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A comparison of ten digestive enzymes reveals a lack of chitinase in a phasmid and the loss of two β-glucanases in a mantid

JOSEPH WOODRING
Department of Animal Ecology II, University of Bayreuth, Bayreuth, Germany

Abstract. In all developmental stages, the phasmid *Peruphasma schultei* (Conle & Hennemann, 2005) is an obligate herbivore, whereas the mantid *Hierodula membranacea* (Burmeister, 1838) is an obligatory carnivore. In *P. schultei*, the luminal activity of all enzymes is approxximately 50% in the crop and 50% in the midgut, which corresponds to the approximate 50 : 50 ratio of volumes of these two regions. These ratios would be expected in insects with a constant feeding rate on an unvaried diet. The enzyme activity and volume ratios in *Hierodula membranacea* vary considerably because of the irregular feeding habits. These differences in activity ratios between phasmids and mantids are not associated with the obligate phytophagous or carnivorous diet. The ratio of membrane bound to luminal aminopeptidases and disaccharidases in the midgut of both species are not significantly different and are within the normal range of other paurometabolous insects. Cellobiase and other plant cell wall digesting enzymes, laminarinase and cellobiase, are present in the phasmid but totally lacking in the mantid. The obligate carnivorous feeding habits of mantids could represent a selective factor leading to the loss of the ability to produce β-glucanases. Chitinase is a moulting enzyme in all insects, whereas, in *H. membranacea*, chitinase also occurs as a luminal digestive enzyme. This modified enzyme function requires production and secretion in another tissue, namely the midgut.

Key words. Bound enzyme ratio, caecal region, enzyme distribution, feeding behaviour, loss of enzymes.

Introduction

To investigate the possible reduction or enhancement of activity of individual enzymes, including the possible loss of some enzymes, in response to an obligatory leaf diet or an obligatory meat diet over hundreds of million years, the digestive enzymes of the phasmid *Peruphasma schultei* (Conle & Hennemann, 2005) and the mantid *Hierodula membranacea* (Burmaster, 1838) are compared. The hexapod orders are assumed to have radiated into the Ametabola (Thysanura), Paleoptera (Ephemerita and Odenata), Paurometabola (cockroaches and locusts), Polyneuroptera (Hemiptera) and Holometabola (Wheeler *et al.*, 2001). The paurometablous insects are terrestrial and eat a relatively consistent diet during all development stages. They evolved early into two major lines: the Blattopteroidea and the Orthopteroidea (Terra & Ferreira, 2012). The blattopteroids gave rise to the mantids and the cockroaches–termites. The phasmids, along with the grasshoppers and crickets arose from a common orthopteroid ancestor. Fossils of phasmids and mantids, with their distinctive body forms, indicate that these insects, and presumably their feeding habits, have probably existed as separate evolutionary lines subsequent to the early Mesozoic (Schang *et al.*, 2011; Hörnig *et al.*, 2017).

The morphology of the gut and the physical breakdown of food during passage through the gut of *Peruphasma schultei* and *Hierodula membranacea* are compared aiming to determine whether some aspect might be associated with an obligatory leaf or meat diet. The main purpose of the present study, however, is to determine whether digestive enzymes can be lost or acquired in the course of evolution in insects, and the ideal test insects would be two related species of the Paurometabola, one an obligate herbivore and the other an obligate carnivore.

Correspondence: Joseph Woodring, Department of Animal Ecology II, University of Bayreuth, 95447 Bayreuth, Germany. Tel.: 48 0921 853204; e-mail: joseph.woodring@uni-bayreuth.de

The cell wall digesting enzymes, cellulase and laminarinase, are β-glucanases, and are found in the lumen of in cockroaches, crickets and walking sticks (Genta *et al.*, 2003; Shelomi *et al.*, 2014a; J. Woodring, unpublished data). Cockroaches and crickets are omnivorous, and walking sticks are phytophagous insects. The mantid *H. membranacea* is an ideal insect for determining whether a carnivorous insect might have β-glucanases.

Chitinase is secreted by epidermal cells of the cuticle and epithelial cells of the fore- and hind gut to initiate the digestion of the endcuticle (apolysis) of the exoskeleton and cuticular lining of the gut at the beginning of moulting in all insects (Kramer & Kuthukrishnan, 1997; Fillho *et al.*, 2002; Weidlich *et al.*, 2013). Chitinase is reported as a digestive enzyme in the gut lumen in grain beetles (Fukamizo *et al.*, 1985; Genta *et al.*, 2006), mosquito larvae (Souza-Neto *et al.*, 2003) and termites (Arquette & Rodriguez, 2013). A digestive chitinase would be expected to occur in a carnivorous insect, although this still remains to be reported. An obligatory carnivore such as a preying mantid could provide an example of a digestive chitinase in a carnivorous insect.

