Phagostimulatory dynamics of adenine nucleotides in mosquitoes
- advancing a taste-based delivery method for vector control agents

Matthew Lukenge
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Matthew Lukenge
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Plant Protection Biology
Alnarp

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Abstract

The mosquitoes *Aedes aegypti* and *Anopheles gambiae* are vectors of major medical importance, incriminated in over half a million lives lost annually. While there has been significant focus on understanding the olfaction mechanisms underlying host-seeking in mosquitoes for the development of odour-based control tools, little is known about the mechanisms underlying gustation in blood feeding, the ultimate behaviour in the change of events leading up to disease transmission. An increased understanding of these gustatory mechanisms may be vital for advancing taste-based alternative vector control tools. Using a no-choice membrane feeding assay (papers II, III, and IV) and a spectrophotometric analysis (paper II), the feeding response of the two vectors to blood-derived adenine nucleotide diets, as proxies, and a reliable signal, for blood, was examined. While these experiments revealed that *Ae. aegypti* is more sensitive than *An. gambiae* (paper II) to the adenine nucleotide diets, both species maintained the same selectivity to the diets, with a dose-dependent bimodal feeding pattern. The latter expands the all-or-none blood-feeding theory for haematophagous arthropods to include an initial tasting step (paper II). Adenine nucleotides also regulated the proportion of *An. gambiae* prediuresing in a dose-dependent manner, but did not affect the volume engorged or prediuresed (paper II). A transcriptome analysis of the labrum identified putative genes that are involved in the detection and assessment of blood modalities (paper III). A shift from a high abundance of genes with structural function in the teneral age libraries to genes with cellular, neuro-communicative and modulatory function, including putative ATP receptors, in the 3 days-post-eclosion libraries was observed, providing blood feeding mosquitoes with a structurally sturdy and chemosensory-competent blood feeding organ (paper IV). The assessment of whether the ATP sensory pathway may be a viable way of overcoming the aversive effects of antifeedants and toxicants revealed its superiority over the sugar sensory pathway. ATP induced a reflexive engorgement on toxic meals, which were directed to the midgut, in contrast to sugar-induced meals, which were directed to the crop, an observation that correlated with the rapid mortality rates. Taken together, this study expanded our mechanistic understanding of the phagostimulatory dynamics of adenine nucleotides in *Ae. aegypti* and *An. gambiae*, and the associated putative labral detection receptors in *Ae. aegypti*, with the establishment of a novel workflow for advancing taste-based vector control tools.

Keywords: *Ae. aegypti*, *An. gambiae*, blood feeding, attractive toxic baits, ATP, purinoceptor, transcriptome, labrum, gustation, feeding deterrents
Dedication

To my family; Patience, Keisha and Nicole

... “Quitters never Win and Winners never Quit: Never measure the height of a mountain until you have reached the top, then you will see how low it is” ... Dag Hamerskjold.
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II. Matthew Lukenge, Rickard Ignell, Sharon Rose Hill. Differential sensitivity and specificity of *Aedes aegypti* and *Anopheles gambiae* to adenine nucleotides – an all-or-none response? (submitted manuscript).

III. Matthew Lukenge, Rickard Ignell, Sharon Rose Hill. Age-depandant differential gene abundance in the labrum correlates with its structural maturation and onset of blood-feeding in the yellow fever mosquito, *Aedes aegypti* (manuscript).


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The contribution of Matthew Lukenge to the papers included in this thesis was as follows:

I. Wrote the chapter section “Phagostimulants drive the acceptance of a blood meal in disease vectors” of the book *Sensory ecology of disease vectors*, with input from co-authors Rickard Ignell and Sharon Rose Hill.

II. Participated in the conception and design of the study, as well as performed the experiments. Analysed and interpreted the data. Wrote the manuscript together with the co-authors.

III. Participated in the conception and design of the study, as well as performed the experiments. Analysed and interpreted the data. Wrote the manuscript together with the co-authors.

IV. Participated in the conception and design of the study, as well as performed the experiments. Analysed and interpreted the data. Wrote the manuscript together with the co-authors.
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Abbreviations

ADP  Adenosine diphosphate
ANOVA Analysis of variance
AMP  Adenosine monophosphate
ATP  Adenosine triphosphate
ATSB Attractive toxic sugar bait
cAMP Cyclic adenosine monophosphate
CSPs Chemosensory proteins
dpe  days-post-eclosion
GPCR G-protein coupled receptor
GRNs Gustatory receptor neurons
Gr   Gustatory receptor
Ir   Inotopic receptor
LIPS Lipid binding protein of the saliva
OBP  Odorant binding protein
Or   Odorant receptor
ppk Pickpocket receptor
RBCs Red blood cells
TRP Transient receptor potential
VOCs Volatile organic compounds
ZT   Zeitgeber time
1. Introduction

Despite their minute size, mosquitoes are by far the deadliest animals to humans (Schmidt, 2005), implicated in nearly half of the casualties of the approximately 108 billion people who have populated the earth to date (Winegard, 2019). The Culicidae family is home to close to 3,578 mosquito species (Yee et al., 2022). However, only 9.3% of these are implicated in disease transmission, 2.5% of which are known vectors, sustaining the disease pathogens within the human population (Knight and Stone, 1959; Scott and Takken, 2012; Yee et al., 2022).

Approximately 17% of the mortalities registered under infectious diseases are due to vector-borne diseases, yet malaria (~619,000) and dengue (~40,000) alone make up more than 50% of this burden, with an associated 247 million and 96 million annual cases, respectively (CDC, 2020; World malaria report, 2021), placing the African malaria vector, *Anopheles gambiae sensu stricto* (s.s.) (*An. gambiae* henceforth) and the dengue vector, *Aedes aegypti*, as mosquitoes of major medical importance. Irrefutably, the marked debilitating effects associated with mosquito vectors on human health led to decades-long research on the behaviours leading up to the transmission of pathogens, which have guided the development of the available mosquito control interventions (Clements, 1992).

The sequence of behavioural events conducted by a female mosquito that culminate in a blood meal, during which pathogens may be transmitted, is initiated by the detection of host volatile cues at long and close ranges (Clements, 1992; Takken and Wijnholds, 1997; Takken and Knols, 1999; Grant and Dickens, 2011). In the quest to imbibe a blood meal, the vector ultimately utilises its sensory feeding appendage, the labrum, to assess the quality and palatability of the blood meal through taste (paper I; Lee, 1974). While extensive research has been dedicated to understanding the olfactory aspects of the host-seeking behavioural events (Gillies, 1980; Majeed et al.,
2014; Takken, 1991; Takken and Knols, 1999), the gustatory mechanism involved in blood feeding is largely understudied.

To evade a potential assault from the host while acquiring a blood meal, mosquitoes must swiftly detect and assess the acceptability of the meal based on the blood phagostimulatory components, particularly adenine nucleotides (paper I; Clements, 1992). While the detection process is heavily dependent on the efficiency of the sensory machinery of the labrum (Lee and Craig, 1983; Liscia et al., 1993; Clements, 1999), the specific labral receptors for detecting the blood phagostimulants remain elusive. This information may be vital for refining and advancing innovative taste-based vector control tools. As such, the current study aimed at enhancing our understanding of the underlying dynamics of blood phagostimulation by adenine nucleotides in *Ae. aegypti* and *An. gambiae* from a behavioural and molecular perspective. Additionally, this study aimed at providing foundational proof of concept for using stable adenosine triphosphate (ATP) analogues as alternative feeding drivers (phagostimulants) in Attractive Toxic Bait (ATB) technologies.
2. Background

For a mosquito, the process leading up to blood feeding unfolds in a sequence of events initiated by the detection of a host at long range, locating the host over increasing shorter ranges and then landing on the preferred host (DeGennaro et al., 2013; Spitzen et al., 2013; McMeniman et al., 2014; van Breugel et al., 2015; Hawkes and Gibson, 2016; Cardé, 2015; Hinze et al., 2021). These behaviours are regulated by various host-derived cues, including volatile organic compounds (VOCs), such as carbon dioxide (CO₂), lactic acid and 1-octen-3-ol, as well as visual and thermal signatures, and moisture (Clements, 1992; Cardé, 2015). However, the ultimate decision to accept and imbibe a blood meal is primarily determined by the sensory parts of the blood-feeding mouthpart, the labrum, which also serves as the food canal (Day, 1954; Lee, 1974). Species-specific variations are observed in the mosquito responses to blood and blood-derived diets, based on the blood-associated cues that are detected by the labrum as reliable indicators of an acceptable meal (paper I). These variations may further be influenced by several intrinsic and extrinsic factors, such as the pH of the meal, tonicity, blood group of the host, size of the red blood cells (RBCs) and the bore size of the labrum of the feeding vector (reviewed in paper I).

Over the past decades, there have been significant advances in understanding the mechanisms underlying olfaction in mosquito vectors, which laid a basis for the advancement of semiochemical-based surveillance and control tools (Wooding et al., 2020). Although gustation-based approaches are a promising strategy in integrated vector control management (Fiorenzano et al., 2017; Baik and Carlson, 2020), our understanding of the underlying blood phagostimulatory dynamics in blood-meal detection and assessment remains under-investigated, thereby limiting further progress.

Upon perforation of the skin of the host, the labrum shears through the blood vessel walls (Born and Kratzer, 1984; Jové et al., 2020), gaining access to the non-cellular and cellular blood components, as well as intracellular molecules, predominantly the adenine nucleotides (Clements, 1992; Day, 1954). The labrum detects individual key blood phagostimulatory modalities, but only through combinatorial sensory integration is engorgement elicited (Jové et al., 2020). Among the blood modalities, haematophagous vectors use adenine nucleotides, predominantly adenosine triphosphate (ATP), as reliable signals for a high-quality blood meal (paper I; Hosoi, 1958). To date, the molecular machinery in the labrum that detects these blood modalities is not fully described. An increased understanding of the molecular aspects related to blood feeding, particularly, the identification
of the ATP receptors, may offer promising genetic manipulation targets in the control of haematophagous vectors, given the crucial relevance of blood feeding in their reproductive cycle.

While earlier research endeavoured to improve our comprehension of the blood phagostimulatory dynamics underlying blood feeding, the available information is generally difficult, if not impossible, to compare due to differences in the experimental design, such as the age of exposed vectors, pH of the solutions, feeding membranes used and the duration of exposure to the meals (Hosoi, 1958, 1959; Galun et al., 1963; Galun, 1967; Galun et al., 1985a,b; Galun and Vardimon-Friedman, 1992). Such information is vital in the establishment of species-targeted and generalised tools aimed at a wider scope of disease vectors.

Through an ecological and behavioural understanding of sugar-feeding in mosquitoes, the taste-based attractive toxic bait technology using sugar as a feeding stimulant was developed (Fiorenzano et al., 2017). Limitations set by this technology, such as the effect on non-target organisms, the sugar-feeding driver being prone to resistance and prone to competition with natural sugar sources, as well as not targeting obligate blood feeders (see section 2.5.3), have opened up an avenue for exploring blood-based alternative feeding drivers such as ATP-stable analogues (Fiorenzano et al., 2017). Consequently, to advance the use of taste-based control tools and improve strategies during their field application, there is a need to expand our mechanistic understanding of the blood phagostimulatory dynamics in mosquito vectors through an increased understanding of their ecology and life history.

2.1 Life history of Ae. aegypti and An. gambiae

Both Ae. aegypti and An. gambiae undergo complete metamorphosis, progressing from egg through four larval instars to pupae, all of which are aquatic stages (Clements, 1992). Teneral adults then emerge 24 – 36 h post-pupation, initially dedicating the first hours to resting and acclimatising to terrestrial life by hardening their cuticular exoskeleton (Christophers, 1960; Clements, 1992). Approximately 1 day-post-eclosion (dpe), both mosquito sexes begin to seek for floral and extra-floral nectaries, (fruits, plant tissues and honeydew) (Takken and Knols, 1999; Stone and Foster, 2013; Nyasembe et al., 2014) to gain the energy required to meet the metabolic needs for flight and reproduction (Nayar and Van Handel, 1971). This behaviour also significantly influences the longevity, flight range, insemination rates, egg maturation, ovarian development and overall
vectorial capacity (Clements, 1992; Klowden, 1995; Nyasembe et al., 2014). Depending on the accumulated energy reserves during the aquatic phase, females typically begin to seek a vertebrate host for a blood meal around age 3 dpe, a behaviour which peaks between age 5-7 dpe (Clements, 1992, 1999; Klowden, 1995).

2.1.1 Host seeking leading to blood feeding in mosquitoes

Host seeking in female mosquitoes is a multimodal process of locating a suitable host during the search for a blood meal and relies on the integration of chemosensory, visual and hygrothermal information (Spitzen et al. 2008; McMeniman et al., 2014; Hinze et al., 2021; van Breugel et al., 2022). Following mating, females engage in host seeking (Clements, 1999), with human and animal-exhaled CO₂ serving as a long-range cue, which is detectable at distances exceeding 50 meters (Gillies, 1980; Zollner et al., 2004; Lorenz et al., 2013). Aedes aegypti, and the majority of mosquito vectors, are activated by and attracted to CO₂ (Dekker et al., 2005; McMeniman et al., 2014; van Breugel et al., 2015; Webster et al., 2015; Vinauger et al., 2019), unlike An. gambiae, in which CO₂ induces negligible activation and attraction, unless present in combination with host odours (Takken, 1991; Takken et al., 1997; Spitzen, et al., 2008; Hinze et al., 2021). In all species studied, CO₂ enhances the attraction to the host by gating the behavioural response of the vector to host odour, visual and thermal cues (Spitzen, et al., 2008; McMeniman et al., 2014; Hinze et al., 2021; van Breugel, Jewell and Houle, 2022). At the intermediate distance, approximately 5-10 m from the host, mosquitoes not only encounter CO₂ but also host-derived volatile organic compounds (VOCs), such as 1-octen-3-ol, sulcatone, ammonia and lactic acid (Clements, 1992; Grant and Dickens, 2011; van Loon et al., 2015). As the mosquito approaches the host, the host-derived VOCs appear to become more crucial than CO₂ in determining the location of the host (Bidlingmayer and Hem, 1980; Dekker and Cardé, 2011; van Breugel et al., 2015). This additional layer of information prompts a stereotypical zig-zag flight motion directed towards the host (Kennedy, 1940; Dekker and Cardé, 2011; van Breugel et al., 2015; Hinze et al., 2021). Despite having lower visual acuity compared to other insects (Land, 1997; Kawada et al., 2006), mosquitoes can perceive objects the size of a human host (Bidlingmayer and Hem, 1980) and discern achromatic and chromatic visual features, further assisting the mosquito in the assessment of the target host (Alonso et al., 2022; van Breugel et al., 2022).
When the mosquitoes approach the host at close-range, hygrothermal signatures and low-volatile VOCs become the most relevant cues for the mosquitoes, and provide critical information about the landing sites on the host (Spitzen et al., 2008; McMeniman et al., 2014; Hinze et al., 2021; van Breugel et al., 2022). Upon landing, a gustatory gating process is initiated, during which the mosquito employs its tarsal and labellar sensory apparatus to locate a suitable spot for perforating the skin with the fascicle comprised of six styles (see section 2.3.1) (Jones and Pilitt, 1973; Jones, 1978; Klowden, 1995). Through guided manoeuvres, the labrum gains entry into the blood capillary bed and assesses the quality and palatability of the blood meal, ultimately leading into blood feeding (Jones and Pilitt, 1973; Jones, 1981).

2.2 Blood feeding
Blood feeding, or haematophagy, in arthropods, including mosquitoes, is a crucial behaviour for acquiring the proteins required for successful vitellogenesis (Clements, 1992). However, this behaviour is also highly risky as it exposes the vectors to potential harm from the host (Lehane, 2005). As such, its execution must be swift and discrete to minimise the defensive responses of the host. To counteract the haemostatic processes of the host, mosquitoes employ a strategic approach by first releasing anti-haemostatic compounds from their saliva into the host (Clements, 1992; Ribeiro, 1995; Arcà and Ribeiro, 2018). While saliva serves as a conduit for the transfer of disease pathogens, its main role is to dampen the pro-inflammatory and coagulation immune response factors from the host (Clements, 1992; Arcà and Ribeiro, 2018). The following sections explore the evolution of haematophagy in arthropods, and the events leading up to blood feeding, with a focus on the dynamics surrounding phagostimulation as the ultimate step to blood feeding.

2.2.1 Evolution of haematophagy
Haematophagy is a specialised feeding behaviour in arthropods presumed to have arisen in response to nutrient resource limitations, particularly the need for a protein-rich meal to support egg development as seen in mosquito vectors (Huff, 1929; Klowden, 1995; Lehane, 2005; Azar and Nel, 2012) or, to acquire salt, as seen in Calyptra moths feeding on salt-limited fruits (Zaspel, 2008). This unique feeding strategy has evolved independently at least five times at the order level within the past 150-200 million years (Lehane, 2005), resulting in the emergence of over 400 haematophagous
arthropod genera (Ribeiro, 1995; Mans, 2011). This evolutionary process, characterised by several morphological, physiological and behavioural adaptations (Lehane, 2005), likely occurred through convergent evolution (Snodgrass, 1945; Adams, 1999).

Two pathways have been proposed for the evolution of the blood-feeding habit in haematophagous arthropods. The first involves a prolonged and close association between the arthropod and a vertebrate host, in which arthropods with chewing mouthparts, feeding on hair, feathers and skin-sloughs, unintentionally gained access to the blood of the host (Waage, 1979; Lehane, 2005; Peach and Gries, 2020). Pool-feeding insects, such as tsetse flies and lice, are thought to have followed this evolutionary pathway (Waage, 1979). The second theory is premised on the existence of insects that were morphologically pre-adapted with piercing-sucking mouthparts, which accidentally fed on vertebrate hosts (Waage, 1979; Lehane, 2005; Peach and Gries, 2020). Selection subsequently favoured insects with physiological and behavioural adaptations that enhanced their ability to locate hosts and digest blood meals (Waage, 1979; Lehane, 2005; Zaspel, 2008; Hill et al., 2010). This conceivably facilitated the development of facultative blood feeders that had an occasional association with the hosts (Waage, 1979; Lehane, 2005). Moreover, a prolonged and close association with a vertebrate host is presumed to have facilitated the transition of the initial facultative feeders to becoming obligate blood feeders (Waage, 1979; Peach and Gries, 2020).

Mosquitoes are believed to have evolved following a process outlined in the second theory. Elongated mosquito mouthparts suggest ancestors that may have been originally feeding on either plant fluids or on plants and insect haemolymph (Waage, 1979; Peach and Gries, 2020). The shift from either plants and/or other insects to vertebrate hosts is believed to have been facilitated by the substantial overlap in floral, larval and vertebrate odour cues (Ignell and Hill, 2020; Peach and Matthews, 2022). Female mosquitoes that subsequently adopted blood-feeding likely evolved as a result of a close association with vertebrates and accidental feeding on these hosts (Waage, 1979; Lehane, 2005). Irrespective of the pathway taken, most haematophagous arthropods share commonalities in the sensory mechanisms used to detect and evaluate the quality and palatability of the blood meal (paper I).
2.3 The peripheral mosquito gustatory system

Gustation, or contact chemosensation, broadly involves the detection of non-volatile compounds using sensory appendages associated with taste, predominantly the proboscis and legs (Lee, 1974; Rossignol and McIver, 1977; Stocker, 1994; Clements, 1999; Sparks and Dickens, 2017). The legs play a vital role in the initial detection of either a sugar meal or in locating the piercing site on the skin of the host to access a blood meal (Clements, 1992). However, the final assessment of the sugar or blood meal is attributed to sensory structures on the labellum and labrum and in the cibarium (Christophers, 1960; Clements, 1992). Therefore, gustation during feeding in female mosquitoes involves structurally dimorphic appendages and functionally distinct sensory programs (Lee and Craig, 1983, 2009; Klowden, 1995; Jové et al., 2020), which are involved in the detection and evaluation of cues leading to either sugar-feeding or blood-feeding (Christophers, 1960; Klowden, 1995). While the labrum is also involved in the sugar-feeding process, the focus hereafter is on the aspects of blood feeding.

