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Mechanisms of Systemic Osmoregulation in Insects

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Abstract

Water is essential to life. Terrestrial insects lose water by evaporation from the body surface and respiratory surfaces, as well as in the excretory products, posing a challenge made more acute by their high surface-to-volume ratio. These losses must be kept to a minimum and be offset by water gained from other sources. By contrast, insects such as the blood-sucking bug *Rhodnius prolixus* consume up to 10 times their body weight in a single blood meal, necessitating rapid expulsion of excess water and ions. How do insects manage their ion and water budgets? A century of study has revealed a great deal about the organ systems that insects use to maintain their ion and water balance and their regulation. Traditionally, a taxonomically wide range of species were studied, whereas more recent research has focused on model organisms to leverage the power of the molecular genetic approach. Key advances in new technologies have become available for a wider range of species in the past decade. We document how these approaches have already begun to inform our understanding of the diversity and conservation of insect systemic osmoregulation. We advocate that these technologies be combined with traditional approaches to study a broader range of nonmodel species to gain a comprehensive overview of the mechanism underpinning systemic osmoregulation in the most species-rich group of animals on earth, the insects.

INTRODUCTION

The ability to homeostatically control internal water abundance is a fundamental prerequisite of animal life. This is particularly true for insects, who are at a physiological disadvantage due to their small size and large surface-to-volume ratio, which creates a large potential for rapid water loss. Managing their internal ion and water budgets thus requires insects to efficiently limit water loss to the environment, as well as effectively extract water from food and even the atmosphere (**Figure 1**). However, despite the potential for dehydration, insects display a considerable capacity for dealing with water deficiencies and excesses and have evolved sophisticated mechanisms by which they can maintain osmotic balance even when exposed to extreme environmental challenges.

In 1931, in a series of back-to-back papers in the *Journal of Experimental Biology*, Sir Vincent Wigglesworth, the founding father of modern insect physiology, set out to introduce a new model for the study of insect excretion (167–169). The goal of these papers was to provide a complete description of the process of excretion in a single insect species—the kissing bug, *Rhodnius prolixus* (Hemiptera)—including an anatomical description of its excretory system and an analysis of the chemical composition of its urine, and to chart the histological changes during the period of diuresis. In doing so, he set the stage for a century of subsequent work on insect excretion and systemic osmoregulation, including pioneering works on the physiology and endocrine control of the Malpighian tubule and hindgut (93, 94, 97, 136–139, 172).

The classical studies in the field tend to encompass a taxonomically broad range of species, covering various aspects of osmoregulation based on both anatomical and physiological investigations. By contrast, contemporary work has focused predominantly on a taxonomically narrower range of species, with the fruit fly *Drosophila melanogaster* (Diptera) being most dominant. The *D. melanogaster* genome was the first insect genome to be sequenced (2), which, coupled with

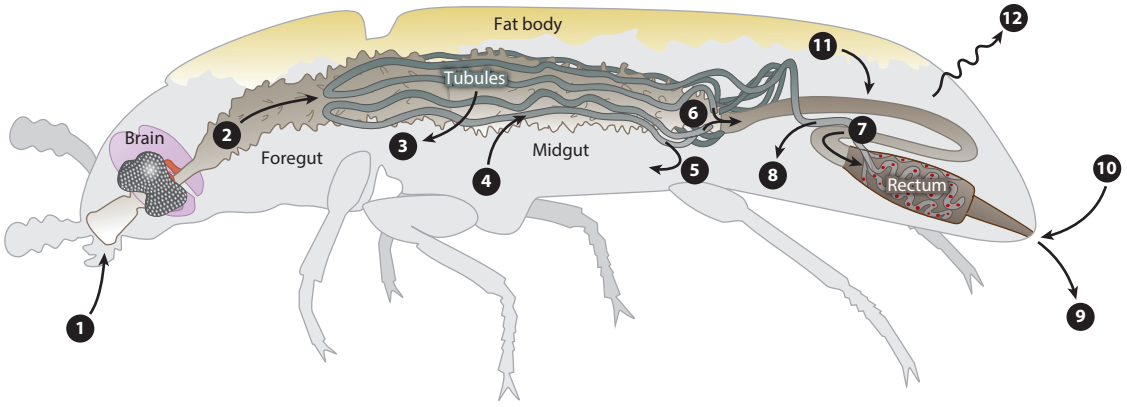


Figure 1

Overview of water, ion, and solute fluxes in a stereotypic insect. (*Step 1*) Nutrients and water are ingested and (*Step 2*) passed into the foregut and (*Step 3*) later into the midgut, where nutrients and water are absorbed. (*Step 4*) The Malpighian tubule main segment secrets a primary urine, along with metabolic waste and toxic solutes. (*Step 5*) The urine is modified in the lower segment of the tubule by selective reabsorption prior to (*Step 6*) being discharged into the junction between the mid- and hindgut, where it is mixed with gut contents before (*Step 7*) the waste is passed into the rectum. (*Step 8*) Water and ions are selectively reabsorbed in proportion to the needs of the animal, and recycled back to the insect by anal papillae (e.g., *Drosophila melanogaster*) or via the perirectal tubules (e.g., *Tribolium castaneum*). (*Step 9*) The remaining waste is then excreted. (*Step 10*) In insects with the cryptonephridial condition, water can further be extracted from the atmosphere through a mechanism called water vapor absorption. (*Step 11*) Water is also generated internally through breakdown of energy reserves from the fat body to produce metabolic water and (*Step 12*) is lost to the atmosphere via body and respiratory surfaces.

its long and rich history as a genetic model and the unparalleled genetic toolbox available, has provided substantial molecular insight into the roles of genes involved in insect osmoregulation. While *D. melanogaster* remains the preferred model to dissect the molecular and cellular basis of osmoregulation, many more insect genomes have now been sequenced, and key advances in new technologies have become available in the past decade that have democratized the scope of functional analyses to include a much wider range of insect species. These new approaches have already started to inform our understanding of the diversity and conservation of the mechanism underpinning systemic osmoregulation in insects (22, 61, 77, 79, 110). The purpose of this review is to provide an integrative, whole-animal perspective on ion and water balance across a broad range of insect species and to identify key questions that remain unanswered to help frame the direction of the field of insect osmoregulation over the coming years.

ONE CELL OR TWO? FUNCTION AND CONTROL OF THE INSECT MALPIGHIAN TUBULE

The Fastest Secreting Epithelium in Biology, the *Rhodnius* Tubule

On a per-cell basis, the upper Malpighian tubule cells in *R. prolixus* are the fastest secreting cells known; each cell is able to secrete its own volume of fluid every 15 seconds (100, 104). *Rhodnius prolixus* is an obligate hematophagous bug capable of taking on a blood meal that is more than 10 times its own body weight, which requires that excess fluid and ions are excreted swiftly. As part of a postingestive homeostatic program, postprandial diuresis is rapidly initiated, raising the rate of fluid secretion by the Malpighian tubules to a thousand times above the unstimulated level, thus allowing the insect to eliminate excess ions and fluid at a rate equivalent to its original body weight every 20–30 minutes (167). Remarkably, this feat is not unusual, as the cells in the *D. melanogaster* tubule are able to secrete at a similarly impressive rate (39).

