Malpighian tubule function in desert locusts in relation to ecology, disease and plasticity

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration with others except where specifically indicated in the text. No part of this dissertation has been submitted to any other university in application for a higher degree.

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Summary

Malpighian tubules play a fundamental role in insect osmoregulation and the extrusion of potentially noxious substances, which is essential for coping with ingested toxins or metabolic waste. Potentially harmful compounds, such as alkaloids, are actively extruded by ABC transporters, which includes P-glycoprotein transporters. Here, we studied P-glycoprotein transport in the Malpighian tubules of desert locusts, *Schistocerca gregaria*, which have evolved to feed on a broad variety of plants, including those producing toxic alkaloids. This makes desert locusts an excellent system in which to study P-glycoprotein-mediated extrusion of xenobiotic compounds.

We developed a novel method combining a modified Ramsay assay with the application of a specific substrate and inhibitor of P-glycoproteins. We incubated *ex-vivo* Malpighian tubules in solutions containing the P-glycoprotein substrate, rhodamine B, as a proxy for toxins, in combination with the P-glycoprotein inhibitor, verapamil. By measuring the size and colour of the droplets secreted, we quantified the fluid secretion rate, the rhodamine concentration within the droplet, and the net rhodamine extrusion. We found that locust tubules express P-glycoproteins, the tubules' surface area positively correlating with their fluid secretion rate, and the fluid secretion rate positively increasing the net rhodamine extrusion.

Desert locusts exhibit density-dependent phenotypic plasticity, existing as two extreme phenotypes (with intermediate forms) that differ in their ecology and behaviour depending upon their density. Phenotypic plasticity occurs on multiple time scales; morphology and physiology change within a lifetime or over several generations, while behaviour can change in a few hours. Low densities produce solitarious phase locusts that avoid ingesting potentially harmful compounds, whereas high densities trigger a shift towards a gregarious phase with a broad diet that includes plants

producing harmful compounds. During the transition from solitary to gregarious, locusts show active preference for toxic plants. Since P-glycoproteins are inducible transporters and their activity regulated by diet, we compared the net rhodamine extrusion between solitarious, gregarious and transiens phases fed on a diet containing the alkaloid atropine, and on an alkaloid-free diet. We found no difference in the net rhodamine extrusion between gregarious, solitarious and transiens locusts fed on alkaloid-free diet. However, gregarious and transiens locusts upregulated their P-glycoprotein after only three days of exposure to the diet containing the alkaloid, and gregarious locusts reared on the alkaloid diet downregulated their transporters after being switched to an alkaloid-free diet.

Parasitic infection can also induce phenotypic plasticity. We studied the performance of Malpighian tubules infected by the protozoan *Malpighamoeba locustae*, an infection that is known to damage their structure. Infected tubules had greater surface area and higher fluid secretion rate than uninfected ones. Infection also reduced the net rhodamine extrusion per unit of surface area, suggesting that the damage of the brush border likely reduces the P-glycoprotein density.

Thus, the work presented in this thesis places the physiology of locust Malpighian tubules in the context of ecology, phenotypic plasticity and disease. We suggest that P-glycoprotein transporters expressed by the Malpighian tubules contribute to xenobiotic extrusion, and that their activity is regulated accordingly to diet. In addition, we showed how a parasitic infection can compromise the Malpighian tubule function, potentially leading to water loss, increased energy consumption, and reduced xenobiotic extrusion.

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Publications

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1 General introduction

1.1 Insect-plant interactions

Insects, belonging to the phylum Arthropoda, are the class of animals with the greatest evolutionary success and diversity on Earth, with more than a million species described so far (Grimaldi et al., 2005). This astonishing diversity is thought to be largely driven by their interaction and co-evolution with plants. It is estimated that more than 400,000 of known insect species are herbivores, feeding on over 300,000 species of vascular plant (Schoonhoven et al., 2005). The relationship between plants and insects can also be mutualistic with plants providing food, shelter and reproduction sites, and insects providing defence against herbivores and pollination.

Herbivory has independently evolved several times across various insect orders (Strong et al., 1984). Phytophagous insects are found in the orders Coleoptera (beetles), Lepidoptera (moths and butterflies), Hemiptera (true bugs), Diptera (flies), Hymenoptera (bees, wasps, ants and sawflies), Orthoptera (crickets and grasshoppers), and Phasmida (stick insects). Individuals of the order Phasmida are entirely herbivorous, the orders Lepidoptera and Orthoptera are mostly herbivorous with few carnivorous species, whereas Coleoptera, Diptera and Hymenoptera include a conspicuous number of species with parasitic or predatory habits.

The majority of herbivorous insects are specialised to feed exclusively on one species or genus of plants (monophagous), or on plants belonging to the same family (oligophagous) (Chapman and Joern, 1990; Schoonhoven et al., 2005). Many coleopterans, aphids, and larvae of Lepidoptera are specialist herbivores. For example,

larvae of the cabbage white butterfly, *Pieris brassicae,* feed on different plants within the family Brassicaceae (Chew and Renwick, 1995). At the other end of the spectrum, fewer than 10% of herbivorous insects are generalists (polyphagous), eating plants from three or more different families (Bernays and Graham, 1988). Although generalist species feed on a great variety of plants, they do not accept all plants indiscriminately. For example, the desert locust, *Schistocerca gregaria*, is one of the most polyphagous of insect species but still shows clear preferences, eating some species in small amounts, other species in large amount, and totally rejecting others (Chapman and Joern, 1990).

The relationship between an insect species and its host-plant is neither uniform nor fixed. Variations can be found between sexes (Stockhoff, 1993), and between populations in distinct geographical regions (Maier, 1985) or even within the same region (Singer, 1983). Exogenous factors such as environmental temperature fluctuations (Schalk et al., 1969), and the succession of seasons (Kundu and Dixon, 1995) can alter feeding preferences. In food choice experiments, the Colorado potato beetle, *Leptinotarsa decemlineata,* shows a predilection for foliage of the potato plants, *Solanum tuberosum*, at lower temperatures, but when the temperature rises above 25^{o}C the preference switches to woody nightshade, *Solanum dulcamara* (Bongers, 1970). As an example of seasonal host-plant alternation, some aphids alternate between woody and herbaceous host plants. Most aphid species of the genus *Cavariella* live on willows, *Salix* spp., in winter and spring where sexual reproduction take place, and in summer migrate to their secondary hosts, aromatic flowering plants of the family Apiaceae (formerly Umbelliferae) on which they reproduce parthenogenetically (Kundu and Dixon, 1995) .

Endogenous factors such as changing nutritive needs during development (Stockhoff, 1993), phenotypic plasticity (polyphenism) (Despland, 2005), or associative and non-associative learning can also induce shifts in the host-plant preferences during the life history of insects (Schoonhoven et al., 2005). For instance, the larvae of the corn rootworm, *Diabrotica virgifera,* feed exclusively on corn roots but become polyphagous as adults (Branson and Krysan, 1981). Some insects can even switch from herbivory to carnivory (Fox, 1975), such as the lycaenid caterpillars that are herbivorous in early instars, but predate ant larvae in later instars (Cottrell, 1984). Phenotypic plasticity may also induce a shift in food preference. As an example, *S. gregaria* is a polyphenic species that in its solitarious phase rejects plants containing toxic compounds but in its gregarious phase ingests these plants (Despland, 2005). Locusts and other insects can also learn to avoid a plant after it has induced a temporary malaise (associative learning) (Dethier, 1988; Simões et al., 2013) or can habituate and consume more from a plant containing non-lethal deterrent that they have experienced before (Jermy et al., 1982).

1.2 Arms race between plants and insect herbivores

The co-evolution of herbivorous insects and their host plants is thought to have driven the astonishing diversification of species that we know today (Ehrlich and Raven, 1964). Insect herbivores and plants form dynamic interactions in which plants that evolve physical or chemical mechanisms to reduce insect attack will be favoured. On the other hand, insects that evolve strategies to overcome these defences will be positively selected. Each evolutionary change creates in turn, pressure on insect or plant populations. This continuous cycle of escalating defences and counter-measures is often described as a coevolutionary arms-race (Feeny, 1976).

1.2.1 Plant secondary metabolites

The chemical defences evolved by plants against herbivores are typically called secondary metabolites. These compounds such as alkaloids, phenolics, and sterols are not directly involved in the growth, development and reproduction of plants but rather have important ecological functions (Fraenkel, 1959). The adjective "secondary" implies the existence of "primary" metabolites. These compounds, such as carbohydrates, proteins, and lipids, are involved in the plant primary metabolic processes, and many act as phagostimulants in herbivores being essential nutrients for insect growth (Bernays and Chapman, 1978; Schoonhoven et al., 2005). The distinction between primary and secondary metabolites is arbitrary and the metabolic processes involving them are interconnected (Ruchika et al., 2019).

Secondary metabolites can affect the nutritional physiology of herbivorous insects by: (i) reducing their food intake; (ii) reducing their efficiency of food utilization; and (iii) poisoning or killing them. These mechanisms are not mutually exclusive and secondary metabolites may act through a combination of them. Most plant allelochemicals (the toxic compounds produced for defence), possess repellent odour or unpleasant taste to inhibit the eating behaviour of herbivores. Rejection, acceptance of a plant, or meal size are criteria used to discern between host and non-host plants. For example, the migratory locust, *Locusta migratoria,* and the grasshopper, *Chorthippus parallelus,* either reject entirely or accept only small meals of plants other than grass or grass seedlings (Bernays et al., 1976; Bernays and Chapman, 1970). These authors suggest that is because other plants contain deterrent chemicals.

In an elegant study, Bernays and Chapman (1977) demonstrated that leaves of lettuce (*Lactuca*), ryegrass (*Lolium*) and rose (*Rosa*), that are relatively unpalatable for

L. migratoria, become more palatable after being soaked in chloroform to remove the deterrent compounds. In a reverse experiment, *C. parallelus* rejected velvet grass (*Holcus*) after it was allowed to absorb coumarin from an aqueous solution, demonstrating the deterrent properties of this compound (Bernays and Chapman, 1978, 1975).

Secondary metabolites can also impact the growth and development of herbivorous insects by interfering with food utilization. Although the interference is more pronounced in insects that are not adapted to cope with specific secondary metabolites, even those insects adapted to these compounds can be negatively affected; some insects grow faster on artificial diets than on their own natural host-plant (Bottger et al., 1964; Schoonhoven et al., 2005). For example, the phenolic aldehyde gossypol is present in cotton plants (genus *Gossypium*) negatively affecting both the survival and growth of several insects that feed upon them (Bottger et al., 1964; Shaver and Parrott, 1970). Despite being specialized on the cotton plant, larvae of the Egyptian cotton leafworm, *Spodoptera littoralis,* develop faster when reared on artificial diet with lower concentrations of gossypol (Meisner et al., 1978). Another example comes from the winter moth larvae (*Operophtera brumata*) that feed on the common oak, *Quercus robur,* whose leaves contain tannins. Tannins are phenolic compounds that inhibit the action of enzymes and form indigestible complexes with proteins, reducing the amount of protein available in the food ingested (Feeny, 1969; Goldstein and Swain, 1965). Although winter moth caterpillars include the common oak in their diet, tannins have a detrimental effect on their development, the rate of which is reduced when tannins are added to an artificial diet (Feeny, 1968), or when they are reared on mature oak leaves compared to young leaves, in which the concentration of tannins increased with the age

of the leaves (Feeny, 1970). These two examples indicate that the evolution of specialised feeding on particular host plants by insect herbivores may involve the acquisition of host adaptations.

Lastly, plant allelochemicals can intoxicate and kill insects by acting as natural insecticides. With more than 10,000 compounds described, the alkaloids are one of the most diverse classes of natural substances with pharmacological relevance (Harborne, 2014). To name just a few natural alkaloids, nicotine and atropine are found in the Solanaceae, whereas morphine and codeine are found in Papaveraceae. Nicotine and tropane alkaloids such as atropine (hyoscyamine) (Osman et al., 2013) are lipophilic compounds that can permeate the gut epithelium and blood brain barrier of most insects and act on one particular class of acetylcholine receptors (nicotinic receptors - nAChR) that are present in the insect's nervous system (Murray et al., 1994). These compounds act as an agonist by mimicking the part of the structure of the endogenous neurotransmitter acetylcholine (Dale, 1914; Langley, 1905; Wink, 2006), whereas atropine is an antagonist of a different class of receptors, muscarinic (mAChR), which are also present in locust nervous tissue (Aguilar and Lunt, 1984; Pauling and Petcher, 1970; Trimmer and Berridge, 1985). Other examples of plant secondary metabolites with insecticidal action include the phenolic compound rotenone, produced by some legumes (family Fabaceae), which inhibits the mitochondrial respiratory chain (Wink, 2013), and the pyrethrin neurotoxins produced by the plant Dalmatian chrysanthemum (*Tanacetum cinerariifolium)* which bind to voltage-gated Na^+ channels (Davies et al., 2007). Nicotine was widely used in the past as an insecticide to protect crops, while rotenone and pyrethrins are commonly used today (Walia et al., 2017).

1.2.2 Insect resistance to plant chemicals

Herbivorous insects, exposed as they are to a broad range of potentially toxic substances, have evolved non-mutually exclusive mechanisms to mitigate the effects of these noxious compounds. Some herbivores can elude plant defences by mechanically deactivating them or by avoiding eating specific parts of a plant containing toxins. Others have specialised to deal with these chemicals post-ingestion by detoxifying or excreting them, reducing the permeability of the midgut epithelium or protecting the nervous system with a selectively permeable blood brain barrier. Still others have undergone an evolutionary change in the originally targeted receptor site so that the toxin is no longer able to interact with it, and some even exploit the secondary metabolites by sequestering them to acquire toxicity to deter predators (Frick and Wink, 1995).

