Acid–Base Regulation in Insect Haemolymph

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8.1 Summary

Insects regulate the acid–base balance of their haemolymph by ventilation, buffering and active uptake and excretion of acid–base equivalents with their gut. Haemolymph pH varies widely across ontogeny and between species, varying from acidic (~6.4) to alkaline (~8.0). Terrestrial and aquatic insects are exposed to different acid–base challenges by virtue of their environment and have evolved different mechanisms to cope. Terrestrial insects use bicarbonate buffering and changes in ventilation to respond to metabolic acid loads, as well as clearing excess acid through excretion into the lumen of the hindgut. V-ATPase and Na^+/K^+-ATPase in the hindgut/Malpighian tubule complex generates the transmembrane electrochemical potential necessary to drive uptake and excretion of acid–base equivalents and ions. The activity of these transport processes is under hormonal control. While aquatic insects also use their hindgut for acid–base regulation, they possess additional ion-transporting chloride cells on parts of their cuticle that are in contact with the surrounding water. These cells provide an extra-renal pathway for regulating haemolymph pH and osmolality, relying on ion transport driven directly and indirectly by V- and Na^+/K^+-ATPases. While aquatic insects are among the most pH-tolerant animals on the planet, the mechanisms that allow them to tolerate chronic exposure to highly acidic or alkaline water across a wide range of ionic strengths are incompletely understood and require further investigation.

8.2 Introduction

The acid–base balance of an insect's body fluids results from the interplay between the partial pressure of carbon dioxide (PCO_2) in its body fluids, the buffering action of weak acids and the active pumping of ions across membranes within the insect as well as between the insect and its environment. By actively regulating these variables, insects can maintain their internal pH within a physiologically acceptable range. But the extraordinary diversity of insects and their distribution across both terrestrial and aquatic habitats make summarising their acid–base regulatory strategies no easy task. This diversity also means that the acid–base regulation of only a few insect species have received any particular attention, by virtue of being either economically important agricultural pests (e.g. *Schistocerca gregaria*, *Manduca sexta*) or vectors of disease (*Aedes* and *Anopheles*). While the mechanisms described from among these few exemplars likely apply to most insects, many new and surprising acid–base adaptations among the millions of unstudied insect species are yet to be discovered.

Insects regulate the acid–base balance of two separate extracellular compartments: the lumen of their gut and their haemolymph-filled haemocoel. This chapter reviews the acid–base balance within the insect's haemolymph. It considers the role of respiration and ion exchanging regions in the hindgut and Malpighian tubules in terrestrial insect acid–base balance strategies, comparing these physiological mechanisms with those utilised by aquatic insect species.

8.3 Acid–Base Regulation in Terrestrial Insects

8.3.1 Haemolymph Composition and pH

The two largest reserves of body water in an insect are located in its gut lumen and in its haemolymph. After the gut lumen, the haemolymph represents the second largest fraction of an insect's total body water. For example, it accounts for between 17 % and 21 % of the total body weight of the American cockroach (*Periplaneta americana*) (Wheeler 1963) but tends to be a higher fraction of the total body weight of larval insects, such as the death's-head hawkmoth caterpillar *Acherontia atropos* (34 % of body weight) (Wasserthal 1996). Haemolymph fills the open circulatory system, the haemocoel, and bathes the insect's tissues, supplying them with nutrients, hormones and ions, while acting as a sink for metabolic wastes. It also acts as a pH buffer and as a source and sink of acid–base equivalents. The acid–base balance of haemolymph is determined by three independent variables, namely, the strong ion difference (the difference between the sum of all cations and anions present in solution), the concentration and type of weak acids and the partial pressure of carbon dioxide (PCO_2) (Stewart 1983). Ideally, all three variables should be measured to provide a complete picture of the acid–base balance of a fluid. But as pH (or, more precisely, proton concentration [H^+]) is the variable that is regulated by physiological processes to maintain enzyme function, pH is the usual starting point for considering the acid–base properties of this fluid. An early, and still repeated, assertion is that insect haemolymph is acidic (Chapman 2013; Wigglesworth 1972). However, the data collated in ◘ Table 8.1 shows this to be incorrect. While lepidopteran and hymenopteran larvae appear to possess haemolymph that is uniformly acidic, measurements from other holometabolous insects show alkaline or near-neutral pH, while most of the hemimetabolous insects measured thus far maintain a haemolymph higher than pH 7 (◘ Table 8.1). The accuracy of some of the older measurements of insect haemolymph pH is questionable because measurements made in vitro used fluid pooled from several individuals. This approach potentially introduces errors due to changes in the composition of haemolymph, the clotting of protein and the loss of CO_2. More recent measurements of haemolymph pH, made in vivo by inserting a pH microelectrode or fibre-optic optode directly into the insect's haemocoel, have recorded pH values within a range that is comparable with in vitro measurements on similar insect species (e.g. Hetz and Wasserthal (1993), Matthews and White (2011)). Nonetheless, this data clearly shows that insects regulate their haemolymph pH across a very wide range of values. When considering the regulation of acid–base balance in insects, it is pertinent to note that nearly all haemolymph pH measurements are point measurements, with a few exceptions (Lettau et al. 1977; Matthews and White 2011; Hetz and Wasserthal 1993). Long-term recording from chronically implanted pH probes has shown that haemolymph pH often varies from minute to minute and may vary by more than 0.3 units across the course of a day (Lettau et al. 1977). Thus, at present, it is difficult to say how precisely insects regulate their extracellular pH environment, or whether closely defending intracellular pH is a more common strategy.