2.3.1 Structure of the main blood-feeding gustatory organ

The female labrum is a component of a fascicle with six stylets (Figure 1a), comprising pairs of chewing maxilla and serrated mandibles, and a salivary conduit, constructed from the hypopharynx and the labrum (epipharynx) (Lee, 1974; Lee and Craig, 1983). Serving as a feeding straw, the labrum connects to the cibarium, which acts as a suction pump, leading the meal to pass across the cibarial sensilla (Lee and Craig, 1983). These sensilla are believed to complement the sensory function of the apical and subapical labral sensilla during blood feeding and also determine the final destination of the meals, either to the crop (sugar) or to the midgut (blood) (Day, 1954; Lee, 1974; Klowden, 1995). While all the stylets gain access to the blood meal, it is noteworthy that only the labrum possesses chemosensory abilities.

2.3.2 The labral sensory system

At the distal end of the labrum lies a pair of basiconic apical and subapical sensilla (Figure 1b), which are involved in detecting and assessing the quality of a blood meal (Lee and Craig, 1983, 2009). Positioned approximately 20 µm behind them are the campaniform sensilla, associated with detecting the flow of blood (Lee and Craig, 1983; Jové et al., 2020). Morphologically, the apical and subapical sensilla are short, single-walled, and contain an apical
uni-pored sensilla housing four gustatory receptor neurons (GRNs) and one mechanosensory neuron (Figure 1c). The dendritic projections of the GRNs terminate at the tip of the pore and are bathed in sensillum lymph (Lee, 1974; Lee and Craig, 1983; Jové et al., 2020), while the GRNs project their axons to the antero-ventral sub-oesophageal ganglion of the brain (Jové et al., 2020). The sensillum lymph is generated by the support cells, i.e., trichogen, tormogen, and thecogen (paper I; Lee, 1974; Figure 1c).

The labral sensilla comprises sub-populations of GRNs that detect different blood modalities, categorised as the adenine nucleotide neurons, salt neuron, bicarbonate neuron and integrator neuron (Liscia et al., 1993; Werner-Reiss et al., 1999a; Jové et al., 2020). Different species rely on a specific subset of taste cues to assess the quality of the blood meal, which aligns with the narrow number of gustatory sensilla and GRNs in the labrum.

Figure 1. Mouthparts and sensory apparatus involved in blood feeding of the mosquito *Aedes aegypti*. a) A schematic illustration of the fascicles, i.e., mandible, maxillae, hypopharynx and the labrum, which pierce the skin and provide the tube through which the blood meal is imbibed, housed within the labium of the proboscis. b) Scanning electron micrograph of the distal part of the labrum, together with the apical and sub-apical sensilla. c) Schematic illustration of a taste sensillum housing four gustatory receptor neurons and one mechanosensory neuron (reproduced from Lukenge et al. 2022).

(Lee, 1974; Lee and Craig, 1983; Liscia et al., 1993). While it is unclear for other mosquito species, it is only upon the presence of all key blood ligands that *Ae. aegypti* will engorge on a meal, suggesting a combinatorial coding of the response of the four functionally distinct stylet neuron classes, each
tuned to specific blood components associated with diverse taste qualities (Jové et al., 2020). Besides these GRNs, there is a subpopulation of labral sensilla neurons that does not respond to any of the above modalities, presumably detecting other sensory cues, such as feeding deterrents, noxious compounds, thermal cues, as well as olfactory cues (Lee and Craig, 2009; Jové et al., 2020).

2.3.3 Taste modalities associated with haematophagy

The evaluation of blood quality in disease vectors primarily relies on two human-ascribed taste modalities, salty (e.g., NaCl), sour (e.g., NaHCO), and a non-canonical modality based on the detection of adenine nucleotides (Hosoi, 1958; Galun et al., 1963; Galun and Kabayo, 1988; Galun, et al., 1988; Galun and Vardimon-Friedman, 1992; Jové et al., 2020). Notably, the canonical taste of umami (protein) is not required for engorgement (Kogan, 1990; Gonzales et al., Hansen, 2015), however, modalities for sweet (glucose) and feeding deterrents seem necessary for blood meal assessment and acceptance (Kogan, 1990; Jové, et al., 2020).

2.3.4 Adenine nucleotides are the major blood phagostimulants

In mosquitoes, the ligands activating blood feeding are primarily derived from the cellular components of whole blood (Hosoi, 1958; Galun et al., 1963), especially phagostimulants released from RBCs (Hosoi, 1959; Friend and Smith, 1982). Although plasma components, such as NaCl and NaHCO₃, are generally less potent, they have been reported to be phagostimulatory in certain mosquito species (Galun et al., 1985a), triatomines (Friend and Smith, 1982) and sandflies (Ready, 1978). Liberated from the cellular components of blood, the adenine nucleotides, i.e., ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP) are prominent feeding stimulants (paper I). In particular, ATP, is recognized as a key phagostimulant for several mosquito species, triatomine bugs, horse flies, bed bugs, tsetse flies, rat fleas, stable flies, ticks and blowflies (paper I). As such, ATP, which is abundant within blood cellular components, and is rapidly degraded by blood ecto-nucleotidases to its downstream products ADP and AMP upon cell lysis, serves as an indicator of fresh blood and is thus a reliable signal of an acceptable blood meal.

2.3.5 Variation in adenine nucleotide structure affects phagostimulation

Adenine nucleotides exert their influence on phagostimulation through three key structural components: the phosphate group, the amino group on the
purine (C6) and the hydroxyl groups on the ribose sugar (C2-OH and C3-OH). Manipulations on these active sites vary the levels of phagostimulation in the majority of disease vectors (details in paper I). For instance, the removal or addition of phosphate groups from ATP, resulting in ADP, AMP or adenosine tetraphosphate (AtetraP), demonstrates a positive correlation with feeding stimulation in various blood-feeding arthropods, while complete removal of the phosphate groups eliminates phagostimulation in most blood-feeding arthropods, except for Simulium venestum, which responds to adenosine (Sutcliffe and McIver, 1979). Moreover, non-hydrolysable and stable analogues of ATP, such as adenylylimidodiphosphate (AMP-PNP) and adenylylmethylenediphosphate (AMP-PCP), were demonstrated to be more potent phagostimulants in Ae. aegypti (Galun et al., 1985) and Tabanus nigrovittatus (Friend and Stoffolano, 1983). This highlights their future potential usage in taste-based tools. The strong potency of non-hydrolysable ATP analogues emphasises that ATP-induced stimulation is not energy-dependent and that ATP and its analogues act as phagostimulants with a direct correlation between phagostimulation intensity and binding affinity strength. While ATP is the most potent naturally occurring nucleotide in a wide variety of genera, exceptions exist. For instance, ADP serves as the key phagostimulant in several Culex mosquito species and the black fly S. venestum (Galun et al., 1963). Moreover, sensitivity to ATP may differ between strains (Galun et al., 1985).

2.3.6 Other factors affecting phagostimulation
Apart from the factors discussed earlier, blood contains various components that directly or indirectly influence phagostimulation in mosquitoes. Three such factors are a) the intrinsic ionic environment, b) blood group antigens and c) the source and size of the red blood cells (paper I). For instance, a) the intrinsic ionic environment: Dissolving ATP with inorganic chlorides, especially NaCl and CaCl₂, significantly enhances its phagostimulatory potency compared to other solutions (Galun et al., 1985). Additionally, the salivary enzyme apyrase, responsible for breaking down adenine nucleotides, requires divalent ions, such as calcium and magnesium, as cofactors (Friend and Stoffolano, 1983; Galun et al., 1985b). Consequently, ATP and other adenine nucleotides undergo degradation during feeding in the presence of these ions, diminishing the overall potential of adenylated phagostimulants. Hence, not only the ligands but also the physical and chemical properties of the diet impact phagostimulation during blood
feeding. b) Blood group antigens: Mosquitoes exhibit selective landing and feeding preferences based on ABO blood types, which are characterized by the presence or absence of antigens on the RBCs (Watkins, 1966; Schenkel-Brunner, 1980). Species, such as *Ae. aegypti* (Wood, 1976) and *Anopheles* spp. (Anjomruz et al., 2014a,b) show a general preference for individuals with blood group O over those with A or B antigens. The ecological significance of this preference remains unclear, as there is no evidence suggesting a relative fitness advantage. However, it is conceivable that the preference is linked to the higher concentration of the precursor H antigen in type O blood, potentially selected over evolutionary time. c) The source and size of RBCs: Mosquitoes obtain intracellular protein content within RBCs through haemolysis, either enzymatically in the gut or by mechanical shearing as the RBCs pass through the cibarial armature situated anterior to the foregut (Coluzzi et al., 1982). Mosquitoes with more derived armature are more likely to utilise nucleated blood from birds (e.g., *Culex* spp.) and reptiles (Henning, 1972) compared to mosquitoes with less derived armatures (e.g., *Anopheles* spp.) (Coluzzi et al., 1982). Additionally, *Culex* ssp. have a wide bore diameter of the labrum, a structural modification that may contribute to their host preference (details in paper I).

### 2.4 Molecular basis of the taste function of the labrum

Both behavioural and molecular studies emphasize a dual role of the labrum in gustation and olfaction (Choo et al., 2015; Won Jung et al., 2015; Jové et al., 2020). Not only does the labrum express genes encoding for cuticular proteins linked to its structural modification during blood feeding (Arnoldi et al., 2022), but also membrane-bound proteins, which may be putative receptors for blood-associated ligands. The proper function of the membrane-bound proteins is likely supported by soluble binding proteins, which hypothetically preserve and protect the integrity of the blood ligands, and act as molecular chaperons trafficking the soluble ligands to their respective membrane-bound proteins (Liman et al., 2014; Jové et al., 2020). These membrane-bound proteins/receptors are associated with the detection of the canonical blood-appetitive signals (low salt, sweet and sour) and the blood-non-appetitive signals (high salt), as well as the non-canonical appetitive adenine nucleotides signal (Jové et al., 2020; Liseia et al., 1993; Werner-Reiss et al., 1999a,b). Moreover, recent behavioural findings demonstrate that the labrum detects feeding deterrent compounds (paper III; Jové et al., 2020; Kessler et al., 2014), suggesting the expression of receptors for these compounds in the labrum.
2.4.1 Soluble binding proteins
As observed in the olfactory organs, the labrum expresses soluble binding proteins, i.e., odorant binding proteins (OBPs) and chemosensory proteins (CSPs) (Jové, et al., 2020; Pelosi et al., 2014). The OBPs and CSPs are believed to bind and transport hydrophobic odorants (Pelosi et al., 2014) and tastants (Swarup et al., 2014) within the blood to their respective receptors (Jové et al., 2020), however, functional characterisation studies are still needed to validate this hypothesis. Recently, the salivary D7 proteins, one of the most abundant components in the salivary glands of haematophagous vectors, and demonstrated to enhance blood feeding (Martin-Martín et al., 2020), were reported to be highly expressed in the blood-feeding ages of *Culex quinquefasciatus* and *Ae. aegypti* (paper III; Jové et al., 2020). The D7 proteins are distantly related to the arthropod OBP superfamily, and are part of the salivary anti-haemostatic arsenal of mosquitoes, which target biogenic amines, leukotrienes and adenine nucleotides involved in blood coagulation (Martin-Martín et al., 2020). As such, they are believed to play a fundamental role as molecular chaperons, trafficking ADP and/or ATP as reliable indicators of a fresh blood meal, to their respective neuronal membrane-bound proteins.

2.4.2 Membrane-bound proteins
The neuronal dendrites of the GRNs in the labrum express membrane-bound receptor proteins that act as molecular detectors with variable sensitivity and specificity to blood ligands during blood feeding (Lee and Craig, 1983; Jové et al., 2020). Binding of the ligand by a receptor leads to transduction of the chemical information into an electrical signal, which is sent along the axons to higher brain centres where the information is integrated, ultimately leading to an appropriate feeding behaviour. Previous studies have revealed a diversity of receptor families expressed in the labrum, including the tuning receptors belonging to the gustatory receptors (GRs) odorant receptors (ORs), ionotropic receptors (IRs), transient receptor potential receptors (TRPs) and pickpocket receptors (ppks) (Choo et al., 2015; Jové et al., 2020; Won Jung et al., 2015). Reliable expression of Or and Ir coreceptors i.e., *Orco*, as well as *Ir25a, Ir8a* and *Ir76b*, respectively, has also been demonstrated (Choo et al., 2015; Jové et al., 2020; Won Jung et al., 2015). However, conclusive evidence of their associated ligands is missing. While no functional studies have been done to demonstrate the link between the putative labral receptors and their associated blood modalities, several
deductions have been made based on molecular and behavioural evidence. The detection of NaCl and NaHCO₃ has been linked to the expression of the salt and sour-sensing Ir7y.1 receptor as well as the sour-sensing Ir7a receptor tuned to a broad range of NaHCO₃ concentrations (Jaeger et al., 2018a; Jové et al., 2020). The labrum also expresses odorant receptors sensitive to blood odours, e.g., the Cx. quinquefasciatus receptor CquiOr99 detects 4-ethylphenol, a common phenolic blood-associated VOC (Choo et al., 2015). Similarly, findings from Won Jung et al., (2015) demonstrated the labral expression of Or8 and Or49 and their involvement in the detection of blood alcohols, carboxylic acids and their esters, such as 1-octen-3-ol, cyclohexanol and ethyl acetate in Ae. aegypti.

2.5 Taste-based toxic bait technology
The decline in mosquito-borne diseases over the past decades is mainly credited to efforts that decreased the mosquito populations (WHO, 2022). However, the cardinal tools for controlling the mosquito vectors are facing challenges due to the increase in physiological and behavioural resistance to chemical insecticides, and unintended effects on non-target organisms (Milam et al., 2000; Sanou et al., 2021; Taillebois and Thany, 2022). It is worth highlighting that the primary control methods, i.e., indoor residual sprays and long-lasting treated bed-nets exploit the endophilic and endophagic nature of host-seeking vectors, leaving people more vulnerable when outdoors. This underscores the importance of supplementary and/or alternative eco-friendly and fast-acting tools with alternative modes of action, including those targeting vectors in outdoor environments. In that regard, taste-based control tools are some of the promising supplementary mosquito control ventures that require more attention (Fiorenzano et al., 2017). Although the attractive toxic sugar bait (ATSB) technology in mosquito control had previously received little attention, this has picked up momentum over the past 60 years (Lea, 1965), with further modifications showing promising advances in the efficiency of the technology (Fiorenzano et al., 2017).

2.5.1 Working principle and application of the ATSB
The mosquito ATSB technology capitalises on the metabolic needs, and the ecology of the mosquito to induce oral-based toxicity in the vectors (Fiorenzano et al., 2017). Adult mosquitoes, regardless of sex, intermittently seek for and feed on nectar and other sugar sources, and regularly return to these sources throughout their lives (Clements, 1992; Stone and Foster,
The ATSB technology leverages this nutritional requirement, as it utilises floral cues to attract the vectors towards a sugar-feeding stimulant laced with toxins (Fiorenzano et al., 2017). The sugar induces a feeding response, leading to the ingestion of the incorporated gut toxin, directing the meal to the crop, a storage organ (Xue et al., 2006). As the energy demands increase, small boluses of the meal are released from the crop down to the midgut, in turn disrupting the mosquito midgut epithelium, affecting digestion and metabolism, and/or inducing neurotoxicity (Sippy et al., 2020; Kumar et al., 2022). To maximise the efficiency of the bait, the baits are placed near mosquito resting environments, such as foliage near host habitats, enhancing the likelihood of mosquito contact with the toxins (Fiorenzano et al., 2017).

### 2.5.2 Active ingredients and modifications of ATSBs

Building upon the groundwork by Lea (1965), advancements and modifications of the ATSBs have improved the technology. Notable improvements include 1) diversification of the active ingredients through the establishment of a) chemical compounds with low toxicity, b) synergistic combinations of these compounds, and c) substances with different modes of action, including an array of transgenic agents (dsRNA: RNA interference) (Coy et al., 2012) and para-transgenic bio-pesticides, such as *Bacillus sphaericus* (Xue and Barnard, 2003; Schlein and Müller, 2010, 2015) 2) Enhancements in the design and application strategy have led to ATSBs being sprayed onto vegetation, *i.e.*, on foliar plant parts of flowering plants or directly onto non-flowering plants, and have been successful in controlling several mosquito species outdoors, having decreased the toxic effect on pollinators (Xue et al., 2006; Müller and Galili, 2016; Furnival-Adams et al., 2020).

Carbon dioxide has been incorporated in ATSBs for *Ae. aegypti* and *Ochlerotatus taeniorhynchus* (Xue et al., 2008) to increase the attractiveness of the tool. Replacement of floral attractants with human-associated cues has also been exploited in the ATSB technology. The host kairomones L-lactic acid and 1-octen-3-ol were demonstrated to improve mosquito attraction towards the bait for *Aedes albopictus* and *Ae. aegypti* (Fiorenzano et al., 2017; Scott-Fiorenzano et al., 2017), however, the addition of kairomones as the attractant reduced the efficiency of the ATSBs (Fiorenzano et al., 2017). In the presence of host cues, *Ae. aegypti* reject a sugar meal (Bishop and Gilchrist, 1946; Jové et al., 2020), which is
hypothesised to be a control mechanism so that a vector in need of blood does not accidentally take in sugar and vice versa (Bishop and Gilchrist, 1946; Jové et al., 2020). The enhanced attraction of mosquitoes by host-kairomones in the ATSB technology (Fiorenzano et al., 2017; Scott-Fiorenzano et al., 2017), can potentially be leveraged by using blood-related feeding stimulants, such as ATP-stable analogues. Irrespective of the modifications and advances that have improved the technology thus far, the residual challenges that constrain the effectiveness and acceptability of the technology require close attention.

2.5.3 Challenges of sugar-based attractive toxic bait tools

While sugar-based control tools show promise in vector control, they present several limitations for the ATSB technology; as 1) Sucrose may not efficiently override the aversive effects of the toxicants since it does not induce a reflexive and rapid feeding response as observed when blood-related feeding stimulants are used (paper IV; Christophers, 1960; Kessler et al., 2014); 2) The use of sucrose as a non-selective phagostimulant affects non-target sugar-seeking organisms, e.g., pollinators (Fiorenzano et al., 2017); 3) Sucrose-induced feeding elicits slow toxicity as a result of inducing feeding with small meal volumes that is first directed to the impermeable crop before small boluses of the meal are released down to the permeable midgut as metabolic demands increase. As such, sucrose-induced feeding requires a long time to cumulate lethal doses of the toxicant in the midgut: 4) The reliance on sugar phagostimulants is prone to resistance (Wada-Katsumata et al., 2013, 2018); 5) The readily available natural sugar sources, e.g., floral and extra-floral nectaries, exert intense competition between the bait sugar source and the natural sources (Beier et al., 2012); 6) A number of haematophagous vectors are obligate blood feeders (Rio et al., 2016), and are thus not targeted by ATSBs. Lastly, 7) the intermittent and small-volumes imbibed by mosquitoes (Clements, 1999) appear to necessitate multiple visits to an ATSB station to accumulate a sufficient level of toxicity for lethality (Fiorenzano et al., 2017). In light of these challenges, it is crucial to explore alternative feeding drivers to sugar with higher potency and selectivity. Such attributes are likely to be found in blood-related phagostimulatory compounds, such as ATP-related stable analogues.
3. Aim and objectives

The main aim of this thesis was to expand the mechanistic understanding of the phagostimulatory dynamics of blood-derived adenine nucleotides in *Ae. aegypti* and *An. gambiae s.s.*, including the identification of putative blood detection receptors in the labrum, while advancing the development of a novel taste-based method for the delivery of toxic and/or vector modifying agents.

The first objective was to examine the existing body of literature surrounding blood phagostimulation in haematophagous vectors (paper I).

