REVIEW

Physiological responses of freshwater insects to salinity: molecular-, cellular- and organ-level studies

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ABSTRACT

Salinization of freshwater is occurring throughout the world, affecting freshwater biota that inhabit rivers, streams, ponds, marshes and lakes. There are many freshwater insects, and these animals are important for ecosystem health. These insects have evolved physiological mechanisms to maintain their internal salt and water balance based on a freshwater environment that has comparatively little salt. In these habitats, insects must counter the loss of salts and dilution of their internal body fluids by sequestering salts and excreting water. Most of these insects can tolerate salinization of their habitats to a certain level; however, when exposed to salinization they often exhibit markers of stress and impaired development. An understanding of the physiological mechanisms for controlling salt and water balance in freshwater insects, and how these are affected by salinization, is needed to predict the consequences of salinization for freshwater ecosystems. Recent research in this area has addressed the whole-organism response, but the purpose of this Review is to summarize the effects of salinization on the osmoregulatory physiology of freshwater insects at the molecular to organ level. Research of this type is limited, and pursuing such lines of inquiry will improve our understanding of the effects of salinization on freshwater insects and the ecosystems they inhabit.

KEY WORDS: Aquaporins, Ion transporters, Osmoregulation, Salinization, Septate junction

Introduction

Contamination of freshwater with salt is occurring throughout the world. The sources of contamination are many, but most result from human activities (Rahman et al., 2019; Schuler et al., 2019). For example, mining and industrial effluent, agricultural run-off, irrigation and road de-icing are all sources of salts (Nava et al., 2020; Timpano et al., 2018). In coastal regions, inundation by seawater resulting from sea-level rise and groundwater extraction is also a source of salt contamination, and this may be linked to climate change and human activity (Mastrocicco et al., 2019). Salt contamination of freshwater is detrimental for many reasons. For example, within an ecosystem, the composition of plant and animal species may be altered owing to displacement of salt-sensitive freshwater species by salt-tolerant ones. The various impacts of freshwater contamination with salt all require investigation, but the focus of this Review is on aquatic insects. The Review will begin by covering the sources of salt contamination of freshwater, because salts of different ions can have different effects on aquatic insects (Box 1). We will then cover the osmoregulatory physiology and

effects of salt on freshwater insects at the molecular, cellular, tissue and organ levels and will not focus on whole-organism effects, which have recently been well covered by a number of studies and reviews (Cañedo-Argüelles et al., 2019; Kefford et al., 2016; Orr and Buchwalter, 2020; Scheibener et al., 2016).

Sources of salt contamination of freshwater

Freshwater contains relatively low levels of salts, which can consist of the monovalent cations sodium and potassium, divalent cations calcium and magnesium, and anions chloride, carbonate, bicarbonate and sulphate (Wetzel, 2001). The natural ion composition of inland freshwater rivers, lakes and wetlands depends on the regional geology, proximity to oceans, prevailing wind direction, groundwater hydrology, precipitation and the balance between precipitation and evaporation (Wetzel, 2001). In general, the majority of inland freshwater is relatively high in calcium carbonate with low sodium chloride levels, although exceptions exist (Wetzel, 2001). Freshwater insects have adapted their physiology for life under these conditions, and alterations can profoundly affect their survival. For example, freshwater ecosystems contaminated by salt show a reduction in biodiversity, with a shift towards salt-tolerant species (Hintz and Relyea, 2019; Kefford et al., 2016; Pond, 2012). The salinization of freshwater is now well documented and occurring on a global scale (Cañedo-Argüelles et al., 2019; Haq et al., 2018; Kaushal et al., 2018; Kefford et al., 2016).

There are many different sources of salt contamination and most result from human activities. For example, the intrusion of seawater into coastal aquifers is driven mostly by anthropogenic water usage, lowering the water levels in aquifers (Ma et al., 2019; Rahman et al., 2019; Telahigue et al., 2020). Climate change is leading to a rise in sea level that affects coastal areas (Cook et al., 2016; Zhang et al., 2004), potentially exaggerating this effect (Colombani et al., 2016; Mastrocicco et al., 2019). Furthermore, sea-level rise may significantly increase the salinity of tidal freshwater wetlands (Barendregt and Swarth, 2013). Inland, mining is a significant source of freshwater salinization that introduces a variety of ions depending on the mining activity. For example, surface coal mining results in elevated levels of sulphate, bicarbonate, magnesium and calcium in rivers and streams (Cianciolo et al., 2020; Pond, 2012; Timpano et al., 2018). Potash mining generates a salty waste containing major salts such as sodium and chloride, which is often discharged into rivers (Braukmann and Böhme, 2011). Another major contributor to the salinization of freshwater is agricultural land use (Iglesias, 2020). Replacement of natural, deep-rooted vegetation with crop plants and subsequent irrigation results in the mobilization of salts, which then enter freshwater streams, rivers and lakes (Williams, 2001). This can result in natural freshwater lakes becoming saline lakes (Williams, 2001). Salt is applied to roads and walkways during winter to control ice formation; road salt is predominantly sodium chloride with far lesser amounts of

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Glossary

Ionocytes
Epithelial cells normally found in gills or amongst epidermal cells that are specialized for transport of ions and usually characterized by the abundant expression of energy-consuming ion pumps, Na^+/K^+-ATPase and/or V-type H^+-ATPase.

Paracellular pathway
The extracellular space between adjacent epithelial cells.

Peritrophic membrane
A membrane that lines the luminal surface of the midgut.

Septate junctions
Occluding junctions of epithelia that typically present as belts around the circumference of the lateral cell borders on the apical or outer surface of epithelial cells. Septate junctions regulate the permeability of the paracellular pathway between adjacent epithelial cells.

Septate junction proteins
The proteins that make up the multi-protein complexes that form septate junctions.

Transcellular transport
Transport that occurs through the cell.

Box 1. Ion uptake by freshwater insects
Salinization of freshwater can lead to increased salt uptake by freshwater insects, disrupting osmoregulation and affecting survival (Kefford et al., 2012; Scheibener et al., 2016). This is because ion uptake follows Michaelis–Mentin kinetics; hence, higher levels of environmental salts result in elevated ion uptake (Donini et al., 2007; Patrick et al., 2001; Scheibener et al., 2016). Insects from different orders exhibit different sensitivities to salt, with Ephemeroptera showing the greatest sensitivity (Hassell et al., 2006; Kefford, 2019; Kefford et al., 2012). A study that utilized nine species across four orders demonstrated that the more sensitive species had higher rates of Na^+ uptake and, in some cases, saturation was not achieved, with uptake rates continuing to increase with increases in external ion levels (Scheibener et al., 2016). This raises the possibility that sensitive species (e.g. ephemeropterans) accumulate Na^+ faster and at lower sodium concentrations than less sensitive species. The excess Na^+ (and other ions in general) must then be excreted, and it has been hypothesized that this incurs an energetic cost, which may explain the sensitivity of these species to salinity (Buchwalter et al., 2019; Scheibener et al., 2016; Verberk et al., 2020). The effects of salinity are dependent on the temperature, the type of salt (ion) and whether other particular ions are also present (Orr and Buchwalter, 2020; Poteat et al., 2012; Scheibener et al., 2017). For example, rates of Na^+ uptake are generally far greater than rates of divalent cation uptake (Orr and Buchwalter, 2020; Poteat et al., 2012). There are ion uptake interactions between salts that are present at the same time, where, for example, Na^+ can affect SO_4^- toxicity and the presence of Cd^{2+} affects Ca^{2+} uptake (Gillis and Wood, 2008; Scheibener et al., 2017). Temperature does not uniformly affect the uptake of different ions, and temperature effects on ion uptake are also dependent on the species of insect (Orr and Buchwalter, 2020). There is still much work to be done in order to understand fundamental ion uptake mechanisms in aquatic insects and how these are affected and/or modulated by salinity.

