Ticks and Tick-borne Diseases 10 (2019) 470–481

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Wonders of tick saliva

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ARTICLE INFO

Keywords:
Saliva
Anti-hemostatic
Immunomodulator
Individuality
Mate guarding
Saliva-assisted transmission
Redundancy

ABSTRACT

Saliva of ticks is arguably the most complex saliva of any animal. This is particularly the case for ixodid species that feed for many days firmly attached to the same skin site of their obliging host. Sequencing and spectrometry technologies combined with bioinformatics are enumerating ingredients in the saliva cocktail. The dynamic and expanding saliva recipe is helping decipher the wonderous activities of tick saliva, revealing how ticks stealthily hide from their hosts while satisfying their gluttony and sharing their individual resources. This review takes a tick perspective on the composition and functions of tick saliva, covering water balance, gasket and holdfast, control of host responses, dynamics, individuality, mate guarding, saliva-assisted transmission, and redundancy. It highlights areas sometimes overlooked – feeding aggregation and sharing of sialomes, and the contribution of salivary gland storage granules – and questions whether the huge diversity of tick saliva molecules is 'redundant' or more a reflection on the enormous adaptability wonderous saliva confers on ticks.

1. Composition of tick saliva

Tick saliva is a fluid secretion injected from the salivary glands of ticks into the feeding site where the tick attaches on a vertebrate host. Given the numerous functions of tick saliva (Section 2), not surprisingly the chemical composition of tick saliva is highly complex (Table 1). The complexity is produced by the pair of relatively large and intricate salivary glands formed by groups of cells in grape-like clusters (known as acini) attached to salivary ducts (Kemp et al., 1982) (Fig. 1). Three acini types - I, II, and III - are found in ixodid tick salivary glands and an additional type IV acinus is found in males. Argasid salivary glands comprise two acini types - A and B (or I and II). Constituents of tick saliva derive from three sources: (i) *de novo* production in the salivary gland acini during feeding, (ii) salivary gland products stored as granules in specific acini, and (iii) components in tick haemolymph that pass across the salivary gland wall into the salivary duct.

Most of saliva is water derived from the bloodmeal. As 50% of the ixodid bloodmeal is taken up during the last 12–24 h of feeding, the volume of water excreted (and hence the volume of saliva) is greatest during the final days of engorgement (Kaufman, 1989). Total volume of secreted saliva for large tick species can easily exceed 1 ml (Kaufman, 2010). Just as it is vital ticks excrete water during blood-feeding, so it is important the balance of ions is maintained. Again, this is achieved by ixodid ticks actively excreting via their hyposmotic saliva about 70% of the water and ions taken up in their bloodmeal (Bowman et al., 2008). In argasid ticks, the excretory role of tick saliva is replaced by coxal fluid (Frayha et al., 1974).

Bioactivity of tick saliva is provided by a mixture of proteins, peptides, and non-peptidic molecules (Table 1). Non-peptidic molecules include the endogenous nucleoside, adenosine, identified at concentrations of ~110 pmol/μl in saliva of female *Rhipicephalus sanguineus* harvested at 5–7 days of feeding on dogs, together with the arachidonic acid derivative, prostaglandin PGE_2 (Oliveira et al., 2011). Prostaglandins $F_{2\alpha}$, I_2 (prostacyclin), D_2 and A_2/B_2 have also been identified in tick saliva. Salivary gland prostaglandins are synthesised from dietary arachidonic acid, achieving remarkably high concentrations in saliva (of some tick species) compared with host inflammatory exudate (Bowman et al., 1996). Prostaglandin A_2/B_2 is thought to be largely derived from PGE_2 because of the alkaline nature (pH ~9.5) of tick saliva. Endocannabinoids are also arachidonic acid derivatives and have been found in saliva of *Amblyomma americanum* (Fezza et al., 2003). microRNA (miRNA) has been detected in the salivary glands of several ixodid species. In the saliva of *Ixodes ricinus*, miRNAs carry modifications consistent with an exosomal origin (Hackenberg et al., 2017).

Most proteins and peptides secreted in tick saliva (the 'sialome') are synthesised in the salivary glands during feeding. Currently, ~70,000 protein sequences deposited on GenBank are assigned to tick salivary glands (Ribeiro et al., 2017). Transcriptomic studies indicate ticks of a given species may secrete > 500 different proteins and peptides in their saliva over the course of feeding (Giovanni et al., 2014; Esteves et al., 2017; Perner et al., 2018). These are grouped into several multigene

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https://doi.org/10.1016/j.ttbdis.2018.11.005
Received 11 June 2018; Received in revised form 31 October 2018; Accepted 9 November 2018
Available online 12 November 2018
1877-959X/

Table 1
Composition of tick saliva.

Constituent	Examples
water	(from host bloodmeal)
ions	(from host bloodmeal)
non-peptidic molecules	adenosine, prostaglandins, endocannabinoids, microRNAs
tick peptides	variegins, hyalomins, madanins
tick proteins	chitinases, mucins, ixostatins, cystatins, defensins, glycine-rich, hyaluronidases, Kunitz-type, lipocalins, metalloproteases
host proteins	immunoglobulins, haptoglobin, transferrin
exosomes	may contain miRNA, mRNA, peptides, proteins
known unknowns	proteome/transcriptome unknowns
unknown unknowns	(tick individualism, skin immune system)

families, including lipocalins, Kunitz-domain containing proteins/peptides, metalloproteases, and basic tail secreted proteins, and several families apparently unique to ticks. Similar protein families are found in ixodid and argasid ticks, and in *Nuttalliella namaqua*, suggesting they were present in the ancestral tick lineage (Mans et al., 2016). Reviews on the discovery of proteins in tick saliva and their characterisation include: Steen et al., 2006; Murfin and Fikrig, 2017; Šimo et al., 2017. Many studies involve tick feeding on laboratory hosts that the particular tick species would not encounter naturally, hence limiting interpretation of the results as host species affects expression of saliva proteins and feeding success (Wang et al., 2001b; Anderson et al., 2017; Tirloni et al., 2017).

Proteomic analysis of saliva collected from female *A. variegatum* at 5–7 days of feeding on goats identified 336 proteins of which 236 were shared with other *Amblyomma* species and only 42 were shared with *I. scapularis* while 58 (17%) were uncharacterised proteins (Rodrigues et al., 2018). Saliva from female *Dermacentor andersoni* fed on cattle for 2 days and 5 days revealed 677 proteins of which 372 proteins were detected at both time points (Mudenda et al., 2014). Argasid tick saliva, from male and female *Ornithodoros moubata*, revealed 193 proteins with only 10 of the proteins common to both sexes (Díaz-Martín et al., 2013).

Relatively large amounts of host proteins are found in tick saliva (Oliveira et al., 2013). These host proteins are derived from the bloodmeal, passing through the midgut, haemocoel, and salivary glands to be excreted in saliva. Proteomic analysis of the saliva of *R. microplus* identified 68 bovine proteins (Tirloni et al., 2014). It is unclear why ticks go to such lengths to obtain blood only to inject large parts of it back into their host. Possibly, in the first days of feeding, ixodid ticks select for oligonutrients and lipids and excrete the rest although indications that ticks secrete modified host proteins suggest nefarious intentions (Oliveira et al., 2013).

