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RESEARCH ARTICLE

Physiological characterization of the hematophagy of *Ornithodoros rostratus* (Acari: Argasidae) on live hosts

Gabriel Cerqueira Alves Costa[1,*], Adriana Coelho Soares[1,*], Marcos Horácio Pereira[1,2], Nelder Figueiredo Gontijo[1,2], Maurício Roberto Viana Sant'Anna[1] and Ricardo Nascimento Araujo[1,2,‡]

ABSTRACT

Ornithodoros rostratus is an argasid tick and its importance is based on its hematophagy and the resulting transmission of pathogens such as *Rickettsia rickettsii* and *Coxiella burnetii* to its vertebrate hosts. In the face of a lack of physiological studies related to hematophagy in argasid ticks, this paper aims to identify and characterize the events that occur throughout the feeding by *O. rostratus* on live hosts. Electrical signals and alterations on the feeding site were monitored using intravital microscopy and electromyography. The analyses allowed for the characterization of four distinct events: suction, salivation, chelicerae movements and inactivity. Feeding was divided into two distinct phases: (1) penetration of mouthparts (when only salivation and chelicerae movements occurred) and the formation of the feeding pool (salivation and chelicerae movements with the first signs of suction) and (2) engorgement, during which chelicerae movements ceased and blood intake took place in feeding complexes (salivation followed by suction). Variations in patterns of the electrical signals, suction frequency and salivation showed four distinct sub-phases: (2a) suction with electrical signals of irregular shape, increased suction frequency and decreased salivation frequency throughout blood feeding; (2b) suction with electrical signals of symmetrical shape, high suction rates (3.8 Hz on average) and feeding complexes lasting for 7.7 s; (2c) suction with electrical signals of irregular shape, high suction frequency and feeding complex lasting 11.5 s; and (2d) electrical signals with no profile and the longest feeding complexes (14.5 s). Blood feeding ended with the withdrawal of the mouthparts from the host's skin.

KEY WORDS: *Ornithodoros rostratus*, Blood feeding, Pharyngeal pump, Intravital microscopy, Electromyogram

INTRODUCTION

Ornithodoros rostratus Aragão 1911 is an argasid tick that uses an eclectic array of food sources, including dogs, pigs, cows, peccaries and humans (Cançado et al., 2008; Hoogstraal, 1985; Ribeiro et al., 2013). These soft ticks are of medical and veterinary importance as they give a painful and itchy bite (Aragão, 1936; Estrada-Pena and Jongejan, 1999) and may harbor and transmit pathogens such as *Rickettsia rickettsii* and *Coxiella burnetii*, the causative agents of the Rocky Mountain spotted fever and acute Q fever, respectively, in humans and other animals (Almeida et al., 2012; Hoogstraal, 1985). *Ornithodoros rostratus* has been reported in four countries in South America: Argentina, Bolivia, Brazil and Paraguay (Barros-Battesti et al., 2006; Guglielmone et al., 2003).

During its life cycle, *O. rostratus* has one larval and six nymphal instars, in addition to the adult stage. The tick is an obligatory hematophage in all stages and remains in contact with its host for varying periods, from a few minutes or hours (nymphal instars and adults) to days (larvae) (Costa et al., 2015; Lavoipierre and Riek, 1955; Ribeiro et al., 2013). Although contact with their host is for a short period of time, it is crucial because their success in obtaining blood will affect their survival, development and reproduction (Anderson and Magnarelli, 2008); moreover, the short period of contact also reduces the risk of being perceived and killed by the host (Rossignol et al., 1985). In addition, it is during blood feeding that pathogens circulate between vectors and their hosts, thus interfering with the epidemiology of vector-borne diseases.

Owing to the obvious importance of hematophagy, some techniques, such as intravital microscopy and electronic monitoring systems, have been developed to study the physiology of the feeding process. Intravital microscopy consists of visualizing the feeding site on live hosts, allowing the evaluation of events that occur throughout the blood feeding from the arthropod's point of view (e.g. visualization of movements of their mouthparts) as well as the host's (e.g. visualizing modifications that occur in their skin, microcirculation and tissues on the feeding site) (Soares et al., 2014). Although very informative, intravital microscopy misses many processes taking place inside the bloodsucking arthropod. This problem is addressed by the use of electronic monitoring systems: the electrical penetration graph (Backus, 1994) and electromyogram (Araujo et al., 2011) monitor the electrical signals (variation of resistance or voltage) generated at the arthropod–host interface. The electromyogram records signals generated by the arthropods' muscles. Because bloodsucking arthropods are still during feeding, most of the electrical signals detected come from muscle contractions of the suction and salivary pumps. These muscles are responsible for the generation of negative pressures during blood feeding, thus allowing blood to enter their digestive system. These techniques have been used in the past to study blood feeding in several groups of arthropods such as aphids (McLean and Kinsey, 1964), mosquitoes (Choumet et al., 2012; Griffiths and Gordon, 1952; Kashin and Wakely, 1965), tsetse flies (Margalit et al., 1972), cimicids (Araujo et al., 2009a), triatomines (Smith and Friend, 1970; Soares et al., 2014, 2006) and ixodid ticks (Bockenstedt et al., 2014; Gregson, 1969; Richter et al., 2013; Sweatman and Gregson, 1970; Tatchell et al., 1971; Waladde et al., 1979). Although they have been used for insects and ixodid ticks, there is an obvious lack of information for argasid ticks, as few studies have focused on the physiology of their feeding process. Lavoipierre and Riek (1955) described several feeding aspects of

[1]Physiology of Hematophagous Insects Laboratory, Department of Parasitology, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais 31270-901, Brazil. [2]National Institute of Molecular Entomology, Rio de Janeiro 21941-591, Brazil.
*These authors contributed equally to this work

‡Author for correspondence (rnaraujo@icb.ufmg.br)

R.N.A., 0000-0002-1272-4386

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different argasid species on rodents using intravital microscopy. However, the authors failed to interpret and quantify some processes and events that occurred during blood feeding. Recently, Zheng et al. (2015) detected electrical signals produced while *Ornithodoros turicata* was feeding on artificial membranes and characterized different phases of this process. However, again, feeding events were not quantified.