magnesium chloride and calcium chloride (Schuler and Relyea, 2018). Many studies have linked road-deicing salts to elevated chloride levels in urbanized freshwater systems (Corsi et al., 2015; Jackson and Jobbágy, 2005; Laceby et al., 2019; Nava et al., 2020). Furthermore, sodium chloride liberates other cations – such as calcium, potassium and magnesium – from sediments, further compounding the effects of salinization (Haq et al., 2018). Other products have been developed and are being used for deicing or to prevent ice formation. Some of these are based on agricultural crops such as sugar beet (Fay and Shi, 2012), the juice of which is mixed with liquid chloride salts for application to roads (Gillis et al., 2021). These deicers will also find their way into surrounding freshwater and almost nothing is known about how they may affect the physiology of freshwater insects. As a step towards understanding the effect of salinization on freshwater insects, below, we discuss the regulation of ionic and osmotic balance in these animals, and what we know so far about the effects of excess salts.

The physiology of salt and water balance in freshwater insects

Before we can determine how freshwater insects are affected by salts, we must first understand their normal osmoregulatory physiology. An animal's internal milieu consists of aqueous solutions of organic and inorganic molecules at levels that are normally tightly regulated, which is a necessity for physiological function (Bradley, 1987; Evans, 2008; Griffith, 2017; Krogh, 1939; Moens, 1975). Many cellular functions are maintained by electrochemical gradients that are established by the active transport of ions and solutes, resulting in an unequal distribution across membranes. These gradients provide energy for the secondary transport of important solutes such as sugars and amino acids across cellular membranes. Environmental factors can disrupt solute concentrations and/or electrochemical gradients, and when the disruption is severe, this can lead to death. For example, the cold tolerance of insects is linked to their ability to maintain internal salt and water balance (Andersen et al., 2017a,b; MacMillan et al., 2015). Freshwater animals are faced with the dilution of internal body fluids because the osmotic gradient across body surfaces overwhelmingly favours water entry, whereas the solute gradient favours loss of solutes. In order to counteract this challenge, these animals typically sequester ions (salts) through

active transport, reabsorb ions across the epithelia of excretory organs, maintain relatively impermeable paracellular pathways (see Glossary) and excrete dilute urine (Bradley, 1987; Chasiotis et al., 2012; Jonusaite et al., 2013; Kumai and Perry, 2012; Nowghani et al., 2017). These strategies are achieved by internal organs associated with the gastrointestinal tract in all insects. In addition, freshwater insects have evolved specialized organs that normally protrude externally, which are also involved in osmoregulation. The relevant physiology of the specialized organs is discussed in more detail below.

Tracheal gills, papillae and ionocytes

Most freshwater insects have evolved outward protrusions of the integument or hindgut that are lined with relatively thin and permeable cuticle; these function as respiratory and/or osmoregulatory organs (Table 1). These organs are generally referred to as 'gills' and can be found arranged as tufts or as a group on the segments of the thorax (Plecoptera), abdomen (Megaloptera, Ephemeroptera, Coleoptera, Trichoptera, Neuroptera, Lepidoptera) and/or the caudal region (Diptera, Coleoptera, Odonata, Plecoptera, Trichoptera). Two main types are recognized: those that contain an extensive network of trachea with a small volume of haemolymph (called 'tracheal gills'), and those that have far less tracheation and are filled with a relatively large volume of haemolymph (called 'blood gills' or, more commonly, 'papillae') (Credland, 1976; Thorpe, 1933). Tracheal gills arise from the integument and are sites of oxygen uptake (Apodaca and Chapman, 2004; Erikson and Moeur, 1990; Thorpe, 1933; Wingfield, 1939); however, they also possess chloride cells (ionocytes; see Glossary), which are putative sites for ion uptake (Ahmad, 2017; Buchwalter et al., 2003; Filshie and Campbell, 1984; Kapoor and Zachariah, 1973a,b; Nowghani

Table 1. Description of the gills and osmoregulatory structures present in addition to organs associated with the gastrointestinal (GI) tract in freshwater insects

Order	Type of gills	Location of gills	Gill function in osmoregulation	Osmoregulatory components in addition to GI tract
Ephemeroptera	Tracheal	Abdomen	Yes, ion uptake[1,2]	Ionocytes on gills expressing Na+/K+-ATPase and V-type H+-ATPase; ionocytes on body surfaces[1,2]
Plecoptera	Tracheal	Thorax, caudal	Yes, ion uptake[3,4]	Ionocytes on gills[5]
Trichoptera	Tracheal, blood	Abdomen, caudal[5]	Unknown	Chloride epithelia on body surfaces[6]
Megaloptera	Tracheal	Abdomen[7,8]	Unknown	Unknown
Odonata	Tracheal	Internal/caudal	Yes, ion uptake	Internal chloride epithelial pads and gills[9]
Coleoptera	Tracheal	Elytral surfaces[10]	Unknown	Unknown
Hemiptera	None	–	–	Ionocytes on body surfaces[11]
Neuroptera	Tracheal	Abdomen[12]	Unknown	Unknown
Diptera	Blood	Caudal	Yes, ion uptake[13,14]	Anal papillae (gills)

[1]Nowghani et al. (2017); [2]Nowghani et al. (2019); [3]Kapoor and Zachariah (1973b); [4]Kapoor (1978); [5]Kapoor and Zachariah (1973a); [5]Morgan and O'Neil (1931); [6]Wichard and Komnick (1973); [7]Barclay et al. (2005); [8]Wang and Zhang (2010); [9]Komnick (1978); [10]Kehl and Dettner (2009); [11]Komnick and Wichard (1975); [12]Jandausch et al. (2019); [13]Donini and O'Donnell (2005); [14]Nguyen and Donini (2010).

