presence of closed vesicles is believed whenever a so-called "overshoot" phenomenon is observed, i.e. a transient accumulation of labeled compound at short time (15-60 sec) of incubation greater than that observed after a prolonged time (60-120 min) of incubation. In preliminary experiments performed in *E. stammeri* vesicles, such an overshoot phenomenon was observed in the presence of labeled amino acid, whereas it was not detected using D-[^{14}C]glucose irrespective of the composition of the incubation buffer (not shown). Maximal effects were observed with L-[^{3}H]leucine as tracer and for this reason we decided to further characterize the transport system in these crustaceans.

Figure 1 shows the time-course of 0.05 mM leucine uptake in the presence of an inwardly directed NaCl or KCl gradient (100 mM$_{out}$/~0 mM$_{in}$) at slightly alkaline pH. After one minute of incubation, in the presence of sodium, but not potassium, the intravescicular amino acid concentration was 6.7 higher than that measured at the equilibrium, which suggests the presence of a highly efficient sodium-dependent carrier-mediated amino acid transport system.

To ascertain whether leucine transport exhibited a requirement for chloride, as demonstrated in other crustaceans, or for more alkaline medium pH a second set of measurements were performed (Fig. 2). Results indicated that in the presence of NaCl the uptake rate is 2-fold higher than that measured in the absence of salts (Fig. 2, column *b* vs *a*) or 1.85-fold higher than that measured in the presence of KCl (Fig. 2, column *b* vs *c*). In the presence of sodium ions, the rate increased, albeit slightly, when Cl$^-$ was substituted by the more lipophilic anion thiocyanate (SCN$^-$), suggesting that uptake was in some way sensitive to changes of membrane potential (Fig. 2, column *b* vs *d*). This effect was not observed in the presence of potassium ions. A similar pattern of cation and anion specificity was obtained when medium pH was shifted at value 8.4, even though, in all conditions, uptake rate were significantly lower than the corresponding rates measured at pH 7.4.

Next, the effect of acidic medium pH on leucine uptake was investigated. When extravesicular pH was reduced from 7.4 to 5.0 a decrease of the specific activity of the sodium-dependent leucine uptake was observed, whereas the cation-independent component, measured in the presence of potassium or in the absence of added salts remained unchanged (Fig. 3). *In vivo* measurements of lumen pH in single individuals emphasized a slightly alkaline pH in the midgut. When cresol red + thymol blue became in contact with the inner content of the midgut the yellow color suddenly changed to a deep violet if the midgut content has a pH close to 8.0. The color change was not much evident in *E. stammeri,* which showed a light blue coloration. Accordingly, phenolphthalein failed to change color, indicating that the lumen pH value was well below 10. These data are in agreement with pH dependence of leucine uptake as measured above.

Uptake of 0.01 mM L-[^{3}H]leucine was a linear function of time from 3 to 20 seconds of incubation, whereas that for 0.5 mM L-[^{3}H]leucine was linear until 10 seconds of incubations (not shown). Therefore, 7 seconds of incubation was chosen as a good estimation of initial rate for studies of the effect of substrate concentration on leucine uptake. Then, transport measurements were corrected for non-specific binding by subtraction a blank uptake made by adding simultaneously the radioactive cocktail and the vesicles suspension to the stop solution. Figure 4 shows the effect of leucine concentration (0.001 to 0.5 mM) on L-[^{3}H]leucine influx from an incubation medium buffered at pH 7.4 and containing 100 mM NaCl. Over the
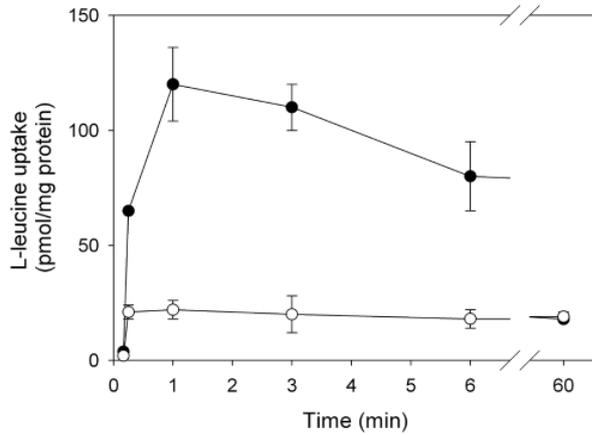
Fig. 1. Time course of leucine uptake into BBMV from *E. stammeri.* One volume of vesicles prepared in 20 mM HEPES-Tris, pH 7.4 and 100 mM mannitol were diluted into four volumes of the same buffer, containing 100 mM NaCl (closed symbols) or 100 mM KCl (open symbols). Incubation buffers included 0.01 mM ^{3}H-leucine. At the indicated times, mixture was blocked by dilution in ice-cold stop solution as described in Material and Methods. Data represents mean ± S.E. of four determinations.
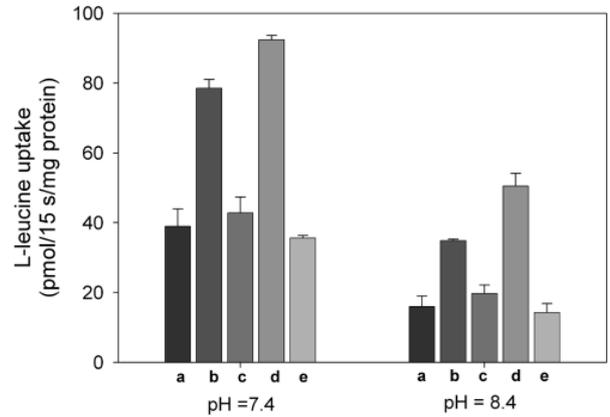

