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REVIEW

The plasticity of extracellular fluid homeostasis in insects

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ABSTRACT

In chemistry, the ratio of all dissolved solutes to the solution's volume yields the osmotic concentration. The present Review uses this chemical perspective to examine how insects deal with challenges to extracellular fluid (ECF) volume, solute content and osmotic concentration (pressure). Solute/volume plots of the ECF (hemolymph) reveal that insects tolerate large changes in all three of these ECF variables. Challenges beyond those tolerances may be 'corrected' or 'compensated'. While a correction simply reverses the challenge, compensation accommodates the challenge with changes in the other two variables. Most insects osmoregulate by keeping ECF volume and osmotic concentration within a wide range of tolerance. Other insects osmoconform, allowing the ECF osmotic concentration to match the ambient osmotic concentration. Aphids are unique in handling solute and volume loads largely outside the ECF, in the lumen of the gut. This strategy may be related to the apparent absence of Malpighian tubules in aphids. Other insects can suspend ECF homeostasis altogether in order to survive extreme temperatures. Thus, ECF homeostasis in insects is highly dynamic and plastic, which may partly explain why insects remain the most successful class of animals in terms of both species number and biomass.

KEY WORDS: Environmental physiology, Hemolymph, Intracellular fluid, Volume expansion, Volume contraction, Isosmotic, Hyperosmotic challenge, Hypo-osmotic challenge, Solute/volume plots, Correction, Compensation, Gut, Malpighian tubules, Rectum, Anal papillae

Introduction

In general, total body water in multicellular organisms makes up 70% of the body weight. The water is distributed largely in two major fluid compartments, with 2/3 in the intracellular fluid (ICF) compartment and 1/3 in the extracellular fluid (ECF) compartment. More than 160 years ago, Claude Bernard recognized that multicellular animals really live in the life-sustaining compartment of the ECF, i.e. the internal environment, and not in the external environment they inhabit (Gross, 1998). The ECF sustains the lives of cells because it buffers the ICF against unpredictable changes in the external environment, and epithelial cells at the surface of the organism adjust their activities to maintain a constant volume and composition of the ECF (Fig. 1). The constancy of the ECF is known as homeostasis. In the case of insects, tracheal tubes provide the ECF (hemolymph) with relatively constant concentrations of oxygen, carbon dioxide and hydrogen ions; the gastrointestinal tract and accessory organs provide relatively constant concentrations of fuel and nutrients; the Malpighian tubules and the gut regulate the volume and composition of the hemolymph; and the circulatory system keeps the hemolymph continuously in motion, from metabolizing cells to the epithelial surfaces and back (Fig. 1A).

Living in the internal environment of the hemolymph, insects have the freedom to inhabit diverse external environments, aquatic and terrestrial. Moreover, insects exhibit considerable capacity for dealing with water deficiencies and excesses. For example, tenebrinoid beetles can survive the loss of as much as 90% of their ECF (Zachariassen and Einarson, 1993). At the other extreme, the kissing bug *Rhodnius prolixus* takes on more than 10 times its body weight in a single blood meal (Buxton, 1930). Although the ingested blood is initially in the transcellular compartment, the lumen of the gut, most of it passes through the ECF before excretion.

This Review examines the regulation of the ECF in insects using solute/volume plots and the ratio of solute to volume – the osmotic concentration or osmotic pressure. The plots trace the effects of environmental osmotic condition and oral fluid intake on the ECF and the physiological response in insects. Environmental challenges may increase or decrease ECF volume (expansion or contraction, respectively) in isosmotic, hyperosmotic or hypo-osmotic ways. The reversal of the environmental challenge in the ECF (e.g. water moving in being balanced by water moving out) is considered a 'correction' (see Glossary), and results in the return of the system to its original osmotic concentration. When correction is not possible, another variable may 'compensate' (see Glossary) for the change (e.g. water in – solute out), and the ECF osmotic concentration decreases as a result.

ECF homeostasis in insects is far more varied than that of vertebrates. It preserves ECF osmotic concentration in most insects and ECF volume in some insects. Other insects minimize or pre-empt ECF challenges by dealing with them in the transcellular compartment, i.e. the lumen of the gut. Still other insects seem to suspend ECF homeostasis altogether in their survival of extreme temperatures. These insects capitalize on the use of so-called organic osmolytes (sugars, amino acids and polyols) to hold on to water in hot climates and to prevent freezing in cold climates. Here, ECF homeostasis is subordinated to the survival of extreme temperatures.

This Review begins with a lesson on the chemistry of concentration, osmotic concentration and osmotic pressure. This basic solution chemistry is then applied to predicting the changes in solute, volume and osmotic concentration (pressure) in both ECF and ICF as Na^+, K^+ and water are added to or removed from the ECF. Armed with this knowledge and referring to solute/volume plots, the Review then examines how insects exercise ECF homeostasis in different habitats and on different diets. ECF homeostasis in the context of temperature regulation is beyond the scope of this Review. However, the role of organic osmolytes in insects surviving thermal extremes has been well reviewed elsewhere (Benoit et al., 2007; Denlinger and Lee, 2010; Teets and Denlinger, 2013; Watanabe et al., 2002; Yancey, 2005).

Osmotic pressure

Solute-free water can be obtained from distillation or reverse osmosis (see Glossary). The addition of solute to distilled water

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Glossary

Anal papilla
In mosquito and other dipterous larvae, one of usually four external appendages at the anus involved in active uptake of Cl^- from low concentrations in fresh water.

Compensation
A physiological response that accommodates a challenge.

Correction
A physiological response that reverses a challenge.

Diuresis
An increase in urine flow.

Distillation
The process that separates the components of a solution (or suspension) by selective evaporation and condensation.

Donnan equilibrium
The electrochemical equilibrium that results from the unequal distribution of non-diffusible and diffusible ions between two solutions separated by a selectively permeable membrane or barrier.

Reverse osmosis
The production of solute-free water by applying a hydrostatic pressure greater than the osmotic pressure.

can lead to the formation of a homogeneous solution when the polar interactions between water and solute keep the solute from precipitating out, i.e. the solute is dissolved. Biological solutions typically contain more than one kind of dissolved solute, and the sum of all dissolved solutes, inorganic or organic, ionized or non-ionized, yields the number 'n' of total dissolved moles, defined as osmoles (osmol); osmoles per liter of solution is the osmolar or osmotic concentration (osmol l^{-1}). Osmoles per kilogram of solvent is the osmolal concentration (osmol kg^{-1}).

Jacobus H. van't Hoff first realized that osmoles in water behave like a gas in a volume (van't Hoff, 1885, 1887), which earned him the first Nobel Prize given for work in Chemistry in 1901. The behavior of a gas is described by the perfect gas law, where P is the pressure, n is the number of gas moles, V is the gas volume, R is the gas constant and T is the absolute temperature (Eqn 1):

$$P = \frac{n}{V}RT. \quad (1)$$

Because R and T are constants, P is a function of the molar gas concentration.

The van't Hoff equation describes the osmotic pressure π as a function of the total solute concentration, i.e. the osmotic concentration (osmol/V, Eqn 2):

$$\pi = \frac{\text{osmol}}{V}RT = [\text{osmol}]\,RT, \quad (2)$$

where V is the volume of the solution. Lachish showed that, in the ideal case, the perfect gas law and the van't Hoff formulation are identical as a direct consequence of the second law of thermodynamics (Einstein, 1905; Fermi, 1956; Lachish, 2007); that is, the osmotic pressure π is equal to the pressure P when the concentration of gas in a volume equals the concentration of solute in solution. The ideal case requires, among other things, dilute solutions, a barrier (membrane) that is permeable to solvent but impermeable to solute, and no chemical interactions between solute and solvent.

Others argue that the perfect gas law and the van't Hoff equation share only similar appearance; that is, Eqns 1 and 2 are only formally analogous (Adam et al., 2009). Nevertheless, it can be shown experimentally that 1 osmol of solute dissolved in 1 liter of water at 0°C exerts a pressure of 22.4 atm, similar to 1 mol of gas exerting a pressure of 22.4 atm in a volume of 1 liter.

Osmotic pressure can be expressed as pressure in atmospheres or as osmotic concentration. The older literature often expresses osmotic pressure as the freezing point depression, one of the four colligative properties of solutions (Ramsay and Brown, 1955), or as equivalent NaCl concentrations (Shaw and Stobbart, 1961). More recent literature uses the Pascal as the unit of osmotic pressure, where a solution containing Avogadro's number of solute (1 Osm) has an osmotic pressure of 2.27 MPa. It follows that the osmotic pressure of 1 mOsm is equivalent to a pressure of 2.27 kPa, 0.0224 atm or 17 mmHg.