Since relatively few reliable measurements have been made on insect haemolymph, much work remains to be done to evaluate what the 'normal' haemolymph pH range is for

Table 8.1 Summary of measured values of haemolymph pH from across eight orders of insects, with an emphasis on more recent measurements

Order	Species	pH	Method	References
Orthoptera	*Melanoplus bivittatus*	7.121	Electrode	Harrison (1988)
	Schistocerca gregaria	7.1	Electrode	Phillips et al. (1987)
	Paracinema tricolor	7.0	Optode	Groenewald et al. (2014)
	Schistocerca gregaria	7.28	Electrode	Stagg et al. (1991)
	Teleogryllus commodus	7.48[a] (fed) 6.76[a] (starved)	Electrode	Cooper and Vulcano (1997)
Blattodea	*Nauphoeta cinerea*	7.084	Electrode	Snyder et al. (1980)
	Nauphoeta cinerea	7.30[a]	Optode	Matthews and White (2011)
	Leucophaea maderae	6.9–7.24[a]	Electrode	Lettau et al. (1977)
Odonata	*Libellula julia*[b, c]	7.58[a]	Electrode	Rockwood and Coler (1991)
	Somatochlora cingulata[b, c]	7.60[a]	Electrode	Correa et al. (1985)
	Uropetala carovei[b, c]	8.2	Electrode	Bedford and Leader (1975)
Hemiptera	*Corixa dentipes*	7.04	Electrode?	Vangenechten et al. (1989)
	Corixa punctata	6.97	Electrode?	
Lepidoptera	*Bombyx mori*[d]	6.60	Electrode	Wyatt et al. (1956)
	Manduca sexta[b]	6.64[a]	Electrode	Moffett and Cummings (1994)
	Agapema galbina[d]	6.45	Electrode	Buck and Friedman (1958)
	Hyalophora cecropia[d]	6.52	Electrode	
	Acherontia atropos[b]	6.9	Electrode	Dow (1984)
	Lasiocampa quercus[b]	6.7	Electrode	
	Lichnoptera felina[b]	6.4	Electrode	
Diptera	*Gasterophilus intestinalis*[b]	6.8	Electrode	Levenbook (1950)
	Chironomus riparius[b, c]	7.2–7.3	Electrode	Jernelöv et al. (1981)
	Chironomus riparius[b, c]	7.7–8.0	ISME	Nguyen and Donini (2010)
	Rhynchosciara americana[b]	7.27	Electrode	Terra et al. (1974)

Table 8.1 (continued)

Order	Species	pH	Method	References
	Ochlerotatus taeniorhynchus[b, c]	7.7	Electrode	Clark et al. (2004)
	Aedes aegypti[b, c]	7.6	Electrode	
	Aedes dorsalis[b, c]	7.55–7.70	Electrode	Strange et al. (1982)
Coleoptera	*Leptinotarsa decemlineata*	6.53–6.74	Electrode	Pelletier and Clark (1992)
	Xylotrupes ulysses	7.0[a]	Optode	Matthews and White (2009)
Hymenoptera	*Neodiprion abietis*[b]	6.59	Electrode	Heimpel (1955)
	Neodiprion americanus[b]	6.54	Electrode	
	Neodiprion lecontei[b]	6.88	Electrode	
	Neodiprion sertifer[b]	6.81	Electrode	
	Neodiprion virginiana[b]	6.84	Electrode	
	Pikonema alaskensis[b]	6.55	Electrode	
	Hemichroa crocea[b]	6.69	Electrode	
	Pristiphora erichsonii[b]	6.64	Electrode	

[a]Indicates in vivo measurement
[b]Indicates larval or nymph/naiad stage
[c]Indicates aquatic habitat
[d]Indicates pupal stage

any given insect. This information is particularly crucial for studies involving isolated or cultured insect tissues. For example, while insect physiological saline is used as an artificial haemolymph in numerous *Drosophila* studies, the precise pH of these solutions varies. Some saline recipes recommend a pH between 6.6 and 6.9, similar to the pH measured in the third instar larvae (Echalier 1997), while other studies on larvae recommend a saline buffered to a pH of 7.0–7.2 (Badre et al. 2005; Stewart et al. 1994). Measurements of heartbeat in larva have been performed at a pH of 7.0 (Gu and Singh 1995), and adult *Drosophila* hearts have been measured at 7.2 (Papaefthimiou and Theophilidis 2001). While all of these values lie close to the values recorded from some other Diptera, the pH of adult *Drosophila* haemolymph still needs to be investigated. It remains to be seen how successfully these physiological salines recreate the normal acid–base environment of the haemolymph in vivo.

8.3.2 Haemolymph Buffering Capacity

Insects can respond to the addition of acid or base into their haemolymph by buffering or by active transport/excretion. Transporting acid–base equivalents between body compartments and/or the environment is a slower, long-term solution, while buffering

immediately limits the extent of the pH shift. Buffering is achieved by the presence of weak acids and their conjugate bases acting as proton donors or accepters. A weak acid and its conjugate base buffer most effectively at the pH that coincides with its dissociation constant (pKa), the pH at which the concentration of weak acid, [HA], is the same as its conjugate base, [A^-]. In biological systems, the most commonly occurring pH buffers are bicarbonate ions, proteins and phosphate (Truchot 1987). The importance of these buffers in maintaining the pH of an insect's haemolymph depends on their concentration in the haemolymph and the nature of the acid–base disturbance. Non-bicarbonate buffers donate and accept protons from non-volatile acids and bases, as well as from carbonic acid produced by the hydration of CO_2. In contrast, bicarbonate ions can only buffer metabolic and non-volatile acid disturbances, as shown by the reversible reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \tag{8.1}$$

