WATER BALANCE IN *MANDUCA SEXTA* CATERPILLARS: WATER RECYCLING FROM THE RECTUM

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Summary

Tobacco hornworm (*Manduca sexta*) caterpillars are able to regulate the water content of their body when fed on diets of markedly different water content. This regulation extends to the water content of food within the gut. Regulation of body water is achieved by adjusting the amounts of water lost with the faeces. The rectum is shown to be the principal site of water reabsorption from the faeces. The rate of rectal water absorption is shown to vary with the water content of the food and thus according to need. Water reabsorbed from the rectal contents is recycled and added to the contents of the midgut. The ultrastructural appearance of epithelial cells in the rectal wall is that expected of a fluid-transporting tissue. The ileum appears to play little or no part in water recycling.

Introduction

The availability of water plays an important role in determining the abundance and distribution of insects (Edney, 1977). Despite the constraints imposed by small size, dry habitats and food sources have been successfully exploited by the evolution of regulatory mechanisms that conserve water, for example by the production of very dry faeces. For caterpillars, which feed on plant material with a high water content, it might be expected that water conservation would be unnecessary, and that any problem related to water balance would be caused by its overabundance. However, plant water content does vary, especially in stress conditions, and it has been suggested that water may be a limiting resource for at least some phytophagous insects (Scriber, 1977; Scriber & Feeny, 1979).

We have previously shown (Reynolds *et al.* 1985) that when tobacco hornworm caterpillars are fed on an artificial diet of standard composition, the water content of the body is maintained at a level greater than that of the food at all times. We suggested that caterpillars might regulate the water content of some or all body compartments by resorbing, or failing to resorb, water from the faeces. A quantitative model of water economy in fifth-instar caterpillars suggested that water is resorbed in the hindgut and recycled *via* the haemolymph to the contents of the midgut.

To test these ideas we fed *Manduca* caterpillars on artificial diets with altered water contents, and determined the degree of hydration of various body compartments. The results indicate that the insects regulate their water content, and provide evidence that the rectum is the site of water resorption in the hindgut.

Materials and methods
Experimental animals

Tobacco hornworms (*Manduca sexta*) were taken from an established culture at the University of Bath. They were individually reared at 25°C under long-day (17 h:7 h L:D) conditions. Relative humidity (RH) in the rearing room was not controlled, but RH inside the closed containers in which the insects and their food were kept was close to 100 %. The larvae were fed an artificial diet, as described by Bell & Joachim (1976). Larvae were raised on the 'normal' artificial diet from hatching and through the first four instars in clear plastic containers with lids (volume 30 ml). During this period the diet was not changed. On the day of ecdysis to the fifth instar (day 0), the larvae were transferred to larger containers (volume 200 ml) when they were given an excess of the experimental diet. In these experiments, fresh supplies of the diet were given each day during the feeding period of the fifth instar.

Experimental diets

These were prepared by altering the amount of the premixed solid food ingredients added to the diet. Four diets were used containing (*A*) 25 %, (*B*) 50 %, (*C*) 100 % and (*D*) 200 % of the normal amount of this premix. To maintain other characteristics of the diet as constant as possible, the amounts of preservatives and setting agent (agar) added to the premix were not changed between diets. For this reason the water content of the diet did not vary in simple proportion to the premix concentration. The water contents of the four diets are listed in Table 1.

Water contents

On each day of the feeding period of the fifth instar, five animals were weighed, anaesthetized with CO_2, and dissected. Fresh masses were determined for (i) fore- and midgut, (ii) ileum, (iii) rectum, and (iv) carcass (i.e. the rest of the body,

Table 1. *Water contents of the experimental diets*

Diet	Water content (%)
A (25 % premix)	90·8 ± 0·06
B (50 % premix)	87·1 ± 0·15
C (normal diet)	79·5 ± 0·78
D (200 % premix)	68·9 ± 0·30

Means ± s.d. ($N = 5$).

including blood). These samples were dried in an oven at 60°C to constant mass (48 h). An initial experiment was performed to determine whether the water content of the midgut varied along its length by dividing it into three equal portions and separately determining their fresh and dry masses. Since the percent dry masses of these three portions did not differ (data not shown), subsequent work simply determined the percent dry mass of the whole fore- and midgut. Water contents of food and faeces were determined using the same method. Where the water contents of individual faecal pellets were determined, these were weighed immediately (i.e. less than 1 min) after their elimination.