This Review will use the term 'osmotic concentration' as the sum of the molar concentration of all dissolved solutes in 1 liter of solution (Osm=osmol l^{-1}) for the following reasons. First, biological barriers are selectively permeable to more than one solute and are not the semipermeable barriers implied in the use of osmotic pressure. Second, the biological literature prefers molar normalization to the volume of 1 liter solution over molal normalization to kg H_2O. Third, the difference between molar and molal concentration is not very large for most biological solutions where the mass of water is far greater than the mass of solute. And fourth, biologists intuitively associate osmosis with the movement of water from a solution of low osmotic concentration to a solution of high osmotic concentration.

Environmental water can be divided into four classes: fresh water, brackish water, saline water and brine. Fresh water has the lowest osmotic concentration, with a salinity <0.5 ppt (parts per thousand). Typical city water in the USA has a salinity <0.1 ppt, or an osmotic concentration of <3.42 mOsm if the salinity stems from NaCl. The salinity of brackish water ranges from 0.5 to 30 ppt. Saline water ranges from 30 to 50 ppt and includes seawater. Standard seawater, i.e. 'Normal Copenhagen Seawater', has a salinity of 34.33 ppt or 1175 mOsm if, for reasons of simplicity, it is considered to consist of completely ionized NaCl alone (Potts and Parry, 1963). However, actual measurements of seawater osmotic pressure are little over 1000 mOsm because the ionization of salts decreases with increasing salt concentration. Brine has a salinity >50 ppt.

Challenges to ICF and ECF compartments

Na^+ and Cl^- are the major electrolytes in the ECF, whereas K^+ and organic anions are the major electrolytes in the ICF (Fig. 1A,B). The unequal distribution of Na^+ and K^+ across cell membranes stems from the operation of (1) the Na^+/K^+-ATPase and (2) the excretory system (Fig. 1A,B). The Na^+/K^+-ATPase maintains high K^+ concentrations and low Na^+ concentrations in the ICF, and the excretory systems (Malpighian tubules, kidneys) maintain high Na^+ concentrations and low K^+ concentrations in the ECF. The concentration differences that drive the diffusion of K^+ out of the cell and the diffusion of Na^+ into the cell provide, respectively, the chemical basis for resting and action potentials in the excitable membranes of nerve and muscle. In addition, the Na^+/K^+-ATPase opposes the Donnan equilibrium (see Glossary) of diffusible ions across cell membranes, such that intracellular and extracellular osmotic concentrations are the same (isosmotic) across cell membranes permeable to water.

Because of the unequal distribution of Na^+ and K^+ across cell membranes, the gain or loss of Na^+, K^+ and H_2O will each affect the ICF and ECF in unique ways. For example, the addition of Na^+ (without water) to the organism will add Na^+ primarily to the ECF,

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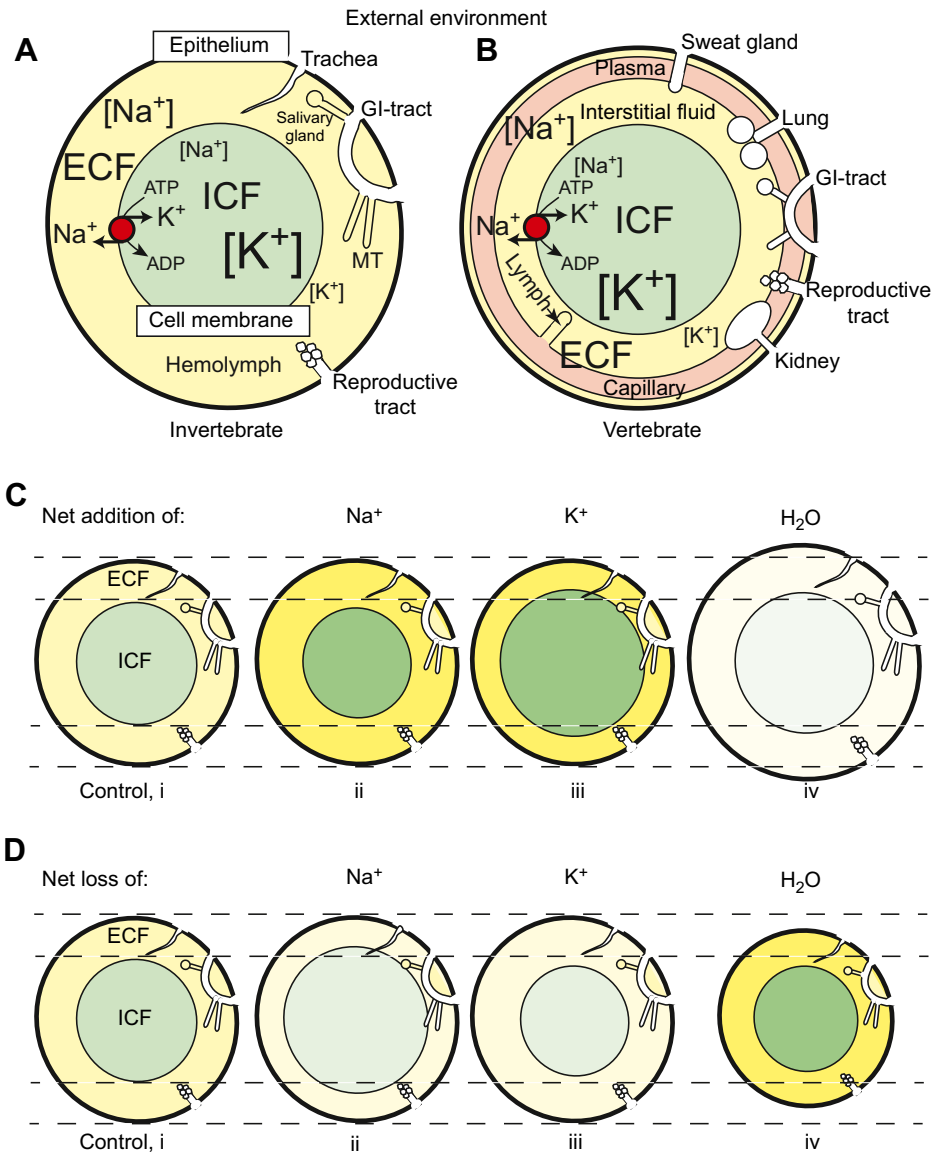

Fig. 1. The major fluid compartments of multicellular animals. All excitable cells (nerve and muscle) are represented by one intracellular fluid compartment (ICF) enclosed by the cell membrane. The concentration of Na^+ is greater in the extracellular fluid (ECF) than in the ICF, whereas the concentration of K^+ is greater in the ICF than in the ECF (as shown by the differences in font size). These differences are maintained in part by the activities of the Na^+/K^+-ATPase (red) and the renal systems (Malpighian tubules in insects and kidneys in vertebrates). ATP, adenosine triphosphate; ADP, adenosine diphosphate. (A) The invertebrate plan. An open circulatory system shuttles the ECF (the hemolymph) between somatic cells and epithelial cells. Epithelial cells of the integument, trachea, salivary gland, gastro-intestinal tract (GI-tract), Malpighian tubules (MT) and reproductive tract line the surface of the animal. (B) The vertebrate plan. A closed circulatory system (enclosed by the capillary endothelium) divides the ECF into a rapidly circulating plasma compartment (containing blood cells) and a slowly circulating interstitial fluid compartment. Lymph returns fluid to the vascular space fluid that has filtered from the plasma. (C,D) The effect of the net addition (C) or loss (D) of Na^+, K^+ and H_2O on extracellular and intracellular volume and osmotic concentration in an invertebrate. The acute changes in the absence of physiological responses are shown. Dashed horizontal lines serve as reference to the control volumes (Ci,Di) of ECF and ICF. Color intensity is proportional to osmotic concentration.

because Na^+ is largely excluded from the ICF by the Na^+/K^+-ATPase (Fig. 1Cii). The ECF thus becomes hyperosmotic to the ICF, drawing fluid from it until both the ECF and ICF are isosmotic again, but hyperosmotic to the control condition (Fig. 1Ci,ii). Opposite volume changes occur upon the net addition of K^+. Moving mostly into the ICF (owing to uptake by the Na^+/K^+-ATPase), K^+ increases the ICF osmotic concentration, which draws water from the ECF until both compartments are isosmotic again, but hyperosmotic to the control condition (Fig. 1Ci,iii). In contrast, the addition of water increases the volume of both the ICF and ECF in proportion to their size. Thus, both fluid compartments become hypo-osmotic to the control condition, and the animal increases in size and weight (Fig. 1Ci,iv).

