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Research



# Association of Salivary Cholinesterase With Arthropod Vectors of Disease

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## Abstract

Acetylcholinesterase (AChE) was previously reported to be present in saliva of the southern cattle tick, *Rhipicephalus (Boophilus) microplus* (Canestrini), with proposed potential functions to 1) reduce acetylcholine toxicity during rapid engorgement, 2) modulate host immune responses, and 3) to influence pathogen transmission and establishment in the host. Potential modulation of host immune responses might include participation in salivary-assisted transmission and establishment of pathogens in the host as has been reported for a number of arthropod vector-borne diseases. If the hypothesis that tick salivary AChE may alter host immune responses is correct, we reasoned that similar cholinesterase activities might be present in saliva of additional arthropod vectors. Here, we report the presence of AChE-like activity in the saliva of southern cattle ticks, *Rhipicephalus (Boophilus) microplus*; the lone star tick, *Amblyomma americanum* (Linnaeus); Asian tiger mosquitoes, *Aedes albopictus* (Skuse); sand flies, *Phlebotomus papatasi* (Scopoli); and biting midges, *Culicoides sonorensis* Wirth and Jones. Salivary AChE-like activity was not detected for horn flies *Haematobia irritans* (L.), stable flies *Stomoxys calcitrans* (L.), and house flies *Musca domestica* L. Salivary cholinesterase (ChE) activities of arthropod vectors of disease-causing agents exhibited various Michaelis–Menten $K_M$ values that were each lower than the $K_M$ value of bovine serum AChE. A lower $K_M$ value is indicative of higher affinity for substrate and is consistent with a hypothesized role in localized depletion of host tissue acetylcholine potentially modulating host immune responses at the arthropod bite site that may favor ectoparasite blood-feeding and alter host defensive responses against pathogen transmission and establishment.

**Key words:** vector-borne disease, salivary-assisted transmission, host–parasite interaction, acetylcholinesterase, cholinergic immunomodulation

Vector-borne diseases result from pathogens that are transmitted by vectors, including hematophagous arthropods such as ticks, mosquitoes, triatomine bugs, sandflies, fleas, blackflies, and others. Vector-borne diseases are responsible for over a million deaths annually, and account for more than 17% of all infectious diseases worldwide (WHO 2016, 2017). Types of pathogens transmitted by arthropod vectors include viruses, protozoa, bacteria, rickettsia, fungi, and nematodes. Gubler (2009) summarized the history of vector-borne diseases and noted that, although many of these diseases were largely controlled by the mid-20th century through judicious use of pesticides, habitat modification, and other control measures, complacency and redirection of resources led to a global re-emergence of vector-borne diseases affecting humans and animals at near epidemic levels (CDC 2018). Although there have been numerous attempts to develop effective vaccines directed toward the causative pathogens (Labuda et al. 2006, Manning and Cantaert 2019), prevention of arthropod-borne disease has largely relied upon vector control (Leitner et al. 2015, Wilson et al. 2020). Arthropod vector control has been predominantly dependent on use of chemical pesticides; however, the development and widespread establishment of pesticide resistance have increasingly compromised efficacy of most insecticides and acaricides used in vector control (Mallet 1989, Angus 1996, Styer et al. 2011, Abbas et al. 2014, Dudley et al. 2017, Klafke et al. 2017, Hancock et al. 2018, WHO 2018). Salivary-assisted

transmission of vector-borne disease is a well-known phenomenon in which pathogen transmission and establishment in the afflicted host is promoted by the presence of saliva from the vector (Jones et al. 1992; Kovár 2004; Nuttall and Labuda 2004, 2008; Andrade et al. 2005, 2007; Thangamani et al. 2010; Fontaine et al. 2011; Styer et al. 2011; Leitner et al. 2011, 2012; Surasombatpattana et al. 2012; Kazimirova and Stibraniova 2013; Briant et al. 2014; Schmid et al. 2016; Wichit 2016; Pingen et al. 2016, 2017; Narasimhan et al. 2017; Kazimírová et al. 2017; Pinheiro et al. 2018); however, the salivary components and mechanisms responsible for salivary-assisted transmission have not been fully elucidated and might be specific to different vector, pathogen, and host combinations. Temeyer and Tuckow (2016) reported acetylcholinesterase (AChE) presence in tick saliva and hypothesized that tick salivary AChE could affect the host immune response to parasitism and infection. Specifically, tick AChE might participate in salivary-assisted transmission by hydrolysis of acetylcholine (ACh) at the tick bite site; this reduction is hypothesized to alter the activation of muscarinic and nicotinic ACh receptors of sentinel and other immune cells and thereby modulate, possibly weakening or redirecting development, of host innate and adaptive immune responses (Temeyer 2018).

ACh is an ancient molecule found throughout the animal kingdom that is active in cholinergic signaling and physiological control, often in an autocrine- or paracrine-like manner, and it has also been reported to have various signaling and regulatory functions in bacteria, fungi, protozoa, algae, and plants (Wessler et al. 2001, Horiuchi et al. 2003, Yamamoto and Momonoki 2012, Taylor et al. 2013, Soreq 2015, Campoy et al. 2016). Most human and mammalian cells express partial or complete cholinergic systems, including ACh synthesis (via choline acetyltransferase [CHAT]), hydrolysis (AChE), and muscarinic and nicotinic ACh receptors (mAChR and nAChR, respectively) (Racké et al. 2006, Beckmann and Lips 2013). Immune cells, including lymphocytes, dendritic cells, macrophages, and mast cells, produce and release ACh when activated (Fujii et al. 2017a, b). Cholinergic systems exert regulatory input for a wide variety of metabolic and physiological activities of cells and tissues, including neural development and function, cellular proliferation, apoptosis, and tumorigenesis, frequently mediated through differential activation of various mAChRs and nAChRs (Fujii and Kawashima 2001, Beckmann and Lips 2013). Different ACh receptors exhibit differential sensitivity to ACh, agonists, and antagonists, and changes in the activation of receptor subsets can alter sensitivity and expression of receptors, which can elicit a wide variety of inter-related cascade reactions (Pope 1999). Cholinergic regulation of immune function in some invertebrates appears to be similar in many respects to that of mammals (Kawli et al. 2010, Liu et al. 2018), suggesting that cholinergic systems may be governing factors when invertebrates are infected by pathogens (Shi et al. 2012, Chen et al. 2016, Pamminger et al. 2017). Cholinergic control is therefore fundamental to aspects of both invertebrate and vertebrate immune system action supporting the hypothesized role of tick salivary AChE as a participant in modulating host immune response (Temeyer and Tuckow 2016, Temeyer 2018). This report explores the possibility that additional hematophagous arthropod species that vector disease-causing agents might secrete AChE-like enzymes in their saliva that contribute to salivary-assisted transmission.