In some experiments the water contents of the ileum and rectum were determined at various times after defecation. The insects were observed until they produced a faecal pellet, following which they were left for a specified time in an incubator at 25°C, when they were rapidly killed and dissected. The contents of the ileum and rectum were removed by gently squeezing with forceps, and the fresh and dry masses determined as above.

Microscopy

Tissue from ileum and rectum of day 2 fifth-instar caterpillars was dissected in *Manduca* saline, cut into small pieces (1–2 mm^2) and fixed in 4 % glutaraldehyde in 0·1 mol l^{-1} cacodylate buffer (pH 7·2) for 12 h. Postfixation was in 1 % osmium tetroxide and embedding was in Araldite. Thin sections were mounted on coated grids, stained in uranyl acetate and lead citrate (Reynolds, 1963) and examined in a JEOL 100 CX transmission electron microscope operated at 80 kV.

Results

Water content of caterpillars fed on diets with altered water content

Fig. 1 shows the water content of three distinct gut regions, and of the remainder of the body (the carcass), on each of days 0, 1, 2 and 3 for insects fed on diets with different water contents. On day 0, in samples taken at the time that the insects were first introduced to the experimental diets, there was no significant variation in the water contents of the carcass or various gut parts among the experimental groups and the data are shown pooled. On subsequent days, however, the insects that had been fed on diets containing a higher percentage of water also contained more water in their bodies. The correlation was significant for all four body compartments sampled, but only for the rectum did the water content change in direct proportion to that of the diet (Fig. 2A). For the midgut, ileum and carcass, the water content changed much less than did that of the diet. This implies that the water contents of these tissues are regulated.

Water content of faeces

To be able to regulate its body water content in the face of an altered intake of water with its food, the insect must be able to alter the amount of water lost with the faeces. We found (Fig. 2B) that the water content of faeces did indeed change

according to the nature of the diet. The relationship between the water contents of faeces and food was in fact steeper than simple equivalence, with the faeces produced by caterpillars feeding on very concentrated food actually being drier than the food itself.

