Nitrogen Excretion and Metabolism in Insects

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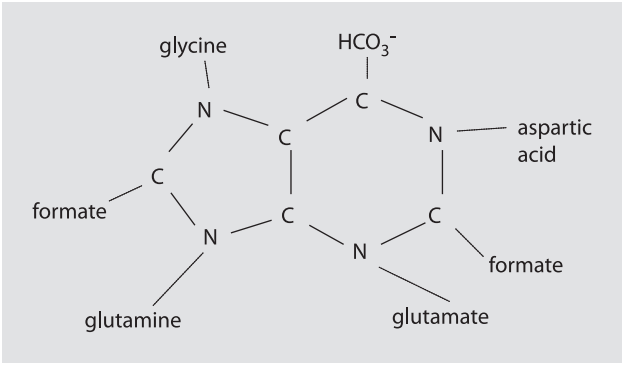
4.1 Introduction

Metabolism of proteins and nucleic acids results in the production of nitrogen-containing compounds. Deamination of amino acids, for example, yields α-keto acids that can be oxidized to carbon dioxide and water, but also ammonia. Ammonia may also be derived from the metabolism of urea or uric acid. Some of the resulting ammonia can be recycled through transamination reactions, by which glutamate is formed from α-ketoglutarate and glutamine is formed from glutamate. Any excess ammonia, however, is toxic; NH_4^+ may interfere with neuronal activity because it can pass through K^+ channels, whereas NH_3 may perturb the pH of cells and organelles, including mitochondria. Although some insects, such as the larvae of the sheep blowfly, tolerate high levels (10–20 mM) of ammonia in their hemolymph (Marshall and Wood 1990), in general, the excretory system functions to prevent accumulation of toxic levels of ammonia. A few species, discussed below, excrete much of their nitrogenous waste as ammonia, but insects generally are viewed as uricotelic, in keeping with the Baldwin–Needham hypothesis that terrestrial animals excrete nitrogenous excretion products with low solubility, such as urea, uric acid, or allantoin, as a means of conserving water (Baldwin and Needham 1934).

4.2 Uric Acid

4.2.1 Transport of Uric Acid by the Malpighian Tubules of Insects

Although uric acid is synthesized from either amino acid nitrogen or nucleic acid nitrogen, the former source is more important because insects generally ingest more protein than nucleic acids. The amino acids Gln, Glu, Asp, and Gly donate the four N-groups to the purine ring during uric acid biosynthesis in the fat body (◘ Fig. 4.1) (Barrett and Friend 1970; Bursell 1967). Uric acid, in spite of the metabolic cost of its synthesis (8 mols ATP/mol uric acid), is an ideal compound for elimination of excess nitrogen because it is only slightly soluble at physiological pH. The low solubility both reduces the toxicity of uric acid and also allows it to be excreted without the loss of much water. Blood-feeding insects must eliminate large amounts of nitrogenous waste produced as the protein-rich meal is metabolized. The tsetse fly, *Glossina morsitans*, for example, eliminates surplus nitrogen by excreting ~ 50 % of the dry weight of the ingested blood in the form of nitrogen-containing compounds (mainly uric acid, but also arginine and histidine) (Bursell 1965). In another blood feeder, *Rhodnius*



◘ **Fig. 4.1** Sources (*dashed lines*) of N and C atoms in the purine ring of uric acid

prolixus, urate is excreted at a rate equivalent to more than 15 % of the unfed body weight per day (O'Donnell et al. 1983). Urate transport has been described for the isolated lower Malpighian tubules of *Rhodnius*, as well as tubules of locusts, butterfly larvae, and mantids, suggesting that the mechanism is widespread (O'Donnell et al. 1983). Calculations of the electrochemical gradients for urate in the lower tubule cells versus the hemolymph and the lumen versus the cells indicate that urate secretion is likely to be an active process across both the basolateral and apical membranes (O'Donnell et al. 1983). Although most studies have dealt with nymphs or adult insects, uric acid deposition in the tubule lumen has been demonstrated in the embryo in *Drosophila*, prior to emergence of the first instar larva (Ainsworth et al. 2000). In *Calliphora*, urate oxidase activity in the Malpighian tubules converts uric acid to a related nitrogenous compound, allantoin, which is then excreted.

Most cockroaches excrete ammonia rather than uric acid, as described below, but a few species in subfamilies Blatellinae and Plecopterinae, as well as the wood cockroach *Cryptocercus*, excrete urates in the form of spherules that are found in the lumen of the Malpighian tubules (Lembke and Cochran 1988). Birefringent materials occur in vacuoles within the tubule cells of these species, suggesting a vesicular transport mechanism for urates.

4.2.2 Contrasts with Mechanisms of Urate Transport in Vertebrate Kidney

The mechanism of urate transport in insects is distinct from that seen in vertebrates; transport of urate by the Malpighian tubules is ouabain-insensitive and results in the precipitation of free uric acid instead of urate salts (Na^+, K^+). In the mammalian kidney, a sodium-dependent phosphate transporter (NPT1) acts as a membrane potential-driven urate exporter across the apical membrane of the kidney (Miyaji et al. 2013). Uptake of urate into the kidney cell may involve organic anion transporters OAT1 and OAT3, which exchange cellular dicarboxylates that are recycled through a Na^+-dependent transporter (Eraly et al. 2008). The transporters responsible for urate secretion by the Malpighian tubules are unknown, but members of the ABCG family are possible candidates. The ABCG family proteins are known as "half-type" ABC transporters comprising a single nucleotide-binding domain in the amino terminus and a single transmembrane domain in the carboxyl terminus. It has been proposed that a heterodimer of two ABCG members, Bm-ok and Bmwh3, are responsible for urate transport by the epidermal cells of the silkworm *Bombyx mori* (Wang et al. 2013). Bm-ok encodes a protein belonging to the ABCG family of ABC transporters, which includes Brown, Scarlet, and White. In *Drosophila*, White encodes an ABCG transporter that is responsible for secretion of cGMP into the Malpighian tubule lumen (Evans et al. 2008). Given the role of ABCG transporters in urate transport in *B. mori*, it will be of interest in future studies to determine the possible role of White and other ABCG transporters in urate secretion by insect Malpighian tubules.

