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RESEARCH ARTICLE

# Blood constituents as phagostimulants for the bed bug *Cimex lectularius* L.

**Alvaro Romero[1,*] and Coby Schal[2]**

## ABSTRACT

Many hematophagous arthropods are stimulated by blood constituents to initiate feeding. We used a membrane-based feeding system to identify chemicals that stimulate acceptance and engorgement responses in various life stages of bed bugs. Water was fortified with a variety of compounds (e.g. salts, amino acids, vitamins, nucleotides, cholesterol and fatty acids) in these bioassays. ATP was the most effective phagostimulant in adults and nymphs, resulting in >70% of bed bugs fully engorging. Addition of NaCl to low ATP solutions that alone elicited <50% engorgement significantly enhanced feeding responses of bed bugs. A comparison of feeding responses with solutions of various adenine nucleotides showed that ATP was more stimulatory than ADP, which was more effective than AMP. Feeding assays with physiological levels of other blood constituents such as D-glucose, albumin, globulin, cholesterol and mixtures of vitamins and amino acids did not stimulate engorgement, suggesting that adenine nucleotides are the most important feeding stimulants in bed bugs. Identification of phagostimulants for bed bugs will contribute towards the development of artificial diets for rearing purposes, as well as for the development of alternative methods to eliminate bed bug infestations.

**KEY WORDS: Bed bug, Blood constituent, Phagostimulant, Adenine nucleotide, ATP, Saline**

## INTRODUCTION

Bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae), are obligate hematophagous insects that have resurged worldwide in the last decade (Krueger, 2000; Doggett et al., 2006; Potter, 2006; Kilpinen et al., 2008). The importance of bed bugs in public health is in large part due to their blood-feeding habits, which can produce several skin clinical syndromes (Fletcher et al., 2002; Ter Poorten and Prose, 2005) including severe bullous reactions that resemble the Churg–Strauss syndrome (deShazo et al., 2012). Chronic blood loss and iron deficiency anemia have also been reported in people who have been continuously exposed to severe bed bug infestations (Venkatachalam and Belavady, 1962; Pritchard and Hwang, 2009; Paulke-Korinek et al., 2012). Bed bugs can also cause psychological disorders because the presence of these insects in intimate places such as beds and bedrooms often creates anxiety, and people who are repeatedly bitten may develop nervous behavior, agitation, stress and sleeplessness (Hwang et al., 2005; Goddard and de Shazo, 2012; Susser et al., 2012). The adverse effects of bed bugs on humans have led the Environmental Protection Agency and Centers for Disease Control and Prevention to consider this pest of significant public health importance (Centers for Disease Control and Prevention and US Environmental Protection Agency, 2010).

Bed bugs are nocturnally active and both host-finding and blood-sucking behaviors occur at night. Onset of nocturnal locomotor activity in bed bugs is driven by hunger and is controlled by the circadian rhythm (Romero et al., 2010). The manner in which bed bugs find a host is poorly understood. Rivnay (Rivnay, 1932) hypothesized that bed bugs search for hosts randomly and only in close proximity to hosts they detect and orient towards heat produced by the host. Marx (Marx, 1955) suggested that besides heat, $CO_2$ produced by hosts may play a role in attracting bed bugs, and this compound is usually incorporated in traps for monitoring bed bug infestations (Anderson et al., 2009; Wang et al., 2012). Although host odors are thought to play a role in the host-seeking process, chemicals collected from human emanations have not been shown to be attractive in behavioral assays (Harraca et al., 2012).

Once on a host, probing is triggered by heat from the skin and the insect penetrates the skin using pairs of mandibles and maxillae, which form a fascicule that leads to the alimentary and salivary canals (Araujo et al., 2011). This fascicule is flexible and readily probes in various directions, until it encounters and enters a vessel of suitable size (Dickerson and Lavoipierre, 1959). Enzymes in the bug's saliva anesthetize the bite site and also prevent clotting. Localization of a blood vessel is followed by an engorgement phase, a process that takes 5–10 min (Usinger, 1966; Araujo et al., 2009). While temperature gradients of the skin guide the localization of a blood vessel (Araujo et al., 2009), the chemical stimuli that trigger the cibarial pump and engorgement are still unknown.