## Materials and Methods

### Arthropod Strains

The cattle ticks, *Rhipicephalus (Boophilus) microplus* (Canestrini) Deutch strain, were obtained from laboratory colonies maintained at the Cattle Fever Tick Research Laboratory, Edinburg, TX (Temeyer et al. 2007). The lone star tick, *Amblyomma americanum*, was obtained from a laboratory colony maintained at the USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL) in Kerrville, TX (Gladney and Drummond 1970, Darrow et al. 1976, Childs and Paddock 2003). Asian tiger mosquitoes, *Aedes albopictus* (Skuse), were obtained from a laboratory colony initiated from wild insects collected at Kerrville, TX. Sand flies, *Phlebotomus papatasi* (Scopoli) (Israeli strain), were from a laboratory colony maintained at the USDA-ARS KBUSLIRL in Kerrville, TX (Temeyer et al. 2013). Biting midges, *Culicoides sonorensis* Wirth and Jones (formerly *C. sonorensis variipennis*), were provided from a laboratory colony maintained at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS (Lehiy and Drolet 2014). Stable flies *Stomoxys calcitrans* (L.), horn flies, *Haematobia irritans* (L.), and house flies, *Musca domestica* L., were reared in laboratory colonies maintained at the KBUSLIRL (Lohmeyer and Pound 2012, Temeyer and Chen 2012, Temeyer et al. 2012).

### Saliva, Salivary Gland Exudate, and Head Extract

Limited quantities of saliva were collected directly in glass capillary micropipettes by placement over the protostomes of newly emerged, unfed adult *R. (Boophilus) microplus* ticks. Salivary exudate was prepared from adult arthropod salivary glands dissected from *R. microplus*, *A. americanum*, *Ixodes scapularis, Ae. albopictus*, *P. papatasi*, *C. sonorensis*, *S. calcitrans*, *H. irritans*, and *M. domestica* as follows. Here, salivary glands collected in phosphate-buffered saline (PBS) pH 7.5 were centrifuged directly, or sometimes with very gentle pressure exerted on the pelleted salivary glands, to empty the salivary gland acini into the PBS, without tissue grinding, to avoid tissue damage and release of membrane-bound AChE from innervating tissue. Salivary gland exudate was collected as the supernatant after 3 min of 10,000 rpm (10,621 × *g*) centrifugation. Preparations from *R. microplus* ticks included unfed adult female salivary exudate (20 pair salivary glands in 250 µl PBS) for measurement of substrate preference, adult female salivary exudate (50 pair salivary glands in 200 µl PBS), and adult male salivary exudate (20 pair salivary glands in 200 µl PBS) for measurements of $K_M$. Preparations from *Ae. albopictus* included salivary gland exudate (25 pair salivary glands in 100 µl PBS) and female head extracts (25 adult female heads after removal of salivary glands in 100 µl PBS). *Phlebotomus*

**Table 1.** Protein concentrations in arthropod salivary preparations utilized in this study

| | Source of salivary glands used in preparation of salivary exudate[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *R. microplus* | *A. americanum* | *I. scapularis* | Mosquito | Sand fly | Biting midge | Stable fly | Horn fly | House fly |
| Protein concentration (mg/ml) | 1.1 | 3.4 | – | 3.4 | 0.78 | 0.98 | 2.0 | 1.05 | 0.55 |

[a]Ticks: *Rhipicephalus (Boophilus) microplus*, *Amblyomma americanum*, and *Ixodes scapularis*; mosquito: *Aedes albopictus*; sand fly: *Phlebotomus papatasi*; biting midge: *Culicoides sonorensis*; stable fly: *Stomoxys calcitrans*; horn fly: *Haematobia irritans*; house fly: *Musca domestica*.

*papatasi* salivary gland exudate was prepared from unfed, adult female flies (50 pair salivary glands in 200 µl PBS for measurements of $K_M$, or from 20 pair salivary glands in 20 µl PBS for determination of substrate preference), as well as from adult male and female flies that were newly emerged, 2 d, and 4 d post-emergence (10 pair salivary glands in 200 µl PBS) to assess relative AChE expression in emergent adult male and female sand flies. Salivary gland exudate was prepared from adult female *C. sonorensis* (20 pair salivary glands in 20 µl PBS). Salivary exudate preparations from stable flies, horn flies, and house flies were obtained from unfed adults (25 pair salivary glands in 100 µl PBS).

### Bovine Serum AChE

Unlike some arthropods, mammals have only one gene encoding AChE; therefore, bovine serum AChE was selected as representative of mammalian host AChE for comparison to AChEs expressed in arthropod salivary preparations. Whole blood was collected from a steer by jugular venipuncture in Corvac serum separator tubes (Medtronic, Minneapolis, MN). Bovine serum was separated by centrifugation for 15 min at 1,200 rpm (10,621 × *g*) and stored at 4°C until use.

