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RNA Interference of the Salivary Gland Nitrophorin 2 in the Triatomine Bug *Rhodnius Prolixus* (Hemiptera: Reduviidae) by dsRNA Ingestion or Injection

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Abstract

Mass sequencing of cDNA libraries from salivary glands of triatomines has resulted in the identification of many novel genes of unknown function. The aim of the present work was to develop a functional RNA interference (RNAi) technique for *Rhodnius prolixus*, which could be widely used for functional genomics studies in triatomine bugs. To this end, we investigated whether double-stranded RNA (dsRNA) can inhibit gene expression of *R. prolixus* salivary nitrophorin 2 (NP2) and what impact this might have on anticoagulant and apyrase activity in the saliva. dsRNA was introduced by two injections or by ingestion. RT-PCR of the salivary glands showed that injections of 15 µg of NP2 dsRNA in fourth-instar nymphs reduced gene expression by 75±14% and that feeding 1 µg/µL of NP2 dsRNA into second-instar nymphs (approx. 13 µg in total) reduced gene expression by 42±10%. Phenotype analysis showed that saliva of normal bugs prolonged plasma coagulation by about four-fold when compared to saliva of knockdown bugs. These results and the light color of the salivary gland content from some insects are consistent with the knockdown findings. The findings suggest that RNAi will prove a highly valuable functional genomics technique in triatomine bugs. The finding that feeding dsRNA can induce knockdown is novel for insects.

Keywords

Triatomine; *Rhodnius prolixus*; RNA interference; Nitrophorin; Salivary gland

1. Introduction

Introduction of double-stranded DNA (dsRNA) induces gene-specific silencing in living organisms producing a knockdown of the corresponding protein (Hammond et al., 2001). As a consequence, RNA interference (RNAi) mediated by dsRNA has emerged as one of the most promising techniques to study gene function in diverse experimental systems, particularly non-model organisms where other methods of investigation are often very limited (Fraser et al., 2000; Gonczy et al., 2000). Most of this success derives from the simple, convenient and inexpensive methods for producing and introducing dsRNA into organisms. The phenomenon was first clearly demonstrated in animals in the nematode *Caenorhabditis elegans* where knockdown can be achieved by injection of dsRNA, by feeding bacteria expressing the dsRNA to the worm or simply by feeding dsRNA directly to the worm (Fire et al., 1998). RNAi has subsequently been adapted for use in insects,

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including *Anopheles gambiae*, *Drosophila melanogaster*, *Manduca sexta*, *Periplaneta americana*, *Oncopeltus fasciatus* (Blandin et al., 2002; Hughes and Kaufman, 2000; Kennerdell and Carthew, 1998; Marie et al., 2000; St. Johnston, 2002; Vermehren et al., 2001; Zhou et al., 2002). The technique was also used for reducing salivary gene expression in the ticks *Ixodes scapularis* (Narasimhan et al., 2004) and *Amblyomma americanum* (Karim et al., 2004).

Triatomine bugs are the vectors of Chagas disease. They take very large blood meals which can take up to 15 min to ingest and so the bugs are likely to have extensive antihemostatic mechanisms. Discovery programs on triatome salivary proteins have been undertaken (Ribeiro and Francischetti, 2003). Among them, mass sequencing of cDNA libraries of *Rhodnius prolixus* have identified several novel genes with unknown functions (Ribeiro et al., 2004). A functional RNAi tool for triatomine bugs would provide a potentially powerful means of investigating the function of the many uncharacterized molecules discovered. In this paper, we describe the development of such a tool using nitrophorins (NPs) as our subject matter. NPs are among the most remarkable proteins in *R. prolixus* saliva. They are salivary hemoproteins with multifunctional activities presenting a reddish color because of the presence of the heme group in the molecule (Ribeiro et al., 1993; Champagne et al., 1995). Four of them, named NP1–NP4, store and transport nitric oxide (NO), which when released in tissues induces vasodilatation and reduced platelet aggregation (Champagne et al., 1995). Recently, two other NPs were described (NP5 and NP6), but anticoagulant activity has been associated only with NP2 (Moreira et al., 2003). To demonstrate that RNAi, achieved by injection or ingestion of dsRNA, can be a functional genomic study tool for triatomine bugs and to further characterize salivary bioactive molecules, we have investigated *R. prolixus* salivary nitrophorin 2 (NP2) and its impact on anticoagulant and apyrase activity in saliva.