Fig. 2. Effect of alkaline pH on leucine uptake into BBMV from *E. stammeri.* Vesicles prepared as in Fig. 1 were diluted in the same buffer (pH 7.4) and in 20 mM Tris-HCl pH 10.8 (final pH 8.4), containing 100 mM mannitol (a), 100 mM NaCl (b), 100 mM KCl (c), 100 mM NaSCN (d) or 100 mM KSCN (e) and 0.01 mM ^{3}H-leucine. Data represents mean ± S.E. of four determinations.
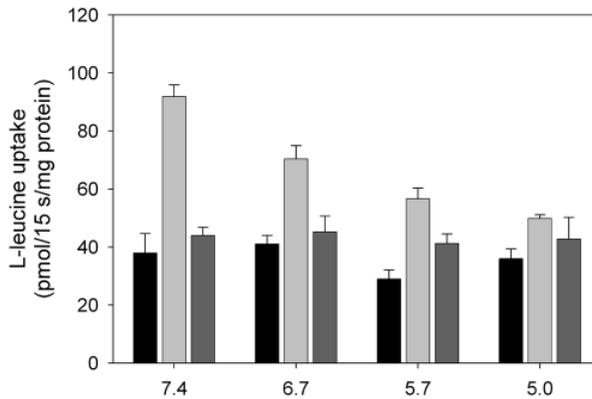

Fig. 3. Effect of acidic pH on leucine uptake into BBMV from *E. stammeri.* Vesicles prepared as in Fig. 1 were diluted in the same buffer (pH 7.4) and in 100 mM MES-Tris solutions buffered at pH 6.5, 5.5 and 4.0 to give the indicated final pH values. Uptake was performed in the presence of 100 mM mannitol (black bar), 100 mM NaCl (light gray bar) or 100 mM KCl (gray bar) and 0.01 mM ^{3}H-leucine. Data represents mean ± S.E. of four determinations.
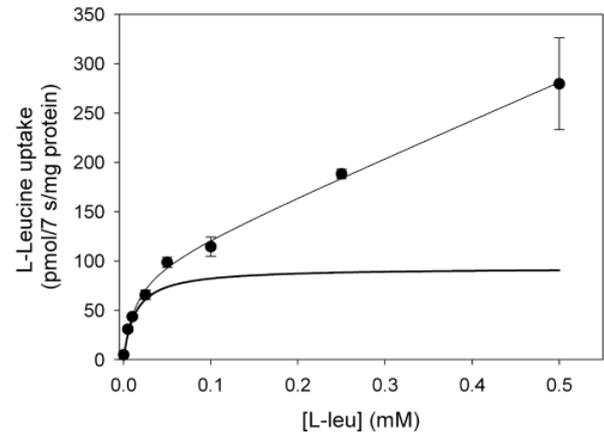

Fig. 4. Leucine uptake as a function of leucine concentration. Initial rates of leucine uptake were measured in the presence of 100 mM NaCl, at pH 7.4, and at the indicated leucine concentrations. Overall uptake was corrected for non saturable component to give hyperbolic component representing the carrier-mediated process (see text for details).

above amino acid concentration range, uptake was a curvilinear function of external leucine concentration and could be described by the sum of at least two independent processes acting simultaneously: i) a carrier-mediated process displaying saturation kinetics and described by the Michaelis-Menten equation, and ii) a linear entry system proportional to external leucine concentration representing a low affinity transport system or simple diffusion. Therefore the overall process is described by the following equation:

$$v_0 = \frac{V_{max} \cdot [\text{Leu}]}{K_{Leu} + [\text{Leu}]} + C \cdot [\text{Leu}]$$