The loss of Na^+ from the organism presents a loss primarily from the ECF compartment (Fig. 1Dii). The loss lowers the ECF osmotic concentration, which shifts water to the ICF until both compartments are isosmotic again, but hypo-osmotic compared with the control condition (Fig. 1Di,ii). Conversely, the loss of K^+ from primarily the ICF renders the ICF hypo-osmotic to the ECF. As a result, water leaves the ICF until both the ICF and ECF are isosmotic again but hypo-osmotic to the control (Fig. 1Di,iii). In the case of dehydration, the loss of water from the organism reduces the volume of both the ECF and ICF in proportion to their size while increasing the osmotic concentration equally in the two compartments (Fig. 1Div). Thus, the animal decreases in size and weight (Fig. 1Di,iv).

In addition to external challenges to the ECF and ICF, there are 'internal' challenges that stem from metabolic activity. For example, the breakdown of one molecule of glycogen may add hundreds of molecules of glucose to the ICF with the osmotic effect of cellular swelling. In contrast, the synthesis of glycogen or any macromolecule (protein, nucleic acid or lipid) removes osmoles from the ICF and causes cells to shrink. Accordingly, cell volume is constantly challenged by changes in catabolism and anabolism.

The regulation of cell volume

A relatively constant cell volume is important for maintaining constant concentrations of intracellular solutes. Constant concentrations of intracellular K^+ and Na^+ are needed for resting and action potentials in excitable cells. Constant concentrations of O_2 and CO_2 are needed to support mitochondrial functions, and constant intracellular pH is required for the proper function of

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enzymes. Moreover, the concentrations of signaling molecules must not be at the whim of changes in cell volume; instead, a constant cell volume allows, for example, the common signaling molecule and activator, Ca^{2+}, to rise and fall transiently with rapid release and rapid re-uptake, respectively. Thus, cells employ mechanisms that prevent large variations in cell volume. The solute/volume plot shown in Fig. 2A illustrates the chemistry of cell volume regulation. The hypothetical spherical animal cell with a radius of 7.1 µm has a control volume of 1.5 pl (1.5×10^{-12} l) and a solute content of 450 fosmol (450×10^{-15} osmol) (Guertin and Sabatini, 2006). The osmotic concentration of the ICF is thus 300 mOsm (300×10^{-3} osmol l^{-1}). V_0 marks this set point of the cell (Fig. 2A). When the ECF osmotic concentration is increased to 400 mOsm, the cell loses water along the iso-solute line, $V_0\rightarrow V_1$, (Fig. 2A). For the ICF to increase from 300 mOsm to 400 mOsm by the removal of water alone, the cell would lose 25% of its volume (0.375 pl; Fig. 2A). The consequent increase in the concentration of every intracellular solute could seriously impair normal cell functions in, for example, excitable cells, which rely on constant K^+ and Na^+ concentrations for communication.

The water loss $V_0\rightarrow V_1$ is reversed or corrected if the cell acquires 0.375 pl water in some way. If, however, correction is not possible, the cell can restore its former volume by adding osmoles to the ICF, which leads to the import of water along the 400 mOsm isosmotic line from $V_1\rightarrow V_2$ (Fig. 2A). Drawing on internal macromolecules, the cell can mobilize small molecules such as taurine, inositols, betaine, polyamines and other organic osmolytes to restore cell volume (Hoffmann et al., 2009). Importing solute from the outside by active transport also serves this purpose. When cells restore their original volumes by importing solutes and/or generating new osmolytes, they are said to exercise regulatory volume increase (RVI) (Burg, 1995; Burg and Ferraris, 2008; Hoffmann et al., 2009).

Compensations sacrifice constancy of one variable for the preservation of another variable. In the above case of RVI, the osmotic concentration of the ICF is sacrificed for the maintenance of volume. This strategy is apparently energetically less costly than maintaining the ICF osmotic concentration below the ECF osmotic concentration.

A 300 mOsm cell in an ECF of 200 mOsm swells on account of the osmotic water load, which increases the ICF volume while diluting all intracellular solutes ($V_0\rightarrow V_3$; Fig. 2A). The cell can restore the original cell volume by (1) exporting solute or (2) removing solute (subunits) through the synthesis of macromolecules. Both mechanisms cause water to leave the cell by osmosis and restore the original cell volume along $V_3\rightarrow V_4$. The compensation is known as regulatory volume decrease (RVD).

The concepts of correction, compensation and regulatory volume changes can also be applied in the study of ECF homeostasis (Fig. 2B). Expansions and contractions of the ECF can be hyperosmotic, hypo-osmotic or isosmotic. In brief, losing water by evaporation generates a hyperosmotic ECF contraction as shown in Figs 1Div and 2B. In contrast, a high-salt diet generates a hyperosmotic ECF expansion (Figs 1Cii and 2B). A NaCl-losing kidney generates a hypo-osmotic ECF contraction (Figs 1Dii and 2B), and excess water intake generates hypo-osmotic ECF expansion (Figs 1Civ and 2B). The loss of salt and water in the proportions isosmotic to the ECF (e.g. through blood donation or diarrhea) produces an isosmotic ECF contraction along the isosmotic line in Fig. 2B. In contrast, infusing the ECF with an ECF-like solution or receiving a blood transfusion generates an isosmotic ECF expansion along the isosmotic line. The following discussion will use this terminology in reviewing ECF homeostasis in insects.

ECF homeostasis in insects responding to environmental challenges

The ECF of insects in desiccating habitats

Insects face desiccation in ambient air; conserving water would therefore appear to be their foremost priority. However, surprisingly, insects can tolerate large swings in ECF volume. For example, eclosion in insects (i.e. the passage of pupa to adult) triggers 'eclosion diuresis' (see Glossary) in aquatic, terrestrial and flightless insects (Bushman et al., 1989; Coast, 1998; Gillett, 1982, 1983; Nicolson, 1976; Strathie and Nicolson, 1993). In the case of the moth *Heliothis zea*, eclosion diuresis reduces body weight by as much as 20%, with

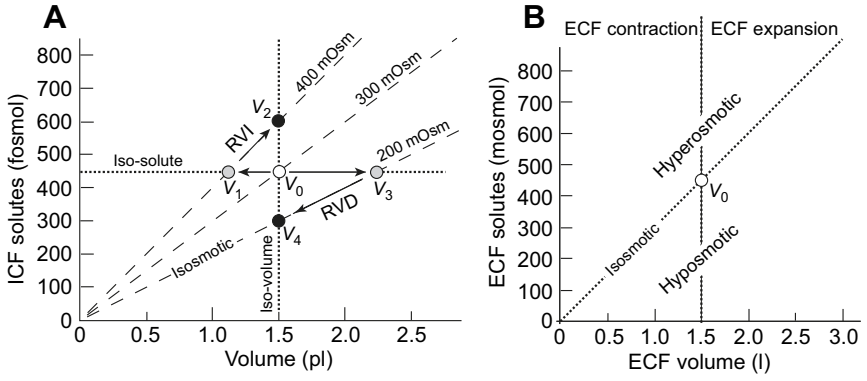

Fig. 2. Solute/volume plots of the ICF and ECF compartments. (A) ICF compartment. The origin connected to any point in the plot delineates the isosmotic line (dashed). The cell has a control volume of 1.5 pl (V_0, 1.5×10^{-12} l), a solute content of 450 fosmol (450×10^{-15} osmol) and an osmotic concentration of 300 mOsm (300×10^{-3} osmol l^{-1}). The cell membrane is semi-permeable (allowing the passage of water but not solute). In hyperosmotic media, the cell loses water ($V_0\rightarrow V_1$). The cell restores volume by accumulating solute ($V_1\rightarrow V_2$) by mechanisms known as regulatory volume increase (RVI). In hypo-osmotic media, the cell gains water ($V_0\rightarrow V_3$). The cell restores volume by losing solute ($V_3\rightarrow V_4$) by mechanisms known as regulatory volume decrease (RVD). For all solute/volume plots, the starting point is shown in white, intermediate points are in gray and the final point is in black. (B) A solute/volume plot of the ECF compartment. The hypothetical multicellular animal has an ECF volume of 1.5 l, a solute content of 450 mosmol (450×10^{-3} osmol), and an osmotic concentration of 300 mOsm (300×10^{-3} osmol l^{-1}) that define the set point of ECF operation (V_0). An ECF volume of <1.5 l is considered to be a contraction, which is hyperosmotic if the ECF loses more water than solute, or hypo-osmotic if the ECF loses more solute than water. An ECF expansion is hyperosmotic if the ECF gains more solute than water or hypo-osmotic if the ECF gains more water than solute. Contractions and expansions may also be isosmotic when both solute and water change in proportion to their presence in the ECF (e.g. by ingestion or infusion of fluids isosmotic to the ECF).

