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# CALCIUM REGULATION IN INTERMOULT *GAMMARUS PULEX*

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## SUMMARY

The compartmentation of calcium was studied in adult intermoult *Gammarus pulex*. The total body calcium concentration was 547 μmol g.wet wt.$^{-1}$, which is weight for weight approximately one half of the calcium concentration found in decapod crustaceans. Over 96% of whole body calcium in *G. pulex* was found in the exoskeleton.

$^{45}$Ca exchange curves for haemolymph and hepatopancreas had similar time constants indicative of a comparatively large pool of freely exchangeable calcium. The parallel exchange of calcium between the haemolymph and other soft tissue compartments confounded the satisfactory use of an algebraic model for indirect calcium influx measurement using haemolymph specific activity. All calcium influx was subsequently measured on a whole-body basis.

Passive absorption of calcium on to the cuticle was eliminated as a significant factor as the metabolic inhibitor 2:4-DNP almost completely inhibited any form of calcium uptake. The active nature of the calcium regulatory mechanism was further illustrated by potential difference measurements indicating that calcium is accumulated against an electrochemical gradient from media having a calcium activity less than 1 mmol l$^{-1}$.

When placed in calcium-free media, intermoult *Gammarus pulex* are able to achieve at least a tenuous calcium balance between 12 and 50 μmol Ca l$^{-1}$ at a density of 1 animal per 10 ml medium. Some specimens show a slow calcium loss. The calcium regulatory mechanism is saturated at an external calcium concentration of 2 mmol l$^{-1}$, which is the normal field calcium concentration for this population, and has a half saturation value of 0·3 mmol l$^{-1}$.

## INTRODUCTION

Freshwater crustaceans maintain their haemolymph calcium level well above that of the external medium. Although the presence of bound calcium in the haemolymph contributes to this concentration difference an active calcium pump has been demonstrated in the freshwater crayfish *Austropotamobius pallipes* (Greenway, 1972). Despite the paucity of direct evidence it is likely that such a mechanism is widespread in aquatic crustaceans. In both freshwater and seawater species the calcium accumulatory mechanism is particularly in evidence following the moult (Robertson, 1960; Greenaway, 1974*c*). Less is known, however, of the activity of the mechanism in intermoult

animals. Although some crustacea are highly permeable to calcium (e.g. Greenaway, 1976) the exoskeleton represents a large reservoir of calcium, capable of making good losses from soft tissues over long periods without impairing skeletal function. Greenaway (1972) has shown that *Austropotamobius* sustains a slow calcium loss throughout the intermoult period which is partially offset by the operation of an active pump. The calcium accumulatory mechanism of the postmoult crayfish is half saturated at an external concentration of 0·13 mmol $l^{-1}$ (Greenaway, 1974*c*). In the shore crab *Carcinus maenas* protein-bound calcium probably accounts for the difference between haemolymph and seawater calcium levels. In dilute seawater *Carcinus* is able to remain in calcium balance or, in some cases, sustain a slight loss (Robertson, 1937; Greenaway, 1976). Under these conditions the haemolymph calcium concentration is maintained at a fairly constant level. On the basis of electrochemical evidence Greenaway (1976) states that, in dilute media, maintenance of haemolymph calcium by uptake from the medium would require active transport of calcium. However, the role of calcium withdrawal from the exoskeleton in maintaining the haemolymph calcium level has yet to be clarified in this case. Vincent (1963, 1969) described two populations of *G. pulex*; one from a soft-water source (Ca, 1·5–1·8 mg $l^{-1}$) and one from a hard-water source (Ca, 100–110 mg $l^{-1}$). The total body and haemolymph calcium levels did not differ significantly between these populations, although it is interesting that postmoult animals from the soft-water environment were able to recoup their full complement of calcium from a medium containing 1 mg Ca $l^{-1}$, whereas similar specimens from the hard-water environment were incapable of living in less than 3 mg Ca $l^{-1}$. This suggests that soft-water animals possess an accumulatory mechanism of higher calcium affinity, although the characteristics of the pump were not investigated.

Although there is a large body of information concerning sodium regulation in gammarid crustacea, including *G. pulex* (Shaw & Sutcliffe, 1961; Sutcliffe, 1967), very little is known about calcium regulation in this important group. In this investigation calcium balance and intercompartmental exchange in intermoult *G. Pulex* is examined and the characteristics of the calcium regulatory mechanism are established.

## MATERIALS AND METHODS

Adult *Gammarus pulex* were collected from the River Pont at Ponteland in Northumberland. Most specimens were between 30–60 mg in weight. The concentrations of major elements in the river water at the collecting site were as follows: Ca, 2·1 mmol $l^{-1}$; Mg, 0·49 mmol $l^{-1}$; Na, 0·89 mmol $l^{-1}$; K, 0·26 mmol $l^{-1}$; Cl, 2·0 mmol $l^{-1}$.