An increase in [H^+] shifts this reaction from bicarbonate towards the production of CO_2. The volatile CO_2 can then be removed from the blood by gas exchange with the surrounding environment. The concentration of HCO_3^- in a fluid is partly determined by PCO_2 (in addition to [SID] and [HA]). The PCO_2 of a terrestrial vertebrate is around 4.7–6 kPa (Truchot 1987), while the PCO_2 of insect haemolymph is generally much lower, around 2 kPa in a cockroach (Matthews and White 2011) and between 1.5 and 2.19 kPa in the desert locust *Schistocerca gregaria* (Gulinson and Harrison 1996; Harrison et al. 1990). As a result, there is generally a lower concentration of bicarbonate available to act as a buffer. The bicarbonate buffering properties of insect blood have been most thoroughly investigated within the Orthoptera, with concentrations of haemolymph bicarbonate levels known from six species, varying between 5 and 13 mmol l^{-1} (Gulinson and Harrison 1996; Harrison et al. 1990, 1995; Harrison 1988, 1989b; Krolikowski and Harrison 1996). In the normal physiological range of haemolymph pH, this equates to a bicarbonate buffer capacity of 12–28 mmol l^{-1} pH $unit^{-1}$ (Harrison 2001). The role of the bicarbonate buffer system in insects has been demonstrated in the desert locust *Schistocerca gregaria*, which was injected with hydrochloric acid (HCl) to simulate a metabolic acidosis. The insect showed a reduction in haemolymph pH of 0.5 units, which caused [HCO_3^-] to drop by half and PCO_2 to double for the first 15 min following injection (Harrison et al. 1992). It was calculated that the bicarbonate buffer neutralised half of the added acid, liberating CO_2 which was completely restored to control levels 1 h after the injection. The remainder was buffered by non-bicarbonate buffers, and ultimately by transport and excretion processes associated with the gut (Harrison et al. 1992). The total buffer capacity of *Schistocerca gregaria* haemolymph at a pH of 7.31 is 33.6 mmol l^{-1} pH $unit^{-1}$, attributed to bicarbonate (60 %), protein (30 %), organic and inorganic phosphate (9 %), with the remainder citrate and histidine (Harrison et al. 1990). One of the few non-orthopteran insects to have the buffer capacity of its haemolymph determined is the larva of the horse bot fly *Gasterophilus intestinalis*. The horse bot fly maggot is a specialist intestinal parasite that lives within the high-CO_2 environment of a horse's stomach, where it regularly experiences a PCO_2 in excess of 66 kPa (Levenbook 1950). Within this unusual environment, the pH buffer capacity of *G. intestinalis* haemolymph at its usual haemolymph pH of 6.8 is 47.0 mmol l^{-1} pH $unit^{-1}$. This buffer capacity is again primarily due to bicarbonate (57.5 %), as well as proteins (30 %), succinate (6 %) and inorganic phosphate (5 %) (Levenbook 1950).

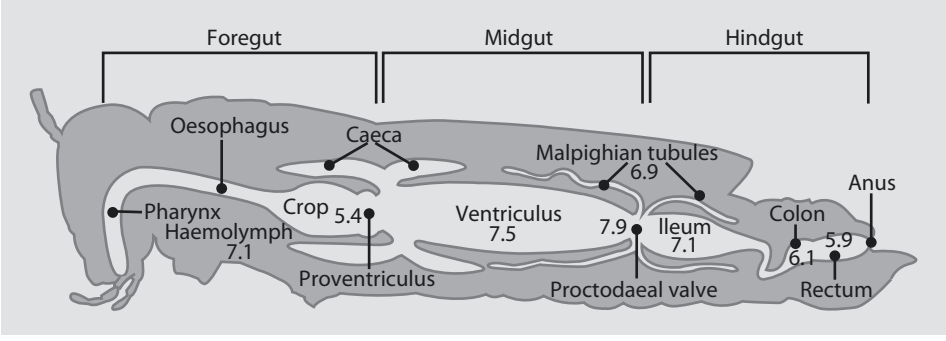
8.3.3 Regulation of Acid–Base Disturbance

Role of the Gut and Malpighian Tubules

Adding a strong acid or base into an insect, either from metabolic by-products, food, or, in the case of aquatic insects, directly from their environment, alters the acid–base balance of its haemolymph. As buffering can only ever limit the change in pH, other mechanisms must come into play to restore the original acid–base balance. This can only be achieved by the active transport of acid–base equivalents across epithelia, either between body fluid compartments within the insect, or between the insect and its environment. In terrestrial insects, this transport occurs between the haemolymph and gut lumen, across the walls of the gut and Malpighian tubules.

A generalised insect gut is divided into several distinct sections, beginning with the foregut, which is made up of the oesophagus, proventriculus, or gizzard, and crop. The midgut comprises the gastric caeca and ventriculus; and the hindgut is made up of the ileum, colon, rectum and anus (◘ Fig. 8.1). The pH of the gut lumen differs along most of its length from that of the surrounding haemolymph. The Malpighian tubules arise between the mid and hindgut regions and extend into the haemolymph, where they take up ions, metabolic wastes and water, excreting this primary urine from the haemolymph into the hindgut, where it is further modified before excretion. In an insect, this hindgut–Malpighian tubule complex is the main site of ion and water regulation and performs essentially the same functions as the vertebrate kidney, including acid–base regulation (Phillips 1981). A series of investigations into the desert locust *Schistocerca gregaria* provides the most complete picture of an insect's acid–base regulation and the central role played by the hindgut–Malpighian tubule complex. However, these studies have focused on the mechanisms used to remove excess acid, and no studies have yet examined the response of the locust to an alkaline challenge. A comprehensive review of this topic can be found in Phillips et al. (1994).

The Malpighian tubules produce the primary urine by actively taking up K^+ and Na^+ (but may preferentially secrete one or the other, depending on the insect species) from the haemolymph, as well as smaller amounts of Mg^{2+}, Cl^-, HCO_3^-, NH_4^+ and other haemolymph solutes (Maddrell and O'Donnell 1992). The transport of inorganic ions is driven by



◘ **Fig. 8.1** Cross section of the locust. The pH of the haemolymph and the various sections of the gut are indicated in bold (Data from Thomson et al. (1988a); image adapted from Phillips (1964))