2. Materials and methods

2.1. Insects

The colony of *R. prolixus* was reared under controlled conditions of temperature (26 ± 2.0 °C) and humidity ($65\pm 5.0\%$) and the insects fed on chickens or rats weekly. The specimens selected for use in the experiments were standardized as 7 days after the last molt and for weight (1.8 ± 0.4 mg for second and 20 ± 2.5 mg for fourth-instar nymphs).

2.2. RNA extractions and RT-PCR

Total RNA was extracted from bug salivary glands using the RNeasy® Micro Kit (Qiagen, USA). Synthesis of cDNA was performed using the M-MLV reverse transcriptase system (Promega, USA) according to the manufacturers instructions. PCR was carried out using specific primers (Table 1).

2.3. dsRNA synthesis

The NP2 gene was PCR amplified from salivary gland cDNA and the ampicillin resistance gene (ARG) was PCR amplified from the pBluescript SK plasmid (Stratagene, USA). PCR was carried out using specific primers conjugated with 23 bases of the T7 RNA polymerase promoter (Table 1). The PCR products, 548 bp for NP2 and 854 bp for ARG, were used as templates for dsRNA synthesis using the T7 Ribomax Express RNAi System (Promega, USA). After synthesis, the dsRNA was isopropanol precipitated, resuspended in ultra-pure water, quantified spectrophotometrically at 260 nm and its purity and integrity were determined by agarose gel electrophoresis. It was kept at -80 °C until use. Before use, the dsRNA was air dried and resuspended in saline or feeding solution in the desired concentration.

2.4. Effect of dsRNA resuspended in saline or water for injection

In order to verify the interference of water or saline in the RNAi phenomenon, we injected two groups of fourth-instar bugs with 15 µg of NP2 dsRNA resuspended in water or saline (0.9% NaCl). Forty-eight hours later the salivary glands were extracted and the expression of NP2 was compared by RT-PCR with the control group injected with saline.

2.5. Injection of dsRNA

Fourth-instar nymphs received two 15 µg injections of NP2 dsRNA (NP2 dsRNA group) in the thorax with a 48 h interval in between. Control groups received 2 injections of 2 µl of saline (0.9% NaCl) alone (saline control) or containing 15 µg of dsRNA for the ARG (ARG dsRNA group).

2.6. Ingestion of dsRNA

Second-instar nymphs were fed with feeding solution (125 mM NaCl, 30 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM NaHCO₃, 2 mM NaH₂PO₄, 1 mM glucose, 1 mM ATP added to 10% Grace's insect medium (Sigma, USA), pH 7.4) containing 1 µg/µl of NP2 dsRNA (NP2 dsRNA group) using an artificial feeder (Sant'Anna et al., 2001) at 39 °C. Control groups ingested feeding solution alone (control) or with 1 µg/µl of dsRNA from ARG (ARG dsRNA group).

2.7. Citrated plasma recalcification time assay

Salivary glands were dissected in saline solution (0.9% NaCl) and transferred to ice-cold microcentrifuge tubes containing 30 µl of HEPES/NaOH buffer (20 mM HEPES–100 mM NaCl, pH 7.5). Glands were disrupted and centrifuged for 3 min at 12,000g. The supernatant containing the saliva was transferred to a new tube and diluted in HEPES/NaOH buffer to 1:4, 1:6 and 1:8 for third, fourth and fifth instars, respectively. For the assay, 30 µl of the diluted samples was added to 96-well plates and incubated with 30 µl citrated (0.38% trisodium citrate) human plasma at 37 °C for 5 min. After incubation, coagulation was triggered by addition of 30 µl of prewarmed 25 mM CaCl₂. Since plasma clotting provokes an increase in the turbidity, it was possible to follow the reaction from ELISAs (adjusted to 37 °C) at 655 nm every 10 s. The coagulation time value was defined as the time taken for the turbidity to achieve an absorbance reading of 0.025 absorbance units, the absorbance at zero time being taken as zero absorbance units (Ribeiro, 2000).