The Molecular Machinery and Epithelial Architecture of Renal Tubules

In his studies on the diuretic response to a blood meal in *R. prolixus* and the mosquito *Aedes aegypti* (Diptera) (136–138), James Arthur Ramsay—another pioneer in the field of insect osmoregulation—identified the circulation of potassium within the body: from hemolymph to tubule and then to midgut and rectum, where it is absorbed back into the hemolymph. He suggested that the secretion of potassium (together with some anion) into the tubule lumen would establish an osmotic pressure, which in turn would promote a passive inward diffusion of water and thus generate the primary urine (136, 138). This hypothesis was strengthened by his demonstration that potassium is actively secreted into the tubule lumen in eight insect species across five different orders (137).

This original hypothesis has since been confirmed, and the molecular machinery for fluid transport in the tubules is now well characterized. The discussion that follows is based on discoveries in *D. melanogaster*, but the general principles are likely to apply to other insect tubules, despite the diversity in the cell types that mediate the transport and movement of ions across the epithelium. The mechanism of fluid secretion is energized by a vacuolar H^+-ATPase (V-ATPase) (64, 165) located in the apical membrane, which establishes a H^+ electrochemical gradient necessary to drive secondary active K^+ secretion via a colocalized H^+/K^+ exchanger (35). K^+ subsequently enters from the hemolymph across the basal membrane and has been shown to be mediated by several distinct transport mechanisms, including the Na^+/K^+-ATPase (37, 157); the inwardly rectifying K^+ channels, Irk1 and Irk2 (173); and the $Na^+/K^+/2Cl^-$ cotransporter, Ncc69 (174). The net movement of potassium ions across the epithelium leads to the accumulation of positive charges

in the lumen, which causes Cl^- to flow down its electrochemical gradient. Cl^- flux is mediated by two chloride channels, Chloride channel-a (ClC-a) in the basal membrane (23) and Secretory Chloride channel (SecCl) in the apical membrane (45). The resulting KCl secretion drives the movement of osmotically obliged water from hemolymph to tubule lumen via both paracellular routes and through the water channels—the basolateral Prip and the apical Drip aquaporins (22). In *D. melanogaster*, but not in all insects, the localization of the V-ATPase and the movement of K^+, on the one hand, and Cl^- flux and water movement, on the other hand, are spatially segregated into two different cell types: the principal and secondary cells, respectively (115).

The lower, proximal segment in *R. prolixus*, *Tribolium castaneum*, and *D. melanogaster* does not have a secretory function, but instead appears to function in ion reabsorption (79, 99, 105, 117, 143). Functional analyses have revealed that significant amounts of K^+, Cl^-, and water are reabsorbed in this segment, implicating the lower tubule in modifying the primary urine prior to passage into the gut. Consistent with these findings, in contrast to the secretory segment, secondary cells are absent from the lower, proximal tubule in *D. melanogaster* (23, 37, 38). The primary roles of the lower tubule, therefore, seem to be to modify fluid passed to it from the main segment and to serve a preparatory function facilitating the handling of excretory fluids by the hindgut.

Neuroendocrine Control of Diuresis

The control of insect diuresis is a complex and highly regulated process that involves multiple hormonal signals that are released from distinct populations of neuroendocrine cells in the central nervous system (and perhaps from the gut enteroendocrine cells) into the circulation to remotely control tubule secretion and/or hindgut reabsorption (**Figure 2**). The cellular targets and intracellular signaling mechanisms of the different hormones have been identified in several species, and recent work has started to uncover the physiological triggers that induce their release, as well as how the different hormonal circuits interact to promote organismal homeostasis.

In all species studied to date, two large families of peptides have been found to act similarly through cyclic AMP (cAMP) to stimulate primary urine production. In *D. melanogaster*, diuretic hormone 44 (DH44)—a homolog of vertebrate corticotropic-releasing factor (CRF)—is a 44-amino-acid-long peptide that acts to stimulate fluid secretion through activation of its receptor, DH44-R2, most abundantly expressed in tubule principal cells (20, 67, 73). Orthologs of the DH44–DH44-R2 hormonal relay have been found in a wide range of species, where they show similar modes of action, indicating that this is a conserved mechanism (8, 24, 63, 85, 122, 152). However, in both Coleoptera and Lepidoptera, two separate CRF-like DH ligands have been found, with each peptide showing different receptor affinities, implying that they might modulate tubule function differently (15, 48, 50, 51, 74, 79). Indeed, in tenebrionid beetles, DH37 and DH47 preferentially activate separate isoforms of the CRF-like receptor, Urn8R, which is exclusively expressed in a subpopulation of tubule secondary cells to stimulate fluid secretion (79). These physiological effects are mediated by four pairs of DH37/47-producing neurons in the beetle brain, which secrete the peptide into circulation via the corpora cardiaca in response to changes in hemolymph osmotic pressure. Thus, in contrast to most other insects, beetles (and perhaps moths) possess two separate peptides that signal exclusively through the smaller secondary cells to modulate diuretic activity (50, 51, 79).

DH31 is a diuretic peptide that is distantly related to vertebrate calcitonin gene–related peptide (29). In *D. melanogaster*, DH31 is produced by several distinct groups of neurons in the brain (82) and is released into the hemolymph via the corpora cardiaca to activate the DH31 receptor in tubule principal cells (49, 61, 73). Interestingly, central neurons producing the neurohormone corazonin carry receptors for both DH44 and DH31 (73), implying that these hormonal circuits