The second objective was to compare the sensitivity and specificity of *Ae. aegypti* and *An. gambiae* in response to feeding on the blood-derived adenine nucleotides (paper II).

The third objective was to identify candidate receptor(s) in the labrum associated with blood detection, predominantly the major phagostimulant adenosine triphosphate in *Ae. aegypti* (paper III).

The fourth objective was to examine the ability of ATP to override the aversive effects of antifeedants and toxicants, and its associated induced toxicity rates in *Ae. aegypti* (paper IV).
4. Materials and Methods

4.1 Mosquito colony maintenance

In the three projects, laboratory colonies of An. gambiae (G3) (paper II) and/or Ae. aegypti (Rockefeller) (papers II, III and IV) were used. Both species were maintained under the same standard rearing conditions, 25 ± 2 °C, 70 ± 5% relative humidity and at a 12 h:12 h light-dark photoperiod, as previously described (Ignell et al., 2010; Omondi et al., 2015). Briefly, eggs of the two species were placed in separate larval trays (23.5 cm × 18 cm × 7.5 cm; filled with ca. 300 ml water) into which the larvae hatched. Each tray contained ca. 300 larvae that were fed daily on Tetramin® fish food (Tetra GmbH, Melle, Germany). The emerged pupae were collected into 30 ml cups (Nolato-Hertila, Åstorp, Sweden) and transferred to Bugdorm cages (30 cm × 30 cm × 30 cm; MegaView Science, Taichung, Taiwan) into which adults emerged. Adult mosquitoes were maintained with ad libitum access to 10% sucrose and females fed on sheep blood (Håtuna Lab, Bro, Sweden) using a membrane feeding system (Hemotek Ltd, Blackburn, UK).

4.2 Preparation of mosquitoes for the experiments

4.2.1 Sensitivity and specificity to adenine nucleotides

While no studies have been conducted to compare the phagostimulatory response of An. gambiae to the four artificial blood-derived adenine nucleotide diets (ATP, ADP, AMP and cAMP), the effect of these phagostimulants has been assessed in Ae. aegypti, yet under various experimental conditions, e.g., various ages of the test mosquitoes, pH of the meals, as well as exposure period and type of membrane used in the feeders.
(Hosoi 1958, 1959; Galun 1967; Galun et al., 1984, 1985a,b). This study analysed both species under similar conditions, using a collagen membrane feeding system for 30 min at blood physiological pH (7.4 ± 0.07). The experiments were conducted within the peak feeding time of each species, i.e., photophase (Zeitgeber time, ZT 8-10; Ae. aegypti: paper II, III, IV) and scotophase (ZT; 13-15; An. gambiae; paper II) (Trpis et al., 1973; Jones 1978, 1981). In brief, 5 days post-eclosion (dpe) non-blood-fed females, previously provided access to 10% sucrose until 4 (dpe), were used. Thereafter, the mosquitoes were starved (22 ± 2 h) with access to water before the start of the feeding experiments (paper II). The same protocol was used for Ae. aegypti for assessing the phagostimulatory efficiency of ATP to override the aversive effects of feeding deterrents and toxicants (paper IV).

4.2.2 Preparation of Ae. aegypti for transcriptome analysis
Mosquitoes prepared for labral tissue dissection, in preparation for total RNA extraction, were divided into three age groups, 1 dpe, 3 dpe and 5 dpe. Teneral (1 dpe) Ae. aegypti were immediately sugar-starved after emergence and maintained on water until the dissection, whereas 3 dpe and 5 dpe mosquitoes were maintained on 10% sucrose for up to two or four days, and then sugar-starved with access to only water, 22 ± 2 h before tissue collection (paper III).

4.3 Behaviour and volumetric response to adenine nucleotides
In a no-choice feeding assay (section 4.2.1 above), starved female mosquitoes of both mosquito species were exposed to adenine nucleotide diets delivered through collagen membrane-covered reservoirs using a membrane feeding system (Hemotek Ltd.). Four adenine nucleotides, i.e., ATP, ADP, AMP and cyclic adenosine monophosphate (cAMP), were dissolved in bicarbonate buffered saline, adjusted to the physiological pH of blood (7.4 ± 0.07) at 37°C. Xylene cyanol was added (1 mg ml⁻¹; Merck, Darmstadt, Germany) to increase the visibility of the meal during engorgement scoring and for assessing the volume imbibed. The experiment was set to run for a 30 min exposure period. The proportion of engorged mosquitoes with a distended blue-coloured abdomen was scored using an established scoring scale for each species (paper II).

The generally accepted dogma in haematophagous arthropods is that blood- or adenine nucleotide-induced feeding is an all-or-none event, in
which even with low-quality diets, partial engorgement is not common (Smith and Friend, 1970; Friend and Smith, 1977; Romero and Schal, 2014). However, this hypothesis has not been verified for all haematophagous vectors. To test this notion in *Ae. aegypti* and *An. gambiae*, the individuals that were scored as engorged and non-engorged on ATP, ADP and AMP diets, across a range of concentrations, were examined for their respective volumes imbibed using a spectrophotometric approach (paper II). Moreover, the effect of the adenine nucleotide feeding stimulant and concentration on the proportion of *An. gambiae* prediuresing, as well as the respective volume engorged and prediuresed, were examined using a modified membrane feeding technique and a spectrophotometric estimation, respectively (paper II).

### 4.4 Age-dependent feeding behaviour and labral transcriptomics in *Ae. aegypti*

To determine how the age of female *Ae. aegypti* mosquitoes influence their response to a blood meal and an artificial ATP diet, as a proxy of blood, age-dependent, no-choice membrane feeding assays were conducted using 1 dpe to 5 dpe females. This age-dependent feeding response was then used to identify relevant age groups for subsequent analysis of differences in abundances of putative labral non-chemosensory and chemosensory genes during the downstream transcriptomics assay. In both experiments, the mosquitoes were sugar-starved with only access to water 22 ± 2 h prior to the experiment (paper III).

#### 4.4.1 Labral extraction of total RNA and differential gene abundance analysis

Total RNA was extracted from 150 labra of 1 dpe, 3 dpe and 5 dpe females, producing four biological replicates of each age groups. The collection of labra was done between ZT 5-7, since RNA transcription is believed to occur 3-6 h prior to the peak blood-feeding time, correlating with the expression of genes associated with blood feeding (ZT 8-12) (Rund et al., 2013; paper III). While females were most prone to take a meal at 5 dpe, the yield of total RNA and the quality of the transcriptomes generated from the labra were low, likely due to the increase in chitinous deposition in this tissue. As such, only the 1 dpe and 3 dpe transcriptome libraries were considered henceforth.

In brief, total RNA extraction was done using an RNeasy microRNA kit (Qiagen, Hilden, Germany), and then immediately stored at -80 °C for
further analysis. The extracted total RNA was used to create ultra-low input transcriptome libraries, which were constructed using NovaSeq Illumina genome sequencing technology (Illumina NovaSeq 6000 S4 PE150 XP). Using the Eurofins proprietary protocol, cDNA library construction was realised, generating paired-end reads of 150 bp coverage and a depth of at least 40 million paired-end reads. The generated raw reads data was cleaned and trimmed to remove adaptors, discarding sequences of Phred score < 20, using CLC Genomics Workbench (version 23.0.1, Qiagen). The cleaned sequences were mapped against the *Ae. aegypti* reference genome (VectorBas: Aaegypti_LVP_AGWG version 61). Variation in the number of transcripts within the individual replicates was then normalised using the trimmed mean of the M-value (TMM) adjusted counts per million (hereafter referred to as CPM).

To establish whether there was an age-dependent enrichment in the transcript libraries at a molecular function level, a gene ontology (GO) analysis ([http://www.geneontology.org/](http://www.geneontology.org/)) was performed on transcripts with a reliable expression level (an average ≥ 1 CPM) in the 1 dpe and 3 dpe libraries, as well as on the differentially upregulated functional enrichment in the 1 dpe and 3 dpe libraries (> 2-fold change; FDR p ≤ 0.05). This was followed by an examination of the most abundant genes in the libraries of either of the age groups, the 25 genes with the highest (top) abundance in 1 dpe and 3 dpe libraries, and those which were differentially upregulated in 1 dpe and 3 dpe libraries (FDR p ≤ 0.05; fold change (FC) ≥ 2).

An assessment of the chemosensory function was conducted for the reliably expressed chemosensory genes and a differential gene expression between 1 dpe and 3 dpe age libraries (FDR p ≤ 0.05; FC ≥ 2), highlighting both the reliable expression of members of gene families previously demonstrated to have functions associated with the detection, regulation and modulation of labral-directed feeding, as well as upregulated genes associated with blood feeding in the 3 dpe libraries. Further still, candidate genes associated with extracellular binding of ATP, highly abundant or orphan genes in the 3 dpe libraries were investigated following a GO term search targeting putative purinoceptors (P1, P2X and P2Y), including the G-protein coupled receptors (GPCR) (P1 and P2Y) and ligand-gated ion channels (P2X) (details in paper III).
4.5 ATP overrides the aversive effects of antifeedants

Being the major blood phagostimulant for a wide range of haematophagous vectors (paper II; Lukenge et al., 2022), ATP was assessed as a model candidate alternative to drive feeding in ATB technology. To test whether ATP can override the aversive effects of feeding deterrents and toxicants, a no-choice membrane feeding assay (see above) was used to compare the proportion of engorged individuals exposed to each of the antifeedant compounds (Table 1) at their threshold concentration, in the presence or absence of ATP at either 0.072 mM (exposure concentration eliciting 50% feeding; EC$_{50}$) or 0.6 mM (exposure concentration eliciting maximum engorgement; EC$_{Max}$).

Table 1. Antifeedant and toxicant compounds and concentration

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS number</th>
<th>Concentration range (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffein</td>
<td>58-08-02</td>
<td>0.1 – 20</td>
</tr>
<tr>
<td>Nicotine</td>
<td>54-11-5</td>
<td>0.1 – 3</td>
</tr>
<tr>
<td>Quinine</td>
<td>130-95-0</td>
<td>0.001 - 2</td>
</tr>
<tr>
<td>Lobeline hydrochloride</td>
<td>134-063-4</td>
<td>0.1 - 3</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>10045-35-3</td>
<td>0.1 – 5</td>
</tr>
<tr>
<td>Boric acid</td>
<td>57-55-6</td>
<td>1.6 - 323.5</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>134-62-3</td>
<td>131.0 - 2628.5</td>
</tr>
<tr>
<td>DEET</td>
<td>404-86-4</td>
<td>0.005 - 53.3</td>
</tr>
</tbody>
</table>

4.6 Knockdown and lethal toxic effects elicited by ATP- and sucrose-induced feeding

The toxic effects elicited by ATP-induced feeding on deterrent and toxicant diets were examined by exposing the individuals to a range of diet concentrations, with and without ATP (0.6 mM EC$_{Max}$). Using a no-choice membrane feeding assay, toxic effects (knock down or lethal) in the exposed mosquitoes were visually examined for up to 24 h after an initial 30 min exposure to the meals. In contrast, the toxic effects resulting from sucrose-induced feeding were examined using cotton wick and open-access continuous meal exposure assays up to 5 days, with 10% sucrose as the feeding driver for the toxicant, boric acid (details in paper IV). To examine the possibility of using lower concentrations of lethal toxicants, a putative synergistic effect of the combination of boric acid and propylene glycol, using ATP as the feeding driver, was examined using combined concentrations as indicated below propylene glycol/boric acid (mM/mM): low concentrations (131.40/16.17); medium (657.10/80.90) and high (13140/161.70).
4.7 Diet destination in ATP- and sucrose-induced feeding on feeding deterrents and toxicants

Sucrose and an artificial ATP meal trigger different feeding programs in mosquito vectors, sending the meals predominantly to the crop or the midgut, respectively. To establish whether the observed differences in toxicity following ATP- or sugar-induced feeding could in part be explained by the diet destination, gut dissections of *Ae. aegypti* were made. Briefly, the ATP-induced feeding assay followed the procedure described in section 4.2.1 and the sugar-induced feeding assay was done by placing cotton balls soaked in the toxic diet warmed at 37 °C for 40 min, placed on top of a bioassay chamber (Semadeni, Koenigstein, Germany), each containing 10 individuals. At the end of the assay, individuals were anesthetised and dissected under a stereo microscope using Ringer’s solution, to ascertain the destination of the meals either in the crop, oesophagus, midgut and or hindgut (paper IV).
5. Summary and discussion of results

5.1 Sensitivity and specificity of *Ae. aegypti* and *An. gambiae* to adenine nucleotides

Species-specific variations in the specificity and sensitivity to blood-derived feeding stimulants are observed across haematophagous arthropods (paper I). While haematophagous arthropods have evolved independently, the majority of them detect select blood-derived adenine nucleotides, predominantly ATP, as indicators of acceptable blood meals, using a conserved mechanism (paper I and II). Previous scholars, who analysed the behavioural response of haematophagous vectors to blood-derived feeding stimulants, used experimental setups that do not allow for direct comparisons across species due to the incomparable experiment setup conditions. To overcome this problem, this study examined the behavioural response of *Ae. aegypti* and *An. gambiae* to the blood-derived adenine nucleotide feeding stimulants (ATP, ADP, AMP and cAMP) under comparable experimental conditions.

The sensitivity and specificity of *Ae. aegypti* and *An. gambiae* were examined by estimating the proportion of individuals that were engorged on the adenine nucleotide diets and the volume of the meals imbibed (Figure 2). Unlike other culicine species that use ADP as a reliable signal for blood quality, *e.g.*, *Culex pipiens* and *Culex univittatus* (Galun et al., 1988; Galun and Vardimon-Friedman, 1992), as well as *Culiseta inornata* (Friend, 1981), both *Ae. aegypti* (paper III; Galun et al., 1985, 1984) and the anopheline *An. gambiae* (paper III) use ATP as the major phagostimulant. It is conceivable that ATP, being a major metabolite in the cellular component in the blood of the host and readily abundant once the mosquito labrum perforates the skin and causes mechanical shear of endothelial cells and RBCs (Rich, 2003; Dunn and Grider, 2022), places it as a reliable indicator of fresh blood.

While the specificity for the adenine nucleotides in this study remained relatively consistent between *Ae. aegypti* and *An. gambiae*, the sensitivity (ED$_{50}$) of the two species differed. Both species maintained an ordered rank response to the adenine nucleotide diets based on the length of the phosphate chain, with the highest sensitivity to ATP and the lowest sensitivity cAMP (paper III; Figure 2). However, *Ae. aegypti* displayed a higher sensitivity to the tested adenine nucleotides compared to *An. gambiae.*
The amino group, the hydroxyl groups and the phosphate group of adenine nucleotides are the key epitopes for the adenine nucleotide receptor (Galun et al., 1985). The avidity of this receptor is dependent on the angle of binding, which is affected by the length of the phosphate chain (Galun et al., 1985). cAMP has a very short phosphate chain, and the active phosphate and hydroxyl groups are concealed by its cyclic structure, limiting the binding of this adenine nucleotide to the receptor, which likely explains the low phagostimulatory effect. Taken together, the findings of this study suggest that the structure of the putative adenine nucleotide receptor is similar in the two species, however, the affinity of the receptors to bind the adenine nucleotides, reflected in the corresponding higher sensitivity, is likely stronger in *Ae. aegypti* than in *An. gambiae*.

![Figure 2](image)

Figure 2. Sensitivity and specificity of *Aedes aegypti* and *Anopheles gambiae* to adenine nucleotide feeding stimulants. a) i-v: The scale used to score unfed to engorged individuals. The dose-dependent response in the proportion of b) *Ae. aegypti* and c) *An. gambiae* engorged on artificial adenine nucleotide diets. The buffer without nucleotides served as the negative control (0 mM; not shown on the log scale). No curves were generated for cAMP since the proportion of mosquitoes engorging was below the 50% proportion level. The error bars indicate the standard error of the mean. The number of replicates for each diet was 90 for *Ae. aegypti* and 50 for *An. gambiae*.

The current working model for blood constituent detection, based on results obtained from *Ae. aegypti*, stipulates a multi-modal taste integration, using the four GRNs within the labral apical and subapical sensilla. In concurrence with Jové et al. (2020), labral electrophysiological findings in *Ae. aegypti* identified four labral neuronal cells, with one being responsive to blood-derived adenine nucleotides (Werner-Reiss et al., 1999c). However, this cell was able to discern neither the structure nor the concentrations of the various adenine nucleotides above the threshold stimulation, indicating a likely additional sensory integration for the assessment of the blood meal
quality, probably at the cibarial region (Day, 1954; Lee and Craig, 1983; Werner-Reiss et al., 1999c).

The feeding response of *Ae. aegypti* in this study are concomitant with the multimodal integration model, since the exposure of bicarbonate buffered saline alone does not elicit engorgement until combined with ATP (paper II, III and IV). While culicine mosquitoes do not engorge on the bicarbonate buffered saline, *An. gambiae* readily engorged on the buffer (paper II), although at lower proportions than previously reported (Galun et al., 1985a; Kessler et al., 2014). This behavioural response suggests that the neuronal mechanism encoding behavioural specificity for blood modalities is different in *An. gambiae* and requires further investigation. Taken together, the study findings suggest species-specific variations in the putative purinoceptor and the neuronal encoding properties for blood components, likely affecting the affinity of cognate ligands, subsequent feeding rate and volumes imbibed.

5.2 A bimodal feeding pattern in *Ae. aegypti* and *An. gambiae*

The currently accepted theory of feeding in haematophagous arthropods is an all-or-none feeding response (Smith and Friend, 1970; Friend and Smith, 1977; Romero and Schal, 2014), in which once blood-feeding arthropods are stimulated by the phagostimulants at a certain threshold, above which the GRNs respond in a dose-independent manner, the arthropods feed to engorgement. This feeding theory has previously been reported in obligate haematophagous vectors, *i.e.*, *Cimex* (Romero and Schal, 2014), *Rhodnius* and *Glossina* species (review by Friend and Smith, 1977), and has been suggested in *Culex nigripalpus* (Edman et al., 1975). In the current study, however, both species displayed a dose-dependent bimodal feeding pattern on adenine nucleotide-containing meals, in which individuals either tasted (at lower concentrations) or, following tasting, proceeded to engorge (at higher concentrations) (paper III; Figure 3a,b). These results expand the current theory to include an initial sampling phase. In an ecological sense, the initial tasting of a meal may be an evolutionary precautionary step required to safe guard an organism against potentially toxic food substances before imbibing huge volumes of the meal, which appears to support the capability of blood-feeding mouthparts to detect such risky compounds.
Whether this bimodal feeding pattern also exists in other mosquito vector species remains for further investigation.

5.3 Effect of adenine nucleotides on prediuresis in An. gambiae

The phenomenon of prediuresis, a physiological process associated with thermoregulation and erythrocyte concentration, has been documented in various Anopheles species, but is notably absent in Aedes species (paper II; Galun et al., 1985; Islam et al., 2019; Lahondère et al., 2013; Vaughan et al., 1991). For example, An. gambiae exhibits a propensity to continue feeding beyond engorgement, with pronounced prediuresis, a behaviour modulated by heat (Lahondère et al., 2013). Aedes aegypti, unlike An. gambiae, expresses an abundance of heat shock protein 70, acting as heat molecular chaperons to control thermostress (Gross et al., 2009; Benoit et al., 2011). In contrast, An. gambiae leverages mechanical evaporative thermoregulation enhanced by the standing angle of inclination away from the hotter body of the host and deploys prediuretic behaviour (Lahondère et al., 2013). Blood physico-chemical stimulants, such as feeding stimulants, have also been postulated to modulate prediuresis in An. gambiae (Arsic and Guerin, 2008).

While the precise role of adenine nucleotide phagostimulants in prediuresis is not fully understood, their modulatory function is commensurate with the second theory of erythrocyte concentration (Vaughan et al., 1991; Clements, 1992). Since adenine nucleotides are
reliable indicators of blood meals, it is conceivable that their presence initiates the prediuretic mechanism to respond to the concentrating nitrogen and haemoglobin. However, the observation that the concentration of the nucleotides does not affect the volume engorged nor prediuresed is counterintuitive and remains to be further investigated.

