Blood feeding in juvenile *Paragnathia formica* (Isopoda: Gnathiidae): biochemical characterization of trypsin inhibitors, detection of anticoagulants, and molecular identification of fish hosts

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SUMMARY

The 3 post-marsupial juvenile stages of the gnathiid isopod, *Paragnathia formica*, are haematophagous ectoparasites of fishes that may, in heavy infestations, cause host mortality. Protein digestion in fed stage 3 juveniles is accomplished by cysteine proteinases, but what bioactive compounds attenuate host haemostatic, inflammatory and immunological responses during feeding is unknown. Trypsin inhibitory activity and anticoagulant activity were detected in crude extracts of unfed *P. formica* stage 1 juveniles; fractionation of stage 1 crude extracts by ion exchange chromatography resulted in 3 preparations each displaying these bioactivities. Further characterization revealed anti-thrombin activity in 2 of these preparations, whilst the third displayed the strongest anticoagulant activity that targeted a factor of the intrinsic coagulation pathway. Three trypsin inhibitors (18 kDa, 21 kDa, and 22 kDa) were also detected using reverse zymography. In parallel, homogenates of fed stage 2 and 3 juveniles were used to identify their fish hosts by amplifying the 16S mitochondrial rDNA and 18S genomic rDNA vertebrate gene regions. Blood from at least 4 fish families had been ingested by separate individuals during feeding. This study demonstrates that trypsin inhibitors and anticoagulants are present in *P. formica* juveniles which could suppress host haemostatic, inflammatory and immunological responses during feeding, and that juveniles are not host specific.

Key words: *Paragnathia formica*, fish ectoparasite, blood feeding, host-parasite interactions, serine protease inhibitor, anticoagulants.

INTRODUCTION

Gnathiid isopods (Crustacea; Isopoda; Gnathiidae) have biphasic life cycles with free-living adults and parasitic, post-marsupial juvenile stages feeding on fishes. Although approximately 187 gnathiid species have been described world-wide, inhabiting estuaries, intertidal zones, coral reefs, and the deep sea, the impact of blood feeding by their juvenile stages is difficult to assess since they mostly target wild fishes (see Smit and Davies, 2004; Jones *et al*. 2007; Tanaka, 2007; Manship, 2009; Ferreira *et al*. 2009, 2010). The parasite-host association can cause host stress, lesions, secondary infections, anaemia, and mortalities in captive environments (see Hayes *et al*. 2011). Like some terrestrial haematophagous arthropods, gnathiids have also been implicated in the transmission of blood dwelling apicomplexans between fishes, though the route of transmission remains uncertain (see Davies and Smit, 2001; Smit and Davies, 2004; Smit *et al*. 2006).

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The present study focuses on the gnathiid *Paragnathia formica* (Hesse, 1864), which inhabits mud microcliffs in estuaries across Western Europe, North Africa and the Mediterranean Basin (Monod, 1926; Upton, 1987; Cadée *et al*. 2001; Silva *et al*. 2006; Kirkim *et al*. 2008). Adult and juvenile stages can be collected readily from galleries in the mud banks, making this species an excellent gnathiid model for study. Mature females, in contact with water, release unfed zuphea 1 juveniles. These attach to host fish epithelia and, using piercing mouthparts, penetrate host tissues, feeding on whole blood, plasma and possibly mucus, to become inflated praniza 1 stages (Smit and Davies, 2004). As in other gnathiids, three zuphea (unfed) and three praniza stages (fed), as well as adults, occur in the life cycle of *P. formica*.

In fed praniza forms of *P. formica*, the bloodmeal undergoes initial breakdown in the anterior hindgut and protein digestion by cysteine-like proteinases in paired digestive glands (Manship *et al*. 2008). These processes provide amino acids that enable growth and moulting of praniza stages 1, 2, and 3 to subsequent zuphea 2, and 3 stages, or to adults; they also sustain survival and reproduction of non-feeding adults, and

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support embryological development (see Smit and Davies, 2004; Manship *et al*. 2011). In some respects the feeding behaviour of gnathiids resembles that of ticks (Ribeiro *et al*. 1985); *P. formica* for example can remain attached to the host for hours to days (Stoll, 1962). This association suggests that gnathiids, like ticks, must evade host defence responses, including haemostasis, which limits blood loss by vasoconstriction, platelet aggregation and blood coagulation (Ribeiro and Francischetti, 2003). Blood coagulation in fishes resembles that in higher vertebrates, with an extrinsic pathway stimulated by a tissue factor mostly found in skin, and thrombocytes replacing mammalian platelets in the intrinsic pathway (Tavares-Dia and Oliveira, 2009; Doolittle, 2011).

While haematophagous aquatic ectoparasites of fishes likely release anti-haemostatic compounds into their hosts during feeding, research on such compounds in these animals is scarce, limited largely to a report by Romestand and Trilles (1976*a*,*b*) demonstrating anti-thrombin activity towards fish blood in the salivary glands of adult cymothoid isopods *Meinertia oestroides* (Risso, 1826) and *Anilocra physodes* (Linnaeus, 1758) (Isopoda: Cymothoidae). Because zuphea 1 stages of *P. formica* have well developed salivary glands, even before hatching from females (Manship *et al*. 2011), we hypothesized that these glands might produce pharmacological compounds such as anticoagulants to facilitate blood feeding by *P. formica*. Here, we demonstrate for the first time the partial purification of trypsin inhibitors, and anticoagulant activities partly targeting thrombin, in recently-hatched *P. formica* zuphea 1 juveniles.