1.2.2.1 Behavioural avoidance of plant toxins

Insect avoidance of (or preference for) plant toxins can be genetically inherited or learnt. Oviposition preference is genetically determined in most butterflies and beetles that show a predisposition to lay their eggs on a particular host-plant to ensure that their offspring have access to suitable plants (Fox et al., 2004; Thompson, 1988). Insects can also learn to avoid plants that cause malaise, as in the case of the caterpillars of two moth species that reject *Petunia* plants after consuming them has caused illness (Dethier, 1988). A remarkable behaviour that allows insects to use a particular host plant is by disarming the plant's defences before feeding (Després et al., 2007; Dussourd and Eisner, 1987; Dussourd, 2017; Helmus and Dussourd, 2005). For instance, to circumvent the latex defence of the milkweed, *Asclepias syriaca*, the chrysomelid beetle, *Labidomera clivicollis* first bites repeatedly at lateral veins next to the leaf midrib to induce latex drainage, and then starts eating the edge of the leaf distal from the cuts

(Dussourd and Eisner, 1987). Similar strategies have been shown in other insects that feed on laticiferous plants, such as the caterpillar of the monarch butterfly, *Danaus plexippus* (Dussourd and Eisner, 1987; Dussourd, 2017; Helmus and Dussourd, 2005). Interestingly, there are some generalist insects that would usually avoid latex-producing plants but may take advantage of latex-free leaves that have not been completely consumed by the milkweed insects feeding secondarily (Dussourd and Eisner, 1987).

1.2.2.2 Target site insensitivity

Some insects have developed target-site insensitivity, that is an alteration of the receptor site, targeted by a toxin, that prevent its binding, thereby reducing or eliminating its deleterious effect (Berenbaum, 1986). Some plants such as milkweeds produce the cardenolide oubain, a toxin that blocks the Na^+/K^+ ATPase pump responsible for maintaining transmembrane cell potentials. Specialist herbivores feeding on these plants, such as the monarch butterfly, two species of leaf beetle (*Chrysochus auratus* and *C. cobaltinus*), and the milkweed bug (*Oncopeltus fasciatus*), show a convergent adaptive evolution in which an amino-acid mutation in the gene for the Na^+/K^+ ATPase pump confers resistance to oubain (Dobler et al., 2015; Holzinger and Wink, 1996; Labeyrie and Dobler, 2004; Moore and Scudder, 1986).

1.2.2.3 Enzymatic detoxification and conjugation

Probably the most common form of defence against chemical plant defences involves the enzymatic neutralization of allelochemicals. Enzymes of the family cytochrome P450 monooxygenases can metabolise a variety of substrates, making them particularly useful to herbivores that may encounter a broad range of secondary metabolites (Schuler, 1996). The P450 enzymes convert the plant toxins, as well as synthetic pesticides, into less toxic compounds that are further metabolised by other enzymes

(Pellmyr and Herrera, 2002). The enzymatic detoxification is a plastic process, since the synthesis of enzymes is often induced by the presence of allelochemicals in the diet (Glendinning, 2002). Another important superfamily of enzymes are the Glutathione S-transferases, that catalyse the conjugation of glutathione to toxic compounds, producing water soluble metabolites to facilitate the excretion (Habig et al., 1974; Yu, 1996). Lastly, some enzymes can be produced by microbial symbionts harboured in the gut, and in some insects they confer protection against pesticides (Hammer and Bowers, 2015; Werren, 2012).

1.2.2.4 Selectively permeable gut, blood brain barrier, and excretion

A selective permeable midgut epithelium can reduce or prevent the uptake of secondary metabolites. In one such adaptation, the epithelial cells of the gut are linked together by junctions that form a physical barrier to prevent the paracellular movement of polar substances (Fiandra et al., 2006). In a different adaptive approach, the uptake of non-polar substances, which can passively permeate the cells, can be reduced or blocked by efflux carriers such as ABC transporters that have affinity for a broad variety of allelochemicals (Dermauw and Van Leeuwen, 2014). For instance, the oleander hawkmoth *Daphnis nerii* that is an oleander specialist, encounters high concentrations of cardenolides, but contrary to other specialists its Na^+/K^+ ATPase pump is susceptible to cardenolides (Petschenka and Dobler, 2009). This moth instead relies on the selective permeability of its gut that excludes almost completely polar and non-polar cardenolides, the latter being blocked by the expression of P-glycoprotein, an ABC transporter (Abe et al., 1996; Dobler et al., 2015) (see heading 1.5). Similarly, generalist species such as *S. gregaria* and the American cockroach (*Periplaneta americana*) have

guts that are impermeable to polar and non-polar cardenolides (ouabain and digitoxin respectively) (Scudder and Meredith, 1982).

A further defence is provided by the blood brain barrier that protects the nervous system from neurotoxic compounds (Hindle and Bainton, 2014). For example, caterpillars of *D. nerii* and the tobacco hawkmoth, *Manduca sexta,* express P-glycoproteins in their blood brain barrier to prevent the ingress into the central nervous system of non-polar cardenolides and nicotine, respectively (Murray et al., 1994; Petschenka et al., 2013).

Herbivores feeding on toxic plants can also rapidly excrete the ingested allelochemicals before a toxic dose can accumulate in the haemolymph. For example, the polyphagous caterpillar of the green hairstreak butterfly, *Callophrys rubi*, can rapidly excrete the quinolizidine alkaloids found in both its host and non-host plants, *Genista tinctoria* and *Lupinus polyphyllus* respectively (Fiedler et al., 1993). Caterpillars of the specialist *M. sexta* have evolved different mechanisms to protect themselves from the highly poisonous nicotine produced by their host plant. As a first defence, nicotine uptake is reduced by the expression of P-glycoproteins in the gut epithelium (Petschenka et al., 2013). To cope with the small amounts of nicotine that still permeate into their haemolymph, the P-glycoproteins on the blood brain barrier protects the nervous system (Murray et al., 1994; Petschenka et al., 2013), and the concentration of nicotine in the haemolymph is maintained low by its active excretion through the Malpighian tubules (Gaertner et al., 1998) (see heading 1.4.1).

1.2.2.5 Further use of plant allelochemicals: Antipredator strategies and self-medication

Certain herbivorous insects have evolved mechanisms to exploit plant chemicals by using the allelochemicals as an antipredator defence, or even as a form of self-medication. Many species of lepidopteran such as Arctiidae, Papilionidae and Danainae actively sequester the plant allelochemicals found in their host-plant species thus becoming unpalatable to predators (Nishida, 2002). Often this strategy is associated with aposematic coloration, whereby insects signal their toxicity to their potential predators by displaying conspicuous colours (Harvey and Paxton, 1981). For instance, aposematic larvae of the monarch butterfly, *D. plexippus*, sequester the cardenolide ouabain in their integument from the milkweed on which they feed. The adult butterflies, which retain their aposematic colourations, also maintain their toxicity because ouabain is transferred from the caterpillar to the imago in the integument and wings (Frick and Wink, 1995). Sequestration of secondary metabolites has also been shown in five species of beetle of the genus *Longitarsus* feeding on plants containing iridoid glycosides (Willinger and Dobler, 2001).

Some orthopterans have additional defences when attacked by predators. For example, koppie foam grasshoppers of the genus *Dictyophorus* extrude a toxic foam through the spiracles by combining haemolymph with air (Chapman and Joern, 1990), whilst the armoured ground cricket, *Acanthoplus discoidalis,* can squirt haemolymph from seams at the base of the legs (Bateman and Fleming, 2009), and *S. lineata* can regurgitate its gut content (Sword, 2001).

Bioactive properties of plants allelochemicals can also be exploited for self-medication against pathogens and parasites. For instance, the bumblebees, *Bombus*

terrestris and *B. impatiens,* may exploit nectar toxins such as nicotine, gelsemine, and anabasine to reduce the infection of the gut protozoan, *Crithidia bombi* (Baracchi et al., 2015; Manson et al., 2010; Richardson et al., 2015). Others, such as the generalist tiger moth caterpillar, *Grammia incorrupta*, increase the consumption of plants containing pyrrolizidine alkaloids when infected with lethal endoparasitic tachinid flies (Singer et al., 2009).

1.3 Insect polyphenism

Phenotypic plasticity describes the ability of a single genotype to produce a broad range of different phenotypes in response to environmental variables (Fusco and Minelli, 2010; Pigliucci et al., 2006; West-Eberhard, 2003). Polyphenism is a special form of phenotypic plasticity, in which the phenotypic outputs are not continuous, but rather discrete, and therefore a single genotype exhibits two or more alternative distinct phenotypes in response to external factors (Hardie and Lees, 2013; Nijhout, 1999). Polyphenism is thought to be one of the keys to the success of insects (Simpson et al., 2011).

Holometabolous insects, which show a complete metamorphosis during their life cycle, have a partitioned life history with larvae adapted to acquire nutritional resources, and adults adapted for reproduction and dispersal (Rolff et al., 2019; Wiegmann et al., 2009). Thus, larvae and adult can be considered discrete phenotypic outputs from the same genome and, consequently, an example of polyphenism albeit developmental polyphenism. In eusocial insects such as Hymenoptera (bees, ants, wasps) and Isoptera (termites) a single genome regulates distinct castes to allow the division of labour, and environmental factors can act as cues for the production of specific castes (Miura, 2005;

Nowogrodzki, 1984; Nutting, 1969; O'Donnell, 1998). For example, the Formosan termite, *Coptotermes formosanus*, produces more soldiers in the warm season, in response to the increased temperature (Waller and La Fage, 1988). This may improve the reproductive success by providing more soldiers to protect the alates during dispersion in the warm season (Nutting, 1969). Seasonal polyphenism enables some aphids and lepidopterans to exhibit a different phenotype to cope with predictable environmental changes, such as seasonal variation in temperature, photoperiod and food availability (Shapiro, 1976). For instance, the caterpillars of the oak specialist emerald moth (*Nemoria arizonaria)* show two distinct seasonal phenotypes which provide camouflage with the food they feed upon to reduce the risk of predation (Greene, 1989; Simpson et al., 2011). In spring, the caterpillars feeding on oak catkins develop into catkin morphs, whereas in the summer, when the catkins have fallen, the caterpillars feeding on the leaves develop into twig morphs. In this case, the cue inducing the phenotypic diversification of the caterpillars lies in the concentration of tannins contained in the food. (Greene, 1989; Simpson et al., 2011).

Locusts, some lepidopteran larvae and also some planthoppers exhibit a density-dependant phase polyphenism that allows them to show distinct phenotypes based on population density (Kazimirova, 1992; Pener and Simpson, 2009). Under low density conditions, individuals show 'solitarious' features such as cryptic colouration and slow movements, whereas under high density population they show 'gregarious' features such as conspicuous coloration and active behaviour (Rowell, 1972; Sword, 1999). It is generally assumed that these density-dependent phenotypes evolved in response to intraspecific food competition and increased predation pressure (Cullen et al., 2017; Simpson and Sword, 2008). However, because a high density population increases the

probability of becoming infected by a pathogen, it may be that these changes are also associated with enhanced disease resistance, via increased investment in immune defence, a phenomenon known as density-dependent prophylaxis (Wilson and Cotter, 2009). For example, caterpillars of the African armyworm moth (*Spodoptera exempta*) exist in two phases: a solitarious cryptic form and an active black gregarious phase (Faure, 1943). Caterpillars raised in crowded conditions are less susceptible to a virus commonly encountered in nature than their solitarious counterparts (Reeson et al., 1998).

The best studied models of density-dependent phase polyphenism are *S. gregaria,* and *L. migratoria*. Locusts are a type of grasshopper belonging to the family Acrididae that show a solitary and a swarming phase (Pener and Simpson, 2009). The two phases differ extensively in size, colour, physiology, endocrinology, ecology and behaviour (Ahmed et al., 2005; Despland and Simpson, 2005a; Pener, 1991; Pener and Simpson, 2009; Rowell, 1972; Simões et al., 2016; Uvarov, 1977; Uvarov and Zolotarevsky, 1929). Cryptic solitarious locusts move slowly and are repelled by other locusts, whereas gregarious locusts are brightly coloured, more active and are attracted by conspecifics (Ellis and Pearce, 1962; Roessingh et al., 1993; Simpson et al., 1999). Population density can increase after a transitory period of rainfall, which promotes egg development and allows the vegetation to flourish. Under these conditions the population of solitary locusts grows, the vegetation is rapidly depleted, and the increased food competition leads to forced crowding and physical contact that triggers gregarization (Culmsee, 2002; Despland et al., 2000; Simpson et al., 1999; Uvarov, 1977). Within a few hours the crowding induces behavioural changes, including altered feeding preferences. Locusts are generalist herbivores, but solitarious locusts avoid plants

containing toxins, whereas during the transformation transiens locusts actively show a preference towards plants containing toxic compounds, and once completely gregarized they continue to ingest toxic vegetation (Despland and Simpson, 2005a; Simões et al., 2013). The switch to aposematic colouration combined with the ingestion of toxic plants contributes to antipredator protection by making locusts unpalatable, and by enabling them to regurgitate their toxic gut content when disturbed (Sword, 2001).

1.4 Insect excretory systems

The remarkable success of insects in colonising highly diverse habitats, from those that are extremely arid and hot to those that are humid and cold (Grimaldi et al., 2005), and the exceptional diversity of feeding habits including herbivory, carnivory, detritivory and hematophagy, can be partially attributed to the robustness of their excretory and osmoregulatory systems (Chapman et al., 2013). Living cells can function efficiently only within a narrow range of ion concentrations, pH and nutrients. Therefore, it is crucial that the internal environment remains stable despite external environmental fluctuations. This is achieved in part by the excretory system that homeostatically maintains the composition of the haemolymph, and excretes potentially toxic substances ingested or that are waste from metabolic processes.