The current study aimed at contributing an insight into the prediuresis phenomenon and demonstrated a general dose-dependent response in the proportion of individuals exhibiting prediuresis across three adenine nucleotide diets, with ATP showing the highest proportion of prediuresing individuals (Figure 4a). Notably, the structure and concentration of adenine nucleotides did not impact the volume engorged by prediuresing individuals (Figure 4b). However, a dose-dependent response in the average prediuresed volume was observed, with no significant variation between ligands (Figure 4c). Available results thus indicate that the structure and concentration of the stimulating blood-derived adenine nucleotide are key factors in modulating the probability of prediuresis, but not the volume engorged in prediuresing individuals, and likely not the volume prediuresed. Taken together, this study has expanded our understanding of how An. gambiae differentially responds to blood meal constituents.

Figure 4. The effect of blood-derived adenine nucleotides diet on prediuresis in Anopheles gambiae. a) The percentage of prediuresing individuals, b) the volume engorged among prediuresing individuals and c) the average volume prediuresed. The letters indicate the levels of significance of the response of An. gambiae within concentrations of the same diet. The single and double apostrophes differentiate levels of significance for the ADP (’) and ATP (’’) diets, respectively. The asterisks indicate significant differences between ligands determined by a two-way ANOVA (*P < 0.05; *** P < 0.001; ns = non-significant).
5.4 Molecular determinants in blood-meal assessment and feeding

5.4.1 Age-dependent shift in labrum gene abundances between 1 dpe and 3 dpe transcriptome libraries

Post-emergence, teneral female mosquitoes rest shortly before engaging in floral- and sugar-seeking behaviour, behaviours not requiring hardened stylets (Clements, 1992; Foster, 1995; Lomelí and Dahanukar, 2022). However, behavioural evidence from this study revealed an age-dependent increase in the propensity of *Ae. aegypti* to feed on blood (paper III; Klowden, 1995) and artificial ATP diets (Figure 5a), correlating with the age-related variation in transcript abundance between non-blood-feeding and blood-feeding labrum libraries (Figure 5b).

![Figure 5. Age-dependent feeding on blood and ATP, and the variation in read abundance of 1 dpe and 3 dpe labral transcriptomes of *Aedes aegypti*.](image)

**a)** A comparison of the proportion of individuals engorged on blood or artificial ATP meals across different age groups. The upper- and lower-case letters indicate significant differences among the age groups feeding on blood and ATP, respectively. The error bars indicate the standard error of the mean. **b)** A principal component analysis representing the variation in overall read abundance in the labral transcriptomes of female *Ae. aegypti* at 1 and 3 days post-eclosion (dpe). The uppercase letters indicate the replicate transcriptomes for each age group.

This study highlights the dominance of chitin and collagen cuticle-associated genes in teneral female labral libraries (Figure 6a,b), emphasising their important role in cuticular structural development and maturation of the labrum in preparation for skin perforation, as mosquitoes reach the blood-feeding age (Vincent and Wegst, 2004; Henriques *et al.*, 2020). Among the cuticular protein genes that are abundant in both 1 dpe and 3 dpe libraries, *cuticular protein 19* (*CP19*) stands out (paper III; Figure 6a,b). The *CP19*
encodes a labral protein, which upon binding with the labrum-interacting protein of the saliva 2 (LIPS-2), expressed in the labral libraries of 3 dpe, but not 1 dpe, females, regulates structural changes in the labrum. These changes lead to more rapid intradermal probing during the initiation of blood feeding (Arnoldi et al., 2022), which would reduce the time spent on the host, thus minimising the likely risk of assault.

The abundance of the cuticular structural genes was reduced in the 3 dpe labral libraries compared to the 1 dpe libraries with a differential upregulation of cellular, neuro-communicative and neuromodulatory genes in the 3 dpe libraries (Figure 6). As such, at 3 dpe, the structurally hardened labrum is at this age of the mosquito poised as a sturdy appendage for skin perforation, with a sufficiently mature chemosensory apparatus for blood-meal assessment and feeding (paper III; Figure 6).
5.5 Chemosensory function of the labrum during blood-feeding

5.5.1 Putative chemosensory role of the labrum in the detection of blood vessels and the blood meal

While blood meal assessment requires contact chemosensation, it necessitates the labrum to use sensory-guided flexible manoeuvres to move through the skin and locate the blood vessels upon skin perforation (Loke et al., 2009; Choumet et al., 2012). This guided labral manoeuvre could in part be supported by the reliable expression of sensory receptors. The upregulation of the thermoreceptors TRPA1 and Ir25a in the 3 dpe libraries (Figure 7a,b) (Corfas and Vosshall, 2015; Zhang et al., 2022; Morita et al., 2023) supports this hypothesis. The demonstrated detection of short-range blood volatile compounds, e.g., modified terpenes (Loke et al., 2009;
Himmel et al., 2019), along with the reliable expression of both Ors and Irs, including their co-receptors (Orco, Ir8a, Ir25a, and Ir76b), suggest that VOCs may assist in this task.

5.5.2 Putative olfactory role of the labrum in blood detection

Of the reliably expressed genes encoding for tuning Ors and Irs, only Or10 and Or14 have been functionally characterised and demonstrated to detect phenolic compounds, e.g., phenol, 4-methylphenol and 4-ethylphenol (Hughes et al., 2010; Zeng et al., 2019; Ni 2021), which are omnipresent in human blood (Loke et al., 2009). The detection of phenolic compounds may be a conserved function of the labrum across mosquitoes, as a 4-ethylphenol receptor, CquiOr99, has also been demonstrated to be expressed in the labrum of the southern house mosquito Culex quinquefasciatus (Choo et al., 2015).

In blood, alcohols, carboxylic acids and their esters, including 1-octen-3-ol, cyclohexanol, acetic acid and ethyl acetate, have been demonstrated to be detected by Or8 and Or49, expressed in the labrum of an insecticide-resistant strain of Ae. aegypti (Won Jung et al., 2015). However, neither of these Ors were reliably expressed in the Rockefeller (this study) or Liverpool strains (Jové et al., 2020). In further support of the olfactory roles of the labrum, was the expression of the OBP and CSP carrier protein gene families, which are often highly expressed in taste organs (Sánchez-Gracia et al., 2009) (Figure 7c). While the majority of the genes in this class were upregulated in the 3 dpe libraries, their functional roles in taste organs are yet to be characterised. However, together with the highly expressed OBP-distantly related D7 proteins, which are distantly related to OBPs, it is likely that they play a role in binding and trafficking of odorants and testants to the putative chemosensory receptors (Pelosi et al. 2014, 2018; Swarup et al., 2014). Taken together, the reliable expression of genes encoding for tuning olfactory receptors, co-receptors and odorant carrier proteins in the labrum emphasises an important role of this organ in blood meal detection.

5.5.3 Putative labral receptors for detecting the key blood taste modalities

The labral GRNs detect taste modalities, including salt (NaCl), sour (NaHCO₃), sweet (glucose) and adenine nucleotides (paper I; Baik and Carlson, 2020; Jové et al., 2020), that are detected or putatively detected by chemosensory proteins encoded among the reliably expressed range of
chemosensory gene families (paper III; Figure 7a). Salt and sour tastes are
detected by GRNs expressing Ir7y.1 (AAEL014686; previously Ir7f), which
is narrowly tuned to a range of concentrations of NaCl and NaHCO₃, and is
upregulated in 3 dpe libraries (Figure 7b). A different population of sour-
sensing GRNs, expressing Ir7a (NM_001358741.1; chromosome 3:
37734383-37736188), is tuned to a broader range of NaHCO₃ concentrations
(Jaeger et al., 2018a). The identification of distinct putative receptors for
NaCl and NaHCO₃, which in combination with ATP elicit the feeding
response, is in concurrence with the proposed multimodal integration of the
blood-feeding signal (Jové et al., 2020).

Pickpocket receptors, a group of the degenerin/epithelial sodium ion
channels, have also been demonstrated to be closely associated with salt
detection (Matthews et al., 2019). Although this study did not detect the
previously identified salt-sensitive receptor (ppk301) in the labrum of Ae.
aegypti, four other putative ppks were reliably expressed: ppk103 and ppk316
were not regulated, while ppk203 and ppk314 were upregulated in the 1 dpe
libraries (Figure 7b). Moreover, the Gr homologue to the salt and sugar
detecting Gr64f in Drosophila melanogaster (Jaeger et al., 2018b; Dey et al.,
2023), Gr13 (Kent et al., 2008), was upregulated in the labrum with age,
correlated with the development and function of the integrator GRN in labral
sensilla, which detects blends of NaCl, NaHCO₃ and glucose in blood-
feeding mosquitoes (Jové et al., 2020). Taken together, the upregulation of
salt sensing ppks in the 1 dpe and not the blood-feeding age, suggests that
these ppks are not involved in the detection of salt during blood feeding.
Whether Gr13 plays a role in salt and sugar detection remains to be
investigated.

5.5.4 Detection of feeding deterrents in blood

Aversive compounds are also detected by the labrum (Kessler et al., 2014;
Lukenge et al., 2023) and are likely mediated by select Grs and/or Trps.
Homologues of genes of D. melanogaster encoding for receptors detecting
bitters (Gr17) (Kent et al., 2008), as well as noxious substances and irritants
(TrpA1, Trpl and painless) (Tracey et al., 2003; Kang et al., 2010; Zhang et
al., 2013) were reliably expressed in the labrum of Ae. aegypti and regulated
with age (Figure 7ab). Moreover, the labrum of Cx. quinquefasciatus
expresses CquiOR136, which detects DEET, a compound that also elicits
feeding deterrence in Ae. aegypti (paper IV; Dennis et al., 2019). Jové et al.
(2020) previously demonstrated that among the population of neurons
identified in the labrum, there was a population that was non-responsive to the blood phagostimulatory modalities and suggested that these neurones may be expressing receptors for other modalities, such as feeding deterrents and pungent irritants. While the latter supports the notion of the labrum expressing feeding deterrent receptors, functional characterisation of these receptors is required.

5.5.5 Labral purinoceptor expression

Purinoceptors are a class of receptors that bind adenosine or the adenine nucleotides (Abbracchio and Burnstock, 1994). Non-insect purinoceptors are classified among the GPCRs (P1 and P2X) and ligand-gated ion channels (P2Y) (Abbracchio and Burnstock, 1994; Kennedy, 2021), which contain a rich set of targets to screen to identify candidates for the putative purinoceptors in mosquitoes, particularly the ATP receptor. A screen of GPCRs revealed 78 reliably expressed genes, the majority of which were significantly more abundant in the 1 dpe libraries (70%), with the only purinoceptor previously identified in Ae. aegypti, the adenosine receptor (Liu et al., 2008) (P1: AAEL026043) being reliably expressed in both ages (paper III). While a significant number of GPCRs are expressed in the labrum, their likelihood of functioning as purinoceptors during blood feeding is therefore limited.

The screen for ligand-gated ion channel protein receptor genes (P2Y) revealed 83 reliably expressed receptor genes, with similar level of abundance in the 1 dpe and 3 dpe libraries. This suggests an unlikely role of these genes during blood feeding. In contrast, among the genes encoding for P2X-like ligand-gated ion channels, Ir100c.2 and Ir7n.1 (Figure 7b) were found to be upregulated in the 3 dpe labral libraries, and may thus be strong candidates for future purinoceptor function investigations. Investigation in the ATP binding (GO:0005524) gene ontology category identified 617 reliably expressed genes, of which 30 were significantly upregulated in the 3 dpe libraries. All genes identified in this group were associated with enzymatic and degrading functions that require ATP binding but do not act as transmembrane extracellular binding proteins, and are therefore unlikely to be receptors for extracellular ATP. Taken together, the current screen indicates a reliable expression of the purinoceptor classes in the labrum, with only a few promising candidates identified for further studies. Noteworthy, while the function of chemosensory tissues in insects can be modulated through changes in the level of gene expression and neural sensitivity, these
two aspects can be influenced via various neuromodulatory pathways (Zhao et al., 2022).

### 5.6 Putative neuromodulatory roles of the labral sensory machinery

The dynamic range of OSN and GRN sensitivity in insects is modulated by the degree of starvation experienced by the insect during early adult maturation, and is then maintained throughout the adult stage, via the endocrine hedgehog signalling pathway between the gut and the peripheral chemosensory organs, as demonstrated in *D. melanogaster* (Sanchez et al., 2016; Zhao et al., 2022). Downstream members of the hedgehog signalling pathway, *patched* and *smoothened*, are reliably expressed in the labral libraries of *Ae. aegypti*, at both 1 dpe and 3 dpe, with the abundance of *smoothened* showing a trend of reduction in 3 dpe libraries. This is in line with the proposed model, in which endocrine hedgehog signalling modulates the sensitivity of the chemosensory neurons by regulating the expression of chemosensory genes, particularly the receptors (Sanchez et al., 2016; Zhao et al., 2022). Chemosensory neuronal sensitivity is also regulated through the short neuropeptide F and sulfakinin pathways in response to feeding state (Lee et al., 2004; Christ et al., 2017, 2018; Guo et al., 2021; Tinoco et al., 2021).

The reliable expression of the *short neuropeptide F* and *sulfakinin* receptors in the labrum suggests that its sensory neurons are being modulated at the peripheral level, as well as at the first synaptic level in the stomatogastric and central nervous systems. Taken together, the concerted feeding response of the labrum is an interplay of a matured and sturdy piercing and feeding straw doubling as a chemosensory organ during the blood-feeding age.

### 5.7 ATP overrides the aversion of feeding deterrents in *Ae. aegypti*

The ATSB technology relies on sugar as the phagostimulant for the toxin-laden meals in vector control. The inherent disadvantages of sugar as a feeding driver (details in section 2.6.1) in the ATB technology warrant the search for alternative phagostimulants. This study demonstrated that ATP is a potent feeding driver activating the adenine nucleotide pathway, which
overrides the aversive effects of several structurally divergent antifeedants and toxicants (Table 1; Figure 8ab) in a dose-dependent manner (Figure 8c). The behavioural response to the antifeedants and toxicants suggests that chemosensory neurons in sensilla on the labrum, or the cibarium, differentially detect these compounds (French et al., 2015a,b). However, whether antifeedants and toxicants modulate the response of sensory neurons in these sensilla in mosquitoes, or act through a dedicated pathway, is currently unknown.

5.8 Toxic effects of ATP-induced and sucrose-induced feeding on feeding deterrent meals

Mosquitoes, like other flower-visiting arthropods, have evolved the ability to detect aversive compounds, presumably due to the perceived toxicity of nectar sources (French et al., 2015; Stevenson, 2020; Lomelí and Dahanukar, 2022). Similarly, mosquitoes appear to have been under selection pressure to detect antifeedant compounds in blood (paper IV; Dennis et al., 2019; Kessler et al., 2014). In addition, obligate haematophagous animals are able to detect these compounds through antifeedant-sensitive receptors (Ziegler and Behrens, 2021). The blood of vertebrate hosts, however, does not appear to contain high concentrations of antifeedant compounds as mosquitoes readily engorge on this resource. This study demonstrated that meals containing antifeedants, in addition to ATP, induce rapid toxic effects (< 3 h) relative to the over 5-day period observed in response to sucrose-induced feeding. Thus, the ATP sensory pathway appear to override the aversive effects of antifeedants and toxicants and inducing a reflexive feeding response, which leads to rapid toxic effects.

ATP-induced feeding on the naturally existing alkaloids, i.e., nicotine, lobeline and caffeine, elicited a knockdown effect. This effect may hypothetically be caused by the compounds passing across the peritrophic membrane into the haemolymph, and interacting with nicotinic acetylcholine receptors (Unwin, 2013; Alkam and Nabeshima, 2019). In contrast, ATP-induced feeding on the synthetic toxicants, boric acid and propylene glycol, elicited lethal effects, likely through a direct mechanism of action as stomach poisons in the midgut of mosquitoes, disrupting the gut epithelium, affecting metabolism, and potentially acting as neural toxins (Sippy et al., 2020). The combination of two lethal compounds resulted in synergistic effects.
demonstrating the potential of using lower, eco-friendly concentrations of toxicants in field applications.

![Figure 8](image_url)

Figure 8. ATP overrides the aversive response to antifeedants and toxicants in *Aedes aegypti*. a) i-v: The scale used to score unfed to engorged individuals. b) The proportion of *Ae. aegypti* engorged on meals of antifeedant and toxicant compounds, in combination with ATP at EC$_{50}$ (0.072 mM) and EC$_{max}$ (0.6 mM) (n = 50). The behavioural response of the mosquitoes to the antifeedant and toxicant compounds is aversive when these are presented in combination with ATP at EC$_{50}$, whereas ATP EC$_{max}$ is able to override this effect. An ANOVA was used for the pairwise comparisons and asterisks indicate the level of significant difference. c) Mosquitoes engorged on 0.6 mM ATP-containing antifeedant and toxicant diets in a dose-dependent manner. The error bars indicate the standard error of the mean. The number of replicates for each diet and concentration was 50. PRO: propylene glycol.

While vectors possess an innate ability to detect and evade antifeedant- or toxicant-containing meals, this study demonstrated that such avoidance is variably overridden depending on the type of phagostimulant used. This, in turn, influences the destination of the meal and the rate of toxicity with the meals destined to the midgut (ATP-induced) causing faster toxicity compared to meals destined to the crop (sugar-induced) (Figure 9). In summary, the benefits linked to ATP as a phagostimulant strongly indicate that its thermally stable agonists could serve as viable alternatives to sucrose in the development of oral-based vector control technologies. These advancements would enable the oral administration of various vector-modifying agents, including biological substances (such as *Bacillus thuringiensis* toxins), genetic material (such as dsRNA) and chemical agents.
Figure 9. ATP and sucrose variably modulate the final destination of the meal. The guts of mosquitoes fed on antifeedant- and toxicant-laden meals induced by either sucrose or ATP are shown in the upper and lower panels, respectively. The micrographs of gut dissections demonstrate the predominant destination of antifeedant- and toxicant-laced meals (NIC: nicotine; LOB: lobeline; QUI: quinine; CAP: capsaicin; PRO: propylene glycol BOR: CAF: caffeine; BOR: Boric acid; DEET: N,N-Diethyl-meta-toluamide; blue) to the crop (CP), oesophagus (OS), midgut (MG) and hindgut (HG). The control gut dissections for unfed, sucrose-, blood- and ATP-fed mosquitoes are presented in the four leftmost panels. The pie charts represent a semi-quantitative indicator of the distribution of the meals to the four main gut regions in an anticlockwise direction, starting from the top-left quadrant, the CP, OS, MG and HG. The colour intensities in the pie chart (scale to the right) visually rank the average intensity of the blue dye in the gut regions on a scale of 0 (lowest) to 3 (highest; n = 10).
Evaluation of the blood meal by the labrum is the ultimate sensory assessment prior to engorgement is poorly understood. This crucial process to the survival of a mosquito may offer an avenue for advancing taste-based tools, which can supplement the existing integrated vector control management strategies. The findings in this study indicate a novel dose-dependent behavioural response to blood-derived adenine nucleotides in *An. gambiae*, which was less sensitive than in *Ae. aegypti*. Both mosquito species exhibited a bimodal feeding response, thereby introducing an initial tasting step to the conventional theory of a general all-or-none feeding pattern among haematophagous vectors. The shift of labral gene abundance in *Ae. aegypti* from those with structural functions to those with cellular, neuro-communicative and modulatory gene functions as the vector matures into the blood-feeding age, positions the labrum as a sturdy and chemosensory competent organ with gustatory, olfactory and thermoreceptive abilities. The identified putative and orphan transmembrane labral genes may serve as receptors for ATP and other blood phagostimulatory modalities, and set the foundation for future functional genomic studies to characterise the receptors involved in, *e.g.*, ATP detection. The evidence that ATP can effectively override the aversive response to feeding deterrents, and when combined with toxicants, elicit more rapid lethal effects, underscores the potential effectiveness of activating the adenine nucleotide pathway over the traditional sugar pathway in the future development of the ATB technology. Prospectively, the electrophysiological response of both species to phagostimulants and antifeedants should be examined to widen our mechanistic understanding of blood feeding. From an applied perspective, stable analogues of ATP will need to be explored to develop a prototype vector control tool that leverages the use of human odours as lures in the development of an ATP-based toxic bait technology.
7. References


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The world is home to a vast array of mosquito species, with many causing mere annoyance through buzzing and irritating bites. However, a small proportion of mosquitoes play a significant role in impacting human health, the economy and productivity, by transmitting disease pathogens. *Anopheles* mosquitoes, for example, are associated with malaria, a disease that claims the lives of two children every minute, primarily affecting low-income developing countries. Despite ongoing efforts to combat mosquito-borne diseases, the current arsenal of control tools is limited, necessitating urgent development of supplementary and alternative approaches. Understanding the behaviour of disease vectors, particularly blood feeding in female adult mosquitoes is crucial for devising and developing effective control strategies.