4.2.3 Urate Salts as "Ion Sinks" During Dehydration/Rehydration in Cockroaches

Cockroaches can be considered as internally uricotelic but externally ammonotelic and uricotelic (Cochran et al. 1985; Mullins 2015). Although ammonium is the final form of nitrogenous waste in the excreta in many species, such as *Periplaneta americana*

(see below), urate salts play a central role in ionoregulatory and N-balance physiology in these insects. Both K^+ and Na^+ are sequestered in the fat bodies during dehydration and then released during rehydration (Hyatt and Marshall 1977, 1985a). Measurements using X-ray microanalysis confirmed that urate crystals within the urocytes sequester both K^+ and Na^+ in water-deprived *P. americana* (Hyatt and Marshall 1985b). Storage of Na^+ and K^+ thus provides the insect with the ions needed to maintain hemolymph osmolality and ion balance when hemolymph volume increases after rehydration with fresh water.

In some species, insertion of the spermatophore may be accompanied by urates stored in specialized male accessory glands, forming a genital plug (Graves 1969; Roth and Dateo 1965). After the spermatophore is emptied, the female may consume the discarded spermatophore and associated urates, thus contributing to nitrogen balance.

4.2.4 Storage Excretion of Uric Acid in Larval Lepidoptera (Fat Body and Epidermis)

The excretory system is nonfunctional during larval–pupal metamorphosis of holometabolous insects, and uric acid is sequestered during this stage of the life cycle. Lepidopterans accumulate uric acid in the fat body during metamorphosis (Buckner 1982), whereas fly pupae store it in the fat body, intestine, and the Malpighian tubules in response to the cessation of excretion (Schwantes 1990). The switch from excretion to storage during the transition from the feeding to the wandering stage in larval tobacco hornworms (*Manduca sexta*) is hormonally controlled and requires an increase in the titer of 20-hydroxyecdysone and a decrease in the titer of juvenile hormone I (Buckner 1982). Fat body urate levels increase and hemolymph urate concentration declines at this time. Staining with reduced silver has demonstrated that urate is stored as discrete membrane-bound vacuoles in the fat body of *M. sexta* pupae. Electron micrographs reveal that the vacuoles contain tightly coiled fibers of crystalline uric acid or urate salts and that each fiber is enveloped with protein (Buckner et al. 1990). Uric acid is also stored in wings or larval cuticle in Lepidoptera, presumably to provide pigmentation (Tojo and Yushima 1972).

4.2.5 Uric Acid as a Protectant Against Oxidative Stress

Blood-feeding arthropods utilize several mechanisms to minimize the impact of multiple reactive oxygen species produced from reactions involving both the iron and the heme group that are released during digestion of hemoglobin (Graça-Souza et al. 2006). One of these mechanisms involves production of antioxidants of low molecular mass, such as urate. Although the Malpighian tubules of the blood-feeding hemipteran *Rhodnius prolixus* secrete urate at high levels after the blood meal, rates of synthesis and excretion are balanced so that high concentrations of urate (up to 5 mM) are retained in the hemolymph. Urate accounts for almost all of the scavenging of free radicals in the hemolymph (Souza et al. 1997). The synthesis of urate by the fat body increases in response to hemin injection but also when the insects are exposed to hyperoxia, confirming that urate release into the hemolymph is an antioxidant response. The signaling pathway leading to stimulation of urate synthesis by hemin in *R. prolixus* involves activation of protein kinase C (Graça-Souza et al. 1999).

4.3 Nitrogen Recycling

Insects feeding on wood, phloem, plant sap, and other nitrogen-deficient diets make use of microbial symbionts to recycle nitrogen, using ammonia as a substrate for the synthesis of essential amino acids (EAAs) which are then available to the host (Macdonald et al. 2012). In addition, the symbionts may also be capable of nitrogen fixation. At least 60 species of wood-feeding insects from three orders (Blattodea, Coleoptera, and Hymenoptera) harbor symbiotic prokaryotes which fix N_2 (Ulyshen 2015). Screening for the *nifH* gene, which encodes a major component of nitrogenase, suggests that multiple types of N-fixing bacteria and Archaea are present in the guts of wood-eating insects (Ulyshen 2015). Symbiont contributions to the N economies of their hosts thus represent a common solution to the problem of surviving on a diet of wood. The insect host benefits from the metabolic capabilities of the bacteria to fix N and obtains N-containing organic compounds from the endobacteria. The endosymbionts, in return, obtain a stable and protected habitat and a reliable supply of the required nutrients for their reproduction (Orona-Tamayo and Heil 2015). These symbioses not only contribute to insect biomass, but may also make a significant contribution to N-cycling in particular ecosystems (Orona-Tamayo and Heil 2015). As an alternative to bacterial endosymbionts, some insects which feed on wood augment their nitrogen balance by ingesting cord-forming fungi which translocate soil nitrogen into the decomposing wood.