In this study, we use a feeding-membrane based system to screen blood constituents as phagostimulants. We report that adenosine triphosphate (ATP) is a highly effective phagostimulant in bed bugs and that its activity is significantly increased in isotonic saline solutions.

## RESULTS

### Responses to adenosine nucleotides

Overall, significantly more bed bugs engorged when offered ATP than phosphate-buffered saline (PBS) alone (Fig. 1). At least 70% of females engorged at all the ATP concentrations offered and these responses were not significantly different from engorgement on blood (Fig. 1). Similar responses were observed in groups of males and nymphs, although at the lowest concentration of ATP ($0.01\ mmol\ l^{-1}$) there was no significant difference when compared with PBS alone (Fig. 1).

Significant differences in engorgement were observed between groups of adult males offered $1\ mmol\ l^{-1}$ ATP, adenosine diphosphate (ADP) or adenosine monophosphate (AMP) ($F_{4,15}$=81.3; $P$<0.0001) (Fig. 2). Engorgement on ADP and AMP solutions was twofold and eightfold lower, respectively, than engorgement on ATP.

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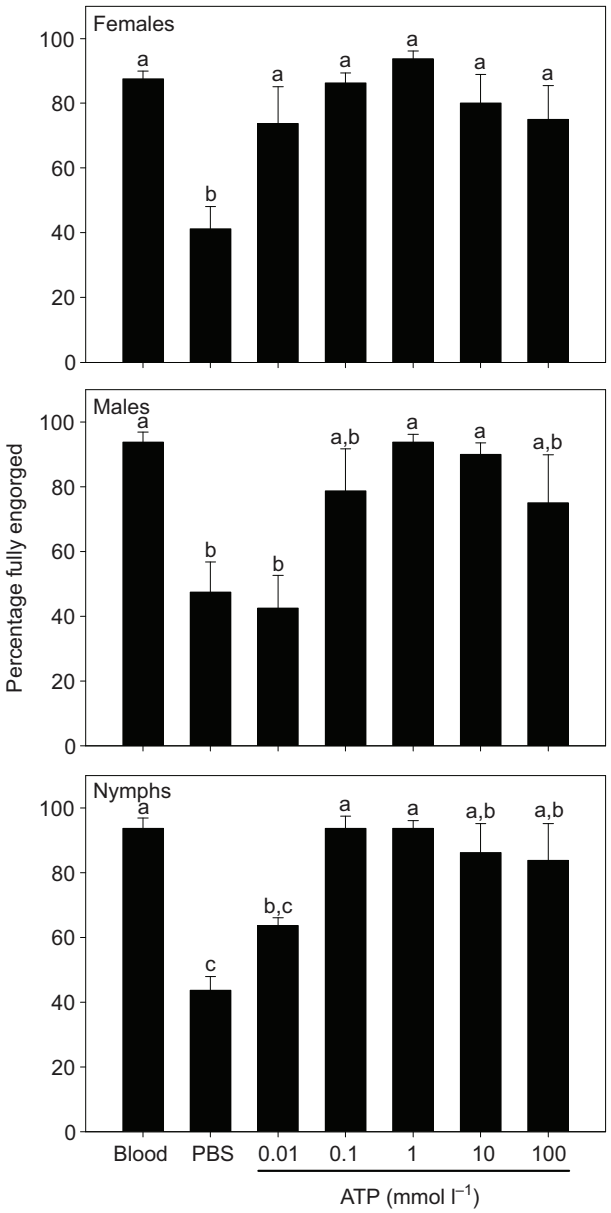


**Fig. 1. Engorgement responses of bed bug (*Cimex lectularius*) adults and nymphs to PBS and ATP solutions.** Defibrinated rabbit blood was used as positive control, while PBS was negative control. Data are presented as means ± s.e.m. Within each panel, bars with the same letter are not significantly different (ANOVA, $P>0.05$).