### AChE Microplate Assay

Biochemical characterization of tick saliva, recombinant tick AChE (rBmAChE1), and exudates of arthropod salivary glands were determined in microplate assays using triplicate samples (Temeyer et al. 2010). Recombinant AChEs of *R. microplus* (rBmAChE1; Temeyer et al. 2010); *P. papatasi* (rPpAChE1; Temeyer et al. 2013); *S. calcitrans* (rScAChE; Temeyer and Chen 2012); and *H. irritans* (rHiAChE; Temeyer et al. 2012) were used as positive controls in AChE assays; negative controls consisted of the assay mixture lacking enzyme (Temeyer et al. 2010). Data for calculation of $K_M$ and $V_{max}$ were analyzed and plotted by nonlinear regression using GraphPad Prism ver. 5.0 (GraphPad Prism, Inc., La Jolla, CA). The Michaelis–Menten constant, $K_M$, is defined as the substrate concentration at which the initial reaction velocity is one half maximal (Lehninger 1975). Comparison of tick salivary gland extract with recombinant BmAChE1 utilized 5 µl per well of either salivary gland exudate or 500-fold dilution (in PBS) of BmAChE1 baculoviral supernatant (Temeyer et al. 2010). Preference for acetylthiocholine versus butyrylthiocholine substrates by enzymatic activity present in salivary gland exudates (Temeyer et al. 2006) was tested at a final substrate concentration of 100 µM. Enzyme activity was measured as the change in optical density over time at 412 nm (Temeyer et al. 2010). A standard curve was constructed using β-mercaptoethanol added to the reaction mix (without enzyme) to calibrate $A_{412}$ versus β-mercaptoethanol. Specific activity was calculated as the micromoles (µmol) of acetylthiocholine hydrolyzed per minute per milligram (mg) of protein.

### Michaelis–Menten Constant (*KM*)

The $K_M$ value is a descriptive biochemical characteristic of an enzyme that is independent of enzyme concentration and is indicative of enzyme affinity for its substrate (Lehninger 1975). The constant is calculated (GraphPad Prism) by nonlinear regression of initial velocities calculated over a range of substrate concentrations encompassing or approaching minimal and maximal velocities.

### Protein Determinations

Protein concentration in saliva and salivary exudate was estimated using protein determination software in a DeNovix DS-11 FX+ spectrophotometer (DeNovix Inc., Wilmington, DE), with correction for nucleic acid ($c_{prot}$ (mg/ml) = 1.55 * $A_{280nm}$ – 0.76*$A_{260nm}$) based on UV absorption 260/280 nm protocol according to Warburg and Christian (1941).

### Statistical Analyses

SE and 95% CIs were calculated for $K_M$ values by GraphPad Prism. For data analyses, Mann–Whitney *U*-test, Kruskal–Wallis followed by Dunn's test of selected pairs of columns or unpaired *t*-test were used. Differences were considered significant for *P* values ≤0.05.

## Results

Previous studies investigating molecular effects of vector saliva on mammalian immune cells have utilized vector saliva or extracts from sonicated salivary glands (Cavassani et al. 2005, Brake et al. 2010, Oliveira et al. 2010, Bizzarro et al. 2013). In the present study investigating vector saliva for the presence of an ACh hydrolyzing enzyme, we were concerned that AChE released from innervating nerves might contaminate salivary exudate prepared when collection of saliva was extremely difficult. We initially tested supernatant from mosquito salivary glands ground in Dounce or Tenbroeck tissue grinders. After centrifugation, significant ACh hydrolyzing activity was present in both the supernatant and pellet fractions suggesting possible presence of AChE in soluble and membrane or cell-bound fractions. In Dipterans, the major function of AChE is associated with neural transmission localized at neural synapses (Kakani et al. 2011); however, soluble monomeric forms with unknown function expressed in non-neuronal tissues have also been reported (Kim and Lee 2013). We reasoned that centrifugation of dissected salivary glands in the absence of tissue disruption by grinding, sonication, or treatment with detergents might result in release of saliva from the salivary acini. Subsequent experiments demonstrated release of soluble protein from salivary glands collected from each of the tested species (Table 1). Microplate assays were used to test each of the arthropod salivary preparations (saliva or salivary exudate) for acetylthiocholine hydrolyzing activity. Application of gentle pressure using a pestle (without grinding) did not result in release of AChE from pelleted salivary glands that were previously negative for AChE activity.

The AChE microplate assays for arthropod saliva and salivary gland exudates of *R. microplus*, *A. americanum, Ae. albopictus*, *P. papatasi*, and *C. sonorensis* permitted the calculation of $K_M$ values of AChE-like activity for each of these arthropods (Table 2). Bovine serum AChE was assayed as an example of mammalian host AChE, yielding a $K_M$ value of 106.80 ± 1.45 µM acetylthiocholine. Tick salivary AChE-like activity exhibited a 44-fold greater substrate preference for acetylthiocholine than for butyrylthiocholine (Fig. 1), consistent with a biochemical identity of AChE and consistent with the lack of butyrylcholinesterase in invertebrates. However, many invertebrates express multiple genes encoding AChE, unlike vertebrates which have one gene encoding AChE and one gene encoding butyrylcholinesterase (Chatonnet and Lockridge 1989), and by determining biochemical properties, including $K_M$ for acetylthiocholine and relative substrate preference of the salivary AChEs in vector saliva, we hoped to elucidate information indicative of which of multiple invertebrate AChEs might be present in saliva. Measured $K_M$ values for acetylthiocholine were 6.33 ± 0.47 µM for rBmAChE1 (recombinant *R. microplus* BmAChE1; Temeyer et al. 2006), 8.04 ± 2.30 µM for adult female salivary exudate (Table 1), and 10.01 ± 5.93 µM for adult male salivary exudate.

**Table 2.** Comparison of Michaelis–Menten $K_M$ values for acetylthiocholine of AChE activities present in salivary preparations from selected arthropod vectors of disease in comparison to a potential mammalian host, *Bos taurus*

| Salivary AChE properties | Salivary AChE source[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *R. microplus* | *A. americanum* | *I. scapularis* | *Ae. albopictus* | *P. papatasi* | *C. sonorensis* | *B. taurus* |
| $K_M$ value[a] | 8.04 ± 2.30 | 48.12 ± 2.18 | 9.99 ± 3.30 | 31.01 ± 1.68 | 63.78 ± 13.29 | 92.00 ± 5.84 | 106.80 ± 1.45 |
| 95% CI | 3.32–12.77 | 43.65–52.58 | 2.81–17.17 | 27.34–34.68 | 36.67–90.89 | 77.90–106.5 | 103.8–109.8 |

[a]The $K_M$ value is the substrate concentration producing half maximal velocity of enzyme activity (µM acetylthiocholine).