The insect excretory system consists of the Malpighian tubules and the hindgut (ileum and rectum) (Figure 1.1). The excretion process is carried out in two phases: the Malpighian tubules produce isosmotic primary urine that carries ions, water, metabolic waste and toxic substances into the hindgut, which secondarily modifies the urine by reabsorbing water, ions and other useful substances (O'Donnell, 2008). The final excreta can be hypoosmotic or strongly hyperosmotic depending on the diet habits of the insect.

Blood sucking insects such as the kissing bug, *Rhodnius prolixus,* secrete hypoosmotic fluid at high rates to eliminate the excess of water (Maddrell and Phillips, 1975), whereas insects feeding on grains such as the mealworm beetle, *Tenebrio molitor*, excrete powder-dry faecal pellets to maximise the water recovery (Ramsay, 1971).

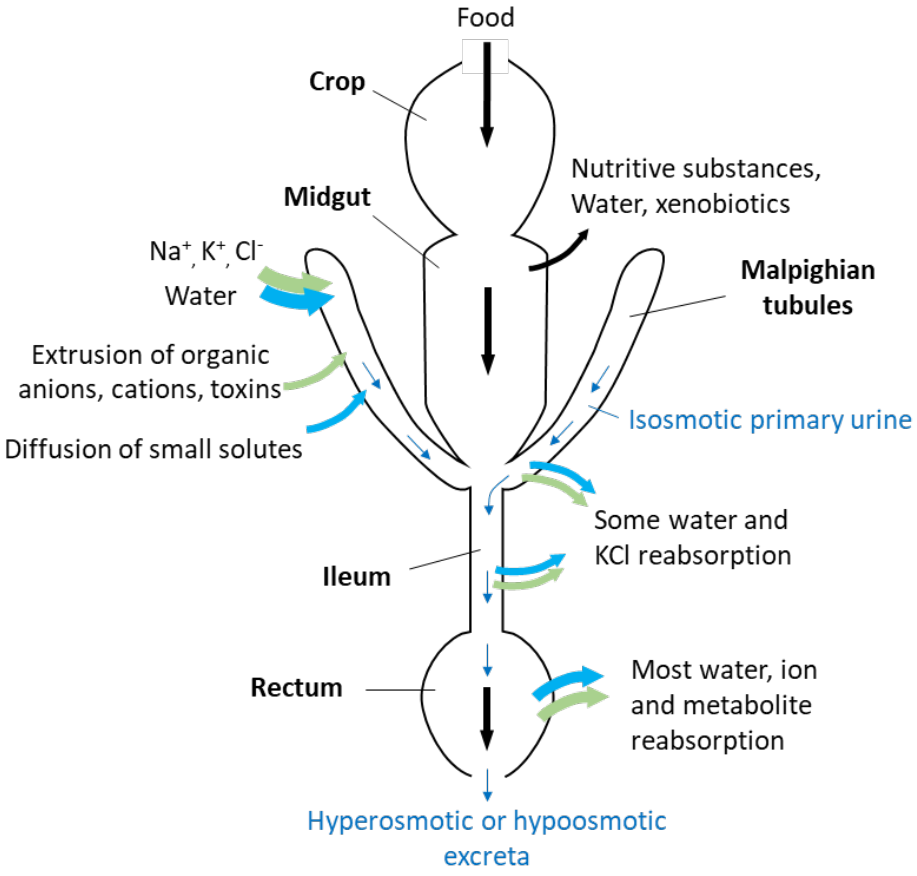

Figure 1.1. Schematic representation of the insect excretory system. Active transport is indicated by green arrows, passive transport by blue arrows, the flow of the urine by thin blue arrows, and the movement of food by black arrows. Nutritive substances, xenobiotics and water are absorbed through the midgut. The Malpighian tubules produce isosmotic primary urine by actively taking up ions that osmotically drive the movement of water. Organic anions, cations and toxins are removed from the haemolymph. Modification of the primary urine, by reabsorption of water and useful substances occurs in the proximal part of the Malpighian tubules, occurs in the Ileum and rectum. The resulting excreta, which can be hyperosmotic or hypoosmotic, is then expelled with the residuals of undigested food with faeces. Figure adapted from Phillips (1981).

1.4.1 Malpighian tubules

The insect Malpighian tubules are analogous to the functional units of the vertebrate kidney, the nephrons. The Malpighian tubules are thin, long structures with a blind end, bathed in the haemolymph, which empty their content into the digestive tract at the junction between the midgut and the hindgut. They were an early specialization of the protostomes already present in tardigrades and myriapods, and are present in all the insects, with the exclusion of aphids which have lost them secondarily (Chapman et al., 2013).

1.4.1.1 Structural variations among insects

The number of tubules is highly variable depending on the insect species, ranging from as few as two in the cotton stainer, *Dysdercus fasciatus*, up to 250 in adult of *S. gregaria*, in which the number increases during the nymph's development (Berridge, 1966; Savage, 1956). Their length varies from 2 to 70 mm, and their diameter from 30 to 100 µm, depending on the insect species (Chapman et al., 2013; Phillips, 1981). The narrow shape of the tubules ensures a large surface/volume ratio in contact with the surrounding environment, to maximize the filtration of the haemolymph and facilitate homeostasis. In large insects such as *S. gregaria* with a considerable number of tubules, the total outer surface area of the tubules can be over 1000 mm^2 per animal (Phillips, 1981).

Depending on the insect species, different morphological regions can be distinguished along the length of the tubule. For example, tubules of the ancient lineage of cricket, *Grylloblatta compodeiformis,* show no regional specialisations (Jarial, 1990), whereas tubules of *Rhodnius* possess two distinct regions: the distal secretory part and the proximal (nearest to the gut) reabsorptive part (Maddrell and Phillips, 1975;

Wigglesworth, 1931). In others, such as the fruit fly, *Drosophila melanogaster*, and *S. gregaria*, the tubules consist of three distinct morphological segments, the secretory distal and middle parts and the reabsorptive proximal part (Garrett et al., 1988; O'Donnell and Maddrell, 1995).

The tubule wall consists of a single layer of epithelial cells, with between two and five cells encircling the lumen (Phillips, 1981). The tubules can be composed of heterogeneous or homogeneous cell types depending on the region and the insect. For instance, the distal segment of *Rhodnius* and the red wood ant, *Formica polyctena*, is composed of a single cell type (Garayoa et al., 1992; Wigglesworth, 1931), whereas in dipterans such as the yellow fever mosquito, *Aedes aegypti* and *Drosophila* it consists of two cell types, principal and secondary (stellate), in a ratio of 5:1 (Beyenbach, 2003; Wessing et al., 1999). The distal and proximal parts of tubules of *S. gregaria* are composed by single distinct cells, whereas the middle segment is composed of principal and secondary smaller cells (Garrett et al., 1988).

The more abundant principal cells are specialised for active transport (see heading 1.4.1.2). They are frequently binucleate, contain concretions, and have long slender microvilli rich in mitochondria (Beyenbach, 2003; Garrett et al., 1988). In contrast, the stellate cells are smaller, have shorter brush border and lack mitochondria (Beyenbach, 2003; Garrett et al., 1988). They express aquaporins to allow water diffusion, and are responsible for chloride transport (Halberg et al., 2015).

Malpighian tubules have high metabolic activity, as suggested by the numerous mitochondria in the principal cells, and by the dense ramifications of tracheoles surrounding the tubules. These tracheoles provide oxygen and remove CO_2 waste both for the active excretory processes, and for the muscles surrounding the tubules. In most

insects the tubules writhe, possibly to help the fluid secretion and to alter the volume of haemolymph they are in contact with (Coast, 1998). In some Orthoptera, Odonata and Hymenoptera fibres of striated muscle coil helically around the tubule (Arab and Caetano, 2002; Garrett et al., 1988; Kukel and Komnick, 1989), in some Coleoptera small muscular fibres are scattered around the tubule perimeter (Hanrahan and Nicolson, 1987), whereas in Hemiptera, Diptera and Lepidoptera the tubules have no muscular supply, apart from a few fibres in the proximal region (Chapman et al., 2013; Wigglesworth, 1931).

1.4.1.2 Fluid secretion and osmoregulation

Most of our knowledge about the mechanisms underlying excretion of ions and water by tubules consisting only of principal cells comes from studies of *Rhodnius* and *Drosophila*, while for those tubules composed of principal and stellate cells our knowledge is largely from *A. aegypti* and *Drosophila*. Overall, the secretion of the primary urine is driven by the active transport of Na^+, K^+ and Cl^- in the distal and middle part of the tubule, and it can be modulated by diuretic and antidiuretic hormones (Larsen et al., 2014).

The apical brush border of the principal cells expresses the vacuolar type H^+-ATPase that provides the driving force for secondary transport of ions by actively pumping H^+ from the cell to the tubule lumen, using the energy released from ATP hydrolysis (Maddrell and O'Donnell, 1992) (Figure 1.2). This creates a favourable electrical gradient that facilitates the movement of Cl^- from the cytoplasm to the lumen through an apical chloride channel (Ianowski et al., 2002; O'Donnell et al., 2003), and energises the apical Na^+/H^+ and K^+/H^+ exchangers that transport Na^+ and/or K^+ from the cell to the lumen in exchange for luminal H^+ (Ianowski and O'Donnell, 2006). In turn, the

decreased intracellular Na^+ concentration creates a favourable gradient for the entry of ions from the haemolymph through the basolateral Na^+:K^+:2Cl^- cotransporter, which transports Cl^- against the negative cellular gradient (Ianowski et al., 2002; Ianowski and O'Donnell, 2004; Scott et al., 2004). A basal Na^+/K^+ ATPase also contributes to maintaining a favourable Na^+ gradient by moving 3 Na^+ out in the haemolymph in exchange of 2 K^+ through ATP hydrolysis (Linton and O'Donnell, 1999; Maddrell and Overton, 1988; Patrick et al., 2006). The accumulated ions in the lumen produce an osmotic gradient that forces the water movement through aquaporins via a transcellular route (Echevarría et al., 2001; O'Donnell et al., 1982).

The transporters described above can vary slightly accordingly to insect species. For example, the exchangers are electrogenic in *M. sexta* and *A. aegypti* by exchanging more than one H^+ for each Na^+ or K^+ (Beyenbach and Wieczorek, 2006; Lepier et al., 1994; Wieczorek et al., 1991), whereas they are electroneutral in *Rhodnius* (Ianowski and O'Donnell, 2006). In some species such as the red wood ant and the black field cricket, *Teleogryllus oceanicus*, basolateral K^+ channels can contribute to the movement of K^+ (Leyssens et al., 1994; Xu and Marshall, 1999). In Diptera such as *Drosophila* and *A. aegypti*, the chloride may be secreted by their stellate cells (O'Connor and Beyenbach, 2001; O'Donnell et al., 1998).

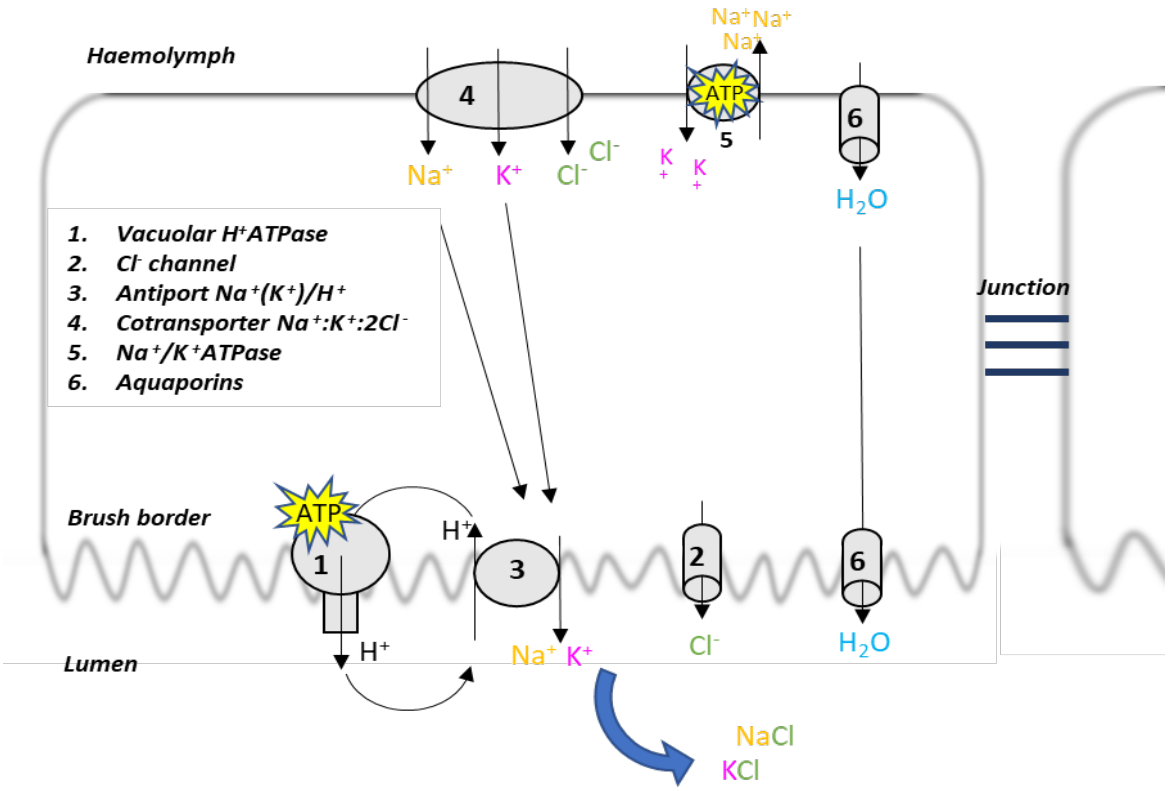

Figure 1.2. Schematic representation of ion transporters and aquaporins in the brush border and basal membrane of principal cells. The vacuolar H^+ ATPase actively moves H^+ from the cell to the lumen (1), creating a favourable electric gradient that moves Cl^- from the cell to the lumen (2), and creates a gradient allowing the Na^+ $(K^+)/H^+$ antiporter to transport Na^+ or K^+ into the lumen in exchange for H^+ (3). The decreased intracellular Na^+ concentration promotes the entry of ions from the Na^+:K^+:2Cl^- cotransporter (4). The Na^+/K^+ ATPase contributes to maintaining a low intracellular Na^+ (5). Water moves transcellularly through aquaporin following the osmotic gradient. Figure adapted from Ruiz-Sanchez and O'Donnell (2015).