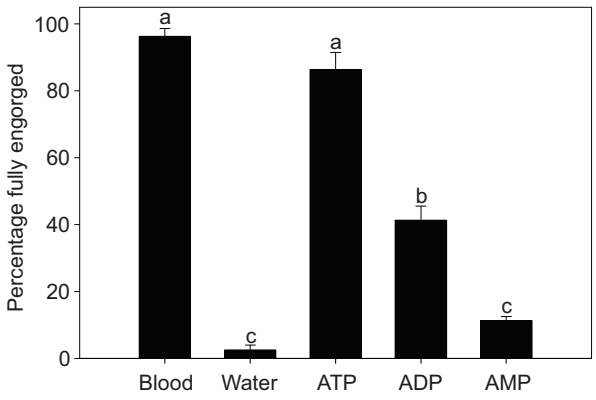


**Fig. 2. Engorgement responses of adult male bed bugs to the adenine nucleotides ATP, ADP and AMP.** The adenine nucleotides were offered at a concentration of 1 mmol $l^{-1}$ in PBS. Bars represent means ± s.e.m. and bars with the same letter are not significantly different (ANOVA, $P>0.05$).

### Responses to NaCl solutions and other compounds

Relatively high engorgement rates on PBS alone suggested that one or more of the constituent salts may stimulate feeding in bed bugs. Engorgement of bed bugs on NaCl solutions varied significantly with concentration ($F_{5,12}=37.62$; $P<0.05$) (Fig. 3). Groups of males that were offered 510 mmol $l^{-1}$ (3%) NaCl engorged the most (72.5±4.78%), significantly more than other groups offered 170 mmol $l^{-1}$ (1%) NaCl (33.3±4.4%; $t=3.71$; $P<0.05$) and 17 mmol $l^{-1}$ (0.1%) NaCl (20.0±7.63%; $t=5.076$; $P<0.05$). None of the insects accepted the 1.7 mol $l^{-1}$ (10%) NaCl solution.

Addition of 170 mmol $l^{-1}$ NaCl (approximately isotonic with human blood) to 0.01 mmol $l^{-1}$ ATP significantly increased (about fivefold) the percentage of engorged bed bugs (91.2±4.27%) when compared with groups of males exposed to 0.01 mmol $l^{-1}$ ATP alone (18.7±3.14%) (Fig. 4). Likewise, addition of 170 mmol $l^{-1}$ NaCl to 0.001 mmol $l^{-1}$ ATP significantly increased (about threefold) engorgement in bed bugs (70.0±4.56%) when compared with groups of males exposed to 0.001 mmol $l^{-1}$ ATP alone (21.2±2.39%) (Fig. 4).

Only minimal engorgement was observed in groups of adult males offered various concentrations of D-glucose and these responses were significantly lower than those observed in response to blood ($F_{4,10}=16.33$; $P<0.05$) (Fig. 5). Similar low feeding responses occurred with solutions containing albumin ($F_{4,10}=114.31$; $P<0.05$), globulins ($F_{4,10}=128.23$; $P<0.05$), mixtures of amino acids ($F_{2,6}=113.43$; $P<0.05$), vitamins ($F_{2,6}=297.49$; $P<0.05$) and cholesterol ($F_{2,6}=115.02$; $P=0.05$) (Fig. 5), when compared with responses to blood.

## DISCUSSION

Hematophagous arthropods use physical and chemical cues to locate hosts. Host localization and blood feeding involve a series of behaviors coordinated by several sensory modalities and mediated ultimately by phagostimulants (Lehane, 2005). In most hematophagous insects, the major stimuli that elicit engorgement are associated with the cellular fraction of host blood (Mumcuoglu and Galun, 1987; Galun et al., 1993; Lehane, 2005), although in some insects plasma components, such as proteins and salts, also contribute to the acceptance and engorgement process (Galun et al., 1985). With some notable exceptions (e.g. Anopheline mosquitoes) (Galun et al., 1985), ATP and ADP are the major phagostimulatory cues for hematophagous insects (Friend and Smith, 1977; Galun, 1987a; Ribeiro, 1987).

