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RESEARCH ARTICLE

Be ready at any time: postprandial synthesis of salivary proteins in salivary gland cells of the haematophagous leech *Hirudo verbana*

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ABSTRACT

Sanguivorous leeches are ectoparasites having access to body fluids of potential hosts only infrequently. During feeding, salivary proteins are released from unicellular salivary glands into the wound. These substances, among them anti-coagulants, anti-inflammatory or anti-microbial agents, allow these animals proper feeding and long-term storage of host blood in their crops for several months. Using histological, protein biochemical and molecular techniques, we investigated whether synthesis of salivary proteins and refilling of salivary gland cells occur immediately after feeding or later when stored nutrients in the crop are getting scarce. The results of the histological analyses showed that gland cell area was significantly smaller right after feeding when compared with those in unfed animals. This parameter recovered quickly and reached the control level at 1 week after feeding. 2D gel electrophoresis and analysis of the abundance of individual proteins in extracts of leech tissues revealed that a subset of proteins that had been present in extracts of unfed animals virtually disappeared during feeding, but re-appeared within 1 week of feeding (most probably secretory proteins) while another subset did not change during the experimental period (most probably housekeeping proteins). Semi-quantitative PCR analysis of hirudin cDNA prepared from leech RNA samples revealed that the amount of hirudin transcripts increased immediately after feeding, peaked at 5 days after feeding and declined to control values thereafter. Our results indicate that bloodsucking leeches synthesize salivary proteins and refill their salivary gland cell reservoirs within a week of a blood meal to be prepared for another feeding opportunity.

KEY WORDS: Sanguivorous leech, Salivary protein synthesis, Blood feeding, Salivary protein cocktail, Hirudin

INTRODUCTION

The European leeches *Hirudo medicinalis* and *Hirudo verbana* are model animals for studying the biology of haemotaphagous leeches in general and for investigating the pharmacological properties of their salivary gland proteins with respect to potential targets in host animals (Kvist et al., 2013; Siddall et al., 2007). These leeches feed on body fluids of amphibians, fish, birds, reptiles and mammals (Ceylan and Erbatur, 2012; Dickinson and Lent, 1984; Kutschera and Roth, 2005; Merilä and Sterner, 2002; Sawyer, 1986). During feeding, leeches release bioactive salivary proteins and peptides (Baskova et al., 2004; Baskova and Zavalova, 2001; Hildebrandt and Lemke, 2011), produced in unicellular salivary glands in the anterior segments of the leech body, into the wound (Hildebrandt and Lemke, 2011). Only a handful of these substances out of up to 100 that may be present in leech saliva (Baskova et al., 2004) are known so far, among them enzymes, anti-inflammatory or anti-microbial agents and inhibitors like anti-coagulants (Ascenzi et al., 1995; Baskova et al., 1992, 2008; Baskova and Zavalova, 2001; Deckmyn et al., 1995; Greinacher and Warkentin, 2008; Gronwald et al., 2008; Hildebrandt and Lemke, 2011; Kvist et al., 2013; Müller et al., 2015; Rigbi et al., 1996; Vilahur et al., 2004). During one blood meal that lasts at least 20–30 min (Lent et al., 1988), up to 1 mg of salivary protein is secreted into the wound (Lemke et al., 2013), resulting in partial or complete emptying of the approximately 40,000 salivary gland cells (Lemke et al., 2013). As the opportunities for leeches to feed on blood are rare (Mann, 1962), they may take up to ninefold their own body weight during one blood meal (Lent et al., 1988). The ingested blood is stored in the large alimentary tract, the crop, composed of 10 pairs of diverticula. The stored blood is digested in small portions (Zebe et al., 1986) over a period of several months to a year (Hildebrandt and Lemke, 2011; Mann, 1962).

To prepare for another blood meal, leeches have to refill their salivary gland cells by synthesis of salivary proteins and peptides. This raises the question (Schremmer et al., 1956) whether synthesis of salivary gland cell proteins and peptides occurs early after feeding (to ensure that every opportunity to feed can be used) or is shifted to a period in which the leech is running short of stored blood in the crop (which would probably be more economical as the energy otherwise required for maintaining and replacing salivary gland cell material during the storage phase could be saved). Such a strategy has been recently suggested for the sanguivorous leech *Hirudinaria manillensis* (Alaama et al., 2014).

To answer this question, we fed animals with commercially obtained citrate blood drawn from pigs and prepared microscopic sections from the anterior body including the salivary gland cells at different times after feeding. We determined the salivary gland cell areas in the images as a means for quantifying the relative filling state of the gland cell reservoirs. In addition, we determined the levels of hirudin cDNA transcribed from RNA samples obtained from leeches at different times after feeding. Furthermore, 2D gel analyses of proteins extracted from the tissue containing the salivary gland cells at different times after feeding revealed the time course of refilling of the gland cell reservoirs at the level of individual proteins.