an electrogenic V-ATPase (O'Donnell and Maddrell 1995), which pumps protons into the tubule, keeping its pH around 0.5 units below that of the haemolymph (Stagg et al. 1991). This proton gradient energises the cation/H^+ antiporters, driving ion transport into the tubule lumen. The reduced pH also causes the PCO_2 in the fluid to increase to three times the haemolymph levels due to the titration of HCO_3^- to CO_2 (Phillips et al. 1994). But the V-ATPase proton pump is a minor contributor to total acid excretion, as most of the protons in the Malpighian tubules are recycled through the cation antiport system (Stagg et al. 1991). The osmotic gradient between the fluid in the tubules and the haemolymph drives the movement of water from the haemolymph into the tubules, flushing the primary urine into the ileum. Both the ileum and the rectum function to reabsorb Na^+, Cl^- and K^+ ions and water while excreting acid or base into the lumen using a range of pathways. In both segments, apical V-ATPase proton pumps actively secrete H^+ into the lumen, progressively lowering the luminal pH from the ileum to the rectum (◘ Fig. 8.1) (Thomson and Phillips 1992). The pH in the rectum is further reduced following the injection of a non-volatile acid, demonstrating that the increased excretion of acid into the rectum occurs to restore the acid–base balance of the haemolymph (Thomson et al. 1988a). Approximately 15 % of the injected acid load was accounted for by an increased transport of ammonium ions (NH_4^+) by the ileum and rectum (Harrison and Phillips 1992). Ammonia (NH_3) is produced primarily from the oxidation of proline, as well as other amino acids, before it is excreted into the gut lumen as NH_4^+ in exchange for the uptake of Na^+ into the haemolymph (Thomson et al. 1988b; Peach and Phillips 1991). It has been estimated that the rectum is responsible for approximately 60 % of all acid excretion in the locust, due to its large surface area (~0.62 cm^2) compared to the ileum, which contributes 30 % (0.4 cm^2), and the Malpighian tubules (less than 0.09 %) (Phillips et al. 1994).

While the acidification of the hindgut occurs predominantly by excretion of H^+ into the gut lumen rather than by the selective reabsorption of bicarbonate (Thomson et al. 1988a), both the ileum and rectum do appear to transport HCO_3^- from the gut into the haemolymph. Experiments on isolated epithelia have shown that these sections of the hindgut secrete a fluid from the gut lumen into the haemolymph that has a HCO_3^- concentration three times higher than that in lumen (Lechleitner et al. 1989). This absorption of bicarbonate by the locust hindgut appears to occur by a process that differs from most other animals, in that it does not rely on a Cl^-/HCO_3^- exchange system. The uptake of Cl^- from the gut lumen appears to be an active process coupled to the passive uptake of K^+ (Hanrahan and Phillips 1983; Phillips and Audsley 1995). Thus, the mechanisms used by the insect hindgut to transport bicarbonate still have not been conclusively identified. It also remains to be seen how the hindgut excretes excess base. Studies on the locust *Taeniopoda eques* has shown that this insect is quite capable of changing from excreting excess acid in the form of titratable acid and ammonium to excreting excess base in the form of bicarbonate, depending on its diet (Harrison and Kennedy 1994). But, again, the mechanisms have yet to be described in detail.

The secretion processes described above are under hormonal control, rather than being influenced directly by the acid–base status of the haemolymph, with rates of ion transport, secretion of H^+ and uptake of HCO_3^- responding to neuropeptides extracted from the corpus cardiacum and ventral ganglia (Phillips et al. 1998). The active component of the corpus cardiacum extract has been identified as a neuropeptide hormone called ion transport peptide (ITP). This hormone has been shown to stimulate Na^+, Cl^-, K^+ and fluid absorption by the ileum, while inhibiting H^+ secretion (Phillips et al. 1998). It is likely that ITP controls the Na^+, Cl^- and K^+ ion pumps by stimulating the intracellular

production of cAMP (cyclic AMP or cyclic adenosine monophosphate). However, while cAMP has been found to inhibit acid secretion in the rectum of *S. gregaria,* it does not appear to have the same effect on the H^+ V-ATPase pumps in the ileum (Thomson et al. 1991), which appear to be inhibited by a different messenger molecule in response to ITP. While the V-ATPase transporters in locust rectum are inhibited by cAMP, a stimulatory effect has been recorded from other parts of the Malpighian tubule–hindgut complex. The V-ATPase in the Malpighian tubules is stimulated by cAMP, with studies on *Drosophila* showing a decrease of 0.4 pH units in the tubule fluid relative to the unstimulated fluid secretion (O'Donnell and Maddrell 1995).

Role of Respiration and Ventilation

The constant production of CO_2 by respiration constitutes the largest continuous influx of acid that most insects must contend with. Given that many flying insects are capable of increasing their rate of CO_2 production over 100-fold when they transition from rest to active flight, it is clear that they must possess effective mechanisms to deal with CO_2 and its potential impact on acid–base balance through carbonic acid production. As CO_2 is a volatile acid, releasing it to the atmosphere is the simplest mechanism to deal with this problem. While vertebrates transport CO_2 from their tissues to gas exchange organs in their blood, insects have a respiratory system that transports O_2 and CO_2 independently from their haemolymph: the tracheal system. Their tracheal system consists of a branching network of air-filled tubes that open to the atmosphere through spiracles, providing a high-conductance conduit between the haemolymph and tissues of the insect and the surrounding atmosphere. While the lack of respiratory pigments within the haemolymph limits the role it can play in transporting O_2 and CO_2 within the insect, it still functions as a significant buffer for respiratory CO_2 and a minor reserve of O_2. The high rate of diffusion and convection within the air-filled tracheal system and the large surface area of the finely branched tracheoles allow for the rapid removal of CO_2 from both tissues and haemolymph. By opening and closing their spiracles to regulate gas exchange between the atmosphere and their tracheal system, insects at rest maintain an internal PCO_2 between 1.8 and 3 kPa. This PCO_2 is considerably lower than that found in an endothermic vertebrate (4–5 kPa) but is similar to values recorded from terrestrial, air-breathing crabs (Wood and Randall 1981; Truchot 1987). Air-breathing vertebrates use their high internal PCO_2 and bicarbonate levels to rapidly neutralise the addition of a fixed acid. The high concentration of bicarbonate reacts with the acid to produce CO_2, which is then exhaled. The low CO_2 and bicarbonate levels reduce the extent to which insects may use ventilation to regulate their internal pH by expelling excess CO_2. Experiments on locusts partially support this view, as the injection of 50 µl of 0.5 mol l^{-1} hydrochloric acid (HCl) into the haemolymph of the locust *Romalea guttata* did not produce a significant increase in ventilation rate, despite significantly reducing pH (Gulinson and Harrison 1996). However, the injection of sodium bicarbonate ($NaHCO_3$) does cause ventilation frequency to increase. This increased gas exchange is unlikely to be a response to correct pH, since the treatment did not significantly alter the pH of the haemolymph, but it may be explained by a significant increase in the average intratracheal PCO_2 during the 10 min following the injection of $NaHCO_3$ (Gulinson and Harrison 1996).