The Malpighian tubules show a remarkable plasticity, and the production of primary urine can be regulated in response to starvation or feeding, by antidiuretic or diuretic hormones. The ion transport and fluid secretion can be altered by different factors such as serotonin and tyramine, or neuropeptides such as kinins and corticotropin-releasing factor-related diuretic hormones (CRF-related DH) (Coast, 2007, 2009). For example, *Rhodnius* must preserve water between infrequent meals, but after a blood meal equivalent to 10 times its body mass, it needs to excrete quickly the excess

water and unwanted ions. In response to the abdominal distension, the diuretic hormone serotonin is released into the haemolymph (Lange et al., 1989; Maddrell, 1964). Serotonin increases the fluid secretion of the distal tubule, by increasing the expression of aquaporins (Martini et al., 2004), and by stimulating the activation of the apical H^+-ATPase, Cl^- channel, and the basolateral Na^+:K^+:2Cl^- cotransporter through the cAMP cascade (Gioino et al., 2014; Ianowski and O'Donnell, 2001).

1.4.1.3 Extrusion and molecular transporters

In addition to regulating homeostasis through secretion of ions and water, the Malpighian tubules excrete nitrogen metabolites such as ammonia, urea and uric acid, and natural or synthetic toxins (O'Donnell, 2008). Several genes for detoxifying enzymes and xenobiotic transporters are expressed in the tubules, so that enzymatic conjugation and the transport system act together to improve the elimination of unwanted compounds (Larsen et al., 2014; O'Donnell, 2008; Wang et al., 2004). A moderately hydrophobic toxin can diffuse into the tubular cells through the basolateral membrane and can be transported unmodified into the lumen by apical ABC xenobiotic transporters such as P-glycoproteins or MRPs (Bard, 2000). Alternatively, the elimination process can occur in three phases (Figure 1.3): In the first phase, oxidase enzymes (e.g. cytochrome P450 family) introduce polar groups on the toxin, and subsequently transferases enzymes (e.g. glutathione transferases) attach to it small polar molecules, forming metabolites that are less toxic and easier to excrete. In the third phase, these metabolites are eliminated through the luminal xenobiotic transporters (Bard, 2000). Exposure to toxins induces a coordinated regulation of both enzymes and transporters, both of which have been implicated in the metabolism of natural and synthetic toxins

(Buss and Callaghan, 2008; Chahine and O'Donnell, 2011; Enayati et al., 2005; Feyereisen, 1999; Ranson and Hemingway, 2005).

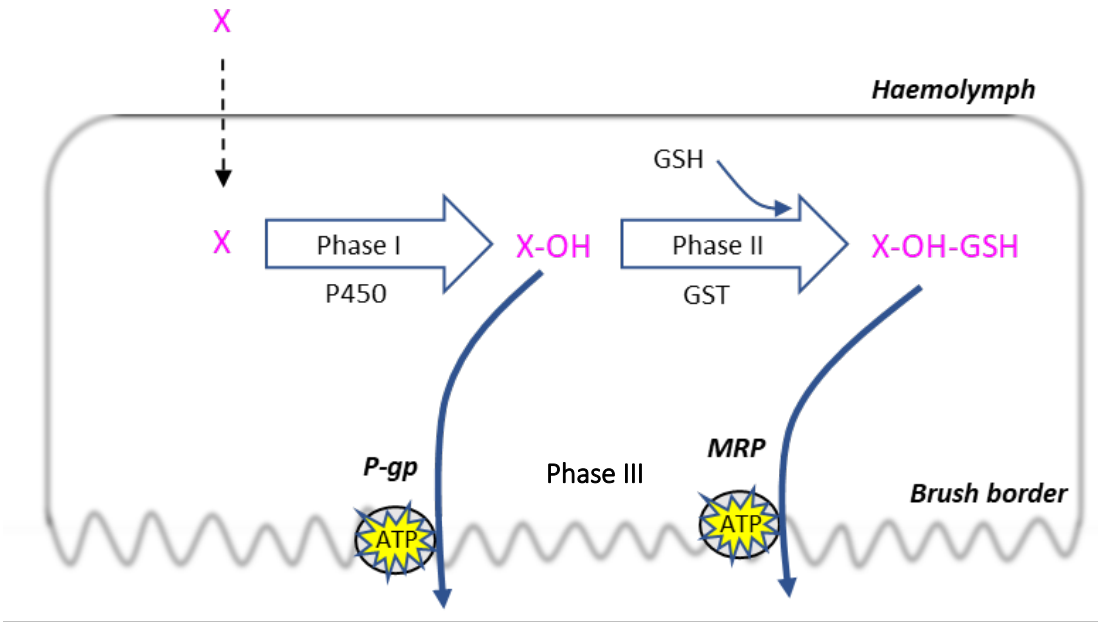

Figure 1.3. Model of the interaction between enzymatic detoxification and excretion through apical ABC tranporters in Malpighian tubule cells. The dashed line indicates passive diffusion, whereas the bold blue lines indicate active transport. A moderately hydrophobic toxin (X) passively permeate the basal side of the cell. It may be transported unaltered into the lumen by P-glycoproteins (P-gps) and/or multidrug resistance associated proteins (MRPs). Otherwise, the toxin can be transported by P-gps or MRPs after being modified by oxidase enzymes (e.g. P450) that indroduce a polar group in the compound (X-OH). Alternatively, the compound can be further modified by transferase enzymes (e.g. glutathione transferases, GST) that attach small polar molecules (e.g.gluthatione GSH), and the resulting metabolite (X-OH-GSH) can be actively transported into the lumen by MRP. Figure adapted from Bard (2000).

Xenobiotic transporters have been studied in various insects, using different techniques including immunostaining, genetic expression, or physiological assays combined with radioactive toxins, or fluorescent substrates and inhibitors (Al-Qadi et al., 2015; Andersson et al., 2013; DeSalvo et al., 2011; Gaertner et al., 1998; Murray et al., 1994). For example, isolated Malpighian tubules of *M. sexta* incubated either in radiolabelled nicotine or vinblastine (a P-glycoprotein substrate), actively concentrated these compounds in the lumen, and both were inhibited by the P-glycoprotein inhibitor verapamil, suggesting that the active excretion was mediated by P-glycoproteins (Gaertner et al., 1998). Another family of transporters identified on the Malpighian tubules of *Drosophila* are the OATPs (organic anion transporting polypeptides) that have affinity with a wide range of organic solutes, including the polar cardenolide ouabain. Interestingly, OATPs are co-localised with the basolateral ouabain sensitive Na^+/K^+-ATPase, therefore preventing the toxin from reaching inhibitory concentrations (Torrie et al., 2004). Another important family of transporters that contributes to the excretion of neutral, cationic or anionic compounds, and conjugated compounds (e.g. substances conjugated with glutathione) are the MRPs (Multidrug Resistance associated Proteins). By using MRP substrates such as Texas red, and MRP inhibitors such as MK571, they have been found on the tubules of *P. americana*, *Drosophila*, and the cricket *Teleogryllus commodus* (Karnaky Jr et al., 2000; Leader and O'Donnell, 2005; O'Donnell and Leader, 2006).

1.4.1.4 Diseases of Malpighian tubules

The midgut and Malpighian tubules of insects can become infected with parasites that can compromise both their ultrastructure and physiology. Adult honey bees and grasshoppers can be infected with the protozoans *Malpighamoeba mellificae* and *M. locustae*, respectively. Infection arises from the ingestion of cysts deposited in contaminated faeces (Bailey, 1968a; Bulger, 1928; Harry and Finlayson, 1975, 1976; King and Taylor, 1936; Taylor and King, 1937). The parasites multiply in the midgut epithelium and the trophozoites migrate to the lumen of the Malpighian tubules, where they cause swelling of the epithelial cells, often destroying their brush borders (Harry and Finlayson, 1976; King and Taylor, 1936; Liu, 1985a,b). Infected tubules become packed with a mix of trophozoites and cysts, and the latter are excreted in the faeces (Bailey, 1968a; Harry and Finlayson, 1976; Liu, 1985a,b). In honey bees, this disease is often associated with *Nosema apis*, a microsporidian parasite that reproduces in the midgut epithelium and releases its spores into the hindgut lumen (Huang and Solter, 2013). *M. mellificae* infection has been suggested to cause dysentery in honey bees (Fyg, 1964), but because of its frequent association with *N. apis*, it remains unclear whether the dysentery is caused by *M. mellificae* itself or by a combination of the two (Bailey, 1968a, 1968b, 1967). Therefore, the physiological effect of *M. mellificae* on Malpighian tubules in terms of homeostasis and excretion needs more investigation. A similar parasite, *Nosema bombi*, infects midgut, brain and muscle tissue of bumble bees, but the most infected tissues are the Malpighian tubules, in which the spores primarily develop (Fries et al., 2001; McIvor and Malone, 1995; Van den Eijnde and Vette, 1993). In heavy infections, the epithelium is destroyed and the spores are released into the tubule lumen (McIvor and Malone, 1995).

In addition to protozoans, the Malpighian tubules can be infected with nematodes. Adults of the nematode *Dirofilaria immitis* parasitise their vertebrate host, the dog, and migrate to a specific location, the heart. There, they produce pre-larvae that circulate in the blood and are then taken up by the female mosquitos of the species *Aedes taeniorhynchus* in which they develop to the third larval stage in their Malpighian tubules. Within two days of contracting the parasite, the ultrastructure of the brush border of the infected primary cells differs from the uninfected ones: the volume of the microvilli is reduced and the mitochondria retract from the brush border (Bradley et al., 1984). The resulting decrease in the surface available for ionic exchange coupled with decreased production of ATP (i.e. fewer mitochondria), leads to an alteration of the tubule physiology. *In vitro* assays of isolated tubules show that the fluid secretion rate of infected tubules is lower compared to uninfected ones, and the decline is proportional to the magnitude of the infection (Bradley and Nayar, 1984). The pre-larvae nematodes are not host-specific for *A. taeniorhynchus*, but can also infect the Malpighian tubules of other mosquitoes such as *A. aegypti* (Palmer et al., 1986). In this species the change in ultrastructure of the principal cells has been observed for 10 days after exposure to the infection. After two days the number of mitochondria and the microvillar volume are reduced, as in *A. taeniorhynchus*. Over time the infection progresses damaging also the basal portion of the principal cells, and eventually eight days post exposure the larvae destroy the apical membrane invading the tubule lumen (Palmer et al., 1986).

1.5 ABC transporters

ATP Binding Cassette (ABC) proteins are ATP-dependent transporters found ubiquitously and conserved in all Kingdoms. They transfer across membranes a broad range of substances, such as amino acids, lipids, polysaccharides, peptides and a large variety of hydrophobic compounds, drugs and toxic metabolites (Dermauw and Van Leeuwen, 2014). These transporters have been broadly studied in bacteria and vertebrates, with a particular focus on mammals due to their role in facilitating multidrug resistance in cancer cells (Gottesman, 2002). Indeed, these transporters increase the extrusion of chemotherapeutic drugs to almost the same speed at which they enter the cells, leading to chemotherapy failure (Schinkel and Jonker, 2003). ABC transporters are also responsible for antibiotic resistance in bacteria, fungicidal resistance in fungi (Steffens et al., 1996), and play an important role in secondary metabolite and insecticide excretion in arthropods (Dermauw and Van Leeuwen, 2014). The best studied ABC transporters implicated in mammalian drug resistance and in xenobiotic excretion in insects are the P-glycoproteins (P-gps, also known as multidrug resistance protein (MDR1), or ABCB1) and the multidrug resistance associated proteins (MRP2, ABCC subfamily) (Dermauw and Van Leeuwen, 2014).

The first evidence implicating P-glycoproteins in the excretion of secondary metabolites in insects came from experiments on *M. sexta*. In 1976, Maddrell and Gardiner identified an active alkaloid pump on the Malpighian tubules of *M. sexta* that extruded not only nicotine, but also atropine and morphine. Twenty years later this pump was identified to be a P-glycoprotein homolog (Gaertner et al., 1998), and it was also found on the blood brain barrier of the *M. sexta* (Murray et al., 1994). P-glycoproteins are now known to transport a broad range of substrates such as organic

cations bigger than 500 Da, hydrophobic and moderately hydrophobic compounds such as alkaloids and quinones (Wright and Dantzler, 2004). Therefore, the P-glycoproteins on the blood brain barrier act as an active efflux barrier by transporting back a variety of non-polar toxins that otherwise would diffuse into the nervous system (Petschenka et al., 2013).