et al., 2017; Wichard and Komnick, 1971). In *Hexagenia rigida* (Ephemeroptera), the ionocytes express the ion-motive enzymes V-type H+-ATPase and Na+/K+-ATPase, and Na+ uptake occurs at the tracheal gills where the ionocytes are located (Nowghani et al., 2017). Papillae are not involved in oxygen uptake but are sites of active ion uptake and passive water uptake, hence they play an important role in regulating salt and water balance (Donini and O'Donnell, 2005; Koch, 1938; Marusalin et al., 2012; Nguyen and Donini, 2010; Stobbart, 1971a,b,c; Wigglesworth, 1932). Apart from tracheal gills and papillae, some aquatic freshwater insects (Ephemeroptera, Plecoptera, Hemiptera, Trichoptera) have ionocytes interspersed amongst epithelial cells of their integument or arranged in fields of transporting epithelia. These function to take up ions from their dilute habitat (Komnick, 1977; Komnick and Wichard, 1975). Some members of Odonata have internal gills with ion-transporting epithelia at their base, which are housed in a specialized area of the rectum and are important for regulating salt and water balance (Green, 1979; Khodabandeh, 2006; Komnick, 1982; Miller, 1994). In this case, water enters and exits the rectal chamber via the anus, a process called rectal ventilation, to facilitate ion exchange (Miller, 1994). All freshwater insects have adapted their physiology for life in a dilute, hypo-osmotic environment where excretion of excess water and scavenging of ions are prioritized. The salinization of freshwater could thus have serious consequences for these insects.

The effect of salt on the physiology of salt and water balance in freshwater insects

Here, we will discuss the effect of salt on the physiology of osmoregulatory organs and structures in freshwater insects. Much of this work has been performed on mosquito or midge larvae, but we aim to discuss data from other freshwater insects where appropriate. It is clear that much research remains to be done in this area before we are fully able to understand the effect of salinization on freshwater insects.

Midgut, gastric caecae and Malpighian tubules

Most of the research on how salinity affects the osmoregulatory functions of the gastrointestinal tract and Malpighian tubules of freshwater insects has been conducted on dipteran larvae. Studies have examined transcellular (see Glossary) and paracellular transport in different parts of the gastrointestinal tract, and a study on mosquito larvae has provided the only information about hormonal responses to salinity in aquatic insects (Clark and Bradley, 1997). Higher levels of the hormone serotonin are present in the

hemolymph of *Aedes aegypti* larvae at higher salinity; combined with knowledge gained from other studies that have examined ion transport mechanisms in the gastrointestinal tract of mosquitoes, this information can help us to understand the responses of freshwater insects to increased salinity. However, studies on freshwater insects of other orders are required because their specific gut physiology may be different from that of dipterans.

The midgut, gastric caeca and Malpighian tubules are organs involved in regulating salt and water balance in insects. In mosquito larvae, the gastric caeca cells actively transport ions and express aquaporins (water channels) (D'Silva et al., 2017a; Misyura et al., 2020; Volkmann and Peters, 1989); the epithelium of the midgut is a site of ion transport (Boudko et al., 2001; Clark et al., 1999; Jagadeshwaran et al., 2010; Onken et al., 2008); and the Malpighian tubules actively transport ions into their lumen, establishing an osmotic gradient that drives water from the haemolymph into the tubule lumen, which produces primary urine (Weng et al., 2003). Serotonin has been shown to stimulate the ion-transport functions of the Malpighian tubules, the midgut and the gastric caeca of mosquito larvae (Clark and Bradley, 1997; Clark et al., 1999; D'Silva and O'Donnell, 2018). At the midgut, this would cause luminal alkalinization and acidification of the anterior and posterior midgut regions, respectively, which is thought to support digestion (Jagadeshwaran et al., 2010; Onken et al., 2008). Alterations in the expression of septate junction proteins (see Glossary) in the midgut in response to changes in salinity have also been noted, but it is not known whether these are a result of tissue responses to serotonin. The septate junction proteins regulate the permeability of the paracellular pathway by determining the permeability properties of the septate junctions (see Glossary; Jonusaite et al., 2016a). In general, it appears that the abundance of various midgut septate junction proteins increases with salinity (Fig. 1A, Table 2) (Jonusaite et al., 2016b, 2017a,b). For example, the protein abundance of Kune (a septate junction protein) in the posterior midgut increases with salinity, and it localizes to the junctional area of adjacent epithelial cells, where the septate junctions are located (Jonusaite et al., 2016b). Furthermore, the transcript abundance of snakeskin and mesh, septate junction proteins that also localize to the junctional areas between adjacent cells, increases with salinity in the midgut, coinciding with an increase in the permeability to polyethylene glycol 400 (PEG-400) (Jonusaite et al., 2017a). Because the midgut is an absorptive organ, this may aid in the absorption of water into the hemolymph to help maintain body fluid volume at higher salinity (Jonusaite et al., 2017a). Gliotactin, another septate junction protein, also increases in abundance in the
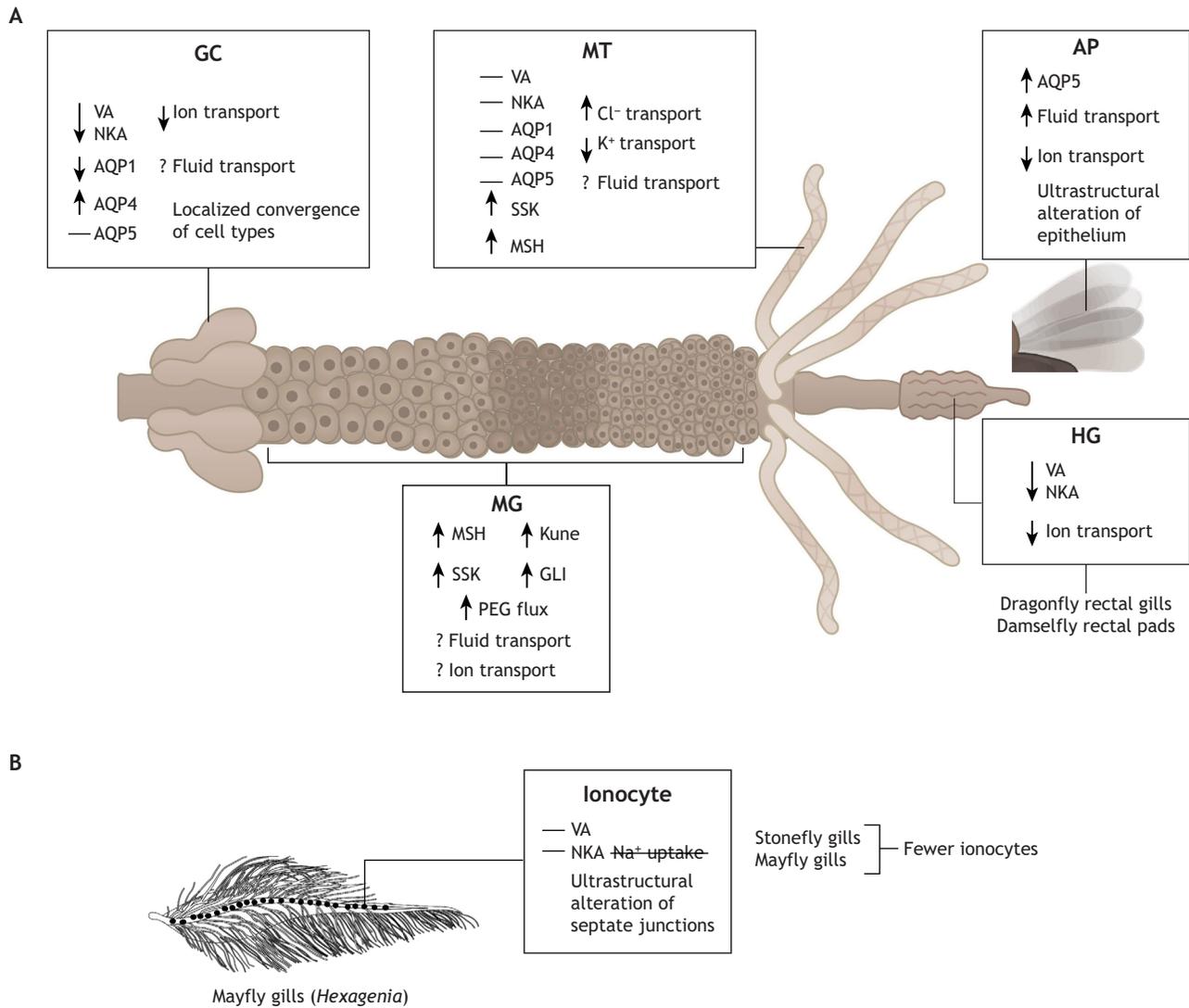
A