After emerging from the pupae, mosquitoes initially seek sugar for energy, then transition to blood feeding, approximately three days later. The process of host seeking, by which a mosquito ultimately acquires a blood meal, involves using odours to locate the host from a distance, followed by landing on the host and using taste sensation to identify suitable blood cues. While much is known about olfaction, from which odour-guided tools have been developed, taste-based tools remain underexplored, although these may provide a novel perspective on vector control management.

The most advanced taste-based control tool presently is the attractive toxic sugar bait technology, capitalizing on the general sugar-seeking behaviour of mosquitoes. However, this technology has drawbacks, including slow toxic effects, non-selective toxicity, competition with natural sugar sources, and does not target obligate blood-feeding vectors. Our study focused on expanding the mechanistic knowledge about taste in two major mosquito vectors, the African malaria mosquito, *Anopheles gambiae*, and the yellow fever mosquito, *Aedes aegypti*. To this end, I used adenine nucleotide
artificial diets, previously identified as the major blood component that regulate feeding in a vast array of haematophagous vectors. Using a comparable experimental design, the feeding response of the two study mosquitoes to adenine nucleotide artificial diets was examined. The results demonstrated that both mosquito species exhibit a similar feeding pattern on the four naturally occurring adenine nucleotides with the highest preference for adenosine triphosphate (ATP). Moreover, this study demonstrated a novel bimodal feeding pattern of the two species. While previous studies have shown that heat modulates prediuresis, a process of passing out urine during blood feeding, in *Anopheles* species, this study also indicated a similar role for adenine nucleotides.

To demonstrate the feasibility of leveraging knowledge about blood-derived feeding stimulants for advancing novel blood-associated taste-based control tools for *Ae. aegypti*, ATP was used as the feeding driver for known feeding deterents and toxicants. Compared to suing sucrose as the feeding driver, ATP was able to override the aversive effects of these compounds resulting in swift toxic effects. This difference in toxicity is likely due to the destination of the induced meals in the intestinal tract of the mosquito.

How ATP and other tastants are detected by the labrum is currently unknown. Using a molecular analytical approach, we analysed the expression of genes putatively involved in this process. This analysis revealed that newly emerged females invest more in the structure development of the labrum but as the mosquito develops the capacity to blood feed, a shift in the expression of genes associated with cellular life processes, as well as sensory functions, including putative ATP receptors, occurs providing the female with a sturdy and sensory competent organ for perforating skin and assessing blood quality.
Populärvetenskaplig sammanfattning

Världen är hem för ett stort antal myggarter, där de flesta endast orsakar irritation genom sitt surrande och irriterandebett. En liten andel myggor spelar dock en betydande roll då de överför virus och parasiter som negativt påverkar människors hälsa, ekonomi och produktivitet. Exempelvis är *Anopheles*-myggor, starkt förknippade med malaria, en sjukdom som kräver två barns liv varje minut, främst i låginkomstländer. Trots pågående ansträngningar för att bekämpa myggburna sjukdomar är den nuvarande arsenalen av kontrollverktyg begränsad, vilket kräver akut utveckling av kompletterande och alternativa metoder. Att förstå beteendet hos sjukdomsvektorer, däribland hur de suger kan leda till utveckling av nya effektiva kontrollstrategier.

Efter att ha kläckts ur sin puppa, söker en honmygga efter en sockermåltid för att få den energi hon behöver för sin överlevnad och reproduktion, och övergår efter ca tre dagar att leta efter en blodmåltid. Denna process börjar med att honan letar efter en värd med hjälp av sitt doftsinne, landar, och sedan använder smaksinnet för att identifiera lämpliga blodsignaler. Idag känner vi till en hel del om vilka dofter som styr myggans beteende, men vår förståelse för hur smak styr de sista stegen i denna process är fortfarande begränsad. En ökad förståelse för dessa mekanismer kan ge ett nytt perspektiv på hur vi kan kontrollera sjukdomsspridande myggor.

adenin-nukleotider, som är rikligt förekommande i blod, och som tidigare visats vara de huvudsakliga blodkomponenterna som reglerar beteendet hos ett stort antal blodsugande insekter. I en jämförande studie kunde jag visa på att båda myggarterna uppvisar en liknande preferens för de fyra naturligt förekommande adenin-nukleotiderna, med den högsta preferensen för adenosintrifosfat (ATP). Dessutom visade studien att myggor använder sig av ett bimodalt mönster då de livnär sig på adenin-nukleotider. Medan tidigare studier har visat att värme modulerar prediures, det vill säga utsöndring av urin, då *Anopheles* myggor suger i sig blod, visade denna studie också en liknande roll för adenin-nukleotider.

För att demonstrera möjligheten att utnyttja kunskap om de mekanismer som styr blodsugning för att utveckla ny teknologi likande ATSB för att bekämpa sjukdomsspridande myggor som *Ae. aegypti*, användes ATP för att trigga upptaget av toxiska substanser. Jämfört med socker, triggade ATP myggorna att suga i sig dessa måltider, vilket resulterade i snabba toxiska effekter. Denna skillnad i toxicitet beror sannolikt på var måltiden hamnande i myggans mag-tarmkanal.

Hur ATP och andra smakämnen i blod detekteras av det organ som penetrerar huden (labrum) är för närvarande okänt. Med hjälp av molekylära metoder identifierade jag de gener som är förmodat involverade i denna process. Denna analys visade att honor som precis kläckts från sin puppa investerar mer i den strukturella utvecklingen av detta organ, men allt eftersom myggan utvecklar förmågan att suga blod, ökar uttrycket av gener förknippade med cellulära livsprocesser, såväl som sensoriska funktioner, inklusive förmodade ATP-receptorer. Detta förser mygghonan med ett robust och sensoriskt kompetent organ för att penetrera hud och bedöma blodkvalitetet.
“Quitters never Win and Winners never Quit: Never measure the height of a mountain until you have reached the top, then you will see how low it was”. Climbing on top of this PhD Mountain has had its very exciting moments, but also challenging moments that quitting would have been the easiest option. It has been upon reaching its peak that I realised how low the mountain is, however, the journey to the top would not have been possible if it were not for the unconditional support from family and friends within and outside my academic circles. First and foremost, heartfelt gratitude extends to my mentors, Sharon and Rickard, for granting me the opportunity to delve into the captivating world of mosquito research. Beyond tapping into your academic expertise, I have learnt invaluable lessons about the influence of patient leadership – leaders who believe in and steadfastly support their teams. Thank you for being invaluable team players on this challenging yet rewarding journey.

To Rickard, my co-supervisor, thanks for the guiding and timely experiment discussions and scientific writing sessions. Of course, I will not forget to ensure “alignment”! Special gratitude is owed to Sharon, my main supervisor, for her ceaseless availability, even extending beyond conventional work hours. Your ability to inspire positivity during challenging situations is infectious, and it has been a guiding light. To my cherished colleagues and friends from the Disease Vector Group, the Unit of Chemical Ecology at SLU, and the Plant Protection Department, you have been my second family away from home. While names may go unmentioned, your impact on both my personal and academic life has been profound. In extending appreciation to my Swedish senior citizen friends, Anders and Barbro, I am reminded of the generosity of your hearts and the joy of sharing the Swedish experience. Your reminders of cherished memories from my home country have been a comforting thread connecting past and present.

To my dad, my mom (no longer with us), brothers and sister, thanks for being such a blessing to me at every step of my academic journey. Saving the best for last is gratitude to my wife, Patience, and our children, Keisha and Nicole. You three have consistently been my wellspring of inspiration. Your prayers, encouragement, and unwavering moral and emotional support, have anchored me, allowing me to weather the storms along this journey to the mountaintop.
Chapter 17

Phagostimulants drive the acceptance of a blood meal in disease vectors

M. Lukenge, R. Ignell and S.R. Hill*

Disease Vector Group, Unit of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, 23054 Alnarp, Sweden; sharon.hill@slu.se

Abstract

Blood feeding is pivotal for the survival and reproduction of haematophagous arthropods, and is intimately linked to the transmission of vector-borne diseases. As such, understanding the dynamics of phagostimulation may identify targets, which can be used in future vector control. The behaviour leading up to the acceptance of a blood meal relies ultimately on the sense of taste, by which disease vectors assess the quality of the meal through blood-related phagostimulatory ligands. Adenylated nucleotides, often in combination with NaCl, NaHCO₃ and other blood-related factors, elicit pronounced, species-specific feeding responses in haematophagous arthropods, an effect reflected in the response of gustatory sensory neurons housed within hairlike sensilla on the mouthparts involved in blood feeding. While there has been progress made to understand the molecular mechanisms regulating the response to blood phagostimulants, there are yet many voids to fill. This chapter gives an account of the existing knowledge about the phagostimulatory dynamics leading up to and during blood-feeding activation in select disease vectors, with emphasis on the blood-related feeding stimulants/mechanisms, which potentially could be targeted for advancing alternative vector control tools.

Keywords: phagostimulation, adenylated nucleotides, purinoceptors, haematophagy, blood feeding

17.1 Introduction

Haematophagy (blood feeding) in arthropods has evolved independently at least five times, at the order level, within the past 150-200 million years, giving rise to over 400 haematophagous genera (Mans, 2011; Ribeiro, 1995). This specialised feeding behaviour has likely arisen as a result of niche expansion due to resource limitation, e.g. the need for a protein-rich meal for egg development and maturation (Azar and Nel, 2012; Huff, 1929; Klowden, 1995; Kogan, 1990; Lehane, 2005), or to salt acquisition, as seen in Calyptra moths (Zaspel, 2008). The blood-feeding habit has evolved through two separate pathways: pre-adaptation of the mouthparts for piercing and sucking/lapping, or a prolonged close association between the arthropod and a vertebrate blood host (Lehane, 2005). Irrespective, most haematophagous arthropods display similar behavioural and physiological adaptations, as a result of convergent evolution (Adam, 1999; Snodgrass, 1945), reflected also in the sensory mechanisms by which these arthropods
detect and evaluate their blood meal (Friend and Smith, 1982; Galun, 1966; Galun and Kabayo, 1988; Galun et al., 1985a,b; Hosoi, 1958, 1959; Liscia et al., 1987; Mitchell, 1976; Werner-Reiss et al., 1999a,b,c).

In this chapter, we review the existing literature on the phagostimulatory dynamics in disease-vector arthropods, including the behavioural events leading up to blood feeding, the morphology of the related sensory apparatus, the blood phagostimulants and their varied phagostimulatory roles across arthropod species, as well as the current knowledge on the sensory and molecular detection of these phagostimulants. Throughout the chapter, mosquitoes will be used as the main exemplar species, due to the breadth of knowledge available for this family. Future perspectives, in which an in-depth understanding of blood phagostimulation may open new insights in viable avenues for the development of supplementary and/or alternative vector-control interventions, are discussed.

17.2 The behavioural events prior to blood feeding

Blood feeding is the ultimate behaviour following the attraction to (Hinze et al., 2022), and the acceptance of, a host (Jones and Pilitt, 1973; Klowden, 1995; Lehane, 2005). This behaviour can be facultative or obligate, and sexually dimorphic (Lee, 1974; Lehane, 2005; Schofield and Dolling, 1993; Vickerman, 1985; Wenk and Raybould, 1972). Irrespective of these factors, blood feeding is a risky venture, which predisposes the arthropod to be attacked and/or predated on by the host. Since random probing for blood vessels increases the risk of a fatal counter response by the host, the process needs to be targeted and executed swiftly and/or stealthily. This process has been well described in mosquitoes, and will therefore be the main exemplar species to describe this behaviour.

Upon landing on the host, a female mosquito first makes contact using the tarsi, which are covered by contact chemosensory sensilla (Jones and Pilitt, 1973). Sensilla are hairlike structures housing the dendrites of sensory neurons, which protrude from the surface of the sensory organs (Figure 17.1) (Pitts et al., 2022). Signals from the tarsal sensory neurons initiate the engagement of the mouthparts, which results in the sensilla on the labellum making contact with the skin to identify an optimal piercing site. The labellum is then retracted, exposing the fascicle, consisting of six specialised mouthparts (stylets) (Figure 17.1A) (Day, 1954; Lee, 1974; Newland et al., 2009; Owen, 1963), which perforates the skin, probes for suitable blood vessels, and ultimately tastes the blood quality prior to its acceptance. Male mosquitoes do not blood feed, and their stylets are rudimentary with conspicuous maxillae and mandibles (Lee, 1974; Wahid et al., 2002, 2007). Moreover, the female labrum is long and tapered with an open point, whereas that of males is shorter, terminating at an abrupt angle with a potentially vestigial, labium-fused immobile hypopharynx (Christophers, 1960; Wahid et al., 2002, 2007). Of the six stylets in the female, the labrum is the specialised sensory organ for the detection of blood phagostimulants, and is apically shaped like a hypodermic needle (Figure 17.1A and B).

Prior to imbibing an acceptable meal, the female mosquito ensures a stable flow of blood by preventing platelet aggregation and coagulation, which are part of the haemostatic response of the host to skin and blood vessel puncture (Chan and Pardes, 2013; Gale, 2011). This is achieved...
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by secreting saliva, which contains general and species-specific anti-haemostatic and anaesthetic agents, thus minimising the risk of alerting the host during the blood meal (Isawa et al., 2002; Islam et al., 2019; Martin-Martin et al., 2020; Ribeiro et al., 1985). Once a suitable capillary bed and blood vessel are located, phagostimulants in the blood regulate subsequent feeding.

17.3 The structure and role of labral sensilla in blood feeding

The decision to accept or reject a blood meal is ultimately determined by the sensory response by the labrum. At the distal end of the female labrum are three pairs of sensilla. The most proximal pair of sensilla are mechanosensory campaniform sensilla (Lee, 1974; Lee and Craig, 1983), and are likely involved in the detection of diet flow (Lee, 1974). The apical and subapical paired sensilla are short, single-walled, apical pore sensilla, characteristic of gustatory sensilla (Figure 17.1B) (Lee, 1974; Lee and Craig, 1983). Each apical and subapical sensillum has four gustatory sensory neurons (GSNs), which terminate in the region of the tip pore, and one mechanosensitive neuron,
which terminates at the base of the sensillum (Figure 17.1C) (Lee, 1974; McLver and Charlton, 1970; Newland et al., 2009). The apical and subapical sensilla are involved in the assessment of a blood meal through the detection of phagostimulants in the blood, primarily adenylated nucleotides, NaCl, NaHCO₃ and albumin (Galun, 1967; Galun et al., 1984, 1985b; Jové et al., 2020; Lee, 1974; Won Jung et al., 2015).

While various species use an array of taste cues to evaluate blood meal quality, each species uses only a fraction of these phagostimulants, corresponding to the small number of gustatory sensilla and GSNs on the labrum (Lee, 1974; Lee and Craig, 1983; Liscia et al., 1993). Individually, or in combination, the key phagostimulant ligands activate sub-populations of the labral GSNs (Jové et al., 2020; Liscia et al., 1993; Werner-Reiss et al., 1999a,b,c). However, in Aedes aegypti, the ultimate decision to imbibe a meal occurs only when all the key ligands are present, suggesting that the ultimate decision to imbibe a blood meal is determined by a combinatorial response of these GSNs (Jové et al., 2020).

17.4 Taste modalities associated with haematophagy

Critical assessment of blood quality in disease vectors is primarily dependent on three human-ascribed taste modalities, including the canonical salty (e.g. NaCl) and sour (e.g. NaHCO₃), and a non-canonical modality based on the detection of adenylated nucleotides (Galun, 1967; Galun et al., 1963, 1985b; Hosoi, 1958, 1959; Jové et al., 2020). Of note, the other canonical umami (protein), sweet and bitter tastes do not appear to be required for blood meal acceptance (Gonzales and Hansen, 2016; Kogan, 1990). While we recognise that this terminology is anthropocentric, and that in arthropods these ligands may signal through multiple channels, we continue to use these terms so that the readers are able to refer back to the existing body of literature. In this section, we review the current knowledge on the detection of the key ligands within these taste modalities, and how they stimulate feeding, predominantly in mosquitoes.

17.4.1 Blood phagostimulatory ligands

The ligands, which activate blood feeding, derive primarily from the cellular components of whole blood (Galun et al., 1985a; Hosoi, 1959), particularly the phagostimulants, which are liberated from erythrocytes/red blood cells (RBCs) (Friend and Smith, 1974; Galun, 1967; Galun and Margalit, 1969; Galun et al., 1984, 1985a; Hosoi, 1959). However, while plasma components are not generally as potent as the cellular components, these have been reported to be phagostimulatory in e.g. Anopheles mosquitoes (Galun et al., 1985a), triatomines (Friend and Smith, 1982) and sandflies (Ready, 1978). The variable contribution of these phagostimulants to induce blood feeding among species is discussed below.

A wide array of nucleosides and nucleotides have been evaluated and observed to be variably stimulatory among vectors, with adenylated nucleotides, i.e. adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), shown to be the most common feeding stimulants (Figure 17.2) (Friend and Smith, 1974; Galun et al., 1963; Galun and Vardimon-Friedman, 1992). Adenosine triphosphate is identified as the key phagostimulant
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for several mosquito species (Galun, 1967; Galun and Vardimon-Friedman, 1992; Galun et al., 1963; Hosoi, 1959; Liscia et al., 1993), triatomine bugs (Friend and Smith, 1974, 1982), horse flies (Friend and Stoffolano, 1983; Lall 1969), bed bugs (Romero and Schal, 2014), tsetse flies (Galun and Margalit, 1969; Mitchell, 1976), rat fleas (Galun, 1966), stable flies (Ascoli-Christensen et al., 1990), ticks (Galun and Kindler, 1968) and blowflies (Liscia et al., 1987).