In order to study in more detail the physiological processes during *O. rostratus* hematophagy on live hosts, groups of fourth instar nymphs were blood fed on mice and the feeding process was monitored using electromyography and intravital microscopy simultaneously. Such an association allowed for the identification and quantification of different electrical signals generated during feeding in order to characterize each phase of the process. Analysis and results presented here allow a better comparison between the feeding patterns of *O. rostratus* and other blood-feeding arthropods.

MATERIALS AND METHODS

Ticks and colony rearing

Ticks used in the experiments were collected in Nhecolândia, located in the wetlands of Mato Grosso do Sul state, Brazil (19°03′S, 56°47′W), and were maintained inside an incubator under semi-controlled conditions of temperature (28±2°C) and humidity (85±10%) (Costa et al., 2015). Ticks were fed Swiss mice (*Mus musculus*) every 20–30 days. Fourth instar nymphs with 27±2 days of starvation were used in the experiments.

Experiment design and ethics

Experiments were performed in two steps. Four nymphs were fed on the ears of mice and monitored using both electromyography and intravital microscopy (Araujo et al., 2011; Soares et al., 2014). The events that occurred during feeding were identified and associated with the recorded electrical signals. Subsequently, the duration and frequency of each event during feeding were determined using intravital microscopy experiments. A second group of eight nymphs were fed on mice abdomen under electromyogram monitoring alone.

All procedures were in accordance with the manuals for experiments using animals and were approved by the Ethics Committee for the Use of Animals (CEUA, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais) under the protocol 301/2013.

Electromyogram

Nymphs were fed on the ventral surface of 6- to 8-week-old male Swiss mice previously anesthetized intraperitoneally (i.p.) with a mixture of 150 mg kg^{-1} ketamine hydrochloride (Cristalia, Itapira, SP, Brazil) and 10 mg kg^{-1} xylazine (Bayer S.A., São Paulo, SP, Brazil). Each animal was placed on a heating pad (Fine Science Tools, Baden-Württemberg, Germany), to maintain body temperature at 37±1°C, monitored by a rectal sensor (Fine Science Tools). Electrical signals were recorded according to Araujo et al. (2011). One electrode was fixed to the host, and a second electrode was connected to a metal mesh, which was placed inside a polystyrene cylinder (0.7 mm diameter×1 cm length) that was used as a landing substrate for the ticks. Captured signals were amplified 210 times, filtered by a low-pass filter and digitalized by an AC-100 plate connected to a computer that recorded the mV variation. Contact between the electrodes (metal mesh) and the tick/host was enhanced using a conductive electrolytic gel (Regisgraf-Gel, São Paulo, SP, Brazil). Care was taken to minimize light, odor, noise, vibration and handling of the ticks before testing. The time of the bite and the end of the feeding process were also visually monitored. The ticks were weighed before and after the feeding experiment in order to determine weight gain (calculated as final weight minus initial weight). Based on records of the electrical signals, the total contact time (TCT) was defined as the length of time the ticks mouthparts remained inserted into the host's skin. Ingestion rate was calculated as the ratio between weight gain and TCT.

Simultaneous intravital microscopy and electromyogram

Hairless Swiss mice (6- to 8-week-old males) were anesthetized and placed on a heating pad as described above. Electromyograms were recorded as above, except that one electrode was fixed to the dorsal surface of the ticks using a small drop of instant adhesive (Cyanocrylate) close to the tip and the feeding site was the convex dorsal surface of the mouse's ear, which was flattened on double-sided adhesive tape against a small Plexiglas platform. Images of the feeding site were recorded at 30 frames s^{-1} using a digital camera (Canon EOS Rebel T3i, Canon, Taiwan) attached to a microscope (Leica DM500, Leica Microsystems, HeerBrugg, Switzerland).

Images recorded during feeding were analyzed using ImageJ software (Abramoff et al., 2004) according to Soares et al. (2014) and Araujo et al. (2011). The image of each movie frame was converted to a binary image (black and white), and the dark area of a selected region (containing the feeding site) was calculated after threshold adjustments, to enable the differentiation between the material leaked to the feeding site during blood feeding and background noise.

Data analysis

The voltage value obtained from electromyograms and the values extracted from image analysis were transferred to SigmaPlot for Windows (Version 12.3) worksheets for graph construction and frequency analysis. Statistical analyses of feeding parameters were performed using GraphPad Prism 5.01, and $P<0.05$ was set as the significance level.

RESULTS

Feeding events and their correspondence to observed electrical signals

The images recorded during the intravital microscopy experiments (Fig. 1) enabled the identification of four events that occurred during feeding – suction, salivation, chelicerae movements and inactivity (Figs 2, 3) – and the association of each of these with the recorded electrical signals. The characteristics of each event were described as follows.

Suction

During hematophagy, a feeding pool was formed surrounding the tick's mouthparts (Fig. 1, Movie 1). Suction occurred when a quick reduction of the reddish area surrounding the tick's mouthparts was seen. Image analysis from the feeding pool generated sequential spikes, each corresponding to one suction event (Fig. 2, Movies 2–4). Electrical signals captured during suction were characterized by sequential and repetitive variations of voltage-forming spikes, with peaks pointing downwards (Fig. 2A–H) reflecting pharyngeal pump muscle contractions and relaxations. The spikes in the electromyograms and image analysis coincided over time (Fig. 2A–H). Suction electrical signals showed three distinct patterns that were characterized by the shape of the spikes: (1) spikes with a symmetric shape (single peak downwards and constant amplitude over time) that formed the pattern herein called