These experiments examining the effect of haemolymph pH on ventilation raise some interesting questions regarding the nature of gas exchange control in insects. If respiratory pH regulation is important, then their respiratory chemoreceptors should respond to changes in pH as well as PCO_2, as they do in air-breathing vertebrates (Milsom 2002).

Using acids such as HCl to change pH did not alter the ventilation rate, but the addition of bicarbonate, and its liberation from the haemolymph as CO_2, did have this effect. Similarly, the spiracles of the housefly *Musca domestica* have been shown to open in response to increases in CO_2, and not to decreases in pH (Case 1957). However, this is not to say that insects do not use gas exchange to regulate the pH of their haemolymph. For example, vigorous hopping in the locust *Melanoplus bivittatus* at 35 °C was sufficient to cause the PCO_2 of its haemolymph to increase from approximately 2.9–5.2 kPa, and the pH of its haemolymph to decrease by 0.14 units (Harrison et al. 1991). While at rest after this exercise, the locust completely reversed the acidification of its haemolymph by employing a sustained period of elevated ventilation to remove the accumulated CO_2, thereby restoring the PCO_2. Thus, regulating ventilation to preserve a constant internal PCO_2 is sufficient to maintain a stable haemolymph pH.

While the stimulatory effect of CO_2 on ventilation is well documented, some studies have also found evidence that decreased haemolymph pH can produce a similar effect. For example, irrigating the central nervous system of the cockroach *Nauphoeta cinerea* with Ringer's solution, either equilibrated with a PCO_2 of 6.2 kPa or acidified to a pH of 6.97 with HCl, resulted in both cases in an equally significant rise in ventilation frequency (Snyder et al. 1980). Other cockroaches have displayed similar responses. The isolated nerve cord of *Blaberus craniifer* could be reversibly stimulated to produce spiking consistent with respiratory activity by exposing it to any of 13 different weakly dissociated acids in saline solution (Case 1961). However, the pH at which the different acids produced an effect varied, indicating that pH per se may not be the main stimulus.

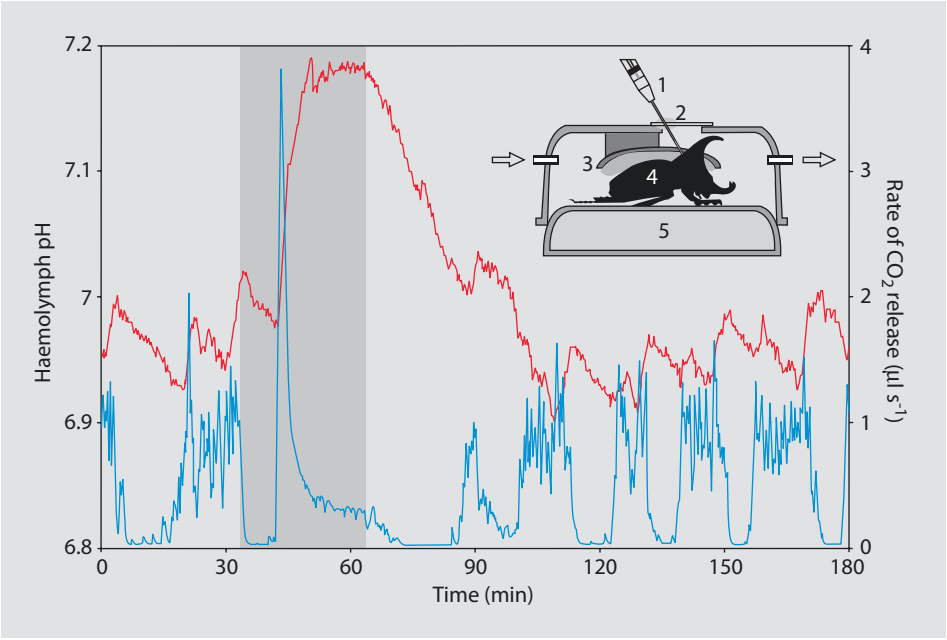
Insects often experience microclimates that have CO_2 concentrations well above normal atmospheric levels. Habitats rich in decaying organic matter, such as dung pats, can have levels of CO_2 up to 20 kPa (Holter and Spangenberg 1997). These environments are challenging to acid–base regulation, as they can increase the PCO_2 of body fluids to dangerously high levels. By increasing their ventilation rate, *Nauphoeta cinerea* cockroaches are capable of maintaining a stable internal pH while breathing air with a PCO_2 of up to 1 kPa (Matthews and White 2011). Exposure to even higher levels of environmental hypercapnia results in a further increase in ventilation frequency. But once the ambient PO_2 exceeds the normal internal PCO_2 of the cockroach, their internal PCO_2 must necessarily increase, associated with a decrease in haemolymph pH. No amount of hyperventilation can restore the pH of the cockroach's haemolymph so long as the PCO_2 remains above the insect's normal PCO_2. The only way to restore the balance is to move the cockroach to a microhabitat with a lower CO_2 level. The locust *Schistocerca nitens* displays the same responses to short-term environmental hypercapnia, with both its abdominal ventilation rate and the PCO_2 of its haemolymph rising as haemolymph pH falls (Harrison 1989a). For some insects, escaping a hypercapnic microhabitat in favour of lower atmospheric CO_2 levels is not an option, and the acid–base effects of chronic hypercapnia must be dealt with. Whether insects in this situation are capable of producing a compensated respiratory acidosis by increasing bicarbonate reabsorption remains unknown.