As in ticks (Pichon *et al*. 2003), gnathiids parasitize a variety of hosts, sometimes demonstrating micropredatory behaviour, though little is known of their host-selectivity. Such knowledge could be vital for assessing the impact of their feeding behaviour in various ecosystems, for instance on the Great Barrier Reef (GBR), Australia (Nagel and Lougheed, 2006; Jones *et al*. 2007), or on the transmission of apicomplexan parasites (Smit and Davies, 2004; Smit *et al*. 2006). Host specificity in some gnathiids of the genus *Gnathia* Leach, 1814 has been partially addressed on the GBR, by sequencing specific vertebrate 12S and 16S rDNA mitochondrial gene regions from fed (praniza) gnathiids (Nagel and Lougheed, 2006; Jones *et al*. 2007). To date, several *P. formica* host fishes have been recorded from the wild (Monod, 1926; Menezes, 1984; Kirkim *et al*. 2008), and in laboratory feeding experiments (Monod, 1926), which suggest that this gnathiid is relatively host non-specific. Here, we verify that several host fishes can be identified from the blood-meal content of praniza juveniles collected from two sites in the UK, using PCR amplification targeting conserved gene sequences, namely 16S rDNA and 18S rDNA gene regions.

MATERIALS AND METHODS

Specimen sampling, maintenance and storage

Paragnathia formica praniza 2 and 3 juveniles ($n = 7$) and gravid females ($n = \sim 600$) were collected from salt marsh banks of the type described by Manship *et al*. (2008, 2011) at Wells-next-the-Sea, Norfolk (Ordnance Survey grid reference, TF 925 438) (see Tinsley and Reilly, 2002) during April and May, and from the Dovey Estuary, Wales (Ordnance Survey grid reference, SN 674 973) during July and September. Live praniza juveniles were rinsed in distilled water, fixed in ice cold 70% ethanol and stored at 4 °C. Live females were maintained on damp tissue paper (in the dark) at 16 °C in an incubator, and were transferred to sea water, once mature, for release of motile zuphea 1 juveniles. Spent females were removed from the sea water, whilst the unfed juveniles were further maintained in sea water for 1 week (Tinsley and Reilly, 2002). After rinsing in distilled water, zuphea 1 juveniles (approximately 1 g in total, or $\sim 53 \times 10^3$ individuals) were collected on absorbent filter paper, snap frozen in liquid nitrogen and stored at −80 °C.

Biochemical analyses and semi-purification of trypsin inhibitors and anticoagulants from unfed zuphea 1 stages

Zuphea 1 forms were selected since only the newly hatched juvenile stages of *P. formica* could be harvested in sufficient numbers to permit biochemical analyses of trypsin inhibitors and anticoagulants. Approximately 1 g of zuphea 1 stages stored at −80 °C (above) were homogenized in a sterile Potter homogenizer on ice in 5 ml of 50 mM Tris-HCl buffer, pH 8·5, containing 20 mM NaCl (Morris and Sakanari, 1994; Azzolini *et al*. 2003), centrifuged at 14 000 g for 5 min at 4 °C, and the crude extract (CE) protein concentrations measured using the Bio-Rad DC microassay according to the manufacturer's instructions.

The inhibitory effect of the zuphea 1 CE (approximate protein concentration = 10–14·5 μg/μl) on the proteolytic activity of trypsin towards the fluorogenic serine protease substrate N-carbobenzoxy-Gly-Pro-Arg-4-methoxy-2-naphthylamine (Z-gly-pro-arg-MNA; Bachem) was then determined according to Morris and Sakanari (1994) and Azzolini *et al*. (2003). Varying amounts of CE (0 μg (control), 0·2 μg, 1 μg, 2 μg, 5 μg 10 μg, 20 μg, 50 μg, and 100 μg) were pre-incubated for 15 min at room temperature with 1 nM (0·006 U) trypsin from bovine pancreas (Calbiochem) in 50 mM Tris-HCl buffer, pH 7·5, within individual wells of a microtitre plate; 10 μl of Z-gly-pro-arg-MNA (0·1 mM final concentration in 100 μl total reaction volume) were then added and the released MNA (excitation 355 nm, emission 420 nm) measured over 2 h at

25 °C using a Fluostar Optima reader (BMG Labtech). The inhibitory capacity of the CE on the hydrolysis of 0·1 mM Z-gly-pro-arg-MNA (Bachem) by thrombin was determined by pre-incubating 50 nM (0·009 U) bovine thrombin (Calbiochem) with 20 μg CE for 15 min at room temperature in 50 mM Tris-HCl buffer, pH 7·5. The reaction was initiated by adding the substrate (0·1 mM final concentration in 100 μl total reaction volume) and inhibition measured as for the trypsin inhibitor assays.

Effects of the zuphea 1 CE on the recalcification time and fibrinogen clotting time of human plasma were assayed according to Ricci *et al*. (2007). For recalcification time, 25 μl citrated human plasma (Sigma) were incubated at room temperature in 50 mM Tris-HCl buffer (final volume 70 μl), pH 7·5, containing 0 μg (control), 14·5 μg, 29·1 μg, or 72·5 μg zuphea 1 CE. After 5 min, coagulation was initiated by adding 5 μl 150 mM $CaCl_2$, and clot formation was continuously measured in microtitre plates at 25 °C for 30 min at 620 nm with a Fluostar Optima reader. For the fibrinogen clotting assay, 6·5 μM fibrinogen from human plasma (Calbiochem) was incubated at room temperature in 50 mM Tris-HCl buffer (total incubation volume of 90 μl), pH 7·5, containing 0 μg (control), 14·5 μg or 72·5 μg CE. After 5 min, 10 μl of a 0·54 μM (2·2 U) solution of thrombin from human plasma (Calbiochem) were added to trigger clot formation, which was measured at 620 nm, as described for the recalcification time; the final absorbances measured were subtracted from values obtained at 0 min.