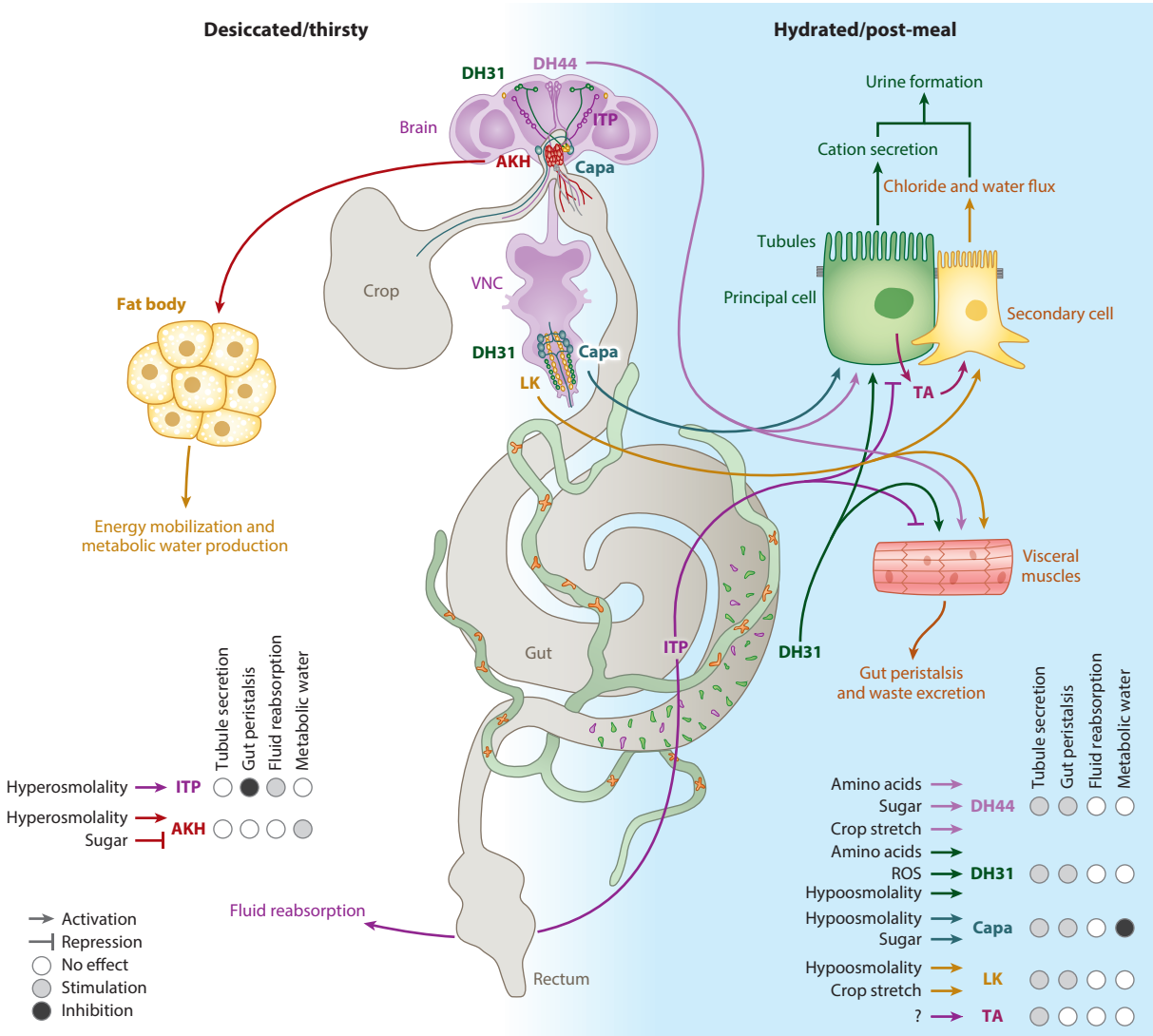


Figure 2

Osmoregulatory networks coordinating systemic water balance in *Drosophila melanogaster*. During desiccation or thirst (*left*), AKH is secreted from the corpora cardiaca to induce breakdown of peripheral fat stores to promote the generation of metabolic water. At the same time, ITP is believed to be released either from central neurons or from gut enteroendocrine cells to suppress tubule secretion and gut peristalsis, as well as to promote rectal fluid reabsorption. By contrast, during periods of fluid excess or after meal ingestion (*right*), DH44, LK, and Capa peptides are secreted from neurosecretory cells in the central nervous system (brain or VNC), whereas DH31 is released from enteroendocrine cells as part of a postingestive homeostatic program that stimulates tubule secretion and visceral muscle contractions to promote fluid and waste excretion. The biogenic amine TA is additionally synthesized and secreted in a paracrine fashion from tubule principal cells to signal to neighboring secondary cells to stimulate urine formation. A white circle indicates no effect, a grey circle indicates stimulation, and a dark circle indicates inhibition. Abbreviations: AKH, adipokinetic hormone; DH, diuretic hormone; ITP, ion transport peptide; LK, leucokinin; ROS, reactive oxygen species; TA, tyramine; VNC, ventral nerve cord.

are all part of a larger osmoregulatory network that controls systemic water abundance (180). For example, the DH31 circuit is part of a hormonal relay that controls sleep and daytime arousal (82), indicating that this hormonal system may mediate the diurnal control of tubule secretion (80). DH31 peptide is also expressed and released by gut enteroendocrine cells (59), and given the close proximity of tubules to the gut, it would be of great interest to investigate the relative importance of gut versus brain DH31 in controlling whole-animal fluid balance.

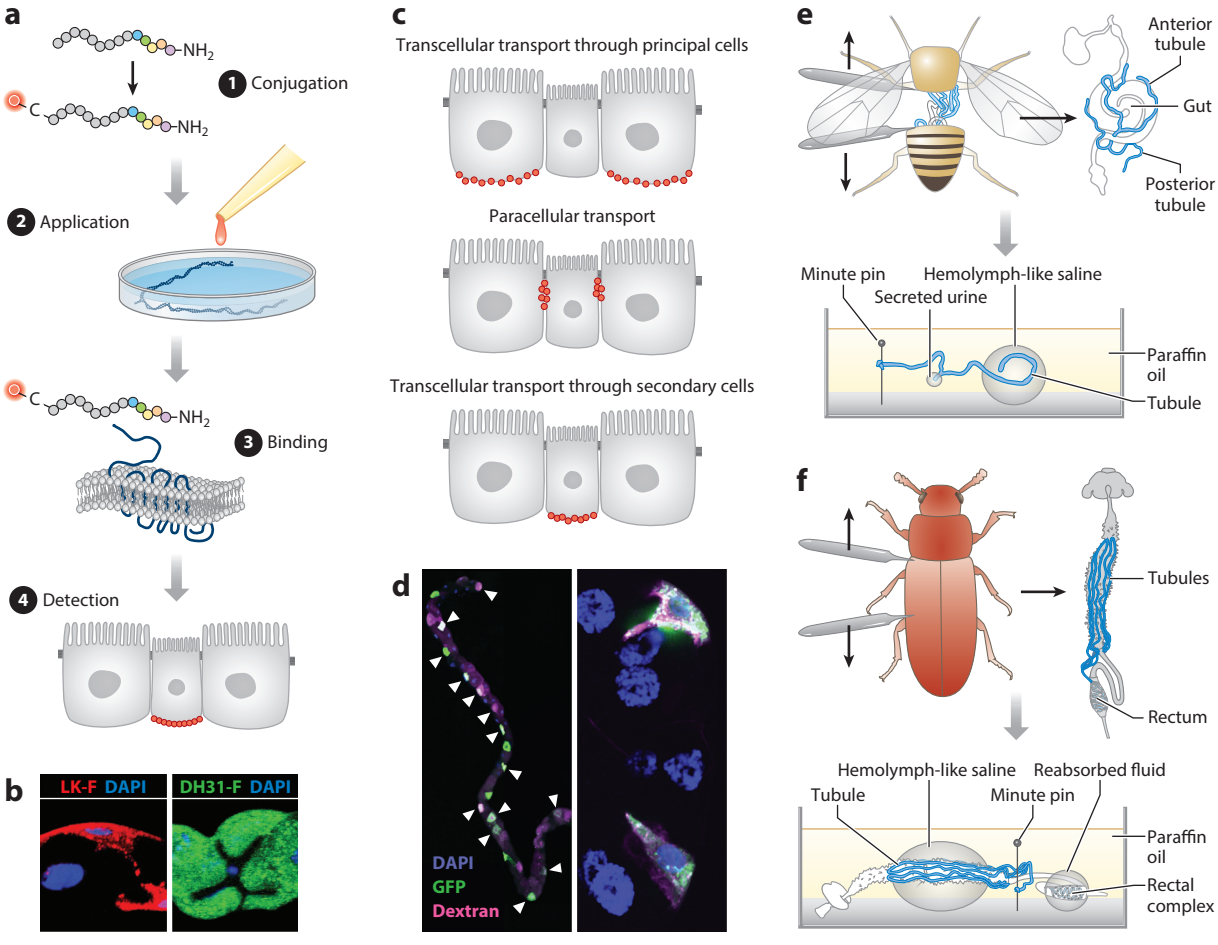
In *D. melanogaster*, the Capa-1 and Capa-2 peptides (including the unrelated Capa-3) are expressed in a single pair of subesophageal ganglion neurons, as well as three pairs of ventroabdominal neurons (81, 132, 163) in the ventral nerve cord (**Figure 2**). Following their release into circulation by the ventroabdominal neurons (147), Capa-1 and Capa-2 bind to the Capa receptor (CapaR), which is also expressed in tubule principal cells (71, 153) (**Figure 3**). Activation of CapaR induces a prominent biphasic Ca^{2+} response (81, 144, 153) that stimulates nitric oxide (NO) synthase and the production of NO. This NO signal subsequently activates a soluble guanylate cyclase to increase cGMP production, leading to activation of the apically localized V-ATPase and thus primary urine production (33, 34). Evidence implicating Capa peptides in the functional stimulation of insect tubules has been reported from a phylogenetically broad range of species (34, 158). However, Capa peptides have also been suggested to act as an antidiuretic signal in *D. melanogaster* (92), and similar reports have been made for the mealworm *Tenebrio molitor* (Coleoptera) (61, 166) and *R. prolixus* (133). Consistent with a role in providing feedback regulation on tubule secretion and fluid balance, several studies have linked Capa/CapaR signaling with organismal survival in response to cold and desiccation stress (92, 153, 155).