ABC transporters are encoded by inducible genes, and their expression can be regulated depending on the amount of allelochemicals encountered with the diet. For instance, *Drosophila* fed on food containing a non-toxic concentration of the secondary metabolite colchicine increased the expression of mdr49 (gene encoding P-glycoprotein) in the brain and gut compared with non-colchicine fed larvae (Tapadia and Lakhotia, 2005). ABC transporters can also be downregulated if toxins are removed from the diet. For example, *M. sexta* feeding on *Nicotiana attenuata* plants that do not produce nicotine express fewer ABC transporters than caterpillars fed on wild type tobacco leaves (Govind et al., 2010).

A body of literature has developed on the role of P-glycoproteins and other ABC transporters in regulating insecticide toxicity (Bariami et al., 2012; Denecke et al., 2017). P-glycoproteins seem to be implicated in conferring insecticide resistance and, therefore, a better understanding of their role in this respect may be useful in the management of pests and pesticide resistance. For instance, populations of the lepidopteran pest tobacco budworm (*Heliothis virescens*) that have become resistant to several pesticides, have a greater expression of P-glycoproteins on the entire body wall compared to susceptible populations, likely to reduce topical absorption (Lanning et al., 1996). In another pest, the cotton bollworm (*Helicoverpa armigera*), P-glycoproteins seems to be involved in the protection of the mitochondria from

insecticide damage, and they have been detected only in the insecticide resistant strain but not in the susceptible one (Akbar et al., 2014; Srinivas et al., 2004).

1.6 Aims and summary of contributions

This thesis makes novel contributions to the field of insect physiology applied to ecology and pathology. Our first aim was to develop a simpler and cheaper method as an alternative to radiolabelled alkaloids, confocal microscopy or liquid chromatography–mass spectrometry to investigate the presence and physiology of P-glycoprotein in Malpighian tubules of *S. gregaria*. To this end, we combined a modified Ramsay assay with the application of the specific P-glycoprotein substrate rhodamine B, as a proxy for toxins, and the specific inhibitor verapamil. By measuring the colour and size of the droplets secreted by isolated Malpighian tubules incubated in solutions containing rhodamine B with or without verapamil, we quantified the fluid secretion rate, the rhodamine concentration of the droplets secreted, and the net rhodamine extrusion as proxy for the presence of P-glycoprotein. We found that tubules express P-glycoproteins, the tubules' surface area positively influences their fluid secretion rate, and the fluid secretion rate positively increases the net rhodamine extrusion.

Our second aim was to investigate the phenotypic plasticity of Malpighian tubules in relation to the exposure to toxic compounds in a natural ecological perspective with a focus on polyphenism. *S. gregaria* provides a good model, with insects in the solitarious phase avoiding ingesting toxic plants in contrast to gregarious insects that ingest them, and transiens showing a preference for them. We compared the net rhodamine extrusion between solitarious, transiens and gregarious phases fed on an alkaloid-free diet, and on diet containing the alkaloid atropine at a concentration

similar to the one found in Egyptian henbane (*Hyoscyamus muticus*), a plant ingested by gregarious and transiens locusts in natural conditions. We found that P-glycoprotein activity is similar in all the locust phases fed on alkaloid-free diet, showing that there were not intrinsic differences induced by the phase shift in the ability of excreting toxins. We also found that gregarious and transiens locusts upregulated their P-glycoprotein after only three days of exposure to the alkaloid diet, and gregarious locusts reared on the alkaloid diet downregulated their transporters after being switched to an alkaloid-free diet.

Our third aim was to study the effect of a parasitic infection on the performance of the Malpighian tubules. To this end, we investigated the physiology of *S. gregaria* tubules infected by the protozoan *Malpighamoeba locustae*, an infection that damages the tubules' cellular structure. We found that infected tubules had greater surface area and higher fluid secretion rate than those uninfected. In addition, the infection reduced the net rhodamine extrusion per unit of surface area, suggesting that the damage of the apical border likely reduces the P-glycoprotein density.

Overall our study provides more understanding of the physiology of locust Malpighian tubules in the context of ecology, phenotypic plasticity and disease. We suggest that the expression of P-glycoprotein on the Malpighian tubules contribute to toxin extrusion, and that the diet regulates the transporter activity. Lastly, we showed how the function of Malpighian tubules can be disrupted by a protozoan infection, potentially leading to reduced xenobiotic excretion, water loss, and increased energy consumption.

2 Transepithelial transport of P-glycoprotein substrate by the Malpighian tubules of the desert locust

2.1 Abstract

Extrusion of xenobiotics is essential for allowing animals to remove toxic substances present in their diet or generated as a biproduct of their metabolism. By transporting a wide range of potentially noxious substrates, active transporters of the ABC transporter family play an important role in xenobiotic extrusion. One such class of transporters are the multidrug resistance P-glycoprotein transporters. Here, we investigated P-glycoprotein transport in the Malpighian tubules of the desert locust (*Schistocerca gregaria*), a species whose diet includes plants that contain toxic secondary metabolites. To this end, we studied transporter physiology using a modified Ramsay assay in which *ex vivo* Malpighian tubules are incubated in different solutions containing the P-glycoprotein substrate dye rhodamine B in combination with different concentrations of the P-glycoprotein inhibitor verapamil. To determine the quantity of the P-glycoprotein substrate extruded we developed a simple and cheap method as an alternative to liquid chromatography–mass spectrometry, radiolabelled alkaloids or confocal microscopy. Our evidence shows that: (i) the Malpighian tubules contain a P-glycoprotein; (ii) tubule surface area is positively correlated with the tubule fluid secretion rate; and (iii) as the fluid secretion rate increases so too does the net extrusion of rhodamine B. We were able to quantify precisely the relationships between the fluid secretion, surface area, and net extrusion. We interpret these results in the context of

the life history and foraging ecology of desert locusts. We argue that P-glycoproteins contribute to the removal of xenobiotic substances from the haemolymph, thereby enabling gregarious desert locusts to become toxic to their potential predators through the ingestion of toxic plants without suffering the deleterious effects themselves.

2.2 Introduction

Insect excretory systems consist primarily of the Malpighian tubules and the hindgut, which act synergistically to regulate haemolymph composition (Maddrell et al., 1974; Ramsay, 1958). Malpighian tubules are blind ended tubules that float in the haemolymph and empty into the gut at the midgut-hindgut junction, secreting primary urine, the composition of which is modified by water and ion reabsorption in the hindgut (Phillips, 1964a,b). The tubules are considered analogous to vertebrate nephrons (Ramsay, 1958). Cells of the epithelium forming the tubule wall express primary and secondary active transporters that move K^+, Na^+ and Cl^- ions into the lumen creating an osmotic gradient that produces water secretion (for a review see O'Donnell, 2008). Insects regulate ion and water secretion according to their feeding habits and ecological niche. For example, haematophagous insects must cope with an excess of NaCl and water after a blood meal (Williams et al., 1983), whereas phytophagous insects must often cope with a diet rich in K^+ as well as with secondary metabolites (Maddrell and Klunsuwan, 1973; Wieczorek, 1992).

In addition to osmoregulation, Malpighian tubules play a fundamental role in the removal of metabolic waste and potentially noxious substances that have been ingested (Maddrell and Gardiner, 1976; Maddrell et al., 1974). Alkaloids and organic anions and cations are actively transported by ATP-dependant transporters such as the multidrug

resistance-associated protein 2 (MRP2) and P-glycoproteins (P-gps, multidrug resistance protein (MDR1) or ABCB1), both members of the ABC transporter family (Gaertner et al., 1998; Karnaky Jr et al., 2000). Multidrug resistance-associated protein 2 (MRP2) transporters are involved in the transport of organic anions (Leader and O'Donnell, 2005; O'Donnell and Leader, 2006), while P-glycoproteins transport type II organic cations (>500 Da), hydrophobic and often polyvalent compounds (e.g. alkaloids and quinones) (Wright and Dantzler, 2004).

The presence and physiology of these multidrug transporters have been explored using specific substrates and selective inhibitors (e.g. Gaertner et al., 1998; Hawthorne and Dively, 2011; Leader and O'Donnell, 2005). In the Malpighian tubules of the cricket (*Teleogryllus commodus*) and the fruit fly (*Drosophila melanogaster*), the transepithelial transport of the fluorescent MRP2 substrate Texas Red is reduced by the MRP2 inhibitors MK571 and probenecid (Leader and O'Donnell, 2005), while the transport of the fluorescent P-glycoprotein substrate daunorubicin is selectively reduced by the P-glycoprotein inhibitor verapamil (Leader and O'Donnell, 2005). The transport of nicotine by P-glycoprotein transporters has also been demonstrated in numerous insect species, including the tobacco hornworm (*Manduca sexta*) (Gaertner et al., 1998), fruit fly (*D. melanogaster*), kissing bug (*Rhodnius prolixus*), large milkweed bug (*Oncopeltus fasciatus*), yellow fever mosquito (*Aedes aegypti*), house cricket (*Acheta domesticus*), migratory locust (*Locusta migratoria*), mealworm beetle (*Tenebrio molitor*), American cockroach (*Periplaneta americana*) and cabbage looper (*Trichoplusia ni*) (Rheault et al., 2006). In insects, the understanding of the interaction between xenobiotics (i.e. insecticides, herbicides, miticides and secondary plant metabolites) and P-glycoprotein transporters is still limited, but there is an increasing interest in

understanding how different xenobiotics can act synergistically to maximize the efficacy of insecticides in pests or impair the xenobiotic detoxification of beneficial insects such as honey bees (Guseman et al., 2016).

Desert locusts (*Schistocerca gregaria*) are generalist phytophagous insects with aposematic coloration in the gregarious phase. They feed on a wide range of plants including those, such as *Schouwia purpurea* and *Hyoscyamus muticus*, that contain toxins to become unpalatable and toxic to predators (Despland and Simpson, 2005a; Mainguet et al., 2000; Pener and Simpson, 2009; Sword et al., 2000). Nevertheless, it is likely that gregarious desert locusts excrete some of the toxins that they ingest, relying instead on their gut contents to maintain toxicity (Sword, 2001, 1999). Two lines of evidence suggest that this excretion is likely to involve P-glycoproteins: (1) they are expressed in the Malpighian tubules of numerous species (e.g. *A. domesticus, L. migratoria, P. americana*) from orthopteroid orders (Rheault et al., 2006); and (2) they are expressed in the blood brain barrier of the desert locust (Andersson et al., 2014). However, P-glycoproteins in the Malpighian tubules of desert locusts have not been studied previously.

Here we show that xenobiotic transport and extrusion in the Malpighian tubules of the desert locust is an active process dependent upon P-glycoprotein like transporters using isolated tubules to perform a modified Ramsay secretion assay (Ramsay, 1954). We evaluated the extrusion of the P-glycoprotein substrate dye rhodamine B (e.g. Eytan et al., 1997; Mayer et al., 2009) with or without the addition of verapamil, a drug used as a P-glycoprotein inhibitor, and as a L-type Ca^{2+} channel blocker (e.g. Abernethy and Schwartz, 1999; Andersson et al., 2014; Cole et al., 1989; Dermauw and Van Leeuwen, 2014; Hamada et al., 1987). Our results suggest that P-glycoprotein transporters may

play an important role in the xenobiotic extrusion in the Malpighian tubules of the desert locust. By using linear mixed effect models to account for repeated observations of single tubules and obtaining multiple tubules from single locusts, we found that tubule surface area more accurately predicts fluid secretion rate than diameter or length. Moreover, this statistical model allowed us to quantify the influence of the surface area on the fluid secretion rate in different treatments, and how it changes over time. We found that the surface area of the tubules positively influences their fluid secretion rate and that the fluid secretion rate influences the net extrusion of rhodamine B. We propose that this assay may be used in future to understand the physiology of the P-glycoproteins when exposed to a wide range of different substances.

2.3 Materials and methods

2.3.1 Animals

Fifth instar desert locusts (*Schistocerca gregaria;* Forskål, 1775) were obtained from Peregrine Livefoods (Essex, UK) and raised under crowded conditions at 28-30°C with 12:12 photoperiod. Locusts were fed with organic lettuce, fresh wheat seedlings and wheat germ *ad libitum*. Fifth instar nymphs were checked daily and, within 24 hours post-eclosion, were marked with acrylic paint (Quay Imports Ltd, Kirkham, Lancashire, UK). Only adult males between 20 and 22 days post-eclosion were used in the experiments.

2.3.2 Saline and chemicals

The saline used was adapted from the Ringer solution of Maddrell and Klunsuwan (1973). Its composition was: 5.73 g/L NaCl (98 mM), 0.30 g/L KCl (4 mM), 2 mL $CaCl_2$ solution 1M (2 mM), 1.86 g/L $NaHCO_3$ (22 mM), 1.09 g/L $NaH_2P0_4{\cdot}2H_2O$ (7 mM), 0.19 g/L

$MgCl_2$ (2 mM), 1.80 g/L glucose (10 mM), 0.83 g/L sodium glutamate (4.9 mM), 0.88 g/L sodium citrate (3.5 mM) and 0.37 g/L malic acid (2.8 mM). The final pH of the saline was 7.15. It was stored at 4°C for a maximum of three days. Stock solutions of rhodamine B (50 mM and 3 mM) and verapamil hydrochloride (20 mM) were prepared in water and diluted to the final concentration in the saline. Rhodamine B was applied at 60 µM and verapamil at 125 µM or 250 µM. All chemicals were purchased from Sigma-Aldrich (UK) or Fisher Scientific (UK).