Cockroaches accumulate uric acid in the fat body, especially on protein-rich diets. The deposits of uric acid are then depleted when the insects are transferred to a low-protein diet (Cochran et al. 1985). Increased gene expression for urate oxidase and glutamine synthetase in cockroaches deprived of dietary nitrogen is consistent with a role for uric acid as a reservoir of nitrogen that is mobilized when dietary nitrogen intake is limited (Patiño-Navarrete et al. 2014). Much of the diet of primitive cockroaches was low in nitrogen and, as a consequence, mechanisms to store excess nitrogen in the fat bodies were probably an adaptive response to exploit occasional nitrogen-rich food sources (Mullins 2015).

Cockroaches benefit from the contributions of two distinct systems of microbial symbionts to their N economy: mycetocytes in the fat body and microbes in the hindgut. The symbionts provide the insects with the capacity to recycle stored urates (Mullins 2015). Three cell types are present in the fat body: whereas the trophocytes function as centers of intermediary metabolism and storage, the mycetocytes contain symbiotic bacteroids implicated in synthesis of essential amino acids, and the urocytes store urates as distinct crystalline spherules. The bacterial symbiont *Blattabacterium* in the mycetocytes produces vitamins and metabolizes sulfur and sulfur-containing amino acids for the host. The bacterium, in turn, relies upon the host's tissues for production of the amino acids Gln, Asp, Pro, and Gly (Patiño-Navarrete et al. 2014). These amino acids are produced through a chimeric metabolic pathway in which enzymes are supplied by both the host and the endosymbiont. Uric acid is degraded to allantoin, allantoic acid and then urea using the host's enzymes in the fat body. The urea is then broken down by urease in the endosymbiont, and the resulting ammonia is synthesized into glutamine using glutamine synthetase supplied by the host.

In the brown planthopper *Nilaparvata lugens* (Hongoh and Ishikawa 1997), endosymbionts enable the host to use uric acid as a nitrogen source during starvation periods, as for cockroaches, but with the difference that the uricolytic activities are supplied by the symbionts. Homopterans such as *N. lugens* feed on plant saps which are characterized by

low levels of N and unbalanced amino acid compositions. They utilize uric acid not just as a nitrogenous waste, but as an N-storage product when they ingest more nitrogen than they need. Uricolysis in the yeast-like fungal endosymbionts can then provide the host with the ammonia needed for amino acid synthesis. In the shield bug *Parastrachia japonensis*, uric acid is excreted by nymphs and reproductive adults but is retained by diapausing adults which survive without feeding for 10 months to 2 years. Uric acid is recycled as an amino acid source with the aid of *Erwinia*-like bacteria found only in the cecum of the midgut, and the uricase functions as a key enzyme during recycling (Kashima et al. 2006).

Endosymbiotic bacteria are also implicated in synthesis of some of the essential amino acids (EAAs) in aphids (Douglas 2006, 2015; Sasaki and Ishikawa 1995); symbionts essentially hydrolyze glutamine and asparagine into glutamic acid and aspartic acid, respectively (Leroy et al. 2011). Bacteria of the genus *Buchnera*, for example, contribute up to 90 % of the essential amino acids required by the pea aphid (Douglas 2006). It is worth noting the ratio of essential amino acids: nonessential amino acids in plant phloem sap are 1:4–1:20, considerably lower than the ratio of 1:1 in animal protein (Douglas 2006). The high levels of free proline and alanine in whiteflies are consistent with known roles for proline as a fuel, via oxidation to alanine, for flight (Gäde 1992). Glutamine metabolism appears to play a pivotal role as aphids adjust to changes in plant nitrogen status. Decreases in the pool of glutamine may allow the maintenance of the proline and alanine pools used for flight muscle metabolism (Crafts-Brandner 2002).

Recent studies have also revealed nitrogen recycling and host–symbiont sharing of biosynthetic pathways for synthesis of EAAs in the pea aphid *Acyrthosiphon pisum* (Macdonald et al. 2012). The bacterial symbiont *Buchnera aphidicola* is restricted to the cytoplasm of the bacteriocyte "host cell" and it supplies the host cell cytoplasm with carbon skeletons derived from the host's nonessential amino acids. Aphid transaminases then combine nitrogen derived from ammonia produced by the host cell metabolism with the carbon skeletons from the symbiont to synthesize essential amino acids. As in mosquitoes, glutamine synthetase/glutamine oxoglutarate aminotransferase (also known as glutamate synthase) (GS/GOGAT) plays an essential role in EAA synthesis.

Gut microbes also make important contributions to metabolic nitrogen utilization in the Asian long-horned beetle *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae: Lamiini). The endosymbionts are important both in nitrogenous waste product (urea) recycling and in nitrogen fixation (Ayayee et al. 2014).

4.4 Ammonia Excretion in Terrestrial Insects

4.4.1 Locusts

Ammonium, in the excreta of the desert locust *Schistocerca gregaria,* is present in the form of a precipitate, so its elimination is compatible with conservation of water. It appears that most NH_4^+ is precipitated with organic anions because the concentration of ammonium in the excreta exceeds that of urate threefold (Harrison and Phillips 1992). The advantage of ammonium urate excretion is twofold; ammonium urate is less soluble than uric acid, and it also provides for the elimination of an additional N relative to uric acid. Whereas urate and organic ions are secreted by the Malpighian tubules, most of the NH_4^+ is transported across the ileum and into the hindgut lumen through amiloride-sensitive

Na^+/NH_4^+ exchange. Although the Malpighian tubules secrete fluids containing as much as 5 mM ammonia, the amount excreted by the tubules is less than 10 % of total NH_4^+ excreted. The ileum is also a major site of acid excretion through H^+ trapped by NH_3 as NH_4^+ (Lechleitner et al. 1989).