The zuphea 1 CE was then applied to a Nap-25 column (GE Healthcare) at 4 °C according to the manufacturer's instructions. Fractions collected from the Nap-25 column displaying inhibitory activity were pooled, and concentrated to 1 ml using a Vivapore® concentrator (Vivascience). In an attempt to purify the bioactive compound(s) from the zuphea 1 homogenate, the concentrated sample was applied to a 1 ml Sepharose anion-exchange chromatography column (HiTrap Q) (GE Healthcare) at 4 °C pre-equilibrated with Tris-HCl wash buffer (Tanaka *et al*. 1999). This resin was used since its working pH range is optimal for the stability of trypsin and trypsin inhibitors. After washing with 50 mM Tris-HCl, pH 8·5, at 1 ml/min, bound proteins were eluted with 50 mM Tris-HCl, pH 8·5, containing a NaCl gradient of 0–0·5 M (in 10 mM increments), and 1 ml fractions collected on ice. Three major peaks (P1, P2, and P3) of trypsin inhibitory activity were obtained and concentrated.

To determine the size of the trypsin inhibitors isolated in the fraction displaying the strongest trypsin inhibitory activity, 6·5 μg P1 were loaded onto 15% SDS-PAGE gels, either containing 0·1% (w/v) gelatin or not, and those with gelatin processed for reverse zymography according to Hanspal *et al*. (1983) and Andreotti *et al*. (2002). The gel was electrophoresed at 4 °C at 140 V, and subsequently washed in 0·1 M Tris-HCl, pH 8·0, containing 2·5% (v/v) Triton X-100 for 1 h. The gel was then rinsed in distilled water and incubated at 37 °C for 90 min in 25 ml of 0·1 M Tris-HCl buffer, pH 8·0, containing 87 μM (125 000 U) bovine trypsin. The gel was then rinsed in distilled water and stained with Coomassie brilliant blue to reveal bands corresponding to the molecular weight(s) of the trypsin inhibitors.

Finally, the effects of the semi-purified preparations on human plasma and fibrinogen clotting times were determined as described above. Briefly, 2·7 μg, 7·6 μg and 8·1 μg of P1, P2, and P3, respectively, were assayed for their effects on recalcification time (at 50% clot formation) and fibrinogen clotting time (at 50% clot formation), against buffer in control wells. In the case of the recalcification time, 2·7 μg P1 (possessing the greatest anticoagulant activity) were boiled for 10 min prior to conducting the assay to verify whether the anticoagulant(s) could be heat denatured, and thus, be proteinaceous.

All enzyme/coagulation assays were carried out in triplicate, and one-way analyses of variance (ANOVA) and post-hoc multiple comparison tests (Least Significant Difference, LSD) were done on data obtained.

Molecular typing of vertebrate DNA from fed praniza juveniles

Zuphea 1, and subsequent zuphea 2 and 3 stages of *P. formica* feed on fishes, becoming pranizae 1, 2 and 3 respectively. Thus, to investigate the source of host blood in the juvenile gnathiid digestive tract, larger fed praniza 2 (2–2·5 mm) and 3 (3–3·5 mm) stages were employed. A set of universal (vertebrate and invertebrate) primers targeting the mitochondrial large subunit ribosomal DNA (16Sar and 16Sbr) (Simon, 1991), as well as the eukaryote conserved 18SA (Blakenship and Yayanos, 2005) primer and a reverse 18S primer modified from Dreyer and Wägele (2001) to anneal the genomic small subunit ribosomal DNA of fishes, were used. DNA was extracted from blood-filled, individual, praniza juveniles using a protocol modified from Sicard *et al*. (2001) and Jones *et al*. (2007). Briefly, specimens were rinsed in distilled water and placed individually in sterile microfuge tubes containing 200 μl DNA extraction buffer (1 M Tris-HCl, 5 M NaCl, 0·5 M EDTA, 0·2% (v/v) β-mercaptoethanol, and 5% (v/v) CTAB). Specimens were then homogenized using sterile plastic pestles (Anachem) and incubated for 1 h at 55 °C, prior to the addition of 200 μl of chloroform. After centrifugation, the aqueous phase containing genomic DNA was added to 200 μl of ice-cold isopropanol to precipitate the DNA. After rinsing in 70% ethanol, precipitated nuclear material was

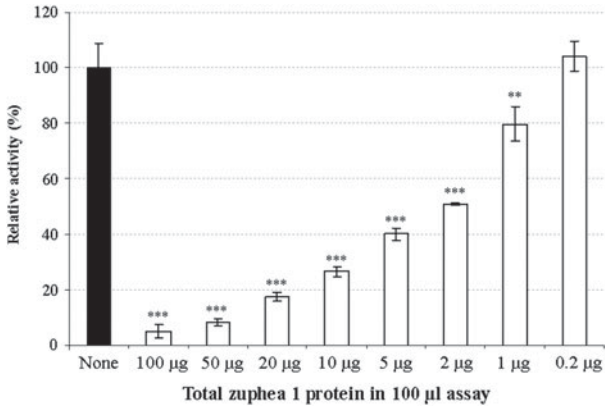

Fig. 1. Effect of varying amounts of *Paragnathia formica* zuphea 1 crude extract (CE) on the hydrolysis of 0·1 mM Z-gly-pro-arg-MNA by 1 nM trypsin. Bars represent mean values of 3 replicates (± S.D.), from both control (■) and CE (□) assays. ** $P<0·01$ and *** $P<0·001$ when compared to control values.

re-pelleted and re-suspended in 15 µl of diethylpyrocarbonate (DEPC)-treated water.

Extracted DNA (5 µl per 50 µl assay) was amplified using the BD TITANIUM™ *Taq* (BD Biosciences), and primers noted above, following the cycling programme used by Jones *et al*. (2007). The ~600–650 bp 16S rDNA and ~300–350 bp 18S rDNA bands were extracted from the agarose gel using a QIAquick gel extraction kit (Qiagen), and sequenced at the Natural History Museum (South Kensington, London).