Because the caterpillars compensated for the dilution of their food by eating more of it (Timmins *et al.* 1988), the total production of faeces in dry mass terms
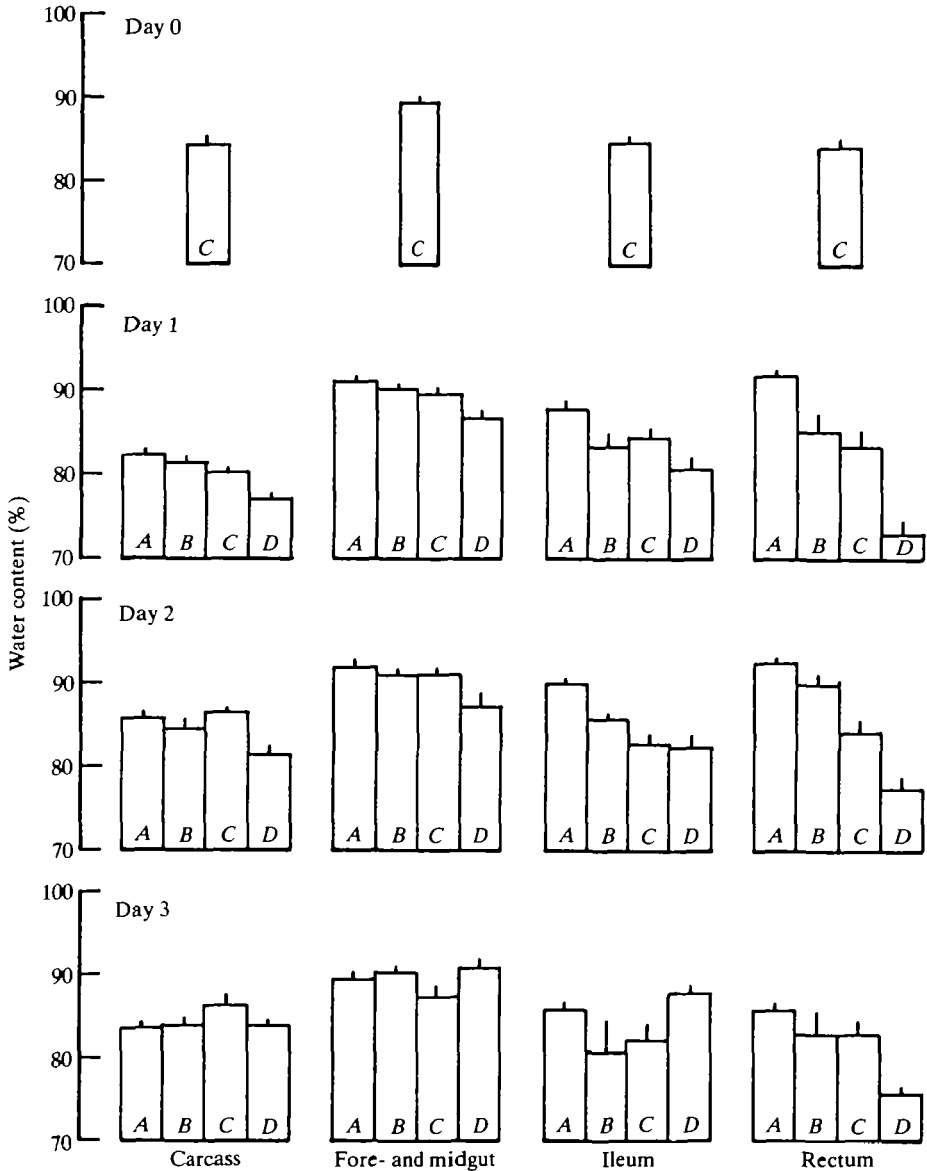

Fig. 1. Water content (%) of gut and carcass on the first 4 days of the fifth instar in *Manduca* caterpillars fed diets of altered water content. Columns show mean + S.E., *N* was 5 in each case.
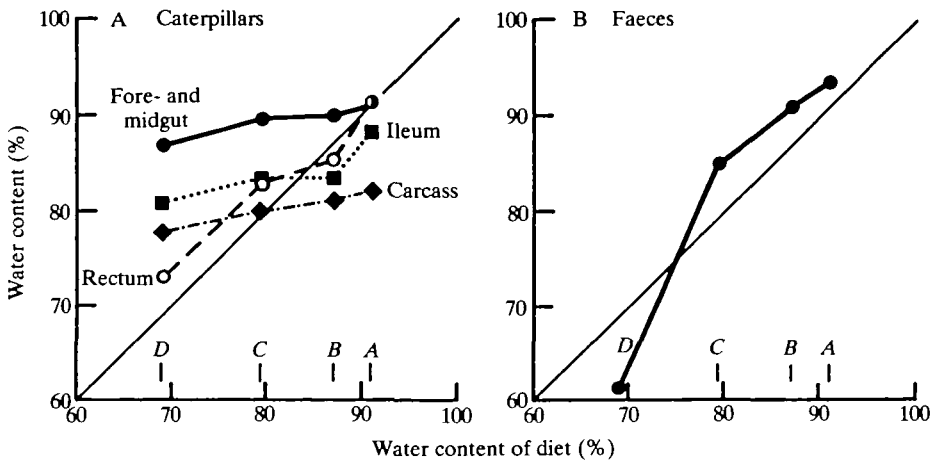
Fig. 2. (A) The relationship between water content (%) of various parts of *Manduca* caterpillars and the water content (%) of the diet. (B) The relationship between water content (%) of the faeces and of the diet. Data was from day 1. Values are means, *N* was 5 in each case. The diagonal lines show equivalence.

did not vary significantly between diets (Fig. 3A). Looking at individual faecal pellets, we found that caterpillars given drier diets produced smaller pellets (lower fresh mass, Fig. 3B), but that these pellets contained more dry matter (Fig. 3C), and were produced less frequently (Fig. 3D).