Transcriptomic and proteomic studies also reveal extensive 'known unknowns' in the composition of tick saliva. For example, of the 677 proteins identified in *D. andersoni* saliva, 80% were of unknown function (Mudenda et al., 2014), and 16% of the reads from adult *Hyalomma excavatum* salivary glands mapped to tick-specific families of unknown function (Ribeiro et al., 2017). Tick genome sequencing, assembly, and annotation are in their infancy, which is a major constraint on data interpretation. In addition, there are most probably 'unknown unknowns' in tick saliva. For example, the hidden complexity within the skin immune system at the tick-host interface (Table 2) together with evidence each individual tick has its own unique salivary repertoire (Section 2.5), are likely to conceal further extraordinary facts about tick saliva. The possibility that saliva exosomes carry novel mediators into the tick-host interface, including miRNAs, and that saliva carries an extracellular ubiquitination mechanism, open further possibilities for as yet unknown unknowns (Díaz-Martín et al., 2013; Hackenberg and Kotsyfakis, 2018; Rodrigues et al., 2018). It is also interesting to consider how bloodsuckers other than ticks control haemostasis and inflammation, and the differences in strategies (Koh and Kini, 2009; Mans, 2011; Chmelař et al., 2012). Are we missing some bioactive tick saliva molecules or is there a reason why ticks have adopted their particular way of controlling their host?

2. Functions of tick saliva

Functional analysis of tick saliva constituents in relation to their host modulatory activity has been comprehensively reviewed (Kotál et al., 2015; Blisnick et al., 2017; Chmelař et al., 2017; Wikel, 2018). Table 3 provides a summary of the various activities of tick saliva and the principal mediators involved. This section aims to take a tick perspective on functionality.

2.1. Water balance

Female ixodid tick salivary glands act as osmoregulators, controlling the hydrostatic pressure within the body of the tick by excreting water and ions taken up in the bloodmeal (Bowman et al., 2008). In addition, 'rehydration saliva' is used to take up water vapour to prevent desiccation of unfed ticks (Needham and Teel, 1986; Kim et al., 2017). Considering that an unfed female *D. andersoni* weighing only 7–10 mg can process approximately 4000 mg of host blood during engorgement, failure to get rid of excess water and ions would be catastrophic (Kaufman, 1982). Hence tick saliva plays an essential role in the homeostasis of ticks.

2.2. Gasket and holdfast

Early observations on ixodid tick feeding reported secretion of a milky white fluid soon after insertion of the mouthparts into the skin (Gregson, 1960; Kemp et al., 1982). This quickly polymerises to form a solid cement cone that acts as a gasket preventing loss of any fluids, and a holdfast securing the mouthparts and enabling the tick to remain attached for many days. Several other functions have been attributed to the cement cone including an antibacterial role [reviewed by (Suppan et al., 2018)]. The observation bioactive saliva molecules may stick to the cement cone, e.g. complement inhibitory proteins (Miles Nunn, *pers com.*), raises the possibility that the cement plug provides a bolus-like function, concentrating bioactive tick saliva molecules at the feeding site.

Ixodid ticks produce cement cones that differ in size, shape and composition depending on species (Moorhouse, 1969; Kemp et al., 1982; Maruyama et al., 2010). Although the typical glycine-rich cement proteins are considered to be biologically inert and non-immunogenic, vaccination with the cement protein, 64TRP, protected mice from lethal challenge with tick-borne encephalitis virus-infected ticks (Labuda et al., 2006). Gene knockdown studies suggest some glycine-rich tick saliva proteins may have additional roles, eg. in embryo development (Leal et al., 2018). Besides its potential as an anti-tick and pathogen transmission modulating vaccine, the natural glue and sealant properties of tick cement suggest the potential for developing new medical adhesives (Suppan et al., 2018).

2.3. Control of host responses

The tick strategy of controlling host responses is highly efficient, maximising effect while minimising the use of finite resources. Tick saliva molecules are characterised by high affinity, avidity and selectivity for their targets, which are often at the apex of a host effector cascade rather than downstream. Several saliva constituents are multifunctional proteins. Multiple functionality provides opportunities for synergism although such effects are largely uncharacterised (Pischke et al., 2017). Although different barriers to tick attachment and feeding – pain induction, haemostasis, inflammation, immunity, and wound healing – are distinguished in this section, in reality they are intertwined creating a level of complexity akin to the 'unknown unknowns'

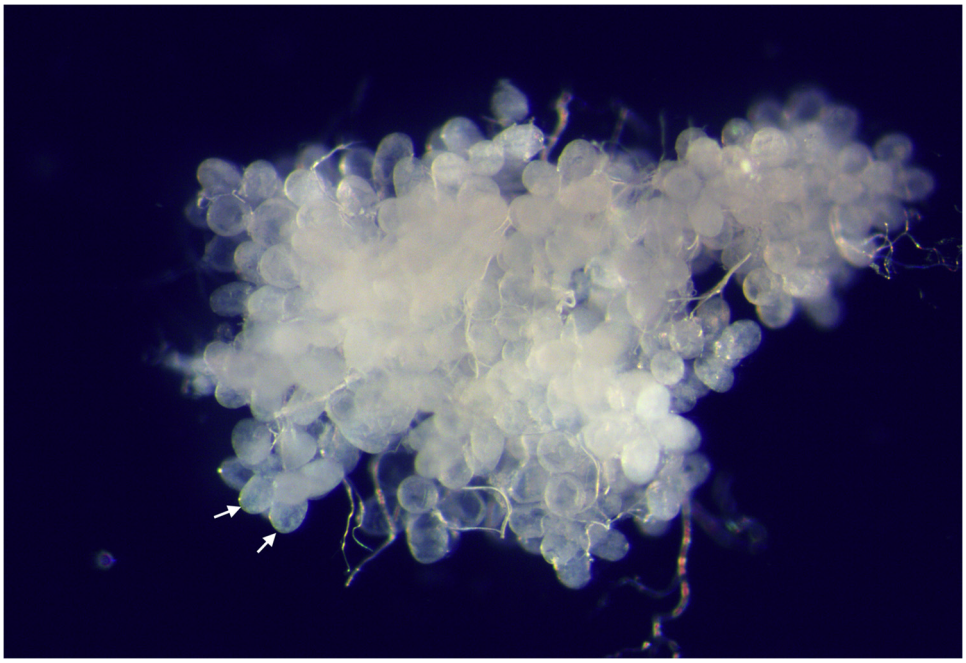

Fig. 1. Ixodid tick salivary glands. Salivary gland from unfed female *Ixodes ricinus*. Arrow indicates acinus. Photo: Ladislav Simo.

Table 2
Constituents of the skin immune system.

Humoral	Cellular
defensins, cathelicidins	keratinocytes
complement and complement regulatory proteins	epidermal Langerhans cells; 'classical' dermal dendritic cells (2 subsets); monocyte-derived dendritic cells[a]
mannose-binding lectins	dermal macrophages
immunoglobulins	granulocytes (neutrophils, basophils, eosinophils)[a]
cytokines, chemokines	mast cells
neuropeptides	vascular/lymphatic endothelial cells
eicosanoids and prostaglandins	T and B lymphocytes[b]
free radicals	

[a] Typically only in inflamed skin.
[b] In mouse, a resident population of gamma-delta T cells; in human, memory T cells; in mouse and human, effector T cells during immune response.

(Section 1).

2.3.1. Pain

Once a tick has found a suitable location to feed, it goes through an elaborate process of attachment. For ixodid species such as *I. ricinus*, this involves shearing the skin epidermis with the hooked chelicerae, burying the cheliceral bundle into the skin, and then replacing the cheliceral bundle with the barbed hypostome through ratchet-like actions of the chelicerae (Richter et al., 2013). Incredibly, this 'bite' of the tick usually goes unnoticed (compare the sharp pain elicited by the bite of a horsefly). Little is known of how the tick so effectively anaesthetises its host though a number of identified saliva molecules may play a role in countering the sensations of pain and itch (Table 3).