Sequences were entered into the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) in order to obtain matching sequences (McGinnis *et al*. 2004). The amplified sequences were subsequently aligned against the closest matching sequences using CLUSTAL W (http://clustalw.genome.jp/) (Thompson *et al*. 1994), and whenever sequence manipulation was required the molecular toolkit program (http://www.vivo.colostate-edu/molkit/manip/) was employed. FishBase (http://www.fishbase.org/) was scrutinised to verify whether matching fish species were compatible hosts for gnathiids inhabiting the Atlantic coast and North Sea coast.

RESULTS

Characterization of trypsin inhibitors and anticoagulants from zuphea 1 extracts

Crude zuphea 1 extracts were assayed for the presence of trypsin or thrombin inhibitors using the serine protease substrate Z-gly-pro-arg-MNA. The proteolytic activity of trypsin towards this substrate was inhibited significantly by over 80% ($P<0·001$) after pre-incubation in buffer containing 20 µg zuphea 1 CE, whereas the proteolytic activity of thrombin towards the same substrate diminished by

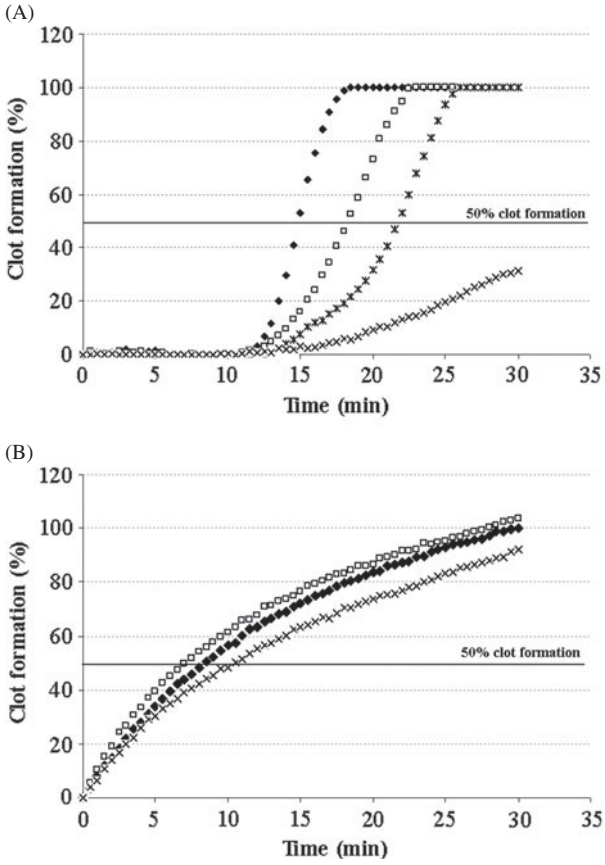

Fig. 2. Anticoagulant properties of *Paragnathia formica* zuphea 1 crude extract (CE). (A) Recalcification time of human plasma in the absence (♦) or presence of 14·5 µg (□), 29·1 µg (*), or 72·5 µg (×) of zuphea 1 CE; (B) Fibrinogen clotting time of human fibrinogen in the absence (♦) or presence of 14·5 µg (□), or 72·5 µg (×) of zuphea 1 CE. Data represent the mean values of 3 replicates.

approximately 30% ($P<0·01$) (data not shown). Owing to the higher level of trypsin inhibition, compared to thrombin inhibition by the zuphea 1 CE, subsequent experiments focused primarily on trypsin inhibition. A dose-dependent inhibition of trypsin activity by the CE was observed; Z-gly-pro-arg-MNA hydrolysis by trypsin was reduced by 50% with 2 µg CE ($P<0·001$), 91·6% with 50 µg CE ($P<0·001$), and over 95% with 100 µg CE ($P<0·001$); in contrast no inhibition was observed with 0·2 µg CE (Fig. 1).

To assess the effect of the zuphea 1 CE on coagulation of human plasma, a coagulation assay estimating the recalcification time of human plasma was conducted. The mean time to reach 50% clot formation in the control sample was 15 min 10 s. Pre-incubation with 14·5 µg or 29·1 µg zuphea 1 CE prolonged 50% clot formation by 3 min 20 s (± 52 s) and 6 min 10 s (± 2 min 30 s), respectively, representing mean increases of 22% and 40·7% (Fig. 2A). Moreover, pre-incubation with, 72·5 µg CE inhibited clot formation entirely over 30 min (Fig. 2A). To substantiate the inhibitory activity of

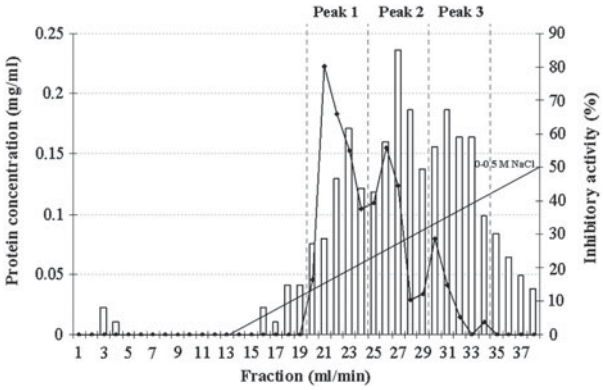

Fig. 3. Protein concentration (bars) and trypsin inhibitory activity (♦) of *Paragnathia formica* zuphea 1 fractions collected from a HiTrap Q ion-exchange chromatography column. Approximately 15 mg of zuphea 1 protein recovered from the Nap-25 gel filtration column were passed through the HiTrap Q column at a flow rate of 1 ml/min. Proteins captured by the column were eluted after 13 min by applying increasing NaCl concentrations (0 to 0·5 M in 0·01 M increments every 30 s). The fractions were recovered, protein concentration measured and 10 μl of each fraction incubated with 1 nM trypsin for 15 min prior to the addition of 0·1 mM Z-gly-pro-arg-MNA to determine trypsin inhibitory activity. Three inhibitory peaks were detected (delineated by the dotted vertical lines), each comprising 5 fractions, between 0·15–0·23 M NaCl (P1), 0·24–0·33 M NaCl (P2) and 0·34–0·43 M NaCl (P3).