We investigated the speed with which caterpillars could respond to a diet of altered composition by altering the water content of their faeces. Fig. 4 shows that when the insects were switched from normal food (diet *C*) to one with greatly increased water content (diet *A*) there was a considerable delay before the water content of the faecal pellets was altered. The change occurred between the fifth and eighth pellets (about 5–6 h after transfer to the new diet), when the water content of the faeces increased sharply. The pellets became slightly larger (in fresh mass terms) at the same time, although their dry mass decreased. This delay is similar to the estimated retention time of normal food in the gut, 5·4 h (Reynolds *et al.* 1985).

The rectum as a site of water resorption

Data presented above suggest that varying resorption of water from the faeces is the means whereby water balance is maintained, and the water content of the body regulated, in the face of altered food water content. We investigated the site of such water resorption by killing caterpillars at various times after defecation, and following the change in water content of the contents of the rectum and ileum. This was done using insects that were feeding on dry food (diet *D*) because water resorption would be expected to be most evident in such insects.

Fig. 5 shows that there is a clear decrease in the water content of excreta within the rectum with increasing time spent there. In contrast, there did not appear to be
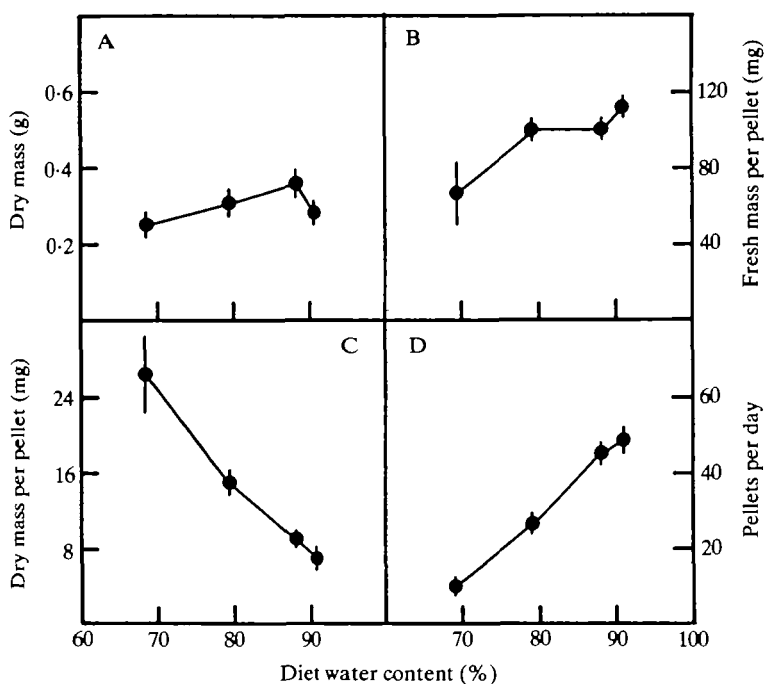
Fig. 3. Production of faeces by *Manduca* caterpillars feeding on diets of altered water content. (A) Total daily production of faeces in dry mass terms. (B) Mean fresh masses of individual faecal pellets. (C) Mean dry masses of individual faecal pellets. (D) Numbers of faecal pellets produced per day. Means ± S.E., *N* was 5 in each case.

any significant change in the water content of the ileum's contents with time since the last defecation. These data are consistent with the rectum being the main site of hindgut water resorption, with the ileum playing at most a minor role in water regulation. It is evident that the rate at which water is removed from the rectal contents declines rapidly with time once the faecal pellet has entered the rectum (Fig. 5B).