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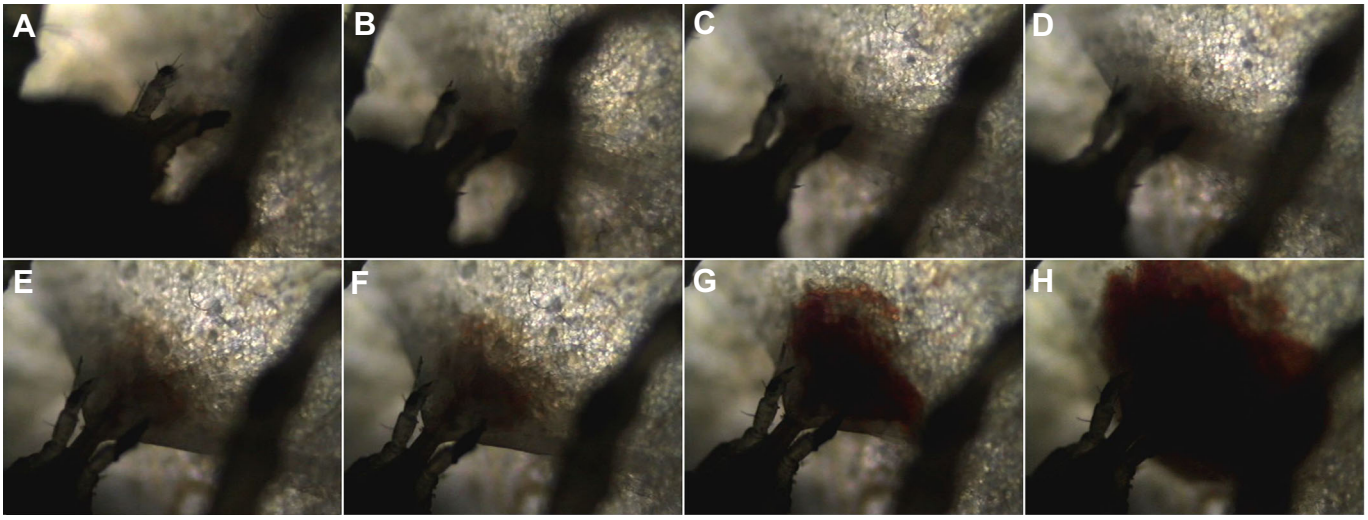

Fig. 1. Bite site and feeding pool of one specimen at different times during *Ornithodoros rostratus* blood feed. (A) Pool formation (phase 1). (B–H) Different moments during engorgement (phase 2). Each image represents the feeding pool at approximately eight equal intervals throughout the feeding period.

Type 1 pattern (Fig. 2A–D); (2) spikes with an irregular shape (double peaks downwards and higher amplitude variation over time) that formed the pattern herein called Type 2 pattern (Fig. 2E–H); and (3) shapeless electrical signals, seen when voltage variation was irregular with no pattern detected (Fig. 2I–L). However, image analysis showed the presence of suction and salivation during shapeless electrical signals (Movie 4).

Salivation

Recorded images showed liquid being regularly egested from the mouthparts of the ticks to the feeding site (Movies 2–4). At the beginning of feeding, the egested material was clear and not conspicuous during image analysis. However, during engorgement, the egested material was red, representing saliva combined with contents of the feeding canal (blood, tissue fluids and cells). At these moments, salivation was characterized by a sudden increase in the pool area represented by the reddish material egested at the feeding site (Fig. 2). Electrical signals captured during salivation were characterized by a sudden increase in voltage (up to ∼2.8 mV) followed by a rapid return to basal voltage (∼1.5 mV) (Fig. 2A–H). The sudden increase in voltage was subtle at times (∼0.2 mV) (third salivation in Fig. 2A; all salivations in Fig. 2C), always containing saliva egested at the feeding site.

Chelicerae movements

At the beginning of feeding, all ticks moved their chelicerae intensely in order to dilacerate tissues and insert their mouthparts into the host's skin (Movies 5, 6). These movements occurred interchangeably with salivation and suction. Electrical signals generated during chelicerae movements showed voltage variations with different, often high-amplitude variations (up to 3 mV) (Fig. 3A,B).

Inactivity

These events were very rare and occurred for short periods (0.1 to 2 s) in any phase of the feeding. They were characterized by a period when ticks remained with their mouthparts in contact with the host's skin without performing any of the characterized events. During these periods, the voltage showed a slight variation (up to 0.8–0.9 mV) (Fig. 3C,D).

Phases of the feeding process

Nymphs that fed on mice remained in contact with the host for an average of 39.1 min and ingested approximately 3.6 times their initial weight (Table 1). The average ingestion rate was 0.7 mg min^{-1} and almost all ticks fed at one site, with only one out of 12 changing to a region next to the initial bite site to resume feeding.

The feeding process was divided into two distinct phases based on the occurrence of feeding events: phase 1 – pool formation; and phase 2 – engorgement (Fig. 4). The characteristics of each phase are explained below.

Phase 1

This is the start of the feeding process that begins with the insertion of the ticks' mouthparts and the formation of the feeding pool. This phase lasted, on average, 5.3% of the TCT (Table 2). All four events occurred during this phase, but with suctions only seen for short periods. The events recorded during phase 1 occurred in a range of frequencies and periods that could be divided into two distinct moments, as follows.

Phase 1a: insertion of the mouthparts

Intense chelicerae movements and salivation (Fig. 3A,B, Movie 5) characterize this phase. Ticks used their chelicerae and saliva to insert their mouthparts into the host's skin in order to cut through tissues and produce the feeding pool (Fig. 1A). Phase 1a lasted 44.7±32.2 s (14–83 s) on average, with no suction events observed.

Phase 1b: pool formation with suction

The second part of phase 1 is characterized by the occurrence of the first suction events (Fig. 3C,D, Movie 6). Initially, suction occurred for short periods (1–3 s) and alternated with intense salivation and sometimes, chelicerae movements. Over time, suction appeared for longer periods and their frequency increased progressively (Fig. 4). Suction electrical signals showed irregular shaped spikes (downward double peaks and variation in amplitude over time).