GC

↓ VA
↓ NKA   ↓ Ion transport

↓ AQP1   ? Fluid transport

↑ AQP4   Localized convergence
— AQP5   of cell types

MT

— VA
— NKA
— AQP1   ↑ Cl⁻ transport
— AQP4   ↓ K⁺ transport
— AQP5   ? Fluid transport
↑ SSK
↑ MSH

AP

↑ AQP5

↑ Fluid transport

↓ Ion transport

Ultrastructural
alteration of
epithelium

MG

↑ MSH   ↑ Kune

↑ SSK   ↑ GLI

↑ PEG flux

? Fluid transport

? Ion transport

HG

↓ VA
↓ NKA

↓ Ion transport

Dragonfly rectal gills
Damselfly rectal pads

B

Ionocyte

— VA
— NKA ~~Na⁺ uptake~~

Ultrastructural
alteration of
septate junctions

Stonefly gills
Mayfly gills ⎤— Fewer ionocytes

Mayfly gills (*Hexagenia*)

Fig. 1. Summary of known salinity-induced molecular and functional changes in osmoregulatory organs of freshwater insects. (A) A representative insect gastrointestinal tract and anal papillae are shown here to illustrate the changes that occur with salinity observed in freshwater insects. (B) A depiction of a single tracheal gill from the mayfly *Hexagenia rigida* with ionocytes (black circles, not drawn to scale). Upward and downward arrows signify an increase and decrease in expression and/or activity, respectively. A hyphen signifies no change in expression and/or activity. Where text is crossed out, this signifies that this function no longer occurs with salinity. 'Fewer ionocytes' means that in salinity, the number of ionocytes decreases. 'Localized convergence of cell types' refers to the two cell types being evenly distributed along the organ's length with salinity. 'Ultrastructural alteration' refers to ultrastructural changes to cells or epithelial structure. AP, anal papillae; AQP1, AQP4 and AQP5, aquaporin 1, 4 and 5; GC, gastric caeca; GLI, gliotactin; HG, hindgut; MG, midgut; MSH, mesh; MT, Malpighian tubules; NKA, Na⁺/K⁺-ATPase; PEG, polyethylene glycol (a paracellular permeability marker); SSK, snakeskin; VA, V-type H⁺-ATPase. Tracheal gill image adapted from Nowghani et al. (2019).

anterior midgut with increased salinity, but gliotactin tightens the permeability of the midgut to PEG-400 (Jonusaite et al., 2017b). Clearly, changes in salinity result in modulation of septate junctions and paracellular permeability in the midgut, which is likely driven by altering the expression of specific septate junction proteins (Table 2). There are many septate junction proteins, only a subset of which have been investigated; thus, this is an important area for future research.

At the gastric caeca, stimulation of ion transport by serotonin seems to be confined to the ion-transporting cells that express V-type H⁺-ATPase on both the apical and basolateral membranes, and that are primarily evident in the distal region of the gastric caeca of freshwater-reared larvae (D'Silva and O'Donnell, 2018). These cells also express at least two aquaporins: AaAQP5, which is localized on the basolateral membrane and has been shown to

transport water, and AaAQP4, which appears as diffuse staining throughout the cells in immunohistochemical sections and preferentially transports solutes such as trehalose (Misyura et al., 2020). In saline-exposed larvae, the ion-transporting cells are interspersed among Na⁺/K⁺-ATPase-expressing digestive cells that, under freshwater conditions, are confined to the proximal regions of the gastric caeca (Table 2) (D'Silva and O'Donnell, 2018; D'Silva et al., 2017b; Volkmann and Peters, 1989). Furthermore, the overall activity of both V-type H⁺-ATPase and Na⁺/K⁺-ATPase is reduced in the gastric caeca of saline-water larvae (Fig. 1A, Table 2), which correlates with a decrease in the density of mitochondria (D'Silva et al., 2017b; Volkmann and Peters, 1989). Functionally, *in vitro,* the gastric caeca of saline water larvae show lower rates of ion transport than those from freshwater larvae (D'Silva et al., 2017b). Furthermore, in brackish-water-reared larvae, serotonin does not

Table 2. Salinity responses of organs associated with the gastrointestinal tract of freshwater insects

Order	Gastric caeca	Midgut	Malpighian tubules
Diptera	Present; cell type localization pattern altered; decreased activity of ionomotive enzymes coincides with decrease in ion transport	Present; increase in SJ gene expression coincides with change in epithelial permeability	Present; increase in chloride transport; increase in SJ gene expression
Plecoptera	Present, physiology unknown	Present, enzymatic physiology studied[1], effect of salinity unknown	Present, ultrastructure studied[2], effect of salinity unknown
Trichoptera	Absent	Present, food types and transit time studied[3], effect of salinity unknown	Present, accumulation of copper in cells observed[4], effect of salinity unknown
Megaloptera	Absent	Present, effect of salinity unknown	Present, effect of salinity unknown
Odonata	Absent	Present, expression of ionomotive enzymes studied[5], effect of salinity unknown	Present, expression of ionomotive enzymes studied[5], effect of salinity unknown
Coleoptera	Absent	Present, effect of salinity unknown	Present, effect of salinity unknown
Hemiptera	Absent	Present, effect of salinity unknown	Present, ion and fluid secretion studied[6], effect of salinity unknown
Neuroptera	Absent	Present, effect of salinity unknown	Present, effect of salinity unknown
Ephemeroptera	Absent	Present, effect of salinity unknown	Present, ion and fluid secretion studied[7], effect of salinity unknown