When skin is cut causing activation of coagulation factor XII (FXII), a cascade of interactions results in release of bradykinin, a pro-inflammatory nonapeptide hormone, which binds to nociceptors creating the pain sensation. Ixodid ticks destroy bradykinin by secreting angiotensin converting enzymes (ACE metalloproteases) (Ribeiro and Mather, 1998). Two ACEs, originally identified in the sialotranscriptome of *A. maculatum* and expressed in the first 120 h of feeding, were shown by gene knockdown to breakdown bradykinin and prevent host agitation to tick feeding on a sheep (Jelinski, 2016).

Endocannabinoids are lipid mediators that bind to and activate cannabinoid receptors found in vertebrates but not in most invertebrates. The finding of endocannabinoids and/or related fatty acid amides in saliva and salivary glands of partially fed female *A americanum* may provide another form of analgesic used by ixodid ticks to hide their presence (Fezza et al., 2003).

Other saliva constituents that may play a role in pain control are adenosine, which can have analgesic or pronociceptive effects depending on the activation of different peripheral receptors (Fontaine et al., 2011), and miRNA as predicted by *in silico* target analysis (Hackenberg et al., 2017).

Table 3
Bioactive tick saliva molecules.

Activity	Principal mediators[a]
gasket & holdfast	cement comprising glycine-rich proteins
analgesic	endocannabinoids, bradykinin inhibitors
anti-haemostatic	prostaglandins, apyrases, metalloproteases, disintegrins, thrombin inhibitors, FXa inhibitors, FVIIa/TF complex inhibitors, contact phase inhibitors, fibrinolysis modulators
anti-inflammatory	histamine and serotonin binding proteins, complement inhibitors
immunomodulatory	immunoglobulin-binding proteins, evasins, dendritic cell modulators
anti-wound healing	growth factor inhibitors
vasoconstriction	uncharacterised phenylalanine-rich peptide

[a] May have more than one activity e.g. tick histamine binders can be anti-haemostatic, anti-inflammatory and immunomodulatory.

2.3.2. Blood clotting

Haemostasis involves 3 major steps: 1) vasoconstriction, 2) temporary blockage of a break in a blood vessel by a platelet plug, and 3) blood coagulation resulting in formation of a fibrin clot. These processes seal the hole until tissues are repaired hence haemostasis is the first step of wound healing. Blood-feeding arthropods have evolved a wealth of anti-haemostatic saliva molecules none more so than ticks (Francischetti, 2010; Fontaine et al., 2011; Mans, 2011).

To counter vasoconstriction, ixodid ticks are thought to use non-peptidic saliva constituents, namely prostaglandins and adenosine (Dickinson et al., 1976; Kemp et al., 1983; Ribeiro et al., 1988; Oliveira et al., 2011). Given that vasoconstriction is one of the first host responses, countering vasoconstriction requires saliva molecules ready for action at the time of tick attachment. It remains to be determined whether adenosine and/or prostaglandins are present at physiologically effective concentrations when a tick first attaches to its host.

Specific tick saliva protein or peptide vasodilators have not been described though a number of characterised proteins may modulate vasoconstriction. These include the ATP-diphosphohydrolase, apyrase, which may counter vasoconstriction by destroying the agonist, ADP (Ribeiro, 1995). Serotonin is a vasoconstrictor hence serotonin-binding saliva proteins may play a role although serotonin is released from damaged cells and blood platelets and therefore is unlikely to be the initial saliva target for countering vasoconstriction (Sangamnatdej et al., 2002). Likewise, histamine is a vasoactive amine hence histamine-binding proteins and histamine-release factor may modulate vascular permeability and increase blood flow (Paesen et al., 1999; Dai et al., 2010). An inhibitor of chymase and cathepsin G, *I. ricinus* serpin-2 (IRS-2), through inhibition of chymase-mediated endothelin processing, may modulate vasoconstriction by indirectly controlling production of potent vasoconstrictors (Chmelař et al., 2011). Argasid ticks (*Ornithodoros* spp.) may target vasoconstriction using lipocalins that scavenge thromboxane A_2, a function that appears ancestral within the moubatin clade of lipocalins (Mans and Ribeiro, 2008). The apparent absence of moubatin-like homologues in *Argas* spp. is consistent with their feeding mostly on birds which lack blood platelets, the primary source of thromboxane.

Besides vasodilator activity, vasoconstriction has been shown with salivary gland extracts from *D. reticulatus* and *R. appendiculatus*, which is not attributable to prostaglandins (vasoconstrictive at high concentrations) but instead may involve a phenylalanine-rich peptide (Pekáriková et al., 2015). Vasoconstrictive activity was detected during the later stages of engorgement and may be a mechanism of controlling blood flow during the rapid feeding phase when female ixodid species take up $\geq$50% of their bloodmeal and/or during detachment.

In contrast to vasoconstriction, lots of tick saliva molecules have been shown to control platelet activation and platelet aggregation (Francischetti, 2010; Chmelař et al., 2012). Activation of blood platelets arises from multiple pathways activated by the tick bite (Yun et al., 2016). Activation leads to loose platelet aggregation as activated platelets adhere to extracellular matrix (via von Willebrand factor, vWF, and exposed collagen), change shape, and de-granulate. Bound fibrinogen or vWF cross-links platelets forming platelet aggregates. Most soluble agonists released by activated platelets (e.g. ADP, thromboxane A_2, and thrombin) trigger platelet activation through G protein coupled receptors, promoting plug formation. Thrombin is the strongest platelet agonist besides converting fibrinogen to fibrin to stabilize the platelet plug. All the platelet signalling events converge upon the final common pathway of platelet activation, the functional upregulation of integrin adhesion receptors, particularly activation of the glycoprotein GP IIb/IIIa receptor. Ticks target many of these steps in platelet activation and aggregation (Table 3).

Apyrases are possibly ubiquitous in argasid and ixodid ticks, enzymatically breaking down ADP released by activated platelets and damaged cells, and consequently neutralising a major agonist of platelet aggregation (Francischetti, 2010). Likewise, thrombin inhibitors are found widely among ticks; they take a variety of forms and may have different roles targeting platelet aggregation and/or blood coagulation (see below). In transcriptomic studies, a relatively large number of transcripts have been identified as disintegrin-like proteins. Typically, these have an arginine-glycine-aspartate (RGD) motif that binds to the αIIbβ3 integrin on platelets, preventing aggregation. At least four families of integrin inhibitors have been identified including a platelet aggregation inhibitor that blocks GP IIb/IIIa (Tang et al., 2015). Platelet activation results in platelet degranulation which releases serotonin and epinephrine. Although individually, these are weak platelet antagonists, when acting synergistically they are comparable to ADP in promoting platelet aggregation (Francischetti, 2010). However, although ticks produce serotonin binders, as yet there is no evidence ticks control epinephrine although an insect bloodsucker, *Rhodnius prolixus*, has evolved a saliva molecule that targets both biogenic amines (Andersen et al., 2003). Ticks (like other bloodsuckers) do not appear to target constitutively expressed receptors (e.g. protease activated receptors, PARs) or plasma proteins that occur at high concentrations (e.g. vWF) despite their critical roles in haemostasis. This conservative strategy contrasts with profligate snake venom toxins that bind vWF and other high concentration plasma proteins (Francischetti, 2010).