Phase 2

The end of phase 1 was conventionally delimited by the occurrence of at least 20 consecutive suction spikes, indicating that the tick had

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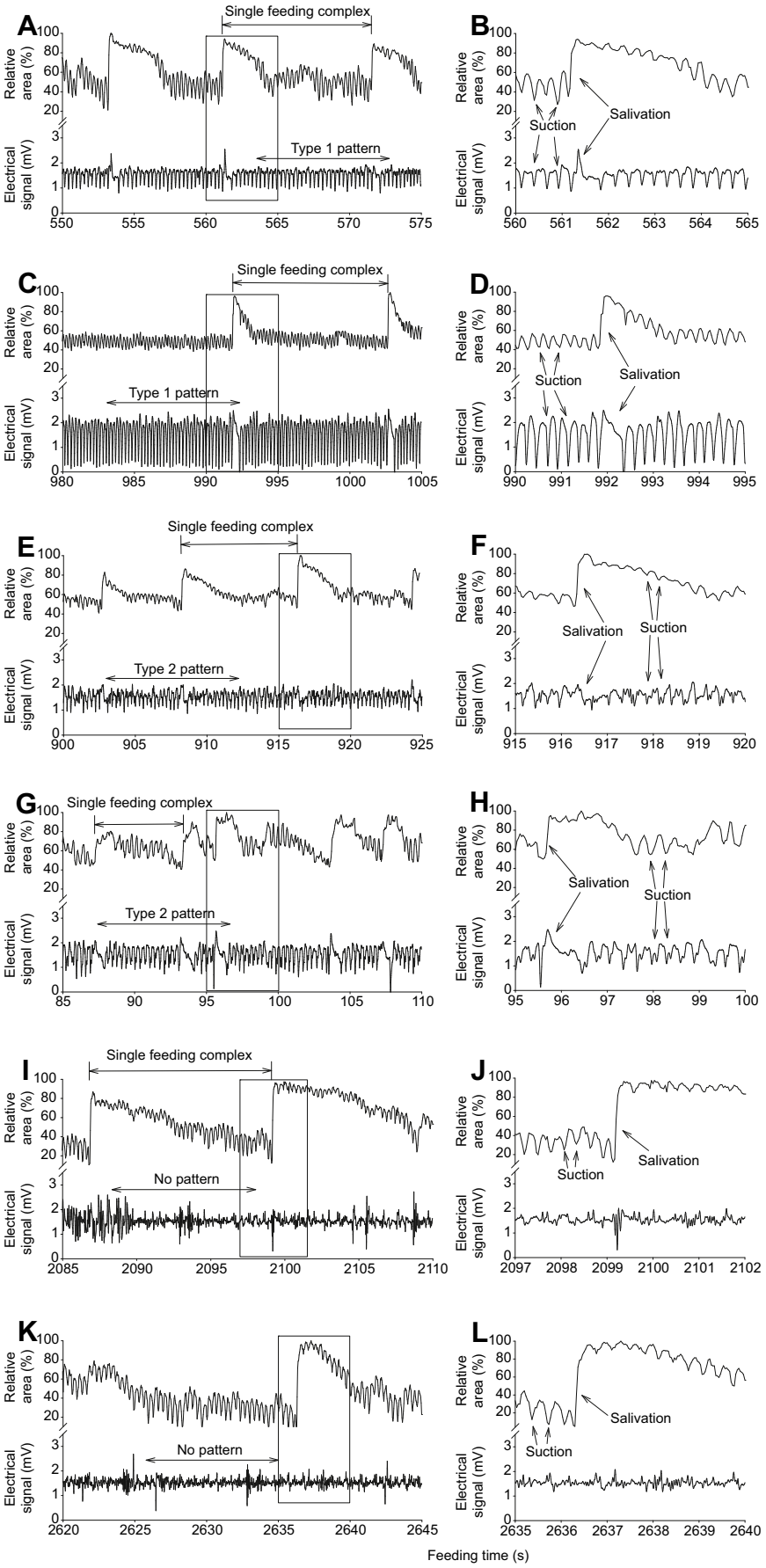

Fig. 2. Image analysis showing variation in the size of the feeding pool (upper line) and electromyogram showing variation in the electrical signals (lower line) recorded during suction and salivation in each phase of *O. rostratus* feeding on mice. (A–D) Suction in Type 1 pattern typical of phase 2b; (E–H) suction in Type 2 pattern typical of phases 2a and 2c; (I–L) suction with no pattern typical of phase 2d. Right panels are close-up views of the areas highlighted inside boxes in left panels.

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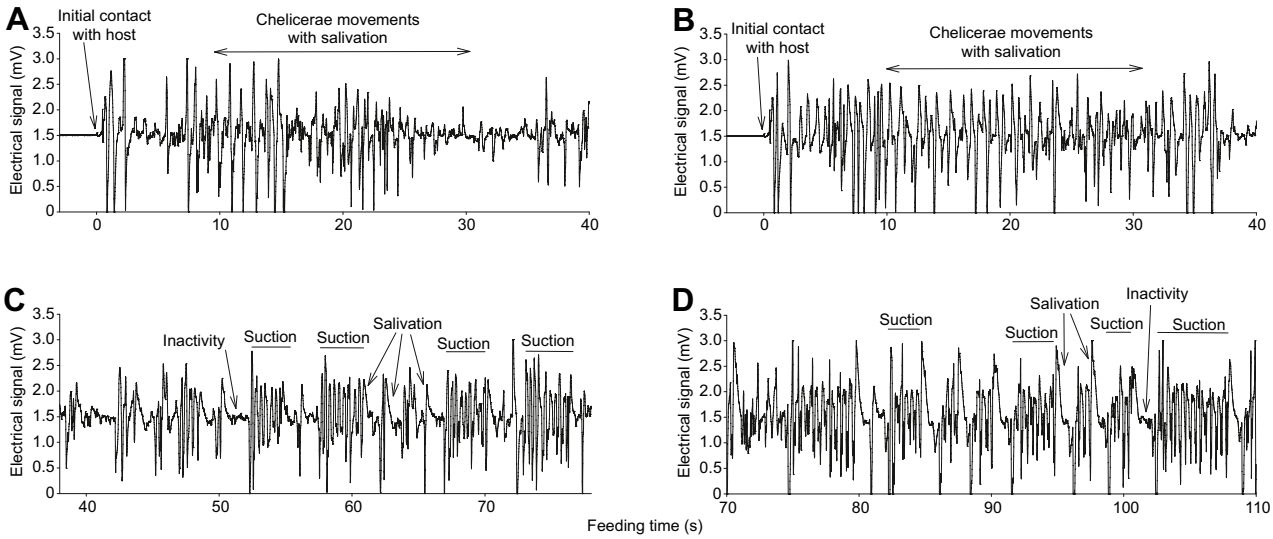