Two factors are known to regulate secondary cell activity, leucokinin and tyramine. Both signals stimulate fluid secretion, albeit through two separate receptors (16, 68, 134). In *D. melanogaster*, leucokinin is a short, 15-amino-acid peptide hormone that is produced by distinct clusters of neurons in the brain and ventral nerve cord of adult flies (154, 179). Leucokinin-producing neurons in the brain are activated by hyperosmotic stimuli to promote formation of water-reward memories (148). By contrast, the leucokinin-expressing neurons in the ventral nerve cord have been reported to be activated by signals related to water ingestion to remotely control tubule secretion (181). Thus, although the two groups of leucokinin neurons both appear to be osmosensitive, they are activated by opposing osmotic stimuli and perform separate functions relating to the systemic control of water balance in flies. The functional stimulation of tubule activity is mediated by the leucokinin receptor, which is only found in the secondary cells of the tubule (134). Activation of the leucokinin receptor causes a rapid increase in intracellular Ca^{2+} levels, which subsequently activates the chloride shunt conductance and increases the fluid secretion rate (23, 45, 115). Interestingly, the biogenic amine tyramine appears to function in a similar fashion to leucokinin, as the physiological effects of activating the tyramine receptor in secondary cells are indistinguishable from those of leucokinin stimulation, suggesting that the two pathways likely converge on the same downstream target in the secondary cell (16, 21).

The Two-Cell-Type Model and Its Evolutionary Origins

As outlined in previous sections, the integrated actions of the insect tubule rely on a two-cell-type model in which the transepithelial movement of cations, anions, and water is functionally segregated into two distinct cell types, with each cell type being regulated by distinct hormonal signals (**Figure 2**). Remarkably, this two-cell-type model is highly conserved in the endopterygote insects, as hallmarks diagnostic of secondary cell function, such as leucokinin action (61), expression of the transcription factor Tiptop (36), and water flux (22), can be mapped to secondary cell–like cells in species representing most higher orders of insects (38). In contrast, secondary cells appear to be

largely absent in the more basal exopterygote insects in which a single secretory cell type is prevalent (61). Taken together, these data imply that a single cell type is the basal condition in insects, whereas the two-cell-type state is a derived trait that evolved approximately 350 million years ago (61). However, a striking exception to this general pattern was recently reported in beetles (79). Beetles lack leucokinin signaling altogether, while other signaling pathways are greatly expanded (86), indicating that the hormonal control of beetle tubules is profoundly different than in other insects. Indeed, we recently discovered a physiologically distinct secondary cell in the tubules of the red flour beetle *T. castaneum* (Coletopera), which, instead of mediating a leucokinin-stimulated Cl^- conductance, has adopted CRF-like (Urinate, Urn8) signaling from the larger principal cells



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Tools democratizing functional studies of systemic osmoregulation in insects. (*a*) Principle of the ligand-receptor binding assay. (*Step 1*) Chemical conjugation (via a cysteine linker, C) of a high-quantum-yield fluorophore to a suitable, less conserved (often N-terminal) region of a synthetic analog of a native peptide, thus generating a fluorescently tagged neuropeptide. (*Step 2*) Application of the fluorescently tagged ligand to acutely dissected tissue ex vivo (e.g., tubules). (*Step 3*) The ligand binds to its endogenous receptor in the target tissue. (*Step 4*) Receptor binding allows rapid detection of receptor-expressing cells and, thus, of the identity of the types of cells that receive the signal. (*b*) Example of fluorophore-labeled leucokinin (LK-F) and diuretic hormone 31 (DH31-F) and their specific binding to secondary cells (*left*) and principal cells (*right*), respectively, in *Drosophila melanogaster* tubules. Panel adapted with permission from Reference 61; copyright 2015 Macmillan Publishers Ltd., CC BY 4.0. (*c*) Principle of the dextran flux labeling technique. To probe the routes of water flux in non-model species, fluorescently labeled dextrans size selected to be too large to cross an epithelium of choice can be applied to a tissue bathed in a solution ex vivo. Dextrans will then accumulate in a compartment diagnostic of the route of water movement, for example, through the principal or secondary cell or the paracellular route between cells in the Malpighian tubule. (*d*) Functional stimulation of *D. melanogaster* tubules with leucokinin (10^{-7} M) coincubated with a 40-kDa fluorophore-coupled dextran (*magenta*) exclusively marks the basal labyrinths of the secondary cells marked by GFP (*green*), demonstrating that this cell type is similar to the major route for water flux in these tubules. Images courtesy of Dr. Anthony Dornan. (*e*) The classic Ramsay fluid secretion assay. A pair of tubules are acutely dissected, then isolated in a drop of hemolymph-like saline under liquid paraffin oil. One of the tubules is then pulled out of the saline and wrapped around a minute pin with the common ureter positioned in the oil. Fluid secreted by the tubule remaining in the drop will form a discrete droplet, the volume of which can be measured with an optical graticule to calculate the volume secreted before and after application of a (anti)diuretic peptide. This technique can be modified to accommodate different sizes, physiologies, and anatomies of insect tubules (61). (*f*) The same principle can be applied to quantify the amount of fluid reabsorbed by the rectum of insects. The entire alimentary canal and associated structures are dissected and isolated under liquid paraffin oil, after which a volume of saline is applied to the midgut (for species with the cryptonephridial condition, such as most beetles, the tubules are further disconnected from the rectal complex). A small droplet of known volume is applied to the rectum, and the preparation is then allowed to accumulate fluid, after which the fluid is removed from the rectum, and the volume is calculated per unit of time. Panel adapted with permission from Reference 110; CC BY 4.0. (*g*) High-throughput in vivo analysis of the excretory physiology of insects can be assessed by allowing animals to feed on food supplemented with a pH indicator, after which the animals are allowed to excrete on a surface. A fecal output scan is then produced, and graphical features of the excreta can then be analyzed by a dedicated software tool, The Ultimate Reader of Dung (TURD) (162). Several aspects of the fecal output profile can be analyzed during data extraction, including the size, number, shape, and hue of the excreta, to provide an integrated readout for gut and tubule functions during different diets and/or environmental conditions.

to control K^+ flux and promote tubule secretion (79). Furthermore, using a ligand–receptor binding assay to localize sites of Urn8R expression (**Figure 3*a***), we mapped DH37/DH47 activity to this novel secondary cell in all tested members of the higher beetle families (Polyphaga); by contrast, this activity was entirely localized to the principal cells in the more basal beetles (Adephaga). These observations imply that, while Urn8 signaling is general to all beetle families, only the polyphagan beetles possess this unique tubule architecture, which likely arose approximately 240 million years ago (79). Although giant leaps have been taken in recent years in our understanding of the evolutionary origins of insect renal function and control (22, 61, 79), the grand challenge of testing the generality of our epithelial models across the vast insect phylogeny still remains.