4.4.2 Cockroaches

Although 90 % of nitrogenous waste in the fecal pellets of some species of cockroaches consists of ammonia, much of the excreted ammonia appears to be derived from the actions of hindgut microflora on urate salts or other N compounds. Ammonium ions are the major cations contained in the feces and are excreted in increasing amounts as the dietary nitrogen levels increase. As a consequence of the relative toxicity of ammonia, water excretion and hence water uptake are increased on N-rich diets. In *P. americana*, for example, a threefold increase in dietary nitrogen is correlated with a 92 % increase in water intake. Ammonia may be secreted by the Malpighian tubules and released into the anterior end of the hindgut, where some of it may be recycled into metabolic nitrogen by gut microbial systems before excretion in the feces. Although the predominance of ammonia as a nitrogenous waste in cockroaches such as *P. americana* has been known since 1972, the means by which ammonia is transported into the hindgut, or is produced therein by microflora, is still unclear. Relative to our understanding of mycetocytes in nitrogen metabolism in the fat body, much less is known of the involvement of gut microbial systems in ammonia metabolism and subsequent absorption of potentially useful materials in the hindgut. Some of the bacteria in the hindgut are involved in nitrogen fixation, and their abundance increases in cockroaches on nitrogen poor diets, suggesting that the gut microbiota complement the activities of *Blattabacterium* in the fat body (Pérez-Cobas et al. 2015). Gut microbes may also be involved in synthesis of acetate and butyrate which may be absorbed across the hindgut wall (Mullins 2015). However, as the latter author noted: "The involvement of gut microbial systems in ammonia metabolism, coupled with subsequent absorption of potentially useful materials achieved by processes within the hindgut, is a much needed area of investigation".

4.4.3 Flesh Fly Larvae

It has been known since the early years of the last century that ammonia constitutes the majority of the nitrogenous waste of the blowfly *Calliphora vomitoria* (Weinland 1906). Subsequent work revealed that larvae reared on sterile media also excrete ammonia resulting from their own metabolism, but that ammonifying bacteria normally present on rotting meat are an additional source of ammonia in the larval environment (Hobson 1932). Larvae of the flesh fly *Sarcophaga bullata* also ingest ammonia as they burrow into and feed upon rotting flesh. Ammonia is absorbed across from the midgut and excreted across the hindgut epithelium. The larvae are also exposed to excess ammonia resulting from protein metabolism. Using an isolated hindgut preparation, Prusch showed that ammonia can be secreted against a lumen–alkaline pH gradient which would oppose NH_3 diffusion (Prusch 1972). NH_4^+ secretion is independent of that for K^+, which is also actively secreted into the lumen. Ammonium secretion is reduced ~ 60 % when either K^+ or Na^+ is removed, consistent with a link between alkali cation transport and NH_4^+ secretion.

4.4.4 *Drosophila*

Ammonia may accumulate to high levels in the medium ingested by larvae of the fruit fly *Drosophila melanogaster*, and the larvae must therefore have means to either detoxify or excrete ammonia. Active secretion of ammonium into the Malpighian tubule lumen is sufficient to maintain concentrations of ~1 mM ammonium in the hemolymph of larvae reared on diets containing 100 mM ammonium chloride (Browne and O'Donnell 2013). The rates of NH_4^+ transport by the Malpighian tubules are sufficient to clear the hemolymph content of NH_4^+ in ~3.5 h suggesting that the tubules play an important role in clearance of ammonia from the hemolymph, although other epithelia such as the hindgut may also contribute.

4.4.5 Mosquitoes

More than 70 % of the amino acids derived from the digestion of blood meal by adult female mosquitoes (*Aedes aegypti*) is catabolized to provide energy and only 10 % is used for egg protein production (Isoe and Scaraffia 2013; Zhou et al. 2004). Potentially toxic loads of ammonia resulting from high rates of amino acid catabolism are avoided through two mechanisms: 1) sequestration of ammonia through synthesis of glutamine and proline and 2) high rates of ammonia excretion in the feces (Goldstrohm et al. 2003; Scaraffia et al. 2005). Ammonia released by subsequent proline catabolism can be excreted, whereas the carbon skeleton is used for synthesis of other compounds or for energy production. Measurements of mRNA expression and the activity of several enzymes implicated in ammonia metabolism suggest that the fat body is the main tissue involved in ammonia detoxification and that both glutamine synthetase and glutamate synthase are important in the process of ammonia storage. Experimental inhibition of glutamine synthetase activity reduces hemolymph glutamine concentration and causes a corresponding increase in proline concentration. Inhibition of glutamate synthase, which contributes to the production of glutamate for proline synthesis, results in an increase in glutamine concentration and a corresponding decrease in proline concentration after feeding. Mosquitoes also synthesize urea through argininolysis and uricolysis. Detailed studies of the metabolic regulation of urea synthesis indicate links to the fixation, assimilation, and excretion of ammonia. For example, glutamine acts as a precursor in uric acid synthesis; measurements using the stable isotope ^{15}N indicate that N from the amide group of two glutamine molecules produces one molecule of uric acid labeled at two positions (Isoe and Scaraffia 2013; Scaraffia et al. 2008). Uric acid is either excreted or degraded by uricolysis to produce allantoin, allantoic acid, and then urea. Proline is used to sequester ammonia and is also linked to arginine metabolism; arginase cleaves arginine into urea and ornithine, which is then used to synthesize proline and several other amino acids (Isoe and Scaraffia 2013).