We screened several constituents of human blood in an effort to identify phagostimulants and begin to understand the physiological basis of blood acceptance and intake in bed bugs. We initially tested chemicals that are normal constituents of blood, at a range of concentrations that included normal physiological levels found in human blood. Bed bugs displayed low feeding responses to glucose, proteins, vitamins, amino acids and cholesterol, indicating that these blood constituents do not appear to serve as phagostimulants for bed bugs. Nevertheless, it is important to consider synergistic interactions, as these compounds might contribute to blood acceptance only in combination with other blood constituents. For example, the addition of albumin to a solution containing NaCl and $NaHCO_3$ significantly stimulated feeding in *Anopheles dirus* (Galun

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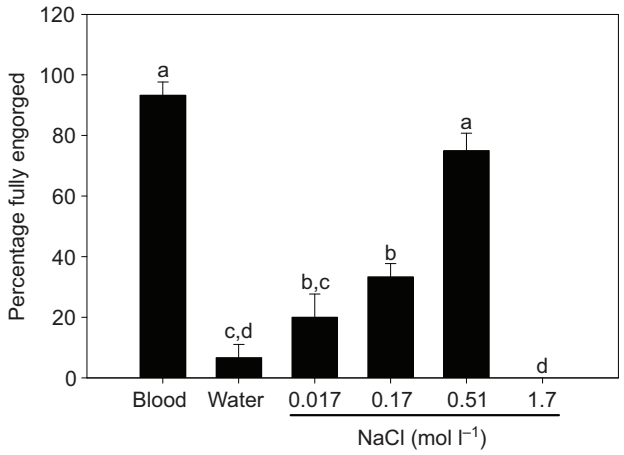


**Fig. 3. Engorgement responses of adult male bed bugs to NaCl solutions.** Bars represent means ± s.e.m. and bars with the same letter are not significantly different (ANOVA, $P>0.05$).

et al., 1985). Similarly, the addition of albumin, NaCl and $NaHCO_3$ to an ATP solution makes the solution as phagostimulatory to *Aedes aegypti* as ATP dissolved in platelet-poor plasma (Galun et al., 1984).

Significant feeding responses in bed bug nymphs and adults (>70% engorgement) were observed with ATP in PBS solution at a wide range of concentrations. For all three life stages, maximal engorgement occurred at 1 mmol $l^{-1}$ ATP, the ATP concentration normally found in human blood (Khlyntseva et al., 2009). Interestingly, engorgement responses in adult males were 50% lower at the lowest concentration than at a middle concentration of ATP tested (0.01 versus 1 mmol $l^{-1}$), whereas in females engorgement responses at these two concentrations were high and not significantly different. The difference in the acceptance threshold of ATP between the sexes reflects the complexity of factors that might control feeding responses in bed bugs. Factors such as sex and nutritional and physiological status influence the responses of many hematophagous insects to phagostimulants (Friend and Smith, 1977). Although our dilution of ATP in PBS precluded quantitative measures of the effective dose of ATP, it is apparent that bed bugs are highly sensitive to micromolar concentrations of this nucleotide.

We also evaluated feeding responses of bed bugs to the adenine nucleotides ADP and AMP, and compared these responses with those observed with ATP. For this comparison, we offered insects solutions of adenine nucleotides at 1 mmol $l^{-1}$, within the range that these nucleotides are normally found in human blood (Khlyntseva et al., 2009). Our results showed that ATP was more stimulatory than ADP, and ADP in turn was more effective than AMP. A decrease in feeding due to reduction of phosphate groups was also reported in the hematophagous triatomine *Rhodnius prolixus* (Smith and Friend, 1976) and the mosquito *A. aegypti* (Galun et al., 1963). Galun et al. (Galun et al., 1963) demonstrated that adenosine tetraphosphate was even more effective than ATP, indicating that the presence of the adenine moiety and number of phosphate groups are important stimuli of the chemosensory system involved in assessing blood quality and engorgement. Moreover, the 5′ position of the phosphates on the ribose was critical for phagostimulatory activity (Galun, 1967).