where v_0 is the initial rate of leucine uptake; V_{max}, the maximal initial uptake rate; K_{Leu} is the affinity constant also called Michaelis-Menten constant for leucine; and C is a constant accounting for the linear component, often referred as "diffusional component". The kinetic parameters estimated from two independent experiments are reported in Table 1. The data indicated that the carrier-mediated sodium-dependent leucine uptake has a strong affinity for its substrate, the leucine, as the affinity is in the micromolar range, and, therefore, saturate at concentration of about 0.2 mM leucine. As a matter of fact at this leucine concentration the ratio $V_{max} \cdot [\text{Leu}]/ (K_{Leu} + [\text{Leu}])$ in equation (1) is close to V_{max}, the maximal velocity. More elevated concentrations could be taken up through less specific transport systems which saturated at high substrate concentration and that in the range of concentration used here are indistinguishable from simple diffusion. Saturation was present only in the presence of sodium e we were unable to demonstrate saturation kinetics in the same range of leucine concentration used above when sodium was substituted by potassium.

Table 1. Kinetics of leucine uptake into BBMV from E. stammeri. Kinetics parameters were calculated by fitting the experimental data illustrated in Figure 3 and in an additional experiment to the equation (1) reported in the text. Estimation was made using a non-linear regression program as cited in Materials and Methods.

Parameter	1st experiment	2nd experiment
K_{Leu}	0.013 ± 0.003	0.014 ± 0.006
V_{max} (pmol/7s/mg protein)	93.1 ± 7.5	95.5 ± 10.9
Diffusional component (min^{-1})	381 ± 19	270 ± 55

Previous vesicles study (Parenti et al. 2000) suggested that leucine uptake can be competitively inhibited by a number of amino acids and analogues. To assay specificity of leucine transport into membrane vesicles from *E. stammeri,* 0.01 mM L-[^{3}H]leucine uptake was measured in the presence of a 20-fold excess of unlabeled amino acids, analogues, and derivatives. Initial uptake rate measurements in the presence of 100 mM NaCl at pH 7.4 were performed. Results are shown in Table 2. Almost all the compounds tested as inhibitors produced a reduction of the specific leucine uptake as determined against a control in the presence of mannitol. From the calculated residual activity (*i*) and assu-

Table 2. Inhibition of leucine uptake in BBMV from E. stammeri. Initial rate of 0.01 mM l-[3H]leucine was measured at pHin = pHout = 7.4, 100 mM NaCl, and in the presence of 0.2 mM of the indicated inhibitor. Residual activity was calculated from a control in the presence of mannitol. The inhibition constant Ki was determined assuming competitive inhibition.

Inhibitor	residual activity (%)	K_i (mM)
L-Leucine	10.1	0.015
L-Phenylalanine	17.0	0.023
L-Methionine	23.4	0.034
L-Alanine	40.5	0.076
L-Proline	60.3	0.172
Glycine	51.1	0.118
L-Lysine	63.7	0.198
L-Glutamic acid	87.7	0.518
β-Alanine	60.0	0.170
BCH	57.4	0.152
Acivicin	32.6	0.055
L-Leucine methyl ester	57.1	0.150
L-Leucine ethyl ester	52.4	0.125
Glycine methyl ester	88.0	0.530
2-hydroxyvaleric ethyl ester	56.3	0.146
3-hydroxybenzoic methyl ester	46.9	0.100
4-hydroxybenzoic methyl ester	45.8	0.095

ming pure competitive inhibition, the mM inhibition constant (K_i) can be estimated according to the following equation:

$$i = \frac{[I]}{[I] + K_i \left(1 + \frac{[S]}{K_{Leu}}\right)}$$

In principle, uncompetitive inhibition could be expected if the inhibitor, by using the same sodium gradient, decrease sodium availability for the transport to be measured. The low concentration (0.2 mM) of the inhibitor and the short time (7 s) selected for uptake determination, ruled out this possibility. As shown in Table 2, leucine uptake was strongly inhibited by hydrophobic amino acids (phenylalanine, methionine, and alanine) and bulky hydrophobic analogues (BCH, acivicin). The transport was also inhibited by hydrophobic amino esters and hydroxyacids, suggesting that the side chain of the amino acid play a major role in the binding to the transport protein. Glycine was a quite good inhibitor, but not its derivative glycine methyl ester, indicating that, as expected, the α-carboxylic

group is important for binding to the carrier protein. Also, a contribution is given by the amino group: β-alanine is a poor inhibitor compared to common alanine. However, α-hydroxyesters are able to significantly inhibit leucine uptake, demonstrating the importance of the presence of a hydrophilic group on the a-carbon of the substrate molecule. Recently (Leonardi et al., 2001), a potent activation mechanism by leucine methyl ester and few related compounds has been described in Insects. As shown in Table 2, these substances produced inhibition rather than activation of leucine uptake in *E. stammeri.*