Formation of the platelet plug is the primary phase of haemostasis; the secondary phase is one of consolidation to form the fibrin plug. During the secondary phase, the platelet plug provides a surface for assembling activated coagulation factors, leading to the formation of a fibrin-stabilised platelet aggregate and thrombus of platelets and erythrocytes. Coagulation factors are core components of the coagulation cascade of reactions resulting in conversion of soluble fibrinogen into insoluble fibrin strands. The most powerful activator of the coagulation system is tissue factor (FIII). Most clotting factors are precursors of proteolytic enzymes (zymogens) that circulate in an inactive form; activation is depicted by the suffix 'a' e.g. activated factor X is designated FXa. An anticoagulant system exerts a regulatory role over procoagulant activity in blood thus localizing the thrombus formation. Traditionally, the coagulation cascade is classified into the extrinsic pathway and the intrinsic pathway. However, the intrinsic pathway is not a parallel pathway but instead augments thrombin generation primarily initiated by the extrinsic pathway. Currently, coagulation is perceived as following steps: initiation, amplification, propagation, and stabilization (Palta et al., 2014).

The largest group of tick saliva anticoagulants are serine protease inhibitors that target thrombin, FXa, and other proteases in the initiation step (Blisnick et al., 2017; Chmelař et al., 2017). Thrombin (FIIa) is the linchpin of coagulation and, not surprisingly, a major target for tick saliva. Many thrombin inhibitors of ixodid and argasid ticks belong to an expanded family of proteins containing one or more Kunitz domains with structural modifications that specify their activity. For example, ornithodorin (which has two Kunitz domains), from *O. moubata*, binds to thrombin active site through its N terminus while the C-terminal helix binds to the fibrinogen recognition exosite I of thrombin, making it a potent and highly selective thrombin inhibitor (van de Locht et al., 1996). The comparative complexity of the Kunitz-type proteins contrasts with the relative simplicity of the peptide thrombin inhibitors. For example, variegin isolated from salivary gland extracts of *A. variegatum*, comprises only 32 residues and yet it interacts with the thrombin active site and exosite I (Cho et al., 2007). Attempts to enhance the potency of variegin showed that sulphation of the tyrosine residue consistently increased the IC_{50} and K_i values of the synthesised variegin variants (Koh et al., 2011). Although tyrosine is not sulphated in native variegin, tyrosine sulphation is present in madanin-1 and chimadanin, two saliva-derived thrombin inhibitors of *Haemaphysalis longicornis* (Thompson et al., 2017). Given that tyrosine sulphation is a natural post-translational modification in eukaryotes, consideration needs to be given to sulphation when producing tick saliva peptides and proteins by synthesis or using bacterial expression systems. Using primers derived from the variegin sequence, a precursor protein was

amplified from a cDNA library of *A. variegatum* salivary glands. This precursor comprised a signal peptide and multiple short peptides, one of which, named avathrin, showed potent thrombin activity (Iyer et al., 2017). Such precursor proteins offer potent inhibition of coagulation over prolonged feeding periods (up to 12 days for *A. variegatum*), minimising the cost of protein synthesis and possibly circumventing immune responses while tuning the inhibitor to suit the feeding conditions.

Factor Xa is involved in converting prothrombin to thrombin. The first protease inhibitor identified in tick saliva, tick anticoagulant protein (TAP), is a specific FXa inhibitor from *O. moubata* and belongs to the Kunitz-type family (Waxman et al., 1990). Interestingly, characterisation of an *I. scapularis* saliva protein, 'Tick Inhibitor of FXa towards FV' (TIX-5) provided a new insight into initiation of thrombin generation by showing FXa activates FV (Schuijt et al., 2013). Another *I. scapularis* saliva protein, Salp14, is a specific FXa inhibitor (Narasimhan et al., 2002). Presumably, this basic tail secreted protein works in conjunction with TIX-5, together with ixolaris, a Kunitz-domain protein from the same tick species that binds to the FVIIa/tissue factor complex, the activator of FX (Francischetti et al., 2002). This multi-targeted approach of an ixodid tick to controlling the initiation of blood coagulation is remarkable and demonstrates the tick strategy of hitting the early steps of a cascade of host reactions. As if this was not enough, ixonnexin, a basic tail peptide from *I. scapularis*, promotes fibrinolysis through the host anticoagulation control system (Assumpção et al., 2018).

The coagulation system and the innate inflammatory response share a common ancestry. They are coupled via common activation pathways and feedback regulation systems such that coagulation triggers inflammatory reactions and inflammation triggers the activation of the coagulation system (Verhamme and Hoylaerts, 2009). Although tick saliva contains specific modulators of haemostasis, many saliva proteins in the same family target host mediators of inflammation; some are multi-functional and do both (Chmelař et al., 2017; Schwarz et al., 2014).

2.3.3. Inflammation

The skin is considered the largest organ of mammals. Besides its role as a physical barrier, the skin acts as a pro-inflammatory organ (Barker, 2005) (Table 2). Epidermal keratinocytes, which comprise 95% of the mass of human epidermis, maintain the barrier and also participate in inflammatory/immune responses through activation of Toll-like receptor 3 (TLR3), for example, and production of cytokines (Heath and Carbone, 2013). Specific interactions between keratinocytes and ticks are largely unexplored except in the context of tick-borne pathogen transmission (Section 2.7).

Mature mast cells are abundant in skin and provide a first line of defence against ticks (Allen et al., 1977). Their cytoplasm is stuffed with electron-dense lysosome-like secretory granules which are filled with an array of preformed compounds, many pro-inflammatory (Wernersson and Pejler, 2014). Tick attachment activates mast cells, causing degranulation and release of granule contents into the extracellular environment (Fig. 2a) and invoking *de novo* synthesis of more bioactive compounds. Ticks neutralise these inflammatory mediators using, for example, lipocalins to mop up histamine and serotonin, and a Kunitz-type protein to block the catalytic activity of tryptase, a mast cell specific protease. Some of these saliva proteins may even stabilise mast cells, preventing degranulation and *de novo* synthesis, thereby efficiently controlling an important host defence (Paesen et al., 1999, 2007; Sangamnatdej et al., 2002). Sialostatin L, targets IRF-4-dependent transcription in murine mast cells resulting in suppression of interleukin-9 (IL-9) production, a primary innate source of this pleiotropic cytokine (Klein et al., 2015). Although the *Ixodes*-secreted cystatin did not affect mast cell degranulation, it had a profound effect on autocrine IL-1β production, a potent costimulator of IL-9 production.

Pro-inflammatory cytokines, such as GM-CSF, IL-1, IL-6, tumour necrosis factor (TNF), and CXCL8 chemokine, are produced by resident skin cells including keratinocytes, fibroblasts (the major cell type in skin dermis), mast cells, Langerhans and other dendritic cells, macrophages, and T lymphocytes. Ticks directly inhibit cytokines by capturing the ligand; the nature of these cytokine inhibitors is known only for the chemokine binders known as evasins (Hajnická et al., 2005; Hayward et al., 2017). A saliva peptide, amregulin, from *A. variegatum*, inhibits secretion of TNFα, IL-1, IL-8 and interferon γ by rat splenocytes and may be a tick model for a pro-inflammatory cytokine inhibitor (Tian et al., 2016).

One of the most potent triggers of inflammation is the complement system. Approximately 40 proteins (soluble or membrane-bound) comprise the three complement pathways – classical, lectin, and alternative (Sarma and Ward, 2011). Almost all plasma complement proteins are synthesised in the liver. The skin epidermis is avascular; in the dermis, passage of large plasma proteins > 40 kD from blood into skin tissue is restricted by endothelial cells and the underlying basement membrane. During inflammation, the absence of plasma complement in extravascular skin tissue is compensated by differentially upregulated synthesis of complement proteins, mainly by keratinocytes and fibroblasts (Asghar et al., 2005). Thus, complement becomes available when the tick bites. The importance of complement for ticks is illustrated by the number and diversity of complement inhibitors in tick saliva (Jore et al., 2016; Šimo et al., 2017; Perner et al., 2018). In the salivary glands of *I. ricinus*, anti-complement activity was detected at all days of feeding and in salivary glands of unfed adult females, suggesting complement inhibitors are prepared before feeding commences and presumably stored within granular acini (Lawrie et al., 1999).