4.4.6 Lepidoptera

Analyses of ingestion and excretion of nitrogen by larvae of the lepidopteran *Mamestra brassicae*, an agricultural pest, have shown that high levels of nitrogen in host plants (e.g., after fertilizer application) enhances N metabolism in the larvae and leads to increased excretion of NH_4^+ (Kagata and Ohgushi 2011). The increase in NH_4^+-N in the frass

originates from N metabolism of the insect, rather than from ammonia in the diet. Ammonia is a common component of the excreta of many terrestrial insects, comprising from ~9 % to ~27 % of total N in the frass. Insect herbivores can thus alter nitrogen dynamics in soil by transforming organic nitrogen into inorganic nitrogen and vice versa. For example, soil quality is enhanced by ammonium release during frass decomposition. Ammonium can then be taken up directly and utilized by plants without the lag associated with the normal breakdown and release of nitrogen from leaf litter (Kagata and Ohgushi 2011).

4.5 Uptake of N-Rich Compounds by Insects

4.5.1 *Manduca*

Two studies have described the uptake of ammonia by the midgut of *Manduca sexta* (Blaesse et al. 2010; Weihrauch 2006). It is proposed that ammonia uptake across the midgut and into the hemolymph provides an additional nitrogen source for rapidly growing larvae as they consume a diet low in protein. A model of active ammonia uptake across the columnar cell epithelium in the median midgut proposes that ammonia enters the columnar cell through one or both of two pathways (◘ Fig. 4.2). Firstly, NH_4^+ may enter via an apical H^+/cation exchanger, driven by the steep pH gradient across this membrane (Dow 1992). *Manduca sexta* Na^+/H^+ exchanger 7, 9 (MsNHE7, 9) is a candidate transporter for this pathway because it exhibits an amiloride binding motif, consistent with observed amiloride sensitivity of ammonia uptake, and it may be localized to the apical membrane. The second pathway involves diffusion of gaseous ammonia along a gradient of P_{NH3} across the plasma membrane, possibly via one of the two identified (Rhesus) Rh proteins in the midgut tissue (GenBank accession nos. AY954627 and DQ864985). Dissociation of cytosolic NH_4^+ to H^+ and NH_3 would then allow diffusion of NH_3 into vesicles acidified by a V-type H^+-ATPase. Alternatively, a NHE capable of H^+/NH_4^+ exchange could sequester NH_4^+ in intracellular vesicles. NHE8, which has been identified as a subapical/vesicular transporter (Piermarini et al. 2009), is a potential candidate for such a role. Rhesus proteins (RhMS) are also richly expressed in the Malpighian tubules and hindgut, suggesting that these tissues contribute to elimination of any excess ammonia (◘ Fig. 4.3).

4.5.2 "Mud-Puddling"

Multiple species of butterflies, moths, and homopterans and at least one orthopteran engage in a behavior known as "mud-puddling" in which they ingest fluids from sources such as moist ground or puddles, sea water, tears, excreta of other animals, or rotting carcasses. Although generally viewed as means of supplementing water intake or lowering body temperature, mud-puddling is also a means to augment the intake of sodium or nitrogen in species whose diet is deficient in these elements. The yellow-spined bamboo locust, *Ceracris kiangsu*, has been observed to visit puddles of human urine on hot summer days. Although the main N compound in human urine is urea, decomposition in high ambient temperatures produces ammonium bicarbonate. Behavioral studies revealed that urea is a repellent to *C. kiangsu*, whereas ammonium bicarbonate is an attractant (Shen et al. 2009). It appears that urine puddles or urine-soaked ground served as a source of both sodium and nitrogen in this species. Thus, although ammonia is generally considered

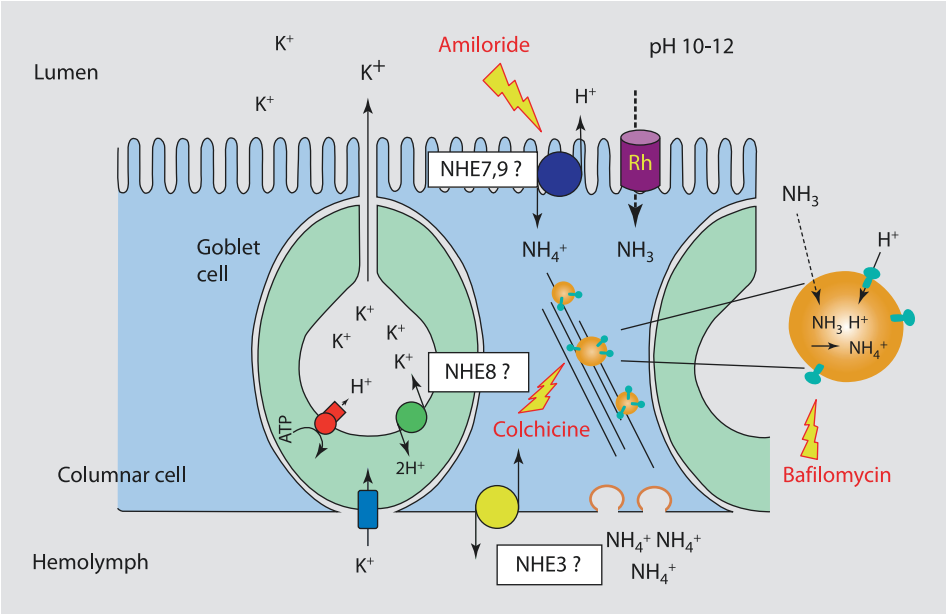


Fig. 4.2 Hypothetical model of active ammonia uptake across the columnar cell epithelium in the median midgut of the tobacco hornworm *Manduca sexta*. On the apical side, ammonia is transported as NH_4^+ via an apical amiloride-sensitive H^+/cation exchanger (possibly NHE7/9) and/or diffuses as gaseous ammonia through an Rh-like protein along an inwardly directed P_{NH3} across the plasma membrane. In the cytoplasm NH_4^+ dissociates into H^+ and NH_3 and gaseous ammonia diffuses into vesicles acidified by a V-type H^+-ATPase. The NH_4^+-loaded vesicles are transported along the microtubules network to the basal membrane, where they fuse with the membrane, releasing NH_4^+ into the hemolymph space. A participation of the basal NHE3 in the ammonia uptake process is unclear. Goblet cells, which are responsible for K^+ excretion, create a high luminal pH and are thereby indirectly involved in ammonia transport. NHE8 was found to be expressed apically/subapically in the goblet, but not in the columnar cells (personal communication, D. Weihrauch). Ammonia may also diffuse paracellularly (not shown). Pharmacological inhibitors of particular components in the ammonia transport mechanism are given with flash symbols pointing at sites of inhibition (Modified from Weihrauch et al. 2012)