Materials and methods

Rearing and sample preparation

The phasmids, *P. schultei*, were fed freshly cut small branches of *Ligustrum vulgare* in a glass terrarium (20 × 25 × 30 cm), misted daily with water, and reared under a 16 : 8 h light/dark photocycle at 28 °C. The mantids, *H. membranacea*, were fed field crickets (*Gryllus bimaculatus*) maintained in plastic boxes (10 × 15 × 15 cm) and reared under a 16 : 8 h light/dark photocycle at 28 °C. Young fed adult females of *P. schulti* and *H. membranacea* were immobilized at 4 °C, ventrally cut open from the anus to the head, pinned open on a Styrofoam surface and flooded with 4 °C *Gryllus* Ringer (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 4 mM Hepes, pH 7.2) (GR). The entire digestive tract was rinsed free of haemolymph and transferred to approximately 3 mL of GR in a plastic Petri dish (diameter 10 cm). The hydrophobic surface tension caused the GR to form a compact puddle and the gut regions could be easily separated and transferred to another puddle with approxmately 350 μL of GR with a minimal loss of lumen contents. The crop, caeca, ventriculus and ileum were slit open, and the lumen contents of each were brought to 500 μL GR and centrifuged at 13 000 ***g*** at 4 °C for 5 min to remove undigested food particles. A few crystals of phenylthiourea were added to inhibit phenoloxidases (prevents darkening) and the tissues were stored at −20 °C until use.

To determine the ratio of bound to luminal enzymes, the rinsed tissues of the caecum and ventriculus of both species were sonicated with a Bronson Ultraschall Sonicator in 500 μL of GR at 4 °C, centrifuged at 13000 ***g*** at 4 °C for 5 min, and then the supernatant was stored at −20 °C. These samples also contain a portion of the cytoplasmic (vesicular) form of the digestive enzymes. The pellet contained almost no enzyme activity. Trypsinogen is also present in tissue samples, whereas activated trypsin occurs only in the lumen. After thawing and before use, samples were again centrifuged and the clear supernatant was used for the enzyme assays.

The salivary glands of *P. schultei* and *H. membranacea* were removed and placed in GR and then the adhering fat body was carefully removed. The salivary glands were touched to a piece of filter paper to remove adhering GR and fat body and were then placed in fresh GR and brought to 500 μL. After centrifugation at 13 000 ***g*** at 4 °C for 5 min, the supernatant was placed in a 1.5-mL Eppendorf tube and stored at −20 °C.

Enzyme assays

Trypsin activity was measured using α-*N*-benzoyl-DL-arginine -*p*-nitroanilide (BApNA) (Sigma, St Louis, Missouri) as a substrate, which is specific for trypsin. A 10 mM BApNA stock solution dissolved in *N*,*N*-dimethylformamide was diluted with 50 mM Hepes buffer, pH 7.2, to a working solution of 1 mM shortly before use. Activity was measured by mixing a 20 μL of sample in 150 μL of 1 mM BApNA in a cuvette at 24 °C and then measuring the nmol pNA released, which was based on a standard curve of dAbs versus nmol pNA at 410 nm. The aminopeptidase activity was measured as the trypsin, except that LpNA (L9125; Sigma) instead of BApNA was used as a substrate.

The lipase activity was measured using the substrate *p*-nitrophenyl palmitate (pNPP), as modified from Pencreach & Baratti (1996). Solution A, comprising 21 mg of pNPP in 5 mL of 2-propanol (16.6 mM), was made fresh immediately before use (warmed up until clear). Solution B, comprising 80 μL of Triton X 100 in 10 mL of 50 mM Hepes buffer (pH 8.0), is stable at 4 °C for weeks. Hepes buffer (24 μL) was added to 20 μL of sample, to which 6 μL of substrate pNPP (mix of solutions comprising one part A and nine parts B) was then added. The total lipase activity was determined by rate of the amount of pNP hydrolyzed from pNPP (nmol min^{-1}), which was based on a standard curve of dAbs versus nmol pNP at 405 nm. The polysaccharidase activities of amylase, cellulase and laminarinase were determined by the hydrolytic action of these enzymes on their corresponding substrates, soluble starch (Sigma), caboxymethyl cellulose (Sigma) and laminaran (L-9634; Sigma), to release a reducing sugar. Next, 6 μL of 1% of the substrate was added to 24 μL of 50 mM Hepes (pH 7.2), which was then incubated at 28 °C with samples of 20 μL for 30 min. The activity of each glucanase was established by the amount (nmol) of maltose produced in 30 min, as determined using the dinitrosalicyclic acid reagent method modified from Bernfeld (1955). Polysaccharidase activity was measured as the amount (nmol) of maltose released. Because some maltase is present in the luminal fluid, a zero time maltase activity of the lumen sample was subtracted for the polysaccharidase assays.