MATERIALS AND METHODS

Experimental animals and feeding

Leeches (*Hirudo verbana* Carena 1820) were obtained from Futura Blutegelzucht, Berlin, Germany. A maximum of 20 animals were maintained in glass containers in artificial pond water (0.5 g sea salt dissolved in 1 litre deionized water) at room temperature (12 h light/dark cycles). Leeches were fed on citrate-supplemented pig blood contained in washed pig intestine (both obtained from Greifenfleisch GmbH, Greifswald, Germany) and heated to 37°C in a water bath.

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Sectioning of paraffin-embedded tissue samples and imaging

Unfed leeches (controls) or leeches that had ingested blood and were held for 0, 1, 3, 5, 7, 14 or 21 days in artificial pond water at room temperature (*N*=3 each) were stretched out on a perforated wooden rod using sewing needles stuck through anterior and posterior suckers and fixed in 4% formaldehyde solution for 12 h at 4°C. The anterior body part containing the salivary gland cells was removed from the rest of the body. The tissue was dehydrated by placing it in 80% (v/v) ethanol in distilled water for 2 h and subsequently for 2×30 min in 96% (v/v) ethanol. The samples were then transferred to a tetrahydrofuran (THF)–ethanol solution (1:1 ratio) for 2 h, pure THF overnight, THF with paraffin for 24 h at 60°C and finally pure paraffin for 24 h at 60°C. Afterwards, liquid paraffin wax (60°C) was poured into a mould, and, using hot tweezers, the sample was longitudinally aligned into the mould and left under a laminar flow hood (24 h, 20°C) for slow cooling. Longitudinal sections (5 µm) of embedded tissue were prepared on a microtome (Microm HM 360 Rotary Microtome, ThermoScientific, Dreieich, Germany). The tissue sections were dried on a heat bench (40°C) overnight, then stained using an Azan staining procedure according to Geidies (Gabe, 1968). Two digital images per sample were obtained using a Nikon Eclipse TE300 microscope and a Nikon DXM 1200 digital camera (Nikon, Düsseldorf, Germany). The salivary gland cell areas were marked and measured using Image Tool Software (UTHSCSA Image Tool 3.0) on a standard laboratory computer.

Extraction of hirudin mRNA and PCR of hirudin cDNA

Leeches were kept for at least 3 months without feeding (unfed leeches, *N*=3) or for 1, 3, 5, 7, 14 and 21 days after voluntary feeding for 30 min (*N*=3 each). To avoid contamination of RNA preparations with residual material from host blood in the crop, the stomach contents of each leech were removed using a cannula inserted through the mouth into the crop 12 h before tissue sampling. Leeches were anaesthetized and frozen by cooling them quickly to −20°C for 10 min. The tissue containing the salivary gland cells was prepared and transferred to 500 µl TRIzol®-reagent (Invitrogen, Karlsruhe, Germany). Tissue was homogenized on ice using a T8 Ultra-Turrax (IKA Labortechnik, Staufen, Germany) for 3 min. Samples were incubated in a water bath (Memmert, Schwabach, Germany) at 25°C for 5 min. Then, 100 µl chloroform (Roth, Karlsruhe, Germany) was added and the tissue sample placed in a water bath for a second time (2 min, 25°C). Subsequently, the sample was centrifuged (15 min, 11,000 ***g***, 2–8°C) using a Biofuge (Heraeus, Hanau, Germany). The aqueous phase containing the RNA was removed and 300 µl isopropanol was added. The mixture was incubated at 20°C for 10 min and centrifuged (10 min, 11,000 ***g***, 2–8°C). The supernatant was carefully removed, and the pellet was washed in 500 µl 70% (v/v) ethanol at −20°C and centrifuged again (5 min, 7000 ***g***, 2–8°C). The alcohol was then poured off the pellet, the pellet was dried (10 min, 37°C), and precipitated material was resuspended in water (bioscience-grade; Roth, Karlsruhe, Germany). The purity and concentration of RNA were determined by measuring the absorption at 230, 260 and 280 nm in a BioPhotometer 6131 (Eppendorf, Hamburg, Germany).