Ultrastructure of ileum and rectum

The conclusion that the rectum is the main site of water resorption is supported by examination of the structure of epithelial cells lining the walls of the ileum and rectum (Fig. 6). A high proportion of rectal cells are apparently specialized for fluid transport, possessing extensive membrane infoldings at both basal and luminal surfaces and large numbers of mitochondria associated with them. In contrast, such cells are absent from the ileum, where epithelial cells have apical microvilli typical of cuticle-secreting cells, but possess no extensive folded stacks of membrane such as are seen in the rectal cells. The ileal epithelial cells have no basal infoldings at all.

Rate of water uptake by the rectum

The percentage of water in the rectal contents was measured as soon as possible after the deposition of a faecal pellet, and again 15 min after the deposition of a pellet. In addition the water content of freshly deposited faecal pellets was
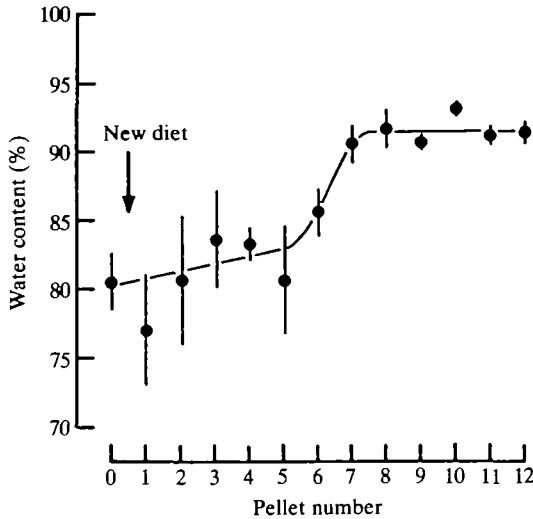

Fig. 4. The time course of adjustment of rectal water reabsorption following a change of diet. The graph shows the water content (%) of successive faecal pellets produced by day 1 caterpillars following a change from an artificial diet of 'normal' composition (19 % dry mass) to one similar to diet *D* (80 % dry mass). Means ± s.e., *N* = 5 in each case.
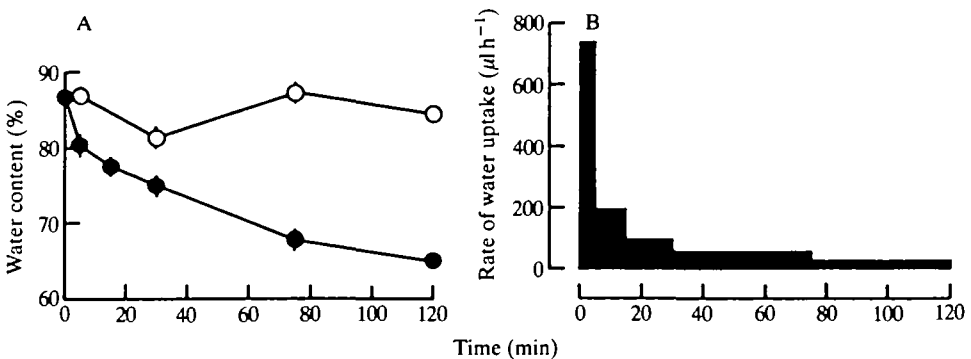