Chitinase activity was determined using 10 mg of Chitin Azure (Sigma) suspended in 30 μL of GR + 20 μL samples of luminal fluid and incubating for 30 min at 40 °C with shaking for 10 min. After centrifugation at 1000 ***g***, the absorbance of the liberated dye remazol brilliant violet 5R (RBW) was measured at 550 nm against a RBW standard curve. The activity of chitinase was expressed as the rate of the amount of RBV released (nmol min^{-1}).

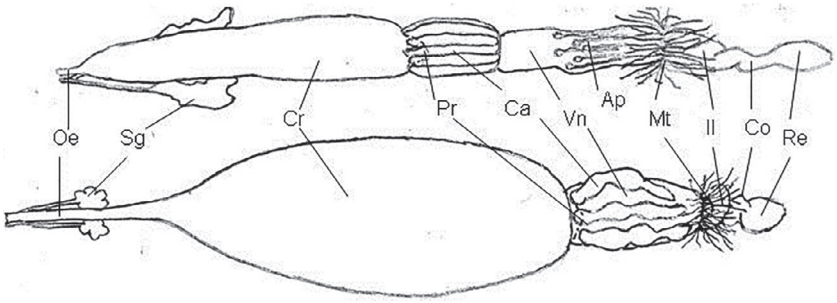

Fig. 1. Digestive tract of adult female of *Peruphasma schultei* (top) and *Hierodula membranacea* (bottom). Level of legs 1–3 (L1–L3), oesophagus (Oe), salivary glands (Sg), crop (Cr), caeca or caecal region (Ca), ventriculus (Vn), malpighian tubules (Mt), ileum (Il), colon (Co), rectum (Re) and ventricular appendices (Ap). The caecal region of the phasmid is the anterior midgut (see text).

The disaccharidase activities of maltase, trehalase and cellobiase were determined by the hydrolytic action of these enzymes on their corresponding substrates maltose, trehalose and cellobiose. Next 6 mg mL^{-1} substrate was added (= 50 µL) to 24 µL of Hepes plus 20 µL of sample, followed by incubation at 28 °C for 30 min. From the 50 µL of incubate, a sample of 5 µL was added to 150 µL of glucose oxidase reagent (G2393; Sigma) and incubated at 28 °C for 10 min. The disaccharidase activity was determined for each substrate by the nmol glucose produced, which was based on a standard curve of dAbs versus nmol glucose at 340 nm. Because some glucose is present in the luminal fluid, a zero-time glucose oxidase activity of the lumen sample was subtracted for the disaccharidase assays. For all assays, a control with heated enzyme (100 °C) was subtracted.

Statistical analysis

WINSTAT for EXCEL (Microsoft Corp, Redmond, Washington) was used to evaluate the data. The mean ± SEM was determined for the wet weight of the gut regions, the activity of the salivary gland enzymes, the percentage of bound enzymes and the luminal activity (U min^{-1}) for trypsin, aminopeptidase, lipase, four polysaccharidases and three disaccharidases in *P. schultei* and *H. membanacea*. Eight fed females that were at least 7 days old were used; therefore, $n = 7–8$ for all assays and the SEM for all enzyme assays was between 7% and 10% of the mean. All calculations were cross-checked using an EXCEL spreadsheet.