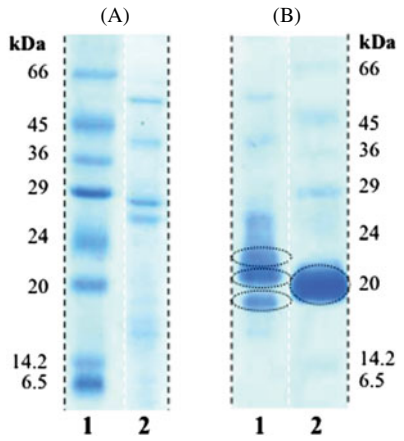

Fig. 4. (A) Coomassie-stained SDS-PAGE gel showing the low molecular weight ladder (lane 1), and 6·5 μg *Paragnathia formica* zuphea 1 peak 1 collected from the HiTrap Q ion-exchange chromatography column (lane 2). (B) Coomassie stained reverse zymography gel showing the trypsin inhibitory activity of 6·5 μg peak 1 (lane 1) and the low molecular weight ladder, in particular the inhibitory activity of SBTI at ~20 kDa (lane 2). Trypsin inhibitors were detected using 15% polyacrylamide resolving gels containing 0·1% gelatin. After electrophoresis at 4 °C, the gels were incubated for 90 min in 87 μM trypsin at 37 °C. Undigested areas by the trypsin produced diffuse inhibition zones, similar to the ~20 kDa band produced by approximately 2·2 μg SBTI contained in the low molecular weight ladder.

zuphea 1 CE towards thrombin seen in the fluorimetric assays, and to characterize further the anticoagulant effect, fibrinogen was pre-incubated with zuphea 1 CE prior to the addition of thrombin. After 30 min, 72·5 μg CE prolonged 50% fibrinogen clot formation by 1 min 40 s (±1 min) compared to the uninhibited control wells that possessed a mean 50% clotting time of 8 min 40 s; no delay was seen with 14·5 μg CE per assay (Fig. 2B).

Zuphea 1 protein (30 mg) was desalted using a Nap-25 column and subsequently applied to a HiTrap Q column. A total of 3–4 mg of zuphea 1 protein was collected in 38 fractions (Fig. 3), and 3 major protein peaks corresponding to 3 separate trypsin inhibitory peaks were eluted off the HiTrap Q column between 0·15–0·23 M NaCl (P1), 0·24–0·33 M NaCl (P2), and 0·34–0·43 M NaCl (P3) (Fig. 3). The fractions within each of these peaks of trypsin inhibition were then pooled. Preparations from peaks 2 and 3 (P2 and P3) had greater protein concentrations 0·76 μg/μl and 0·81 μg/μl, compared to that of P1 (0·27 μg/μl); however, they displayed lower trypsin inhibitory activities. Indeed, 2 μl P2 (1·52 μg zuphea 1 protein) and P3 (1·62 μg zuphea 1 protein) inhibited trypsin hydrolysis by 28·2%±6·4% ($P<0·05$) and 44·1%±2·5% ($P<0·05$), respectively, whereas 5 μl (1·35 μg zuphea 1 protein) P1 reduced trypsin hydrolysis by 84·25%±0·46 ($P<0·001$) (data not shown). Similar levels of trypsin inhibition were obtained using 2·7 μg of the semi-purified preparation P1 to those observed using 50 μg zuphea 1 CE (Fig. 1), indicating that the partial purification of the trypsin inhibitor(s) from the crude extract was successful.

Following the partial purification of trypsin inhibitor(s) in the zuphea 1 CE, reverse zymography was done using protein from the most active peak (P1) to determine the sizes of the trypsin inhibitor(s) present. Soybean trypsin inhibitor (SBTI) (20 kDa) in the molecular weight markers served as a positive control. The large diffuse band obtained (Fig. 4B, lane 2) corresponded to the location of SBTI (Fig. 4A, lane 1) after trypsin digestion of the gelatin within the gel. In the case of the semi-purified zuphea 1 P1, 3 smaller diffuse bands were obtained with apparent molecular weights of ~18 kDa, ~21 kDa and ~22 kDa (Fig. 4B, lane 1), which did not seem to correspond to any of the 6 major bands seen on the SDS-PAGE gel (Fig. 4A, lane 2). This suggests that although present in low concentrations, the inhibitors have potent inhibitory activity.

The anticoagulant properties of the semi-purified fractions were assayed alongside their trypsin inhibitory activities. Of the 3 preparations obtained, preparation P1 possessed the greatest anticoagulant activity, with 2·7 μg zuphea 1 protein delaying recalcification time of human plasma (mean time to 50% clot formation of 15 min 10 s) by 6 min 40 s (±3 min 30 s), representing a 44% ($P<0·05$) increase