[1]López-Rodríguez et al. (2012); [2]Kapoor (2011); [3]Sangpradub and Giller (1994); [4]Darlington and Gower (1990); [5]Khodabandeh (2006); [6]Cooper et al. (1989); [7]Nowghani et al. (2017). SJ, septate junction.

stimulate gastric caeca to the same extent as observed in freshwater larvae; this may be due to the overall reduction and redistribution of ion-transporting cells from the distal region to all areas of the gastric caeca in these larvae (D'Silva and O'Donnell, 2018). Hence, although serotonin levels are elevated in the haemolymph of larvae that encounter higher salinity, the effects of serotonin on the gastric caeca of these larvae appear to be minimal. This reduced ion-transport activity – and the observation that serotonin is likely to have less of an effect on the gastric caeca under these conditions – is likely to relate to the higher levels of salts ingested by saline-water larvae. This reduces the osmotic gradient across the gastric caeca; consequently, lower rates of ion transport are sufficient for osmoregulation and maintenance of digestive processes (D'Silva et al., 2017b). The digestive cells express a water-transporting aquaporin, AaAQP1, on their apical membrane and AaAQP5 on their basal membrane; thus a route for transepithelial water transport is present across these cells, which are distributed along the entire length of the gastric caeca in saline larvae (Misyura et al., 2020).

At the Malpighian tubules, serotonin activates ion transport, which increases fluid secretion and hence urine production (Clark and Bradley, 1997). The expression and relative abundances of three aquaporins, AaAQP1, AaAQP4 and AaAQP5, remain consistent whether larvae develop in freshwater or saline water (Fig. 1A) (Misyura et al., 2020). Together, this suggests that larvae that encounter higher salinity would have higher rates of ion transport and, hence, increased fluid secretion by their Malpighian tubules relative to freshwater larvae. This assumption is supported by the response of tubules to cAMP, the second messenger of serotonin, which is not affected by rearing salinity, thus also indicating that higher levels of circulating serotonin in the haemolymph of higher-salinity reared larvae should result in higher rates of ion and fluid transport by their Malpighian tubules (Donini et al., 2006). This may aid in clearing some of the higher NaCl levels from the haemolymph at higher salinity (Donini et al., 2006). Lastly, larvae reared in relatively high salinity show increased Cl^- secretion by unstimulated Malpighian tubules, along with increases in transcript abundance of snakeskin and mesh, two septate junction proteins that may regulate the paracellular permeability of Malpighian tubules, through which Cl^- has been shown to cross the epithelium (Table 2) (Jonusaite et al., 2017a; Yu and Beyenbach, 2001).

In Malpighian tubules of midge larvae, ion-motive pump activity, fluid secretion rate and composition do not differ between freshwater and saline-water-reared larvae, but fluid secretion rates increase significantly when larvae are reared in ion-poor water (Jonusaite et al., 2013; Zadeh-Tahmasebi et al., 2016). However, the effectiveness of serotonin in stimulating ion and fluid secretion is significantly reduced in Malpighian tubules of salinity-reared larvae (Zadeh-Tahmasebi et al., 2016). This last observation suggests that midge larvae Malpighian tubules differ from those of mosquito larvae in their response to salinity and/or serotonin.

The Malpighian tubules of mayfly nymphs have also been investigated in the context of osmoregulation, but we do not have a clear understanding of their function. The morphological structure of the Malpighian tubules in mayfly nymphs is comparatively complex (Gaino and Rebora, 2000a). The distal portions of the tubules are coiled to varying extents, and connect to the gut either directly or through connecting tubes and trunks (Gaino and Rebora, 2000a,b; Nicholls, 1983; Nowghani et al., 2017). Based on their cellular ultrastructure, the distal portion of the Malpighian tubules may be the site of ion secretion, whereas the trunk may be a site for reabsorption; however, Na^+ secretion and K^+ absorption are detected at both of these regions, suggesting that tubule function is complex (Gaino and Rebora, 2000a; Nicholls, 1983; Nowghani et al., 2017). It is clear that the mayfly Malpighian tubules, like in other insects, play a role in osmoregulation (Table 2); however, much more work is needed to understand their physiology before we can even begin to understand effects of salinity on their function. To our knowledge, the effects of salinity on Malpighian tubules of other freshwater insects, except those covered above, have not been studied.

Hindgut and anal papillae epithelia

The ion-transporting epithelia found in the hindgut and anal papillae of some insects will be discussed together here, because their ultrastructure is similar and the anal papillae embryologically arise from the hindgut. The role of these structures in maintaining salt and water balance has been investigated in Odonata and Diptera.

Dragonfly nymphs (Anisoptera) have a specialized enlarged rectum housing the gills (Rich, 1918). This 'rectal gill chamber', as it is called, is involved in locomotion, respiration, fat storage and ion uptake (Komnick, 1982). For a detailed description of the structure

and morphology of the rectal gill chamber, see Rich (1918) and Komnick (1978, 1982). The rectum of damselfly nymphs (Zygoptera) does not have an enlarged gill chamber because they possess external caudal gills; however, an ion-transporting epithelium arranged as three pads lines the rectal lumen (Khodabandeh, 2006; Komnick, 1978). The ultrastructures of the dragonfly and damselfly rectal ion-transporting epithelia are similar, with extensive apical folding (facing the rectal lumen) and interdigitations of the basolateral membrane, both features serving to increase the surface area for transport (Kukulies and Komnick, 1983). The ultrastructure of the rectal and anal papillae epithelia of dipterans is also similar, although that of the anal papillae is a syncytium in some species (e.g. *Chironomus riparius*, *Aedes aegypti*). In *C. riparius*, *Chironomus tentans* and *A. aegypti*, the papillae have a simple epithelium with basal and apical membrane folding, and the rectal epithelium of *A. aegypti* also exhibits membrane folding (Credland, 1976; Jarial, 1995; Meredith and Phillips, 1973a; Sohal and Copeland, 1966).

In these epithelia, Na^+/K^+-ATPase on the basolateral membrane acts as a route for Na^+ transport into the haemolymph (Del Duca et al., 2011; Jonusaite et al., 2013; Khodabandeh, 2006; Komnick, 1978; Patrick et al., 2006). Not much else is known about the molecular transport machinery of the ion-transporting epithelia of Odonata, but much more is known for dipterans. In *A. aegypti* anal papillae, ion uptake at the apical side is energized by V-type H^+-ATPase (Del Duca et al., 2011; Patrick et al., 2006). Sodium uptake is thought to occur through Na^+ channels driven by the electrochemical gradient established by the ATPases, whereas Cl^- uptake probably occurs through a Cl^-/HCO_3^- exchanger (Del Duca et al., 2011; Stobbart, 1971c). Carbonic anhydrase inhibitors affect NaCl uptake, indicating that carbonic anhydrase provides the necessary H^+ and HCO_3^- for exchange (Del Duca et al., 2011). In *C. riparius*, no transporters have been localized in the anal papillae, but a Na^+/H^{-+} exchanger mediates Na^+ uptake, and carbonic anhydrase supplies H^+ (Nguyen and Donini, 2010). In the rectum of *C. riparius*, V-type H^+-ATPase on the apical membrane drives K^+ reabsorption through K^+ channels (Jonusaite et al., 2013). In Odonata, a putative anion ATPase (Cl^-/HCO_3^- exchange) stimulated by Cl^- has been suggested, and active Cl^- uptake has been shown; however, the molecular identity and localization of this putative anion ATPase remain elusive (Gerencser and Zhang, 2003; Komnick, 1982; Leader and Green, 1978). Furthermore, the possibility of an apical V-type H^+-ATPase operating in concert with carbonic anhydrase to promote Cl^-/HCO_3^- and Na^+/H^+ exchange should be investigated (Kirschner, 2004).