to be a highly toxic waste product for most animals, species of beetle, moth, and mosquito recycle body ammonia as a resource for synthesis of amino acids, which in turn are used for protein biosynthesis (Honda et al. 2012). Both sexes of the swallowtail butterfly *Papilio polytes* utilize ammonia taken in at puddling sites such as surface water, dung, and carrion. Experiments following the fate of ^{15}N taken in during puddling shows that females incorporate the nitrogen into eggs, whereas males use it for protein replenishment in spermatozoa, seminal fluids, and thoracic muscle (Honda et al. 2012).

4.6 Ammonia Excretion in Aquatic Insects

In general, aquatic animals excrete nitrogenous waste primarily in the form of ammonia (NH_3/NH_4^+) (Weihrauch et al. 2012; Wright and Wood 2009; Larsen et al. 2014). This is because ammonia is highly soluble and thus aquatic animals take advantage of the abundance of water in their habitat to excrete nitrogenous waste in the form that requires the

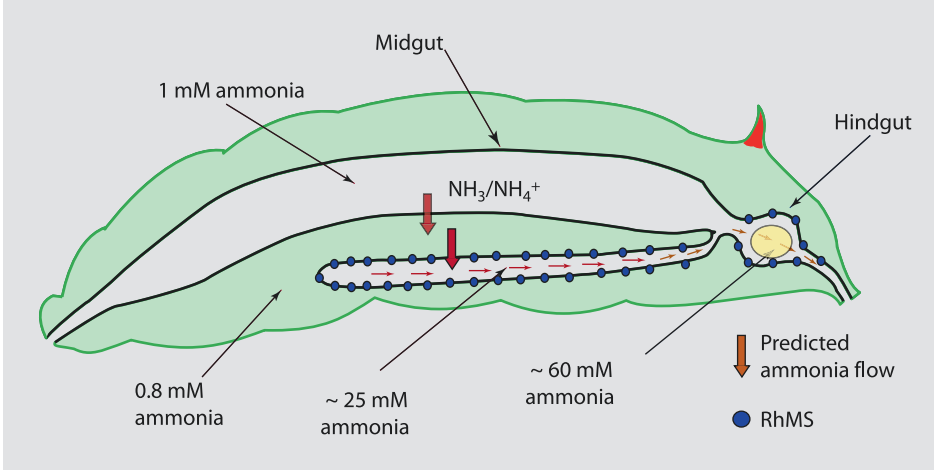


Fig. 4.3 Predicted ammonia flow in the tobacco hornworm *Manduca sexta*. Ammonia is actively transported from the midgut lumen into the hemolymph. Ammonia not utilized for amino acid synthesis is secreted into the Malpighian tubules and transported into the hindgut for final secretion. It is predicted that Rh-proteins are involved in ammonia secretion process and in maintenance of high ammonia concentrations in the hindgut lumen. Concentrations of ammonia in midgut lumen, hemolymph, Malpighian tubules, and hindgut are shown (Modified from (Weihrauch et al. 2012))

least expenditure of metabolic energy. Insects appear no different; however, this is based on measurements of excretory products from a few select aquatic insects in orders Coleoptera, Odonata, Hemiptera, Trichoptera, and Diptera (Delaunay 1930; Staddon 1955, Donini and O'Donnell 2005). In species where specific life stages are aquatic (e.g., larvae) and others terrestrial (e.g., adult), the larvae excrete primarily ammonia, while the terrestrial stages excrete nitrogen waste in other forms such as uric acid (Staddon 1955). An exception is the adult, terrestrial blood-fed female mosquito, *Aedes aegypti*, where the molar ratios of uric acid, urea, and ammonia in the excreta are equal (Briegel 1986). In experiments in which adult females were fed NH_4Cl-rich fluids (80 mM), ammonia was the predominant (>80 %) N waste in the excreta (Scaraffia et al. 2005).

4.6.1 Anal Papillae of Larval Mosquitoes

Excretion in insects is accomplished through the combined actions of the Malpighian tubules and hindgut. In freshwater inhabiting mosquito larvae, the combined actions of these organs produces dilute urine thereby eliminating excess water while conserving ions. The larvae of many mosquito species also possess external organs called anal papillae. These are finger-like projections formed from the eversion of hindgut tissue and they surround the anus. The anal papillae are typically composed of a single cell layer thick syncytial epithelium that is covered by a thin cuticle that faces the aquatic environment (Edwards and Harrison 1983; Sohal and Copeland 1966). In freshwater species like *Aedes aegypti*, the apical membrane of the epithelial syncytium contains very regular and extensive infoldings that give rise to microvilli that project deep inside the cytoplasm, while the basolateral (lumen facing) membrane is also infolded but forms a dense network of

channels that are continuous with the papilla hemocoel and extend into the epithelium. The cytoplasm contains numerous mitochondria which are densely packed near the microvilli of the apical membrane. This ultrastructure is consistent with ion transporting epithelia and functions to increase the surface area of the membranes available for solute exchange. The lumen of the anal papillae contains hemolymph and is continuous with the hemocoel of the body.