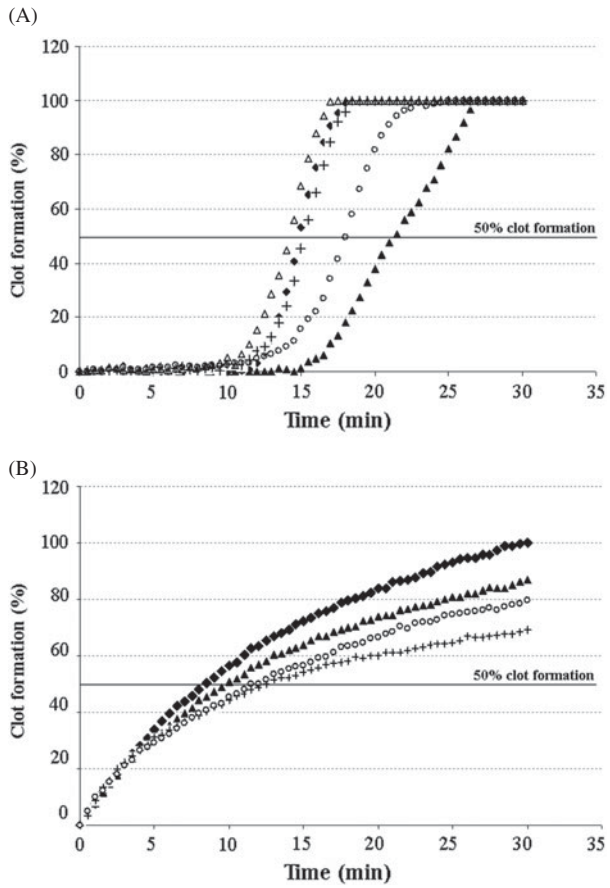

Fig. 5. (A) Effect of *Paragnathia formica* zuphea 1 semi-purified proteins on the recalcification time of human plasma after pre-incubation of plasma with 0 μg zuphea 1 protein from semi-purified peaks (♦), 2·7 μg P1 (▲), 7·6 μg P2 (+), or 8·1 μg P3 (○), as well as 2·7 μg zuphea 1 peak 1 previously boiled for 10 min at 100 °C (Δ). (B) Effect of *P. formica* zuphea 1 semi-purified proteins on the fibrinogen clotting time of human fibrinogen after pre-incubation with 0 μg zuphea 1 protein from semi-purified peaks (♦), 2·7 μg P1 (▲), 7·6 μg P3 (+), or 8·1 μg P3 (○). Data represent the mean values of 3 replicates.

(Fig. 5A). In contrast, 8·1 μg P3 delayed recalcification time by 2 min 50 s (± 1 min) or 18·7%, and 7·6 μg P2 did not delay 50% clot formation compared to control samples (Fig. 5A). However, both P2 and P3 preparations displayed anti-thrombin activities in fibrinogen clotting assays, since the duration to 50% clot formation was delayed by 4 min (± 2 min 10 s) or 46·2%, and by 4 min 10 s (± 4 min 20 s) or 48·1%, respectively compared to the mean control clot formation time (8 min 40 s) (Fig. 5B). Only a slight increase 15·4% (± 5·8%) in delay in fibrinogen clotting time could be attributed to P1, and although the protein concentration in this preparation was lower than in P2 and P3, inhibitory activity towards thrombin in this preparation again appeared to follow that observed in the CE (Figs 2B and 5B). This is supportive of zuphea 1 anti-thrombin compound(s) being present in small amounts and primarily in P2 and P3, and that the predominant anticoagulant(s) contained in the CE were present in P1. Finally, the anticoagulant activity observed in P1 was heat denatured (Fig. 5A) and this no longer inhibited the recalcification time of human plasma, suggesting that the anticoagulant(s) are proteinaceous.

Molecular identification of fish hosts from praniza bloodmeals

Fish DNA sequences amplified best with the mitochondrial 16S rDNA primers from *P. formica* praniza 3 juveniles collected at Wells-next-the-Sea, revealing 3 host families, including the Anguillidae, Clupeidae, and Pleuronectidae (Table 1). Furthermore, the 16S rDNA gene region identified hosts successfully to genus and species level, with over 99% homology to the European eel, *Anguilla anguilla* (Linnaeus, 1758), 95–98% homology to the Atlantic herring, *Clupea harengus* Linnaeus, 1758, and 98–99% homology to the European flounder, *Platichthys flesus* (Linnaeus, 1758), all fish species native to North Sea shores.

Two host families were identified from praniza 2 and 3 specimens from the Dovey Estuary using the 18S rDNA primers (Table 1). Two pranizae had fed on hosts belonging to the Pleuronectidae, the closest matching fish (with over 97% and 99% homology) being the stone flounder, *Kareius bicoloratus* (Basilewsky, 1855), a Pacific Ocean fish (Table 1). However, the European plaice, *Pleuronectes platessa* Linnaeus, 1758, has a shorter 18S rDNA sequence than the stone flounder, but shares similar homologies (99% and 97%, respectively) with the 2 sequences obtained from the gnathiid gut contents (Table 1) and is a much more likely host in this instance. In a similar manner, a third praniza collected from the Dovey Estuary had apparently fed upon a member of the Scombridae, and in this case the closest match was the bullet tuna, *Auxis rochei* (Risso, 1810) (Table 1). Since this tuna occurs on European Atlantic coasts, but does not inhabit estuaries, it appears more likely that the gnathiid had fed on another scombrid. Thus, it seems that the 18 s rDNA gene region selected was too conserved to identify hosts reliably to genus and species level, and the host species listed as the closest matching organisms in the BLAST results were a consequence of better sequence coverage rather than sequence homology.

DISCUSSION

We report for the first time, partial purification by anion-exchange chromatography of different bioactive compounds from homogenates of newly hatched *P. formica* zuphea 1 juveniles which could attenuate host haemostatic, inflammatory and immunological responses. Preliminary trypsin/thrombin

Table 1. Identification of vertebrate fish host from the bloodmeal content of praniza 2 and praniza 3 juveniles of *Paragnathia formica* collected from Wells-next-the-Sea (see Tinsley and Reilly, 2002) and the Dovey Estuary (see Manship *et al*. 2008)

Bloodmeal origin	Targeted genes	Host Family	Homology	Closest matching organism
Wells-next-the-Sea	16S rDNA	Anguillidae	99%	*Anguilla anguilla*[a]
		Pleuronectidae	99%	*Platichthys flesus*[a]
		Clupeidae	98%	*Clupea harengus*[a]
		Pleuronectidae	98%	*Platichthys flesus*[a]
Dovey Estuary	18S rDNA	Pleuronectidae	99%	*Kareius bicoloratus*[b]
		Scombridae	97%	*Auxis rochei*[b]
		Pleuronectidae	97%	*Kareius bicoloratus*[b]

[a] Likely host species; [b] unlikely hosts = possible congener species.

inhibition assays and coagulation assays with zuphea 1 CE, revealed trypsin and thrombin inhibitors and anticoagulants. The anticoagulants delayed the re-calcification time of human plasma, an indicator of blood coagulation *via* the intrinsic pathway (Abebe *et al*. 1996; see Tavares-Dia and Oliveira, 2009), and fibrinogen clotting time, a test estimating thrombin catalysis of fibrinogen into fibrin (Ricci *et al*. 2007; see Tavares-Dia and Oliveira, 2009).