THE OTHER SIDE OF THE COIN: WATER REABSORPTION BY THE HINDGUT AND RECTUM

The upper Malpighian tubule is responsible for generating the primary urine. Although the lower (proximal) tubule is involved in reabsorption in at least some species (70, 79, 117, 168), the hindgut and rectum are the primary sites of solute, ion, and water reabsorption. Yet there is great diversity in how different groups of insects perform and regulate these functions.

Mechanisms of Selective Water and Ion Reabsorption by the Rectal Epithelium and Rectal Glands

Based on anatomical and histological criteria, as well as observations on the duration and behavior of material moving through the hindgut and rectum, Wigglesworth (170) came to the conclusion

that the rectal glands constitute the primary sites of water absorption in all main insect orders. Several subsequent physiological studies on the insect rectum appear to confirm this, and it has been found that, in addition to water, the rectum is an important site for the reabsorption of ions and solutes secreted by the Malpighian tubules (127–129, 135, 140, 151). For example, Phillips (126–129) filled canulated, ligatured recta of the blowfly *Calliphora erythrocephala* (Diptera) and the locust *Schistocerca gregaria* (Orthoptera) with measured quantities of solutions of various ionic composition, including completely ion-free sugar solutions (impermeable to the rectal wall), and was able to show that ions and water were removed from the rectum against their electrochemical and osmotic gradients with respect to the hemolymph. At the time, there was considerable debate as to whether water was actively transported across the rectal epithelium, a hypothesis that Phillips' findings might be seen as lending support to; however, Philips was not in favor of this model and speculated on a range of alternative mechanisms in his discussion (126–129; see also 139, 142). However, based on the structural design of the rectal epithelia (discussed below), it has been argued and generally accepted—albeit not proven conclusively—that the reabsorption of water is a consequence of active transport of solutes into narrow, intercellular spaces between rectal cells, rather than the active transport of water per se (12, 60, 159).

In many terrestrial insects, such as locusts, cockroaches, and blowflies, the rectum is structurally modified for reabsorption (12, 60, 69, 72). The predominant feature lies with the principal cells of the rectum, whose lateral membranes are elaborately folded, interdigitating with neighboring cells (in locusts and cockroaches) or into membrane stacks (in blowflies). The folds and stacks are associated with numerous mitochondria and penetrated by a rich supply of tracheoles for gas exchange. It has been proposed that water reabsorption is a consequence of active transport of ions into these narrow intercellular spaces, resulting in localized fluids of very high concentration, which draw water osmotically from the lumen of the rectum. In support of this, a strong correlation between elaboration of these lateral membranes and the dryness of the habitat has been shown in termites (114).

Water reabsorption in the locust rectum is underpinned by rectal transport of Cl^-, with the uphill entry step for Cl^- being K^+ dependent. The exact mechanism of apical Cl^- transport is still debated, but Cl^- might be reabsorbed via (*a*) H^+ recycling through H^+/Cl^- symporters and apical V-ATPase activity and/or (*b*) direct Cl^- reabsorption via an electrogenic Cl^- pump (56, 62, 123–125). Basolateral Na^+/K^+ ATPase activity contributes to the generation of the osmotic gradient between the rectal lumen and the narrow intercellular spaces (14). Reabsorption in the *D. melanogaster* hindgut has recently been reported and appears to share similar mechanism(s) with that described for locusts (4).

Mechanisms of Selective Water Reabsorption by the Cryptonephredial System

In the cryptonephridial (crypto meaning hidden and nephridial referring to the kidney) arrangement of insect renal systems, the distal regions of the Malpighian tubules—the so-called perirectal tubules—are closely applied to the rectum, forming a so-called rectal complex. In some species, the complex is enclosed within a chamber, the perinephric space, separated from the body cavity by a tissue layer known as the perinephric membrane. The cryptonephridial condition is found in several groups of insects, including Coleoptera, Lepidoptera, Diptera, Hymenoptera (e.g., sawflies, fire ants), and Neuroptera (e.g., lacewings, antlions) (5, 13, 57, 106, 146). There is strong evidence that the Coleopteran rectal complex is a system to conserve water, and further evidence suggests that the system might be used to harvest water from the atmosphere in some species (17, 31, 32, 58, 90, 91, 107, 113, 141, 171). Evidence in support of water recycling by the rectal complex in other Orders is less clear, and while the rectal complex of Lepidoptera is capable of recycling water

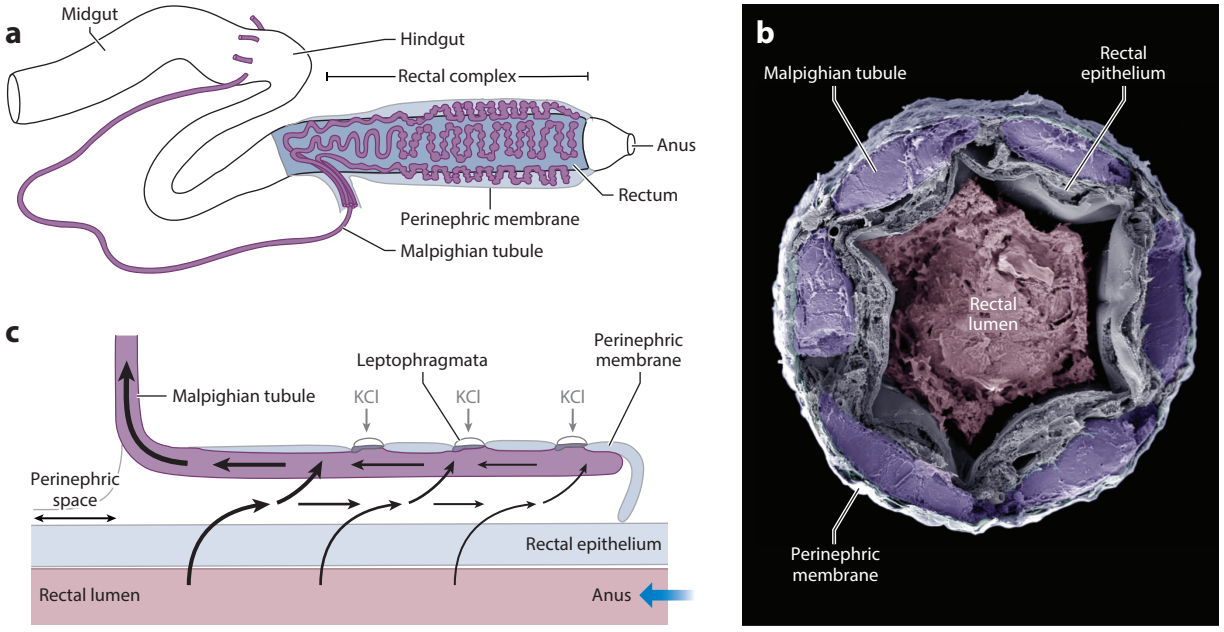


Figure 4

The cryptonephridial complex in beetles. (*a*) Drawing of the tenebrionid rectal complex. (*b*) Scanning electron micrograph showing a cross-section through a *Tribolium castaneum* rectal complex. The picture shows dry feces (*magenta*) in the rectal lumen. The Malpighian tubules (*purple*) surround the rectum (*gray*) and are in close apposition; the complex is wrapped by the perinephric membrane (*light outer layer*). Panel adapted with permission from Reference 110; CC BY 4.0. (*c*) Schematic of the rectal complex as a counter-current exchange system. The Malpighian tubules are intimately associated with the rectum, and they are both surrounded by the perinephric membrane. Fluid flow through the Malpighian tubule runs counter to flow of feces through the rectal lumen. A high concentration of potassium chloride (KCl) is generated in the tubule lumen via transport from the hemolymph through leptophragmata (*gray arrows*). This draws water (*black arrows*) osmotically from the rectum at all points along its length (the thickness of the arrows scales with the water flux rate). Water is recycled back to the body through the distal region of the free tubule, which is not associated with the complex. The perinephric membrane forms a highly impermeable barrier to prevent deleterious hemolymph-to-tubule water flow. The beetle can also take up atmospheric water (*blue arrow*) in conditions where relative humidity exceeds a certain threshold.

back into the body, it is thought primarily to function in the return of ions in the maintenance of acid-base balance (78).