2.3.3 Locust dissection

The locusts were placed in the freezer for 4-5 minutes until sedated. Upon removal from the freezer, the abdomen was cut transversely ~5 mm from the anus, and holding the head with one hand and the thorax with the other hand, the head was pulled away from the remainder of the body (Figure 2.1A) (Maddrell and Klunsuwan, 1973). In this way, the entire gut with the Malpighian tubules attached was removed from the body. The gut was placed onto an 8 cm Sylgard® 184 (Dow Corning, Midland, MI, USA) coated Petri dish, filled with saline. The head was separated from the saline using modelling clay (Plasticine®) (Figure 2.1B). The preparation was pinned at the cut distal end of the gut to prevent it from floating.

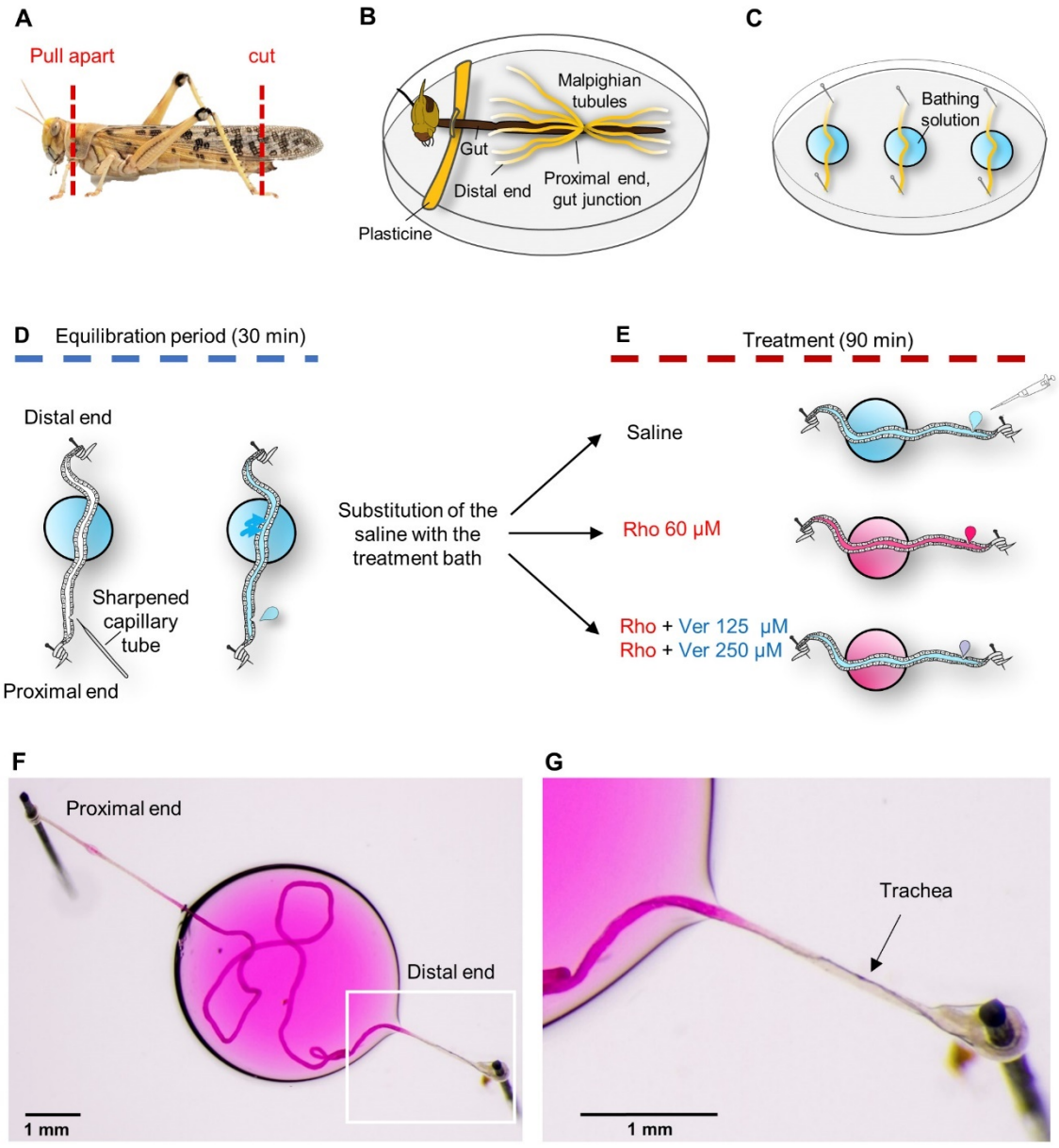

Figure 2.1. Preparation of Malpighian tubules for *ex vivo* experimentation from the desert locust, *Schistocerca gregaria*, and experimental scheme for assaying the presence of P-glycoproteins within the Malpighian tubules. (A) Cutting the posterior tip of the abdomen permits removal of the head and the fully intact gut. (B) The gut is submerged in saline and the Malpighian tubules are exposed. The head is separated from the saline bath by a barrier of modelling clay. (C) Three tubules are removed from each locust and fixed on a Sylgard® surface traversing a small drop of bathing solution, and covered with paraffin oil. (D) The proximal and distal ends of individual tubules are wound around Minuten pins to fix them. Once the saline bath and paraffin oil have been applied, the proximal end of the tubule is punctured with a sharpened capillary tube to allow fluid secretion. After 30 minutes of equilibration the saline bath was replaced by

a bath containing one of the treatments. (E) Every 30 minutes the secreted droplet was removed, placed on the Petri dish and photographed. (F) An Example of an isolated Malpighian tubule to perform a modified Ramsay secretion assay. The middle section of the tubule is immersed in the bathing solution with the respective treatment, while the proximal and distal ends are fixed outside. (G) Detail of the distal end with the trachea visible. Only a small part of the trachea is immersed in the bath.

2.3.4 Malpighian tubules dissection

Using a Nikon SMZ-U (Nikon Corp., Tokyo, Japan) stereoscopic microscope, the Malpighian tubules were removed by gently pulling the distal part to release them from the gut and cutting the proximal end at ~5 mm from the gut). Each isolated tubule was moved immediately into a 30 µL drop of saline on a 5 cm Sylgard® coated Petri dish and covered with paraffin oil to prevent desiccation. Both ends of each Malpighian tubule were pulled out from the saline drop in opposite directions and wrapped around steel pins pushed into the Sylgard® layer (Figure 2.1C,D). Three anterior tubules were removed from each locust. Tracheae coiled around the distal part of the tubule were not removed to prevent any damage of the tubule surface (Figure 2.1F,G).

2.3.5 Fluid secretion (Ramsay) assay

Using a sharpened glass capillary tube, each tubule was punctured near the proximal end to allow the fluid secretion (Figure 2.1D). The tubule was allowed to equilibrate for 30 minutes at which point the saline bath was replaced with 30 µL drop containing one of the four different treatments we tested: (i) saline, (ii) rhodamine B 60 µM, (iii) rhodamine B 60 µM + verapamil 125 µM, (iv) rhodamine B 60 µM + verapamil 250 µM (Figure 2.1E). The first droplet secreted after the bath replacement was discarded. For the subsequent 90 minutes, the secreted droplet was removed at intervals of 30 minutes

(Figure 2.1E,F) using a P10 pipette (Gilson Scientific UK, Dunstable, Bedfordshire, UK) and photographed with a digital camera (Canon EOS 7D; Canon, Tokyo, Japan) mounted with two custom attachments (Best scientific A clamp via 1.6 x Canon mount; Leica 10445930 1.0 x) on the stereoscopic microscope (Nikon SMZ-U; Nikon Corp., Tokyo, Japan). Images were shot in raw format and processed with ImageJ v.1.51p software (Schneider et al., 2012). To prevent the photobleaching of the rhodamine B, we minimised light exposure by conducting the experiment under red light and keeping the sample in a custom designed dark box between measurements.

2.3.6 Droplet measurement

The diameter (µm) of each secreted droplet (Figure 2.2) was measured to calculate its volume (nL) using the sphere formula, where V is the drop volume and d the droplet diameter. The volume was converted from µm^3 to nL using the formula:

$$V = \frac{4}{3}\pi\left(\frac{d}{2}\right)^3 10^{-6}.$$

For each tubule, we calculated the fluid secretion rate (nL/min), given by the droplet volume divided by the time between samples (30 mins). For each droplet, we also measured colour intensity to estimate the rhodamine B concentration (µM) from a calibration curve (see below). To estimate the number of moles of rhodamine B extruded per minute, we calculated the net extrusion of rhodamine B (fmol/min) as the product of the fluid secretion rate and the rhodamine B concentration.

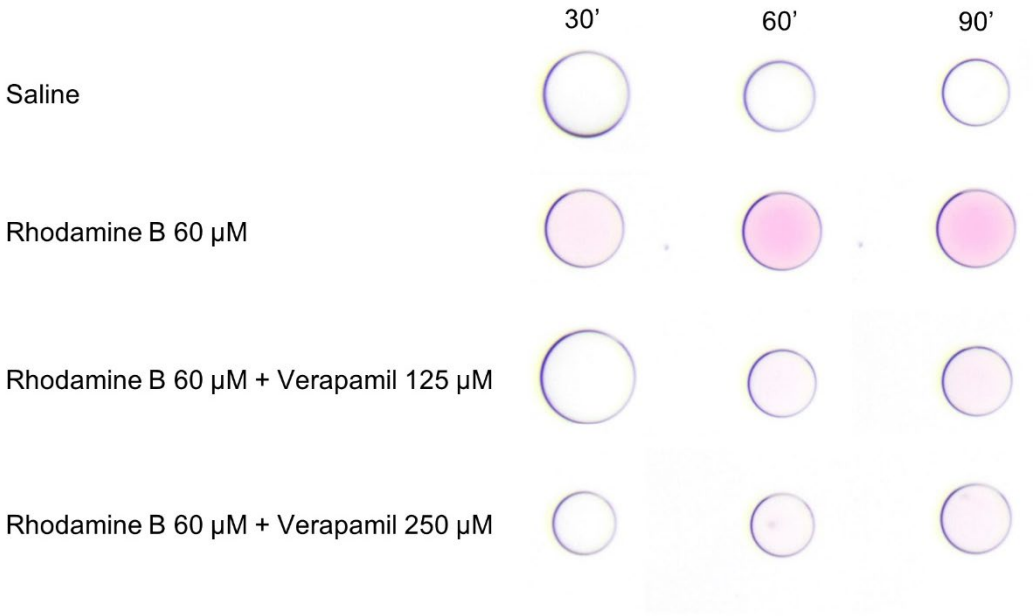

Figure 2.2. Examples of the droplets secreted by the Malpighian tubules of desert locusts during the incubation in each of the different treatments every 30 minutes. The size of each droplet depends upon the fluid secretion rate whilst the colour is determined by the net extrusion rate of rhodamine B.

2.3.7 Rhodamine B calibration curve

The intensity of the droplets depends not only on rhodamine B concentration, but also on the droplet diameter (Figure 2.3; 2.4). So, we constructed a calibration curve for rhodamine B concentration to estimate the rhodamine B concentration of the droplets secreted. We prepared standard solutions of known rhodamine B concentrations: 0, 15, 30, 50, 60, 75, 120, 150, 240 and 480 µM. For each concentration, droplets of different sizes were placed on a Petri dish coated with Sylgard® and covered with paraffin oil. We photographed the droplets against a white background at the same light intensity, and white balancing the camera before shooting. All the images were analysed subsequently using ImageJ v.1.51p software (Schneider et al., 2012).

Droplet colour varied from white (transparent droplet at rhodamine B concentration = 0 μM) to intense pink, depending upon the rhodamine B concentration. We split each image into the component colour channels and measured the intensity of the green channel. To control for the background, we compared the mean intensity inside the droplet with that outside using the formula $I = I_i - I_o$, where I is the intensity, I_i is the intensity inside the droplet, and I_o is the intensity outside the droplet. We used a range of droplet diameters from 138 μm to 999 μm.

To validate the reliability of using the green channel, we also measured the magenta channel and the total intensity using Adobe Photoshop CC v. 19.1.1 (Adobe Systems Incorporated, CA, USA). Both the magenta channel and total intensity correlated with the intensity of green channel (Pearson's correlation, Magenta: $p<0.001$, $df=17$, $R^2=1$; Total intensity: $p<0.001$, $df=17$, $R^2=0.99$).

The relationship between the intensity and the rhodamine concentration depends upon the diameter of the droplet (Figure 2.3). For each droplet diameter rank, the relationship between the intensity measured and the known rhodamine concentration can be described by a linear model. To determine the rhodamine concentration given the intensity and the diameter of the droplets, for each diameter rank we ran a linear regression model forced through the origin, with intensity as the independent variable and rhodamine concentration as the response variable (linear model: rhodamine concentration ~ intensity – 1). Hence, for each diameter rank we obtained the equation that predicts the rhodamine concentration from the intensity measured, given a specific diameter (Figure 2.3).

The slope of the linear equations decreases as the diameter increases, following an exponential decay (Figure 2.4A). To obtain the equation that predicts the slope of the

linear equations for a given diameter, we log transformed both axes and we ran a log-level regression model (Linear model: log (slope) ~ log (droplet diameter); Figure 2.4B). The resulting equation was: $\log(slope)_i = -1.44 \cdot \log(diameter)_i + 10.38$.

Using the predict command in R, we used the model to predict the slope value (back transformed to the original scale) for each diameter of the droplets we collected in the experiment. Finally, we multiplied the slope value for the intensity measured to calculate the rhodamine concentration of each droplet.