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obvious savings in the energetic costs of flying. Because this weight loss derives from the excretion of water, the ECF loses 20% of its volume during eclosion diuresis (Fig. 1Div). Even greater changes of the hemolymph volume are observed in adult fruit flies (Albers and Bradley, 2004), which can live with ECF volumes as little as 20 nl and as large as 84 nl (Fig. 3). Under control laboratory conditions, the fruit fly has an ECF volume of 50 nl with an osmotic concentration of 353 mOsm (Fig. 3A, point 'a'). Under desiccating laboratory conditions (no food, no water, low humidity for 8 h), the fly loses as much as 60% of the hemolymph water without ill effect (Albers and Bradley, 2004). A loss of 60% of the water from the ECF would be expected to increase the hemolymph osmotic concentration to 885 mOsm (Fig. 3A, a→b). However, direct measurement reveals a hemolymph osmotic concentration of only 405 mOsm (Fig. 3A, a→c). Accordingly, the fly must remove solutes (osmoles) from the ECF in order to compensate for the loss of water; this minimizes the effect of desiccation on ECF osmotic concentration.

The rehydration of the same desiccated flies provides compelling evidence that the osmoles removed from the ECF are not excreted but stored. When the desiccated fly is allowed to drink distilled water (~0 mOsm), the fly over-hydrates, increasing the hemolymph volume from 20 nl to 84 nl (Fig. 3B, d→e). The more than fourfold increase in hemolymph water content would be expected to decrease the ECF osmotic concentration from 405 mOsm to 96 mOsm (Fig. 3B, e), but it drops only to 298 mOsm (Fig. 3B, f). Clearly, the flies must mobilize stored osmoles in the absence of ingested solutes. Moreover, the fly mobilizes more solutes than have been removed during the previous desiccation. The product of the osmotic concentration and the ECF volume is the amount of ECF osmoles, which is 17.7 nosmol (17.7×10^{-9} osmol) under control conditions (Fig. 3A, a) and 25 nosmol upon drinking distilled water (Fig. 3B, f).

The obvious advantage of storing hemolymph solutes in an osmotically inactive form is their rapid availability when hemolymph volume is replenished. As a site for storing Na^+, K^+, Cl^-, divalent ions and other solutes, the epithelial cells of Malpighian tubules are likely repositories. Wessing and Zierold have observed ion accumulations as intracellular spherites in the principal cells of Malpighian tubules in *Drosophila melanogaster* (Wessing and Zierold, 1993; Wessing et al., 1992, 1988). Intracellular spherites containing calcium, magnesium, sodium, potassium, aluminium, silicon, phosphorus, sulfur and iron associated with polysaccharides and proteins are also found in epithelial cells of the intestine of the springtail (Humbert, 1978). Spherites are also present in principal cells of Malpighian tubules of the salt marsh mosquito *Aedes taeniorhynchus* and the yellow fever mosquito *Aedes aegypti* (Beyenbach and Hagedorn, 2004; Bradley, 1985; Plawner et al., 1991). They are particularly abundant in principal cells of the tubules in female mosquitoes, which ingest an excessive amount of ions during a vertebrate blood meal (see below).

In summary, *D. melanogaster* compensates for large ECF volume losses by removing solute from the hemolymph and storing it elsewhere, and compensates for large ECF volume increases by adding stored solute to the hemolymph. Both cases of compensation minimize changes in ECF osmotic concentration.

The ECF of insects gorging on fluids

In insects with rigid, non-compliant exoskeletons, large changes in ECF volume would be expected to lead to (1) very low hydrostatic pressures and circulatory collapse in the case of desiccation, and (2) crushing hemolymph pressures for the cells in the case of rehydration. Because the hemolymph volume in *Drosophila* can vary from 20 nl to 84 nl in respective bouts of dehydration and rehydration (Fig. 3), the exoskeleton must be remarkably compliant, akin to an adjustable corset that minimizes hydrostatic pressure changes with large volume changes. Gorging insects also demonstrate the ability to accommodate large fluid volumes with an adjustable exoskeleton, especially in the abdominal segment. Still, insects gorging on the sap of plants or the blood of animals must have mechanisms for rapidly dealing with ingested solute and water loads, especially if they plan on flying and escaping predation (Kwon et al., 2012). Accordingly, the glassy-winged sharpshooter urinates while gorging on xylem (Brodbeck et al., 1993), thereby lightening the flight load. Similarly, mosquitoes begin to urinate even before they have finished their blood meal (Fig. 4). The kissing

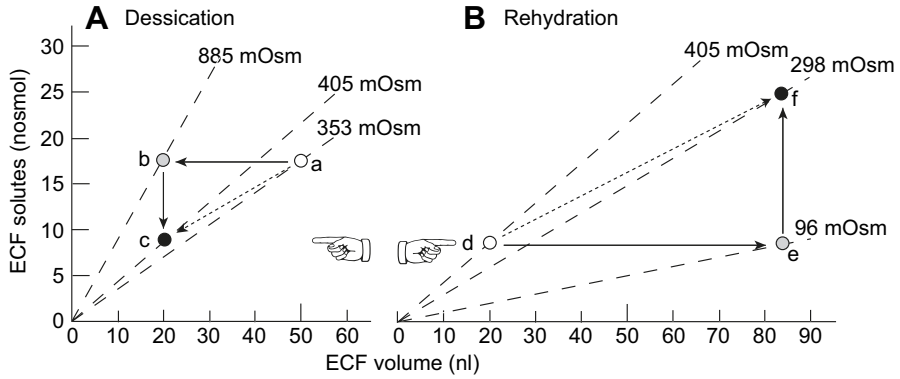

Fig. 3. From hyperosmotic contraction to hypo-osmotic expansion of the ECF in the imago (adult) fruit fly *Drosophila melanogaster*. The abscissa of A and B have the same scale. Dashed lines depict isosmotic change. The dashed arrows (A, a→c and B, d→f) indicate the physiological adaptation, and solid arrows depict the intermediate changes in ECF volume and solute content (not necessarily in that sequence). Note that A, c and B, d are identical in ECF volume and solute content. (A) Desiccation removes 60% of the hemolymph volume. The volume contraction is expected to raise the ECF osmotic concentration from 353 mOsm to 885 mOsm (A, a→b). However, the ECF osmotic concentration increases only to 405 mOsm, indicating that ECF solutes are removed from the circulation and stored (A, c). The reduction in the amount of ECF solute compensates for the large loss of ECF volume in order to minimize the change in ECF osmotic concentration. (B) Upon access to distilled water, the desiccated fly gorges on water, increasing the ECF volume more than fourfold (B, d→e). The volume expansion is expected to reduce the ECF osmotic concentration to 96 mOsm (B, e). However, direct measurement reveals an osmotic concentration of 298 mOsm (B, f), indicating that – in the absence of oral intake – solutes must be mobilized from stores and added to the ECF. The addition of solute compensates for the large increase in volume in order to minimize the change in ECF osmotic concentration. Data taken from Albers and Bradley (2004).

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bug *R. prolixus* takes on blood meals up to 10 times its own body weight (Buxton, 1930; Gioino et al., 2014), and *A. aegypti* takes on twice its body weight (Beyenbach and Petzel, 1987; Williams et al., 1983). Only female mosquitoes take blood meals in order to provide a final nourishing boost for developing eggs (Klowden, 1995). Because mammalian plasma (~290 mOsm) is hypo-osmotic to the hemolymph (354 mOsm), the blood meal causes the hypo-osmotic expansion of the ECF (Williams et al., 1983). This is largely corrected by increasing the excretion of urine that is hypo-osmotic (309 mOsm) to the hemolymph (Beyenbach and Petzel, 1987; Coast et al., 2005; Petzel et al., 1985; Williams et al., 1983). Diuretic hormones that are released from the mosquito head during the ingestion of blood trigger this diuresis (Beyenbach, 2003b; Wheelock et al., 1988).

Female *A. aegypti* mosquitos have an average hemolymph volume of 0.6 µl at an osmotic concentration of 354 mOsm, which defines the ECF set point before the blood meal (Fig. 4, a). In the short time of 2 min, the mosquito can consume 3.5 µl of blood (Williams et al., 1983). On the assumption that the plasma portion of the blood (1.9 µl with an osmotic concentration of 290 mOsm) is first absorbed by the gut, the hemolymph volume increases from 0.6 µl to 2.5 µl (Fig. 4, a→b) and hemolymph solutes increase from 212 nosmol to 763 nosmol (Fig. 4, b→c). As a result, the hemolymph osmotic concentration decreases from 354 mOsm to 305 mOsm (Fig. 4, a→c). The first urine droplets excreted from the rectum (Fig. 4, inset) consist largely of NaCl and water (Williams et al., 1983), which reflects the excretion of the unwanted plasma fraction of the blood. In particular, urine collected during the first 102 min after the blood meal (Williams et al., 1983) has a volume of 0.8 µl containing 244 nosmol (120 nmol Na^+, 13 nmol K^+ and 111 nmol Cl^-). The excretion of this diuretic urine reduces the ECF volume to 1.7 µl with an osmotic concentration of 305 mOsm (Fig. 4, e). As the blood meal diuresis wanes during the next 24 h,