An effort was made to confine the current work to intermoult animals. It is very difficult to determine accurately the exact stage of the intermoult cycle reached without resorting to histological examination (Martin, 1965), although free swimming, actively feeding specimens with hard exoskeletons were found to be generally reliable in this respect. Unpublished work has shown that ecdysis in *G. pulex* is preceded by a period of rapid calcium loss and is succeeded by a period of strong calcium uptake. Similar changes in calcium balance have been found in the freshwater crayfish *Austropotamobius pallipes* (Greenaway, 1974*b*, *c*) and have been used to identify

premoult and postmoult specimens not easily recognized from external characteristics. A similar procedure was adopted in the current work. Prior to use, animals were stored in perspex photographic trays containing water, gravel and decaying leaves from the river.

An artificial stream water was used for experiments having a composition of 0·9 mmol $l^{-1}$ $Na^+$, 0·25 mmol $l^{-1}$ $K^+$, 0·2 mmol $l^{-1}$ $Mg^{2+}$, 0·5 mmol $l^{-1}$ $HCO_3^-$ and 1·05 mmol $l^{-1}$ $Cl^-$. The experimental calcium concentrations varied with different experiments and are described later. The calcium was usually added as the chloride salt. Experimental animals were placed in aerated perspex containers maintained at 10 ± 1 °C.

*Analytical techniques*

A 0–5 mg torsion was used to weigh all tissues to the nearest 0·1 mg. For whole animals a pan balance was used.

Standard planchetting techniques were employed to count $^{45}Ca$ (Radiochemical Centre, Amersham) in tissues and medium using a Panax low background GM counter. Where specific activities were to be measured, soft tissue samples were spread (using a dextrose and soap spreader), counted and dissolved in concentrated $HNO_3$ for spectrophotometric determination of calcium using a EEL 240 AAS (422·7 nm). In the case of calcified tissue, counting and spectrophotometric calcium determination was preceded by ashing in platinum crucibles at 500 °C. In all calcium analyses lanthanum chloride was added to samples to offset anionic interference.

Calcium influx was measured by immersing animals in a $^{45}Ca$-labelled medium for a measured period not longer than 1 h. In most cases whole body counting was employed. A preliminary investigation showed that an indirect method of calcium influx measurement using haemolymph specific activity determinations (Greenaway, 1971*b*, 1976) was unsuitable for this species. Reasons for this are discussed later. In view of the fact that direct whole-body counting was employed, consideration was given to the contribution made by drinking of labelled medium to total radioactivity accumulated over the measured period. The most convenient method of adjustment would have been to apply a constant correction factor based upon a known turnover of gut contents in this species. Data are conveniently available from Sutcliffe (1967) who found an expulsion rate of gut fluid from *G. pulex* into deionized water of 0·56 $\mu$l animal $^{-1}$ $h^{-1}$. If under steady-state conditions this could also be considered as an imbibition rate an overestimate of influx (caused by drinking) of less than 2% is indicated for most of the data presented here. Very often, this value is much lower and, in view of this, this possible source of error has been ignored.

In order to measure calcium efflux, $^{45}Ca$-loaded animals were placed in a small volume of unlabelled medium for a measured period of ½–1 h. The medium was then evaporated to a suitable volume for spreading, drying and counting.

The terms influx, efflux, net uptake and net loss are as used by Greenaway (1972).

Haemolymph and whole body copper was measured by AAS at a wavelength of 324·8 nm. For whole-body copper analysis wet oxidation using nitric acid was employed.

Measurements of calcium were made using an Orion model 92–20 calcium ion

electrode coupled to an Orion model 401 specific ion meter. The electrode was calibrated with solutions having a cation content similar to that of the experimental medium (or haemolymph) but with chloride as the sole anion. Details of the technique have been described by Greenaway (1971*a*, 1972).

Measurements of the potential difference between the haemolymph and the external medium were made using a Vibron Electrometer Model 33B-2 and two calomel:saturated-KCl electrodes. Animals were attached to the head of a bent pin by a small blob of beeswax resin applied to the dorsal surface of the thorax. The exoskeleton was then pierced mid-dorsally with a sharp pin. A small drop of haemolymph is exuded without the need to apply any pressure to the animal. The animal was then mounted dorsal side uppermost on a waxed slide such that the gills and other appendages were immersed in a large drop of experimental medium. High and low resistance electrodes were then applied to the haemolymph and medium respectively using micro-manipulators. Measurements were made at 20 °C.

*Tissue sampling*

To obtain a haemolymph sample an incision was made mid-dorsally with a sharp needle and the bead of haemolymph which was extruded was collected in a standard microcapillary tube prior to planchetting or dilution for chemical analysis. Pressure on the animal was avoided, as this could lead to contamination of haemolymph with material from the hepatopancreas.