Fig. 3. Electrical signals observed at two distinct moments of *O. rostratus* blood feeding on mice. (A,B) Insertion of mouthparts (phase 1a). (C,D) Pool formation with suction alternated with salivation (phase 1b).

started engorgement (phase 2). During engorgement, ticks performed suction and salivation with short, occasional short periods of inactivity where the chelicerae remained still. The ingestion of the pool content occurred in feeding complexes, with a single feeding complex conventionally characterized by the period from saliva ejection followed by suction until the next salivation (Fig. 2). Suction frequency, the pattern of electrical signals during suction, and the duration of the feeding complexes were variable throughout feeding and could be characterized further into four different moments during phase 2, as follows.

Phase 2a

This phase lasted 5.3% of the TCT (Table 2) and was distinguished by continuous blood suction with salivation. Suction electrical signals had the Type 2 pattern (Fig. 2E–H) and suction frequency increased gradually over time, whilst salivation decreased (Fig. 4). The average length of a single feeding complex during phase 2a was shortest during the engorgement phase, lasting 3.1 s on average (Table 2). The end of this phase was conventionally recorded with the occurrence of at least 20 consecutive Type 1 pattern suction signals.

Phase 2b

This was the second longest phase, lasting an average of 23.9% of the TCT (Table 2). Salivation was at a low frequency while suction frequency was high (Fig. 4). The length of a single feeding complex increased to an average of 7.7 s (Table 2). Suction electrical signals had a Type 1 pattern (Fig. 2A–D) and suction frequency reached and remained at its highest level (up to 4.7 and 3.5 Hz when feeding on the mouse's abdomen and ear, respectively) (Fig. 4). Over time, irregular suction spikes appeared and the end of this phase was conventionally marked with the appearance of at least 20 consecutive Type 2 pattern signals.

Table 1. Alimentary parameters of fourth instar nymphs of *Ornithodoros rostratus* fed on mice

	Feeding site		
Feeding parameter	Abdomen	Ear	Abdomen+ear
Initial weight (mg)	6.3±2.6	8.8±4.4	7.2±3.4
Weight gain (mg)	26.9±15.6	23.8±5.6	25.8±12.5
Bites	1.1±0.4	1.0±0.0	1.1±0.3
Total contact time (min)	31.4±10.4	52.7±14.3*	39.1±15.6
Ingestion rate (mg min^{-1})	0.8±0.4	0.5±0.1	0.7±0.4
n	8	4	12

Data are shown as means±s.d.
*Significant difference between feeding sites (two-tailed *t*-test; *P*=0.019).

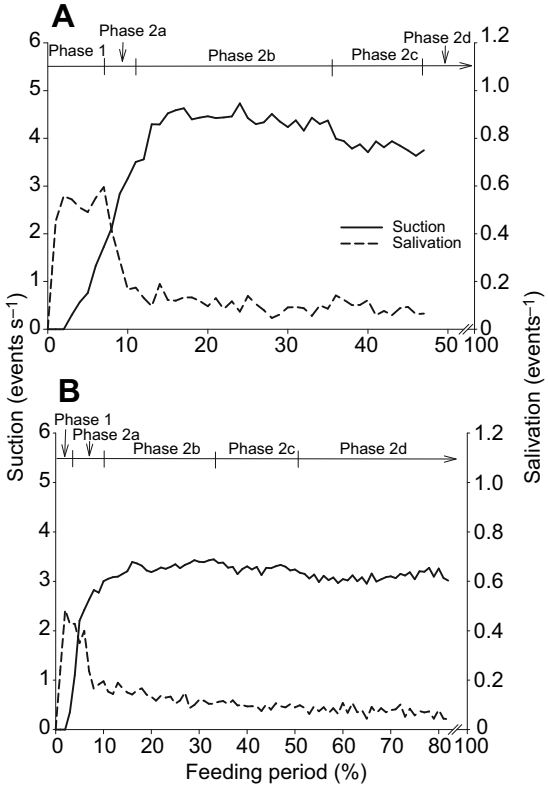

Fig. 4. Suction and salivation frequency during pool formation (phase 1) and different moments of engorgement (phase 2) of *O. rostratus* fourth instar nymphs blood feeding on mice. (A) Abdomen; (B) ear. Each point represents the mean frequency of suction/salivation in 1% intervals of the feeding period. *n*=8 and 4 for A and B, respectively.