[b]Ticks: *Rhipicephalus microplus*, *Amblyomma americanum*, and *Ixodes scapularis*; mosquito: *Aedes albopictus*; sand fly: *Phlebotomus papatasi*; biting midge: *Culicoides sonorensis*; bovine: *Bos Taurus*.

Salivary gland exudate and head extracts from adult female *Ae. albopictus* that had not blood fed yielded $K_M$ values of 31.01 ± 1.68 and 39.09 ± 1.68 µM acetylthiocholine, respectively. Substrate preference for acetylthiocholine over butyrylthiocholine was 3.70-fold for mosquito salivary gland exudate and 8.90-fold for mosquito head extract. Taken together, the differences in values for $K_M$ and relative substrate preference suggest that the principal AChE-like activities found in *Ae. albopictus* head and saliva are of substantially different compositions, likely reflecting differential expression of the two mosquito AChEs (Weill et al. 2002) in head and salivary gland.

Salivary gland exudate prepared from unfed adult female *P. papatasi* sand flies yielded a $K_M$ value of 61.72 ± 12.11 µM acetylthiocholine, which matched the value (63.78 ± 13.29 µM acetylthiocholine) obtained for recombinant PpAChE1 (Temeyer et al. 2013), and consistent with the presence of a single gene encoding AChE in *P. papatasi*. *Phlebotomus papatasi* salivary gland exudate exhibited a 9.60-fold preference for acetylthiocholine over butyrylthiocholine. Newly emergent adult female *P. papatasi* exhibited twice as much AChE-like activity at day 0 as their same age male counterparts, and the AChE-like activity in salivary extracts of females doubled from day 0 to day 2 and remained constant through day 4. Expression of AChE-like activity in salivary gland exudates prepared from male flies increased by less than one third from day 0 to day 2 and doubled from day 2 to day 4.

Collection of salivary glands from *C. sonorensis* midges was very challenging and recovery of the AChE-like activity was low; however, salivary gland exudate yielded a measurable $K_M$ value of 92 ± 5.84 µM acetylthiocholine. Salivary gland exudates prepared from stable flies, horn flies, and house flies did not exhibit detectable AChE-like activity.

## Discussion

Temeyer and Tuckow (2016) identified AChE in tick saliva and proposed an immunomodulatory role in the host's response to blood feeding and pathogen introduction. Salivary AChE was hypothesized to reduce localized ACh concentrations in host blood and tissues at the tick bite site, plausibly functioning to 1) reduce potentially toxic effects of ACh present in the large volume of host blood consumed during rapid engorgement; 2) potentially modulate host immune responses (innate and acquired) to tissue damage, tick antigens, and subsequent tick exposures; and 3) influence tick transmission and establishment of pathogens within the host (Temeyer and Tuckow 2016, Temeyer 2018). Effects on pathogen transmission and establishment within the host may be an inadvertent consequence of selection for parasite protective functions. Regardless, this function is important because salivary assisted transmission is an important aspect of vector-borne diseases. Ticks are vectors for a greater number and variety of pathogens than any other parasite and are second only to mosquitoes (owing to malaria) as vectors of serious human disease (Wikel 2013, Kazimírová et al. 2017). Saliva and salivary gland exudate of *R. microplus* ticks exhibited biochemical properties that were essentially indistinguishable from recombinant BmAChE1 (Temeyer et al. 2010). The biochemical properties of *R. microplus* salivary AChE-like activity are consistent and further supportive of evidence presented by Temeyer and Tuckow (2016), demonstrating similarity and possible identity of the salivary AChE-like activity of *R. microplus* to recombinant BmAChE1 (Temeyer et al. 2006).

Mosquitoes express two different genes encoding AChE (Weill et al. 2002). However, the distinct physiological functions and roles of multiple arthropod AChEs have not yet been fully elucidated. The differences in biochemical properties ($K_M$ and substrate preference) observed for AChE-like activity in salivary exudate and head extract (lacking salivary glands) *of Ae. albopictus* strongly suggest differential expression of the two mosquito AChEs in saliva and in the central nervous system. Female mosquitoes, midges, and sand flies require a bloodmeal for reproduction, while males feed predominantly on plant sap or nectar. We observed that salivary expression of AChE-like activity in newly emerged adult female *P. papatasi* appeared to occur more quickly than in males, suggesting a more important role of AChE-like activity in saliva of females than in the saliva of males.

Salivary-assisted transmission has been reported for a variety of vector-borne diseases. Parasites have coevolved with their hosts to successfully manage or manipulate host immune system responses to promote parasite survival (Adamo 2013, Šimo et al. 2017). In view of the hypothesized role of tick salivary AChE in modulating host immune response, and potentially participating in salivary-assisted transmission of pathogens (Wikel et al. 2017), it seemed appropriate to investigate the possible presence of AChE-like activity in saliva of several different hematophagous arthropod ectoparasites. AChE-like activity was detected in saliva or salivary exudates prepared from arthropod vectors of disease, including *R. microplus*, *A. americanum*, *Ae. albopictus*, *P. papatasi*, and *C. sonorensis*, but not from salivary exudates of non-vector arthropod species, including *S. calcitrans*, *H. irritans*, and *M. domestica*. Ticks (Jones et al. 1990, Narasimhan et al. 2017), mosquitoes (Moser et al. 2016), sand flies (Lestinova et al. 2017), and midges (Drolet et al. 2015) have been reported to exhibit salivary-assisted transmission (Narasimhan et al. 2017), while muscid flies are more typically identified as mechanical vectors of microorganisms. The salivary AChEs expressed by each of these arthropod vectors exhibits a $K_M$ value lower than that of *Bos taurus* (Table 1). This is consistent with the hypothesized local depletion of tissue ACh present in host tissue near the bite site. These vectors penetrate the host skin and feed on internal blood either pooled (telmophagic feeding: ticks, sand flies, midges) or contained in vessels (solenophagic feeding: mosquitoes). Both of these types of blood-feeding involve sucking