The hepatopancreas itself is a four-lobed organ, which may be dissected out by gripping the head of an anaesthetised animal dorsoventrally with fine forceps. With a sharp pull the hepatopancreas may be pulled from the thoracic cavity anteriorly. The lobes may then be severed from the alimentary canal at the anterior end.

Samples of exoskeleton were removed from the dorsal surface of the thorax between the coxal plates.

## RESULTS

*Calcium compartmentation and exchange*

Table 1 shows the calcium concentration in the body tissues of *Gammarus pulex*. These data, together with measurements of the relative proportions of the total body weight represented by these tissues, enable the distribution of calcium within the body of the animal to be calculated. The haemolymph calcium compartment was calculated on the basis of a figure for haemolymph space which was determined as copper space. A haemolymph sample pooled from 21 specimens had a copper concentration of 2·4 mmol $l^{-1}$. The total body copper concentration from the same animals was 0·525 $\mu$mol $g^{-1}$, thereby giving a haemolymph copper space of 22% body weight. This compares favourably with the value of 26% obtained for this species by Butterworth (1968) using inulin. The figure for soft tissue calcium (other than hepatopancreas) is estimated indirectly by subtracting other tissue calcium data from the whole body figure. The comparatively massive calcium compartment in the exoskeleton is to be expected although on a unit weight basis *Gammarus* is considerably less calcified than the decapods *Austropotamobius pallipes* and *Carcinus maenas* both of which has a total body calcium concentration of approximately 1 mmol $g^{-1}$

Table 1. *Calcium distribution in* Gammarus pulex (*where possible possible mean values* ± *standard error* (*n*) *are given*)
(*Dash indicates no measurement made.*)

| Tissue | Tissue Ca concn. ($\mu$mol $g^{-1}$ (wet wt.)) | Tissue weight (% whole body wt.) | Tissue Ca (% whole body Ca) |
|---|---|---|---|
| Whole body | 547 ± 14 (50) | 100 | 100 |
| Exoskeleton | 2157 ± 91 (50) | 24·43 ± 0·95 (8) | 96·33 |
| Hepatopancreas | 128 ± 31 (48) | 5·52 ± 0·37 (13) | 1·29 |
| Haemolymph | 11 ± 2 (25) | 22·0* | 0·44 |
| Other tissues (mainly muscle) | — | 48·05† | 1·94† |

* From copper space determination.
† By subtraction of measured tissue data from whole body figure.

(Greenaway, 1974*a*; Wright, 1977). The low calcium concentration found in the hepatopancreas is in agreement with earlier observations on this species (Graf, 1962, 1964; Martin, 1965) although this tissue has been shown to be a significant calcium store in members of the Talitridae immediately before and after moult (Graf, 1964, 1967).

In order to measure calcium exchange in *G. pulex* small batches of animals were placed in 1 l artificial stream water containing 0·2 m-mol Ca labelled with $^{45}$Ca. Animals were removed at intervals for measurement of the specific activity of calcium in the haemolymph, hepatopancreas, exoskeleton and whole body. The increase in the specific activity of calcium in the tissues and whole body over the experimental period is shown in Fig. 1. In each case the specific activity is expressed as a percentage of external specific activity. Over the period investigated (140 h) $^{45}$Ca appears in the exoskeleton in an essentially linear manner, although because of the large amount of calcium in the tissue, the specific activity only reaches 3·5% of that of the external medium. This is also reflected in the whole body exchange.

From Fig. 1 it appears that even after more than 100 h exposure to the labelled medium the specific activity of the haemolymph ($SA_2$) remains at a level considerably less than that of the external medium ($SA_1$). A similar, although less extreme, situation was described on the freshwater snail *Limnaea stagnalis* where, after 100 h exposure to $^{45}$Ca labelled medium, the ratio $SA_2$: $SA_1$ stabilized at approximately 0·7 (Greenaway, 1971*b*). It was felt that this disparity between $SA_2$ and $SA_1$ may have been partly due to the presence of bound non-exchangeable calcium in the haemolymph. However, in *L. stagnalis* this bound fraction has been shown to be small (van der Borght & van Puymbroek, 1964) and it was concluded that the major reason for the difference between $SA_2$ and $SA_1$ was because that $SA_2$ was the resultant of two fluxes connected 'in series'; $m_1$, the influx from medium to haemolymph, and $m_3$, the flux from haemolymph to exoskeleton such that

$$SA_2 = SA_1 \frac{m_1}{m_1 + m_3}\left(1 - \exp\left(-\frac{m_1 + m_3}{a_2}t\right)\right)$$

(Greenaway, 1971*b*), where $t$ represents the time constant for $m_1$, and $a_2$ the number of calcium ions in the haemolymph. Although some investigation has been made of