2.8. RT-PCR silencing verification

PCR was carried out using cDNA pools of three nymphs (three salivary gland pairs) from each group. Each of these pools represented one replicate. The expression of NP2 and the housekeeping gene UGALT (Ribeiro et al., 2004) was evaluated along with the expression of other NPs (NP1, NP3, NP4) and other *R. prolixus* genes such as RPMYS3, RP ribosomal protein 18S and RPP450 (Ribeiro et al., 2004) (Table 1). The RT-PCR products were analyzed by 2% agarose gel electrophoresis and the reduction in gene expression was measured by densitometric measures of bands using the Alpha DigiDoc 1201TM software (Alpha Innotech).

2.9. Apyrase activity assay

Salivary glands from individual bugs were collected and disrupted in 30 µl of HEPES/NaOH buffer (20 mM HEPES–100 mM NaCl, pH 7.5). For the assay, 0.5 µl of fifth-instar bug samples and 1 µl of third-instar samples were added to HEPES/NaOH buffer to a final volume of 20 µl. These 20 µl aliquots were added to 80 µl HEPES/NaOH buffer containing 2 mM ATP and 5 mM CaCl₂ and incubated at 37 °C for 20 min (Ribeiro et al., 1998). After

incubations, inorganic phosphate was measured using a commercial kit (Labtest Diagnostica, Brazil) based on the method described by Baginski et al. (1967).

2.10. Protein quantification

The total amount of protein in extracted saliva was quantified (Bradford, 1976) using BSA as control.

2.11. Experimental description

Normal transcriptional levels of NP1-4 were measured by RT-PCR in nymphs of all five instars and eggs of *R. prolixus* before and after blood feeding. Then two experiments were performed. In experiment 1, fourth-instar nymphs were injected twice with dsRNA within a 48 h interval. Forty-eight hours after the second injection, nymphs were fed on hamsters and kept in our rearing facility until they molted. For verification of knockdown, RT-PCR was performed on RNA extracted from insects 48 h after the first injection, 48 h after the second injection and 7 days after the molt. Anticlotting activity was measured by citrated plasma recalcification assay carried out with saliva of nymphs 7 days after the second injection and 7 days after molt to fifth instar. Saliva extracted 7 days after molt was also used for apyrase activity assay and protein quantification.

For experiment 2, second-instar bugs were fed dsRNA as described above and 48 h later were fed on hamsters. RT-PCR was carried out on RNA extracted from salivary glands removed 48 h after dsRNA ingestion and 7 days after molt. Salivary glands extracted 7 days after molt had their saliva used for activity tests (citrated plasma recalcification time assay and apyrase activity assay) and for protein quantification.

All glands extracted were photographed using a digital camera Camedia C-4000 (Olympus). This analysis permitted the verification of the color intensity of the glands, focusing on the characteristic cherry red color given by the presence of the NPs (Champagne et al., 1995).

2.12. Statistical analysis

Averages and standard deviation from RT-PCR values were calculated between replicates (pools of three nymph salivary glands). For citrated plasma recalcification time assay, apyrase activity assay a protein quantification, values refer to individual nymph salivary glands. Statistical analysis was carried out with GraphPad Instat™ (Wass, 1998) for Windows. ANOVA was used to verify differences between groups. *T* tests were used to test for differences between two variables.

3. Results

3.1. Expression of NPs

RT-PCR from *R. prolixus* eggs amplified only NP2 transcripts. Except for NP3 expression that could not be detected in unfed first instars, RT-PCR detected transcripts for NP1-4 in both fed and unfed first, second, third, fourth and fifth instars. The 390 bp fragment of the constitutive gene UGALT was also amplified in eggs and all fed and unfed instars and was used as housekeeping control in the experiments.

3.2. Effect of dsRNA resuspended in saline or water for injection

RT-PCR results from three replicates (pools of three pairs of glands each) showed no statistical difference ($p = 0.9345$) in salivary gland NP2 expression between groups injected with NP2 dsRNA resuspended in water and saline (Fig. 1). The UGALT was used as housekeeping gene for the RT-PCR. The reduction in NP2 transcript levels in bugs injected with NP2 dsRNA resuspended in water was 43% while bugs in which NP2 dsRNA was

resuspended in saline was reduced by about 44% when compared to the NP2 expression for the saline-injected controls.