Examination of the gills in the rectal chamber of *Aeshna cyanea* larvae (Odonata) held in different salinities revealed that the size of the ion-transporting epithelia of the gills is dependent on external salinity (Komnick, 1978). Nymphs held in higher salinity have smaller gills than those held in more dilute media (Komnick, 1978). This is indicative of the gills in the rectal chamber actively taking up NaCl when hyper-regulating in a dilute habitat, and also suggests that the ion-transporting epithelia is not as important in nymphs that are chronically exposed to salt. Incidentally, total ATPase and Na^+/K^+ ATPase activity of homogenized rectums from nymphs held in dilute conditions were higher relative to those of nymphs held at higher salinity (Fig. 1A) (Komnick, 1978).

In *C. riparius* (Diptera), the activity of the primary ion-motive pumps, Na^+/K^+-ATPase and V-type H^+-ATPase, is ~10 times higher in the rectum compared with other segments of the gastrointestinal tract and Malpighian tubules (Jonusaite et al., 2013). Ion reabsorption is significantly reduced when these pumps

are pharmacologically inhibited (Jonusaite et al., 2013). The activity of both pumps as well as the magnitude of K^+ reabsorption at the rectum of larvae reared in high salinity are lower than those from freshwater-reared larvae (Jonusaite et al., 2013). Therefore, the function of ion-transporting epithelia in the recta of Odonata and Diptera appear to be altered by salinity in a similar manner.

The anal papillae epithelium of mosquito larvae show ultrastructural alterations caused by differences in external salt levels (Sohal and Copeland, 1966; Wigglesworth, 1932, 1933). Compared with larvae held in freshwater, the apical membrane shows reduced folding and contains fewer mitochondria when larvae are held in salt water, but, paradoxically, the oxygen consumption of anal papillae does not change (Edwards, 1982; Sohal and Copeland, 1966). These ultrastructural changes are associated with reduced ion transport by the papillae of larvae in salt water (Donini et al., 2007). There is some evidence that the size of the anal papillae decreases as salinity increases in *Chironomus oppositus*, which is consistent with an observed increase in anal papillae size of *C. riparius* larvae that are held in ion-poor water (Kefford et al., 2011; Nguyen and Donini, 2010). These observations seem to reaffirm the importance of the anal papillae as sites for ion uptake in dilute water but do not shed light on potential roles in salt water. To our knowledge, nothing else is known about how chironomid anal papillae respond to salinity.

The above studies were performed with freshwater-adapted *A. aegypti* or chironomids. Interestingly, if *A. aegypti* are adapted to high salinity for at least 20 generations, then the length and width of the anal papillae of salt-water-adapted larvae are greater than those of freshwater larvae, but it is unclear whether there are any ultrastructural differences (Surendran et al., 2018). Nevertheless, this suggests that the anal papillae may play an important physiological role in salt-water-adapted mosquitoes, contrary to previous observations that suggested that the anal papillae were a liability when larvae encounter salt water because they are utilized for ion uptake (Wigglesworth, 1933). This is an important observation, because it suggests that alterations to the anal papillae are dependent on whether larvae arise from freshwater- or salt-water-adapted mosquitoes.

The physiological role that anal papillae may play in salt-water-adapted mosquitoes is not known. It would make sense for the anal papillae to excrete ions in salt water to help eliminate the salts that are imbibed. This has been previously suggested because no ultrastructural differences in the anal papillae of a salt-water mosquito species, *Aedes campestris*, were observed when these larvae were held in freshwater or salt water (Meredith and Phillips, 1973b). Another possibility is that the anal papillae are important in regulating water fluxes when larvae are in salt water. It was shown that anal papillae of *A. aegypti* held in salt water are more permeable to water than those of freshwater-held larvae, which coincides with a thinner cuticle and alterations in the transcript abundance of cuticle protein genes (Ramasamy et al., 2021; Wigglesworth, 1933). AaAQP5, which was shown to transport water in Malpighian tubules, is expressed at much higher levels in the anal papillae of salt-water-reared *A. aegypti* larvae (Fig. 1A) (Akhter et al., 2017; Misyura et al., 2017). In addition, the pH of external salt water also changes the size of the anal papillae: alkaline salt water increases the length of the papillae (Clark et al., 2007). Finally, although the anal papillae epithelium in mosquitoes is a syncytium, it expresses septate junction proteins, and the expression of some is altered by salinity (Jonusaite et al., 2016b, 2017a,b). Apart from forming the septate junction complex, septate junction proteins have other cellular functions, such as participating in determining polarity in epithelia.

To summarize, in freshwater, the ion-transporting epithelia of the odonata rectal chamber, the chironomid rectum and the dipteran anal papillae take up ions and, in some cases, water from the animal's dilute habitat. Salinity reduces the activity of ion transporters and can lead to changes in the ultrastructure or size of these structures, which reduces ion uptake. These changes undoubtedly help the insects survive salt exposure by limiting salt accumulation in the body fluids. Potential roles beyond limiting ion uptake for anal papillae in mosquitoes that have inhabited brackish water for several generations is a possibility given some recent findings that these larvae have larger anal papillae and have transcriptome differences highlighted by reduced expression of cuticle protein genes and a thinner cuticle structure (Ramasamy et al., 2021; Surendran et al., 2018). There is also evidence of acclimation to salinity at the organ level from Trichoptera larvae. When transferred to salt water, larvae of *Anabolia nervosa* and *Limnephilus stigma* repeatedly ingest and regurgitate salt water, lose the ability to regulate levels of body fluid and show significantly reduced fluid excretion (Sutcliffe, 1962). These larvae also suffer severe disruption to their alimentary tract: the peritrophic membrane (see Glossary) of the midgut is often expelled, and the rectal wall is everted (Sutcliffe, 1962). These more severe effects on the gut can be limited with gradual acclimation to salt water (Sutcliffe, 1962). Behavioural responses to salinity appear to be determined by the salinity of the Trichoptera larvae's original habitat, suggesting that there is a degree of acclimation that can occur over multiple generations (Carter et al., 2020). Some evidence of acclimation is also seen from measurements of stress biomarkers in Trichoptera larvae collected from areas with different salinity (Sala et al., 2016). More physiological studies at the molecular, cellular and tissue levels of the anal papillae and abdominal chloride epithelium are needed in order to understand the impacts of salinity on the physiology of Trichoptera larvae. Furthermore, an in-depth look at the ion and water transporter genes at the protein level, including their localization in the anal papillae epithelium of mosquito larvae, could help to determine whether anal papillae can secrete ions in brackish water or regulate water fluxes between the insect and its environment.