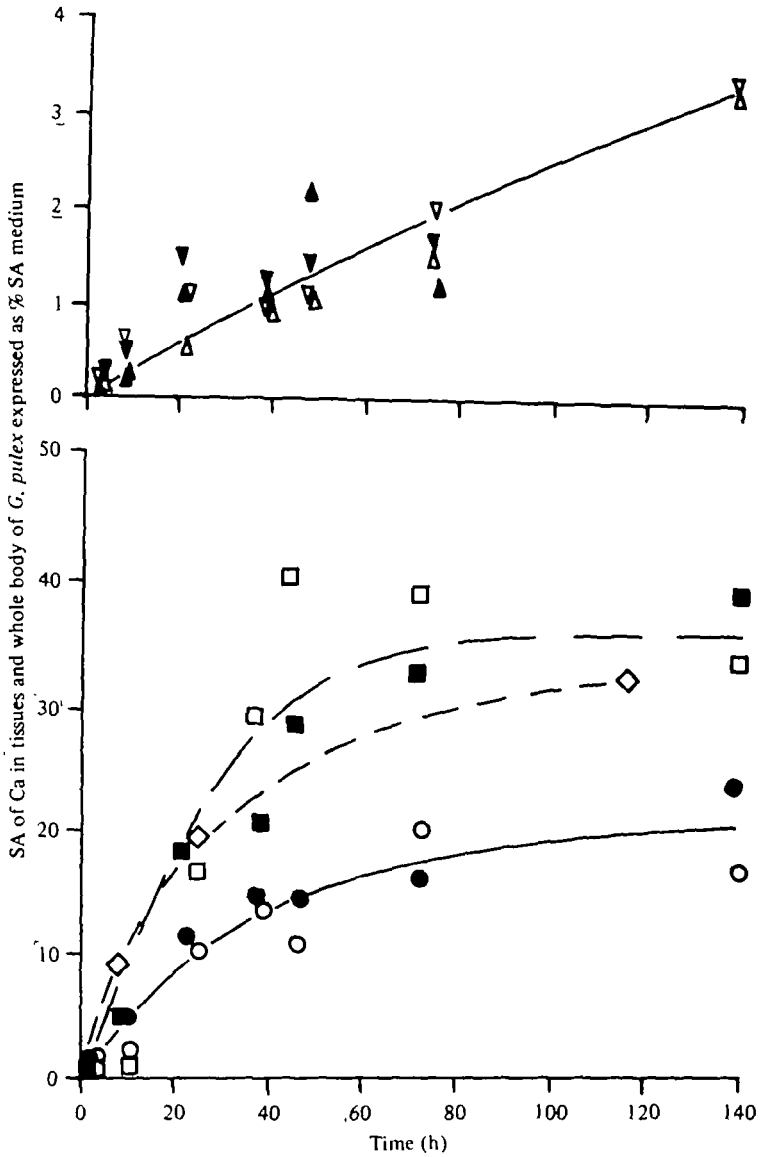


Fig. 1. Calcium exchange in tissues and whole body of intermoult *Gammarus pulex*. Each point represents the mean of 4–6 determinations. Solid symbols represent animals which had been coated with a thin layer of silicone grease (see text). ▲, △, Whole body; ▼, ▽, carapace; ■, □, hepatopancreas; ◆, ◇, ●, ○, haemolymph (two different experimental runs).

bound and ionized calcium in the haemolymph of *G. pulex* (see later) there are no available data concerning the amount of non-exchangeable calcium present. However, it is likely that the bound non-exchangeable fraction is low in crustaceans. Andrews (1967) found that 18% of haemolymph calcium in the freshwater crayfish *Orconectes rusticus* was bound to protein. In *Homarus americanus* the non-diffusible, presumably protein-bound calcium fraction was found to be 12–13% total haemolymph calcium (Hayes, Singer & Armstrong, 1962). Assuming a non-exchangeable fraction as high

as 20% in *G. pulex*, there is still a very large shortfall in $SA_2$ compared with $SA_1$. This is apparent even after allowance is made for a good deal of variability in the data (Fig. 1). The haemolymph data represent two different experimental runs. In one (circles), $SA_2$ appears to have stabilized at little more than 20% $SA_1$, although in a second less complete experiment (triangles), $SA_2$ exceeds 30% $SA_1$ at 120 h. Using the more complete data represented by circles, equation (1) gives a value for $m_1$ of 0·0147 $\mu$mol $g^{-1}$ $h^{-1}$, and a value for $m_3$ of 0·055 $\mu$mol $g^{-1}$ $h^{-1}$. The data differ from those of Greenaway (1971*a*, *b*) in a number of important aspects. Firstly, the large size of $m_3$ with respect to $m_1$ suggests a comparatively rapid exchange between haemolymph and exoskeleton. In *Limnaea* values for $m_1$ and $m_3$ were 0·2 and 0·09 $\mu$mol $g^{-1}$ $h^{-1}$ respectively, considerably higher than in the present study. More important, however, is the discrepancy, in the current work, between $m_1$ calculated using equation (1) and whole body calcium influx measured directly. For example, the whole body exchange data shown in Fig. 1 were obtained from eactly the same animals as the haemolymph exchange data (circles). However, calcium influx measured directly using whole animals gave a figure of 0·106 $\mu$mol $g^{-1}$ $h^{-1}$. This differs from $m_1$ as calculated by a factor of approximately 7.