Discussion

MacIntosh et al. (1994) reported for the first time an experimental procedure aimed to obtain purified brush border membranes from whole invertebrates of tiny size. This methodology combined the well-known Mg^{++} precipitation-differential centrifugation protocol (Wolfersberger et al. 1987) with low speed centrifugation and filtration on cheesecloth. Since then it has been used both for binding studies (Abdul-Rauf & Ellar 1999) and transport experiments (Parenti et al. 2001). In this paper, we present results on solute transport capability in a membrane preparation obtained from whole animals of the freshwater crustacean species *E. stammeri.* This preparation was apparently composed of closed vesicles able to take up amino acids, as evidenced by the radiolabeled tracer L-[^{3}H]leucine. Uptake was sodium-dependent, chloride-insensitive and pH-dependent, with an optimum at slightly alkaline values around pH 7.4. In addition, leucine transport was mediated by a specific carrier protein, which saturated at about 0.2 mM leucine and can use, as substrates, additional amino acids, mainly hydrophobic. If external leucine concentration was further increased, the relationship between the uptake rate and the concentration followed a quasi-linear pattern, suggesting that other mechanisms, probably diffusional or low affinity systems, could be involved in amino acid absorption.

Most of what we know about molecular physiology of nutrient transport processes in the crustacean gastrointestinal tract came from studies on hepatopancreas brush border membrane vesicles from the lobster *Homarus americanus* (Wright & Ahearn 1997). These studies were a consequence of a series of physiological data demonstrating a minimal role of the crustacean intestine in total nutrient transport (Ahearn 1982). Vesicles obtained from lobster hepatopancreas using the magnesium precipitation method were employed to characterize several organic solute transporters, including D-glucose, a number of L-amino acids and inositol. A summary of the findings of these studies indicated the presence of at least seven transport systems, of which four are sodium-dependent and three are sodium-independent. The former include the carrier systems for D-glucose, inositol, L-leucine, and L-glutamate, the latter group contains the transporter for L-alanine (able to recognize also L-lysine and L-arginine), L-proline, and a second system for L-leucine. In the case of L-glutamate and L-leucine sodium-dependent systems, a stimulatory effect of chloride ions was observed. Moreover, a reduction of pH down to 4.0 had a marked stimulatory effect on uptake rate of both Na^{+}-dependent and Na^{+}-independent transport systems.

The main result reported in the present paper is that, despite differences in ionic concentrations between marine and freshwater habitat, *Echinogammarus* appears to use a strategy to move organic molecules into its cells similar to that present in *Homarus.* Important differences are, however, present. Variations between amino acid uptake from the scud *E. stammeri* and that reported in the lobster *H. americanus,* can be related to the ecological niche occupied by the two species and their feeding behaviour. A number of variations are quantitative in nature, such as the very high affinity found in *Echinogammarus* (the K_{m} for leucine is one order of magnitude lower in the scud compared to that measured in the lobster). However, some differences are qualitative, such as the decrease of the specific transport activity at the acidic pH observed in *Echinogammarus* compared to data obtained in *Homarus.* The sensitivity to medium pH appears the main feature, which distinguishes *Echinogammarus* from *Homarus.* Gastric content of *Homarus* is acidic and is periodically flushed through hepatopancreas tubules, where they come into contact with apical surfaces of absorptive cells (Wright & Ahearn 1997). Thus protons can be used as driving force to further increase nutrient transport rate and absorption efficiency. *Homarus* is primarily carnivorous and there are evidences that when proteins are the primary food material the lumen pH tends to be slightly acidic (O'Riordan 1969). The ultrastructure of hepatopancreatic caeca has been examined in *Gammarus minus* (Schultz 1976) and the author concluded that within the Malacostraca the hepatopancreatic epithelium of Amphipoda is more similar to that of Decapoda than that of Isopoda. Despite these structural similarities, our results show that *Echinogammarus* does not use protons as driving force for amino acid uptake. The trophic ecology of gammarids has been recently revisited by MacNeil et al. (1997). They concluded that, although gammarids are typical-

ly viewed as archetypal shredders and their herbivorous nature has been emphasized in several studies, this trophic classification must be treated with caution when applied to this group of macroinvertebrates. Gammarids are potentially omnivorous, able to utilize a wide range of food resources, and showing high herbivore/predator plasticity switching between foods as they became available. This feature seems compatible with the presence of basal sodium-dependent transport systems for organic solutes. Therefore, for amino acid uptake, *Echinogammarus* did not evolve specializations, such as the use of protons, as in *Homarus,* or potassium ions, as in phytophagous insects (Giordana et al. 1998). This absence of specialization may contribute to the ability to assimilate a diverse suite of foods, which in turn could contribute to the capability of the gammarids to persist in and colonize new habitats, and to live in disturbance-prone aquatic ecosystems such as rivers where food resources may be highly transitory. The effect of changing environment on membrane transport of organic solute in freshwater invertebrates is completely unrecorded and *E. stammeri* could represent a good model to investigate these aspects.

Acknowledgement

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