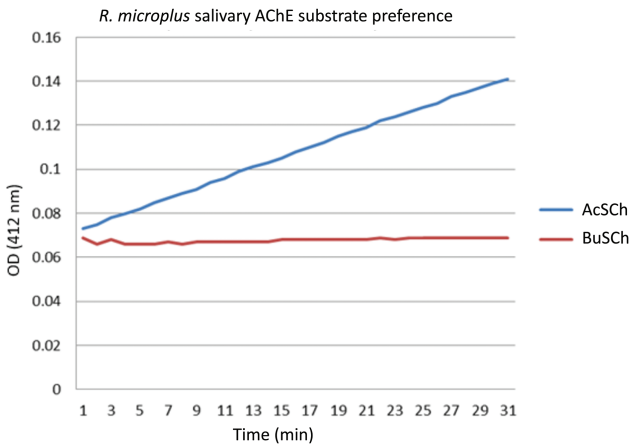


**Fig. 1.** Saliva collected from unfed adult female *R. (B.) microplus* ticks was assayed using a microplate cholinesterase assay with 100 µM acetylthiocholine (AcSCh) or butyrylthiocholine (BuSCh) as substrate as described in Temeyer et al. (2006). Relative substrate preference was calculated as the relative ratio of the slopes of hydrolytic activity.

blood from within the host. Hemostasis occurs within minutes of vascular injury, and inhibition of hemostasis is imperative for success of rapid and slow feeders (Narasimhan et al. 2017). Localized ACh tissue depletion produced by parasite salivary AChE would be expected to differentially reduce activation of nAChRs because mAChRs are generally more sensitive to lower concentrations of ACh compared to nAChRs (Pope 1999). The resulting differential cholinergic activation of muscarinic receptors in microvasculature of the mouse has been reported to result in nitric oxide production, producing vasodilation and thereby promoting blood acquisition from within the host (Hamel 2004). Cholinergic activation of muscarinic or nicotinic receptors is capable of mediating either pro or anti-inflammatory effects and the balance affects every aspect of immune function (Kawashima et al. 2012a,b; Beckmann and Lips 2013; Nizri and Brenner 2013; Zdanowski et al. 2015). Cholinergic competence of many immune component cell populations suggests that these cells have the capacity to maintain homeostatic local concentration of ACh, thereby providing a limitation on response to changes in ACh receptor activation and preventing runaway immune responses to temporal immune stimulation. Further studies may require choice and separation of immune cell subsets and may be critical to identify regulatory changes in production of cytokines, interferons, tumor necrosis factors, or other immunoregulatory products. Localized presence of arthropod salivary AChE possessing a lower $K_M$ than mammalian AChE would therefore be expected to prevent return to homeostatic balance as long as the arthropod AChE remained present. It is reasonable to conclude that arthropod vector salivary AChE works together with other immunomodulatory components of arthropod vector saliva to modify the resulting immune response of the host. In particular, the reduced local concentration of ACh might be expected to support the pro-inflammatory vasodilation response, helping to maintain availability of host blood to the arthropod vector, and coincidentally modulating host innate and adaptive immune responses to favor successful pathogen transmission and establishment within the host. Elucidation of molecular interactions at the vector bite site where vector saliva, pathogen introduction and host responses are initiated may provide new opportunities to reduce incidence and impacts of vector-borne diseases (Manning and Cantaert 2019).

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## References Cited

**Abbas, R. Z., M. A. Zaman, D. D. Colwell, J. Gilleard, and Z. Iqbal. 2014.** Acaricide resistance in cattle ticks and approaches to its management: the state of play. Vet. Parasitol. 203: 6–20.

**Adamo, S. A. 2013.** Parasites: evolution's neurobiologists. J. Exp. Biol. 216: 3–10.

**Andrade, B. B., C. R. Teixeira, A. Barral, and M. Barral-Netto. 2005.** Haematophagous arthropod saliva and host defense system: a tale of tear and blood. An. Acad. Bras. Cienc. 77: 665–693.

**Andrade, B. B., C. I. de Oliveira, C. I. Brodskyn, A. Barral, and M. Barral-Netto. 2007.** Role of sand fly saliva in human and experimental leishmaniasis: current insights. Scand. J. Immunol. 66: 122–127.

**Angus, B. M. 1996.** The history of the cattle tick *Boophilus microplus* in Australia and achievements in its control. Int. J. Parasitol. 26: 1341–1355.

**Beckmann, J., and K. S. Lips. 2013.** The non-neuronal cholinergic system in health and disease. Pharmacology 92: 286–302.

**Bizzarro, B., M. S. Barros, C. Maciel, D. I. Gueroni, C. N. Lino, J. Campopiano, M. Kotsyfakis, G. P. Amarante-Mendes, E. Calvo, M. L. Capurro, et al. 2013.** Effects of *Aedes aegypti* salivary components on dendritic cell and lymphocyte biology. Parasit. Vectors 6: 329.

**Brake, D. K., S. K. Wikel, J. P. Tidwell, and A. A. Pérez de León. 2010.** *Rhipicephalus microplus* salivary gland molecules induce differential CD86 expression in murine macrophages. Parasit. Vectors 3: 103.

**Briant, L., P. Despres, V. Choumet, and D. Misse. 2014.** Role of skin immune cells on the host susceptibility to mosquito-borne viruses. Virology 464–465: 26–32.

**Campoy, F. J., C. J. Vidal, E. Muñoz-Delgado, M. F. Montenegro, J. Cabezas-Herrera, and S. Nieto-Cerón. 2016.** Cholinergic system and cell proliferation. Chem. Biol. Interact. 259: 257–265.

**Cavassani, K. A., J. C. Aliberti, A. R. Dias, J. S. Silva, and B. R. Ferreira. 2005.** Tick saliva inhibits differentiation, maturation and function of murine bone-marrow-derived dendritic cells. Immunology 114: 235–245.