One possible explanation is that there is a component in influx which bypasses the haemolymph compartment; for example, the adsorption of calcium on to the exoskeleton directly from the medium. This was anticipated in the first exchange experiment (Fig. 1), where half the animals were coated with a thin layer of silicone grease. The fact that there were no apparent differences in calcium exchange between greased and ungreased specimens, argues against there being a significant medium/exoskeleton exchange.

In the foregoing calculations the term $a_2$ has been calculated on the basis of 22% haemolymph space as defined by the copper space determination. In view of the discrepancy between calcium influx measured directly and indirectly, measurements of calcium space were attempted, using an adaptation of the $^{45}Ca$ injection technique used by Greenaway (1976), in order to define $a_2$ more accurately. Using this technique, extrapolation of $^{45}Ca$ disappearance curves (Greenaway, 1976) reveals that a meaningful value for haemolymph calcium space cannot be obtained for this species, and it seems likely that a parallel calcium exchange between haemolymph and soft tissues is primarily responsible for this. All subsequent measurements of calcium influx were therefore made directly by whole body counting.

*Effect of 2:4-dinitrophenol upon calcium influx*

The size of the passive component of calcium influx was further investigated by exposing to a metabolic poison prior to influx measurement. The assumption was made that although active processes would be inhibited using DNP, any passive process such as absorption would remain unimpaired. Stobbart, Keating & Earl (1977) found that 0·2 mmol $l^{-1}$ DNP reduced *Daphnia magna* to a moribund state when the influx of radioactive sodium was severely inhibited. In the current investigation seven animals were exposed to 0·4 mmol $l^{-1}$ DNP solution for 5 h prior to placing them in a $^{45}Ca$-labelled medium containing 0·2 mmol Ca $l^{-1}$ for 40 min. By reference to a control group it was found that this treatment caused a reduction in calcium influx from $0·193 \pm 0·047$ to $0·0138 \pm 0·0036$ mmol $kg^{-1}$ $h^{-1}$. It is reasonable

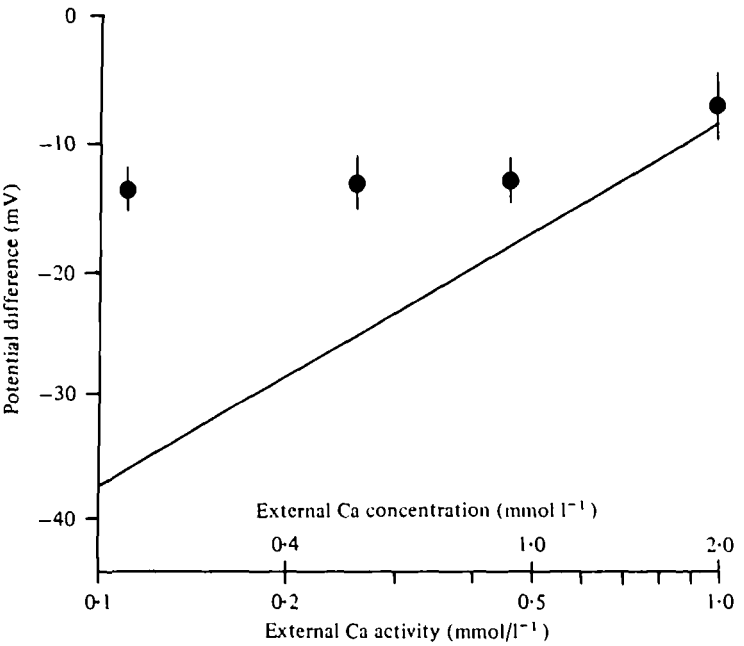


Fig. 2. The relationship between transepithelial potential difference in intermoult *Gammarus pulex* and the external calcium activity and concentration. ●, Represent mean potential difference from 6 to 8 animals. Vertical lines represent standard errors of the means. The calculated equilibrium is indicated by the unbroken line.

to assume that this fall of 93% has been caused by the inhibition of active processes. Passive adsorption of calcium onto the exoskeleton can therefore be effectively ruled out as a significant pathway, and it is reasonable to state that calcium taken up by the animal follows the route: medium → gills (gut) → haemolymph → other tissues (exoskeleton).