The volume of RNA suspension for each of the reverse transcriptase reactions was adjusted to 1.2 µg of RNA per assay; 2 µl Oligo-dT primer and the RNA sample were mixed. Water (see above) was added to reach a final volume of 14 µl per assay. The samples were incubated in a thermocycler (Biometra, Göttingen, Germany) at 70°C for 10 min. Afterwards, they were cooled on ice and centrifuged briefly. A volume of 4 µl of a 5× reaction buffer for reverse transcription (RT) (ThermoScientific, Schwerte, Germany) and 2 µl dNTPs (Roth) were added to the sample and incubated at 37°C for 5 min in a T-gradient thermocycler (Biometra, Göttingen, Germany). Addition of 1 µl of reverse transcriptase (RevertAid™ Reverse Transcriptase M-MuLV, ThermoScientific) started the RT reaction (42°C for 1 h, 70°C for 10 min). For each PCR reaction, 2.5 µl Taq buffer solution+$(NH_4)SO_4$-$MgCl_2$ and 2.5 µl $MgCl_2$ (Fermentas, St Leon-Rot, Germany), 1 µl dNTPs (Roth), 2.5 µl cDNA template, 11.5 µl water and 0.8 µl DNA Taq polymerase (ThermoScientific) were combined in a PCR tube. Either β-actin primers (*Anas_Act_FW1* forward primer, 5′-GGCTACAGCTTC-ACCACCACAGC-3′, and *Anas_Act_Rev1* reverse primer, 5′-TG-CTTGCTGATCCACATCTGCTGG-3′, 2.5 µl each) or hirudin primers (*Hiru_FW2* forward primer, 5′-CTTACACTGATTGTAC-AGAATCGG-3′, and *Hiru_Rev2* reverse primer, 5′-TATTGGTA-AATAGCTTAGCTATGG-3′, 2.5 µl each) were used. The PCR reactions (30 cycles) were performed using annealing temperatures of 48°C. Equal volumes of PCR products were transferred to 2% agarose gels and electrophoresis was carried out at 80 V. For better comparison, all samples of a given time point were loaded onto the same gel. Agarose gels were incubated in ethidium bromide solution (0.0001% w/v ethidium bromide in TAE buffer) for 25 min at room temperature. For detection of PCR products using UV light, the Quick Store Plus system (MS Laborgeräte, Wiesloch, Germany) was used. Densitometric analyses were performed using Phoretix 1 D (NonLinear Dynamics, Newcastle upon Tyne, UK). The relative band densities of hirudin-related PCR products were normalized to those of β-actin in the same cDNA preparation.

2D gel-based semi-quantification of salivary proteins

Leeches were prepared for salivary gland cell protein extraction at 1, 3, 5, 7, 14 or 21 days after feeding or after being kept for at least 3 months without feeding (unfed leeches). Protein extracts from tissue containing the salivary gland cells were prepared and processed by 2D gel electrophoresis as described elsewhere (Lemke et al., 2013). Spot detection after silver staining and densitometric analyses were performed using Phoretix 2D (NonLinear Dynamics). To avoid inequalities based on silver staining, the densitometric volume of each spot was normalized to that of a protein spot that was consistently detected in each gel (spot 1).

Data presentation and statistics

Means (±s.d.) were calculated from double determinations on tissue preparations of different animals (*N*). Differences of individual means were tested for significance using Student's *t*-test or the Wilcoxon test using the program package R. Significant differences of means were assumed with $P<0.05$.

RESULTS

Emptying and refilling of salivary gland cells

As previously reported (Hildebrandt and Lemke, 2011), and confirmed in this study (Fig. 1), leeches secrete most of the material stored in the cell bodies of their salivary gland cells during one round of feeding. Many salivary gland cell bodies were visible in Azan-stained longitudinal sections of leech tissue from the anterior body part of unfed animals (Fig. 1A). In leeches that were prepared for sectioning immediately after having consumed a blood meal (Fig. 1B), the areas covered by salivary gland cell bodies in images of microscopic sections (arrows) were much smaller than those in unfed animals. Taking this parameter as a proxy for the filling state of the gland cell bodies, analyses were

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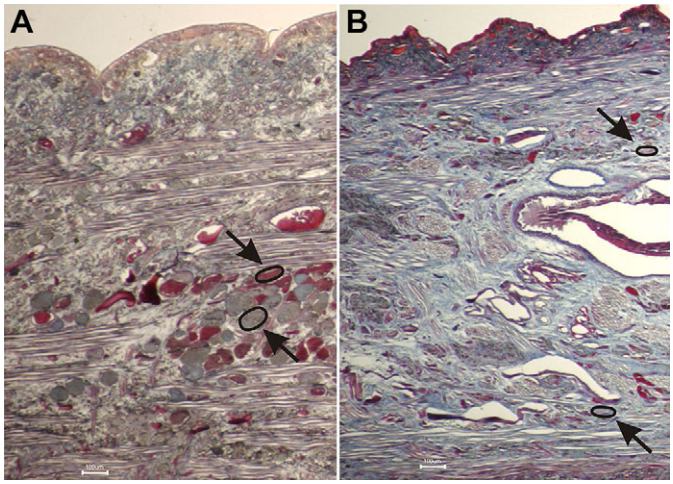

Fig. 1. Comparison of Azan-stained longitudinal paraffin sections prepared from the anterior body part of *Hirudo verbana*. (A) Example image of leech tissue prepared from an unfed animal. (B) Example image of leech tissue prepared from an animal immediately after feeding. In unfed leeches, salivary gland cells (examples encircled to indicate the cell area in the section) are filled with salivary material (blue or red staining, arrows). Immediately after feeding, salivary gland cells (see encircled examples) appear partially or completely empty (smaller encircled area). Scale bar, 100 µm.