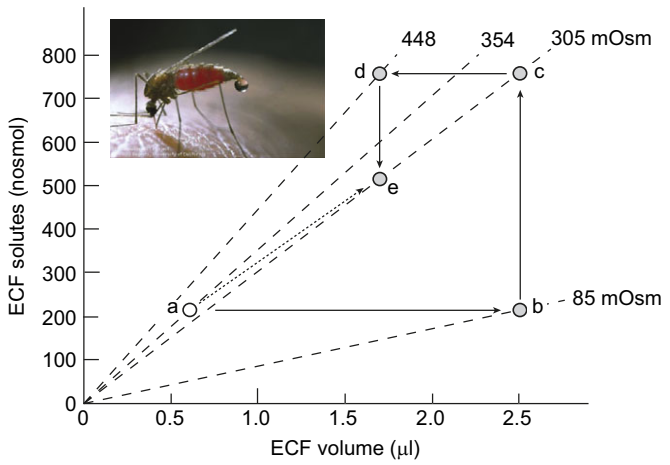

Fig. 4. The hypo-osmotic expansion of the ECF in the yellow fever mosquito, *Aedes aegypti*, gorging on mammalian blood. Dashed lines show isosmotic changes. The dashed arrow (a→e) indicates the physiological change in ECF osmotic concentration 102 min after the blood meal, and solid arrows depict the intermediate changes in ECF volume and solute content (not necessarily in that sequence). The ingestion of a blood meal triggers a diuresis even before the blood meal has been finished (inset; photo courtesy of Jack Kelly Clark, University of California). The solute/volume plot of the ECF shows that the hypo-osmotic expansion of the ECF (a→b→c) has been partially corrected by the excretion of urine isosmotic to the ECF (c→d→e) 102 min after the blood meal. As the correction continues beyond 102 min, the ECF volume, solute and osmotic concentration of the hemolymph approach the original set point 'a'. Data from Williams et al. (1983).

and as solute and water are also transferred to developing eggs, the ECF volume, solute content and osmotic concentration continue to move towards the preprandial set point (Fig. 4, a).

Malpighian tubules are the primary mediators of the blood meal-initiated diuresis (Fig. 4, c→e). At least two diuretic hormones correct the ECF expansion in the first two post-prandial hours (Beyenbach and Petzel, 1987; Beyenbach and Piermarini, 2011). One is the natriuretic hormone, a calcitonin-like peptide (Coast et al., 2005; Petzel et al., 1985, 1986) that increases the Na^+ conductance of the basolateral membrane of principal cells, which increases the transepithelial secretion of Na^+ (Sawyer and Beyenbach, 1985). The stimulation of active transepithelial secretion of Na^+ hyperpolarizes the transepithelial voltage to more lumen-negative voltages, thus increasing the paracellular secretion of Cl^- (Petzel et al., 1987; Williams and Beyenbach, 1983, 1984). Water follows across the epithelium by osmosis. The other hormone, aedeskinin, is chloruretic. It increases the Cl^- conductance of the paracellular pathway in *Aedes* Malpighian tubules (Beyenbach, 2003a; Beyenbach et al., 2010). The release of both diuretic hormones leads to apparently synergistic effects on the transepithelial secretion of NaCl and water (O'Donnell et al., 1996).

In summary, the blood meal produces a hypo-osmotic expansion of the ECF (Fig. 4, a→c), which is largely corrected by the excretion of urine that is hypo-osmotic to the preprandial hemolymph (Fig. 4, c→e). The correction minimizes changes in ECF osmotic concentration while reducing volume. The initial diuresis rids the mosquito of the unwanted NaCl and water fraction of the ingested blood (Fig. 4, c→e) within 102 min of the blood meal. Thereafter, the diuresis shifts to the excretion of K^+ obtained from digested mammalian blood cells (Williams and Beyenbach, 1983; Williams et al., 1983).

The ECF of insect larvae in fresh water

Of nearly 1 million known species of insects, 30,000 are considered freshwater insects (Springer, 2009). Around 95% of the 3500 species of mosquito develop in fresh water (Bradley, 1987) where osmotic concentrations are <20 mOsm. In this strongly hypo-osmotic environment, the larvae gain water by osmosis and lose salt (osmoles, ions) by diffusion (Fig. 5A). Reducing the permeability of the exoskeleton (cuticle) is perhaps the best strategy to limit the water load and the loss of solute (Beament, 1961; Nicolson and Leader, 1974; Stobbart, 1971a; Stobbart and Shaw, 1974), which leaves the anal papillae (see Glossary) as the major cuticular sites of osmotic water uptake and diffusive salt loss (Ramsay, 1950; Wigglesworth, 1933a,b,c). The aquaporin proteins *Aa*AQP4 and *Aa*AQP1b are present in the anal papillae of the mosquito *A. aegypti*, and mercury, which blocks aquaporin water channels, inhibits the uptake of water by the papillae (Marusalin et al., 2012). Although the oral uptake of water has been considered to be negligible in the past (Bradley, 1987; Wigglesworth, 1933c), recent studies (Clark et al., 2007) have uncovered appreciable drinking rates in freshwater larvae of the mosquito *A. aegypti* (Fig. 5A).

To get rid of water, Malpighian tubules secrete fluid into the tubule lumen at rates higher than those of osmotic water entry. The secreted fluid is isosmotic to the ECF and consists primarily of KCl, NaCl and water (Bradley, 1987; Clark and Bradley, 1998; Ramsay, 1950). The tubules empty their secretions into the pyloric chamber between the midgut and hindgut (Fig. 5A), from where tubular fluid may be passed upstream to the midgut if water needs to be conserved or downstream to the hindgut if water needs to be eliminated (Bradley, 1987). In freshwater larvae, the tubular fluid is passed downstream through the ileum without much modification

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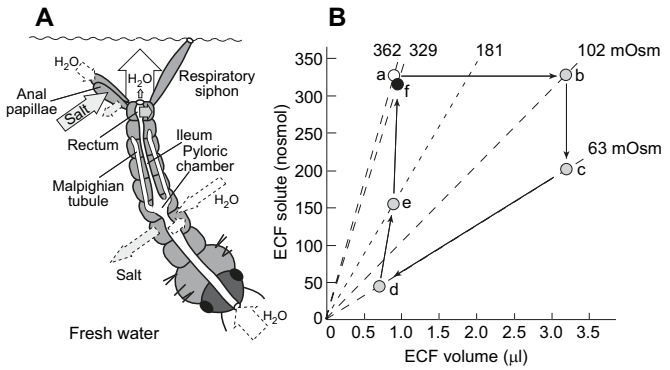

Fig. 5. The hypo-osmotic expansion of the ECF in larvae of the mosquito *A. aegypti* dwelling in fresh water. (A) Environmental challenges and physiological responses. Dashed arrows indicate diffusion and oral uptake; solid arrows indicate the participation of active transport mechanisms. White arrows show the movement of water and gray arrows show the movement of salt. (B) Hypothetical solute/volume plot of the ECF. Dashed lines show isosmotic changes. The physiological adaptation proceeds from a to f; solid arrows depict the intermediate changes in ECF volume and solute content (not necessarily in that sequence). The hypo-osmotic expansion (a→b→c) is corrected by (1) the Malpighian tubules secreting solutes and water (c→d), (2) rectal reabsorption of solutes with little water (d→e) and (3) the absorption of NaCl from the low concentrations present in fresh water (e→f) by powerful active transport mechanisms in the anal papillae (Del Duca et al., 2011). The sequence a→f defines the outer boundary of ECF changes. The physiology takes place within that boundary. Data taken in part from Chambers and Klowden (1990); Chapman (1998); Clark et al. (2009); and Clark et al. (2007).

(Bradley, 1987; Meredith and Phillips, 1973). However, beyond the ileum, the rectal epithelium reabsorbs NaCl and KCl with little water, leaving behind a dilute fluid of 24 mOsm for excretion (Chapman, 1998; Ramsay, 1950). The excretion of urine with an osmotic concentration of 24 mOsm constitutes a solute loss (Fig. 5A). The intake of food replaces some solute; however, the availability of food cannot always be relied upon, and pupal mosquitoes cease feeding altogether. Therefore, solutes must be acquired by other means. The anal papillae serve this role (Bradley, 1987; Del Duca et al., 2011; Edwards, 1983; Koch, 1938; Phillips and Meredith, 1969; Stobbart, 1967, 1971a,b, 1974; Wigglesworth, 1933b, 1939; Wright, 1975). The papillae are capable of absorbing NaCl and KCl from very low concentrations in fresh water (Fig. 5A). The absorption replaces ions that are lost by diffusion and sacrificed in the renal excretion of water. The size of the anal papillae increases with decreasing osmotic concentration of the external environment, which is thought to reflect increased transport activity (Bickley, 1945; King et al., 1939; Nayar and Sauerman, 1974; Wigglesworth, 1939).