However, insects exhibit great diversity in their chemosensory responses to various adenine nucleotides, despite their ubiquitous sensitivity to these compounds. Unlike bed bugs, triatomines, *Aedes* mosquitoes, the mosquito *Culex pipiens* and the blackfly *Simulium venustum* respond to AMP as a more potent engorgement stimulus than to ATP (Hosoi, 1959; Smith and Friend, 1982).

Exposure of bed bugs to adenine nucleotides might initially occur at the biting site, while insects probe the skin in search of a blood vessel. Adenine nucleotides are constituents of the inflammatory factors released by platelets and injured cells (Ribeiro, 1987). However, locally produced adenine nucleotides might be degraded by the action of the enzyme apyrase, a component of bed bug saliva (Valenzuela et al., 1998; Francischetti et al., 2010). Other sources of adenine nucleotides include blood cells, such as platelets and erythrocytes, which contain high concentrations of these compounds. However, it is not clear how cellular adenine nucleotides would act as phagostimulants for bed bugs as 99% of adenine nucleotides are normally found intracellularly where it would not stimulate the insect's chemosensory system. We hypothesize that adenine nucleotides might be released from blood cells in response to deformation of the cell membrane during the cell's passage through the bed bug's food channel. Shear-induced ATP release has been documented in response to vasoconstriction of blood vessels (Wan et al., 2008). The occurrence of this mechanism in bed bugs is plausible as the diameter of the food channel at the tip of the proboscis is slightly narrower than the diameter of a red blood cell (Dickerson and Lavoipierre, 1959). If so, the chemosensilla that respond to ATP would be expected to be within the food canal rather than on the outer surface of the mandibles and maxillae.

Osmotic pressure of ionic compounds plays a role in inducing feeding in most hematophagous insects. Saline solutions elicit engorgement in sand flies (Ready, 1978) and triatomines (Guerenstein and Núñez, 1994). However, limited feeding responses have been observed when the osmotic pressure of solutions is increased with non-sodium ions such as potassium, calcium or magnesium (Galun et

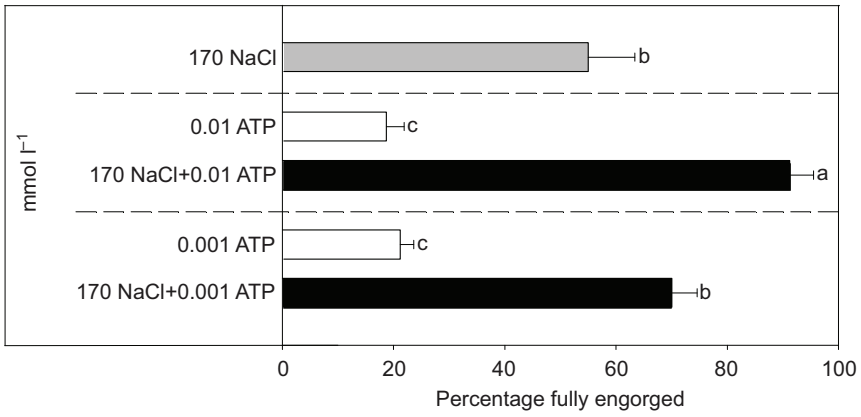


**Fig. 4. Effect of isotonic saline solution (1% NaCl) on the engorgement responses of adult male bed bugs to low concentrations of ATP (0.01 and 0.001 mmol $l^{-1}$).** Bars represent means ± s.e.m. and bars with the same letter are not significantly different (ANOVA, $P>0.05$).