In addition to regulating CO_2 removal, insects must also maintain O_2 uptake. Exposure to environmental hypoxia elicits hyperventilation, increasing the clearance rate of CO_2 from the insect's body fluids above rates of production. This decreases the internal PCO_2, shifting the bicarbonate/CO_2 equilibrium towards CO_2, which is then exhaled. The end result is a pH increase indicative of respiratory alkalosis. Exposure to a PO_2 of 5 kPa caused hyperventilation in the cockroach *Nauphoeta cinerea*, causing a rise in haemolymph pH of 0.34 units (Matthews and White 2011), and the same PO_2 caused pH to rise

0.15 units in the rhinoceros beetle *Xylotrupes ulysses* (unpublished data ◘ Fig. 8.2). The data from *X. ulysses* also shows the corrective response when returned to normoxic air. Once internal PO_2 has been restored, the beetle begins a breath-hold period that allows CO_2 to accumulate within its body fluids, restoring its haemolymph pH to pre-hyperventilation levels.

Effects of Discontinuous Gas Exchange

Some insects at rest breathe intermittently, alternately accumulating CO_2 while they hold their spiracles shut, then expelling the accumulated CO_2 in a burst or bout of ventilation. This pattern of gas exchange is best known from lepidopteran pupae, but also occurs in the Orthoptera, Blattodea, Coleoptera and Hymenoptera (Marais et al. 2005). The acid–base balance of the insect's haemolymph fluctuates during these discontinuous gas exchange cycles (DGCs), with in vivo measurement of haemolymph pH revealing periodic decreases of around 0.06 pH units below pH 6.74 in a butterfly pupa (Hetz and Wasserthal 1993) and 0.11 pH units below ~7.3 in a cockroach (Matthews and White 2011). In vitro measurements made on lubber grasshoppers (*Taeniopoda eques*) displaying DGCs by extracting haemolymph samples immediately preceding and following a CO_2 burst found DGCs caused even smaller changes in haemolymph pH of between 0.030 and 0.037 units (Harrison et al. 1995). The pH change observed during DGCs is due entirely to the accumulation and release of CO_2. An example of the relationship between intermittent gas exchange and pH fluctuation is shown in ◘ Fig. 8.2. Simultaneous record-



◘ **Fig. 8.2** Haemolymph pH (*red*) and exhaled CO_2 (*blue*) before, during and after exposure to hypoxia (5 % PO_2, grey rectangle) including an inset of experimental setup showing (*1*) syringe mounted optical pH probe, (*2*) respirometry chamber including wax seal around needle, (*3*) putty-filled restraint, (*4*) rhinoceros beetle, (*5*) temperature controlled water jacket maintained at 25 °C. Arrows indicate direction of airflow through the chamber to the CO_2 analyser

ing of haemolymph pH, using a fibre-optic pH optode implanted in the haemocoel of a rhinoceros beetle (*Xylotrupes ulysses*) in concert with the rate of CO_2 release from the insect measured using flow-through respirometry, clearly shows the decrease in haemolymph pH during periods where CO_2 is not released. The high intracellular buffering capacity estimated from whole body homogenates, relative to that of the haemolymph, suggests that pH changes associated with DGCs are insignificant within the insect's tissues (Bridges and Scheid 1982).

8.4 Acid–Base Regulation in Aquatic Insects

Terrestrial insects have reinvaded the aquatic environment numerous times throughout their evolutionary history, evolving either partially or completely aquatic lifecycles (Wootton 1988). Aquatic insects spend their lives in intimate contact with an aqueous environment with an ionic composition and pH that varies more or less from the internal fluids of the insect. This has two important consequences for acid–base regulation: First, it means that aquatic insects can excrete or take up acid–base equivalents directly from their surrounding environment across ion permeant epithelia. Second, because water has a far lower capacitance for O_2 than for CO_2 when compared with air (Dejours 1981), life in water can have a dramatic impact on an insect's internal PCO_2 and bicarbonate concentration. Aquatic animals must ventilate large volumes of water to extract sufficient O_2, and in the process, they quickly expel their respiratory CO_2 (Dejours 1988). In contrast, terrestrial animals can easily obtain O_2 from the air and so ventilate less and consequently retain more CO_2. This means that water-breathers have a much lower body fluid PCO_2 and $[HCO_3^-]$ than air-breathers, reducing the importance of the bicarbonate buffer system as a mechanism for regulating internal pH (Truchot 1987). Despite the fact that at least seven orders of insects have independently evolved tracheal gills with which to breathe water, it remains to be seen whether they show the expected reduction in PCO_2 and $[HCO_3^-]$. There are some evidences that this may not be the case. Measurements of total CO_2 in the haemolymph of a water-breathing damselfly nymph (*Austrolestes* sp.) show concentrations similar to those of air-breathing insects and higher than those of vertebrate water-breathers (Cooper 1994).

8.4.1 Water-Breathing Aquatic Insects

Acid–base balance and regulation of osmolarity are inextricably linked in aquatic animals. This is because ion transporters couple the movement of H^+ and HCO_3^- with that of Na^+ and Cl^-, respectively. In fish and crustaceans, these Na^+/H^+ and Cl^-/HCO_3^- antiporters, in addition to H^+ V-ATPases, are mainly found on gill epithelia (Claiborne et al. 2002; Henry and Wheatly 1992). Water-breathing insects also possess ion-transporting cells on their tracheal gills, but they are frequently encountered on other parts of their bodies as well (Wichard et al. 1972). The thin epithelia and large surface area of the tracheal gills enable them to function as ion exchange organs and to participate in acid–base regulation, in addition to their respiratory function. Chloride cells occur over the entire body surface of mayflies (Ephemeroptera) and stoneflies (Plecoptera) but occur in higher abundance on the surface of the tracheal gills (Komnick 1977). In comparison, the chloride cells of dragonflies and damselflies (Odonata) are restricted to the rectum, which is modified into a

gas exchange organ (a rectal gill) in dragonflies (Wichard and Komnick 1974; Komnick and Achenbach 1979). In order to maintain a hypertonic haemolymph with respect to their freshwater environment, aquatic insects rely on the pumping action of chloride cells to counteract the constant passive loss of Na^+ and Cl^- ions into the surrounding water across permeable regions of their cuticle and expelled from their gut. However, exposure to acidic water has the potential to disrupt osmoregulation by interfering with the function of V-ATPase proton pumps, thereby preventing the Na^+/H^+ exchanger from functioning. There is evidence that many aquatic insects exposed to low pH succumb due to the disruption of Na^+ uptake and subsequent decrease in internal [Na^+] due to ion efflux, rather than a loss of pH homeostasis. This has been observed in stoneflies (Lechleitner et al. 1985) and dragonflies (Rockwood and Coler 1991) which both show a significant decrease in haemolymph [Na^+] following prolonged exposure to acidic water (pH 3 and 2.3, respectively). Exposing the stonefly nymph to alkaline water with a pH of 8.0 did not significantly change [Na^+] (Lechleitner et al. 1985). Given that osmoregulation is a serious challenge in acidified water, it follows that an aquatic insect's ability to withstand highly acidic aquatic environments is enhanced by a low cuticular permeability (Havas and Advokaat 1995). This can be more easily achieved if the aquatic insect breathes not water, but air. Without the need for tracheal gills and the large permeable surface area they represent, air-breathing aquatic insects can potentially maintain a much lower cuticular permeability.