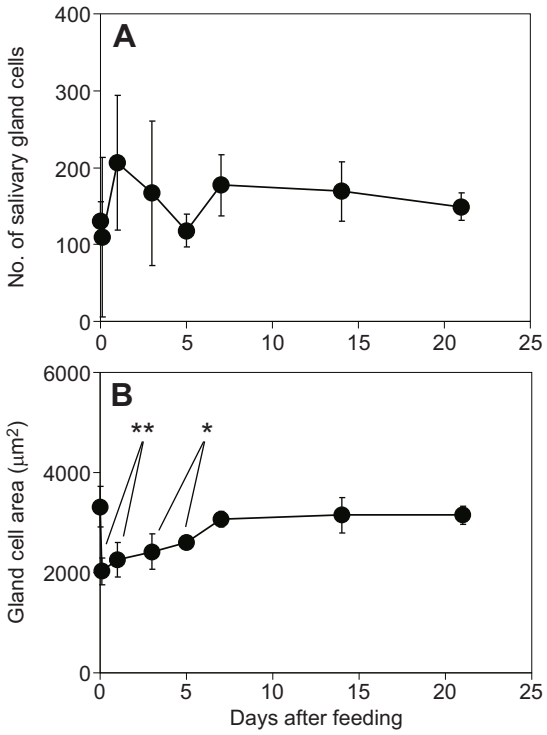

Fig. 2. Number of visible salivary gland cells and relative cell area as determined in digital images of Azan-stained microscopic sections. Tissue sections were prepared from unfed leeches (day 0) and at 1, 3, 5, 7, 14 or 21 days after feeding (N=3 each). In two microscopic images per animal, the number of detectable salivary gland cells was counted (A) and the gland cell area was measured (B). Data are shown as means±s.d. with levels of significance of *P<0.05 or **P<0.01.

performed on microscopic sections prepared from unfed leeches (0 days after feeding), and leeches prepared immediately after feeding (0.1 days after feeding) or 1, 3, 5, 7, 14 or 21 days after feeding (N=3 animals per time point). Approximately 150 salivary gland cells per time point were measured. As shown in Fig. 2, the area in microscopic images of tissue sections covered by salivary gland cell bodies decreased significantly by approximately 40% during feeding. Measurements of this parameter during the 3 weeks post-feeding revealed that the area values slowly increased during the first week after feeding to reach a level that was not significantly different from that in unfed leeches on day 7 after feeding (Fig. 2).

Feeding-associated changes in abundance of individual salivary proteins

Protein extracts prepared from central tissue cylinders excluding the body wall muscle layers from anterior portions of leech bodies were used for 2D analyses of changes in abundance of individual proteins during feeding and during refilling of the salivary gland cell bodies after feeding. Proteins that could be consistently detected in silver-stained gels of protein extracts from unfed leeches were individually numbered (Fig. 3A) and followed over a period of 3 weeks after feeding. The example gel images shown in Fig. 3B–F illustrate the characteristic protein spot patterns that were observed at 1, 3, 5, 7 or 21 days after feeding, respectively. Some protein spots were highly reduced or even absent in samples from recently fed animals, while others were consistently present at all times. This indicates that one fraction of the extracted proteins represented secretory proteins, while the other represented housekeeping proteins from salivary gland cells and surrounding tissue whose abundance was not affected by the feeding process. Densitometric analyses (density of protein spots in silver-stained gels normalized to the density of the reference protein, spot 1) revealed that a subgroup of proteins in the extracts underwent characteristic changes in abundance. Proteins represented by spots 4, 5, 7, 9, 10, 13, 19, 20, 21, 25, 36 or 39 among others were significantly reduced in abundance compared with the control samples obtained from unfed animals (Fig. 4) on days 1, 3 and, in some cases, 5, and increased afterwards to reach levels that were not distinguishable from the control levels in unfed animals at day 7 onwards after feeding. Another set of proteins in the extracts represented by the spots 2, 3, 6, 11, 17, 18, 31 or G, among others, did not show any significant changes in their abundance over time (Fig. 5).

Changes in hirudin transcript abundance in leech tissue after feeding

As the identities of the proteins represented by the silver-stained gel spots are still unknown, we were not able to analyse their transcript abundance, which can be used as a proxy for the rate of gene transcription and synthesis of the respective proteins. However, as hirudin is one of the few salivary proteins that have been characterized at the molecular level, we performed RT-PCR reactions on total RNA extracts prepared from central tissue cylinders excluding the body wall muscle layers from anterior portions of leech bodies to semi-quantify the abundance of hirudin transcripts in unfed leeches and in fed leeches at 1, 3, 5, 7, 14 or 21 days after feeding (N=3 animals each).