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Table 2. Suction and salivation frequencies (means±s.d.) in each phase of the feeding of fourth instar nymphs of *O. rostratus* fed on mice

	Duration (% of TCT)			Suction frequency (Hz)			Salivation frequency (Hz)			Single feeding complex (s)		
Feeding phase	A	E	AE	A	E	AE	A	E	AE	A	E	AE
1	7.0±5.6	3.5±1.3	5.3±3.7	0.3±0.3	0.1±0.1	0.3±0.3	0.44±0.20	0.45±0.03	0.44±0.16	na	na	na
2a	4.0±4.4	6.6±5.1	5.3±5.4	2.5±0.7	2.2±0.7	2.3±0.7	0.54±0.09	0.21±0.06	0.33±0.18	1.9±0.4	4.7±1.2	3.1±1.8
2b	24.5±12.5	23.2±17.6	23.9±16.1	4.3±0.3	3.3±0.1	3.8±0.6	0.13±0.12	0.13±0.03	0.13±0.08	7.8±4.2	7.6±1.5	7.7±3.4
2c	11.0±9.4	17.3±6.5	14.1±10.4	3.8±0.1	3.3±0.1	3.5±0.3	0.07±0.02	0.10±0.01	0.09±0.02	13.9±3.2	10.3±1.2	11.5±4.9
2d	53.5±41.0	49.4±35.0	51.5±38.1	na	3.1±0.1	3.1±0.1*	na	0.07±0.02	0.07±0.02*	na	14.5±5.3	14.5±5.3*
n	8	4	12	8	4	12	8	4	12	8	4	12

TCT, total contact time; A, abdomen; E, ear; AE, abdomen+ear; na, not applied.
*Values based on image analysis until 83% of the feeding period had elapsed; *n*=4.

Phase 2c

This phase lasted for approximately 14.1% of TCT (Table 2) and presented a suction electrical profile similar to the ones seen in the second phase (Fig. 2E–H). The average suction frequency (Hz) decreased or remained relatively constant over time in ticks fed on the mouse's abdomen or ear, respectively (Table 2, Fig. 4). Salivation frequency was lower (0.09 Hz; Table 2) than suction frequency and a single feeding complex lasted longer (11.5 s on average) than in phases 2a and 2b. Over time, suction electrical signals became increasingly irregular, ending with the occurrence of electrical signals with no pattern for at least 10 s.

Phase 2d

The final phase of engorgement was the longest, corresponding to 51.5% of the TCT (Table 2). Captured electrical signals were extremely irregular (Fig. 2I–L) and therefore suction and salivation frequencies could not be quantified using an electromyogram alone (Fig. 4A). Image analysis under intravital microscopy indicated that suction and salivation frequencies were 3.1 and 0.07 Hz, respectively, with single feeding complexes lasting, on average, 14.5 s (Table 2). Frequencies were calculated during phase 2d only until 83% of the feeding period had elapsed. After that, the excess of blood at the feeding site (Fig. 1G,H, Fig. 5) made it impossible to measure the feeding frequency using image analysis. This phase ended with the withdrawal of the mouthparts from the host's skin.

Feeding pool and feeding efficiency

The size of the formed feeding pool throughout the feeding process was also analyzed. It was absent during the first feeding phase (Fig. 1A,B) and was formed at the beginning of the engorgement phase (Fig. 1C). The feeding pool area increased slowly, and up to 3.8 times its initial size (Fig. 5) throughout feeding, until approximately the last 17% of the feeding period, when an intense bleeding occurred (Fig. 1G,H, Fig. 5, Movie 1). Bleeding continued even after the removal of the mouthparts from the host's skin.

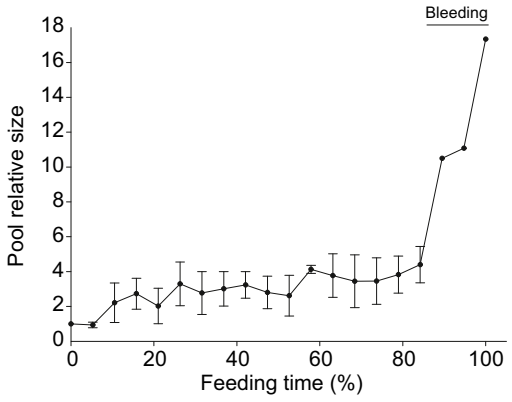

Fig. 5. Relative size of the feeding pool at different moments during *O. rostratus* fourth instar nymphs blood feeding on mouse ear. The size of the pool at the beginning of feeding was conventionally set to 1. *n*=4.

When comparing feeding sites, ticks that fed on the ear had a poorer feeding efficiency as they needed a significantly longer TCT ($P<0.05$) to ingest similar amounts of blood in comparison to ticks that fed on the abdomen (Table 1). These ticks also had lower suction frequencies during all feeding phases and presented a smaller variation in suction frequency between phases 2b and 2c (Table 2, Fig. 4) than those that fed on the abdomen.

DISCUSSION

When used together, electromyography and intravital microscopy are complementary techniques that promote a significant improvement in several aspects of arthropod blood-feeding analysis. Using these techniques, we were able to acquire a detailed description of the events that occurred during *O. rostratus*' blood feeding and determine how often different events occurred along the feeding process.

The feeding parameters (e.g. weight gain, TCT, ingestion rate) observed for *O. rostratus* fourth instar nymphs fed on mouse abdomen were similar to those observed in previous reports (Costa et al., 2015; Ribeiro et al., 2013). *Ornithodoros rostratus* was more efficient when feeding on the abdomen of mice than on their ears. Although ticks are pool feeders, such differences are in line with findings previously reported for the vessel feeders *Cimex lectularius* and *Rhodnius prolixus*, suggesting that for these insects, blood feeding from a mouse's ventral surface or lateral tail vein is more effective than feeding from its dorsal surface (Araujo et al., 2009a,b). These differences can be attributed to the size of the blood vessels located in those body parts, which directly reflects the volume of blood available for the arthropod to ingest. The external surface of a mice ears were used as a feeding ground for *O. rostratus* nymphs in the intravital experiments as it was more suitable, having a translucent flat surface with easily accessible vessels, without the need for disrupting the skin's integrity (Bockenstedt et al., 2014).