*Haemolymph calcium activity and potential difference measurements*

The nature of the calcium regulatory mechanism was further examined by considering the relationship between the transepithelial potential difference and the calcium activity of the external medium. The potential difference between the haemolymph and the external medium was measured over a range of external calcium activities from 0·1–1·0 mmol $l^{-1}$. Results are shown in Fig. 2 along with equilibrium potentials derived from the Nernst equation $E = (RT/ZF)\ l_n(a_i/a_o)$, where $a_i$ and $a_o$ represent internal and external calcium activities. Technical difficulties precluded the measurement of haemolymph calcium activity in individuals used for potential difference measurement, and $a_i$ was obtained from a pooled sample collected under liquid paraffin from 50 specimens taken from the same experimental batch. The total calcium concentration of this pooled sample, measured by atomic absorption spectroscopy, was 9·0 mmol $l^{-1}$. Measurement of ionized calcium concentration was made empirically using $CaCl_2$ standards made up in haemolymph Ringer containing chloride as the sole anion. This gave a figure of 5·6 mmol $l^{-1}$ and indicated that 62·2% of the haemolymph calcium was present in ionized form. Calcium activity, calculated according to the Debye–Hückel theory using an activity coefficient of

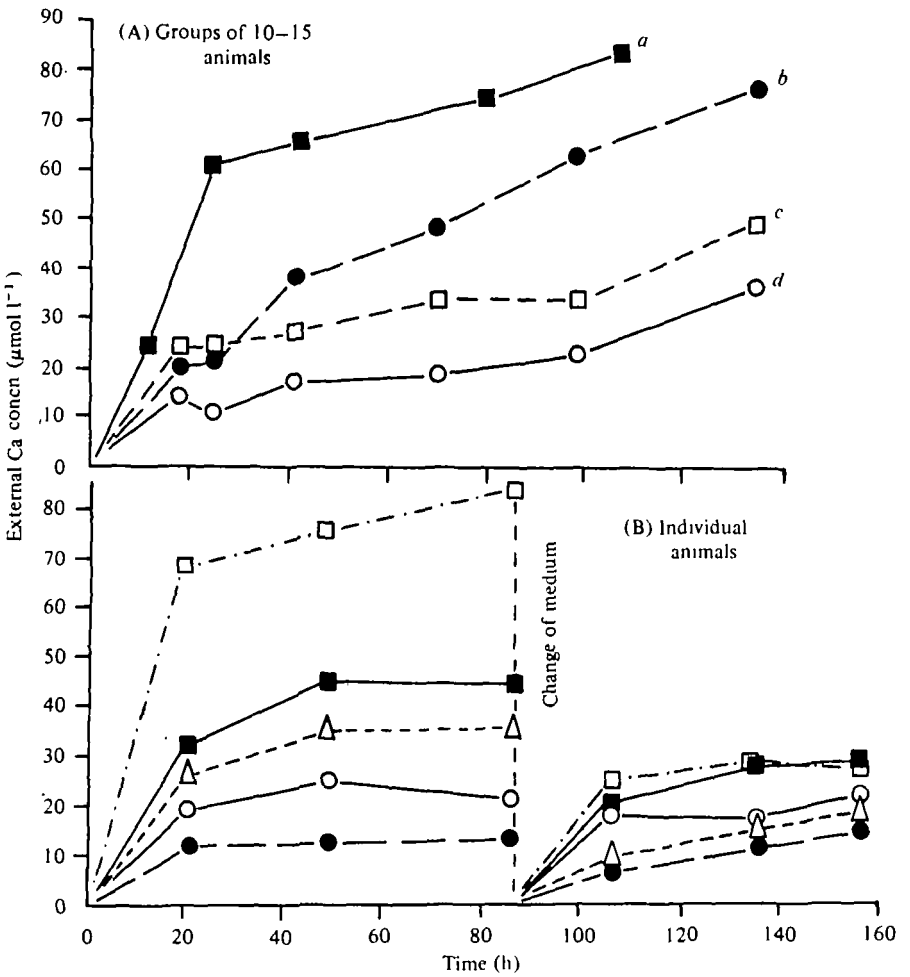


Fig. 3. Calcium balancing ability of individuals or batches of 10–15 intermoult *Gammarus pulex* exposed to calcium-free medium at a density of 1 animal per 10 ml.

0·35 and based upon a haemolymph ionic strength of 190 mmol $l^{-1}$, was found to be 1·96 mmol $l^{-1}$. Potential difference measurements (Fig. 2) are similar to those obtained from the crayfish *Austropotamobius* by Greenaway (1972, 1974*b*, *c*) who detected no significant alteration in potential difference throughout the life-cycle of this species. The discrepancy between measured and calculated potential differences indicates that net calcium uptake by intermoult *G. pulex* occurs against an electrochemical gradient over the range of external activities used.