Adenosine triphosphate is a key component in metabolic processes, and is abundant within cellular components of blood (Rich, 2003), and thus, a reliable signal of an acceptable blood meal. This phagostimulant is liberated into plasma as an inflammatory marker (Di Virgilio and Vuerich, 2015; Trautmann, 2009), upon mechanical shearing of endothelial and blood cells during labral perforation, as well as during vasoconstriction (Born and Kratzer, 1984; Forsyth et al., 2011). While ATP is a reliable signal, it is also an ephemeral one, being highly labile (Fang et al., 2015; Wang et al., 2005), which has motivated investigation into more stable analogues as an avenue for alternative vector control tools, e.g. as a driver to induce engorging in attractive bait control agent delivery systems (see conclusions) (Ascoli-Christensen et al., 1990; Friend and Smith, 1982; Friend and Stoffolano, 1983; Galun and Kabayo, 1988; Galun and Margalit, 1969; Galun and Vardimon-Friedman, 1992; Galun et al., 1963). While promising stimulatory analogues to ATP have been assessed in various species (Friend and Smith, 1982; Friend and Stoffolano, 1983; Galun and Vardimon-Friedman, 1992; Galun et al., 1985a,b; Hosoi, 1959; Romero and Schal, 2014), further exploration of thermally and/or environmentally stable analogues is necessary to make these a cost-effective viable option.
Adenylated nucleotides have three main structural groups affecting phagostimulation, i.e. the phosphate group, the amino group on the purine (C6) and the hydroxyl groups on the ribose sugar (C2-OH and C3-OH). The addition or removal of phosphate groups to/from ATP, generating ADP, AMP or adenosine tetraphosphate ($A_{tetra}P$), demonstrates a positive correlation between feeding stimulation and the length of the phosphate chain in several blood-feeding arthropods (Friend and Stoffolano, 1983; Galun and Kabayo, 1988; Galun et al., 1963, 1985b). Complete removal of the phosphate groups abolishes phagostimulation in all blood-feeding arthropods, except for *Simulium venestum*, which is responsive to adenosine (Sutcliffe and McIver, 1979). Increasing the stability of the phosphate chain using non-hydrolysable ATP analogues, e.g. adenylylimidodiphosphate (AMP-PNP) and adenylylmethylenediphosphate (AMP-PCP) (Figure 17.2), enhances phagostimulation in *Ae. aegypti* (Galun et al., 1985b) and *Tabanus nigrovittatus* compared with ATP (Friend and Stoffolano, 1983). This demonstrates that stimulation by ATP is not an energy-dependent event, i.e. does not require energy to be released from the hydrolysis of ATP (Galun et al., 1985b). Rather, ATP and its analogues act as ligands, with the direct correlation between the intensity of phagostimulation and the strength of binding affinity (Friend and Smith, 1982; Galun and Kabayo, 1988; Galun and Margalit, 1969; Galun and Vardimon-Friedman, 1992; Galun et al., 1985b). Insertion of imido and methylene groups between select phosphate groups increases the distance between the amino group ($NH_2$) and the phosphate group (Galun et al., 1985b). While this distance enhances the binding affinity and potency of the adenine nucleotides, the complete removal of the $NH_2$ group from C6 of the adenine purine breaks this key bond with the receptor, rendering the ligands inactive, as evidenced in *Aedes* and *Culex* mosquitoes (Galun and Vardimon-Friedman, 1992; Galun et al., 1985b).

The removal of the hydroxyl groups from the ribose sugar at either C2 (2’d ATP) or C3 (3’d ATP) generates analogues that are approximately half as potent as ATP in eliciting phagostimulation in *Ae. aegypti* (Friend and Smith, 1982; Galun and Kabayo, 1988; Galun and Margalit, 1969; Galun et al., 1985b), similar to the replacement of the ribose group with its stereoisomer arabinosyl (Galun et al., 1985b). However, removal of both hydroxyl groups (2’,3’-dideoxyadenosine triphosphate) from the ribose increases phagostimulation in *Ae. aegypti* 10-fold compared to ATP (Galun et al., 1985b). The structural changes in 2’,3’-dideoxyadenosine triphosphate further exposes the phosphate chain, and thereby likely increase the ability of the ligand to rotate in a putative receptor pocket, thus improving binding affinity of the amino group and the terminal phosphates (Friend and Smith, 1982; Galun et al., 1985b). *Culex* and *Aedes* mosquitoes respond differentially to these structural changes in ATP (Galun and Vardimon-Friedman, 1992). In contrast to the behaviour of *Ae. aegypti*, feeding stimulation in *Culex* spp. is abolished with the removal of both hydroxyl groups (2’d ATP or 3’d ATP) (Galun and Vardimon-Friedman, 1992; Galun et al., 1988). Therefore, while adenylated nucleotides are important in phagostimulation leading to blood meal acceptance, the mechanism by which this is achieved varies among blood-feeding arthropods.

While ATP is the most potent naturally existing nucleotide in a wide variety of genera, there are exceptions to this rule. For example, ADP is the key phagostimulant in select mosquito species and the black fly *S. venestum* (Table 17.1). Moreover, the sensitivity to ATP may differ between strains,
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as demonstrated for *Ae. aegypti* (Galun et al., 1984, 1985b). While adenylated nucleotides are key phagostimulants in disease vectors, their potency is affected by various structural modifications, and the dissolving solvent is a key factor known to modulate and synergise the response during blood feeding. A plausible explanation for the interspecific differences in sensitivity to ATP and ADP may be linked to an adaptation of some species to increase the rate of blood uptake, and limit the duration of probing, in response to increased predation risk (Ribeiro et al., 1985). For example, ornithophilic mosquitoes (e.g. *Culiseta inornata*, *Culex pipiens* and *Culex univittatus*) are more sensitive to ADP than ATP (Table 17.1).

### 17.4.3 Other phosphate-related phagostimulants in blood

A number of non-adenylated and non-nucleotide phosphate-rich ligands have been demonstrated to elicit significant phagostimulation in triatomines and malaria vectors (Emami et al., 2017; Friend and Smith, 1982; Galun et al., 1985a). Phosphorylated compounds, which modify oxygen-haemoglobin affinity in birds (phytic acid) and mammals (2,3-diphosphoglyceric acid) are highly phagostimulatory in triatomines (Friend and Smith, 1982). In fact, *Rhodnius prolixus* appears to be flexible in which phosphate components in blood are required for phagostimulation, as pyrophosphates and phosphate buffers, in addition to the aforementioned compounds, can induce feeding (Friend and Smith, 1982). Moreover, a phosphorylated compound found in the isoprenoid 2-C-methyl-D-erythritol 4-phosphate (MEP) biosynthetic pathway of apicomplexan parasites, *(E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate* (HMBPP), has been found to be released into the blood by the malaria parasite *Plasmodium falciparum*, and is highly phagostimulatory in *Anopheles* spp., *Culex* spp. and *Ae. aegypti* (Emami et al., 2017; Stromsky et al., 2021). Thus, while the presence and/or length of the phosphate group(s) appears crucial for phagostimulation, variation in the stimulation is species dependent.

### Table 17.1. The ranked sensitivity to adenosine phosphates and their analogues of select haematophagic disease vectors.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ranking1</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
<td>AMP-PNP  = AMP-PCP &gt; A₆₇₋₇₋₇ &gt; ATP &gt; ADP &gt; AMP &gt; cAMP</td>
<td>Galun (1967); Galun et al. (1985b)</td>
</tr>
<tr>
<td><em>Glossina palpalis</em></td>
<td>A₅₋₅₋₇ ≥ ATP = 2’3’dATP ≥ ADP = 2’3’dADP &gt; AMP-PNP &gt; 3’3’dATP ≥ 2’3’d3ATP &gt; AMP-PCP &gt; adenosine 5’-triphosphate 2’3’dialdehyde &gt; AMP-CPP ≥ AMP</td>
<td>Galun and Kabayo (1988)</td>
</tr>
<tr>
<td><em>Tabanus nigrovittatus</em></td>
<td>ATP &gt; ADP &gt; 2’3’dATP &gt; ATP-y-S = 2’3’dADP &gt; AMP-PCP = A₅₋₇₋₇ &gt; AMP-PNP =</td>
<td>Friend and Stoffolano (1983)</td>
</tr>
<tr>
<td><em>Rhodnius prolixus</em></td>
<td>A₅₋₇₋₇ ≥ ATP &gt; AMP-PNP &gt; ADP &gt; AMP-PCP &gt; cAMP &gt; AMP</td>
<td>Friend and Smith (1982)</td>
</tr>
<tr>
<td><em>Culex pipiens</em></td>
<td>ADP &gt; ATP &gt; AMP-PNP &gt; AMP-PCP &gt; AMP</td>
<td>Galun et al. (1988); Galun and Vardimon-Friedman (1992); Liscia et al. (1993)</td>
</tr>
<tr>
<td><em>Culiseta inornata</em></td>
<td>ADP &gt; AMP-PNP &gt; ATP &gt; AMP-PCP &gt; AMP-PCP &gt; AMP-S</td>
<td>Galun et al. (1988)</td>
</tr>
<tr>
<td><em>Simulium venestum</em></td>
<td>ADP &gt; ATP &gt; AMP &gt; Adenosine &gt; cAMP</td>
<td>Sutcliffe and McIver (1979)</td>
</tr>
</tbody>
</table>

1 AMP-PNP = adenylylimidodiphosphate; AMP-PCP = adenylylmethylenediphosphate; 2’3’d3ATP = 2’3’-dideoxyadenosine 5’-triphosphate; AMP-CPP = α,β-methyleneadenosine 5’-triphosphate; ATP-y-S = adenosine-5’-(γ-thio)-triphosphate; APS = adenosine-phosphosulfate.
17.4.4 Salty and sour taste modalities

Non-cellular components of blood, i.e. plasma, contain salts of various forms, which are classified among the salty and sour taste modalities. Sodium chloride ensures the isotonic balance in blood, and stimulates feeding in most haematophagous arthropods, e.g. mosquitoes (Galun and Vardimon-Friedman, 1992; Galun et al., 1985a,b; Hosoi, 1959; Liscia et al., 1993), kissing bugs (Friend and Smith, 1974; Guerenstein and Nunez, 1994; Pontes et al., 2017; Smith and Friend, 1970) sand flies (Ready, 1978) and tsetse flies (Galun and Kabayo, 1988). For additional information, please see Chapter 18 (Barrozo et al., 2022). Sodium bicarbonate, and to a lesser extent other phosphate buffers used as replacements for plasma in RBC cultures, enhance phagostimulation (Friend and Smith, 1982; Galun, 1967; Galun et al., 1985b; Romero and Schal, 2014). A plausible explanation for this is the release of soluble carbon dioxide, through the dissociation of the buffer (Fischler et al., 2007; Galun and Margalit, 1969; Galun et al., 1985b; Werner-Reiss et al., 1999c), which enhances probing in the vectors (Jové et al., 2020; Khan and Maibach, 1966). While these taste modalities are central in the blood matrix, individually these do not elicit feeding in most vectors.

17.4.5 Synergy among phagostimulants

Mixtures of ATP, NaCl, NaHCO₃ and other blood components elicit pronounced feeding responses, similar to that of whole blood, in several species of mosquitoes (Friend, 1985; Galun and Kabayo, 1988; Galun et al., 1985b; Hosoi, 1959; Jové et al., 2020; Moskalyk and Friend, 1994; Werner-Reiss et al., 1999a,b,c), tsetse flies (Galun and Margalit, 1969), triatomines (Friend and Smith, 1982; Guerenstein and Nunez, 1994; Pontes et al., 2017; Smith and Friend, 1970), tabanids (Friend and Stoffolano, 1983; Lall, 1969) and bedbugs (Romero and Schal, 2014). Depending on the taxa, however, the importance of individual phagostimulants in the mixtures may vary. For example, Galun and colleagues (1985a) demonstrated that a mixture of ATP, NaCl and NaHCO₃ elicited minor or negligible increase in feeding over NaCl and NaHCO₃ alone in several Anopheles species, whereas in Ae. aegypti, the feeding response to ATP, NaCl and NaHCO₃ was shown to be dose-dependent (Galun et al., 1985b). In stable flies, however, the addition of ATP reduces the potency of saline (Ready, 1978). The fact that several anophelines readily engorge on plasma, with or without ATP, suggests an evolutionary adaptation of other accessible ligands in the blood plasma (Galun et al., 1985a).

Evaluation of blood fractions containing either cellular components or higher molecular weight substances, e.g. albumin, supports the role of these factors as additional phagostimulants (Galun et al., 1984, 1985a; Hosoi, 1959). In fact, several vector species will not readily gorge on plasma alone, but require the presence of either the cellular component of the blood, or its component parts, e.g. culicine mosquitoes (Galun and Vardimon-Friedman, 1992; Galun et al., 1988; Hosoi, 1959), tsetse flies (Galun and Margalit, 1969; Galun and Kabayo, 1988) and tabanids (Friend and Stoffolano, 1983; Lall, 1969; Stoffolano, 1990). In Ae. aegypti, ATP-supplemented platelet-poor plasma induces higher feeding rates than either ATP-supplemented filtered plasma or ATP in saline alone (Galun et al., 1984). This suggests that plasma contains higher molecular weight substances, e.g. albumin, which improves phagostimulation. The addition of serum albumin to the ATP, NaCl and NaHCO₃ mixture enhances stimulation of Ae. aegypti feeding to a level similar to that of ATP in unfiltered plasma.
plasma (Galun et al., 1984). A similar role of albumin has been demonstrated for Anopheles dirus (Galun et al., 1985b). There is, therefore, an increased support for combinatorial coding playing a role in phagostimulation and its resulting blood-feeding behaviour.

### 17.5 Other factors affecting phagostimulation

Blood includes various components in addition to those discussed above, which directly or indirectly modulate phagostimulation, and further potentiate the effects of circulating phagostimulants. Examples of such factors include the intrinsic ionic environment, blood group antigens and the size of the RBCs.

#### 17.5.1 Intrinsic ionic environment

Phagostimulation is more pronounced when ATP is dissolved in solution with inorganic chlorides, particularly NaCl and CaCl₂ (Hosoi, 1958). Isosmotic NaCl is the most effective electrolyte in this regard (Galun, 1967; Galun and Kindler, 1968; Galun et al., 1963; Hosoi, 1959). The salivary enzyme apyrase, which degrades nucleotides, however, requires divalent ions, i.e. calcium and magnesium, to act as a cofactor for its activity (Friend and Stoffolano, 1983; Galun et al., 1985b). Thus, ATP and other nucleotides, are degraded during feeding in the presence of calcium and magnesium ions, which reduces the overall potential of adenylated phagostimulants. Therefore, it is not only ligands that directly affect phagostimulation during blood feeding, but also the physical and chemical properties of the diet.

#### 17.5.2 Blood group antigens

Mosquitoes demonstrate selective landing and feeding preferences based on the ABO blood types, defined by the presence or absence of antigens on the red blood cells (Watkins, 1966; Schenkel-Brunner, 1980). Aedes aegypti (Wood, 1976), Ae. albopictus (Shirai et al., 2004) and Anopheles spp. (Anjomruz et al., 2014b) preferentially select and feed on people with blood group O more often than on those with either A or B antigens. The mechanism by which mosquitoes are able to discriminate among blood type meals, and why, is so far unclear, especially in the light of the lack of a clear fitness advantage associated with this preference (Anjomruz et al., 2014a; Prasadini et al., 2019; Wood, 1974; Wood et al., 1972). The likely explanation for this meal preference may relate to the high level of the H antigen in type O blood (Blancher, 2013; Yamamoto, 1995), which may have been selected for over evolutionary time.

#### 17.5.3 The source and size of red blood cells

Mosquitoes access the intracellular protein content within the RBCs through haemolysis, either by enzymatic lysis in the gut or by mechanical shearing as the RBCs pass through the cibarial armature, situated anterior of the foregut (Coluzzi et al., 1982). The type of cibarial armature is species dependent, with the more derived armature leading to enhanced haemolysis (Chadee et al., 1996; Coluzzi et al., 1982). In addition, the size of the RBCs varies considerably across the vertebrate hosts (Gulliver, 1870; Martinho, 2012; Wintrobe, 1933), and mosquitoes with derived
armatures are more likely to be able to make use of the nucleated blood from birds (e.g. *Culex* spp.) and reptiles (Henning, 1972), compared to those with less developed cibaria (e.g. *Anopheles* (Coluzzi *et al.*, 1982; Sinton and Covell, 1927). This type of morphological adaptation, along with the bore diameter of the labrum are potential drivers for host preference.

### 17.6 Mechanism underlying the detection of phagostimulants

Phagostimulants are detected by receptors on the dendritic surface of GSNs housed in the apical and sub-apical labral sensilla in mosquitoes (Figure 17.1) (Galun *et al.*, 1985b; Jové *et al.*, 2020; Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999c) and the labella of pool feeders, e.g. tsetse flies (Ascoli-Christensen *et al.*, 1990; Michtell, 1976; Rice *et al.*, 1973). The physiological response of these GSNs to various phagostimulants has been characterised and shown to be age- and state-dependent (Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999a,b,c).

### 17.6.1 Physiological responses of labral sensilla in mosquitoes

The current understanding of the sensory basis for blood-phagostimulant detection is based primarily on fundamental analyses of the labral-apical sensilla of mosquitoes (Jové *et al.*, 2020; Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999a,b,c). The labral sensilla in mosquitoes house four GSNs (Figure 17.1C), each of which has a different tuning profile. Functional imaging of the labral tip of *Ae. aegypti*, including the apical and sub-apical gustatory sensilla, revealed that each of the four types of GSNs have unique tuning profiles to ATP, NaHCO₃ and NaCl, or a mixture of NaHCO₃, NaCl and glucose (Jové *et al.*, 2020). While all GSNs respond to adenylated nucleotides (Werner-Reiss *et al.*, 1999a,b,c), each differs in sensitivity reflecting the variation in behavioural activity to these ligands (Galun *et al.*, 1985a,b; Hosoi, 1959). A mixture of ATP, NaHCO₃, NaCl and glucose stimulates the labral GSNs in a manner similar to blood (Jové *et al.*, 2020). While the results obtained by functional imaging reflect that which was previously found using electrophysiology in *Ae. aegypti* (Werner-Reiss *et al.*, 1999a,b,c), there appears to be significant variation in tuning profiles across species (Mitchell, 1976; Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999a,b,c), as has also been observed in behaviour (Friend and Stoffolano, 1990; Friend and Smith, 1982; Galun, 1967; Galun and Kabayo, 1988; Galun and Margalit, 1969; Galun and vardimon-Friedman, 1992; Galun *et al.*, 1985a,b; Hosoi, 1959). While interaction between stimuli is not explicit in the imaging results, presentation of ATP in combination with either saline, bicarbonate buffer or sugar in electrophysiological studies resulted in the potentiation or dampening of the response to ATP alone in several GSNs across species (Ascoli-Christensen *et al.*, 1990; Jové *et al.*, 2020; Liscia *et al.*, 1993; Werner-Reiss *et al.*, 1999a,b,c). These studies emphasise that the combinatorial code relayed by the labral GSNs is reflected in the feeding behaviour of the different vectors, as is the species-dependent variability in these responses.

### 17.6.2 Gustatory plasticity

Gustatory plasticity in blood feeding is exemplified by the variable behavioural responses to blood or blood-related phagostimulants, and is influenced by both intrinsic, e.g. age (Werner-Reiss *et al.*, 1999c), and previous experience and learning (Mwandawiro *et al.*, 2000; Tomberlin *et al.*, 2006;
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Vantaux et al., 2014, as well as extrinsic factors, e.g. heat (Bodin et al., 2009; Fresquet and Lazzari, 2011). In Ae. aegypti, the sensitivity of the labral apical GSNs increases as the mosquito ages, i.e. with a higher neural response rate in females 17 days post-emergence compared with 24 h post-emergence, while there is no response to ATP 5 h post-emergence (Werner-Reiss et al., 1999c). These changes in blood-feeding behaviour reflect the developmental propensity of mosquito to host seek (Brown et al., 1994; Davis, 1984). While the modulatory mechanism regulating host seeking has received some attention, identifying neuropeptides involved in regulating the refraction to host odour (Christ et al., 2018; Duvall et al., 2019), additional research is required to elucidate the role of these, and/or other neuromodulators, in regulating the gustatory system in mosquitoes.

17.6.3 Molecular mechanism of blood phagostimulation

While a variety of chemosensory receptors, including gustatory receptors (GRs), ionotropic receptors (IRs), odorant receptors (ORs), transient receptor potentials (Trps) and pickpocket receptors (Ppks) (Ruel and Bohbot, 2022), have been shown to be expressed in the fascicles of mosquitoes (Choo et al., 2015; Jové et al., 2020; Won Jung et al., 2015), the molecular mechanism underlying the detection of blood phagostimulants remains largely unknown. Here, we provide a short précis of the current understanding of phagostimulant detection by the labrum in mosquitoes.