Ionocytes of tracheal gills and body surfaces

The nymphs of Ephemeroptera, Trichoptera and Plectoptera possess tracheal gills that can have respiratory and/or osmoregulatory functions (Brittain, 1982; Kapoor and Zachariah, 1973a,b; Morgan and O'Neil, 1931; Wingfield, 1939). The osmoregulatory function of tracheal gills was postulated when putative chloride cells (ionocytes) that resembled those of fish gills were discovered on their surface (Wichard and Komnick, 1971). A specialized, thinner area of cuticle (the porous plate) covers the apex of the ionocytes, presumably facilitating the passage of small solutes such as ions, and ionocytes have been implicated as sites of NaCl uptake (Filshie and Campbell, 1984; Komnick and Stockem, 1973; Wichard et al., 1972). More recent experiments on the tracheal gills of the mayfly *Hexagenia rigida* demonstrated that these ionocytes express the ion-motive pumps Na^+/K^+-ATPase and V-type H^+-ATPase, which probably drive NaCl uptake (Nowghani et al., 2017). Furthermore, Na^+ uptake occurs along the central axis of tracheal gills where the ionocytes are located, providing further evidence for the role of these cells and the tracheal gills in ion uptake (Nowghani et al., 2017, 2019). In the caddisflies, the tracheal gills are important in carbon dioxide elimination (Morgan and O'Neil, 1931), but have not yet been shown to take up ions; the anal papillae are likely to function in ion uptake in these insects (Vuori, 1994). To the best of

our knowledge, there are no studies that directly implicate the gills of stoneflies in ion uptake; however, it has been observed that stonefly larvae are able to sequester ions from the surrounding water: individuals without food in stream water are able to maintain haemolymph osmolality and ion levels, whereas those in deionized water show reduced haemolymph osmolality and ion levels (Colby, 1972). Cadmium uptake has also been observed in stoneflies (Buchwalter et al., 2008).

In mayflies, ionocytes are found across most of the body surfaces, whereas in caddisflies there are fields of ion-transporting epithelia on abdominal segments; in waterbugs (Hemiptera), ionocytes are distributed among the regular epithelial cells of the integument and share ultrastructural features with ionocytes on tracheal gills of other insects (Komnick and Abel, 1971; Komnick and Wichard, 1975; Wichard and Komnick, 1973). In caddisflies, the abdominal ion-transporting epithelium is thicker than the surrounding epithelium and underlies an area of the cuticle that is thinner than the surrounding area (Wichard and Komnick, 1973). The epithelial cells possess a greater abundance of mitochondria than the cells of the surrounding hypodermis; the mitochondria are concentrated in the elaborately folded apical region, where they can provide ATP to energize transport (Wichard and Komnick, 1973). Chloride precipitation occurs at the surface of this epithelium, indicating that these cells are sites of chloride uptake (Wichard and Komnick, 1973). In the hemipterans, the number of ionocytes on the thorax increases over successive instars; however, this is dependent on salinity – fewer ionocytes are present on insects in higher salinities (Komnick and Wichard, 1975). This suggests that ionocytes are important in dilute conditions, presumably taking up ions for osmoregulation, but this has yet to be shown directly.

The effects of salinity on the freshwater insects that possess tracheal gills and/or ion-transporting cells and epithelia on body surfaces has been limited mostly to whole-animal studies (Box 1), with very little research conducted at the molecular, cellular or organ level. Furthermore, mayflies have received the vast majority of attention in these studies because they are generally more sensitive to salinization than other freshwater insects, but the reasons for this are not clear (Beermann et al., 2018; Dowse et al., 2017; Kefford, 2019; Kefford et al., 2012; Pond, 2012). Although this Review is aimed at covering studies undertaken below the whole-organism level, in the following we present information from a subset of whole-organism studies to show that this important work can inform research at lower hierarchical levels.

A comparison of Na^+ influx in a mayfly and a caddisfly showed that the caddisfly reaches maximum rates of influx at a lower external sodium concentration than the mayfly, suggesting that mayflies would continue to take up Na^+ at relatively high rates as salinity increases, which would be detrimental to survival (Scheibener et al., 2016). The species and combination of ions are also important when considering effects of salts; for example, Na^+ is antagonistic to SO_4^- uptake in the mayfly *Neocloeon triangulifer* (Scheibener et al., 2017). Furthermore, temperature and oxygen levels can interact with salinity to affect how an insect allocates energy to important processes such as growth and homeostasis (Verberk et al., 2020). Temperature alone has profound effects on ion-transport rates and salt toxicity; in general, higher temperature leads to increased ion uptake (Orr and Buchwalter, 2020). That said, results depend on the ion measured and the species of insect being assessed. For example, the mayflies *Maccaffertium* spp. and *Isonychia sayi* show increased Ca^{2+} uptake with higher temperature, whereas *N. triangulifer* does not, but both *N. triangulifer* and *I. sayi* show higher Na^+ uptake with higher temperature and *Maccaffertium*

spp. do not (Orr and Buchwalter, 2020). Furthermore, survival of *N. triangulifer* in SO_4^--contaminated water is temperature dependent (Orr and Buchwalter, 2020). These studies undertaken at the whole-animal level illustrate the complexity and variability of the osmoregulatory mechanisms in freshwater insects and the effects of salinization on these mechanisms. They also inform future research, which can be aimed at the molecular to organ levels. For example, research at lower levels of organization has been mostly limited to the effects of NaCl (see below); however, whole-animal studies clearly show that other salts need to be considered, as does the interaction of different salts and how these affect ion-transporting epithelia and ionocytes of freshwater insects.

Existing research on ionocytes on the tracheal gills of stoneflies shows that high concentrations of salt water (leading to 80% mortality) result in degeneration of the ionocytes of the gills of survivors, which suggests that the insects are responding by removing ionocytes in an attempt to limit ion uptake (Fig. 1B, Table 3) (Kapoor, 1978). In a similar study, in surviving nymphs of the mayfly *Callibaetis coloradensis* exposed to high levels of salt water, a significantly lower number of ionocytes were present compared with nymphs in more dilute freshwater, suggesting that mayflies might be able to regulate ionocyte numbers to limit salt uptake (Wichard et al., 1973). However, no effect on the number of ionocytes, ionocyte surface area or ionocyte fractional surface area was found in gills of *Hexagenia rigida* nymphs exposed to similar levels of NaCl for 7 days, an exposure that was determined to be sub-lethal (Nowghani et al., 2019). This high, but sub-lethal, salinity exposure does not alter ion-motive enzyme activities or ion-motive enzyme immunoreactivity of ionocytes; furthermore, it does not alter the ultrastructure of ionocytes on the gills (Nowghani et al., 2019). Interestingly, despite the lack of effects on ultrastructure and ion-motive enzymes in the ionocytes, the gills of salinity-exposed nymphs secrete Na^+, in stark contrast to the uptake of Na^+ measured at the gills of freshwater nymphs (Table 3) (Nowghani et al., 2019). In freshwater nymphs, the regions of paracellular occlusion – including septate junctions in the epithelia of the tracheal gills – possess a more convoluted structure with more septa than observed in the salinity-exposed nymphs (Nowghani et al., 2019). This suggests that the tracheal gill epithelia of salinity-exposed nymphs

is 'leakier' than that of freshwater nymphs; this may be a mechanism to secrete excess salt that accumulates in the body (Jonusaite et al., 2016a; MacMillan et al., 2017). More research at the molecular and ultrastructural level is needed on ionocytes and ion-transporting epithelia on body surfaces of freshwater insects in order to understand how salinity affects their osmoregulatory physiology.