3.3. Experiment 1

Fourth-instar nymphs injected with NP2 dsRNA had NP2 expression reduced by $38\pm 7\%$ ($n = 5$) after the first injection and by $75\pm 14\%$ ($n = 7$) after two injections when compared to the controls (Fig. 2). Both reductions were statistically significant ($p = 0.004$ and 0.005 , respectively). Except for NP4 expression, reduction for other NPs was also verified after two injections of NP2 dsRNA. NP1 had its expression reduced by about 69% while NP3 was reduced by about 83%. Their expression was not decreased in the groups injected with ARG dsRNA when compared to the saline controls. Levels of transcripts for RPMYS3, RP ribosomal protein 18S and RPP450 were similar in the NP2 dsRNA injected, ARG dsRNA and saline controls. When RT-PCR was performed with glands extracted 7 days after the molt to fifth instar, expression had returned to normal and levels of NPs transcripts were similar between groups.

To determine whether RNAi of NP2 affected the anticlotting activity, saliva from bugs was tested in citrated plasma recalcification time assays. Coagulation time from the saliva of knockdown bugs showed no statistical difference ($p = 0.265$) from ARG dsRNA and saline injected when a citrated plasma recalcification time assay was performed 7 days after the second injection (Fig. 3). Thus, the group injected with NP2 dsRNA prolonged the initiation of the coagulation by 928 ± 301 ($n = 13$) s while ARG dsRNA and saline control prolonged the initiation by 1111 ± 392 ($n = 12$) and 1142 ± 433 ($n = 14$) s, respectively. However, when the assay was performed 7 days after the molt to fifth instar, significant differences ($p < 0.001$) were observed between the group that was injected with NP2 dsRNA and the controls (Fig. 4). The coagulation time for the saliva of nymphs from saline control and ARG dsRNA was now prolonged by 1013 ± 404 ($n = 16$) and 1162 ± 538 ($n = 13$) s, more than four-fold higher than knockdown bugs saliva that initiated coagulation by only 219 ± 163 ($n = 17$) s.

Apyrase activity and protein quantification was also verified (Fig. 4). Results showed that the knockdown and ARG dsRNA groups from fifth-instar nymphs had a slightly lower average of released inorganic phosphate and amount of total proteins in the saliva when compared to the saline control group, but this was not statistically significant ($p = 0.3193$ and 0.6355).

Visual observation of the glands extracted from experiment 1 and a drop in the characteristic cherry red color also indicates a reduction of NPs in the saliva of knockdown nymphs. Fifth-instar nymphs from NP2 dsRNA-injected groups, dissected 7 days after molt, showed three different phenotypes (Fig. 5). Among the 17 silenced bugs, five had reduction in the color content of one gland only, three showed reduction in the color of both glands and the remaining nine appeared visually normal. In order to verify differences in bugs presenting differences in the color of the glands, they were separated into two groups (light and dark red gland content) and the citrated plasma recalcification time assay and apyrase activity assay results from the groups were compared. Results showed that bugs with light and dark color contents have no statistical difference in apyrase activity ($p = 0.2380$), coagulation inhibition ($p = 0.2816$) or protein content ($p = 0.1670$) (Fig. 6).

3.4. Experiment 2

Second-instar nymphs ingested about 13 mg of feeding solution on average, equivalent to about $13\text{ }\mu\text{g}$ of dsRNA. RT-PCR of knockdown group salivary glands, 48 h after ingestion, demonstrated a significant decrease ($p = 0.0054$) of $42\pm 10\%$ ($n = 4$) in the NP2 expression

when compared to controls (Fig. 7). Expression levels of UGALT transcript remained the same in knockdown and both feeding solution control and ARG dsRNA groups. Again, except for NP4, decreased expression of other NPs was also observed 48 h after ingestion of NP2 dsRNA. NP2 knockdown group showed 15% and 28% decrease in NP1 and NP3 transcripts, respectively, when compared to controls. Levels of UGALT expression remained similar in all groups, as well as the levels of NP1, NP3 and NP4 between groups that ingested feeding solution alone and with ARG dsRNA. Expression of other genes was also verified and knockdown bugs had no reduction in RPMYS3, RP ribosomal protein 18S and RPP450 expression when compared to controls. The transient effect of RNAi was also verified in experiment 2 nymphs. RT-PCR from glands extracted 7 days after molt to third instar showed no difference in expression of NPs between knockdown and control groups.