The pioneering studies of Ramsay and colleagues in the 1960s established the broad principles of rectal complex function for the mealworm beetle *T. molitor* (58, 141) (**Figure 4**). In *T. molitor*, water is drawn osmotically from the contents of the rectal lumen via the generation of a steep osmotic gradient between the rectal contents and the perinephric space. This is achieved by the secretory activities of the distal ends of Malpighian tubules, the perirectal tubules, which generate high concentrations of KCl in their lumen. Water is drawn from the rectum into the perinephric space and then into the tubule lumen, from which water is recycled back to the body via the distal region of the free tubule (79). The perinephric membrane forms a water-tight seal around the complex, isolating the perinephric space and tubules from the rest of the hemocoel, thus preventing deleterious movement of water from hemolymph into the complex (141). However, there is evidence that water might be at least partly recycled back to the body directly from the perinephric space at the anterior-most end of the complex, where the perinephric membrane is bound less tightly; specifically, it has been proposed that such an unsealed sleeve could act as a valve, allowing excess fluid to escape while restricting backflow into the complex (58, 141) (**Figure 4**).

The perinephric membrane is pierced regularly with small windows under which a specialized group of tubule secondary cells, known as leptophragmata, sit. The leptophragmata were initially identified as the main sites for the hemolymph-to-tubule movement of chloride ions (88), an observation that we have since confirmed (110). Chloride ions are thought to move passively into the tubule lumen, driven by a lumen positive potential established through the active transport of K^+ into this compartment (91, 116). Indeed, we have recently identified a cation/proton antiporter (NHA1) that is expressed in the leptophragmata of tenebrionid beetles, which plays a key part in the transport machinery required for establishing the osmotic gradients necessary to extract water from the rectal lumen to maintain organismal water balance. Electrophysiological characterization of NHA1 indicates that it acts as a potassium/proton antiporter, and expression analysis reveals that it is transcriptionally upregulated in response to desiccation. It is likely that transport is energized by a V-ATPase proton pump in the principal cells of the perirectal tubules, as components of the pump are expressed in and localize to the luminal membrane of these cells (110). The V-ATPase proton pump as a driver for epithelial secretion in the perirectal tubules of the beetle is equivalent to the tubules in those species without the cryptonephridial condition (e.g., *D. melanogaster*). Together, these data strongly imply that NHA1 mediates K^+ transport from the hemolymph into the rectal complex through leptophragmata (110) in a manner similar to that observed in the free tubules (79). K^+ transport by secondary cells is likely a derived feature of beetle Malpighian tubules, as K^+ transport occurs exclusively via principal cells in species such as *D. melanogaster* (115).

Leptophragmata have been identified as a specialized group of tubule secondary cells based on their expression of the transcription factor Tiptop, as well as their smaller cell and nuclear size relative to principal cells (36, 77, 110). Leptophragmata, but not secondary cells in the free tubule, express the transcription factor Dachshund, suggesting that leptophragmata can be homologized to the bar cells found in *D. melanogaster* tubules that also express Dachshund (149; R Beaven, KV Halberg and B Denholm, unpublished manuscript). Dachshund is required in leptophragmata to establish their identity relative to secondary cells of the free tubule (R Beaven, KV Halberg and B Denholm, unpublished manuscript).

The cryptonephridial condition is extremely common in insects (approximately 400,000 species), being particularly prevalent in Coleoptera and the larvae of Lepidoptera, where it is present in some of the most species-rich clades (e.g., *Ditrysia* in Lepidoptera). It appears to have evolved a minimum of seven times: once each in Diptera, Neuroptera, and Lepidoptera and twice each in Coleoptera and Hymenoptera. The widespread occurrence of insect rectal complexes, their association with species-rich clades, and the fact that they have evolved convergently several times throughout evolution strongly suggest that the cryptonephridial system has been an important driver in insect evolution. A first step in understanding how the cryptonephridial condition might have evolved from the ancestral, noncryptonephridial state is to determine the mechanisms that underpin its development. We have begun to unpick these mechanisms for the *T. castaneum* rectal complex.

The *T. castaneum* rectal complex has evolved via a radical reorganization of the internal anatomy: The rectum and Malpighian tubules are brought into close apposition in countercurrent configuration and are isolated within a chamber by a unique tissue of unusual structure and unknown origin—the perinephric membrane. The arrangement of these features with respect to one another is essential for the function of the rectal complex (58; R Beaven, KV Halberg and B Denholm, unpublished manuscript). We have recently discovered that a dynamic and intricately woven crosstalk among the three major organs of the complex—the rectum, the Malpighian tubule, and the perinephric membrane—underpins the assembly of the rectal complex during beetle embryogenesis. This crosstalk is mediated by two fibroblast growth factor (FGF) ligands

acting through two FGF receptor isoforms. FGF signaling directs the migration of perinephric membrane precursor cells onto the rectum, the migration of the distal tubules to the posterior end of the rectum (so that they lie in counter-current configuration with respect to material that will ultimately flow throw them), and the envelopment of tubules and rectum by the perinephric membrane. We also find that the perinephric membrane—a tissue layer with an unusual structure resembling the myelin sheath of vertebrate neurons—develops from a distinct population of mesodermal cells that has not been identified previously (58; R Beaven, KV Halberg and B Denholm, unpublished manuscript). Therefore, in the beetle at least, it appears that evolution of the rectal complex has involved (*a*) the dramatic reorganization of existing organs (rectum and Malpighian tubules) into a novel configuration and (*b*) the recruitment and elaboration of a novel tissue (the perinephric membrane), with FGF signaling being central to these processes. It will be interesting to determine if FGF signaling has been co-opted in the independent evolution of the cryptonephridial condition in other insects.

It is worth noting that some insects without a rectal complex are capable of water uptake from the atmosphere through their rectum, including the firebrat *Thermobia domestica* (Zygentoma) (10, 112, 113). In fact, water uptake can occur at much lower humidities in the firebrat compared to *T. molitor*, as low as 45% relative humidity in *T. domestica* compared to 88% in *T. molitor* (10, 107, 112).

MECHANICAL INTEROCEPTION AND FEEDBACK CONTROL OF OSMOREGULATORY RESPONSES

As Maddrell (101, p. 131) observed, "Rhodnius is a blood-sucking bug of immoderate feeding habits. Given the opportunity, this insect will quickly, skillfully and painlessly remove from its host a volume of blood sufficient to increase its own weight by about tenfold." During the postfeeding diuresis that ensues, fluid is pumped across the midgut into the hemocoel; the distal portions of the Malpighian tubules then rapidly remove from the hemolymph a relatively K^+-rich fluid of similar osmolarity to the hemolymph, which is then modified in the proximal tubule by reabsorption of a hyperosmotic KCl solution (44, 98, 105). These processes occur rapidly, such that, within two to three hours, a volume of fluid equivalent to approximately 10 times that of the insect's hemolymph is excreted. How do insects sense engorgement, and what are the feedback mechanisms that enable the insect to return to a homeostatic condition?