17.6.4 Salty and sour taste modalities

Salty and sour are the only canonical taste modalities, which so far have been associated with phagostimulation in haematophagous insects (Galun and Vardimon-Friedman, 1992; Galun et al., 1985b; Hosoi, 1958; Pontes et al., 2017; Smith and Friend, 1970; Werner-Reiss et al., 1999c). While the mosquito labral apical sensilla house both low and high salt-sensitive GSNs (Liscia et al., 1993; Werner-Reiss et al., 1999c), a chemosensory receptor gene encoding a putative salt receptor has yet to be identified in the labrum. Proposed to modulate both the appetitive low salt sensing (Zhang et al., 2013), and the high salt aversive response in Drosophila (Lee et al., 2017), the ionotropic co-receptor IR76b, is widely expressed in various taste-associated organs, including the mosquito stylet, and is thus a candidate receptor for salt sensing and blood feeding in the labral gustatory neurons (Ye et al., 2021). Similarly, the pickpocket receptor AePPK301 responds to water and low salinity, and has been demonstrated to be involved in oviposition-site choice in Ae. aegypti, however transcriptome analysis does not indicate its expression in the labrum (Jové et al., 2020; Matthews et al., 2018, 2019). For an in-depth discussion on salt detection, please see Barrozo et al. (2022).

The sour taste modality is mediated by soluble carbon dioxide (CO₂), which is present in blood predominantly as HCO₃⁻ (Centor, 1990; Xu et al., 2020). A triad of mosquito gustatory receptors involved in CO₂ detection have been postulated to also detect bicarbonates (Jones et al., 2007; Kwon et al., 2007), however, none of these subunits are reported to be expressed in the labrum (Jové et al., 2020). While IR56d, as well as the IR co-receptors, IR25a and IR76b, have been reported to be involved in the detection of carbonation in Drosophila (Sánchez-Alcañiz et al., 2018), these IRs also have not been reported to be expressed in the labrum of mosquitoes (Jové et al., 2020).
However, the bicarbonate-responsive neurons in the labrum do express an IR, IR7f, in Ae. aegypti, which hitherto has not been shown to be carbonate-sensitive (Jové et al., 2020). Further analysis is required to elucidate the molecular mechanism for the salty and sour modalities in the labrum.

17.6.5 Adenylated nucleotides

The molecular mechanism underlying the binding and detection of adenylated nucleotides is in the process of being described, with individual components of the pathway identified and/or characterised (Mitchell, 1976; Galun et al., 1985a, b; Galun and Vardimon-Friedman, 1992; Liscia et al., 1993; Werner-Reiss et al., 1999a,b,c; Jové et al., 2020; Martin-Martin et al., 2020). The recent identification of a member of the salivary protein D7 family, CxD7L1, in Cx. quinquefasciatus as an adenosine nucleotide binding protein, besides inhibiting haemostatic vasoconstriction and platelet aggregation, has increased our knowledge of the families of molecules involved in blood feeding on mammalian hosts (Martin-Martin et al., 2020). This study suggests a role for these binding proteins in degradation protection, transport and detection of ATP and ADP by GSNs in the labrum, similar to the role of odorant binding proteins in the olfactory system of insects (Vogt and Riddiford, 1981; Leite et al., 2009). While the identity of the membrane-bound receptors for adenylated nucleotides in arthropod vectors is unknown, several studies have inferred the structure of the putative receptor based on ligand and agonist activation of the GSNs in comparison with vertebrate purinoceptors (Liscia et al., 1993; Mitchell, 1976; Werner-Reiss et al., 1999a,b,c).

Vertebrate purinoceptors are transmembrane receptors activated by extracellular ATP and purine ligands (Burnstock, 1972, 1980), and are subclassified as P_1 and P_2 depending on their response to variations in the length of phosphate chains, and a preference for either adenosine or ATP/ADP, respectively (Burnstock and Kennedy, 1985). The P_2 receptors may be further classified (Abbracchio and Burnstock, 1994; Gordon, 1986; Murgia et al., 1993), including the ligand-gated ion channels (P_{2x}) (Benham and Tsien, 1987) and the G-protein-coupled receptors (P_{2y}) (Cusack, 1993), which are relevant for most haematophagous insects (Galun et al., 1988). For example, G. palpalis has a potency order of A_{tetra}P ≥ ATP = 2'd ATP ≥ ADP =2' ADP > AMP-PNP > 3'd ATP ≥ 2' 3'ddATP > AMP–PCP > AMP–CPP >> AMP and is categorised as a P_{2y} receptor (Galun et al., 1988). In addition, Stomoxys calcitrans has a potency order of CH_3–S–ATP = ATP > ADP > AMP > adenosine (Ascoli-Christensen et al., 1991) and is categorised as a P_{2x} receptor (Burnstock, 1972). However, there are exceptions to the vertebrate classification of purinoceptors, e.g. Cx. pipiens and Cu. inornata, which have a uniquely strong response to ADP, and are inhibited by changes in the length of the phosphate chain (Hosoi, 1959; Galun et al., 1988; Liscia et al., 1993). Thus, these vectors either lack the P_1 (>AMP) and P_2 (>ATP) purinoceptor type system or express both types of receptors, the additive effect of which would result in the demonstrated response to ADP (Galun et al., 1988). The P_{2x} receptor type is not indicated to be present in mosquitoes. With improved functional genomic tools, and increasing availability of curated vector genomes, we are likely on the cusp of identifying the vector arthropod purinoceptors.
17.7 Conclusions

Knowledge concerning blood-related phagostimulants provides a mechanistic insight into vector feeding and viable avenues for the improvement and development of complementary vector control tools. To this end, identification of stable analogues of known blood phagostimulants, e.g. ATP, may enhance arrestment, acceptance and diet uptake to increase the efficacy of existing control tools, such as attractive toxic sugar baits (Barbosa et al., 2019; Maia et al., 2018; Revay et al., 2014). By varying these analogues, these tools may be targeted against specific species using a variety of control agents, which may also be tailored to different species or taxa, e.g. RNA interference (Pillai et al., 2017; Whitten, 2019). Identification of such analogues may be benefited by an enhanced understanding of the molecular and physiological characteristics of the gustatory system of disease vectors from an evolutionary perspective. Ultimately, much remains to be explored concerning taste associated with haematophagy and understanding its underlying mechanisms might bring new invaluable insights that can be used for vector control.

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Chapter 17 – Phagostimulants drive the acceptance of a blood meal in disease vectors


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M. Lukenge, R. Ignell and S.R. Hill


Adenosine triphosphate overrides the aversive effect of antifeedants and toxicants: a model alternative phagostimulant for sugar-based vector control tools

Matthew Lukenge1, Rickard Ignell1 and Sharon Rose Hill1*

Abstract

Background  Sugar, when used as the phagostimulant in attractive toxic bait control tools, limits the efficacy and selectivity of this technology. Thus, more potent and selective phagostimulants than sugar are required to improve this technology. The potency of adenosine triphosphate (ATP) as an alternative model phagostimulant was assessed to determine its capacity to override the aversive effects of select antifeedants and toxicants. How ATP and sucrose modulate the rate of toxicity in the yellow fever mosquito Aedes aegypti was also examined.

Methods  A no-choice feeding assay was used to investigate the phagostimulatory ability of ATP to override the aversive effects of structurally divergent antifeedant and toxicant compounds, and to modulate the rate of toxicity over 24 h. Binary combinations of antifeedant and toxicant compounds, at various concentrations, were similarly assessed for enhanced lethal potency. In comparison, no-choice open access and cotton wick feeding assays were used to determine the phagostimulatory role of sucrose in the ingestion of boric acid-laced diets. Dissections of the guts were performed to determine the diet destination as dependant on the phagostimulant.

Results  ATP is a potent phagostimulant that dose dependently overrides aversion to antifeedant and toxicant tastants. Feeding on antifeedant- or toxicant-laced diets that was induced by ATP selectively resulted in rapid knockdown (nicotine, lobeline and caffeine) or death (boric acid and propylene glycol), with a combination of the two lethal compounds inducing a synergistic effect at lower concentrations. ATP- and sucrose-induced feeding predominantly directed the antifeedant- or toxicant-laced meals to the midgut and the crop, respectively.

Conclusions  ATP is an efficacious alternative model phagostimulant to sucrose that overrides the aversive effects of antifeedants and toxicants, resulting in rapid toxic effects. Furthermore, this study demonstrates that variation in the rate of toxicity between ATP- and sugar-induced feeding is at least partly regulated by the differential feeding response, volume imbibed and the destination of the meals. Additional research is needed to identify structurally related, stable analogues of ATP due to the ephemeral nature of this molecule. For future applications, the workflow presented in this study may be used to evaluate such analogues for their suitability for use in attractive bait stations designed to target a broad range of hematophagous arthropods and prevent off-target species’ feeding.

Keywords  Adenosine triphosphate, Taste, Toxins, Aedes aegypti, Feeding, Mosquito, Attractive toxic sugar bait

*Correspondence:  Sharon Rose Hill  sharon.hill@slu.se
Full list of author information is available at the end of the article
Background
Interventions targeting arthropod vectors are the most efficient strategies in the fight against vector-borne diseases [1]. With a limited arsenal of vector control tools, and those that are available becoming less effective [1, 2], the World Health Organization advocates for the development of new tools with different modes of action to be used in future integrated vector management strategies [3]. Although taste-based arthropod control tools date back as far as the eighteenth century [4], the model taste-based tool, the attractive toxic sugar bait (ATSB), has received insufficient attention. Improvements in ATSB efficiency and efficacy offer a viable avenue to increase their usefulness for integrated vector management strategies.

Current ATSB technologies have several limitations, primary among which is the use of sucrose as a non-selective phagostimulant, targeting select disease vectors and beneficial insects, e.g. pollinators, alike [5]. In addition, sucrose may not be an efficient phagostimulant to override the aversive effects of natural antifeedants and synthetic toxicants, as reliance on sugar phagostimulants is prone to resistance [6, 7]. Moreover, many disease vectors are obligate blood feeders that do not imbibe sugar meals [8], and are thus not targeted by ATSBs. For mosquito vectors, which tend to imbibe small sugar meals at a time, it may also require multiple visits to an ATSB station to accumulate a sufficient level of a toxin for it to be lethal. Thus, to mitigate the limitations highlighted, a more potent and selective phagostimulant, to which the cost of evolutionary pressure is too high for the vector to develop resistance, needs to be identified.

A model alternative phagostimulant for this purpose is adenosine triphosphate (ATP), a highly potent and strongly selective phagostimulant for the majority of blood-feeding arthropods [9]. The likelihood of blood-feeding vectors developing resistance to ATP is low because ATP is an indicator of a blood meal, which is required to secure fitness-related behaviours. Due to the labile nature of ATP [10], it cannot be used in current ATSB technology. An increased understanding of the signalling pathway and how this modulates the uptake of toxic antifeedants may, however, provide guidance for the identification and/or development of stable ATP analogues for future use in attractive toxic ATP-analogue bait technology. In this study, ATP was assessed for its ability to drive the feeding of the yellow fever mosquito *Aedes aegypti* on a variety of structurally different antifeedant and toxicant compounds that elicited varying degrees of toxicity, ranging from knockdown to lethal effects. Depending on the phagostimulant used, i.e. ATP or sugar, the diet destination differed, correlating with different rates of action of the toxic compounds. We discuss the use of ATP as a model for alternative phagostimulants to sugar as a driver for toxic baits in vector control tools.

Methods
Mosquito rearing
*Aedes aegypti* (Rockefeller strain) was reared at 25 ± 2 °C, 70 ± 2% relative humidity and a 12 h:12 h light:dark photoperiod. Briefly, eggs were hatched in plastic trays (23.5 cm × 18 cm × 7.5 cm; ca. 300 ml water), with ca. 300 larvae per tray. The pupae, which were collected in small plastic cups (30 ml water), were transferred into BugDorm-4E1515 cages (17.5 cm × 17.5 cm × 17.5 cm; Megaview Science, Taichung, Taiwan). Emerging adults were provided with ad libitum access to 10% weight/volume (w/v) sucrose up to 4 days post-eclosion, and then starved for 22 ± 2 h with ad libitum access to water prior to the feeding experiments.

Bioassay to assess ATP-induced feeding on antifeedant and toxicant compounds
To make the feeding test solutions, stock solutions of 12 mM ATP [Chemical Abstracts Service (CAS) no. 34369-07-8; Merck, Darmstadt, Germany] were prepared by dissolving ATP in bicarbonate buffered saline [150 mM sodium chloride (Merck) and 10 mM sodium bicarbonate (Merck)] at pH 7.4 ± 0.07, and then stored at −20 °C. To examine whether ATP overrides feeding deterrence and induces feeding on natural antifeedants or synthetic toxicants, a panel of tastants, previously classified as antifeedants, toxicants or feeding deterrents, were assessed at a range of concentrations (Table 1). The natural antifeedants were the plant-derived alkaloids caffeine [11, 12], quinine, nicotine [11, 13, 14], lobeline [13] and capsaicin [15, 16], while the toxicants were the synthetic compounds *N,N*-diethyl-meta-toluamide (DEET), a major insect repellent [13], and propylene glycol [17],...
and the mineral acid boric acid, which are known insecticides [16, 18–20] (Table 1). Stock solutions of the tastants were prepared as follows: water-soluble compounds, i.e. caffeine (80 mM), propylene glycol (10.5 M), DEET (209 mM), nicotine (20 mM) and boric acid (1.3 M), were dissolved in the bicarbonate buffered saline. This was done at room temperature, except for boric acid and quinine that required heating at 42 °C to fully dissolve. In contrast, lobeline hydrochloride (100 mM) and capsaicin (100 mM) were dissolved in 50% (volume/volume; v/v) ethanol, while quinine (20 mM) was dissolved in 30% (v/v) ethanol. Working dilutions were prepared for lobeline hydrochloride and capsaicin at 5 mM, and for quinine at 2 mM in the bicarbonate buffered saline. The final concentrations contained less than 2.5% (v/v) ethanol. Ethanol at 2.5% (v/v) ethanol was examined for its effect on ATP phagostimulation in a membrane feeding assay, as described below, which demonstrated that it had no effect on ATP potency \( U_{(y)} = 10.50, Z = 0.30, P > 0.99 \).

Each stock solution of the tastant compounds was serially diluted using the pH-controlled bicarbonate buffered saline to which was added an equal volume of ATP to a final concentration of 0.6 mM, a concentration that elicited maximum phagostimulation in \( \text{Ae. aegypti} \), as determined in a preliminary analysis, through the generation of a dose–response curve with nine doses between 0.005 and 0.6 mM (data not shown), and supported by a previous study [21]. In addition, xylene cyanol FF (CAS no. 2650-17-1; Merck) was added to a final concentration of 1 mg ml \(^{-1} \) to aid visualisation of engorged individuals. The final concentration ranges of the tastants are indicated in Table 1. A positive control (0.6 mM ATP in buffer) and two negative controls, i.e. buffer alone and buffer together with the lowest concentration of each antifeedant and toxicant, were used, in which avid probing, but no engorgement, was observed.

To confirm whether the mosquitoes detected the antifeedants and toxicants, a concentration at, or close to, the threshold of behavioural response was used. The assay included a negative control (antifeedant or toxicant “tastant” alone) and two positive ATP controls, the 50% and fully effective concentrations (respectively, \( EC_{50} 0.072 \text{ mM and } EC_{\text{max}} 0.6 \text{ mM} \), as determined in preliminary studies; see above), as well as the tests, i.e. combinations of the tastant with either of the ATP concentrations \( (n = 5, n = 50) \).

Ten female mosquitoes at 5 days post-eclosion were gently aspirated into bioassay chambers (tall polystyrene Petri dishes, 12 cm diameter x 6 cm height; Semadeni, Ostermundigen, Switzerland) covered with fine mesh, for each of the controls and tests, and for five replicates. Using a membrane feeding system (Hemotek, Blackburn, UK), and feeding reservoirs (0.3 ml; Hemotek) filled with 200 µl of the diets under a collagen membrane (Hemotek), the mosquitoes were exposed to the diets for 30 min at 37 °C. Since \( \text{Ae. aegypti} \) is a diurnal feeder, the assays were performed at Zeitgeber time 6–9, within the peak activity period [22]. Mosquitoes that scored iv or v on the feeding scale were considered engorged, while those that scored above ii were considered fed (Fig. 1a), and used as such in further analyses.

**Toxic effects of antifeedant and toxicant diets imbibed during ATP-induced feeding**

To examine the toxic effect of the antifeedant and toxicant compounds, a no-choice feeding assay was used as described above in which 10 mosquitoes were exposed to the antifeedant and toxicant diets and observed for 24 h without access to sugar and water. This experiment was replicated 5 times. Unlike in previously conducted sugar-bait toxic assays, in which the mosquitoes had access to the toxic diet ad libitum for the duration of the experiment [19, 20, 23], the mosquitoes in this experiment had access to the ATP diet for 30 min, since ATP is thermostable and rapidly loses its integrity. Lethal and knockdown effects were scored after exposure to the diets for up to 24 h.

**Toxicity of sucrose-induced feeding on boric acid**

Taste-based toxic baits commonly include sucrose as a feeding stimulant. To compare our results of ATP-induced feeding on boric acid by \( \text{Ae. aegypti} \) to those of previous studies, an assessment of the sugar-induced feeding [10% (w/v) sucrose] on a boric acid diet was conducted. For this purpose, 10 female mosquitoes (5 days post-eclosion) were placed in a BugDorm cage and then exposed to the diet via either of two methods: a cotton wick soaked in a 1% (w/v) boric acid, 10% (w/v) sucrose (Merck) and xylene cyanol (1 mg ml \(^{-1} \)) diet contained in a 5-ml glass vial; or an open-access feeding assay, in which 200 µl of the same solution was added to a Hemotek feeding reservoir (without a membrane), as described above, with refills every 20–24 h. In both experiments, eight replicates of the treatments and two replicates of the control [10% (w/v) sucrose plus dye (xylene cyanol)] were conducted. The survival of the exposed individuals was scored for up to 5 days.

**Synergistic effect of ATP-induced feeding on combined antifeedant and toxicant diets**

In our panel of toxicants, boric acid and propylene glycol elicited lethal effects, while the antifeedants nicotine, lobeline and caffeine induced knockdown followed by recovery. We hypothesised that a combination of lower concentrations of each of the compounds would result
in a synergistic lethal effect. To address this hypothesis, the same membrane feeding assay as described above was performed with 10 female mosquitoes, and was replicated 5 times, using a combination of boric acid and propylene glycol, nicotine or caffeine (Additional file 1: Table S1). In all cases, the mosquitoes were stimulated to feed using 0.6 mM ATP, which also served as the positive control.

**Destination of ATP- and sucrose-induced diets**

Having observed that the rate of toxicity in ATP-induced feeding was, by far, much faster when compared to sucrose-induced feeding, we hypothesised that this difference was in part due to the destination of the meals in either instance. To test this hypothesis, the lowest concentrations that induced the highest level of engorgement while still eliciting a toxic effect were considered for each individual compound. In brief, for the ATP-induced meal, the membrane feeding assay, as described above, was used. For comparison, a sucrose-induced feeding assay was used, in which cotton balls soaked with the diet were placed on top of bioassay chambers (Semadeni) and heated to 37 °C for 40 min. Each BugDorm contained 10 mosquitoes per diet. After exposure, the mosquitoes were anaesthetised on ice and their guts dissected in Ringer's solution under a stereo microscope (x10, Nikon SMZ100; Nikon, Stockholm, Sweden) equipped with a 64 MP Android camera (Tecno Camon 19 Pro; Tecno, Shenzhen, China). The experiment was repeated until the guts of 10 fed individuals per diet had been successfully dissected.