Fig. 5. (A) Water content (%) of the contents of the ileum (○) and rectum (●) at various times after the last defecation for day 1 fifth-instar *Manduca* caterpillars fed on diet *D* for the previous 24 h. Means ± s.e., *N* = 5 in each case. (B) Rate of water resorption from rectal contents plotted against time. Rates of resorption were calculated from the mean values shown in A, and thus no statistical estimates of their accuracy can be made. Calculations assume that the dry mass of the rectal contents was 25 mg (see Fig. 3C).
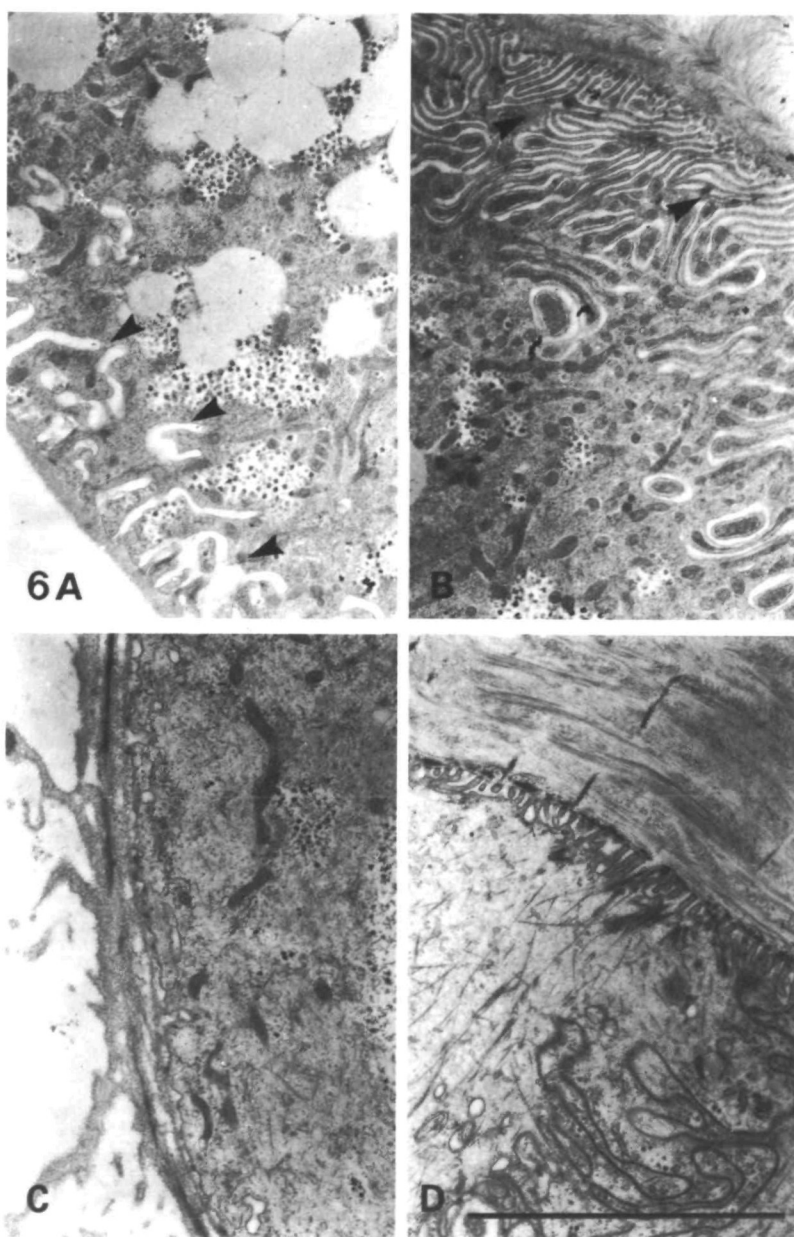
Fig. 6. Ultrastructure of hindgut epithelial cells in *Manduca* caterpillars fed 'normal' artificial diet. (A) Rectal epithelial cell, basal surface infolds are marked with arrowheads. (B) Rectal epithelial cell, apical surface. Apical infolds are marked with arrowheads. (C) Ileal epithelial cell, basal surface. Note absence of basal infolds. (D) Ileal epithelial cell, apical surface. Note absence of apical infolds. Scale bar, 5 μm.

determined (Table 2). These measurements, which were made only for diets *A*, *C* and *D*, allowed the estimation of the initial rate of water removal from the diet (i.e. 0–15 min) and the overall rate of water removal (i.e. over the whole period that the faecal pellet remains within the rectum), taking account of the differing sizes of faecal pellets according to diet (see Fig. 3). It can be seen (Table 2) that the overall rate of water uptake is greater for insects feeding on drier foods. The overall rate of resorption of caterpillars fed on diet *D* is three times that of those fed on diet *A*. The initial rates of resorption are apparently modulated even more strongly, with the estimated rate of uptake from caterpillars fed on diet *D* being almost six times that of those fed on diet *A*.