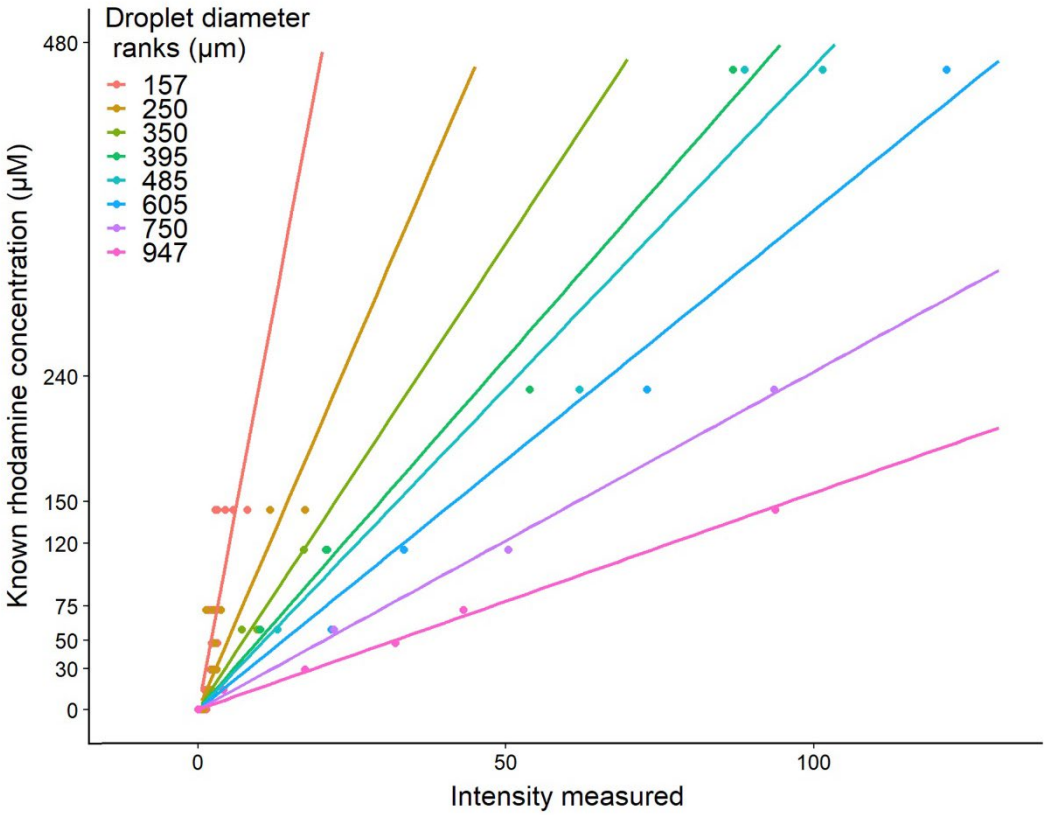

Figure 2.3. Calibration lines used to determine the rhodamine B concentration in the droplets secreted by the Malpighian tubules. For each droplet diameter rank there is a linear relationship between the rhodamine concentration of the droplet and the colour intensity measured. The slope of the lines decreases as the diameter increases. We estimated the rhodamine B concentration of the droplets secreted by measuring the colour intensity and the diameter of each droplet. Each line represents the linear regression fit for each mean diameter rank.

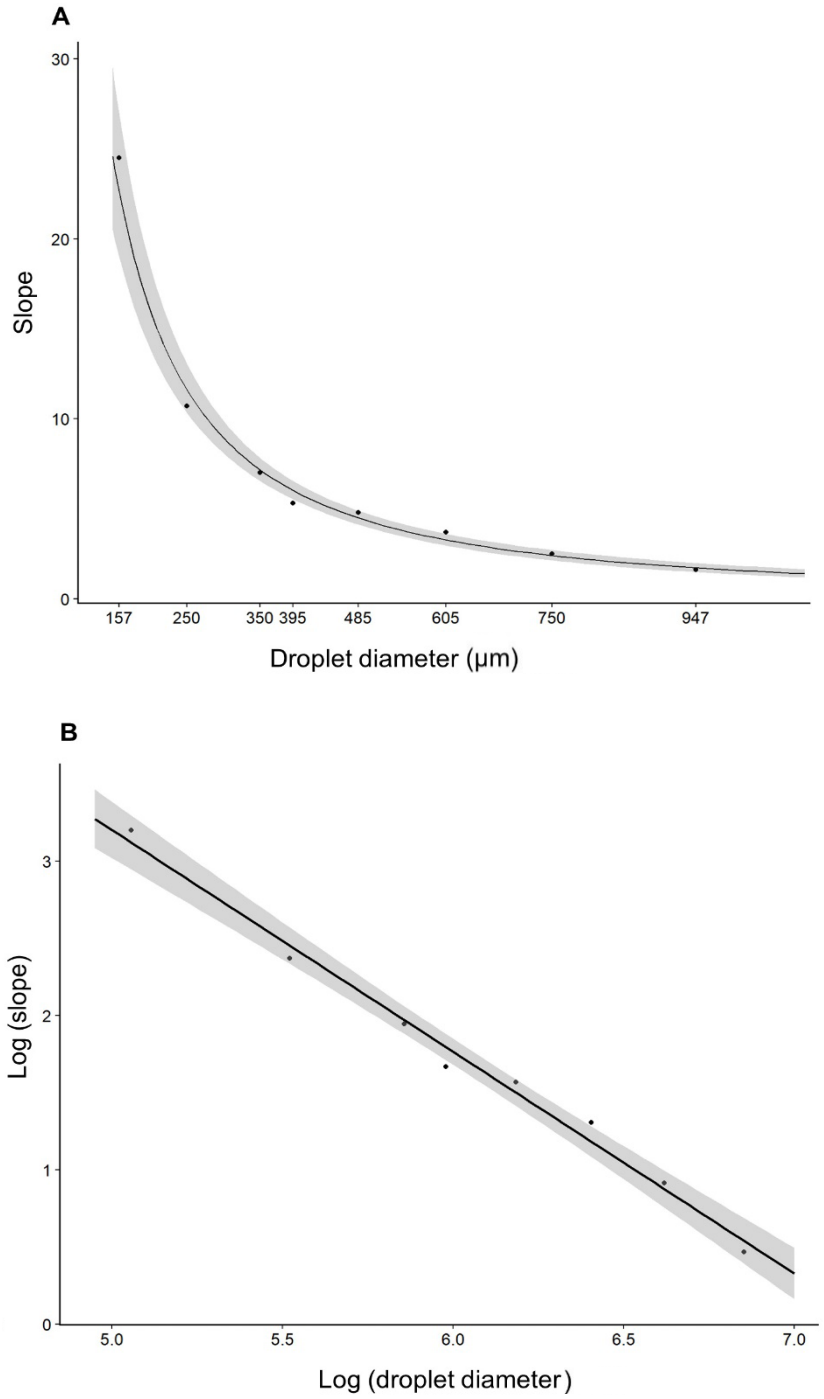

Figure 2.4. The relationship between intensity and rhodamine B concentration depends upon droplet diameter. (A) The slope decreases as the diameter increases, following an exponential decay. (B) After log transformation the relationship becomes linear. Using this linear equation for each droplet diameter measured, we predicted the slope of the line equation that link the colour intensity to the rhodamine concentration.

2.3.8 Malpighian tubule measurement

At the end of the assay, the tubule was photographed to measure its diameter (µm). The length (mm) of the tubule in contact with the treatment solution, was measured by cutting off the two extremities of the tubule outside the bath, laying the remaining section of tubule flat on the Sylgard® base, and photographing it. The surface (mm^2) of the tubule in contact with the bath, was calculated from the cylinder formula:

$S = 2\,\pi \left(\frac{d}{2}\right) l$, where S is the tubule surface, d is the tubule diameter, and l is the tubule length.

2.3.9 Statistical Analysis

All the statistical analysis was conducted in R version 3.4.1 (Team R Core, 2013). We performed Linear Mixed Effect Models (LMEM) by restricted maximum likelihood (REML) estimation by using the lmer function from the 'lme4' package (Bates et al., 2015). We used the Akaike information criterion (AIC) (Akaike, 1987) for model selection. Significances of the fixed effects were determined using Satterthwaite's method for estimation of degrees of freedom by using the anova function from the 'lmerTest' (Kuznetsova et al., 2017). The non-significant interactions (P>0.05) were removed. However, we retained all the main effects even if they were not statistically significant to avoid an increase in the type I error rate (Forstmeier and Schielzeth, 2011). Estimated marginal means and pairwise comparisons were obtained using the 'lsmeans' package (Lenth and Lenth, 2018) and the p value adjusted with the Tukey method. All plots were made using the 'ggplot2' package (Wickham, 2016).

To investigate the effect of the treatments on the fluid secretion rate and the net extrusion of rhodamine B, we analysed the interaction between the treatment

(categorical), time of incubation (categorical) and the surface area (continuous (mm^2)). For the rhodamine B concentration, we analysed the interaction between treatment (categorical) and time (categorical). To account for the nested structure of data, we included the individual locust as random intercept in the model. We also included tubule identity as a random intercept and time as random slope to account for the repeated measurements on the same individual tubule. To investigate the effect of the fluid secretion rate on the net extrusion of rhodamine B we analysed the interaction of the variables secretion rate, treatment and time including as before the individual locust as random intercept, and tubule identity as a random intercept and time as random slope. To simplify the interpretation of the regression estimates, we centred the surface variable on its mean. Therefore, all the estimates and comparisons are referred to a tubule with a mean surface area.

2.4 Results

We prepared three Malpighian tubules from each locust (see Materials and Methods; Figure 2.1). Each tubule was punctured near the proximal end to allow the luminal fluid to be secreted and then they were allowed to equilibrate in the saline bath for 30 minutes (Figure 2.1D). The saline bath was then replaced with one of four treatments: saline (control); rhodamine B 60 µM (R60); rhodamine B 60 µM + verapamil 125 µM (V125); and rhodamine B 60 µM + verapamil 250 µM (V250) (Figure 2.1E). Six locusts were used for each of the treatments except for the R60 treatment in which eight locusts were used. Every 30 minutes the droplet secreted by the tubule during the Ramsay assay was removed.

2.4.1 Fluid secretion rate and surface area

We determined the fluid secretion rate of each tubule from the volume of the droplet secreted after each 30-minute interval up to 90 minutes after the start of the treatment. Thus, for each tubule we had three measurements of the secretion rate in each of the four treatments. In total, there were 233 treatment observations (one droplet was lost after 60 minutes for the V250 treatment) from 78 Malpighian tubules.

To determine whether the surface area of the Malpighian tubules exposed to the bath solution influences the fluid secretion rate, we measured the length and diameter of each tubule immersed in the saline or treatment. By comparing linear mixed effect models that incorporated these measurements of length, diameter or surface area, we determined that surface area was the best explanatory variable (Table 2.1). There was no difference in the surface area of Malpighian tubules exposed to the bathing solution among the treatments ($F_{3,22.02}$=0.488, p=0.694; Control: 2.00 ± 0.09 mm^2 (mean ± S.E.); R60: 2.01 ± 0.06 mm^2; V125: 2.27 ± 0.06 mm^2; V250: 2.29 ± 0.07 mm^2).

The surface area of the tubule exposed in the bathing solution influenced the fluid secretion rate depending on the treatment ($F_{3,66.29}$=3.25, p=0.027; Figure 2.5; Table 2.2A). Throughout the whole period of incubation, the surface area positively influenced the fluid secretion of tubules incubated in R60, V125 and Saline, while the V250 treatment the tubules showed no significant correlation between surface area and fluid secretion rate (Figure 2.5C; Table 2.2A). Having incorporated tubule surface area into our statistical model, we were able to compare the fluid secretion rates of our control and treatments objectively. The fluid secretion rate decreased over time irrespective of the treatment ($F_{2,82.36}$=46.12, p=<.001; Time 60 vs Time 30: -0.12 ± 0.01 nL/min, p<.001; Time 90 vs Time 60: -0.04 ± 0.01 nL/min, p=0.013; Figure 2.6, Table 2.2B) and at each

time point there was no significant difference between treatments (Figure 2.6, Table 2.2C).

Table 2.1. Comparison of linear mixed effect models incorporating length, diameter or surface area. Based on the lowest AIC parameter, the surface area was the best explanatory variable for the secretion rate. The row in bold indicates the model with the lowest AIC. Only the fixed effects are shown.

model	df	AIC	BIC	LogLik	deviance
secretion rate ~ diameter * time + treatment * time	20	-318.14	-249.12	179.07	-358.14
secretion rate ~ length * time + treatment * time	20	-312.61	-243.59	176.30	-352.61
secretion rate ~ surface * time + treatment * time	**20**	**-326.81**	**-257.79**	**183.40**	**-366.81**

Table 2.2. Outcomes of the linear mixed effect model investigating the effect of time of incubation, treatment, and surface area on the fluid secretion rate (SR) (nL/min) of Malpighian tubules. The model applied was (Secretion rate ~ surface * treatment + time + (1| locust) + (1+time|tubule)). (**A**) Estimates of the influence of each unit of surface (mm^2) on the fluid secretion rate for each treatment. (**B**) Summary of the mean values for each treatment at each time point. (**C**) Pairwise comparisons between treatments for each time of incubation.