During image analysis, four events were identified throughout feeding (suction, salivation, chelicerae movements and inactivity) and their electrical signal profiles were characterized. Suction was the most common event. It started at pool formation (phase 1b) and occurred more intensely throughout engorgement (phase 2). The typical electrical signal during suction is a single spike downwards that indicates the contraction of the pump muscle bundles and blood filling/emptying into/out of the suction pump chamber (Araujo et al., 2011). Suction also generated electrical signals with different shapes such as double peaked spikes and voltage variations with no pattern. During suction in triatomine bugs, when the pump is

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operating without difficulty, electrical signals exhibit spikes with regular shapes (single peaks) (Araujo et al., 2009b; Soares et al., 2014). Irregular shaped spikes may indicate a lack of synchrony between the muscle bundles responsible for operating the suction pump. Double peaks may form because different muscle bundles contract asynchronously. In *Rhipicephalus microplus*, Tatchell et al. (1971) described secondary or subsidiary spikes on the leading edge of the suction signal that represents the opening of the pharyngeal valve. These signals were not observed in the present study.

Salivation was another very common event. Electromyograms revealed salivation signals characterized by a sudden increase in voltage (with different amplitudes) followed by a return to the baseline. Although electrical signals during salivation had different shapes, these differences could not be explained, as image analysis showed similar events. These observations differed from those presented by Sweatman and Gregson (1970) in a study on the ixodid *Hyalomma aegyptium*. They found that different electrical signals were associated with different actions of the salivary apparatus during blood feeding, including ejection of saliva, a flexing and an inactive open salivarium. These results may indicate a different mechanism between ixodid and argasid tick salivation, as argasids need a much higher salivation frequency owing to their shorter contact time with their hosts and faster blood ingestion.

Inactivity could be seen at different moments of feeding, but always for very short periods. The inactivity period during blood feeding varies amongst bloodsucking arthropods. In triatomine bugs, inactivity periods are rare and, when present, they occur for short periods throughout the engorgement phase (Araujo et al., 2009b; Sant'Anna et al., 2001). Ixodid ticks have various inactivity periods (also named resting periods), which can last several minutes (Gregson, 1969; Sweatman and Gregson, 1970; Tatchell et al., 1971). In argasids, Lavoipierre and Riek (1955) reported long periods of inactivity at the beginning of feeding (mainly for *Ornithodoros moubata* and *O. tholozani* var. *typicus*) that occurred shortly after the bite (especially after the salivation), whereas Zheng et al. (2015) did not comment about inactivity periods of *O. turicata* feeding on artificial feeders with cattle blood. In the present study, as observed for most blood-feeding insects, inactivity during feeding was very rare and short.

The overall description of blood feeding by *O. rostratus* enabled the identification of two distinct phases: pool formation and engorgement. Blood feeding begins with contact with the host followed by the insertion of the tick's mouthparts through chelicerae movements, with salivation. Saliva is important for the inhibition of physiological reactions triggered by the host during pool formation such as hemostasis, inflammation and immune responses (Francischetti et al., 2009; Ribeiro, 1987).

In phase 1b, ticks made their first suction events, which were combined with salivation and chelicerae movements. At this point, there is apparently no blood ingestion as the feeding pool is still clear and does not have the reddish color indicating the presence of blood. It is possible that these early suction events aim to evaluate the quality of the feeding pool, in order to determine whether the feeding process should proceed to the engorgement phase. Similar events are seen in the triatomine species *Rhodnius prolixus*, which presents suction events during the probing phase in an attempt to evaluate the nutritional value of the blood at the feeding site (Friend and Smith, 1971).

Over time, chelicerae movements are always linked with salivation and decrease in frequency while suction events increase until they become continuous. At this point, the engorgement phase begins and remains until the end of feeding. Chelicerae movements were not observed during the engorgement phase. However, Lavoipierre and Riek (1955) reported occasional chelicerae movements after the beginning of blood suction in argasid ticks.

During engorgement, the suction pump is intensely active to allow blood flow into the arthropod's gut; blood intake occurs by repeated feeding complexes. The engorgement by feeding complexes is a finding previously reported by several authors studying blood feeding by argasid and ixodid ticks (Lavoipierre and Riek, 1955; Sweatman and Gregson, 1970; Tatchell et al., 1971). However, while the feeding complex identified here has only suction and salivation periods, other authors have also identified resting periods as part of the single feeding complex. In a study looking at *O. moubata* and *O. tholozani* feeding on rats, Lavoipierre and Riek (1955) reported short resting periods at the beginning of the blood feed which got progressively longer throughout, but the length of these resting periods were not measured. In ixodid ticks, the duration of the resting period is considerably longer (from 22 s to over 1 min) (Sweatman and Gregson, 1970) and resting times can represent up to 96% of a single feeding complex (Tatchell et al., 1971). The duration of feeding complexes can be influenced by several factors such as period of daylight and darkness, female size, host and diet temperature, and diet composition, among others (Gregson, 1969; Sweatman and Gregson, 1970; Tatchell et al., 1971; Waladde et al., 1979).

Different patterns of electrical signals during suction were observed in the present study and, as stated above, these signals may reflect the effort to ingest blood at each feeding phase. Phase 2a presented signals with a Type 2 pattern and intense salivation, which is suggestive of difficulties during blood suction. At this point, it is likely that difficulty occurs because the feeding pool is not in a condition that is conducive for blood intake, perhaps reflecting a high blood viscosity. In insects, viscous diets significantly increase difficulty in suction, thus reducing pump suction frequency and the average volume of blood ingested per stroke (Smith, 1979). Salivation usually occurs at a higher frequency in this phase to inhibit the host's hemostasis and increase the fluidity of the diet.

Phase 2b represents the time during feeding when suction can be performed at higher frequencies and the pharyngeal pump works, without any major difficulty, generating electrical signals with a Type 1 pattern. This suction pattern indicates that the pool is fluid and is being supplied by a large volume of liquid from surrounding tissues. It probably represents the time when *O. rostratus* ingests larger quantities of blood.