Discussion

The results obtained here show that *Manduca* caterpillars can regulate their body water content in the face of varying water availability, so that the water contents of the fore- and midgut, the ileum and the carcass all vary much less than that of the food. This ability to regulate is not complete, and changes in water content of all body compartments are evident after only 1 day's feeding on the altered diets. Nevertheless, these changes are limited and it is clear that the insects respond to more concentrated food by conserving water, and to more dilute food by losing more water, with their faeces.

Previous work by Scriber (1977) on *Hyalophora cecropia* and by Reese & Beck (1978) on *Agrotis ipsilon* larvae has indicated some degree of regulation of whole-body water content similar to that reported here. Ramsay (1976) investigated the response of *Pieris brassicae* larvae to water-depleted leaves. Although he concluded that the caterpillars showed little ability to remove water from their faeces, his own figures show that the insects markedly reduced the water content of their excreta under these conditions, producing faeces that were drier than their food. In the same paper he showed that the water content of the midgut of *Manduca* was much greater than that of either its food or its faeces. Although the ability of *Manduca* to produce very dry faeces is clearly much less highly developed than that of *Tenebrio molitor* (see Grimstone *et al.* 1968), rectal water resorption and water recycling nevertheless occur and are essential to the exploitation of plant food of varying water content.

Faecal water content closely parallels that of the food. An interesting consequence of this is that caterpillars feeding on dilute food produce faecal pellets more frequently than do those feeding on food that contains less water. Although faecal pellets are smaller when the insects are given food containing less water, the change is much less marked than is the decrease in water content. As a consequence of this, each faecal pellet contains more dry matter than when the caterpillars are feeding on normal food. Since the caterpillars compensate for diluted food by eating more (and thus maintaining their throughput of dry matter; Timmins *et al.* 1988), this means that insects eating food with a high water content

Table 2. *Rates of water resorption from the rectum in caterpillars fed diets of altered water content*

Diet	Water content of rectal contents at t = 0 min (%)	Water content of rectal contents at t = 15 min (%)	Water content of faecal pellets (%)	Initial rate of water resorption (0–15 min) (μl h^{-1})	Overall rate of water resorption (μl h^{-1}) (time)
A	95·7 ± 0·9	95·4 ± 0·5	92·8 ± 0·4	57	14·0 (0–45 min)
C	89·3 ± 1·6	86·5 ± 1·0	82·0 ± 0·6	116	37·6 (0–90 min)
D	86·8 ± 0·4	77·6 ± 1·0	61·6 ± 1·6	327	43·6 (0–150 min)

Water contents are given as means ± S.E., N = 5 or 6.
Rates of water resorption were calculated from the mean water content values and thus no statistical estimates of their accuracy can be made.

must produce more faecal pellets than those eating more concentrated foods, even though the amount of dry matter eaten is the same. The relationship between the number of faecal pellets produced and the food's water content is linear over the range we investigated (Fig. 3D). This is an important point, since it means that food intake cannot be estimated simply by counting the number of faecal pellets produced, unless the water content of the food is known not to vary.

The principal site of water resorption from the faeces appears to be the rectum. Two lines of evidence support this conclusion. The first is physiological. According to Reinecke & Adams (1977), the contents of the *Manduca* midgut are continuously packed into the ileum by the action of the pyloric musculature of the midgut–hindgut junction. A faecal pellet is formed in the ileum by the compressive action of these muscles, together with those of the ileum's thick, muscular walls. This pellet is then moved on to the rectum only when the rectal lumen is vacated by the voiding of the previous faecal pellet in the series. Our finding that the water content of faecal pellets in the rectum decreases with time since defecation, whereas that of pellets in the ileum does not, clearly implicates the rectum as the major site of water resorption.