*Calcium balance*

The ability of the intermoult specimens of *Gammarus pulex* to attain calcium balance in a low calcium environment was tested by following the calcium concentration of an initially calcium-free medium after the introduction of animals at a density of about 1 animal per 10 ml. Animals were used singly or in batches of 10–15. Results indicate that some degree of calcium balance is attained after about

Table 2. *Some values for calcium influx and efflux from* Gammarus pulex *in* 0·2 *mmol Ca* $l^{-1}$

| Influx (mmol $kg^{-1}$ $h^{-1}$) | Efflux (mmol $kg^{-1}$ $h^{-1}$) |
|---|---|
| 0·405 | 0·251 |
| 0·224 | 0·302 |
| 0·260 | 0·165 |
| 0·180 | 0·280 |
| 0·307 | 0·604 |
| 0·207 | 0·506 |
| 0·608 | 0·206 |
| 0·464 | 0·595 |
| 0·132 | 0·505 |
| 0·086 | 0·482 |
| 0·122 | 0·146 |
| 0·621 | 0·895 |
| 0·268 | |
| 0·115 | Mean 0·411 ± 0·065 (S.E.) |
| Mean 0·287 ± 0·051 (S.E.) | |

60 h at an external calcium concentration between 12–50 $\mu$mol $l^{-1}$ (Fig. 3), although a slow calcium loss occurred in some cases. Individuals performed slightly better than groups in this respect and, furthermore, showed a decrease in the rate of calcium loss following a change of medium.

*Calcium influx and efflux*

Table 2 gives values for calcium influx and efflux in animals maintained in an experimental medium having a calcium concentration of 0·2 mmol $l^{-1}$. Efflux values were obtained from animals which had been loaded for several weeks in the same $^{45}$Ca-labelled medium used for influx measurements. Influx and efflux values did not differ significantly, indicating that a steady state had been reached in these animals. These fluxes are considerably higher than in the freshwater crayfish *Austropotamobius pallipes*. For intermoult crayfish in a medium containing 0·5 mmol Ca $l^{-1}$ Greenaway (1972) found influx and efflux rates of 0·014 mmol $kg^{-1}$ $h^{-1}$, although a loss rate of 0·046 mmol $kg^{-1}$ $h^{-1}$ was recorded from late intermoult animals in 1 mmol Ca $l^{-1}$.

The effect of external calcium concentration on calcium flux is shown in Fig. 4. The results indicate the presence of a saturable influx component similar to that which characterizes the sodium regulatory mechanism (Sutcliffe, 1967). The relationship between external calcium concentration and calcium influx is reasonably compatible with the Michaelis–Menten equation for enzyme kinetics, and indicates a calcium carrier mechanism which is half saturated at approximately 0·3 mmol $l^{-1}$ and which approaches saturation in concentrations greater than 2 mmol $l^{-1}$. By reference to the calcium concentration of water from the collecting site (Materials), it may be seen that for this population the calcium mechanism is just saturated in the natural environment. Records of this species from softer waters (Vincent, 1960) give rise to the possibility that populations may exist at ambient calcium level

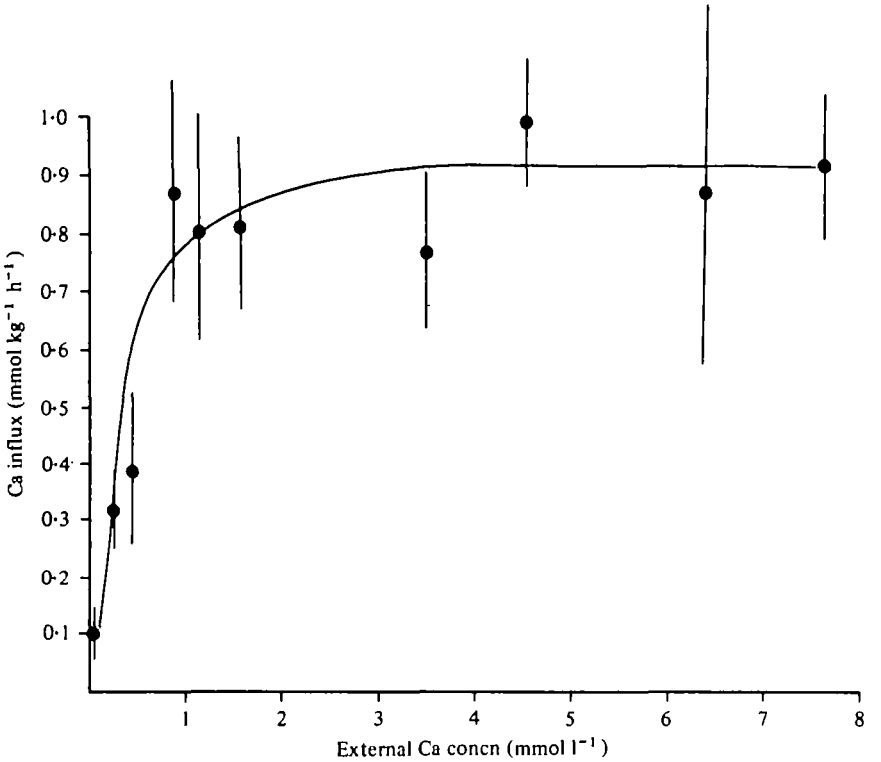


Fig. 4. The effect of increasing external calcium concentration on the rate of calcium influx in intermoult *Gammarus pulex*. ●, Mean influx values from 6–8 animals. Vertical lines show the standard errors of the means.

apparently below the saturation level of the calcium mechanism. However, the possibility of some adjustment to the calcium affinity of the mechanism in such low-calcium populations cannot be overlooked.