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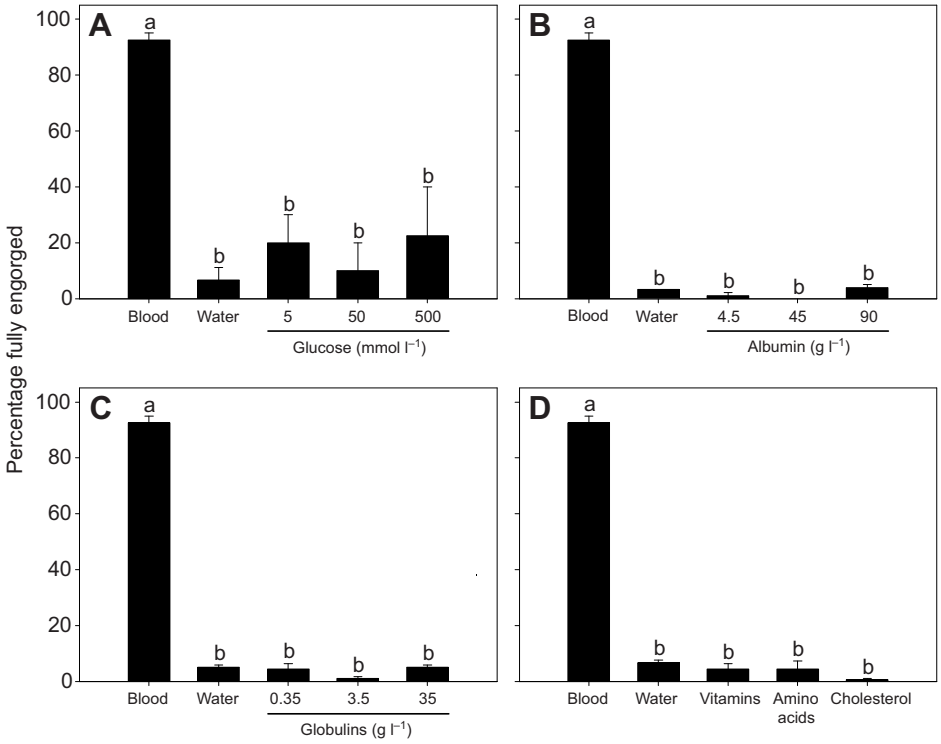


**Fig. 5. Effect of various blood constituents on the engorgement responses of adult male bed bugs.** Engorgement responses of adult male bed bugs on (A) D-Glucose, (B) albumin, (C) globulin and (D) mixtures of vitamins, amino acids or cholesterol. Bars represent means ± s.e.m. No significant differences were observed between the responses to water and to the different concentrations of any mixture or compound tested (ANOVA, $P>0.05$).

al., 1963). Thus it seems that in addition to assessing osmotic pressure, hematophagous insects also specifically require sodium for optimal feeding responses (Galun et al., 1963). In our study, much greater engorgement responses were observed when bugs were offered a PBS solution rather than water alone, indicating that bed bugs might be stimulated by one or more of the constituent salts. Bed bugs engorged on NaCl solutions in a dose-dependent manner, but they refused to ingest a 10% NaCl solution (1.7 mol $l^{-1}$). These findings suggest that bed bugs have gustatory receptors on their mouthparts that mediate acceptance of low NaCl concentrations and deterrence to high NaCl concentrations, as in many other insects. Surprisingly, however, bed bugs exhibited a low feeding response to physiological concentrations of NaCl (1%; 170 mmol $l^{-1}$) and much higher responses to 3% (510 mmol $l^{-1}$) NaCl. These results suggest that there might be an additive interaction between the phagostimulatory effects of NaCl and ATP at near-physiological concentrations. We therefore offered bugs a mixture of 1% NaCl and 0.01 or 0.001 mmol $l^{-1}$ ATP; both compounds are within the range normally detected in plasma (Gorman et al., 2007), but each alone induced marginal engorgement in male bed bugs at these concentrations (Fig. 1). These results are similar to work with *A. aegypti*, showing that the feeding responses to an ATP solution buffered by bicarbonate (the major natural buffer in blood) were fivefold higher than to ATP buffered by phosphate (Galun et al., 1984). Significantly enhanced feeding of various hematophagous insects with mixtures of compounds indicate that multiple appetitive chemosensory channels (also including osmotic pressure and pH detectors) contribute additively to the engorgement response. Nevertheless, whereas *Aedes* mosquitoes do not feed on pure salt solutions and require the addition of ATP to an NaCl solution buffered with bicarbonate (Werner-Reiss et al., 1999a), >50% of bed bugs accept NaCl alone.