Results from citrated plasma activity assay showed that coagulation time in samples from bugs that ingested NP2 dsRNA was significantly ($p < 0.001$) lower than in control groups (Fig. 8). Bugs that ingested feeding solution alone or with ARG dsRNA prolonged initiation of plasma coagulation by 711 ± 301 ($n = 14$) and 839 ± 324 ($n = 15$) s, respectively, while bugs that ingested NP2 dsRNA prolonged initiation of coagulation by only 114 ± 109 ($n = 14$) s.

Apyrase activity assays and protein quantification of the saliva of each group showed no significant difference between groups ($p = 0.3136$ and 0.6772 , respectively), although the average of inorganic phosphate released and the protein content were slightly lower in the ARG dsRNA and knockdown groups when compared to the control that ingested feeding solution alone (Fig. 8).

When dissected, no distinction in the color of salivary gland contents between knockdown and control insects was verified in bugs from experiment 2 seven days after molt to the third instar.

4. Discussion

NPs regulate the levels of NO which, when released in the feeding wound, induces vasodilatation and reduced platelet aggregation (Ribeiro et al., 1993; Champagne et al., 1995). Anticoagulant activity has been associated only with NP2 (Moreira et al., 2003). The above results support these findings, showing that introduction of NP2 dsRNA significantly reduces the anticoagulant activity of *R. prolixus* saliva and that this reduction can be achieved by injection or ingestion of the dsRNA. Although RNAi has been shown in many species, including hemipteran insects (Hughes and Kaufman, 2000), this is the first time it has been used for triatomine bugs.

Hematophagous insects must overcome host hemostasis, which they do by producing a sophisticated cocktail of potent biological compounds in their saliva. Recent advances in transcriptome and proteome research allow an unprecedented insight into the complexity of these compounds, indicating that their molecular diversity as well as the diversity of their targets is even larger than previously thought (Ribeiro et al., 2004). Several tools have emerged for post-genomic studies and among them there is great interest in exploring RNAi via the use of dsRNA as a means of assessing specific function(s) of genes (Hannon, 2002). The main hallmarks of RNAi are its simplicity and the ease with which the method can be applied to an organism in vitro or in vivo (Fjose et al., 2001).

NPs are hemeproteins corresponding to a considerable proportion of the proteins in the saliva of *R. prolixus* (Champagne et al., 1995). The NPs start being produced in the salivary glands in different stages and once started, they continue being produced until they reach the adult stage. Moreira et al. (2003) found that NP2 proteins are the sole NPs in N1 salivary glands, with NP4 also found in N2 saliva and NP1 starts to appear in N3. NP3 protein is the

last to be identified and is only found in N5 saliva. Using northern blot analysis, Sun et al. (1998) did not see NP3 transcripts in N4 instars. In this work, RT-PCR carried out with salivary glands identified that RNA encoding NP3 is not found in unfed first instar, while all the other NPs are transcribed in all instars.

The results obtained with injections of dsRNA resuspended in water or saline induced about the same level of inhibition in triatomine bugs. This is an important point because the volume of the injections (2 μ l) corresponds to around 10% of the weight of the fourth instar, suggesting that water could cause any kind of osmotic damage to the nymphs. For these reasons, we opted for using saline instead of water.

In salivary gland genes from hematophagous arthropods, RNAi was previously demonstrated in ticks (Karim et al., 2004; Narasimhan et al., 2004). But different from ticks, triatomines have the saliva stored in a reservoir comprised of the epithelial and muscular cells of the salivary gland (Meirelles et al., 2003). During the blood meal, only half of the salivary proteins are lost and a single blood meal allows the bug to molt to the next stage, when the next feed occurs usually after the 3rd–5th day (Nussenzweig et al., 1995). For this reason, reduction in protein levels are only observed after molt to the next instar while reduction in RNA expression occurred as early as 48 h after dsRNA introduction. Loss of salivary activity is only seen after new saliva is resynthesized, without or with low levels of the knockdown protein, to refill the saliva reservoir (Nussenzweig et al., 1995). In addition, there is a considerable expansion of the glands after molt, as observed for other triatomine species, whose reservoir more than doubles its size and the amount of saliva as well (Guarneri et al., 2003).