Conclusions and perspectives

Freshwater salinization is an important challenge that is occurring throughout the world (Iglesias, 2020; Rahman et al., 2019; Schuler et al., 2019). Work in the last two decades has examined the effects of salinization on freshwater invertebrate fauna at the whole-organism, population and community levels (Benbow and Merritt, 2004; Blasius and Merritt, 2002; Braukmann and Böhme, 2011; Hassell et al., 2006; Hills et al., 2019; Hintz and Relyea, 2019; Hintz et al., 2017; Pond, 2012), revealing that different groups of invertebrates have different sensitivities to the various sources of salt water contamination (Iglesias, 2020; Kefford et al., 2012). Recently, researchers have continued to examine the effects of salt contamination on the osmoregulatory physiology of freshwater insects at the whole-organism level (Orr and Buchwalter, 2020; Scheibener et al., 2016; Scheibener et al., 2017). This sheds light on how ion uptake and excretion rates differ for specific ions and between different insects. This work has also led to the theory that the observed effects of salinity on freshwater insects may result from increased energy requirements needed to rid the insect of unwanted salts (Verberk et al., 2020).

What is lacking is the understanding of how salinity affects osmoregulation at the molecular, cellular, organ and organ system levels in freshwater insects. In this respect, there is some information available on the fundamental freshwater osmoregulatory physiology of some groups such as Diptera, Odonata and Ephemeroptera, with the most comprehensive information on mosquitoes and midges; however, even in these insects, our understanding is not complete. There is even less information on how salinity affects osmoregulatory physiology at the molecular to organ system levels in these insects; however, again, most knowledge has been accrued for mosquitoes and midges. Nevertheless, we can make some general conclusions with the limited information that we have, and

Table 3. Salinity responses of hindgut, anal papillae and ionocytes of freshwater insects

Order	Hindgut	Anal papillae	Ionocytes on gills/body surfaces
Diptera	Reabsorption of ions and ATPase activity decreases with salinity[1]	Salinity results in decrease in cell membrane surface area, decrease in ion transport, alterations to size of papillae dependent on length of salinity exposure[2,3,4]	Absent
Plecoptera	Effects of salinity unknown	Effects of salinity unknown	Salinity decreases number of ionocytes[5]
Trichoptera	Abrupt salinity exposure sometimes results in hindgut eversion[6]	Effects of salinity unknown	Effects of salinity unknown
Megaloptera	Effects of salinity unknown	Absent	Effects of salinity unknown
Odonata	With gills or rectal pads in rectal chamber	Absent	Gills or rectal pads for osmoregulation in rectal chamber, decreased size of gills and lower ATPase activity with salinity[7]
Coleoptera	Effects of salinity unknown	Absent	Effects of salinity unknown
Hemiptera	Effects of salinity unknown	Absent	Effects of salinity unknown
Neuroptera	Effects of salinity unknown	Absent	Effects of salinity unknown
Ephemeroptera	Effects of salinity unknown	Absent	Salinity decreases number of ionocytes in at least one species but not in others[8,9]; salinity reverses sodium uptake to sodium secretion[9]; salinity reduces number of septate junctions[9]

[1]Jonusaite et al. (2013); [2]Sohal and Copeland (1966); [3]Donini et al. (2007); [4]Surendran et al. (2018); [5]Kapoor (1978); [6]Sutcliffe (1962); [7]Komnick (1978); [8]Wichard et al. (1973); [9]Nowghani et al. (2019).

these are summarized in Fig. 1. Epithelia derived from the hindgut (rectal epithelia, rectal gills, anal papillae) generally exhibit indicators of decreased ion transport activity when insects are exposed to salt, which can include decreased expression of ion transporters, ultrastructural changes that limit surface area for exchange and reduced ion fluxes. Because these epithelia are all associated with absorption of ions in freshwater insects, this must be a mechanism to limit salt uptake when insects are faced with elevated salinity. Epithelia of the Malpighian tubules continue to function in ion and fluid transport, but results suggest that this function is modulated such that certain ions are favoured (e.g. Cl^-) over others (e.g. K^+). In the midgut epithelium, salinity alters the permeability of the paracellular pathway, but we do not know what changes, if any, occur in transcellular transport (e.g. ion transporters). In the gastric caecae, ion transporters and ion flux are decreased with salinity and, similar to the hindgut epithelium, this is likely to prevent salts from accumulating in the haemolymph. In structures that possess distinct ionocytes, such as the mayfly tracheal gills, there is evidence that ion uptake ceases at high salinity. The numbers of ionocytes in these structures may be reduced, although this remains to be resolved, and functional changes are at least partly due to alterations in paracellular permeability. In fact, there is ample evidence that the septate junctions may play a significant role in adjusting epithelial function in response to salinity, because changes in septate junction protein expression and occluding junction (including septate junctions) ultrastructure and/or paracellular permeability have been noted in Malpighian tubules, midgut and ionocytes of tracheal gills. In tracheal gills of the mayfly *H. rigida*, the changes in junctional ultrastructure coincide with a reversal in the direction of net Na^+ transport from uptake in freshwater to secretion in salt, which presumably would be largely passive in nature (Nowghani et al., 2019). Coupled with the observation that there is no change in the activities of the predominant energy-consuming, ion-transporting enzymes, this suggests that, at least in this species, no increase in energy demand is needed to rid the insect of excess salt. By contrast, exposure to salinity in the larval stage results in increased energy consumption of adults of the damselfly *Lestes macrostigma* (Lambret et al., 2021).

In order to improve our understanding of the physiological responses of freshwater insects to salinization, research utilizing some well-established experimental protocols, such as electron transmission microscopy for ultrastructure, enzymatic assays and ion-flux assays, should continue to be applied, along with more novel molecular techniques, such as transcriptomics. Furthermore, endocrinological studies assessing neural and hormonal factors are almost entirely lacking in this area. We believe that research should be directed to these types of studies so that we can understand whether and how specific freshwater insects will cope with salt contamination of their freshwater habitats.

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