**CDC. 2018.** Vital signs – illnesses on the rise from mosquito, tick, and flea bites. Downloaded 22 August 2018. https://www.cdc.gov/vitalsigns/pdf/vs-0518-vector-borne-H.pdf.

**Chatonnet, A., and O. Lockridge. 1989.** Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem. J. 260: 625–634.

**Chen, H., Z. Zhou, L. Wang, H. Wang, R. Liu, H. Zhang, and L. Song. 2016.** An invertebrate-specific miRNA targeted the ancient cholinergic neuroendocrine system of oyster. Open Biol. 6: 160059.

**Childs, J. E., and C. D. Paddock. 2003.** The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu. Rev. Entomol. 48: 307–337.

**Darrow, D. I., W. J. Gladney, and C. C. Dawkins. 1976.** Lone star tick: mating behavior of gamma-irradiated males. Ann. Entomol. Soc. Am. 69: 106–108.

**Drolet, B. S., L. M. Reister, C. J. Lehiy, P. A. Van Rijn, and R. A. Bowen. 2015.** Effect of *Culicoides sonorensis* salivary proteins on clinical disease outcome in experimental bluetongue virus serotype 8 infection of Dorset sheep. Vet. Ital. 51: 379–384.

**Dudley, D. M., C. M. Newman, J. Lalli, L. M. Stewart, M. R. Koenig, A. M. Weiler, M. R. Semler, G. L. Barry, K. R. Zarbock, M. S. Mohns,**

**et al. 2017**. Infection via mosquito bite alters Zika virus tissue tropism and replication kinetics in rhesus macaques. Nat. Commun. 8: 2096.

**Fontaine, A., I. Diouf, N. Bakkali, D. Missé, F. Pagès, T. Fusai, C. Rogier, and L. Almeras. 2011**. Implication of haematophagous arthropod salivary proteins in host-vector interactions. Parasit. Vectors 4: 187.

**Fujii, T., and K. Kawashima. 2001**. An independent non-neuronal cholinergic system in lymphocytes. Jpn. J. Pharmacol. 85: 11–15.

**Fujii, T., M. Mashimo, Y. Moriwaki, H. Misawa, S. Ono, K. Horiguchi, and K. Kawashima. 2017a**. Expression and function of the cholinergic system in immune cells. Front. Immunol. 8: 1085.

**Fujii, T., M. Mashimo, Y. Moriwaki, H. Misawa, S. Ono, K. Horiguchi, and K. Kawashima. 2017b**. Physiological functions of the cholinergic system in immune cells. J. Pharmacol. Sci. 134: 1–21.

**Gubler, D. J. 2009**. Vector-borne diseases. Rev. Sci. Tech. 28: 583–588.

**Gladney, W. J., and R. O. Drummond. 1970**. Mating behavior and reproduction of the lone star tick, *Amblyomma americanum*. Ann. Entomol. Soc. Am. 63: 1036–1039.

**Hamel, E. 2004**. Cholinergic modulation of the cortical microvascular bed. Prog. Brain Res. 145: 171–178.

**Hancock, P. A., A. Wiebe, K. A. Gleave, S. Bhatt, E. Cameron, A. Trett, D. Weetman, D. L. Smith, J. Hemingway, M. Coleman, et al. 2018**. Associated patterns of insecticide resistance in field populations of malaria vectors across Africa. Proc. Natl Acad. Sci. USA 115: 5938–5943.

**Horiuchi, Y., R. Kimura, N. Kato, T. Fujii, M. Seki, T. Endo, T. Kato, and K. Kawashima. 2003**. Evolutional study on acetylcholine expression. Life Sci. 72: 1745–1756.

**Jones, L. D., E. Hodgson, and P. A. Nuttall. 1990**. Characterization of tick salivary gland factor(s) that enhance Thogoto virus transmission. Arch. Virol. Suppl. 1: 227–234.

**Jones, L. D., E. Hodgson, T. Williams, S. Higgs, and P. A. Nuttall. 1992**. Saliva activated transmission (SAT) of Thogoto virus: relationship with vector potential of different haematophagous arthropods. Med. Vet. Entomol. 6: 261–265.

**Kakani, E. G., S. Bon, J. Massoulié, and K. D. Mathiopoulos. 2011**. Altered GPI modification of insect AChE improves tolerance to organophosphate insecticides. Insect Biochem. Mol. Biol. 41: 150–158.

**Kawashima, K., T. Fujii, Y. Moriwaki, and H. Misawa. 2012a**. Critical roles of acetylcholine and the muscarinic and nicotinic acetylcholine receptors in the regulation of immune function. Life Sci. 91: 1027–1032.

**Kawashima, K., T. Fujii, Y. Moriwaki, H. Misawa, and K. Horiguchi. 2012b**. Reconciling neuronally and nonneuronally derived acetylcholine in the regulation of immune function. Ann. N.Y. Acad. Sci. 1261: 7–17.

**Kawli, T., F. He, and M.-W. Tan. 2010**. It takes nerves to fight infections: insights on neuro-immune interactions from *C. elegans*. Dis. Model. Mech. 3: 721–731.

**Kazimirova, M., and I. Stibraniova. 2013**. Tick salivary compounds: their role in modulation of host defenses and pathogen transmission. Front. Cell. Infect. Microbiol. 3: 43.

**Kazimírová, M., S. Thangamani, P. Bartíková, M. Hermance, V. Holíková, I. Štibrániová, and P. A. Nuttall. 2017**. Tick-borne viruses and biological processes at the tick-host-virus interface. Front. Cell. Infect. Microbiol. 7: 339.

**Kim, Y. H., and S. H. Lee. 2013**. Which acetylcholinesterase functions as the main catalytic enzyme in the class insecta: insect biochem. Mol. Biol. 43: 47–53.

**Klafke, G., A. Webster, B. Dall Agnol, E. Pradel, J. Silva, L. H. de La Canal, M. Becker, M. F. Osório, M. Mansson, R. Barreto, et al. 2017**. Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul state, Southern Brazil. Ticks Tick. Borne. Dis. 8: 73–80.