Fig. 5B illustrates the adaptation to fresh water on the assumption that the larva starts out with the following hemolymph variables: 0.9 µl in volume, 326 nosmol of solute and an osmotic concentration of 362 mOsm (Fig. 5B, a). Each day, the larva gains approximately 2.3 µl of water along the iso-solute line – 2.1 µl by ingestion (Clark et al., 2007) and, hypothetically, 0.2 µl by osmosis – and loses ∼125 nosmol of salt (Patrick et al., 2001). The water load decreases the ECF osmotic concentration from 362 mOsm to 102 mOsm (Fig. 5B, a→b), and the salt loss reduces the ECF osmotic concentration even further, to 63 mOsm (Fig. 5B, b→c). Responding to the serious hypo-osmotic volume expansion, Malpighian tubules secrete 2.5 µl of isosmotic fluid per day into the tubule lumen (Fig. 5B, c→d). This rate of fluid secretion is ∼0.35 nl min^{-1} per Malpighian tubule, i.e. well below 0.5 nl min^{-1}, the secretion rate measured in isolated Malpighian tubules of larval mosquitoes (Clark and Bradley, 1998). Because the secreted fluid is isosmotic to the hemolymph, it reduces the ECF solute content by 157 nosmol to 44 nosmol without a change in ECF osmotic concentration (Fig. 5B, c→d). Subsequently, downstream, the rectal epithelium hypothetically reabsorbs 110 nosmol of mostly KCl and NaCl with 0.15 µl water, which raises the ECF osmotic concentration to 181 mOsm and the ECF volume to 0.85 µl (Fig. 5B, d→e). Active transport mechanisms in the anal papillae may absorb 159 nosmol of electrolytes with 0.10 µl water, to bring the ECF to the new set point at 0.95 µl volume and an osmotic pressure of 329 mOsm (Fig. 5B, e→f); namely, the values measured in freshwater mosquitoes by Clark et al. (2009).

The above balance of ECF inputs and outputs shows that, in fresh water, the mosquito excretes 2.35 µl and 47 nosmol from the rectum every day. Accordingly, excreted urine has an osmotic concentration of 20 mOsm, which is within the diluting capability of the rectum (Clark et al., 2009; Ramsay, 1950). Furthermore, the balance of inputs and outputs yields 733 mOsm as the osmotic concentration of the fluid absorbed in the rectum (Fig. 5B, slope d–e) which again is within the diluting capacity of the rectum (Chapman, 1998; Ramsay, 1950). Likewise, these considerations yield 1590 mOsm as the osmotic concentration of the fluid absorbed by the anal papillae (Fig. 5B, slope e–f). The strongly hyperosmotic absorbate suggests that the relative rates of active ion uptake in the anal papillae are significantly greater than those of passive osmotic water entry, as indicated in studies by others (Koch, 1938; Stobbart, 1967, 1974).

In summary, in freshwater mosquito larvae, the hypo-osmotic expansion of the ECF is largely corrected by the renal and rectal excretion of more water than solute and by the uptake of more salt than water by the anal papillae. These corrections preserve ECF osmotic concentration and ECF volume in fresh water.

The ECF of insect larvae in brackish water

Nearly all insects breeding in fresh water can also be found in brackish water (Osburn, 1906). The adaptation of mosquito larvae to brackish water has been studied extensively in two mosquito genera: *Culex* and *Culiseta* (Bradley, 1987; Garrett and Bradley, 1984, 1987; Patrick and Bradley, 2000). The two mosquitoes survive in water with osmotic concentrations less than ∼300 mOsm using mechanisms similar to those of freshwater mosquito larvae, i.e. they osmoregulate (Figs 5 and 6A). In contrast, at ambient osmotic concentrations above ∼400 mOsm, the ECF osmotic concentration rises in parallel with the environmental osmotic concentration, i.e. the ECF osmotic concentration 'conforms' to the ambient osmotic concentration (Fig. 6A). In particular, *Culex* larvae allow ECF concentrations of Na^+ and Cl^- to rise (Garrett and Bradley, 1987). More importantly, the larvae accumulate organic osmolytes – such as proline (124 mmol l^{-1}), serine (54 mmol l^{-1}), leucine (23 mmol l^{-1}), trehalose (37 mmol l^{-1}) and other organic solutes (90 mmol l^{-1}) – in the hemolymph. Together, organic osmolytes account for >50% of the ECF osmotic concentration when mosquito larvae inhabit brackish water with an osmotic concentration of 600 mOsm (Garrett and Bradley, 1987). It should be noted that not all mosquito larvae osmoconform in this way – others, such as those of *A. taeniorhynchus*, osmoregulate in seawater and brine by maintaining remarkably constant ECF osmotic concentrations over a wide range of environmental salinity (Fig. 6B).

By allowing ECF osmotic concentration to conform with the environmental osmotic concentration by the addition of osmolytes

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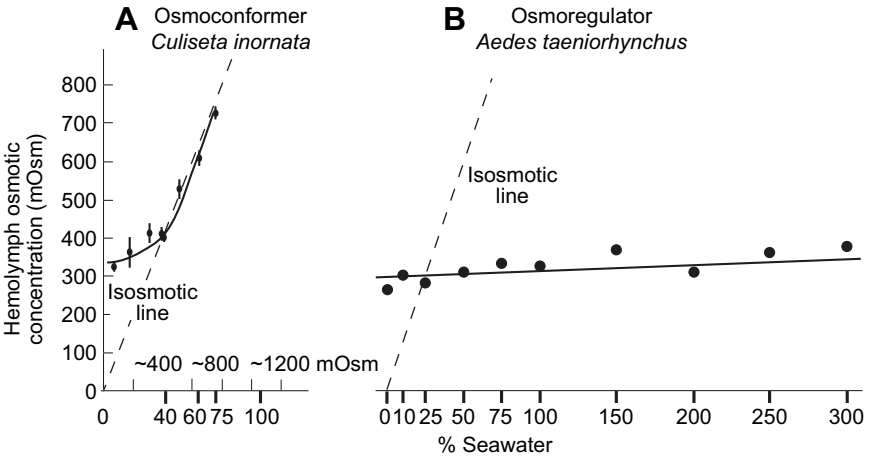

Fig. 6. Osmoconformation and osmoregulation. (A) The winter mosquito *Culiseta inornata* regulates the hemolymph in ambient media of <400 mOsm but conforms to environmental osmotic concentrations in media with concentrations >400 mOsm by accumulating organic osmolytes in the ECF. However, in 75% seawater (~750 mOsm), the larvae begin to die. Data are means±s.d.; redrawn from Garrett and Bradley (1984). (B) The salt marsh mosquito *Aedes taeniorhynchus* regulates hemolymph osmotic concentration over a wide range of external osmotic concentrations (fresh water to 300% seawater). In threefold concentrated seawater (300% seawater), the larvae begin to die. Data from Nayar and Sauerman (1974), redrawn and converted to mOsm using NaCl activity coefficients. The abscissa of A and B have the same scale.

to the ECF, the costs of maintaining water balance are eliminated (Fig. 6A). The functions of Malpighian tubules are largely reduced to removing excess electrolytes and unwanted organic solutes from the ECF (Garrett and Bradley, 1984). Fluid excreted from the rectum is either isosmotic or slightly hyperosmotic to the hemolymph (Bradley, 1987; Garrett and Bradley, 1984) (Figs 6A, 7A).

The ECF chemistry of adapting from 5% seawater to 60% by way of osmoconforming is outlined in Fig. 7B, based on the studies of the mosquitoes *Culex tarsalis* and *Culiseta inornata* (Garrett and Bradley, 1984, 1987). In 5% seawater, the larva of *Culex* has an ECF osmotic concentration of 275 mOsm (Garrett and Bradley, 1987).