The introduction of NP2 dsRNA in *R. prolixus* nymphs reduced NP2 expression, but also reduced levels of other NPs. This low specificity could be due to the considerable homology observed between the NPs (Andersen and Montfort, 2000). It has already been observed for other insect species that RNAi can also trigger the destruction of mRNAs that contain significant stretches of sequence identity (Jackson et al., 2003).

The activity assays performed and the protein content suggest that introduction of unspecific dsRNA induce slight alterations in the gland machinery, but do not compromise the functioning of the glands, which enforces the idea of specificity of the RNAi. It is important to highlight the variation observed among specimens in each of the experiments and the constraints that this will place on future experimental design.

An important finding of this work was the verification that RNAi can be achieved by ingestion of dsRNA in triatomine bugs. Ingestion inducing RNAi was previously achieved in nematodes (Hunter, 1999; Timmons and Fire, 1998) and in ticks (Soares et al., 2005). In *C. elegans*, RNA can be absorbed through the gut and distributed to somatic tissues and germ line (Kamath et al., 2001; Timmons and Fire, 1998). However, the same methodology has not been shown in insects. Curiously, host intact immunoglobulin G has been reported to occur in the hemolymph of insects with marked differences in digestive tract anatomy and physiology such as ticks (Ackerman et al., 1981; Ben-Yakir, 1989; Tracey-Patte et al., 1987), some mosquitos (Hatfield, 1988; Lackie and Gavin, 1989; Ramasamy et al., 1988), fleshflies (*Sarcophaga falculata*) (Schlein et al., 1976) and the blood-fed fly *Haematobia irritans* (Allingham et al., 1992), showing that molecules as big as immunoglobulin G can cross the gut epithelium with no damage even in flies that have the peritrophic membrane.

As observed in *C. elegans* (Hunter, 1999), ingestion of dsRNA is less effective in inducing RNAi in triatomines than injection. While two injections of 15 μ g of dsRNA for NP2 in fourth-instar *R. prolixus* was shown to reduce gene expression in 75%, an experiment in which two ingestions of 80 μ g of NP2 dsRNA were offered to the same instar showed no

reduction at all (data not shown). But delivering dsRNA by ingestion is less traumatic to the nymphs than injections. After ingestion, nymphs remain healthier and the mortality is considerable lower. Ingestion has another great advantage, it is easier to perform in first and second-instar nymphs in which injection would need special equipment and result in a high mortality.

In the past years, our laboratory has been working in identifying and characterizing novel genes expressed in the salivary glands of triatomines (Sant Anna et al., 2002). The optimization of the RNAi technique and the possibility of introduction by ingestion in early nymph stages will greatly help at the functional characterization of new genes and better analyze the functions of the NPs in the feeding process. Similar methodology will be used in future experiments with *R. prolixus* and other triatomine bugs. After silencing, bugs saliva will be used in in vitro assays for specific activity and knockdown bugs used for in vivo feeding behavior experiments, such as electric monitoring of cibarial pumps and intravital microscopy, in order to precisely analyze the lack of the protein in the feeding process.

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Fig. 1. Effect of dsRNA resuspended in water or saline for injection. (A) NP2 expression in fourth-instar nymph salivary glands from saline injected control and knockdown groups injected with NP2 dsRNA resuspended in water and saline. UGALT was used as housekeeping of the RT-PCR. (A) Shows the results from replicate 1. Three replicates were performed and each corresponds to a pool of three nymphs. Average and standard deviation are shown in (B). Asterisks indicate significant statistical difference ($p < 0.05$).