Following fractionation by ion-exchange chromatography, 3 different bioactive peaks were isolated (P1, P2, P3). The first preparation (P1), displayed the strongest anti-trypsin activity, and reverse zymography revealed 3 trypsin inhibitors in this fraction with apparent molecular weights ~18 kDa, ~21 kDa and ~22 kDa. These trypsin inhibitors might target serine protease factors of the coagulation cascade (Watanabe *et al*. 2010) or serine proteases involved in other physiological processes (see Rimphanitchayakit and Tassaanakajon, 2010) in this gnathiid isopod. Indeed, serine protease inhibitors from the haemolymph of other crustaceans have been implicated in the immune response of these organisms, including melanization processes, and haemolymph clot formation (Kanost, 1999). Furthermore, serine protease inhibitors regulate digestive serine proteases (Morris and Sakanari, 1994; Azzolini *et al*. 2005), although in *P. formica*, digestion of the bloodmeal results primarily from cysteine protease activities (Manship *et al*. 2008). Characterization of trypsin inhibitors from homogenates of haematophagous ectoparasites, such as ticks and the horn fly (see Azzolini *et al*. 2003, 2005), have led to the identification of potential vaccine targets (see below).

Alongside these trypsin inhibitors, an anticoagulant(s) contained in the P1 fraction significantly delayed recalcification time of human plasma. Heating of the P1 preparation destroyed this anticoagulant activity, suggesting that the anticoagulant compound(s) present are proteinaceous; since P1 only slightly reduced thrombin interaction with fibrinogen, we hypothesize that the anticoagulant(s) inhibit a different factor(s) in the intrinsic pathway. However, the limited amount of gnathiid material available (1 g total represented $\sim 53 \times 10^3$ zuphea 1 juveniles) restricted further characterization of the anticoagulant(s) found, and the specific factor(s) in the coagulation cascade targeted by these compounds. In terrestrial blood-feeding arthropods, anticoagulant compounds identified so far predominantly target common factors to both intrinsic and extrinsic pathways, such as factor Xa or thrombin (see Stark and James, 1996; Valenzuela, 2002; Koh and Kini, 2009), and since thrombin activity was not reduced by the bioactive compounds contained in P1, these may inhibit factor Xa activity.

Finally, the semi-purified preparations P2 and P3 reduced fibrinogen-clotting time, and may contain the thrombin inhibitor(s) detected in the CE using a fluorogenic substrate Z-gly-pro-arg-MNA. Although not further characterized, it appears that both fractions contain different bioactive compound(s), eluted off the anion-exchange chromatography column at differing NaCl concentrations, which prevent the thrombin-fibrinogen interaction. These compounds, could possibly affect exosite I on thrombin, the specific fibrinogen active-binding site on thrombin (see Ciprandi *et al*. 2006; Ricci *et al*. 2007; Koh and Kini, 2009; Tanaka-Azevedo *et al*. 2010). In the case of the CE, the thrombin inhibitor(s) attenuated hydrolysis of the peptide synthetic substrate by thrombin; they may have bound the catalytic site of thrombin (Horn *et al*. 2000), or to different regions that prevented interaction of the catalytic site with the substrate (see Koh and Kini, 2009; Tanaka-Azevedo *et al*. 2010). The thrombin inhibitor(s) present in peaks P2 and P3, may function alongside anticoagulants present in P1 to inhibit clot formation, and/or may reduce thrombin mediated thrombocyte aggregation to the site of lesion (see Mann and Lorand, 1993; Dahlbäck, 2000; Tavares-Dias and Oliveira, 2009; Tanaka-Azevedo *et al*. 2010).

It is probable that the anticoagulant(s) identified in the present study are contained in the salivary glands of this gnathiid, in a similar manner to parasitic cymothoid isopods (Romestand and Trilles, 1976*a*, *b*), or they may be divided between the salivary glands

and digestive tract (anterior hindgut) as reported in *Rhipicephalus microplus* (syn, *Boophilus microplus*) (Canestrini, 1888) ticks (Ricci *et al*. 2007) where they delay clot formation during digestion in the midgut. In larger blood-feeding ectoparasites, for example mosquitoes, tabanid flies or ticks, similar biochemical investigations have been conducted using proteins exclusively extracted from the salivary glands (Ribeiro *et al*. 1995; Stark and James, 1995, 1996; Kazimírová *et al*. 2001, 2002), or proteins collected after pilocarpine-induced salivation in ticks (Ciprandi *et al*. 2006). However, the size of the gnathiid juveniles and in particular the unfed zuphea 1 stage (1 mm each) (Upton, 1987), prevented dissection and separation of the salivary glands ($\sim$100–200 μm in size) of *P. formica*, and therefore specific localization of the anticoagulant(s) could not be achieved. A recent report detailing the recovery of salivary secretions from the honeybee mite *Varroa destructor* (approximate size of 1–1·8 mm wide by 1·5–2 mm long) after inducing salivation using pilocarpine (Richards *et al*. 2011) could help future investigations into the localization and extraction of bioactive compounds from gnathiid isopods. In this context, a comparative study of anticoagulants present in unfed and fed stages of gnathiid juveniles, may reveal whether these bioactive compounds are restricted to a given stage, as previously reported for the cysteine-like proteolytic enzymes, which are more abundant in *P. formica* praniza stages (Manship *et al*. 2008). Interestingly, experiments conducted on anticoagulants of blood-feeding female mosquitoes revealed a strong factor Xa inhibitor in salivary gland extracts, whilst extracts of non-haematophagous male mosquitoes produced no delay in the recalcification time of human plasma (Stark and James, 1995). A comparative study of anticoagulant compounds found in the haematophagous juvenile stages and non-feeding males of *P. formica* (Monod, 1926) might offer an insight into the function of the male salivary glands.