The ultrastructure of freshwater mosquito anal papillae matches their physiological function. The anal papillae play an important role in osmotic and ionic regulation by actively transporting ions into the hemolymph from the dilute freshwater environment (Stobbart 1971; Donini and O'Donnell 2005), and this function is regulated in response to salinity changes in the water in which the larvae reside (Donini et al. 2007). In addition to substantial measureable influx of Na^+, Cl^-, and K^+, relatively large effluxes of NH_4^+ and H^+ were detected at the anal papillae (Donini and O'Donnell 2005). The NH_4^+ efflux amounted to an estimated 360 nmol cm^{-2} h^{-1} which compares with 580 nmol cm^{-2} h^{-1} detected at the isolated locust hindgut (Thomson et al. 1988) and 220 nmol cm^{-2} h^{-1} for the cockroach (Mullins 1974). Therefore, the anal papillae of mosquito larvae play an important role in nitrogen waste excretion and are functionally similar to the gills of fish and crustaceans (Henry et al. 2012; Evans et al. 2005).

There are two families of ammonia transporters, the Rhesus glycoproteins (RhGP) which are found in all animals and implicated in ammonia excretion, and the methylammonium/ammonium transporters (MEP/Amt) found in unicellular prokaryotes, eukaryotic cells, plants, and invertebrate animals. The mosquito *Aedes aegypti* has two RhGPs (AeRh50-1, AeRh50-2) and at least one Amt (AeAmt1) (Weihrauch et al. 2012; Chasiotis et al. 2016). The AeAmt1 protein is expressed in the anal papillae and localized to the lumen-side membrane along with Na^+/K^+-ATPase. Although the transcripts of the RhGPs have been detected in the anal papillae (Weihrauch et al. 2012), the proteins have not been localized. Knockdown of AeAmt1 protein expression reduces ammonia excretion at the anal papillae (Chasiotis et al. 2016). It is suggested that NH_4^+ is driven into the cytoplasm through the AeAmt1 protein by the negative electrical gradient established by Na^+/K^+-ATPase (◘ Fig. 4.4). The NH_4^+ may then exit the apical side through the NHE3 or, if dissociated into NH_3 and H^+, through one of the RhGPs (Chasiotis et al. 2016). This is facilitated by the H^+ transporting functions of an apical V-type H^+-ATPase (VA) which pumps H^+ out of the cytoplasm creating an acidified boundary layer. This ensures that NH_3, exiting the anal papillae, is converted into NH_4^+ thereby maintaining a favorable partial pressure gradient for NH_3 to exit (Chasiotis et al. 2016). Both scenarios are supported by pharmacological inhibition of NHE3 and VA which results in reduced ammonia excretion by the anal papillae. Furthermore, pharmacological inhibition of carbonic anhydrase (CA) also reduces ammonia excretion suggesting that CA provides H^+ for the VA.

4.6.2 Ammonia Excretion by Larval Alderflies, Dragonflies, Stoneflies and Backswimmers

Although ammonia excretion in some aquatic insects has been measured, there are only a few studies that have gone beyond this to begin examining the physiology of ammonia excretion. The alderfly nymph, *Sialis lutaria*, excretes ~86 % of nitrogen waste as ammonia (Staddon 1955). The fluid in the hindgut contains relatively high amounts of ammonia (~136 mg N/100 mL), whereas fluid in the foregut had no measureable ammonia.