2.3.4. Immunity

Ticks are masters of controlling their host's immune response. They produce an awesome repertoire of immunomodulators targeting innate and acquired immune elements, both acellular and cellular (Kotál et al., 2015; Šimo et al., 2017; Wikel, 2018). The pressure to control immunity is illustrated by the effects observed when *R. appendiculatus* nymphs were fed on guinea pigs immunised with 64TRP, a protein derived from the tick's cement cone that cross reacts with epitopes in the midgut (Trimnell et al., 2002). Immunised animals showed a pronounced inflammatory response in the skin around the feeding site (Fig. 3). Adult female *R. appendiculatus* fed on 64TRP-immunised guinea pigs had difficulty feeding; those that engorged became solid, blackened and died consistent with antibody + complement-mediated rupture of the midgut observed in *R. microplus* ticks fed on cattle immunised with Bm86, a tick midgut antigen (Rand et al., 1989). The observations using 64TRP as a vaccine antigen indicate that when the tick host has immunological memory of a tick saliva protein, the host creates a hostile environment in the skin that impedes/deters feeding (Fig. 2) and that specific antibody (probably in combination with complement) can be lethal to ticks.

The major acellular component of skin immunity is the cytokine family, a diverse group of small extracellular proteins that includes chemokines and growth factors. Cytokines orchestrate the immune response through binding to specific cell receptors; ticks control cytokines by outcompeting with the cell signalling receptors. As there is often more than one receptor per cytokine, the tick strategy of secreting cytokine-binding proteins is more cost effective than targeting multiple cytokine receptors, especially when considering the host diversity of a tick species. Tick-derived cytokine binders of chemokines are called evasins and they appear to be unique to ixodid ticks. At least 265 putative evasins have been identified from *Rhipicephalus*, *Amblyomma*, *Dermacentor*, and *Ixodes* spp. (Hayward et al., 2017). Evidently, the long duration of ixodid tick feeding requires ruthless control of chemokine activity whereas the relatively quick feeding argasids do not. In skin, chemokines are produced by keratinocytes, mast cells, and endothelial cells, as well as dendritic cells, in order to recruit discrete T-cell subsets and other leukocytes to the site of tick feeding. The targets of specific

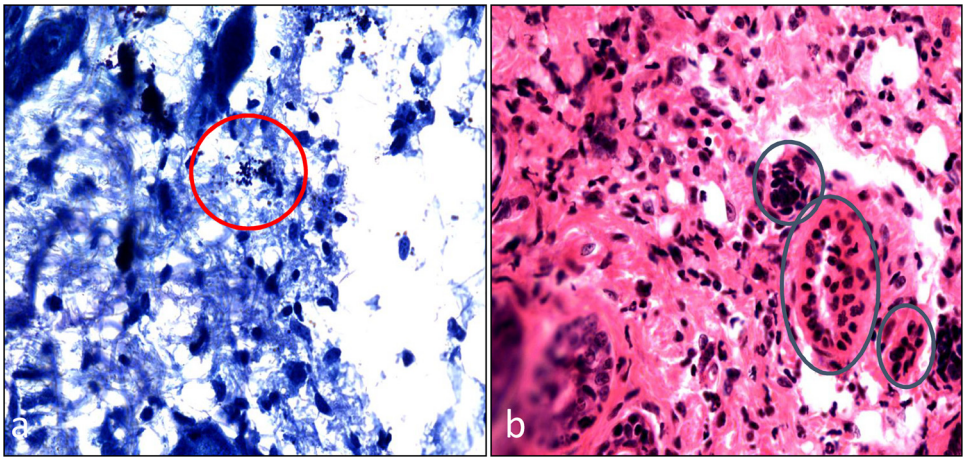

Fig. 2. Hostile environment in the skin feeding site where ticks fed on a guinea pig immunised with recombinant 64TRP tick cement protein. (a) mast cell degranulation (circled) and (b) perivascular cuffing (circled). Photo: Adama Trimnell.

evasins therefore identify the immune cells ixodid ticks seek to avoid. For example, evasin-1 reduces neutrophil recruitment in mice by targeting CCL3 (Vieira et al., 2009).

Dendritic cells are often described as immune sentinels. Their role is to sense danger and send warning signals to other immune cells that deal with the threat. As they sit at the apex of an immune cascade, they are obvious targets for ticks. Several subtypes of dendritic cells occur in mammals of which three distinct subsets are resident in murine skin: Langerhans cells in the epidermis, and two dermal populations (Heath and Carbone, 2013) (Table 2). In addition, blood monocytes can generate dendritic cells in skin; such monocyte-derived dendritic cells contribute to both innate and adaptive immune responses (Austyn, 2016). Metastriate ixodid ticks have evolved a unique family of saliva lipocalins that target monocyte-derived dendritic cells (Preston et al., 2013). Japanin, the prototype of this family, reprogrammes dendritic cell responses to a wide variety of stimuli *in vitro*, radically altering the cells' expression of co-stimulatory and co-inhibitory transmembrane receptors, and their secretion of pro-inflammatory, anti-inflammatory, and T cell polarising cytokines. In addition, Japanin inhibits the differentiation of human dendritic cells from blood monocytes (Preston et al., 2013). The effect of Japanin on other types of dendritic cells, in the dermis and regional lymph nodes, remains to be tested.

Prostriate ixodid ticks have a different strategy for controlling dendritic cells compared with metastriate ixodid species. Sialostatin L, from *I. scapularis*, alters murine bone marrow-derived dendritic cell cytokine secretion and co-stimulatory molecule expression in response to *in vitro* lipopolysaccharide treatment; the secreted cysteine protease inhibitor also affects T cells and inhibits cathepsin L1 (Kotsyfakis et al., 2006; Sá-Nunes et al., 2009). Salp15 (salivary gland protein 15), the prototype of an *Ixodes*-specific multigene family, was originally recognised as inhibiting $CD4^+$ T cell activation (Anguita et al., 2002). However, the 15-kD saliva protein is multi-functional, additionally affecting dendritic cells by interacting with DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin, a type II C-type lectin) resulting in the inhibition of pro-inflammatory cytokines and suppression of T cell activation (Hovius et al., 2008). Argasid ticks do not appear to produce dendritic cell modulators, probably because they feed relatively quickly.

By controlling dendritic cells, ticks exert influence on T lymphocytes. However, there is also ample evidence tick salivary gland products (particularly of *Ixodes* species) directly target T cells (Kotál et al., 2015). The total number of T lymphocytes present in normal human skin is estimated at $\sim 2.0 \times 10^{10}$, almost twice the number in the circulation (Clark et al., 2006). If other species are similar, there is a large pool of memory T cells in normal skin that can initiate and perpetuate immune reactions in the absence of T cell recruitment from the blood. In the epidermis, these are human $CD8^+$ cytotoxic T cells or murine dendritic epidermal T cells (DETCs), and in the dermis, $CD4^+$ T helper cells and dermal γδ T cells (Heath and Carbone, 2013). Salp15 inhibits activation of naïve $CD4^+$ T cells through binding to the first domain of the CD4 co-receptor, impeding cell activation and inducing a long-lasting increasing immunomodulatory effect; in addition, Salp15 affects regulatory T cells (Tregs), inducing increased production of adenosine, a recognised immunosuppressive product (Garg et al., 2006; Tomás-Cortázar et al., 2017).