Bioactive salivary compounds of arthropods of medical, veterinary and economic importance have been studied in detail (see Valenzuela, 2002; Ribeiro and Francischetti, 2003; Koh and Kini, 2009). A rationale for characterizing these salivary proteins is the design of arthropod and arthropod-borne control strategies, now advanced for anti-tick vaccines with successful applications in field studies (see Willadsen, 2006). In aquatic environments, such studies have been conducted only on the haematophagous marine ectoparasite *Lepeophtheirus salmonis* Krøyer, 1837 (Copepoda: Caligidae). Prostaglandin E_2 (an anti-inflammatory molecule) was identified in secretions of the copepod salmon louse (see Wagner *et al*. 2008), and knockdown of the prostaglandin E synthase 2 gene was attempted (Campbell *et al*. 2009) to identify targets for salmon vaccine development (Raynard *et al*. 2002). Gnathiid isopods cause tissue damage to their fish hosts whilst feeding (see Smit and Davies, 2004; Hayes *et al*. 2007, 2011). It is thus likely that as in other blood feeding ectoparasites, the isopod saliva contains a variety of immunomodulatory compounds (Singh and Girschick, 2003), such as prostaglandin E_2 secreted by the salmon louse *L. salmonis* (see Wagner *et al*. 2008), alongside the potential trypsin inhibitors and anticoagulant(s) detected in this study.

A fuller understanding of host-preference or specificity is also vital in estimating the ecological and economic impact of gnathiid blood feeding. Molecular determination of host origin from the dietary content of invertebrates has been reported for predatory beetles (Zaidi *et al*. 1999), spiders (Agustí *et al*. 2003), blood-feeding ticks (Pichon *et al*. 2003), scavenging amphipod crustaceans (Blankenship and Yayanos, 2005), and recently the juveniles of gnathiid isopods from the Great Barrier Reef (GBR) (Nagel and Lougheed, 2006; Jones *et al*. 2007). The latter authors amplified fish DNA from the bloodmeal contents of 2 nocturnal species of gnathiids from the GBR, identifying 21 different host families, and finding differences in host-preference between these species.

Results presented here, reinforce the relevance of using molecular markers for identifying gnathiid host families and species, and it is surprising how many fish families/species were identified from relatively few gnathiids inhabiting mud banks at Wells-next-the-Sea and the Dovey estuary. It is generally assumed that 18S genomic rDNA evolves more slowly than 16S mitochondrial rDNA, and is thus, highly conserved across a wide range of taxa (Pichon *et al*. 2003). This might explain our results, since some fish species apparently shared a high level of 18S rDNA sequence conservation, and gnathiid hosts could be assigned only to their respective fish families. Sequences obtained using the universal (vertebrate and invertebrate) 16S rDNA primers (Simon, 1991) were all of fish origin, corroborating findings by Blankenship and Yayanos (2005) that these primers preferentially amplify vertebrate DNA. Importantly, fish hosts were identified to the species level, by this marker, making it ideal for studying gnathiid host-preferences as reported by Jones *et al*. (2007).

In the present study, hosts of *P. formica* pranizae belonged to different fish families to those reported for gnathiids on the GBR (Jones *et al*. 2007). Early laboratory feeding experiments using *P. formica* juveniles (Monod, 1926) and recent observations of gnathiids from the GBR (Nagel and Grutter, 2007), suggest adaptability by these isopods to available fish hosts. In the case of *Paragnathia*, this monotypic genus has been reported, as noted earlier, across Western Europe, North Africa and the Mediterranean basin (Monod, 1926; Upton, 1987; Menezes, 1984; Cadée *et al*. 2001; Silva *et al*. 2006; Kirkim *et al*. 2008) where juveniles likely feed on a variety of fish hosts according to availability. The host families

obtained here agree with the findings of Monod (1926), who noted that *P. formica* fed on members of the Pleuronectiformes and Perciformes, and on European eels. Severe pathological effects on eels have been reported as well as host death in heavy infestations, or in captive environments (Mugridge and Stallybrass, 1983). Monod (1926) also observed an accidental association between *P. formica* and the European sprat, *Sprattus sprattus* (syn, *Clupea sprattus*) (Linnaeus, 1758), a species sharing over 98% 16S rDNA homology with *Clupea harengus* (Atlantic herring). However, Monod's (1926) "accidental association" may not be so, since *C. harengus* DNA was amplified from 2 juvenile gnathiids at Wells-next-the-Sea. Finally, unidentified gnathiid isopods have recently been recorded on the European flounder off the coast of Portugal (Cavaleiro and Santos, 2009), and this fish appears to be a common host for *P. formica* (see Menezes, 1984), as reported here. The success of the host typing technique could lead to future identification of gnathiid hosts from larger specimen collections, to determine whether they are host-specific or respond to host availability. This is important since Marino *et al.* (2004) suggested that the opportunistic behaviour of juvenile gnathiids could result in infestations of captive or cultured fish worldwide, leading to economic loss.

In conclusion, this research was undertaken to provide important insight into the host-parasite interactions that occur between the post-marsupial stages of a gnathiid isopod and their host fishes. Together with our previous work on protein digestion in *P. formica* (see Manship *et al.* 2008), this constitutes the first thorough investigation of blood ingestion and host origin in *P. formica* juveniles, advancing considerably our understanding of feeding in this extraordinary gnathiid isopod.

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