Transcripts in total RNA preparations were transcribed into cDNA. The cDNAs of hirudin and β-actin were amplified by PCR using specific primers as previously described (Müller et al., 2015). The PCR reactions were performed using a limited number of cycles to prevent the samples going into saturation, and the PCR products were separated on agarose gels and stained using ethidium bromide. The gel images were digitized and the staining densities of amplificates were determined. The band densities of hirudin

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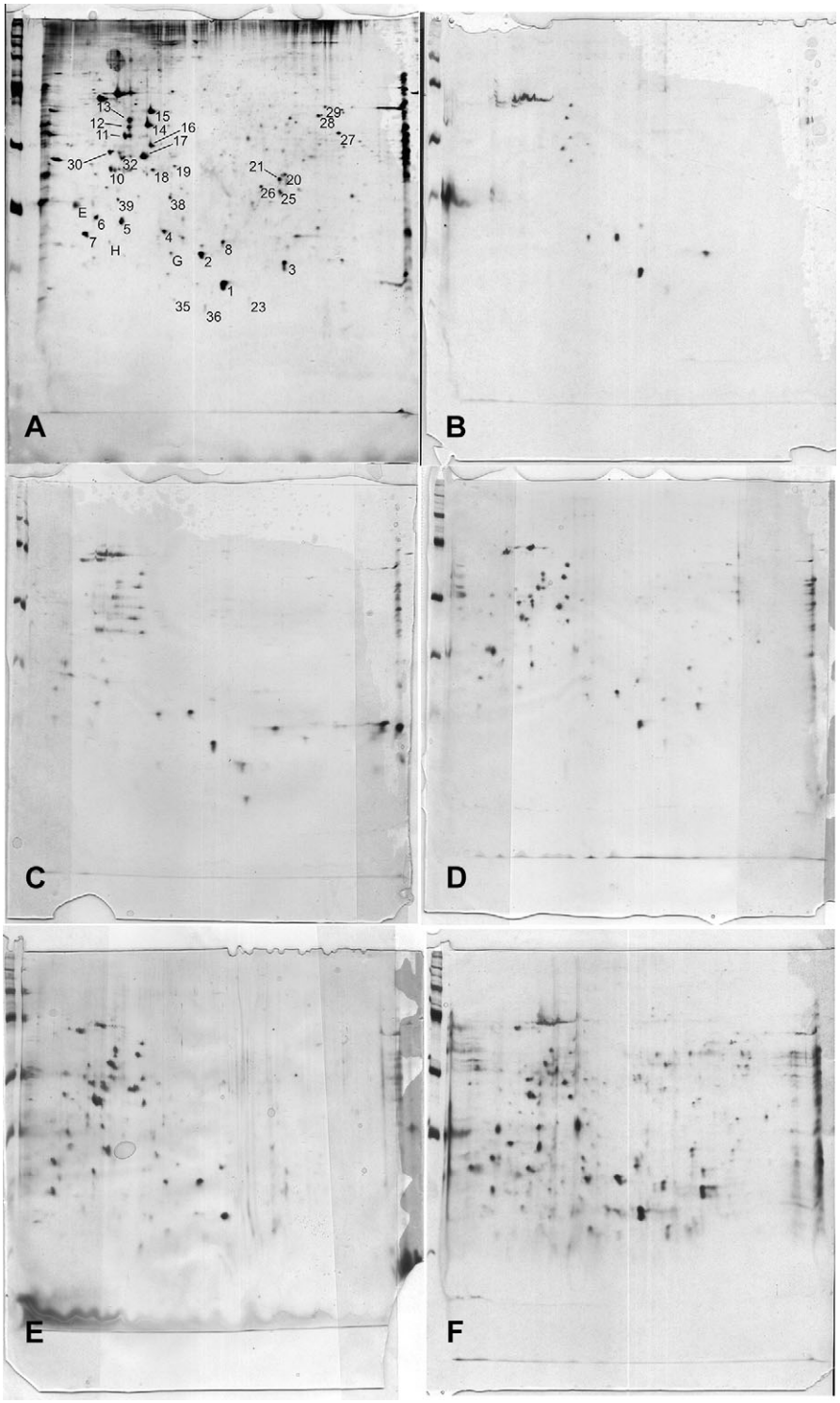

Fig. 3. Representative 2D gels prepared using protein extracts from the salivary gland cell tissue of adult *H. verbana*. Protein samples were prepared from leeches that were unfed (A, control), or had been fed 1 (B), 3 (C), 5 (D), 7 (E) or 21 days (F) before protein preparation. Proteins were separated according to their pI (horizontal axis) and their molecular mass (vertical axis) and were silver stained. Some of the spots detected in unfed leeches (A) were transiently reduced in volume (density×area) or even absent in samples from fed leeches. Within 1 or 2 weeks (E,F), most of these spots returned to volumes that were similar to those in unfed leeches.

cDNA were normalized to those of β-actin cDNA. These analyses showed significant differences in hirudin cDNA levels in fed leeches at days 3 and 5 after feeding compared with those in unfed leeches (Fig. 6). The highest level of hirudin cDNA occurred at 5 days after feeding. At 14 and 21 days after feeding, the transcript level was back to the control level in unfed leeches.