Through its effect on T cells, Salp15 exerts an indirect effect on the ability of B cells to produce antigen-specific antibodies (Anguita et al., 2002). Direct inhibitors of B cells have been recorded in tick saliva: BIP (B cell inhibitory protein) from *I. ricinus* and BIF (B cell inhibitory factor) from *H. asiaticum* (Hannier et al., 2004; Yu et al., 2006).

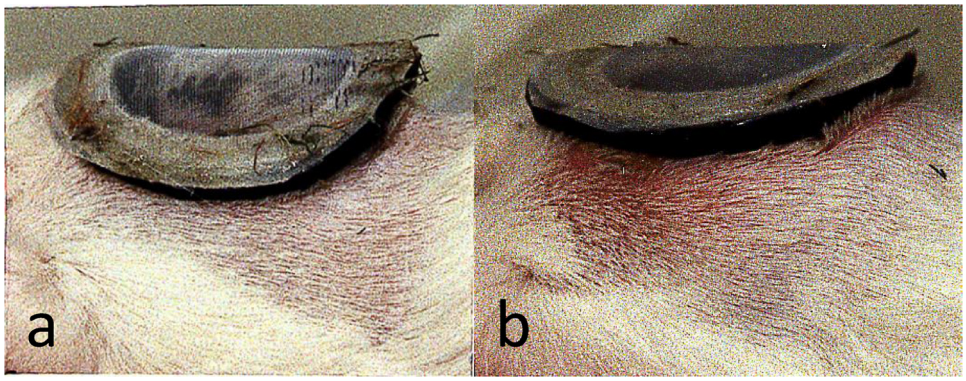

Fig. 3. Tick feeding site on guinea pigs immunised with (a) control glutathione S-transferase fusion protein or (b) recombinant 64TRP tick cement protein fragment 2. Images show gauze-covered neoprene chambers containing nymphal ticks on shaved flank of guinea pigs revealing inflammation in (b) but not in the control (a). Photo: Adam Trimnell.

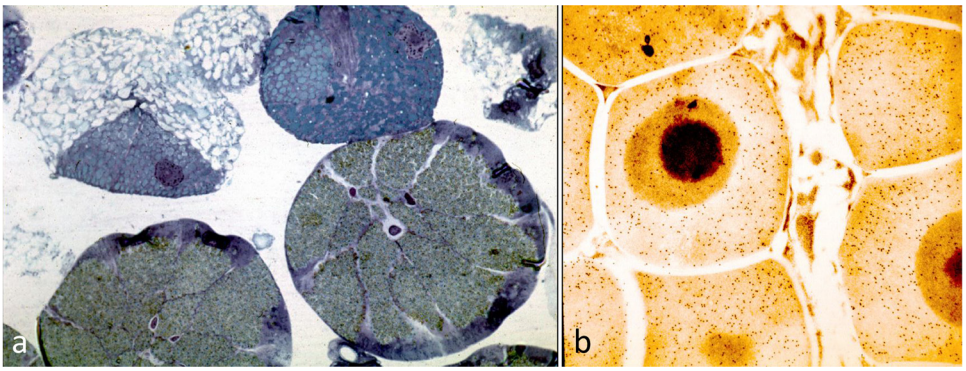

Fig. 4. Male specific immunoglobulin binding protein localisation in granules of type IV acinus of adult male *R. appendiculatus*. (a) immunohistochemistry of salivary glands showing brown staining of the protein within type IV acini and (b) electron micrograph showing immunogold particle localisation of the protein within granules of a type IV acinus. Photo: Hui Wang.

Although B lymphocytes occur infrequently in normal skin under homeostatic conditions, growing evidence suggests they fulfil both antibody-dependent and independent roles in the maintenance of skin immunity, migrating to skin with the aid of skin-homing chemokine receptors (Egbuniwe et al., 2015). In unperturbed sheep skin, specific B cell populations have been detected which could play a role in skin surveillance against ticks (Geherin et al., 2012). One indirect mechanism of controlling B cell function is through the production of immunoglobulin-binding proteins, which appear to be common in ixodid species but have not been recorded in argasid species (Wang and Nuttall, 1999).

2.3.5. Wound healing

During the attachment process in which ticks inflict skin wounding (Section 2.3.1), epidermal keratinocytes activated via TLR3 induce inflammation in response to endogenous RNA released by damaged cells. Inflammation is needed for wound healing while overt inflammation slows the repair process and can be harmful (Heath and Carbone, 2013). In addition, disruption of the defensive skin barrier exposes the epidermis and dermis to pathogens. The process by which ticks control the repair process and help prevent skin infections that are not tick-borne, is little understood (Šimo et al., 2017). Some insight is provided by the discovery ticks produce growth factor inhibitors, though the nature of these apparent cytokine binders is unknown (Hajnická et al., 2011). A correlation was observed between ixodid tick hypostome length (and hence depth of skin penetration) and activity against platelet-derived growth factor (PDGF) (Slovák et al., 2014). Although this observation suggests anti-PDGF activity is a feature of ticks possessing long mouthparts, it does not hold for other growth factors that are inhibited by metastriate but not prostriate (*Ixodes*) ixodid tick species. Despite having much shorter feeding strategies, argasid ticks may manipulate host wound healing responses. The mechanisms are unknown and the effect may be peculiar to *O. brasiliensis*, which creates a feeding lesion notably slow to heal (Reck et al., 2013).

2.4. Dynamic activity

Dynamic changes in salivary bioactivity mirror changes in host response, and the chemical and cellular mediators involved, influenced by sex and gluttony (Kaufman, 2007). Dynamism is illustrated by differential anti-chemokine activity during the 10-day blood-feeding of adult *A. variegatum*, one of the largest tick species: different activities wax and wane at different rates as feeding progresses (Vančová et al., 2007). At the level of saliva composition, comparison of unfed adult female *D. andersoni* with 2- and 5-day fed ticks identified 140 proteins unique to day 2 and 165 for day 5, and 372 proteins for both time points (Mudenda et al., 2014). Dynamic differential expression of saliva genes is substantially greater in adult female compared with male ixodid ticks, reflecting the different goals of feeding females (maximise bloodmeal size to maximise egg production) and males (mating and mate guarding) (De Castro et al., 2017) (Section 2.6). Transcriptomic analysis at the level of individual salivary glands confirmed expression of saliva genes in defined clusters as feeding progresses for adult female *I. ricinus* (Perner et al., 2018). The term 'sialome switching' has been coined to describe the changes in saliva composition as engorgement progresses (a physiological feeding 'clock') although it could also refer to changes in feeding environment resulting from e.g. a change of host or an acquired immune response (Wang et al., 2001b; Karim and Ribeiro, 2015). Little is known about the mechanism of timed regulation of saliva gene transcription during feeding; possibly, epigenetic regulation is involved mediated by histone modification and chromatin remodelling (Kotsyfakis et al., 2015; Cabezas-Cruz et al., 2016).

The dynamic nature of saliva bioactivity is demonstrated when blood-feeding is interrupted and the feeding tick prematurely drops off its host. This may arise, for example, as a result of grooming, host immune responses, or death of the host. When blanket dragging for questing *I. ricinus* it is not uncommon to find partially fed nymphs. If the partially fed tick has not achieved a 'critical weight' (~10 times the unfed weight), it will seek a new host to complete engorgement (Kaufman, 2007). In experimental studies, deliberate interruption of feeding resulted in re-programming of salivary gland protein expression (Wang et al., 1999a). As yet, this ability has not been explored at the level of sialome switching. It could be of particular importance in pathogen transmission as re-attachment of partially fed ticks may lead to accelerated pathogen transmission (Wang et al., 1999a).