The locations and functions of chemosensilla that detect phagostimulatory compounds, including adenine nucleotides, are poorly known. Because the gustatory responses to phagostimulants have evolved independently many times, it is possible that receptors also reflect high divergence in location, structure and function. In some blood feeders, such as the tsetse fly *Glossina* that has sponging mouthparts, phagostimulant-responsive sensilla have been identified on the labella, and have been recorded from using extracellular tip recordings (Galun and Margalit, 1969; Mitchell, 1976). Similar recordings from labral apical sensilla of *C. pipiens* (Liscia et al., 1993) and *A. aegypti* (Werner-Reiss et al., 1999a) have identified NaCl, $Na_2HPO_4$, L-alanine and ATP responsive sensilla; notably, these chemosensilla are present only in female mosquitoes. Insects with sucking mouthparts have sensilla possibly involved in the detection of phagostimulants inside the food channel, particularly on mandibles and maxillae and within the cibarium (Bernard et al., 1970; Ascoli-Christensen et al., 1990; Werner-Reiss et al., 1999b). Morphological evidence supports the existence of putative chemosensilla in the cibarial region of the esophagus that could play a role in determining acceptability of ingested food (Rice, 1970; Lee and Craig, 1983). In some hemipterans such as Triatominae and Cimicidae, a cibarial pump is associated with this region and electromyogram recordings from this structure indicate that insects sample blood before the engorgement phase commences (Friend and Smith, 1971; Araujo et al., 2011). If so, sampling would enable the blood to come into contact with cibarial sensilla. The repeated sampling seen during the probing phase of bed bugs and the detection of small amounts of phagostimulant-free dye solution in the esophagus is consistent with this suggestion (A.R., unpublished observation).

The use of adenine nucleotides as cues to recognize a blood meal by many unrelated blood-feeder arthropods indicates that hematophagy evolved independently (Galun, 1987b; Ribeiro, 1987). Despite the convergent evolution of hematophagy, studies of feeding responses of various hematophagous arthropods to artificial diets containing adenine nucleotide analogues show that there is a great diversity of structure–activity relationships of adenine nucleotide receptors (Friend and Smith, 1982; Galun et al., 1985; Galun and Kabayo, 1988; Galun, 1989; Friend and Stoffolano, 1990). Further

characterization of structural requirements for triggering putative adenine nucleotide receptors will provide insights on the nature of these receptors as well as how it relates to other blood-feeder arthropods. This information along with genome analysis will provide insights on how hematophagy evolved in Cimicidae. From a practical perspective, identification of phagostimulants for bed bugs will contribute to the development of artificial diets for rearing purposes, as well as for the development of alternative methods to eliminate bed bug infestations. The development and future deployment of toxic baits against bed bugs will depend not only on the use of effective attractants, but also on maximizing acceptance and ingestion of toxicants with effective phagostimulants.

## MATERIALS AND METHODS

### Insects

Bed bugs were obtained from a colony maintained at 25°C, 50±5% relative humidity, and a photoperiod of 12 h:12 h (light:dark). This colony was originally established from bed bugs collected in an apartment in Jersey City, NJ, USA in 2008. Insects were fed in the laboratory through a parafilm-membrane feeder with defibrinated rabbit blood heated to 37°C by a circulating water bath (Montes et al., 2002). Experimental insects (third and fourth instar nymphs, and adult females and males) were tested unfed, 7 days after emergence. Assays were conducted in a dark room at ambient temperature between 22 and 25°C.