DISCUSSION

Haematophagous leeches like *H. verbana* feed on body fluids of fish, amphibians, reptiles, birds and mammals (Ceylan and Erbatur, 2012; Davies and McLoughlin, 1996; Dickinson and Lent, 1984; Kutschera and Roth, 2005; Merilä and Sterner, 2002; Sawyer, 1986). Mammalian blood in particular is an important resource for leeches as it contains hormones and other components the parasites need for reaching sexual maturity (Wilkin and Scofield, 1991). When the residual crop contents of the previous blood meal have been digested and leeches get hungry, they become extremely sensitive to water movements which may indicate potential prey. In close range of a potential host they are attracted by body heat and chemical clues, and attach themselves to a suitable portion of the

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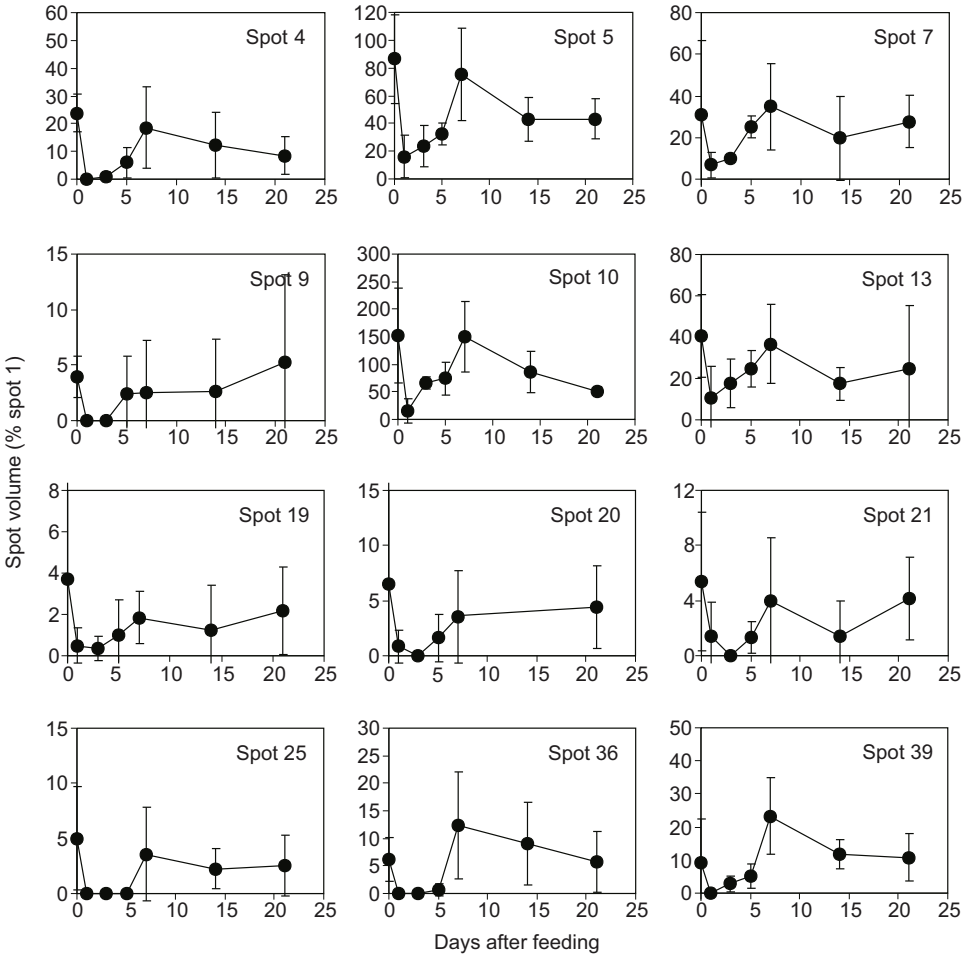

Fig. 4. Relative abundance of individual secretory proteins in tissues of the anterior body part of *H. verbana*. Relative spot volumes were determined using 2D gel electrophoresis in unfed leeches (day 0) and in leeches prepared at 1, 3, 5, 7, 14 and 21 days after being fed with pig blood. While the abundance of the proteins represented by spots 4, 5, 7, 9, 10, 13, 19, 20, 21, 25, 36 and 39 was significantly reduced immediately after feeding, it gradually increased afterwards and mostly reached the levels of those in unfed leeches at 1 week after feeding. For all spots, at least one of the means at time points 1, 3 or 5 days after feeding was significantly different from that of the control or the mean at day 7.

host's body surface and start biting through the skin (Dickinson and Lent, 1984). Feeding on blood, however, requires that substances are applied to the wound to prevent the host from noticing the parasite (anaesthetics), and to prevent wound closure and haemostasis (anti-thrombotic agents) or blood clotting (anti-coagulants). Furthermore, the leech has to make sure that the blood components in its crop are