In examining the dynamism of tick blood-feeding, one important component is often overlooked: the contribution of granules stored in salivary gland acini of ixodid and argasid ticks (Binnington, 1978; Coons and Roshdy, 1981). As already noted (section 2.3.3), anti-complement activity is present in salivary glands of unfed *I. ricinus* female ticks (Lawrie et al., 1999). Male specific immunoglobulin-binding protein is stored in granules within type IV acini of unfed male *R. appendiculatus* (Wang et al., 1998) (Fig. 4). A cement protein observed in type III acini of female *D. variabilis* was more abundant in salivary gland granules of unfed compared with ticks fed for 2 days (Jaworski et al., 1992). Migration inhibitory factor (MIF) is detectable in salivary glands of unfed *A. americanum* (Bowen et al., 2010). These examples indicate that tick saliva is ready for action as soon as the tick attaches and commences feeding. Determining the contents of salivary gland granules should help elucidate the first tick-host interactions at the feeding site and identify early targets for anti-tick vaccine development.

2.5. Molecular individuality

The first hint of tick individuality came from a comparison of the protein profiles of salivary gland pairs of single adult ticks. Protein profiles of each gland comprising a pair were identical for individual males and females of three ixodid species; however, no one individual was the same as another of the same species and sex (Wang et al., 1999b) (Fig. 5). As the ticks in this study were unfed (3 months post moult and therefore in a resting state), the protein profiles presumably reflect the contents of salivary gland products stored in granules of

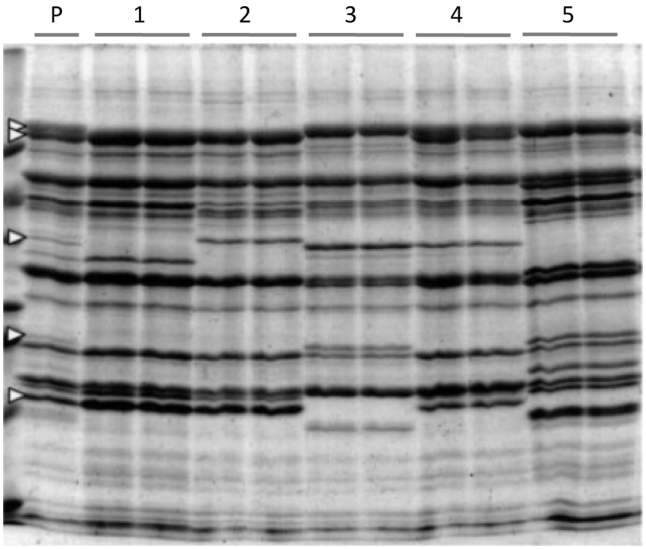

Fig. 5. Salivary gland protein profiles of individual unfed male *Rhipicephalus appendiculatus* compared with a pool of the same salivary glands. P, pooled salivary glands; 1 to 5, salivary gland of each pair from five individual ticks. Modified from Wang et al., 1999b.

specific acini (Sections 1 and 2.4). The molecular individuality observed in unfed tick salivary glands was maintained when feeding commenced (Wang et al., 2001b). At no stage did the protein profile of pooled salivary glands resemble the profile of individuals.

Examination of salivary glands from single female *I. ricinus* after 24 h, 48 h, and 72 h feeding on rabbits by RNA sequencing confirmed the individuality of tick sialomes: single ticks were found to express unique clusters of genes (Perner et al., 2018). The pooling of 'quantum sialomes' has obvious benefits for ticks. Ticks often feed in aggregates, clustered together in a common site on their host which is often a spot difficult to groom. Experimental studies comparing feeding success of adult female *R. appendiculatus* fed on guinea pigs as pairs or in groups (10 pairs) showed that group-fed females fed more rapidly and reduced the time to mating and repletion compared with singletons; however, fast-feeding ticks appeared to impair blood-feeding success of slower feeders (Wang et al., 2001a). Greater understanding of the costs and benefits of feeding aggregation may help improve tick control strategies.

2.6. Mate guarding

Most metastriate ixodid species mate on the host (Feldman-Muhsam, 1986). Males and females attach separately on the same host and then, after a few days of feeding, the male detaches, mates with a female, and then reattaches adjacent to its mate and continues feeding. This curious behaviour may be a form of physical mate guarding whereby the male tick wards off other amorous males. However, when adult *R. appendiculatus* were fed experimentally on guinea pigs immunised with a male-specific immunoglobulin-binding protein, the feeding performance of the females was adversely affected whereas there was no detrimental effect on male ticks (Wang et al., 1998). Differences in the saliva protein composition of males and females have been shown in *R. pulchellus*, including immunoglobulin-binding proteins overexpressed or only detected in males (Tan et al., 2015). By helping his mate to feed, the male maximises perpetuation of his genes, the ultimate goal of mate guarding, but in this case the male is protecting his mate against rejection by the host through the action of male-specific saliva molecules.

2.7. Saliva-assisted transmission

The first indication saliva plays a role in tick-borne pathogen transmission (beyond that of merely providing a physical medium for virus transfer) was the demonstration of non-viraemic tick-borne virus transmission (Jones et al., 1987). Follow-up studies showed that non-viraemic transmission can be mimicked by co-inoculation of virus with saliva or salivary gland extract from partially fed uninfected ticks (Jones et al., 1989, 1992). These initial observations, considered highly controversial when first published, are now widely substantiated: tick-borne pathogens exploit the immunomodulatory effect of tick saliva to promote their transmission (Nuttall and Labuda, 2008; Wikel, 2013; Šimo et al., 2017).

A number of tick saliva molecules have been implicated in saliva-assisted transmission. These include: Salp15 and TSLI (tick salivary lectin pathway inhibitor) of *I. scapularis*, which protect *Borrelia burgdorferi* from antibody- and complement-mediated killing, respectively (Ramamoorthi et al., 2005; Schuijt et al., 2011); IrSPI, a serine protease inhibitor from *I. ricinus* affecting *Bartonella henselae* salivary gland infection and feeding success (Liu et al., 2014); and sialostatin L2, a cysteine protease inhibitor of *I. scapularis*, which facilitates growth of *B. burgdorferi* in murine skin and reduces inflammasome activation during *Anaplasma phagocytophilum* infection (Kotsyfakis et al., 2010; Chen et al., 2014). Sialostatin L2 also inhibits interferon-β antiviral activity resulting in enhanced tick-borne encephalitis virus replication in dendritic cells although sialostatin L2 is not from a natural vector species of the virus (Lieskovská et al., 2015). Genes encoding specific saliva proteins can be upregulated by tick-borne pathogens, as reported for Salp15, TSLI and IsSPI (Ramamoorthi et al., 2005; Schuijt et al., 2011; Liu et al., 2014). Saliva proteins that facilitate pathogen transmission have been identified as candidates for development of anti-tick vaccines (Wikel, 2013; Liu and Bonnet, 2014). However, protection against lethal tick-borne challenge with tick-borne encephalitis virus by immunisation of mice with 64TRP, a tick cement protein, demonstrates that provoking the appropriate inflammatory/immune response is sufficient to change the outcome of tick-borne infection (Labuda et al., 2006). Changing the benign feeding site regulated by the tick to a hostile environment governed by the host could be the key to success in controlling tick-borne infections (Fig. 2) (Kazimírová et al., 2017). This approach may be particularly effective if exosomes secreted in tick saliva play an important role in tick-borne virus transmission (Zhou et al., 2018).

Although saliva-mediated control of host responses is the obvious function of tick saliva contributing to saliva-assisted transmission of tick-borne pathogens, other saliva functions may play a role (Fig. 6). The cement cone may contribute to the phenomenon if it provides a bolus of bioactive saliva molecules (Section 2.2). Dynamic changes in saliva composition and bioactivity affect saliva-assisted transmission, as shown in early studies with Thogoto virus (Jones et al., 1989). However, the significance of molecular individuality (Section 2.5) has not been explored in the context of pathogen transmission. One particularly intriguing question is whether saliva-assisted transmission affects the feeding success of infected ticks.