Using an ex vivo preparation of *R. prolixus* tubules (**Figure 3*e***), Maddrell (97) was able to pinpoint the source of the diuretic factor by assessing which tissues increased tubule secretory activity when added into the drop of hemolymph in which they rested. He discovered that the central nervous system, and in particular the fused meso- and metathoracic ganglia and all of the abdominal ganglia, was more potent than other tissues in inducing diuresis and went on to refine the location of the source to two small populations of cells in the posterior of this mass that had neurosecretory characteristics (97, 156). This region of the nervous system was subsequently shown to be the seat of diuretic activity, with diuretic hormones such as serotonin and CRF-like diuretic factors being released into the hemolymph very soon after feeding to stimulate fluid section in the Malpighian tubules (9, 46, 83, 95, 96, 102, 103, 109, 118, 119).

In further experiments, Maddrell (93) demonstrated that hormone release was stimulated by mechanical distention of the abdomen due to the swelling of the gut after a meal, rather than by a change in osmolarity or temperature associated with the meal, and that signals of abdominal distention were relayed to the neurosecretory cells by afferent neurons projecting from the abdomen. This work highlighted the presence of pressure receptors in the abdomen of *R. prolixus*. The location and mechanism of these receptors were subsequently discovered (25, 26). The receptor is

a single chordotonal-like sense cell located in the ventral abdominal body wall, where it responds tonically to pressure applied internally and communicates information about this pressure via the abdominal nerves to the secretory cells in the mesothoracic ganglia, identified as the release site for diuretic activity. The receptors are located in regions subject to forces related to the size and movement of the gut and therefore ideally positioned for long-term monitoring of gut size (25, 26). The molecular nature of this mechanosensitive system, i.e., mechanosensitive ion channels, is not known. Other components of the feeding response, including the regulation of absorption of a NaCl-rich fluid from the midgut lumen into the hemolymph and reabsorption of KCl from the primary urine by the lower portions of the Malpighian tubule, may also be influenced by this mechanosensor–neuronal axis (44, 105). There is evidence to suggest that a similar axis controls postprandial diuresis in other hematophagous insects (54, 55, 111).

INTERACTIONS OF METABOLIC PROGRAMS AND OSMOREGULATORY CIRCUITS

Coordination of Postprandial Physiology

As might be expected, insect osmoregulatory circuits interact intimately with other homeostatic programs, and in recent years, it has become clear that hormonal pathways involved in osmoregulation also play key roles in coordinating systemic metabolism. For example, in *D. melanogaster*, DH31 and DH44 are both secreted into circulation in response to nutrient ingestion, where these hormones, in addition to their role in controlling tubule secretion (20, 29), orchestrate distinct feeding behaviors, as well as promoting gut motility and excretion (11, 40, 176). Similarly, leucokinin and Capa hormones are released systemically from subpopulations of neurons in the ventral nerve cord in response to both hemolymph hypoosmolality and circulating sugar levels (81, 181). Whereas leucokinin has been implicated in controlling meal size through a stretch-dependent feedback circuit (3), Capa has been shown to inhibit release of adipokinetic hormone (AKH)—a key metabolic hormone involved in promoting lipid mobilization from the fat body (76)—to avoid postfeeding hyperglycemia (81). Both peptides also facilitate digestion by stimulating intestinal contractions and reducing gut transit time (61, 81). Finally, ion transport peptide (ITP) was recently shown to promote drinking, as well as to reduce gut motility, suppress food intake, and modulate AKH sensitivity (52, 53). Taken together, these reports suggest that the coordinated control of food intake, intestinal contractions, energy storage, and renal secretion are intimately linked and that the regulation of these responses has likely been co-opted into intertwined networks throughout evolution to promote integrated homeostasis. These observations further reveal an unexpected principle by which the convergence of internal signals of nutrient and water availability onto the same neuronal effectors is an important mechanism by which the endocrine system executes the appropriate organ functions to regulate postprandial physiology. Intriguingly, orthologs of these neuropeptide–receptor systems have been shown to mediate similar responses across a range of insects, including beetles, locusts, and moths (6, 63, 75, 79), showing that these integrated network functions are widely conserved across insect Orders. It would be of great interest to understand the relative contributions of the individual hormonal circuits during various environmental challenges in different insects. Adopting a more global view of the organ-specific actions controlled by each hormonal circuit, as well as a detailed understanding of the cellular sensors that activate each system, will help resolve this issue.

Unique Mechanisms for Water Production in Arid Environments

Despite their small size, many insects thrive in arid environments, as they are able to engage physiological responses that allow them to cope with permanent or intermittent periods of water

deprivation (41). Remarkably, some xeric species can even survive their entire life cycle without access to water (and feeding on dry grains) yet still maintain water balance (hemolymph osmolality and volume) within nonlethal limits (1, 47, 79). These observations imply that the obligatory water loss through respiration (spiracle opening) and waste excretion (urine production and gut excretion) must be balanced by water gain through noncanonical routes, one of which could be metabolic (oxidative) water production. Indeed, classic studies on a diverse range of species have shown that the metabolic rate of animals exposed to dry conditions is markedly higher than that of animals at normal humidities (18, 19, 47, 89, 107, 164), suggesting that some insects may actively adjust metabolic water production to oppose the effects of desiccation. Consistent with this notion, work on *D. melanogaster* has shown that a perceived lack of water caused by blocking of water taste perception was sufficient to activate AKH signaling to increase metabolic water production and desiccation tolerance (161) (**Figure 2**). Conversely, a recent study found that Capa hormones are released from ventroabdominal neurons as part of a postingestive response to inhibit AKH release during periods of fluid excess (79). Taken together, these studies imply that AKH release and metabolic water production are under active homeostatic control by extrinsic signals, which bidirectionally control metabolic water production to maintain body fluid balance. There is thus mounting evidence that points to a physiologically relevant role of metabolic water in the overall water budget of insects during periods of water stress; however, further work needs to be done to elucidate the molecular, cellular, and network mechanisms underpinning metabolic water production, particularly in species colonizing xeric environments.

NEW TECHNOLOGIES, NEW HORIZONS—DEMOCRATIZING INSECT PHYSIOLOGY

Although *D. melanogaster* remains the preferred model when it comes to dissecting the molecular and cellular bases of osmoregulation, new technologies have become available that have enabled functional analyses across a much wider range of insect species. These include fluorescent tools to map sites of hormone action and water transport, ex vivo approaches to assess organ activities, and in vivo assays to quantify whole-animal excretion (**Figure 3**). Furthermore, cell- and tissue-specific expression atlases are becoming available for an increasing number of species as public resources and, in combination with genetic manipulations using RNA interference and CRISPR/Cas technologies, have greatly improved our understanding of the genes that underpin various osmoregulatory responses. In this section, we outline how these new approaches can help expand and accelerate our understanding of systemic osmoregulation in insects.