It is noteworthy that the water content of the ileum and its contents is less than that of the midgut and its contents, even immediately after defecation. This is consistent with the suggestion of Dow (1986) that some water may be mechanically 'squeezed' from the midgut contents as they pass into the ileum as a result of compression in the pyloric region. This water would remain in the midgut, where it could be used to increase the degree of hydration of food entering the midgut from the foregut. Such local recycling of water within the midgut would lessen the need to recycle water from hindgut to midgut *via* the haemolymph (Reynolds *et al.* 1985). Measurement of the amount of water recycled by resorption from the rectum (see below) suggests, however, that the contribution of such mechanical squeezing is relatively small – of the order of 10 % of the total recycled.

It is clear from the data of Fig. 5 that a law of diminishing returns operates for the time course of water resorption from the rectum, the rate of uptake falling steeply with time once the faecal material has entered the rectum. This may represent the increasing difficulty of extracting water from the progressively more concentrated faecal material. Alternatively, the rate of water resorption may be regulated to change with time in this way, for example by the release of a resorption-stimulating factor at the time of defecation.

The second line of evidence implicating the rectum in water resorption is anatomical. It is well known that lepidopteran larvae possess a cryptonephric rectal complex in which the distal ends of the Malpighian tubules are closely applied to the rectum and enclosed with it in a special chamber, the perinephric space (Wall & Oschman, 1975). A similar arrangement in the beetle *Tenebrio molitor* has been proposed as the basis for the production of the extremely dry faeces which that insect produces when living on very dry food (Grimstone *et al.* 1968). Additionally, we show here that the ultrastructure of many of the epithelial cells lining the rectum is typical of cells involved in fluid transport, with extensive

basal and apical infoldings, the latter closely associated with a high density of mitochondria. These infoldings presumably serve to increase membrane surface area for transport, and to generate local osmotic gradients that allow water to follow passively (Wall & Oschman, 1975). In contrast, the epithelial cells of the wall of the ileum do not show such membrane specializations, having neither basal nor apical infoldings, and they are thus unlikely to be involved in active fluid transport.

Ramsay's (1976) study of the rectal complex in *Pieris* and *Manduca* led him to conclude that the primary role of the cryptonephric condition in caterpillars was in the regulation of ion balance, rather than in water resorption from the faeces. Although the rectum undoubtedly has the former function, it is clear that Ramsay's conclusion that 'any ability to remove water from the faeces...is not brought into use under their natural dietary regime' is incorrect. The rectum is quantitatively important in its role as the source of recycled water. The rate of rectal water resorption estimated here for day 1 fifth-instar larvae on normal diet ($37.6\,\mu l\,h^{-1}$ or $0.90\,g\,day^{-1}$) represents 90% of the total amount previously estimated to be recycled by day 1 insects (Reynolds *et al.* 1985).

It is evident that the amount of water resorbed in the rectum is regulated. An initial hypothesis might be that the extent to which faecal pellets are dehydrated would simply depend on the length of time that they spend in the rectum, just as in the collembolan *Petrobius brevistylus* the water-absorbing abdominal vesicles are only everted for as long as necessary to absorb sufficient water (Houlihan, 1977). This is in line with our finding that the frequency of faecal pellet production increases with faecal water content. A direct check of this hypothesis suggests, however, that additional controls are present. The overall rate of water uptake from the rectal contents was increased by a factor of three between diets *A* and *D*, whereas the initial rate of resorption (measured over the first 15 min after the faecal pellet enters the rectum) was increased by a factor of six.

The apparent modulation of the rate of resorption of water by the rectum provides strong circumstantial evidence for the existence of a neural or hormonal control system. An increase in the rate of rectal resorption might be achieved either by decreasing the level of a diuretic factor or by increasing the level of an antidiuretic factor. There is evidence for the existence of both diuretic and antidiuretic hormones in many insects (Raabe, 1982), although none has yet been described for a caterpillar. The results presented here suggest that it might be worthwhile to look for one.

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