## DISCUSSION

In a small animal such as *Gammarus* the direct measurement of whole-body calcium influx is comparatively easy, although in a large decapod such as *Carcinus* whole-body counting would be a considerably more cumbersome operation and the adoption of an indirect technique for measurement of calcium influx (Greenaway, 1976) is understandable. However, as current results show, there are a number of potential problems associated with this approach.

The equation described here relies on there being a discrete haemolymph compartment exchanging 'in series' with a discrete exoskeleton compartment, and requires that calcium entering the animal equilibrates fully with the haemolymph compartment before significant exchange with other tissues occurs. Although the necessary conditions are fulfilled in *Limnaea stagnalis* (Greenaway, 1971*b*) and *Carcinus maenas* (Greenaway, 1976) calcium space determinations in the current work were confounded by 'parallel' exchanges occurring between haemolymph and other tissues,

for example, Fig. 1 shows that $^{45}Ca$ exchange with the hepatopancreas has a similar time constant to the haemolymph exchange curve, thus introducing a freely exchanging calcium pool 60% the size of that of the haemolymph (Table 1). If other tissues (as described in Table 1) are also found to have a similar time constant for $^{45}Ca$ exchange then these, too, must be included in the freely exchanging calcium pool, raising this to approximately five times the size of the haemolymph calcium pool. In view of such complications there is clearly no reasonable substitute for direct measurement of whole body calcium influx in *Gammarus*. In this regard an important finding of the current work is that nearly all the calcium influx is dependent upon metabolic energy. The figure of 93% found here using DNP may be a considerable underestimate in view of the fact that all animals analysed, although moribund, were still alive.

The experiments described here establish that *Gammarus pulex* possesses a calcium regulatory mechanism capable of retaining body calcium in low calcium media against an electrochemical gradient.

Although the calcium mechanism has broad similarities with that of the crayfish *Austropotamobius* (Greenaway, 1972), perhaps the most surprising difference is the apparently greater calcium permeability possessed by *G. pulex*. On a weight-for-weight basis calcium turnover in *G. pulex* is an order of magnitude higher than in the crayfish and flux values are closer to those described for *Carcinus* (Greenaway, 1976). Thus, it seems that the balance or near-balance situations demonstrated in very dilute media represent a substantially dynamic equilibrium with the comparatively large efflux component offset by an influx of similar magnitude. The size of the influx component in *G. pulex* can be conveniently illustrated by comparison with the sodium regulatory mechanism described for the same population by Sutcliffe (1967). It may be seen that, on a molar basis, the maximum calcium influx attained by *G. pulex* (0·9 mmol $kg^{-1}$ $h^{-1}$) is as high as 43% of the maximum sodium influx rate (2·1 mmol $kg^{-1}$ $h^{-1}$). By contrast, a similar comparison of sodium and calcium influx data from the freshwater crayfish (Shaw, 1959; Greenaway, 1972) shows that the sodium influx is at least $\times 20$ faster than calcium influx in this animal. On the other hand, Greenaway (1976) has suggested that calcium influx in the shore crab *Carcinus maenas* is probably higher than the sodium flux in the same species.

The haemolymph calcium concentration of 9 mmol $l^{-1}$ obtained from a large pooled sample compares quite well with the value of 7·65 mmol $l^{-1}$ given by Vincent (1969) for *G. pulex* from an environment of similar calcium concentration, although experience has shown that this is subject to a good deal of variation and effect of the moult cycle on haemolymph calcium concentration requires further investigation. Although the haemolymph calcium activity measured here is lower than that of the crayfish the ionized calcium in *G. pulex* represents a higher percentage of the total haemolymph calcium, i.e. 62% compared with a figure for intermoult *Austropotamobius* of 51% (Greenaway, 1972, 1974*b*).

In view of the comparatively high saturation concentration of the calcium mechanism in *G. pulex* it would be particularly interesting to investigate whether this aspect of the calcium pump is capable of altering in populations of this species collected from low calcium environments. In such environments it is clear that calcium uptake would be against an electrochemical gradient and would involve the

penditure of metabolic energy. Although calcium uptake from low calcium media is clearly implicated in this investigation and in unpublished work, it must be remembered that the population of experimental animals used here were taken from a comparatively high calcium environment having a calcium activity of approximately 1 mmol $l^{-1}$. In such an environment calcium regulation would involve, for this species, a very low energy budget in view of the proximity of the measured transepithelial potential difference and the calculated equilibrium potential.

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