8.4.2 Air-Breathing Aquatic Insects

Many aquatic insects do not possess tracheal gills or breathe water, but remain air-breathers, either by maintaining contact with atmospheric air using a snorkel, by intermittently contacting the surface to refresh a bubble of atmospheric air carried on their body or by having a layer of air trapped permanently over their spiracles. For example, mosquito larva use a respiratory syphon to connect their tracheal system with the atmosphere while resting at the surface of the water, while many aquatic bugs (Hemiptera) and beetles (Coleoptera) carry a bubble of air on their body while diving. As a result, these insects do not bring water into direct contact with their thin respiratory epithelia and are likely to possess a PCO_2 and [HCO_3^-] similar to terrestrial insects. But, again, this assumption remains untested. The absence of gills also reduces the degree of contact between the surrounding water and respiratory epithelia, limiting opportunities for both passive and active ion movement across the body wall. It is likely that this increases the importance of the gut in regulating acid–base balance and osmolality, as has been observed in the larvae of diving beetles (Komnick 1977). However, even air-breathing aquatic insects possess ion-transporting chloride cells on parts of their cuticle that remain in contact with the surrounding water, providing a direct pathway for ionic regulation with the aquatic environment in addition to the gut (Komnick 1977; Komnick and Schmitz 1977). Water boatmen (Corixidae) are aquatic hemipterans that carry a bubble of air on their ventral surface while underwater. While they do possess chloride cells on their head and legs (Komnick and Schmitz 1977), they lack tracheal gills and have very low rates of sodium efflux (Vangenechten et al. 1989; Witters et al. 1984). Consequently, they are highly tolerant of life in acidic water, showing insignificant decreases in haemolymph [Na^+] in water with a pH as low as 3.0 (Needham 1990). Several species of corixids also show a high degree of tolerance to alkaline conditions, living in so-called athalassohaline lakes that are rich in

carbonates and have very high pH (9–10). While the Malpighian tubules of these insects are capable of producing an alkaline primary urine when stimulated by cAMP (Szibbo and Scudder 1979), excretion of HCO_3^- in exchange for Cl^- and Na^+ for H^+ by the chloride cells are likely to play a central role in acid–base regulation (Cooper et al. 1987).

Dipteran larvae, including mosquitoes, midges and alkali flies show some of the most impressive acid–base regulation abilities in the animal kingdom. Of these insects, mosquito larvae are among the most capable pH regulators known. Larvae of both *Aedes aegypti* and *Ochlerotatus taeniorhynchus* are able to complete their development in water with a pH ranging from 4 to 11, and, more impressively, they can maintain the pH of their haemolymph to within 0.1 pH units or less across this range (Clark et al. 2004). This constancy of internal pH is reflected by the mosquito larva's ability to maintain internal osmolality in both very dilute (20 μmol l^{-1} NaCl) and hypersaline (>300 mmol l^{-1} NaCl) water (Garrett and Bradley 1984; Patrick et al. 2002). Rather than having chloride cells scattered over their entire cuticle, many dipteran larvae instead possess dedicated ion exchange organs called anal papillae. The anal papillae are finger-like projections of the cuticle that lie next to the anus and are involved with ion exchange rather than respiratory gas exchange. They are similar to gills in that they are also highly water permeable but afford a far smaller surface area for the passive movement of ions and water (Wigglesworth 1933). Restricting cutaneous ion and water transfer to a dedicated ion exchange organ allows the rest of the cuticle to be water and ion impermeable. The transport mechanisms used by mosquito larvae to achieve their acid–base and ionoregulatory feats are not yet completely understood, but the measurements of ion fluxes across the anal papillae and immunolocalization of the ion pumps on the anal papillae (Patrick et al. 2006) and along the alimentary canal (Okech et al. 2008) have greatly advanced our understanding of ion transport and pH regulation in these insects.

The presence of V-ATPase and Na^+/K^+ ATPase in the epithelium of anal papillae has been confirmed using immunolocalization techniques (Patrick et al. 2006) while the presence of a Cl^-/HCO_3^- transporter is currently only inferred from pharmacological experiments (Del Duca et al. 2011). Curiously, though, there is no evidence for the presence of a Na^+/H^+ ATPase on the papillae. The uptake of sodium into the papillae appears to be driven primarily by the electrical potential generated by apically located V-ATPase pumping protons into the surrounding water, assisted by a basal Na^+/K^+-ATPase (Patrick et al. 2006). It is not yet clear whether this V-ATPase is actively involved in pH regulation, and there are some lines of evidence that suggest otherwise. In particular, the observation that larvae of *A. aegypti* do not upregulate mitochondrial density in their papillae when reared in high or low pH suggests that this organ does not actively increase acid or base excretion in response to ambient conditions (Clark et al. 2007). Mitochondrial density is, however, upregulated in response to low salt levels (Sohal and Copeland 1966). Regardless, the presence of a V-ATPase demonstrates that the anal papillae are a site of active H^+ excretion. The movement of other ions across the anal papillae of *A. aegypti* have been measured by recording ion gradients adjacent to the epithelium using ion-selective microelectrodes. This has revealed a net influx of Na^+, Cl^- and K^+ and net efflux of acid and NH_4^+ (Donini and O'Donnell 2005). Unlike other animals that rely on the secretion of bicarbonate to drive Cl^- uptake, mosquitoes also appear to use a novel Cl^- channel that is stimulated by exposure to acidic conditions and by the inhibition of the apical V-ATPase. This Cl^- pathway has only been observed in insects and shows an enhanced rate of Cl^- uptake during exposure to low pH. It has been suggested that the Cl^- transport is driven by an electrodiffusive gradient that is enhanced by either a reduction in the rate of H^+

excretion increasing the electrical gradient for Cl^- entry through a Cl^- channel or by a steeper H^+ gradient driving uptake via a Cl^-/H^+ co-transporter (Patrick et al. 2002). But while the transport of H^+ is clearly required for ion uptake, there is little evidence supporting the anal papillae as significant organs of pH homeostasis. This leaves the mosquito's hindgut as the next most likely candidate.