**Kovár, L. 2004**. Tick saliva in anti-tick immunity and pathogen transmission. Folia Microbiol. (Praha) 49: 327–336.

**Labuda, M., A. R. Trimnell, M. Ličková, M. Kazimírová, G. M. Davies, O. Lissina, R. S. Hails, and P. A. Nuttall. 2006**. An antivector vaccine protects against a lethal vector-borne pathogen. PLoS Pathog. 2: e27.

**Lehiy, C. J., and B. S. Drolet. 2014**. The salivary secretome of the biting midge, *Culicoides sonorensis*. Peer J 2: e426.

**Lehninger, A. L. 1975**. Biochemistry. 2nd ed. Worth Publishers, Inc., New York.

**Leitner, W. W., A. Costero-Saint Denis, and T. Wali. 2011**. Immunological consequences of arthropod vector-derived salivary factors. Eur. J. Immunol. 41: 3396–3400.

**Leitner, W. W., A. Costero-Saint Denis, and T. Wali. 2012**. Role of immune cell subsets in the establishment of vector-borne infections. Eur. J. Immunol. 42: 3110–3115.

**Leitner, W. W., T. Wali, R. Kincaid, and A. Costero-Saint Denis. 2015**. Arthropod vectors and disease transmission: translational aspects. PLoS Negl. Trop. Dis. 9: e0004107.

**Lestinova, T., I. Rohousova, M. Sima, C. I. de Oliveira, and P. Volf. 2017**. Insights into the sand fly saliva: blood-feeding and immune interactions between sand flies, hosts, and *Leishmania*. PLoS Negl. Trop. Dis. 11: e0005600.

**Liu, Z., L. Wang, Z. Lv, Z. Zhou, W. Wang, M. Li, Q. Yi, L. Qiu, and L. Song. 2018**. The cholinergic and adrenergic autocrine signaling pathway mediates immunomodulation in oyster *Crassostrea gigas*. Front. Immunol. 9: 284.

**Lohmeyer, K. H., and J. M. Pound. 2012**. Laboratory evaluation of novaluron as a development site treatment for controlling larval horn flies, house flies, and stable flies (Diptera: Muscidae). J. Med. Entomol. 49: 647–651.

**Mallet, J. 1989**. The evolution of insecticide resistance: have the insects won? Trends Ecol. Evol. 4: 336–340.

**Manning, J. E., and T. Cantaert. 2019**. Time to micromanage the pathogen-host-vector interface: considerations for vaccine development. Vaccines 7:10.

**Moser, L. A., P. Y. Lim, L. M. Styer, L. D. Kramer, and K. A. Bernard. 2016**. Parameters of mosquito-enhanced West Nile virus infection. J. Virol. 90: 292–299.

**Narasimhan, S., T. R. Schleicher, and E. Fikrig. 2017**. Saliva-assisted pathogen transmission, pp. 265–273. *In* S. K. Wikel, S. Aksoy and G. Dimopoulos (eds.), Arthropod vector: controller of disease transmission, vector saliva-host interactions, vol. 2. Academic Press, Elsevier, San Diego, CA.

**Nizri, E., and T. Brenner. 2013**. Modulation of inflammatory pathways by the immune cholinergic system. Amino Acids 45: 73–85.

**Nuttall, P. A., and M. Labuda. 2004**. Tick-host interactions: saliva-activated transmission. Parasitology 129(Suppl.): S177–S189.

**Nuttall, P. A., and M. Labuda. 2008**. Saliva-assisted transmission of tick-borne pathogens, pp. 205–219. *In* A. S. Bowman and P. A. Nuttall (eds.), Ticks – biology, disease and control. University Printing House, Cambridge, UK.

**Oliveira, C. J. F., W. A. Carvalho, G. R. Garcia, F. R. S. Gutierrez, I. K. F. de Miranda Santos, J. S. Silva, and B. R. Ferreira. 2010**. Tick saliva induces regulatory dendritic cells: MAP-kinases and Toll-like receptor-2 expression as potential targets. Vet. Parasitol. 167: 288–297.

**Pamminger, T., C, Botias, D. Goulson, and W. O. H. Hughes. 2017**. A mechanistic framework to explain the immunosuppressive effects of neurotoxic pesticides on bees. Funct. Ecol. 32: 1921–1930.

**Pingen, M., S. R. Bryden, E. Pondeville, E. Schnettler, A. Kohl, A. Merits, J. K. Fazakerley, G. J. Graham, and C. S. McKimmie. 2016**. Host inflammatory response to mosquito bites enhances the severity of arbovirus infection. Immunity 44: 1455–1469.

**Pingen, M., M. A. Schmid, E. Harris, and C. S. McKimmie. 2017**. Mosquito biting modulates skin response to virus infection. Trends Parasitol. 33: 645–657.

**Pinheiro, L. J., L. F. Paranaíba, A. F. Alves, P. M. Parreiras, N. F. Gontijo, R. P. Soares, and W. L. Tafuri. 2018**. Salivary gland extract modulates the infection of two *Leishmania enriettii* strains by interfering with macrophage differentiation in the model of *Cavia porcellus*. Front. Microbiol. 9: 969.

**Pope, C. N. 1999**. Organophosphorus pesticides; do they all have the same mechanism of toxicity? J. Toxicol. Environ. Health B Crit. Rev. 2: 161–181.

**Racké, K., U. R. Juergens, and S. Matthiesen. 2006**. Control by cholinergic mechanisms. Eur. J. Pharmacol. 533: 57–68.

**Schmid, M. A., D. R. Glasner, S. Shah, D. Michlmayr, L. D. Kramer, and E. Harris. 2016**. Mosquito saliva increases endothelial permeability in the skin, immune cell migration, and dengue pathogenesis during antibody-dependent enhancement. PLoS Pathog. 12: e1005676.