Mapping Sites of Hormone Action

There are more insect species on the planet than all other animal groups combined, so obtaining general insights into the endocrine control of osmoregulatory responses in insects is a grand challenge. In the early days of the postgenomic era, the primary structure of many neuropeptides representative of most peptide families was resolved for an increasing number of species, allowing insights into the evolutionary divergence, novelty, and loss of peptidergic signaling in insects (86, 108). These efforts revealed that most neuropeptide families were defined by an active core that was highly conserved (61, 65, 86, 177), thus explaining why many neuropeptide systems are able to cross-activate receptors in species separated by millions of years of evolution (28, 61, 66, 79). These facts, in combination with the emergence of high-quantum-yield fluorophores, raised the possibility that rational design of fluorescently coupled archetypal neuropeptides could be used to directly visualize ligand–receptor interactions in dissected tissues and thus provide a fast and efficient tool for mapping systemic sites of hormone action across Insecta (**Figure 3*a*,*b***). This

approach has already provided unparalleled insights into the evolution of insect renal function and control (61, 79) and represents a convenient method with which to identify cells that respond to a given hormonal signal in nonmodel species to help generate an evolutionary overview of the neuroendocrine control of physiological functions.

Determining Routes of Water Flux

Mapping routes of water movement across epithelia is key to understanding the mechanics of fluid transport of individual tissues and across the whole organism. The complex branched glucose polymers, the dextrans, are easily labeled with fluorescent dyes and are available in molecular sizes that are too large to move across epithelia. Co-incubation of any epithelium of choice with fluorescently labeled dextrans, optimally in conditions that functionally stimulate the tissue, will thus cause the dextrans to be swept along by bulk fluid flow and subsequently accumulate in compartments diagnostic of the routes of water flux (**Figure 3*c*,*d***). Combining this method with fluorescent imaging can thus readily identify sites of high water permeability across a range of species and can further be used as a convenient readout to assess cell-specific effects of aquaporin depletion, in combination with reverse genetic approaches (22).

Quantifying Fluid Transport

In the classic paper, published almost three quarters of a century ago, Ramsay (139) introduced a novel method by which one can quantify rates of fluid transport from the tubule of the stick insect *Dixippus morosus* (Phasmatodea) (**Figure 3*e***). This method (the Ramsay secretion assay) has since been expanded to accommodate different tubule anatomies and to work across a range of species including flies, beetles, moths, bees, and bugs (39, 61, 79, 121). The method relies on the native dissection of tubules, which are isolated in a hemolymph-like solution under liquid paraffin oil with one end drawn out into the oil to allow secreted fluid to accumulate as a discrete droplet. Using an ocular graticule, the diameter of the growing droplet can be measured and a volume calculated at distinct time intervals before and after application of potential bioactive compounds to the hemolymph solution. Strikingly, this classic technique has become even more relevant in the postgenomic era, as it allows the quantification of renal output following genetic manipulation and can be adapted to allow quantification of hindgut water reabsorption (4, 110) (**Figure 3*f***).

Assessing In Vivo Excretory Behavior

In addition to quantifying cell- and organ-specific responses, it is further possible to gain detailed insights into in vivo excretion of insects by feeding them a diet supplemented with a dye and allowing them to excrete onto a surface that can be imaged and subsequently analyzed digitally (30, 162). In this way, one can get quantitative measures of the number, size, shape, and intensity of excreta that can be directly related to the integrated output of gut and tubule physiology (**Figure 3*g***). This approach was originally developed and used for *D. melanogaster* (30, 81, 162), but we recently adapted it for *T. castaneum* (79, 110), and it can easily be adapted to work in a broad range of species. Importantly, this approach can be combined with large-scale functional screens aimed at understanding the genes underlying whole-animal excretory physiology.

Tissue-Specific Expression Atlases: Revolutionary Tools in Insect Physiology

Cataloguing the relative strength and specificity of gene expression across different tissues and life stages of an organism can provide valuable insights into most biological functions. Transcriptomic atlases have therefore become powerful tools in the functional genomics arsenal by enabling the

annotation of physiological mechanisms and developmental processes on a gene-by-gene basis (7, 27, 84, 160). For example, the first comprehensive analysis of the adult *D. melanogaster* tubule transcriptome (160) provided a detailed list of genes, including genes that underlie physiological processes already studied (e.g., genes encoding the V-ATPase subunits); that encode proteins with predicted roles in tubule transport function but whose identity was unknown at the time (e.g., the inward rectifier family of potassium channels) (43); that encode transcription factors mapping to anatomically and physiologically defined regions and distinct cell types (that have since been shown to pattern different physiological activities) (36); that encode transporters for almost every class of organic and inorganic solute (which invited a substantial revision of our understanding of the tubule and its mechanisms as an excretory epithelium); and that are related to human genes for which mutations cause disease, including several with renal phenotypes (suggesting that fly and human renal function may be more similar than commonly considered and highlighting the Malpighian tubule as a model for human renal disease). Since this pioneering paper, advances in transcriptome technologies have progressed and now include single-cell and single-nucleus RNA sequencing, which provide higher resolution of cell differences in a heterogeneous tissue like the Malpighian tubule. The recently published single-nucleus RNA sequencing of the *D. melanogaster* Malpighian tubule provides a rich resource to mine the function of this organ at single-cell resolution (175).

Subsequent Malpighian tubule transcriptome analysis of other common pest and disease vector species including *R. prolixus*; *T. castaneum*; the cotton bollworm (*Helicoverpa armigera*); the leafhopper *Psammotettix striatus*; and the mosquitos *Aedes aegypti*, *Anopheles gambiae*, and *Aedes albopictus* (42, 87, 110, 120, 122, 178), among others, could pave the way to the discovery of novel and specific pest control strategies. Pharmacological approaches that target osmoregulation in a range of insects have met with considerable success in the past (130, 131, 145).

Some of the work highlighted in this review was underpinned by data from the recently published BeetleAtlas resource (**https://BeetleAtlas.org**) (110). BeetleAtlas, a transcriptomic resource that maps gene expression across a wide number of distinct tissues at different life stages in *T. castaneum*, is proving to be a powerful tool to understand osmoregulation in beetles. The picture that is beginning to emerge shows that beetle osmoregulation appears to operate in a radically different way to that of all other insects studied to date (79). In addition, BeetleAtlas is starting to provide fundamental insight into the development, evolution, and molecular mechanisms that underpin the function of one of the most powerful water-conserving mechanisms in nature, the beetle cryptonephridial complex (110; R Beaven, KV Halberg and B Denholm, unpublished manuscript).

CONCLUSIONS AND FUTURE DIRECTIONS

We have come a long way since Wigglesworth introduced *R. prolixus* as a model for insect excretion nearly 100 years ago (167–169). Since then, classical studies have taught us a great deal about osmoregulation in a phylogenetically broad range of species, including various aspects of the anatomy and physiology of different osmoregulatory responses. However, with the emergence of powerful transgenics and sophisticated genetic approaches, *D. melanogaster* has since become the predominant model for dissecting the molecular, cellular, and network mechanisms underpinning systemic osmoregulation in insects. Research into the osmoregulatory strategies of *D. melanogaster* has been extremely illuminating. Yet, with an estimated 5–10 million insect species on the planet (150), there is undoubtedly a lot of interesting biology yet to be discovered in the field. Recent studies in *T. castaneum* and other beetle species discussed in this review are excellent cases in point. Key advances in new technologies have enabled functional analyses to be carried out across a much wider range of insect species. In this review, we provide several examples of

how these new approaches have started to increase our experimental sampling and may ultimately enable us to tame insect biodiversity. We advocate that these novel technologies be combined with those classic technologies traditionally used in the field (such as the Ramsay secretion assay) and be brought to bear on a broader range of nonmodel species, in particular, members of the underrepresented Hemimetabola. Adopting such a strategy that combines state-of-the-art technologies with classical approaches has the potential to generate an unprecedented overview of the osmoregulatory mechanisms present in insects, as well as shedding light on how these adaptations have enabled the insects to colonize virtually every habitat on Earth.

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