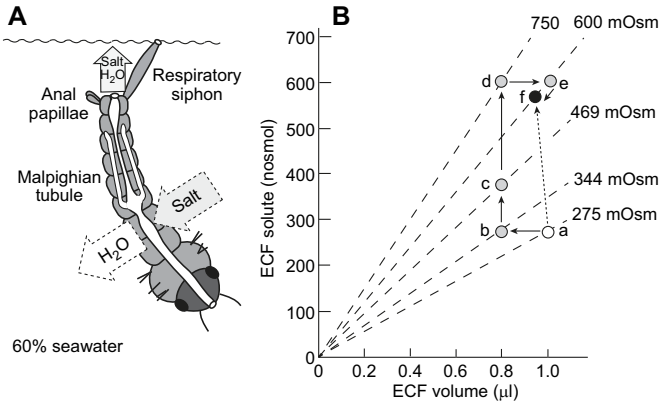

Fig. 7. The hyperosmotic contraction of the ECF in larvae of the encephalitis mosquito *Culex tarsalis*, an osmoconformer, inhabiting brackish water. (A) Environmental challenges and physiological responses of the adaptation from 5% seawater (~50 mOsm) to 60% seawater (~600 mOsm). Dashed arrows indicate diffusion; solid arrows indicate the involvement of active transport mechanisms. (B) Solute/volume plot of the adaptation to 60% seawater. Dashed lines show isosmotic changes. The dashed arrow (a→f) indicates the physiological change in ECF osmotic concentration, and solid arrows depict the intermediate changes in ECF volume and solute content (not necessarily in that sequence). The mosquito larva counters the hyperosmotic volume contraction (a→b→c) by raising the ECF osmotic concentration. The mosquito retains some of the NaCl load in 60% seawater, and, more importantly, adds organic osmolytes to the ECF (c→d). Together, inorganic and organic osmolytes raise the ECF osmotic concentration to 750 mOsm (d), which results in the import of water, until the ECF is at osmotic equilibrium with 60% seawater (d→e). The adaptation to 60% seawater exemplifies compensation that sacrifices ECF osmotic concentration in order to preserve ECF volume. The secretion of fluid (isosmotic to the ECF) by Malpighian tubules and its excretion from the rectum (e→f) eliminates part of the diffusive salt load (b→c). Data taken from Garrett and Bradley (1987).

This osmotic concentration and the assumption of an ECF volume of 1 µl place the initial set point of ECF homeostasis at point 'a' in Fig. 7B. During adaptation to 60% seawater, the larva hypothetically loses water (0.20 µl) by osmosis (Fig. 7B, a→b) and gains electrolytes (100 nosmol) by diffusion (Fig. 7B, b→c). As a result, the ECF osmotic concentration rises from 275 mOsm to 469 mOsm (Fig. 7B, c). The addition of 225 nosmol of organic osmolytes (Garrett and Bradley, 1987) to the ECF raises the ECF osmotic concentration to 750 mOsm (Fig. 7B, c→d), which allows 0.20 µl of water to move in by osmosis, thereby matching the ECF osmotic concentration to the environmental osmotic concentration of 600 mOsm (Fig. 7B, d→e). The larva is now at osmotic equilibrium with the external medium. Of the 100 nosmol of electrolytes gained by diffusion in 60% seawater, the larva retains 70 nosmol (Garrett and Bradley, 1987). The remaining 30 nosmol are secreted by the Malpighian tubules and excreted from the rectum in a volume of 0.05 µl (Fig. 7B, e→f). Accordingly, excreted urine has an osmotic concentration of 600 mOsm (30 nosmol/0.05 µl), consistent with measured osmotic concentrations of rectal excretions in *Culiseta* (Bradley, 1987; Garrett and Bradley, 1984). The excretion of 0.05 µl of urine reduces the ECF volume to 0.95 µl without changing the ECF osmotic concentration (Fig. 7B, e). The excretion of only 0.05 µl urine illustrates that, compared with osmoregulation, osmoconformation reduces the workload on renal and excretory systems.

In summary, the hyperosmotic contraction of the ECF in 60% seawater triggers primarily a compensatory response in osmoconformers although the excretion of most of the salt load in 60% seawater constitutes a correction. By adding organic osmolytes to the ECF and retaining some of the salt load, the mosquito ECF becomes isosmotic to the external medium. Thus, ECF osmotic concentration is sacrificed to maintain ECF volume. The adoption of a new, substantially increased ECF osmotic concentration is expected to trigger the mechanism of RVI in the cells of the larva.

The ECF of insect larvae in seawater

The number of insects that can be considered marine comes to only several hundred (Springer, 2009). But truly marine species (namely, those in remote offshore regions of the open ocean) are very few – all belonging to the genus *Halobates* of ocean striders or sea skaters (Springer, 2009). Other marine insects (Diptera, Coleoptera and Hemiptera) are found in mangrove swamps, estuaries, saltmarshes and intertidal zones. Among the mosquitoes (Diptera), only 5% have larvae that can survive and grow in 100% seawater (Bradley, 1987). Here, ambient osmotic concentrations are much greater than

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those of the hemolymph; thus, the larvae must deal with the challenges of losing water and gaining salt (Fig. 8A). Mosquito larvae of the genera *Aedes*, *Anopheles* and *Opifex* handle these challenges by regulating the volume and composition of the ECF (Fig. 6B). For example, larvae of *Aedes* maintain the hemolymph osmotic concentration at ∼350 mOsm with little or no use of organic osmolytes even in 300% seawater (Asakura, 1980; Bradley, 1987; Bradley and Philips, 1977c; Nayar and Sauerman, 1974) (Figs 6B, 8). The larvae handle the water loss by drinking seawater and generating new hemolymph from it, and they handle the salt load by producing excretory fluids that are hyperosmotic to the ambient medium (Beadle, 1939; Bradley, 1987).

The rate at which seawater is consumed by a larva is more highly correlated with the surface area of the larva than with the osmotic concentration of the external medium (Bradley and Phillips, 1975, 1977a). Although the exoskeleton is two orders of magnitude less permeable to water in seawater than in fresh water, larvae in seawater constantly lose water by osmosis (Bradley, 1987; Bradley and Phillips, 1977a; Nicolson and Leader, 1974). Replacing hemolymph water begins with drinking seawater (Fig. 8A) (Asakura, 1980; Beadle, 1939). The volumes ingested can be substantial: *A. taeniorhynchus* ingests more than twice its own body weight in a single day (Bradley, 1987; Bradley and Phillips, 1977a). Apparently, most of the ingested seawater is absorbed from the midgut (Bradley, 1987; Kiceniuk and Phillips, 1974), which restores hemolymph volume but presents a considerable salt load.

Malpighian tubules begin the process of eliminating salt from the hemolymph by secreting an isosmotic fluid rich in Na^+, Cl^-, Mg^{2+}, SO_4^{2-} and other ions ingested with seawater (Beyenbach and Hagedorn, 2004; Bradley, 1985; Donini et al., 2006; Kiceniuk and Phillips, 1974; Phillips and Maddrell, 1974; Ramsay, 1951). The ECF volume processed by Malpighian tubules must approximate the ingested volume of seawater (Fig. 8B). Fluid secreted by the Malpighian tubules enters the hindgut and remains isosmotic with hemolymph until it arrives in the rectum (Bradley, 1985, 1987; Ramsay, 1950). Here, Na^+, K^+, Cl^-, Mg^{2+}, SO_4^{2-} and other solutes are added to the secreted fluid by active transport mechanisms with little or no transport of water from the hemolymph, which renders rectal fluid hyperosmotic to seawater (Bradley, 1985; Bradley and Phillips, 1975, 1977a,b,c). Accordingly, the production of hyperosmotic fluids in the rectum generates free water for the hemolymph, i.e. it dilutes the hemolymph (Fig. 8B). Thus, gut, Malpighian tubules and the rectum 'distill' fresh ECF from ingested seawater.

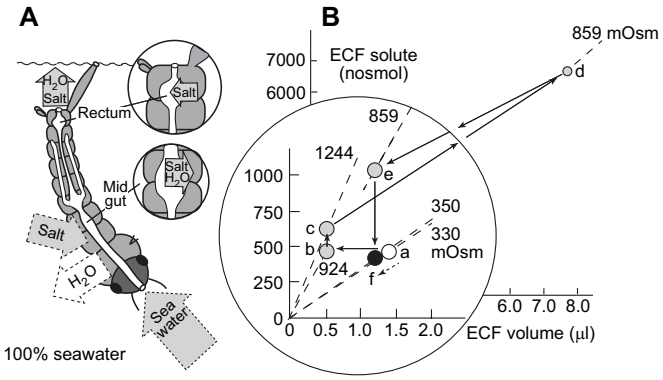

Fig. 8. The hyperosmotic contraction of the ECF in larvae of the salt marsh mosquito *A. taeniorhynchus*, an osmoregulator, inhabiting seawater. (A) Environmental challenges and physiological responses. Dashed arrows indicate diffusion or oral ingestion; solid arrows indicate the involvement of active transport mechanisms. In seawater, the mosquito corrects the hyperosmotic volume contraction by (1) generating new ECF from ingested seawater and (2) excreting the salt load by secretory mechanisms in the rectum. (B) Solute/volume plot of the adaptation. Dashed lines show isosmotic changes. The dashed arrow (a→f) indicates the physiological change in ECF osmotic concentration, and solid arrows depict the intermediate changes in ECF volume and solute content (not necessarily in that sequence). In seawater, the ECF loses water (a→b) and gains salt (b→c). To restore hemolymph volume, the larvae drink and absorb a large amount of seawater (c→d). Malpighian tubules secrete most of this volume (d→e) and present this fluid to the rectum, where solute is added (e→f). Importantly, the rectal secretion of solute (with little or no water; e→f) generates solute-free water for the hemolymph. Thus, mechanisms of correction mediate the hypo-osmotic ECF regulation of *A. taeniorhynchus* in seawater. Numerical estimates derive from the adaptation of *A. taeniorhynchus* to 100% Vancouver seawater with an osmotic concentration of 832 mOsm (Bradley and Phillips, 1977a).