**Shi, X., Z. Zhou, L. Wang, F. Yue, M. Wang, C. Yang, and L. Song. 2012**. The immunomodulation of acetylcholinesterase in Zhikong scallop *Chlamys farreri*. PLoS ONE 7: e30828.

**Šimo, L., M. Kazimirova, J. Richardson, and S. I. Bonnet. 2017**. The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. Front. Cell. Infect. Microbiol. 7: 281.

**Soreq, H. 2015**. Checks and balances on cholinergic signaling in brain and body function. Trends Neurosci. 38: 448–458.

**Styer, L. M., P. Y. Lim, K. L. Louie, R. G. Albright, L. D. Kramer, and K. A. Bernard. 2011**. Mosquito saliva causes enhancement of West Nile virus infection in mice. J. Virol. 85: 1517–1527.

**Surasombatpattana, P., S. Patramool, N. Luplertlop, H. Yssel, and D. Missé. 2012**. *Aedes aegypti* saliva enhances dengue virus infection of human keratinocytes by suppressing innate immune responses. J. Invest. Dermatol. 132: 2103–2105.

**Taylor, P., A. De Jaco, D. Comoletti, M. Miller, and S. Camp. 2013**. Cholinesterase confabs and cousins: approaching forty years. Chem. Biol. Interact. 203: 10–13.

**Temeyer, K. B. 2018**. Molecular biology of tick acetylcholinesterases. Front. Biosci. (Landmark Ed) 23: 1320–1337.

**Temeyer, K. B., and A. C. Chen. 2012**. Acetylcholinesterase of *Stomoxys calcitrans* (L.) (Diptera: Muscidae): cDNA sequence, baculovirus expression, and biochemical properties. Vet. Parasitol. 184: 92–95.

**Temeyer, K. B., and A. P. Tuckow. 2016**. Tick salivary cholinesterase: a probable immunomodulator of host-parasite interactions. J. Med. Entomol. 53: 500–504.

**Temeyer, K. B., J. H. Pruett, P. M. Untalan, and A. C. Chen. 2006**. Baculovirus expression of BmAChE3, a cDNA encoding an acetylcholinesterase of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 43: 707–712.

**Temeyer, K. B., J. H. Pruett, P. U. Olafson, and A. C. Chen. 2007**. R86Q, a mutation in BmAChE3 yielding a *Rhipicephalus microplus* organophosphate-insensitive acetylcholinesterase. J. Med. Entomol. 44: 1013–1018.

**Temeyer, K. B., J. H. Pruett, and P. U. Olafson. 2010**. Baculovirus expression, biochemical characterization and organophosphate sensitivity of rBmAChE1, rBmAChE2, and rBmAChE3 of *Rhipicephalus (Boophilus) microplus*. Vet. Parasitol. 172: 114–121.

**Temeyer, K. B., D. K. Brake, and K. G. Schlechte. 2012**. Acetylcholinesterase of *Haematobia irritans* (Diptera: Muscidae): baculovirus expression, biochemical properties, and organophosphate insensitivity of the G262A mutant. J. Med. Entomol. 49: 589–594.

**Temeyer, K. B., D. K. Brake, A. P. Tuckow, A. Y. Li, and A. A. Pérez de León. 2013**. Acetylcholinesterase of the sand fly, *Phlebotomus papatasi* (Scopoli): cDNA sequence, baculovirus expression and biochemical properties. Parasit. Vectors 6: 31.

**Thangamani, S., S. Higgs, S. Ziegler, D. Vanlandingham, R. Tesh, and S. Wikel. 2010**. Host immune response to mosquito-transmitted chikungunya virus differs from that elicited by needle inoculated virus. PLoS One 5: e12137.

**Warburg, O., and W. Christian. 1941**. Isolierung und Kristallisation des Gärungsferments Enolase. Biochem Z. 310: 384–423.

**Weill, M., P. Fort, A. Berthomieu, M. P. Dubois, N. Pasteur, and M. Raymond. 2002**. A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the ace gene in *Drosophila*. Proc. Biol. Sci. 269: 2007–2016.

**Wessler, I. H. Kilbinger, F. Bittinger, and C. J. Kirkpatrick. 2001**. The biological role of non-neural acetylcholine in plants and humans. Jpn. J. Pharmacol. 85: 2–10.

**WHO. 2016**. Vector-borne diseases. Downloaded 12 September 2017 from http://www.who.int/mediacentre/factsheets/fs387/en/.

**WHO. 2017**. Vector-borne diseases. Downloaded 22 August 2018 from http://www.who.int/en/news-room/fact-sheets/detail/vector-borne-diseases.

**WHO. 2018**. Global report on insecticide resistance in malaria vectors: 2010–2016. Downloaded 21 August 2018 from http://www.who.int/malaria/publications/atoz/9789241514057/en/.

**Wichit, S., P. Ferraris, V. Choumet, and D. Missé. 2016**. The effects of mosquito saliva on dengue virus infectivity in humans. Curr. Opin. Virol. 21: 139–145.

**Wikel, S. 2013**. Ticks and tick-borne pathogens at the cutaneous interface: host defenses, tick countermeasures, and a suitable environment for pathogen establishment. Front. Microbiol. 4: 337.

**Wikel, S., S. Aksoy, and G. Dimopoulos. 2017**. Arthropod vector: controller of disease transmission, vol. 2, 1st ed. Academic Press, New York.

**Wilson, A. L, O. Courtenay, L. A. Kelly-Hope, T. W. Scott, W. Takken, S. J. Torr, and S. W. Lindsay. 2020**. The importance of vector control for the control and elimination of vector-borne diseases. PLoS Neg. Trop. Dis. 14: e0007831.

**Yamamoto, K., and Y. S. Momonoki. 2012**. Tissue localization of maize acetylcholinesterase associated with heat tolerance in plants. Plant Signal. Behav. 7: 301–305.

**Zdanowski, R., M. Krzyzowska, D. Ujazdowska, A. Lewicka, and S. Lewicki. 2015**. Role of alpha-7 nicotinic receptor in the immune system and intracellular signaling pathways. Cent. Eur. J. Immunol. 40: 373–379.