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

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SUMMARY

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. Adenosine triphosphate 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 toward 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 bugs, blood constituents, phagostimulants, adenine nucleotides, adenosine triphosphate, saline.
INTRODUCTION

Bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae), are obligate hematophagous insects that have resurged worldwide in the last decade (Doggett et al., 2006; Kilpinen et al., 2008; Krueger, 2000; Potter, 2006). 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 (Paulke-Korinek et al., 2012; Pritchard and Hwang, 2009; Venkatachalam and Belavady, 1962). Bed bugs can also cause psychological disorders because the presence of these insects in intimate places such as beds and bedrooms often create anxiety, and people who are repeatedly bitten may develop nervous behavior, agitation, stress and sleeplessness (Goddard and de Shazo, 2012; Hwang et al., 2005; 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 U.S. 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 (1932) hypothesized that bed bugs search for hosts randomly and only in close proximity to hosts they detect and orient toward heat produced by the host. Marx (1955) suggested that besides heat, CO₂ 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 (Araujo et al., 2009; Usinger, 1966). 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.

MATERIAL AND METHODS

Insects

Bed bugs were obtained from a colony maintained at 25°C, 50±5% RH, and a photoperiod of 12:12 (L:D) h. This colony was originally established from bed bugs collected in an apartment in Jersey City, NJ 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 (3rd and 4th 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). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (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} M;
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} M), folic acid (10^{-6} M), D-pantothenic acid hemicalcium salt (10^{-6} M),
niacinamide (10^{-3} M), cobalamin (10^{-6} M), biotin (10^{-6} M), choline chloride (10^{-5} M), and
riboflavin (10^{-6} M). Bovine albumin and globulin were diluted in water at 4.5, 45 and 90 g/L, and
0.35, 3.5 and 35 g/L, 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 at 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. (2002). The
system consisted of several custom-made water-jacketed glass feeders with a synthetic
membrane (Nescofilm, Alfresa Pharma Corp., 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). We removed the bottom of the jar and replaced it with a plankton mesh
(BioQuip Products, Rancho Dominguez, CA) 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 3rd-4th 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).

**RESULTS**

**Responses to adenosine nucleotides**

Overall, significantly more bed bugs engorged when offered ATP than 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 to PBS alone (Fig. 1).

Significant differences in engorgement were observed between groups of adult males offered 1 mmol l⁻¹ ATP, ADP, or AMP (F₄,₁₅=81.3; P<0.0001) (Fig. 2). Engorgement on ADP and AMP solutions was two-fold and eight-fold lower, respectively, than engorgement on ATP.

**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 (F₅,₁₂=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 five-fold) 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.
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(18.7±3.14) (Fig. 4). Likewise, addition of 170 mmol l⁻¹ NaCl to 0.001 mmol l⁻¹ ATP significantly increased (about three-fold) 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 to 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 (Galun et al. 1993; Lehane, 2005; Mumcuoglu and Galun, 1987), 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., Anopheine 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 appear to not 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 NaHCO₃ significantly stimulated feeding in Anopheles dirus (Galun et al., 1985). Similarly, the addition of albumin, NaCl and NaHCO₃ to an ATP solution makes the
solution as phagostimulatory to *A. 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 vs. 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 to 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 *Aedes aegypti* (Galun et al., 1963). 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 (Francischetti et al., 2010; Valenzuela et al., 1998). 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 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 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
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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 *Aedes aegypti*, showing that the feeding responses to an ATP solution buffered by bicarbonate (the major natural buffer in blood) were 5-fold 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 recorded from using extracellular tip-recordings (Galun and Margalit 1969; Mitchell, 1976). Similar recordings from labral apical sensilla of *Culex pipiens* (Liscia et al., 1993) and *Aedes aegypti* (Werner-Reiss et al., 1999a) have identified NaCl, Na₂HPO₄, 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 (Ascoli-Christensen et al., 1990; Bernard et al., 1970; 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 (Lee and Craig, 1983; Rice, 1970). In some hemipterans such as Triatominae and Cimicidae, a cibarial pump is associated with this region and electromyogram recordings from this structure indicates that insects sample blood before the engorgement phase commences (Araujo et al., 2011; Friend and Smith, 1971). 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. Romero, unpublished).

The use of adenine nucleotides as cue 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; Friend and Stoffolano, 1990; Galun et al. 1985; Galun and
Kabayo 1988; Galun 1989). 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 toward 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.

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

COMPETING INTEREST
No competing interests declared.

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**Figure Captions**

Fig. 1. Engorgement responses of bed bug adults and nymphs to phosphate-buffered saline (PBS) and ATP solutions. Defibrinated-rabbit blood was used as positive control while PBS was negative control. Data are presented as mean±SEM. 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 adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). The adenine nucleotides were offered at a concentration of 1 mmol l\(^{-1}\) in PBS. Bars represent mean±SEM and bars with the same letter are not significantly different (ANOVA, \( P>0.05 \)).

Fig. 3. Engorgement responses of adult male bed bugs on NaCl solutions. Bars represent mean±SEM and bars with the same letter are not significantly different (ANOVA, \( P>0.05 \)).

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 mean±SEM and bars with the same letter are not significantly different (ANOVA, \( P>0.05 \)).

Fig. 5. 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 mean±SEM. No significant differences were observed between the responses to water and to the different concentrations of any mixture or compound tested (ANOVA, \( P>0.05 \)).
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Figure 1

The figure shows the percentage of fully engorged females, males, and nymphs across different ATP concentrations (Blood, PBS, and concentrations of ATP in mmol l⁻¹). The bars are labeled with letters (a, b, c) to denote statistical significance, with similar letters indicating no significant difference. The ATP concentrations tested were 0.01, 0.1, 1, 10, and 100 mmol l⁻¹.
Figure 2

Percentage fully engorged

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Water</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
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<tr>
<td>a</td>
<td>a</td>
<td>c</td>
<td>a</td>
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The figure shows the percentage of fully engorged samples for different substances: Blood, Water, ATP, ADP, and AMP. The bars are labeled with letters (a, b, c) to indicate statistical differences.
Figure 3

Percentage fully engorged

NaCl concentration (mmol l⁻¹)

Blood

Water

0.017

0.17

0.51

1.7
Figure 4

Percentage fully engorged

170 NaCl

0.01 ATP

170 NaCl + 0.01 ATP

0.001 ATP

170 NaCl + 0.001 ATP

mmol l⁻¹

a

b

c

Percentage fully engorged
Figure 5

A. Percentage fully engorged in Blood and Water with different concentrations of Glucose (mmol l⁻¹)

B. Percentage fully engorged in Blood and Water with different concentrations of Albumin (g/L)

C. Percentage fully engorged in Blood and Water with different concentrations of Globulins (g/L)

D. Percentage fully engorged in Blood and Water with different concentrations of Vitamins, Amino acids, and Cholesterol