Fig. 2. RT-PCR verification of NP2 expression from fourth-instar nymph salivary glands from saline injected control, ARG dsRNA injected and NP2 dsRNA injected. UGALT was used as housekeeping of the RT-PCR. (A) Shows the reduction in NP2 expression after one or two injections from replicate 1. Five replicates were performed for 1 injection and seven for 2 injections. Each replicate consists of a pool of three nymphs (three pairs of glands). Average and standard deviation from replicates are shown in (B). Asterisks indicate significant statistical difference ($p < 0.05$).

Fig. 3.

Citrated plasma recalcification time assay from salivary glands of fourth-instar nymphs from saline control ($n = 12$), ARG dsRNA ($n = 14$) and knockdown ($n = 13$) groups. Glands were extracted 7 days after the second injection. Asterisks indicate significant statistical difference ($p < 0.05$). Coagulation time was defined as the time taken for the turbidity to achieve an absorbance reading of 0.025 absorbance units, the absorbance at zero time being taken as zero absorbance units.

Fig. 4.

Citrated plasma recalcification time assay (A), apyrase activity assay (B) and protein quantification (C) from salivary glands of nymphs that were injected twice with saline ($n = 14$), ARG dsRNA ($n = 12$) or NP2 dsRNA ($n = 13$). Glands were extracted 7 days after molt to fifth instar. Asterisks indicate significant statistical difference ($p < 0.05$). Coagulation time was defined as the time taken for the turbidity to achieve an absorbance reading of 0.025 absorbance units, the absorbance at zero time being taken as zero absorbance units.

Fig. 5. Photography of salivary glands from groups injected twice with saline (A), ARG dsRNA (B) or NP2 dsRNA (C and D) showing the color of the content. Glands were extracted 7 days after molt to fifth instar. Arrows indicate light red content salivary glands.

Fig. 6.

Citrated plasma recalcification time assay (A), apyrase activity assay (B) and protein quantification (C) from nymph salivary glands that were injected twice with NP2 dsRNA, which had light ($n = 8$) and dark ($n = 9$) red gland content after molt. Glands were extracted 7 days after molt to fifth instar. Asterisks indicate significant statistical difference ($p < 0.05$). Coagulation time was defined as the time taken for the turbidity to achieve an absorbance reading of 0.025 absorbance units, the absorbance at zero time being taken as zero absorbance units.

Fig. 7. RT-PCR verification of NP2 expression from second-instar nymph salivary glands that ingested feeding solution alone (control), with ARG dsRNA or with NP2 dsRNA groups. UGALT was used as housekeeping of the RT-PCR. (A) Shows the results from replicate 1. Four replicates were performed. Each replicate consists of a pool of three nymphs (three pairs of glands). Average and standard deviation from replicates are shown in (B). Asterisks indicate significant statistical difference ($p < 0.05$).

Fig. 8.

Citrated plasma recalcification time assay (A), apyrase activity assay (B) and protein quantification (C) from salivary glands of nymphs that ingested feeding solution alone (control, $n = 14$), with ARG dsRNA ($n = 15$) or with NP2 dsRNA ($n = 14$). Glands were extracted 7 days after molt to third instar. Asterisks indicate significant statistical difference ($p < 0.05$). Coagulation time was defined as the time taken for the turbidity to achieve an absorbance reading of 0.025 absorbance units, the absorbance at zero time being taken as zero absorbance units.

Table 1

Primers used for RT-PCR

Gene	Forward	Reverse	Amplicom
Ampicilin resistance gene (ARG)	gaactggatctcaacag	ggatcttcactagatc	808
Nitrophorin 1 (NP1)	tttgctgcagtggtgtaag	agttgcccgcgttacatct	334
Nitrophorin 2 (NP2)	gccgtgaccattctctgtct	tcacggcgcttttaacttt	502
Nitrophorin 3 (NP3)	tggaaaccgtactcagctttg	gcgtttttaaccgttgact	520
Nitrophorin 4 (NP4)	cgtagccattctgtgcctgt	gcagatacggcgctttta ac	501
UGALT	atggcactccagtgggtag	aagaaaggcgaggcattgta	390
RPMYS3	actgtgcctggattggagtc	tgcaagaagtatttaaagg	547
RP ribosomal protein 18	cctgcggcttaattgactc	gtacaaaggcgaggacgta	468
RPP450	gcatgcagctaacgtttga	atgccaacattgtctcttc	593