The role that the mosquito larvae's hindgut–Malpighian tubule complex plays in the ionic and acid–base regulation of the haemolymph is currently incomplete. Previous studies examining the responses of *A. aegypti* to aquatic pH have found that drinking rates varied inversely with pH, being highest in acidic water (pH 4) and lowest in alkaline (pH 11) (Clark et al. 2007). From this study, it was concluded that acid excretion by the Malpighian tubules associated with increased fluid clearance through the hindgut and rectum was responsible for maintaining haemolymph pH homeostasis under acid conditions. Low fluid ingestion rates have also been documented for mosquitoes naturally adapted to alkaline, high carbonate water (Strange et al. 1982), indicating that both alkaline-tolerant and alkaline-adapted mosquito larvae share a similar response to high pH water. But the nature of this response and the associated processes used to maintain pH homeostasis in alkaline water are unknown. Immunolocalization of V-ATPases in the Malpighian tubules and posterior rectum of *A. aegypti* and *Anopheles gambiae* larvae (Okech et al. 2008; Patrick et al. 2006) and the observation that the rectum is acidified ($pH < 6.2$) in both acidic and alkaline water (Clark et al. 2007) indicate that acid is excreted through the hindgut. However, an acidified rectum raises an interesting issue in regard to pH regulation in *Aedes dorsalis* mosquito larvae inhabiting carbonate-rich hypersaline lakes. The first investigations into this species concluded that the rectum was responsible for excreting HCO_3^- to maintain a haemolymph pH of ~7.7 in alkaline water with a pH above 8.85 (Strange et al. 1982, 1984). But if this species is like other *Aedes* mosquito larvae, then they should maintain an acidic rectal lumen, regardless of their alkaline environment. Thus, if they were to regulate their internal pH in alkaline water by excreting bicarbonate into their acidic rectum, it would react with the acid and be converted to CO_2, subsequently diffusing into the surrounding water and producing a net loss of acid from the insect (Clark et al. 2007). Clearly, a great deal more research is required to produce a complete picture of the acid–base regulation of mosquitoes and aquatic insects in general.

8.4.3 A Comment on Mosquitoes and Midges

The non-biting midges (Chironomidae) and mosquitoes (Culicidae) both have aquatic larvae that are highly competent iono- and acid–base regulators, as well as being similar anatomically. However, they differ in their mode of respiration. Mosquito larvae are primarily air-breathers, using their respiratory syphon to access atmospheric oxygen. This abundant source of O_2 is further supplemented by the trans-cuticular diffusion of oxygen and carbon dioxide between the larvae and surrounding water, particularly across their thin anal papillae (Wigglesworth 1933). In contrast, the benthic chironomid larvae are exclusively water-breathers, taking up dissolved oxygen across their entire cuticle (Fox 1921). As chironomids not only lack access to the atmosphere but also live burrowed into stagnant sediment, they fill their haemolymph with high oxygen affinity haemoglobins (Weber 1980). While these respiratory proteins facilitate oxygen uptake, their ability to bind reversibly with protons also allows them to contribute to the pH buffering capacity of the haemolymph (Jernelöv et al. 1981). It has been suggested that populations of

Chironomus riparius living in acidic streams have elevated levels of haemoglobin precisely for this reason (Jernelöv et al. 1981). Furthermore, the reversible binding of H^+ by haemoglobin alters the oxygen affinity of the protein (i.e. induces a Bohr shift), such that oxygen delivery is altered by changes in their internal pH (Weber et al. 1985). As mosquito larvae rely on the same air-filled tracheal oxygen delivery system as other insects, their respiration is not linked to pH in this manner nor do they possess this additional pH buffer. Beyond this obvious difference, there may be yet more subtle divergences between the acid–base and ionoregulatory systems of mosquitoes and chironomids. Experiments using carbonic anhydrase inhibitors have revealed a surprising difference between how these insects take up Cl^-. The currently enigmatic Cl^- uptake mechanism observed among mosquitoes is insensitive to acetazolamide, indicating that bicarbonate originating from the hydration of CO_2 in the papillae is not involved in Cl^- transport (Patrick et al. 2002). In contrast, chironomids exposed to methazolamide show a dose-dependent decrease in Cl^- uptake, indicating that they couple the uptake of Cl^- across their anal papillae with HCO_3^- excretion (Nguyen and Donini 2010). The significance of these differences is currently unknown. But future efforts to compare and contrast the respiratory physiology, iono- and acid–base regulation of these two insects, and more broadly across the diverse infraorder Culicomorpha, have the potential to reveal much about how insects evolve different solutions in response to environmental challenges.

8.5 Future Directions

Our understanding of insect acid–base physiology continues to advance, with studies on the location and action of ion transporter proteins in the gut and anal papillae of mosquitoes and locusts further refining earlier observations of ion transport made on isolated epithelia. However, it is clear that while this research has provided a better framework for understanding osmoregulation and pH regulation, much remains to be done to understand how both aquatic and terrestrial insects regulate their extracellular acid–base balance in response to environmental and dietary challenges. In particular, while the processes used to excrete excess acid have been well investigated, there have been very few studies that specifically investigate how insects, aquatic or otherwise, eliminate excess base. It also remains to be determined why some aquatic insects are capable of regulating internal pH homeostasis in the face of highly acid or alkaline water, while other species have such a low sensitivity to these conditions.

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