**Statistical analysis**

To test whether the mosquitoes detected the antifeedant and toxicant compounds, a one-way ANOVA was used to test the proportion of individuals that engorged on ATP (EC_{50}) and those that fed on a combination of ATP at EC_{50} whereas ATP EC_{max} is able to override this effect. ANOVA was used for the pairwise comparisons; ns non-significant, * P<0.05, ** P<0.01. Mosquitoes engorged on 0.6 mM ATP-containing antifeedant and toxicant diets in a dose-dependent manner. The error bars indicate the SEM. The number of replicates for each diet and concentration was 50. DEET N,N-diethyl-meta-toluamide; PRO propylene glycol.
was the response variable, while the diets were the predictor variables. An extra sum-of-squares F-test was used to compare whether the best fit values of select parameters (log $EC_{50}$ Hill slope, top value) differed between individual antifeedant and toxicant datasets. To assess the mortality rates of both ATP-induced (24 h) and sucrose-induced (5 days) toxicity, the probability of survival was visualised using Kaplan–Meier survival curves, and the rate of survival analysed using the log-rank Mantel–Cox test. To test for differences in the potency of the knockdown effects, a Kruskal–Wallis test followed by Dunn’s pairwise post hoc comparison was used based on the 30-min timepoint as the earliest peak knockdown time point. In contrast, the comparison of mortality as a result of boric acid alone and that of boracic acid in combination with either nicotine or caffeine was analysed using the Mann–Whitney U-test based on data at the 24-h timepoint. All of the analyses and generated graphs were done using GraphPad Prism software (GraphPad Prism, v. 8.0.0; GraphPad Software, San Diego, CA) and variance is indicated on the graphs, where appropriate.

**Results**

**ATP overrides the aversive effect of antifeedant and toxicant compounds**

To assess whether the mosquitoes were able to detect the antifeedant and toxicant compounds, no-choice feeding assays were performed, which demonstrated that all tastants elicited an aversive response when combined with ATP at $EC_{50}$ (0.072 mM; Fig. 1a, b). In contrast, when tested at the same concentration, the antifeedant and toxicant compounds in combination with ATP at $EC_{max}$ (0.6 mM) elicited no aversive response (Fig. 1b), demonstrating that ATP is able to override the aversive effect of these tastants. A comparison of the proportion of females enrgoing in response to a range of concentrations of antifeedant and toxicant compounds together with ATP ($EC_{max}$) demonstrated an overall significant difference in sensitivity to various antifeedant and toxicant compounds [$F_{(7,212)} = 30.54, P < 0.001;$(Fig. 1c]. More specifically, the antifeedants and toxicants were ranked with respect to female mosquito sensitivity as follows: DEET ($EC_{50}=0.39$ mM) > quinine ($EC_{50}=0.23$ mM; $F_{(1, 61)}=1.19, P=0.28$) > lobeline ($EC_{50}=1.14$ mM; $F_{(1, 61)}=23.03, P<0.001$) > nicotine ($EC_{50}=1.25$ mM; $F=0.21, P=0.65$) > capsaicin ($EC_{50}=3.07$ mM; $F=57.15, P<0.001$) > caffeine ($EC_{50}=9.34$ mM; $F=51.37, P<0.001$) > boric acid ($EC_{50}=321.80$ mM; $F=16.50, P<0.001$) > propylene glycol ($EC_{50}=1429$ mM; $F=8.11, P=0.0070;$(Fig. 1c).

The observed volume of antifeedant- and toxicant-laden diets imbibed due to ATP phagostimulation was higher (stage iv–v) than that observed in the sugar-induced feeding (stage ii–iii; Fig. 1a). However, the volume of nicotine imbibed due to ATP-induced feeding was lower than that of the other antifeedants or toxicants tested, ranking on average as fed (stages ii–iii) compared to engorged (stages iv–v), respectively (Fig. 1a). In general, the females that fed on the antifeedant- and toxicant-laden meals displayed similar windows of dynamic response to the antifeedant and toxicant compounds, as indicated by similar slopes of the linear parts of the curves ($F=2.87, P=0.0070;$(Fig. 1c). While similar, the slopes associated with feeding on DEET (slope $=−0.92$) and propylene glycol (slope $=−1.21$; $F_{(1, 44)}=0.39, P=0.53$) demonstrated wider dynamic windows for these antifeedants and toxicants when compared to the rest of the compounds, which shared similar dynamic windows [$F_{(3,168)}=0.49, P=0.80;$(Fig. 1c].

**Feeding on toxicant-laced diets elicits toxic effects**

ATP-induced feeding on boric acid and propylene glycol elicited rapid lethal effects in individuals that engorged (Fig. 2a, b), as well as in those that had fed, but not engorged, on the diets (Additional file 2: Fig. S1a, b). Boric acid ($\chi^2 = 247.50, df=5, P<0.001$) and propylene glycol ($\chi^2 = 163.00, df=4, P<0.001$) elicited significant mortality in engorged individuals in a dose-dependent manner, with maximum mortality observed within the first 3 h for the two and three highest doses tested of the respective toxicants (Fig. 2a, b). Among the total individuals exposed to the highest concentration of propylene glycol, mortality was observed in those that had engorged as well as in those that imbibed less than a complete meal (Additional file 2: Fig. S1b).

The highest doses of boric acid and propylene glycol that did not result in a reduction in the proportion of mosquitoes engorging (boric acid, 16 mM; propylene glycol, 131 mM; Fig. 2c) caused no significant effect on mortality alone, but when combined, elicited a synergistic effect on mortality (Fig. 2d). The second lowest combined doses, eliciting both engorging and feeding (boric acid, 81 mM; propylene glycol, 657 mM), elicited an effect similar to that of propylene glycol alone (propylene glycol, 657 mM; Fig. 2e; Additional file 2: Fig. S1c), while at the highest doses tested (boric acid, 162 mM; propylene glycol, 1314 mM), mortality reduced with the combined diet compared with the individual toxicants (Fig. 2f), likely due to the reduced diet intake (Fig. 2c).

**ATP-induced feeding on antifeedants and boric acid diets elicits knockdown toxic effects**

In addition to the observed lethal effects, knockdown effects were observed in response to feeding on nicotine, lobeline and caffeine in a dose-dependent manner (Fig. 2g; Additional file 2: Fig. S1d–f). Nicotine
Fig. 2a–h ATM-induced feeding elicits rapid antifeedant- and toxicant-associated toxic effects in Aedes aegypti. Kaplan–Meier probability of survival curves for a ATP and boric acid (BOR) and b ATP and PRO in the engorged individuals. c Comparisons of the proportion of engorged individuals on ATM-induced feeding on BOR and PRO, and combinations thereof, over various concentrations. Comparison of Kaplan–Meier survival curves in response to ATM-induced feeding on BOR and PRO, and combinations thereof, over the low (d), middle (e) and high (f) concentrations. g Proportion of mosquitoes knocked down at the most potent concentrations of nicotine (NIC), caffeine (CAF) and lobeline (LOB) following exposure to ATM-containing meals. h The probability of survival of sugar-induced feeding on BOR in open access (dotted line) and cotton wick (solid line) exposure assays. A log-rank (Mantel-Cox) statistical test was used to analyse the probability of survival, while a Kruskal–Wallis test was used to analyse the proportion of engorged mosquitoes; * P < 0.001. The colour intensities represent the increase in concentrations, while the lowercase letters indicate significant differences among the individual concentrations tested and the combinations thereof. For other abbreviations, see Fig. 1.
elicited a more rapid knockdown effect, peaking after 30 min, compared with lobeline and caffeine ($H = 10.76, df = 2, P < 0.001$; Fig. 2g). Dunn's post hoc comparison indicated a significantly higher knockdown effect for nicotine than caffeine ($Z = 2.20, P = 0.030$) or lobeline ($Z = 3.21, P = 0.0010$), whereas the knockdown effects of caffeine and lobeline were not significantly different ($Z = 1.010, P = 0.31$; Fig. 2g).

To assess the possible synergistic effect of nicotine and boric acid on mortality, mosquitoes were fed on combined diets, which demonstrated that the reduction in engorgement elicited by nicotine at the two highest doses tested resulted in reduced mortality in the combined diets compared to that induced by boric acid alone (Additional file 3: Fig. S2a, b). The lowest doses tested elicited no difference in engorgement and no mortality in response to the combined diet (Additional file 3: Fig. S2a, b). To assess the potentiation effect of caffeine on mosquitoes engorging on boric acid, 5 mM caffeine, the concentration eliciting the highest level of engorgement alone, was used together with various concentrations of boric acid in the toxicity assay. There was no increase in the proportion of mosquitoes feeding [$F_{(2, 30)} = 1.53, P = 0.23$; Additional file 3: Fig. S2c], nor was there an increase in the mortality within the corresponding combinations ($\chi^2 = 9.5, df = 5, P = 0.091$; Additional file 3: Fig. S2d–f), when combined caffeine and boric acid diets were analysed.

**Sucrose-induced feeding on boric acid-containing diets elicits slow lethal effects**

To compare the mortality rates in response to ATP- and sugar-induced diets, three modes of sugar meal delivery were assessed. While membrane feeding did not elicit sufficient numbers of fed mosquitoes within 30 min to compare with ATP-fed individuals, open source and cotton wick feeding ad libitum resulted in diet uptake and significant mortality (comparison between sucrose plus boric acid vs. sucrose alone, $\chi^2 = 108, df = 2, P < 0.001$; comparison between open source vs. cotton wick feeding, $\chi^2 = 2.51, df = 1, P = 0.11$; Fig. 2h). The maximum mortality in response to feeding on boric acid-laced sugar diets occurred at or before 5 days (Fig. 2h).

**Diet destination is modulated by the type of phagostimulant**

To assess whether ATP and sucrose modulate the destination of the antifeedant and toxicant diet in the mosquito, gut dissections were performed. In the mosquitoes that were induced to feed on blood or ATP-containing diets the meals were predominantly directed to the midgut, whereas in those induced to feed on sucrose-containing diets the meals were predominantly directed to the crop (Fig. 3). It is noteworthy that caffeine induced an increase in the volume imbibed when combined with sucrose (stage iv) compared to the other diets (stage ii, iii), while feeding on both caffeine and DEET, in combination with sucrose, led to variable distribution of the meal to different destinations in the gut (Fig. 3).

![Fig. 3](image-url) ATP and sucrose variably modulate the final destination of the meal. Exemplar micrographs of gut dissections of *Aedes aegypti* demonstrate the predominant destination of antifeedant- and toxicant-laced meals [NIC, LOB, quinine (QUI), capsaicin (CAP), CAF, BOR] to the crop (CP), oesophagus (OS), midgut (MG) and hindgut (HG). The control gut dissections for unfed, sucrose-, blood- and ATP-fed mosquitoes are presented in the four leftmost panels. The guts of mosquitoes fed on antifeedant- and toxicant-laden meals induced by either sucrose or ATP are shown in the upper and lower panels, respectively. The pie charts represent a semi-quantitative indication of the distribution of the meals to the four main gut regions. The colour intensities in the pie chart (scale to the right) visually rank the average intensity of the blue dye in the gut regions on a scale of 0 (lowest) to 3 (highest) ($n = 10$). For other abbreviations, see Figs. 1 and 2.
Discussion
The ATSB technology used in the control of mosquito vectors relies on sugar as the phagostimulant for the toxin-laden meals. A general food substrate for many non-target organisms, sugar induces intermittent feeding by mosquitoes [24], requiring multiple visits to the control tool, which results in a low rate of toxicity [5]. In this study, we evidenced that ATP is a potent model-alternative phagostimulant to sucrose, overriding the aversive effect of a range of structurally divergent antifeedant and toxicant compounds in a dose-dependent manner. The ATP-induced feeding on a toxic diet predominantly directed the meal to the midgut and rapidly elicited both knockdown and lethal effects, contrary to the slower acting sugar-induced toxicity (this study; [5, 17]). The combination of ATP and binary mixtures of toxic compounds resulted in either synergistic or additive lethal effects at low concentrations. In sum, we identified a novel and more effective pathway for delivering toxic and/or modifying agents in mosquito vector control tools.

Feeding on ATP-containing meals induces rapid ingestion of high volumes of the meal that are directed to the midgut, as part of a chain of reflexes initiated during host seeking [24], while feeding on sucrose induces a slower ingestion of smaller meal volumes, which are directed predominantly to the crop [15, 25–28]. This difference in feeding strategy likely relates to the risk of assault associated with host-seeking and blood-feeding [29, 30] compared to feeding on nectar resources. Both ATP and sucrose dose dependently override the aversive response induced by antifeedant and toxicant compounds, with ATP driving the meal uptake at a more rapid rate and to a greater volume than sucrose (this study; [15, 26]). The behavioural response to the antifeedant and toxicant compounds suggests differential sensitivity of the pathways detecting aversive compounds, similar to that observed in Drosophila, in which antifeedant and toxicant compounds can activate antifeedant- and toxicant-sensitive receptors and/or suppress the response of sugar-sensitive neurons [31, 32]. Behavioural evidence in mosquitoes (this study; [13, 33]) suggests that chemosensory neurons in sensilla on the labrum, or in the cibarium, differentially detect antifeedants and toxicants. However, whether antifeedant compounds modulate the response of sensory neurons in these sensilla in mosquitoes, or act through a dedicated pathway, is currently unknown. As with other flower-visiting insects, mosquitoes have evolved the ability to detect aversive compounds as a response to their presumed toxicity in nectar sources [32, 34, 35]. Similarly, there appears to be a selection pressure to detect antifeedant compounds in blood (this study; [13, 33]), as evidenced by the ability of obligate haematophagous animals to maintain antifeedant-sensitive receptors over an evolutionary timescale [36].

The risk associated with naturally occurring alkaloids likely explains the higher sensitivity to these tastants compared to boric acid, the most common lethal agent [5], and the synthetic compound propylene glycol, a potentially safer toxicant [17, 37] for use in ATSBs. Of the alkaloids tested in this study, nicotine, lobeline and caffeine elicited a knockdown effect, likely by acting as agonists of the nicotinic acetylcholine receptors [38, 39]. While the cholinergic antagonist quinine [40] had no effect in Aedes aegypti (this study; [13]), in Anopheles gambiae, blood meals containing quinine induced knockdown effects [33], likely due to differences in sensitivity to quinine. In contrast, the mechanisms of action of boric acid and the breakdown products of propylene glycol are direct, with the former acting as a stomach poison in the midgut of mosquitoes, disrupting the gut epithelium, affecting metabolism, and potentially acting as a neural toxin [41]. While the ability to detect and evade antifeedant- or toxicant-containing meals is an innate response, this can be overridden by the use of a phagostimulant, which in turn influences the meal destination and the rate of toxicity.

ATP-induced feeding elicited a more rapid toxic effect compared to sucrose-induced feeding (this study; [17–19, 23, 42]), as a result of directing the meals containing the toxic agents almost exclusively to the midgut, as opposed to the crop. The destination of the meals was not influenced by the type of the antifeedant or toxicant compound contained within, with two notable exceptions, caffeine and DEET, when combined with sucrose. This suggests that the detection of select antifeedants and toxicants has the potential to affect diet destination. Caffeine combined with boric acid was predicted to potentiate the lethal effects of boric acid when feeding was induced by ATP; however, this was not observed in the present study. In contrast, combinations of low doses of boric acid and propylene glycol significantly and synergistically enhanced the lethal effect. Combinations of the most potent knockdown-inducing compound, i.e. nicotine, with boric acid, did not increase the lethal effect of the meal, likely due to nicotine regulating the ingestion of low diet volumes at the high concentrations. Overall, the available results suggest that phagostimulants triggering feeding via the ATP-pathway may be used to improve the efficacy and efficiency of the technology in the control of disease vectors.

ATP as a model phagostimulant in taste-based control tools provides additional advantages compared with sucrose. In principle, not only would ATP agonists increase selectivity for haematophagous vectors, these
would also include obligate blood feeders [8] that are currently not targeted by sugar-based control tools. Resistance is a major factor limiting the usefulness of currently available vector control tools [43–45]. Moreover, resistance to glucose, caused by the misexpression of a sugar receptor in the aversive sensory neuron, has led to a loss of efficacy of ATSBs used to control cockroaches [7]. The evolutionary cost of a mutation in the ATP-detection pathway would detrimentally affect vector fitness and is thus highly unlikely to be a “selected for” trait. Taken together, the advantages associated with ATP as a phagostimulant strongly suggest that its thermostable agonists represent a set of alternative phagostimulants to sucrose for future oral-based vector control technologies. Such technologies would allow for the oral delivery of other vector-modifying agents, including biological material (e.g. *Bacillus thuringiensis* toxins), genetic material (e.g. double-stranded RNA) and chemical agents [5, 46–48].

**Conclusions**

*Aedes aegypti*, used as a representative haematophagous vector, responds reflexively to ATP and demonstrates variable sensitivities to ATP-laced antifeedant and toxicant meals. ATP non-specifically overrides the aversive effect of a range of structurally divergent antifeedants and toxicants in a dose-dependent manner. Based on the combined aspects of ATP-driven feeding responses, i.e. reflexive engorgement, high meal volume and midgut diet destination, ATP serves as a model alternative phagostimulant delivering more efficient toxic effects than sucrose when used in available ATSBs, which non-selectively affects off-target organisms, and does not target obligate blood-feeding vectors. Being an ephemeral molecule, rapidly degrading at room temperature, further studies are required to identify structurally related, stable analogues of ATP, such as the non-hydrolysable ATP analogues adenyl imidodiphosphate and adenyl methylene diphosphate [49], from among the currently known and commercially available ATP analogues [50, 51]. Such analogues may allow for the use of more lethal and eco-friendly toxic compounds, which can be used individually or synergistically, in future attractive toxic bait technologies. Further still, to ascertain the wide application of ATP analogue-based toxic baits, the stable analogues should be examined with a wider array of representative haematophagous vectors under both laboratory and field conditions. For this purpose, the workflow presented in this study is ideal, and is amenable to the recently developed bait stations designed to prevent off-target species feeding [52].

**Abbreviations**

ATP  Adenosine triphosphate  
ATSB  Attractive toxic sugar bait  
DEET  N,N-diethyl-meta-toluamide

**Supplementary Information**

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**Additional file 1. Table S1**: The concentrations (millimolar; mM) of combined antifeedants and toxicants and their corresponding percentages. The tabulation indicates combinations of propylene glycol (PRG) and boric acid (BOR), BOR and nicotine (NIC), as well as BOR and caffeine (CAF).

**Additional file 2. Figure S1**: Toxic effects of adenosine triphosphate (ATP)-induced feeding on toxicant- and antifeedant-laced meals by *Aedes aegypti*. The probability of survival among all of the individuals having been exposed to diets containing ATP (0.6 mM) together with *a* BOR, *b* PRG and *c* a combination of the two toxins. The proportion of individuals knocked down in response to having been exposed to diets containing ATP and *d* nicotine, *e* lobeline and *f* caffeine. Different lowercase letters indicate significant difference among treatments and concentrations.

**Additional file 3. Figure S2**: Evaluation of the potentiation effect of combing antifeedant compounds inducing knockdown with BOR. *a* The proportion of engorged individuals on BOR and NIC, as well as combina-

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**Author contributions**

ML, RI and SRH conceived and designed the study. ML performed the experiments and analysed the results. ML drafted the manuscript and ML, RI and SRH critically revised the manuscript. All authors approved the final version of the manuscript.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article and Additional files 1–3.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

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The authors declare that they have no competing interests.

**Author details**

1Disease Vector Group, Unit of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Alnarp, Sweden.


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To determine the quality and palatability of a blood meal, mosquitoes use the labrum to detect blood-associated feeding stimulants, including adenine nucleotides. I investigated the underlying phagostimulatory mechanism of adenine nucleotide detection in *Aedes aegypti* and *Anopheles gambiae*. Species-specific feeding responses were observed, placing ATP as the major phagostimulant, which acted as a strong model taste-based feeding driver for mosquito toxic agents. Putative ATP receptors were identified in the labrum, which were upregulated at a time when mosquitoes need to blood feed.

Matthew Lukenge completed his doctoral education at the Department of Plant Protection Biology, SLU, Alnarp, Sweden. He received his M.Sc. in *Medical Research, Infection biology, and M.Sc. in Zoology, Entomology* from Uppsala University, Sweden and Makerere University, Uganda.

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