In phase 2c, the presence of spikes with irregular shapes (Type 2 pattern) suggests that the tick experiences some difficulty during feeding. Difficulty may arise when pumping blood into the digestive system, mainly owing to an increased blood volume inside their gut and cuticle expansion that might be causing reverse pressure, therefore reducing blood intake (Guarneri et al., 2000). Secretion of coxal fluids may also affect contact with the electrodes. In this study, two out of 12 ticks were found to secrete coxal fluids during feeding in phase 2c. However, the visualization of secreted coxal fluid did not coincide with the beginning of phase 2c, suggesting that other phenomena may be occurring or inconspicuous small amounts of secreted coxal fluid altered the electrical signals during suction.

The lack of a pattern in electrical signals observed in the second half of the engorgement phase (phase 2d) onwards may represent an increased interference in those signals or more difficulties faced by the ticks to pump blood into their digestive system. During phase 2d, we observed that ticks ingested considerable amounts of blood;

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thus, any disturbance would trigger the end of feeding. This was also discussed by Lavoipierre and Riek (1955), who concluded that ticks that were filled up with blood were easily bothered. When ticks are feeding on anesthetized hosts with no apparent disturbances, they keep in contact with the host even if they have ingested enough blood to molt to the next stage. In nature, where ticks feed on active hosts, this phase probably would be considerably shorter than that under laboratory conditions, and the end of the meal would occur earlier. Moreover, *O. rostratus* is known to have a painful and itchy bite (Aragão, 1936; Estrada-Pena and Jongejan, 1999), which increases its chances of being perceived by its host. As a result, these ticks have probably evolved to feed for shorter periods.

The quality of the feeding pool seems to be of utmost importance for efficient blood pumping. After the pool is formed, it grows slowly until approximately 83% of the feeding time has elapsed, when blood starts to accumulate around the bite site, probably because suction has stopped at this point and there is a continuous leakage of blood from the host's tissues. The blood leakage to the feeding pool is probably assisted by anti-hemostatic molecules present in the tick's saliva (Francischetti et al., 2009; Ribeiro, 1987).

In a study by Zheng et al. (2015) with *O. turicata*, the electrical signals recorded throughout feeding were very similar to those recorded here for *O. rostratus*. The signals interpreted by Zheng et al. (2015) as penetration of the mouthparts and salivation coincide in timing and profile to those in this study called 'chelicerae movements with salivation'. The higher suction frequency in *O. turicata* (>6 Hz) and lack of salivation signals during the engorgement phase (Zheng et al., 2015) could be a consequence of feeding on an artificial system where host hemostasis is absent and blood is always presented in a suitably low viscosity. Zheng et al. (2015) did not discuss the different suction profiles throughout feeding.

This is the first work to provide a wide-range analysis of the feeding pattern of *O. rostratus* on live hosts. This study presents new information on the feeding events and their frequency of occurrence. Similar analyses based on intravital microscopy coupled with electromyography need to be applied to other arthropod species in the future to evaluate parameters that may influence their feeding performance on vertebrate hosts.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
R.N.A. and M.H.P. designed the study; G.C.A.C. and A.C.S. carried out the intravital microscopy and electromyogram experiments; R.N.A., G.C.A.C. and A.C.S. carried out the graph construction, figure preparation and wrote the manuscript; all authors contributed to the data analyses, discussion of results and manuscript revision.

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Data availability
Raw data will be made available upon request.

Supplementary information
Supplementary information available online at
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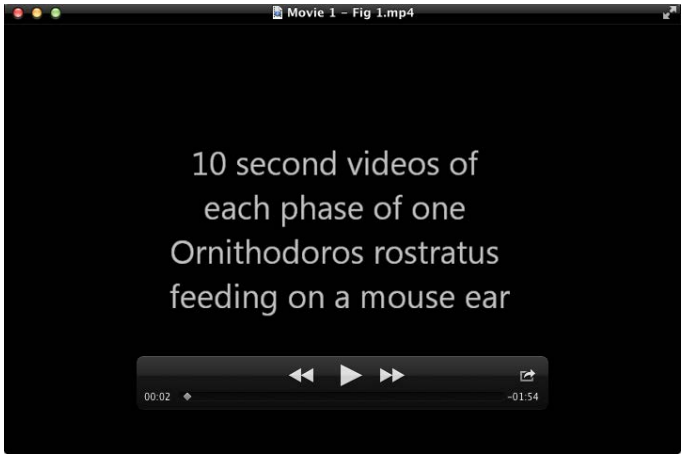
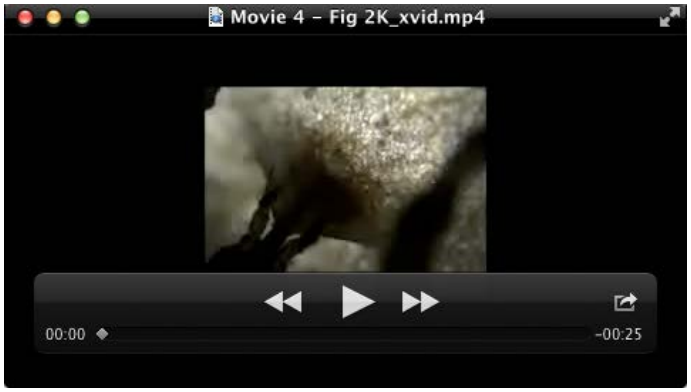


Movie 1. Images of bite site and feeding pool of one specimen at different moments during *Ornithodoros rostratus* blood feed. Movie shows 10 second videos of different moments of phase 1 and 2.

Movie 2. Variation in size of the feeding pool during phase 2b of *Ornithodoros rostratus* engorgement on mice. Video shows images represented in figure 2C.

Movie 3. Variation in size of the feeding pool during phase 2a of *Ornithodoros rostratus* engorgement on mice. Video shows images represented in figure 2G.



Movie 4. Variation in size of the feeding pool during phase 2d of *Ornithodoros rostratus* engorgement on mice. Video shows images represented in figure 2K.

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Movie 5. Images of the bite site during phase 1a of *Ornithodoros rostratus* feeding on mice. Video shows images represented in figure 3A.

Movie 6. Images of the bite site during phase 1b of *Ornithodoros rostratus* feeding on mice. Video shows images represented in figure 3D.