Results

The guts of *P. schultei* and *H. membranacea* have a quite similar morphology (Fig. 1). The proventriculus in both species narrows down to a conical projection that protrudes into the anterior end of the mid gut. The proventricular teeth are small but the muscular cardiac valve is well developed. The hind gut of both species is relatively small, coiled and similar in appearance. The crop of fed mantids is much larger than that of fed phasmids. The mantid crop weighs up to 320 mg compared with 60–80 mg for the caecae plus ventriculus, giving a ratio of approximately 4:1. In phasmids, the crop weighs 210 mg compared with 80 mg for the fused caecae (anterior midgut) plus 130 mg for the ventriculus, giving a ratio of approximately 1:1. The caecae have fused together to forms separate gut region in phasmids (Fig. 1). The diameter of the anterior midgut is approximately the same as its length and the wall appears to be pleated, with very small caecae projecting from the anterior end. The relatively large, fast cuboidal, frothy appearing endothelial cells secrete a peritrophic gel, as described by Terra (2001). The posterior hind gut (ventriculus) of the phasmid is three times longer than the anterior midgut. The columnar endothelial cells are smaller with almost no vacuoles and these cells secrete a laminated type II peritrophic membrane. In *H. membranacea*, six tubular caecae arise from the anterior end of the ventriculus and extend almost to the posterior end. They adhere lightly to the ventricular surface. In the phasmid, a dozen appendices arise from the ventriculus, which is a unique feature of this group, forming a novel system of excretion (Shelomi & Kimsey, 2014). In newly moulted mantids, the caecae are short, have a light brown colour, are relatively thin and have a uniform diameter. In fed mantids, the caecae are longer, their diameter doubles, the contents become dark brown and they have bubble-like expansions along their length. These expansions move by peristaltic contractions, although so slowly that it is difficult to determine with certainty the direction of movement.

There were essentially no differences observed in the breakdown processes of leaf tissue or prey tissue during the passage though the gut in either species. Relatively larges pieces of intact leaves were observed at the anterior end of the crop, which gradually decreased in size and structure, with a colour change from green to brown, as the food bolus was churned in its passage through the crop. Likewise, recognizable pieces of legs, cuticle and muscle were observed at the anterior end of the crop, although only small pieces of cuticle were present in the mush that passed into the ventriculus.

The enzyme activity of trypsin, lipase, three polysaccharidases, chitinase, aminopeptidase and three disaccharidases in the lumen of the caecum ventriculus and ileum of fed adult females of *P. schultei* and *H. membranacea* was determined (Table 1). The ratio of the luminal activity of all enzymes examined was approximately 50: 50 in the crop and midgut (caecum + ventriculus) of *P. schultei*. The ratio of the luminal enzyme activity between the crop and midgut of *H. membranacea*, on the other hand, varied considerably. In the mantid crop, only 10% of the lipase and 40% of the trypsin

Table 1. Digestive enzyme activity in *Peruphasma schultei* and *Hierodula membranacea.*

		Try	Lip	Amy	Cel	Lam	Chit	Amp	Mal Tre	Ceb	WW	
Phasmid												
Salgl		32	0	0	0	0	ND	13	15	0	0	ND
Crop		1125	31	22	10	7	0	385	70	15	28	250
Caecum	958	8.6	18	9.7	3.5	0	222	6	2.5	15	85	
Ventriculus	362	26	0	0	0	0	178	29	1.4	5	150	
Ileum		42	4.6	0	0	0	0	124	0	0	0	40
Mantid												
Salgl		8.3	0	4	0	0	ND	0	54	62	0	ND
Crop		1623	20	142	0	0	29	137	365	206	0	460
Caecum	1126	74	ND	0	0	3	373	91	46	ND	30	
Ventriculus	2871	159	62	0	0	5	596	127	77	0	90	
Ileum		617	33	2	0	0	0	52	23	4	0	30

All enzyme activities are as U animal^{-1} min^{-1}. Trypsin and aminopeptidase U = nmol pNA; lipase U = nmol pNP; polysaccharidase U = mg maltose; and disaccharidases U = mg glucose. Amp, aminopeptidase; Amy, amylase; Ceb, cellobiase; Cel, cellulase; Chit, chitinase; Lam, laminarinase; Lip, lipase; Mal, maltase; Salgl, salivary gland; Tre, trehalase; Try, trypsin; WW, wet weight (mg); ND, not determined. $n = 7–8$ in all assays. The SEM for all enzyme assays was between 7% and 10%.

Table 2. Ratio of lumen to membrane bound enzymes in the midgut of *Peruphasma schultei* and *Hierodula membranacea*.