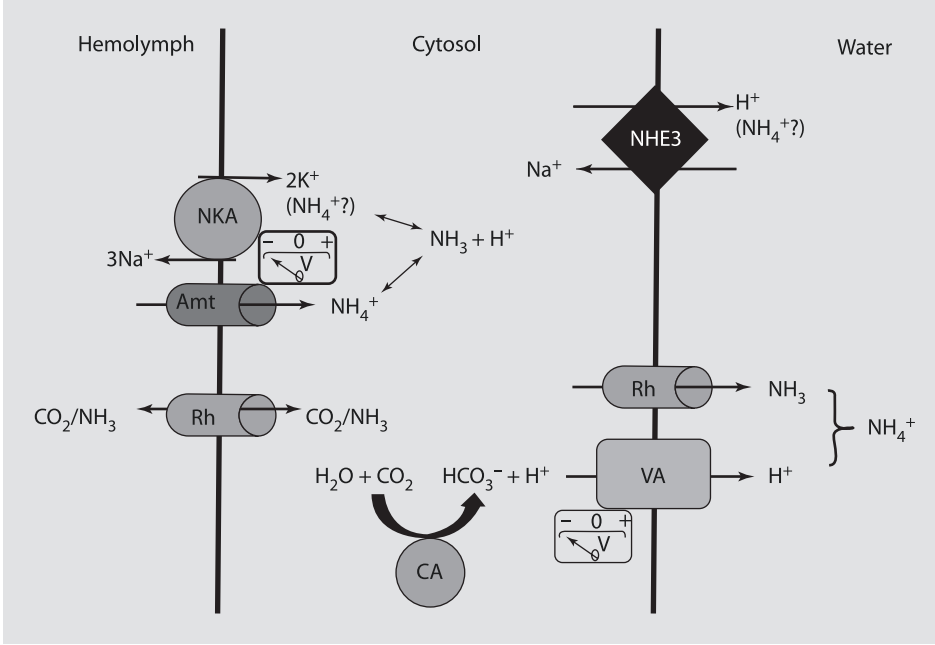


Fig. 4.4 Proposed mechanism of ammonia excretion by anal papillae of larval *Aedes aegypti*. Na^+/K^+-ATPase (*NKA*) provides a cytosol negative voltage potential which serves to drive NH_4^+ from the hemolymph to the cytosol through AeAmt1 (*Amt*). NH_4^+ could also cross from hemolymph to cytosol directly through NKA substituting for K^+. CO_2 and NH_3 may enter the cytosol through one of the two RhGP-like proteins, AeRh50-1 and/or AeRh50-2 (Rh), if one or both are expressed in the basal membrane (not yet determined). NH_4^+ in the cytosol exits from the apical side to the water through the NHE3 in exchange for a cation (e.g., Na^+). NH_3 in the cytosol exits the apical side via one of the two RhGP-like proteins (*Rh*) (localization not yet determined) with the aid of an ammonia trapping mechanism, whereby apical V-type H^+ ATPase (*VA*) acidifies the papilla boundary layer. NHE3 may also participate in this mechanism by moving H^+ into the water. This would sustain the outward directed NH_3 gradient by converting NH_3 to NH_4^+. A cytoplasmic carbonic anhydrase (*CA*) can supply H^+ to the VA which is also likely participating in generating the cytosol negative potential that drives NH_4^+ into the cytosol on the basal side (Proposed model from Chasiotis et al. 2016)

Although fluid from the Malpighian tubules was not measured, it was suggested that the tubules were the source of ammonia excretion where the fluid would then pass into the hindgut for periodic expulsion (Staddon 1955). No ammonia excretion occurs across the body surface of the nymph. Furthermore, if excretion is prevented, there is no accumulation of ammonia in either the hemolymph or tissues, suggesting that *S. lutaria* nymphs can store ammonia in another form (e.g., glutamine) if excretion is not feasible. This is significant in that alderfly nymphs leave the water for some time prior to pupation.

Ammonia accounts for ~87 % of the nitrogenous waste excreted by the dragonfly nymph *Aeshna cyanea* and uric acid makes up 8 % (Staddon 1959). Exposure to aluminum or low pH results in a large reduction in ammonia excretion in another dragonfly nymph *Somatochlora cingulata*, resulting in the accumulation of ammonia and glutamate levels in tissues (Correa et al. 1985b). In contrast, exposure to trichloroacetic acid at environmentally

relevant levels doubles the ammonia excretion rates (Correa et al. 1985a). The underlying physiological mechanisms behind these observations remain unstudied. The stonefly larva *Klaptopteryx kuscheli* is a leaf-litter shredder and shown to regulate internal carbon/nitrogen (C:N) at a level of ~5.5 regardless of the C:N ratio of the food (Balseiro and Albariño 2006). It was shown that as the C:N ratio decreases, the larvae excrete more ammonia.

In a study examining the regulation of water balance of a backswimmer *Notonecta glauca*, a carnivore, the relationship of water output and ammonia output exhibited a linear trend (Staddon 1963). This might be expected since ammonia is highly soluble and aquatic insects exploit the abundance of water in their habitat to excrete ammonia. The ammonia levels in the rectal fluid were ~75 mmol l^{-1} and contributed to ~75 % of total nitrogen in the fluid. In contrast, a similar study in another species of backswimmer *Corixa dentipes*, it was noted that the rectal fluid contained very variable and in some cases relatively low amounts of ammonia ranging from 21 to 85 mmol l^{-1} with an average of ~ 50 mmol l^{-1} (Staddon 1964). Little difference in ammonia levels were observed whether the fluid was forced out or allowed to be expelled naturally by the insect leading to the suggestion that the rectum plays no role in modification of the rectal fluid. Although it might be expected that water output, is at least in part, dependent on ammonia output, this was not the case in *Corixa dentipes*. Furthermore, the rate of water output far exceeded the volume required for the observed rate of ammonia output. The independence of water and ammonia output could be due to the fact that this insect is omnivorous and ingests mainly a liquid diet resulting in a copious amount of water ingestion which needs to be expelled regardless of ammonia levels; however, the results of the study on water intake of the insect were inconclusive (Staddon 1964). If this were the case, it could explain the relatively low concentrations of ammonia in rectal fluid of this species.

4.7 Excretion of Free Amino Acids

Silverleaf whiteflies (*Bemisia tabaci* Gennadius) and other phloem-feeding insects such as aphids excrete amino acids, especially nonessential amino acids such as glutamine, asparagine, glutamate, and aspartate (Douglas 2006). Whiteflies reared on well-fertilized cotton plants excrete an amount of amino nitrogen that exceeds by nearly twofold the total amino acid pool in the body. However, when flies are fed plants with low nitrogen levels, pools of individual amino acids in the body are adjusted and amino nitrogen is no longer excreted at significant rates (Crafts-Brandner 2002). Total nitrogen content of whiteflies reared on plants with reduced nitrogen content does not decline significantly, relative to whiteflies reared on plants with higher levels of nitrogen. However, the free amino acid content of whiteflies feeding on low nitrogen plants declines ~90 % relative to controls. It is important to note that the amino acid pools in tissues of whiteflies feeding on high nitrogen cotton plants are not closely related to the amino acid composition of the phloem sap. Whereas the predominant amino acids in whiteflies are glutamine (26 % of the total), alanine (19 %), proline (13 %), and glutamate (10 %), the predominant amino acids in phloem sap from high nitrogen cotton plants are aspartate, glutamate, and arginine, with relatively large amounts of glutamine and asparagine, and the excreted honeydew contains mostly asparagine (46 % of total amino acid content) and glutamine (12 %). These differences indicate that dietary amino nitrogen is rapidly assimilated into metabolites or protein.

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