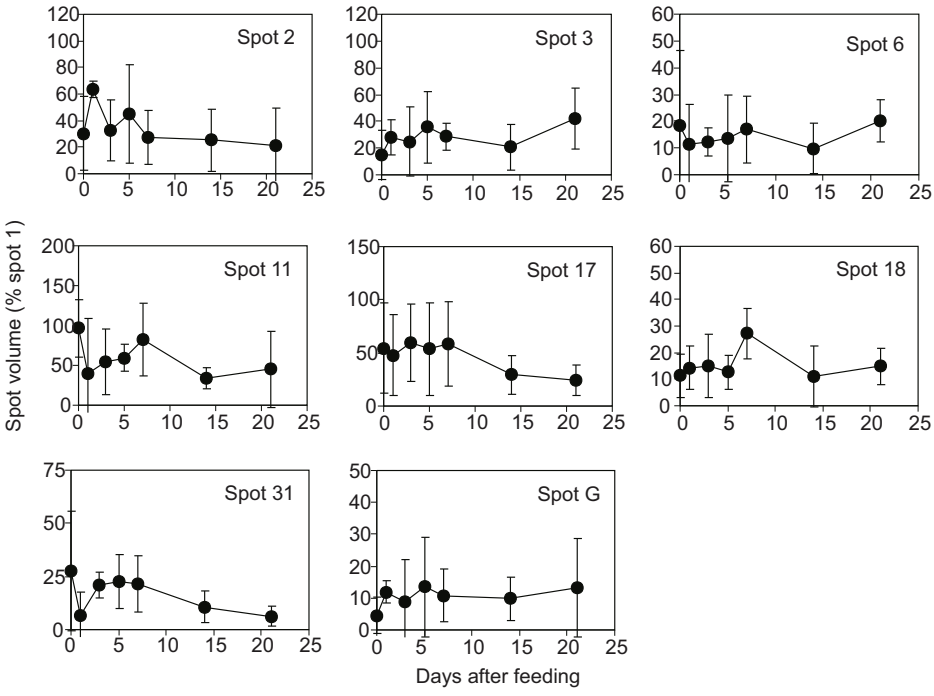

Fig. 5. Relative abundance of individual housekeeping proteins in tissues of the anterior body part of *H. verbana*. Relative spot volumes were determined using 2D gel electrophoresis in unfed leeches (day 0) and in leeches prepared at 1, 3, 5, 7, 14 and 21 days after being fed with pig blood. The abundance of the proteins represented by spots 2, 3, 6, 11, 17, 18, 31 and G was similar to that of unfed leeches at all time points after feeding.

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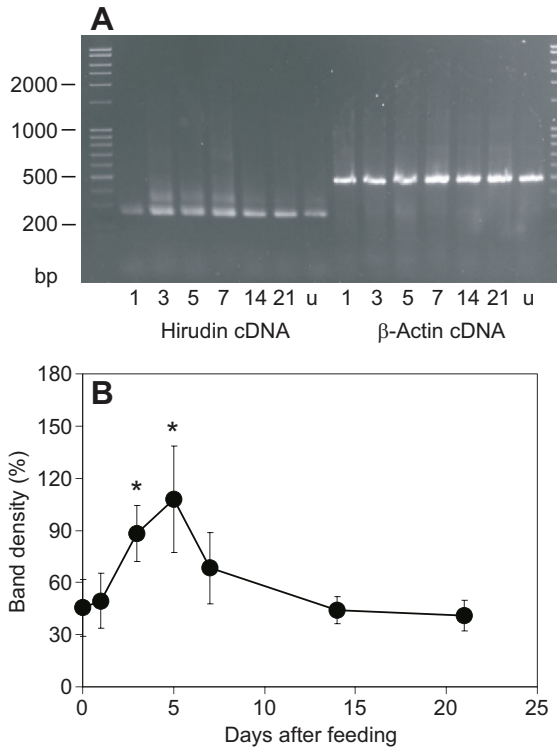

Fig. 6. Abundance of hirudin transcripts in leech salivary gland tissue before and after feeding. (A) Example gel showing PCR products using primers selective for hirudin or β-actin cDNA using cDNA preparations from unfed (u) or fed (1, 3, 5, 7, 14 and 21 days after feeding) leeches as templates. (B) Semi-quantification of hirudin PCR products. Band densities of hirudin PCR products prepared from RNA samples obtained from unfed leeches (day 0) or leeches at 1, 3, 5, 7, 14 or 21 days after feeding (N=3 each) were normalized to those of β-actin cDNA at the same time points. Data are shown as means±s.d. with levels of significance of *P<0.05.