Central to the production of hyperosmotic and hypo-osmotic fluids in the rectum is the transport of solute with little or no water. Epithelial cells are capable of this; for example, in renal tubules of the vertebrate kidney, the apical membranes of epithelial cells facing the tubule lumen can be absolutely impermeable to osmotic water flow (Beyenbach, 1984; Cabral and Herrera, 2012). In insect larvae, the apical membrane of rectal epithelial cells shows little or no permeability to water, thus allowing the production of dilute urine in freshwater larvae (Fig. 5) and concentrated urine in seawater larvae (Fig. 8).

Fig. 8B illustrates the daily water and solute input and output of a larva of the mosquito *A. taeniorhynchus* adapting to Vancouver seawater (832 mOsm), as studied by Bradley and Phillips (1975, 1977a,b). Initially, the larva has a hypothetical hemolymph volume of 1.4 µl, a solute content of 462 nosmol and an ECF osmotic concentration of 330 mOsm (Fig. 8B, a). In 832 mOsm seawater, the larva loses 0.9 µl by osmosis (Fig. 8B, a→b) (Bradley and Phillips, 1977a), and hypothetically gains 160 nosmol of solute by diffusion (Fig. 8B, b→c). As a result, the hemolymph osmotic concentration increases from 330 mOsm to 1244 mOsm (Fig. 8B, a→c). On a daily basis, the ingestion and absorption of seawater adds as much as 7.2 µl in volume and a salt load of as much as 5990 nosmol to the ECF (Bradley and Phillips, 1977a), reducing the ECF osmotic concentration to 859 mOsm (Fig. 8B, c→d). Malpighian tubules respond to the hyperosmotic volume expansion by secreting a volume of 6.5 µl and 5582 nosmol of solutes into the tubule lumen (Fig. 8B, d→e). As a result, the ECF volume decreases to 1.2 µl but the ECF osmotic concentration remains at 859 mOsm (Fig. 8B, e). Once this fluid arrives in the rectum, epithelial secretion adds 610 nosmol with little or no water (Fig. 8B, e→f). As a result, urine with a volume of 6.5 µl containing 6192 nosmol of solutes is excreted from the rectum. The osmotic concentration of excreted urine is thus 963 mOsm, i.e. 14% higher than the ambient osmotic concentration (832 mOsm) of Vancouver seawater and well within the concentrating ability of the rectum (Bradley and Phillips, 1975, 1977a). As rectal secretion of solutes without water concentrates the urine, it dilutes the ECF, lowering the osmotic concentration from 859 mOsm to 350 mOsm (Fig. 8B, e→f), the osmotic concentration that is measured in *A. taeniorhynchus* larvae adapted to 832 mOsm Vancouver seawater (Bradley and Phillips, 1977a). Although the anal papillae have been suggested to secrete electrolytes in seawater larvae (Asakura, 1980; Bradley, 1987; Garrett and Bradley, 1984; Phillips and Meredith, 1969), direct evidence for this hypothesis is lacking.

By drinking and processing seawater with a volume up to eight times greater than that lost from the ECF (Bradley and Phillips, 1977a), the excretory organs turn over large volumes of ingested

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seawater. This strategy is necessary because of the limited concentrating ability of the rectum. If the larval rectum was able to generate fluid that was 28% hyperosmotic to seawater instead of 14% (Bradley and Phillips, 1977a,c), the larva could reduce the oral intake of seawater by half.

In summary, the hyperosmotic contraction of the ECF in seawater triggers mostly corrections of changes in ECF variables. The volume loss is corrected by distilling new body fluid from ingested seawater, and the salt load is corrected by salt secretion in the rectum. In addition, the small rise in ECF osmotic concentration reflects some compensation, which has the benefit of reducing osmotic and ionic gradients between ECF and seawater. The increase of the ECF from 330 mOsm to 350 mOsm must trigger compensatory mechanisms in cells to preserve cell volume.

Insects without Malpighian tubules

Above, I have presented the usual mechanisms for maintaining homeostasis of the ECF compartment in insects. As so often occurs in biology, one can find exceptions to the rule. One group of insects – the aphids – deals with challenges to the ECF in a transcellular compartment: the lumen of the gut. The absence of Malpighian tubules in aphids may have selected for increasing homeostatic roles of the gut. How well aphids protect the ECF without Malpighian tubules is underscored by their handling of huge sugar loads.

Aphids feed on phloem sap. Because phloem sap is a poor source of quality protein, aphids must consume large quantities in order to satisfy their nutritional requirements for nitrogen. The dominant solute in phloem sap is sucrose, which is present at concentrations as high as 2000 mOsm (Douglas, 2003, 2006). The presence of such a hyperosmotic fluid in the lumen of the gut presents the threat of hyperosmotic ECF volume contraction. Aphids reduce this threat by (1) breaking down sucrose in the gut lumen to fructose and glucose (Cristofoletti et al., 2003; Price et al., 2007, 2010), (2) absorbing fructose for metabolism and lipid synthesis, and (3) packaging glucose as oligosaccharides that are then excreted from the rectum as ‘honeydew’, which is isosmotic to the hemolymph (Ashford et al., 2000; Downing, 1978; Rhodes et al., 1997; Wilkinson et al., 1997). The formation of oligosaccharides from glucose is thought to render the luminal fluid in the distal intestine hypo-osmotic to the fluid in the stomach (Shakesby et al., 2009). Because the distal intestine loops around the stomach, water can move from the former to the latter (Douglas, 2003). The water transfer is expected to dilute the ingested phloem sap, with the benefit of reducing the threat of hyperosmotic ECF volume contraction. Consistent with this model is the fact that knockdown of the aquaporin water channel (ApAQP1) in the stomach and distal intestine reduces the water flux from the distal gut to the stomach (Shakesby et al., 2009). As a result, ingested phloem sap is not diluted and the ECF osmotic concentration increases from 453 mOsm in control aphids to 590 mOsm in ApAQP1-deficient aphids (Shakesby et al., 2009).

In summary, aphids counteract the threat of ECF hyperosmotic contraction that results from the presence of strongly hyperosmotic phloem sap in the lumen of the gut by digesting sucrose, absorbing fructose and forming oligosaccharides from glucose remaining in the gut for excretion as honeydew. The synthesis of oligosaccharides lowers the osmotic concentration of the gut contents with the benefit of minimizing the osmotic water loss from the ECF. This chemical strategy of ECF homeostasis probably reflects the increased homeostatic role of the gastro-intestinal tract in aphids, which lack Malpighian tubules.

Conclusions

In this Review, I began by showing that the osmotic concentration of a fluid is determined by the quantity of solute (osmol) and the volume of the solution. Accordingly, plots of solute versus volume show how the two variables affect the osmotic concentration. Challenges to ECF solute and volume can be corrected by simply reversing the challenge (i.e. what comes in must go out), in which case the osmotic concentration remains unchanged. However, if corrections are not possible, then changes in an alternative variable may compensate, in which case the osmotic concentration changes. In general, maintenance of the osmotic concentration receives priority in ECF homeostasis; however, ECF osmotic concentration can be sacrificed for the preservation of ECF volume as in osmoconformers. The preservation of ECF solute as the target of ECF homeostasis has not been observed. On the contrary, electrolytes and organic osmolytes may be added to or removed from the ECF, thereby increasing compensatory capacity. Furthermore, insects use organic osmolytes to prevent the ECF from freezing at low temperatures and to minimize the evaporative loss of ECF at high temperatures; thus, ECF homeostasis cannot be dissociated from temperature adaptation in some insects. Feeding habits (starving and gorging), nutritional needs and limits in the diet, and the energetic costs of flying all affect ECF homeostasis. Finally, the apparent absence of Malpighian tubules in some insects means that other organs (such as the gut) must take on additional roles in ECF homeostasis. Thus, ECF homeostasis in insects is not a matter of strictly regulating the ECF compartment around a target osmotic concentration as it is in mammals. Instead, the study of ECF homeostasis in insects demonstrates a high degree of physiological freedom and resourcefulness to cope – remarkably – with diverse challenges. Undoubtedly, the diversity and mechanism of ECF homeostasis is part of the evolutionary success of insects.

Acknowledgements

I thank Allan Weinstein, David Dupee, Peter M. Piermarini, Helmut Wieczorek, Timothy Bradley and the anonymous reviewers of JEB for the constructive scientific review of this manuscript.

Competing interests

The authors declare no competing or financial interests.

Funding

I thank the National Science Foundation, USA, for supporting research in my laboratory for many years.

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