	Amp	Mal	Tre
Phasmid	400 : 140	35 : 9	3.9 : 1.6
Mantid	509 : 113	218 : 69	123 : 28

Amp, aminopeptidase; Mal, maltase; Tre, trehalase. $n = 7–8$ in all comparisons. The SEM for all enzyme assays was between 7% and 10%.

activity was found, in contrast to 65–70% of amylase, maltase and trehalase activity. The ratio of luminal to membrane bound enzymes (aminopeptidase, maltase and trehalase) in the caecum-ventriculus was approximately the same for all three of these enzymes for each species, although it was somewhat higher in *P. schultei* (20–29%) than in *H. membranacea* (10–24%). There was no difference in total trypsin activity between the two species but, surprisingly, the amylase activity was higher in the mantid than in the phasmid.

Another aspect of the distribution of enzymes concerns the percentage of aminopeptidases and disaccharidases bound to the epithelial membrane in the midgut. In *P. schultei*, 26% of the aminopeptidase, 20% of the maltase and 29% of the trehalase are bound and, in *H. membranacea*, 18% of the aminopeptidase, 25% of the glucase and 19% of the trehalase (cellubiase is lacking) are bound (Table 2).

The low enzyme activity found in the salivary gland homogenates of both *P. schultei* and *H. membranacea* indicates a minimal role of salivary enzymes in hydrolysis of nutrients, and no association with the diet. There was no visible accumulation of microorganisms in any gut region of either species. A comprehensive investigation of the microbiology in the gut of several other phasmid species indicated no contribution of any microorganisms to digestion (Shelomi *et al.*, 2015).

The most striking difference between the distribution of enzymes of *P. schultei* and *H. membranacea* was the complete lack of cellulase, laminarinase and cellubiase in the gut of the mantid. Chitinase activity was found in the lumen of the crop, caecal arms and ventriculus of *H. membranacea*, although it was totally lacking in *P. schultei*.

Discussion

The morphology of the gut of *P. schultei* and *H. membranacea* is quite similar, although some differences in the morphology are observed. The most conspicuous differences are the larger crop of the mantid and the fused caecae (anterior midgut) of the phasmid. The mantid crop is almost four times larger than the midgut and, for some enzymes, the ratio of enzyme activities is several times higher in the midgut than in the crop. The large mantid crop is probably an adaptation to the often huge, irregular meals and is not associated with a carnivorous feeding habit. Phasmids eat a relatively constant amount of food, resulting in an equal sized crop and midgut and an approximately 50 : 50 ratio of the activity of all enzymes in the crop and midgut.

The caecae of *P. schultei* forms a distinct region of the midgut, which is termed anterior midgut by Shelomi *et al.* (2015). This anterior midgut is similar to the caecal region of crickets (Woodring & Lorenz, 2007; Biagio *et al.*, 2009). The caecal region of crickets and phasmids appears to form from the fusion of the anterior tube-like caecae of the orthopteroid ancestors. The caecal region of cricket extends across the midline and forms a pair of lateral pockets with longitudinal folds, which are remnants of separate caecae. The anterior midgut of phasmids and caecal region of crickets are lined with a secreted peritrophic gel, and the typical peritrophic membrane is secreted by the ventriculus. The caecal region is fragile and the cells break apart easily, in contrast to the robust ventriculus. The caecae of *H. membranacea* arise from the anterior end of the ventriculus and resemble those of cockroaches and locusts. In conclusion, neither the large crop of the mantid, nor the fused caecal region (anterior midgut) of the phasmid is associated with an obligate phytophagous or carnivorous diet.

The major aim of the present study is to compare the activity and distribution of the digestive enzymes of a phasmid and

a mantid and to determine whether any differences could be associated with an obligatory diet of leaves or insect tissues. The first comparison is the ratio of bound to unbound enzymes in the midgut. In the holometabolous insects, all aminopeptidases and disaccharidases are membrane bound (Terra & Ferreira, 2012). In paurometabolous insects, some of these enzymes also occur free in the lumen. The percentage bound to unbound aminopeptidase and maltase in grasshoppers (Ferreira *et al.*, 1990), cockroaches (Tamaki *et al.*, 2014) and crickets (Biagio *et al.*, 2009; Woodring, 2017) ranges from 5% to 80%. In *P. schultei*, the bound enzymes in the midgut is10-24%, whereas, in another phasmid species, *Cladomorphus phyllinus*, it is approximately 70% (Monteiro *et al.*, 2014). The ratio of bound enzymes in the midgut of *H. membranacea* is 20–29%. Thus, the ratio of membrane bound to luminal aminopeptidase and maltase in paurometabolous insects is highly variable and is not associated with either a plant-eating or meat-eating diet.