2.8. Redundancy

Host physiological systems antagonised by tick attachment and blood-feeding are highly redundant: many different host genes, molecules, and pathways achieve similar outcomes. To counteract such nuanced host responses, ticks "either have to find their host's 'Achilles heel,' or they have to use a similarly redundant counter-offensive system" (Ribeiro, 1995). Current sequence-based approaches and bioinformatics reinforce evidence ticks have opted for a 'redundant' system (Ribeiro et al., 2012; Díaz-Martín et al., 2013; Esteves et al., 2017; Hackenberg et al., 2017). Thus, the same host homeostatic mechanism is targeted by more than one tick saliva molecule.

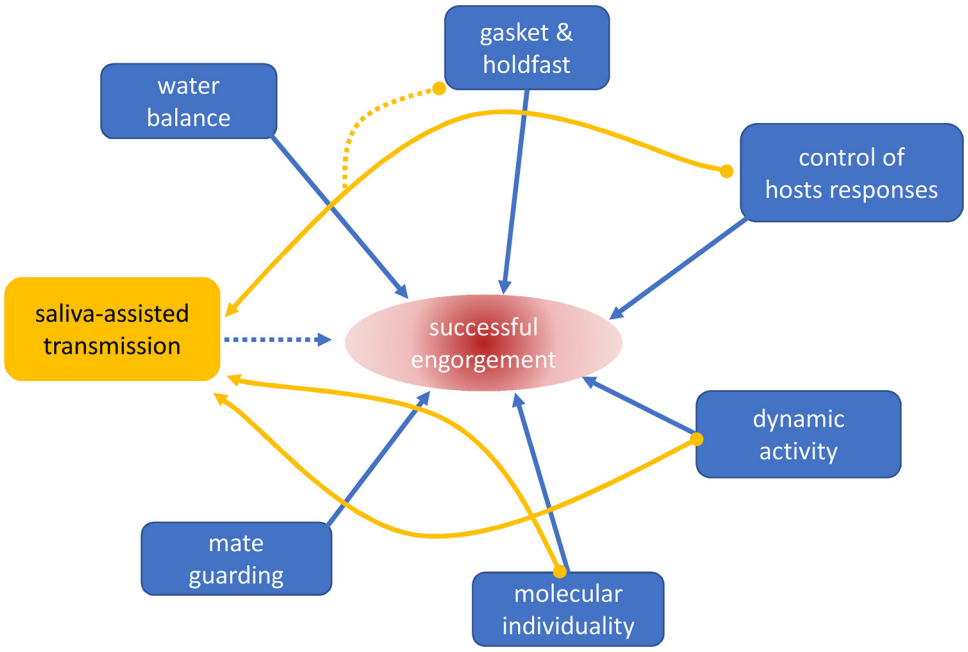

Fig. 6. Major functions of tick saliva contributing to successful engorgement. Orange arrows indicate saliva functions contributing (solid line) or potentially contributing (dashed line) to saliva-assisted transmission of tick-borne pathogens. Blue dashed line indicates potential for saliva-assisted transmission to contribute to feeding success of infected ticks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Does tick saliva really show redundancy? Ticks cope with a multitude of host protection mechanisms, but they also have sufficient biodiversity within their saliva to tackle diverse host species, even across taxa. For example, *I. ricinus* can feed successfully on reptiles, birds, and mammals during its life cycle. Although saliva molecules generally target conserved elements which are often ligands rather than cell surface receptors, there is still a massive challenge to cope across species let alone taxa. For example, histamine is conserved throughout the animal kingdom whereas, in humans, histamine exerts its effects through binding to G protein-coupled histamine receptors of which there are at least four different types in humans, let alone other species. Ticks are efficient and thrifty: they target the histamine ligand rather than the histamine receptors. Although adult *R. appendiculatus* produce at least three histamine-binding proteins, they are not redundant: one is specific for males and the other two are found in female saliva but appear to show different strategies with one binding extracellular histamine while the other targets histamine within mast cell granules (Paesen et al., 1999).

In contrast to histamine, dendritic cells differ both within species and between species (Table 2). Hence targeting dendritic cells is a potentially costly strategy for ticks as it requires an added level of diversity in molecular mediators demonstrated by the seemingly different strategies adopted by prostriate compared with metastriate ixodid species (Section 2.3.4).

In addition to host diversity and the pressure for ticks to have seemingly redundant countermeasures, ticks are individuals (Section 2.5). Until recently, analysis of tick salivary glands and saliva has relied on pooled material. A pool can be very different from an individual, as illustrated by comparing protein profiles of salivary glands from pools with individual tick salivary glands (Fig. 5). Nevertheless, sequence analysis of single pairs of salivary glands confirms 'redundancy' at the level of the 'tick quantum sialome' while revealing individual uniqueness (Perner et al., 2018). Hence, redundancy cannot simply be explained by the pooling of unique individual sialomes though sialome pooling creates even greater molecular diversity and helps explain the benefits of feeding aggregation, a common trait in ticks (Section 2.5).

Host redundancy as a driver of diversity in tick saliva constituents also helps explain how ticks are able to adapt to changed feeding environments. Changed conditions may arise from interrupted feeding (Section 2.4), host change (Wang et al., 2001b; Tirloni et al., 2017), or immune pressure (Anderson et al., 2017; Vora et al., 2017; Perner et al., 2018). Again, sialome pooling through feeding aggregation may aid adaptation to changed conditions (Section 2.5). Thus, if the sialome of one tick is more in tune with the feeding environment compared with that of its feeding neighbour, the neighbour may benefit from the more effective sialome. Such sialome sharing benefits may also extend to immature ticks in species showing aggregated feeding (Rechav and Nuttall, 2000).

Diversity in saliva proteins provides a possible means of immune evasion (Francischetti et al., 2009). By secreting small quantities of many antigenically different, but functionally similar saliva proteins, at different times of feeding, low dose tolerance and/or antigenic competition may impair cell activation (Chmelař et al., 2016). This hypothesis is compatible with male *versus* female differences in saliva molecules and male and female co-feeding post-copulation (Section 2.6), and with the pooling of sialomes through aggregated feeding (Section 2.5). Nevertheless, the ability of certain natural hosts to tolerate repeated tick infestations while atypical hosts reject them, challenges this hypothesis (Ribeiro, 1989; Dizij and Kurtenbach, 1995; Carvalho et al., 2014; Anderson et al., 2017). Bioinformatic analysis suggests tick miRNAs are similarly redundant: many target genes in the same host pathway appear to be regulated by more than one saliva-specific miRNA (Hackenberg et al., 2017). If tick miRNAs are not subjected to the same immune pressure experienced by tick saliva proteins, their apparent redundancy is most likely a reflection of redundancy in host homeostatic mechanisms. On balance, host redundancy combined with adaptability and differing roles of males and females (at least within ixodid species) appear to be the main evolutionary drivers of the extraordinary molecular diversity of tick saliva.

3. Conclusions and future directions

Tick saliva is complex and has many different functions. The complexity and functionality change as feeding progresses, at least in ixodid species that take days to complete engorgement. Further comparative studies on the composition and activity of saliva from ixodid and argasid species, and the intriguing evolutionary relic, *N. namaqua*, should provide important new insights into how tick saliva has evolved with the different life strategies of this successful superfamily of obligate ectoparasites. Most intriguing of all is what new insights tick saliva can bring to understanding the host response mechanisms of their vertebrate hosts – the skin haemostatic, inflammatory and immune systems

as seen by ticks.

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