not degraded during the storage period of up to a year (Hildebrandt and Lemke, 2011; Lent et al., 1988; Mann, 1962), a task that is supposedly assisted by symbiotic bacteria in the crop and by protease inhibitors released into the wound and in part swallowed together with host blood. These bioactive substances are produced and stored in unicellular salivary gland cells in the anterior body part of the leech and secreted during feeding (Ascenzi et al., 1995; Baskova et al., 1992; Baskova and Zavalova, 2001; Hildebrandt and Lemke, 2011; Kvist et al., 2013; Müller et al., 2015). We have recently shown (Hildebrandt and Lemke, 2011), and confirmed in this study (Fig. 1), that leeches transfer most of the salivary gland cell contents into the wound during a single blood meal. As leeches are unable to feed again without first refilling their salivary gland reservoirs, they have to prepare for another blood meal by synthesizing salivary proteins and peptides and accumulating the products in the salivary gland cell reservoirs. This raises the question, in which period after feeding are salivary proteins synthesized? The refilling could proceed along with the digestion of residual stored blood at the end of the storage period (up to 1 year after feeding) or soon after feeding. Both strategies would be biologically meaningful. In the first case, leeches could invest energy and building material from host blood into processes associated with general growth and maturation of the sexual organs, while not spending resources on the production and maintenance of salivary proteins and peptides they do not really need while their crop is still filled with host blood from a previous meal. Alternatively, leeches may want to be prepared to successfully obtain blood from any available host at any time of their digestive phase as their chances of finding a proper host are very limited.

To evaluate these possible alternatives, we investigated the degree of salivary gland cell filling using histological techniques in unfed leeches and in fed leeches at different times after feeding, studied the changes in salivary protein abundance in leech tissue using quantitative protein analysis by 2D gel electrophoresis and silver staining, and determined the transcript abundance of a small example protein in leech saliva, hirudin.

The salivary gland cell areas of fed animals were at least 40% smaller than those of unfed leeches (Fig. 2). To investigate this further, we analysed protein extracts from leech tissue containing salivary gland cells for changes in protein abundance using 2D gel electrophoresis. There were at least 30 proteins that could be consistently detected in such preparations (Fig. 3). Most of these proteins were absent or at least reduced in their abundance immediately after feeding (Figs 3, 4), indicating that these proteins are secretory proteins, while others displayed a stable presence over the entire experimental period (Figs 3, 5), indicating that these are housekeeping proteins in the salivary gland cells or in the surrounding tissue. As the protein spots representing the secretory proteins re-appeared in samples obtained during the first week after feeding (Figs 3, 4), we assume that these proteins had been newly synthetized and stored within the salivary gland cell bodies. All of these proteins had a very similar time course of abundance (Fig. 4), which indicates that the signals mediating the elevation of synthesis rates of salivary proteins are switched on immediately after feeding. Moreover, these signals are probably not specific for individual proteins, but are overall inducers of salivary gland protein synthesis.

Signals inducing increases in protein abundance may induce elevations in the rates of gene transcription or in translation rates. To better understand the mechanisms underlying the observed increases in protein synthesis in salivary gland cells, knowledge about potential changes in the amount of transcripts (mRNAs) for these proteins is required. However, as genome sequences are not yet available for many leech species and genetic background information is only available for a very limited number of salivary proteins, we were not able to analyse transcript abundance on a broad scale. Instead, we selected one well-known salivary protein, the anti-coagulant hirudin, for analysing transcript levels and their potential changes following feeding of the animals.

Densitometric analyses of relative band densities of PCR products obtained using hirudin cDNA derived from RNA preparations of leech tissue as a template (normalized to β-actin cDNA) showed that hirudin transcript levels were significantly increased at 3 and 5 days after feeding (Fig. 6). After this period, transcript abundance returned to the control level in unfed animals. We conclude that the signals mediating synthesis of salivary gland proteins and re-filling of the gland cell reservoirs may induce transcription of the hirudin gene, resulting in elevated levels of hirudin mRNA in the cells. Considering the time course of transcript level changes (Fig. 6), it seems clear that the synthesis of hirudin occurs within a few days after feeding like all of the proteins represented by the gel spots shown in Fig. 4. It remains unclear, however, whether synthesis of all these salivary gland cell proteins is a result of increased transcription rates of their genes or of regulation of the translation rates at unchanged levels of their transcripts.

On first sight, our data seem not to confirm to the results previously obtained using *H. manillensis* (Alaama et al., 2014), which indicated that the highest protein concentrations in saliva

secreted during feeding were reached only at 12–15 weeks after the previous blood meal. The results of our study indicated that synthesis of salivary gland proteins starts within days of feeding and results in virtually complete refilling of the gland cell reservoirs within 1 week of feeding. This strategy of being prepared at all times for taking up food seems to be well adapted to the condition in which finding suitable hosts and consuming sufficient amounts of blood are rather rare events in the lifetime of a leech in its natural environment.

While our data point to the early re-instatement of the potential to secrete large amounts of salivary proteins, the data reported by Alaama et al. (2014) may better indicate what recently fed leeches actually do if they get another opportunity to take up blood. It is an interesting hypothesis that leeches having residual amounts of host blood in their crops may not secrete the full load of salivary substances during another round of feeding, while really hungry leeches do not hold back any of these substances during feeding to enhance their chances of taking up maximum loads of host blood.

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Competing interests
The authors declare no competing or financial interests.

Authors contributions
S.L., C.M. and J.-P.H. jointly conceived the concept of the study. C.M. introduced S.L. to the molecular biology techniques, J.-P.H. introduced S.L. to the biochemical assays. S.L. performed the practical work for this study. S.L. and J.-P.H. wrote the manuscript.

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