The second comparison is the activities of the plant cell wall digesting enzymes, which are all β-glucanases. The activities of cellulase, laminarinase and cellubiase are completely lacking in *H. membranacea* but present in *P. schultei*. Several dozen cell wall digesting enzyme encoding genes, including cellulases and pectinases, are expressed in the anterior midgut of *P. schultei* (Shelomi *et al.*, 2014b). Endogenous cellulase, laminarinase, licheninase and cellobiase are also found in the omnivorous cockroach *P. americana* (Genta *et al.*, 2003) and cricket *G. bimaculatus* (J. Woodring, unpublished data). Therefore, the common ancestors of mantids, cockroaches and termites very likely possessed these enzymes. The mantid line of the Paurometabola separated at least 100 millions years ago from the main line of Blattopteroidea (Hörnig *et al.*, 2017) and it is possible that the capacity to synthesize and secrete certain enzymes has been lost over time, either by loss of these genes or their expression. The β-glucanase cellulase is found in lumen and tissues of 68 species representing eight orders (Oppert *et al.*, 2010), although cellulase is apparently lacking in some Diptera such as *Drosophila* and *Anopheles* (Kunieda *et al.*, 2006). An obligatory meat eater has no need for β-glucanses and perhaps the loss of these enzymes or the expression of the genes could have selective value.

The third comparison is chitinase activity. Chitinase is secreted by the epidermis of the cuticle and by the epithelium of the fore- and hindgut, which initiates the digestion of the endocuticle (apolysis) in all insects (Kramer & Kuthukrishnan, 1997. Chitinase is an enzyme involved with moulting and is not usually associated with nutrient digestion; however, chitinase as a digestive enzyme is reported in stored grain beetles (Fukamizo *et al.*, 1985; Genta *et al.*, 2006), mosquitoes (Fillho *et al.*, 2002; Souza-Neto *et al.*, 2003), crickets (Weidlich *et al.*, 2013) and termites (Arquette & Rodriguez, 2013). Interestingly, none of these insects are carnivorous. To function as a digestive enzyme, the chitinase genes must be expressed in another tissue, specifically the midgut epithelium, and must be secreted into the lumen. This evolutionary modification appears to have occurred several times in insects. The characteristics of the luminal chitinase of *T. molitor* indicate a considerable modification from the chitinase involved with moulting (Genta *et al.*, 2006), possibly to protect the peritrophic membrane. The presence of chitinase in the crop and midgut lumen of the carnivorous *H. membranacea* is the first report of a digestive chitinase in a carnivorous insect. It would be interesting to examine other entomophagous insects for luminal chitinases.

The obligate carnivorous feeding habits of mantids could have been a selective factor leading to the loss of the ability to produce β-glucanases. In the holometabolous insects, it is not unusual for many or even all of the enzymes present in the larval stage to be lacking in the digestive tract of the adult, which is possible because the adult stage of these insects can live from the fat body stores produced by the larvae. Although cellulase is lacking in *Drosophila* and *Anopheles* larvae (Kunieda *et al.*, 2006), there are no reports of a total loss of a group of enzymes in all developmental stages of any species of holometabolous insects. Thus, *H. membranacea* is the first insect found that has lost the capacity to produce an entire group of digestive enzymes in all stages of development. The energy saved by not producing β-glucanases could provide the necessary selective pressure allowing the obligatory meat eating mantids to lose these enzymes. It would be interesting to investigate other obligatory meat eating insects, such as dragon flies, carrion beetles and flesh flies, to determine whether mantids are unique among insects with regard to the loss of β-glucanases.

The fourth comparison is the total enzyme activities of proteases, lipases and amylase. Although the total wet weight of the gut of both species is similar, trypsin activity is 2,3 times higher, lipase activity is four times higher and amylase activity is five times higher in the mantid than in the phasmid. One possible explanation for this difference is the higher nutrient value of the mantid diet compared with the phasmid diet, and both species adjust their digestive enzyme activity to the composition of their diet. This appears to be the case in the grasshopper *Oedaleus asiatius*, where the ratio of chymotrypsin, lipids and amylase activities varies strongly with respect to the type of grass eaten (Huang *et al.*, 2017). The diet of the mantid (crickets) contains 15% protein, 5% lipids and 2,3% carbohydrates (Woodring *et al.*, 1977) and leaves comprise approximately 2–3% protein, 0.5% lipids and 1–7% carbohydrates. The gut of *P. schultei* thus contains a food of much lower nutrient value and the phasmid could save energy by secreting a reduced amount (activity) of enzymes compared with that of *H. membranacea*.

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The authors declare that they have no conflicts of interest.

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