### Chemicals and solutions

Modified Dulbecco's phosphate-buffered saline (PBS; 8 mmol $l^{-1}$ sodium phosphate, 2 mmol $l^{-1}$ potassium phosphate, 140 mmol $l^{-1}$ sodium chloride, 10 mmol $l^{-1}$ potassium chloride, pH 7.4) was from Pierce/Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). ATP, ADP and AMP were diluted in PBS. ATP was tested at 0.01 to 100 mmol $l^{-1}$. Bed bug feeding responses to ADP and AMP were evaluated at a concentration of 1 mmol $l^{-1}$. Sodium chloride (NaCl) was dissolved in water to yield final concentrations of 0.1% (17 mmol $l^{-1}$), 1% (170 mmol $l^{-1}$), 3% (510 mmol $l^{-1}$) and 10% (1.7 mol $l^{-1}$). Mixtures of aqueous solutions of 170 mmol $l^{-1}$ NaCl and either 0.01 or 0.001 mmol $l^{-1}$ ATP were also tested. All concentrations of the mixtures fall within the range of these compounds normally detected in blood (Gorman et al., 2007). Glucose was dissolved in water to final concentrations of 5, 50 and 500 mmol $l^{-1}$.

Groups of nine amino acids or eight vitamins were mixed in water at concentrations that fall within the range of these compounds normally detected in blood [amino acids=$10^{-7}$ mol $l^{-1}$; vitamins at various concentrations (see below)] (http://www.mayomedicallaboratories.com/test-catalog/alphabetical/V). The amino acids tested were: L-arginine hydrochloride, L-cystine dihydrochloride, L-histidine monohydrochloride monohydrate, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-threonine and L-tyrosine. The vitamins screened were: thiamine hydrochloride ($10^{-7}$ mol $l^{-1}$), folic acid ($10^{-6}$ mol $l^{-1}$), D-pantothenic acid hemicalcium salt ($10^{-6}$ mol $l^{-1}$), niacinamide ($10^{-3}$ mol $l^{-1}$), cobalamin ($10^{-6}$ mol $l^{-1}$), biotin ($10^{-6}$ mol $l^{-1}$), choline chloride ($10^{-5}$ mol $l^{-1}$) and riboflavin ($10^{-6}$ mol $l^{-1}$). Bovine albumin and globulin were diluted in water at 4.5, 45 and 90 g $l^{-1}$, and 0.35, 3.5 and 35 g $l^{-1}$, respectively. Cholesterol was initially dissolved in 0.1% Tween 20 (in water) and then diluted in water to 0.1, 1 and 5 mmol $l^{-1}$. All solutions were prepared fresh before each experiment.

### Feeding assays

Test solutions were warmed to 37°C with a water bath circulator system, and offered to the insects in a feeding membrane system similar to the one used by Montes et al. (Montes et al., 2002). The system consisted of several custom-made water-jacketed glass feeders with a synthetic membrane (Nescofilm, Alfresa Pharma Corporation, Osaka, Japan) stretched across the bottom through which the insects fed.

Bed bugs were placed into 2 oz (60 ml) clear round wide-mouth jars (Consolidated Plastic, Stow, OH, USA). We removed the bottom of the jar and replaced it with a plankton mesh (BioQuip Products, Rancho Dominguez, CA, USA) which was attached to the jar using methylene chloride to melt the plastic. Bugs were provided with strips of paperboard folder paper of the same length as the jar to allow them to climb to the mesh and reach the test solution.

Groups of 20 bed bugs (females, males or third to fourth instar nymphs) were used each time and allowed to feed for 20 min. Only males were used for the evaluations with non-ATP nucleotides, mixtures of physiological saline and ATP, and other blood constituents. Engorgement was determined by visual inspection, as the feeding response in bed bugs is usually an all-or-none phenomenon, as reported also in some hematophagous triatomines (Friend and Smith, 1977). The percentage of insects that fully engorged was used as a measure of the phagostimulatory quality of test solutions. Unless otherwise stated, three replicates (20 insects each) were used with each test solution. Engorgement responses to test solutions were compared with responses to defibrinated rabbit blood (positive control) and with responses to PBS or distilled water (negative control).

### Statistical analysis

The number of engorged insects was divided by the number of insects in each replicate (20), and the square root of this proportion was arcsine transformed before analysis of variance (ANOVA) using Mixed Procedure (SAS Institute, 2002) and Tukey's pairwise comparison (at 5% level of significance).

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#### Competing interests

The authors declare no competing financial interests.

#### Author contributions

A.R. and C.S. designed the study and wrote the manuscript. A.R. performed all the experiments and analyzed the data.

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