A.	**Treatment**	**Time 30, 60, 90 min**	
		surface trend ± se	P-value
	R60	0.16 ± 0.04	**<.001**
	V125	0.14 ± 0.05	**0.006**
	V250	0.02 ± 0.04	0.569
	Saline	0.16 ± 0.04	**<.001**

B.	**Treatment**	**Time 30 min**	**Time 60 min**	**Time 90 min**	**SR decrease between 30 and 60 min**	**SR decrease between 60 and 90 min**
		Mean ± se	Mean ± se	Mean ± se		
	R60	0.44 ± 0.05	0.32 ± 0.05	0.28 ± 0.04	-27%	-13%
	Saline	0.46 ± 0.05	0.33 ± 0.05	0.30 ± 0.05	-28%	-9%
	V125	0.36 ± 0.05	0.24 ± 0.05	0.20 ± 0.05	-33%	-17%
	V250	0.30 ± 0.05	0.18 ± 0.05	0.15 ± 0.05	-40%	-17%

C.	**Treatment**	**Time 30, 60, 90 min**	
		estimate ± se	P-value
	V125 vs R60	-0.08 ± 0.07	0.662
	V250 vs R60	-0.13 ± 0.07	0.213
	Saline vs R60	0.02 ± 0.07	0.995
	V250 vs V125	-0.06 ± 0.07	0.850
	Saline vs V125	0.09 ± 0.07	0.572
	Saline vs V250	0.15 ± 0.09	0.182

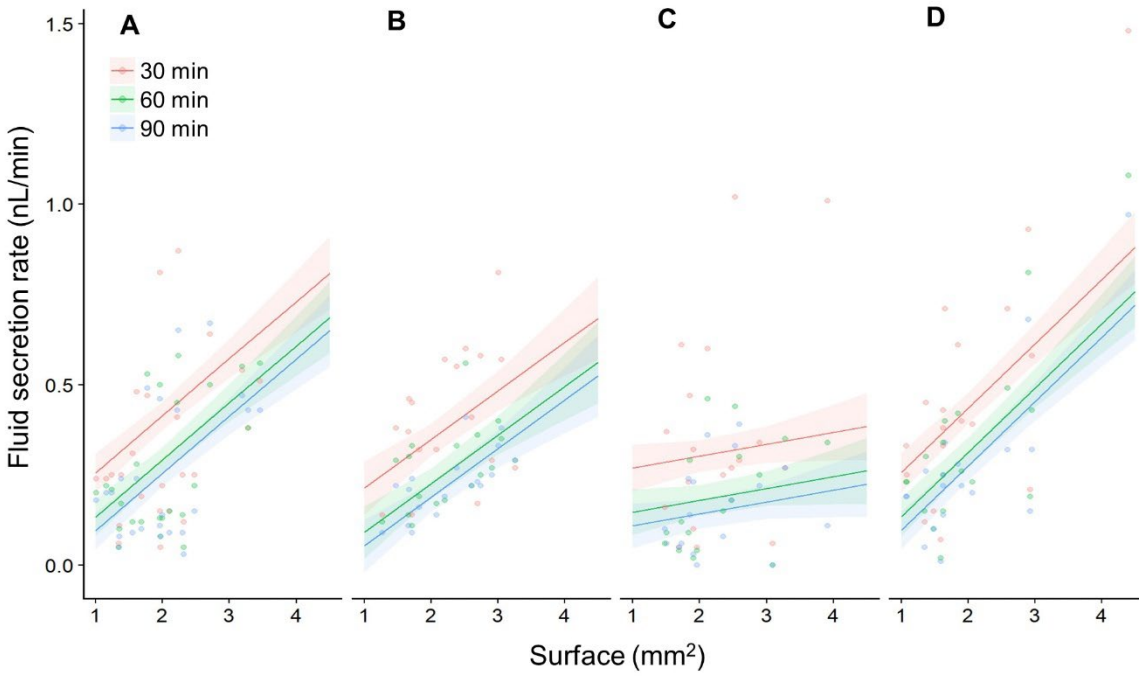

Figure 2.5. Tubule surface area positively influences the fluid secretion rate. (A) A plot of the surface area of the tubule exposed to the bath versus the fluid secretion rate for the R60 treatment every 30 minutes. (B) As in 'A' but for the V125 treatment. (C) As in 'A' but for V250 treatment. (D) As in 'A' but for the saline treatment. Small circles indicate the fluid secretion rates of individual tubules at a particular time point. Each line with the shaded area around represents the mixed effect model fit with the standard error.

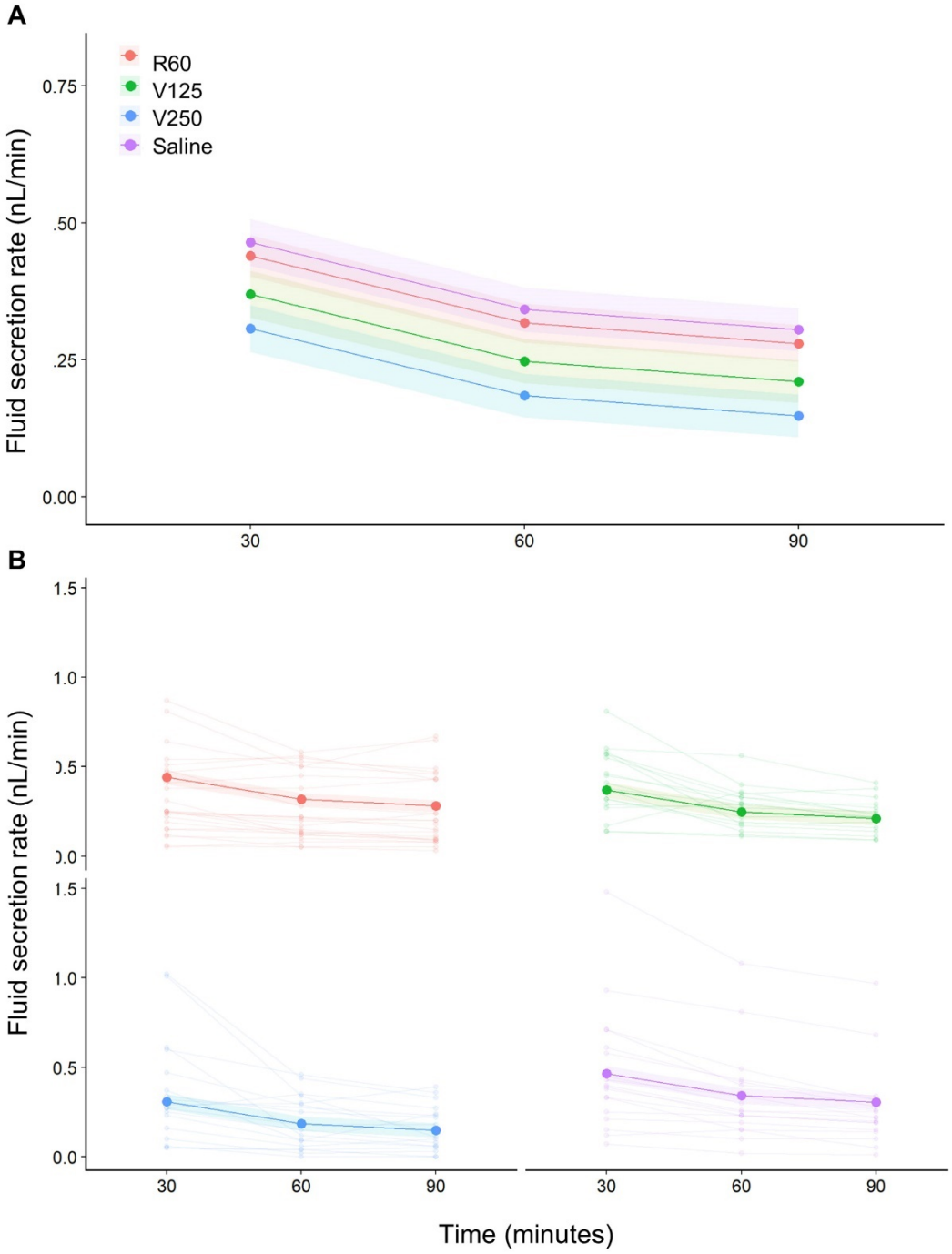

Figure 2.6. Mean fluid secretion rate of Malpighian tubules during the incubation period. (**A**) The mean fluid secretion rate decreased after 60 minutes of incubation in all the treatments, while it remained steady for the next 30 minutes in all the treatments except in the saline one. Each line with the shaded area around represents the mixed effect model fit with the standard error. (**B**) As in 'A', but with the fluid secretion rate of individual tubules shown by pale lines linking smaller dots.

2.4.2 Renewing bath saline increases the fluid secretion rate

To exclude the possibility that the decrease in the fluid secretion rate was caused by damage to the tubules during the Ramsay assay, we replaced the saline bath after 90 minutes with fresh saline, to determine whether tubules would increase their fluid secretion rate to previous levels. We removed three tubules from six locusts (17 tubules in total, one tubule excluded) and incubated them in saline for 90 minutes, removing the droplet secreted every 30 minutes. After 90 minutes the saline bath was removed, and replaced with fresh saline. The tubules were then incubated for further 90 minutes removing the droplet secreted every 30 minutes. The secretion rate decreased after 60 and 90 minutes (Figure 2.7, Table 2.3), but increased after 120 minutes following replacement of the saline (Figure 2.7, Table 2.3).

Table 2.3. Outcomes of the linear mixed effect model investigating the effect the replacement of the saline with a fresh saline after 90 minutes of incubation upon the fluid secretion rate. The model applied was (Secretion rate ~ surface + time + (1| locust) + (1+time|tubule)). The rows in bold indicate the first observation after the saline has been replaced. (**A**) Summary of the mean values of fluid secretion rate at each time point. (**B**) Pairwise comparisons between subsequent times of incubation.

A

		Time	Mean ± SE
Fluid secretion rate (nL/min)	Saline 1	30	1.54 ± 0.15
	Saline 1	60	0.91 ± 0.13
	Saline 1	90	0.63 ± 0.12
	Saline 2	**120**	**0.90 ± 0.10**
	Saline 2	150	0.53 ± 0.09
	Saline 2	180	0.40 ± 0.09

B

Contrasts	estimate ± SE	P-value
60 min vs 30 min	-0.63 ± 0.08	**<.001**
90 min vs 60 min	-0.28 ± 0.08	**0.006**
120 min vs 90 min	**0.28 ± 0.08**	**0.008**
150 min vs 120 min	-0.37 ± 0.08	**<.001**
180 min vs 150 min	-0.14 ± 0.08	0.500

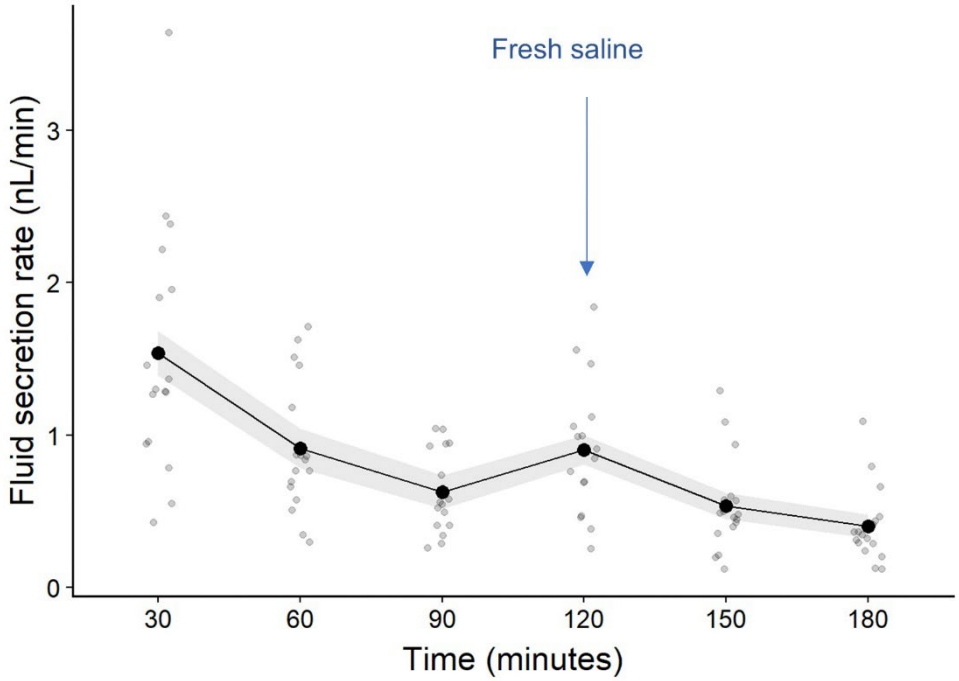

Figure 2.7. Mean fluid secretion rate of Malpighian tubules increases with fresh saline. The tubules were incubated in saline but after 90 minutes the saline bath was removed and replaced with fresh saline. The arrow indicates the first measurement taken after the saline had been replaced. Grey points indicate the fluid secretion rate of individual tubules at a particular time point.

2.4.3 Malpighian tubule integrity during the Ramsay assay

To exclude the possibility that manipulation during the Ramsay assay altered the diameter of the tubules, we measured the diameter of the tubules *in vivo*, at the beginning, and at the end of the assay. We found that the diameter of the tubules was unaffected by the assay and was comparable to the tubule's diameter *in vivo* (Figure 2.8, Table 2.4A,B).

Table 2.4. Outcomes of the linear mixed effect model investigating the diameter of the tubules in different moments during the Ramsay assay. The model applied was (diameter ~ assay time + (1| locust) + (1+time|tubule)). (**A**) Summary of the mean diameter of Malpighian tubules in vivo, at the beginning of the essay and at the end of the essay after 180 minutes of incubation. (**B**) Pairwise comparisons between different moments of the assay.

A

		Mean ± SE
Diameter (µm)	In vivo	71.0 ± 1.6
	Beginning assay	69.3 ± 1.6
	End assay	70.4 ± 1.6

B

	estimate ± SE	P-value
Beginning assay vs in vivo	-1.7 ± 1.1	0.260
end assay vs in vivo	-0.6 ± 1.1	0.847
end assay vs beginning assay	1.1 ± 1.1	0.551

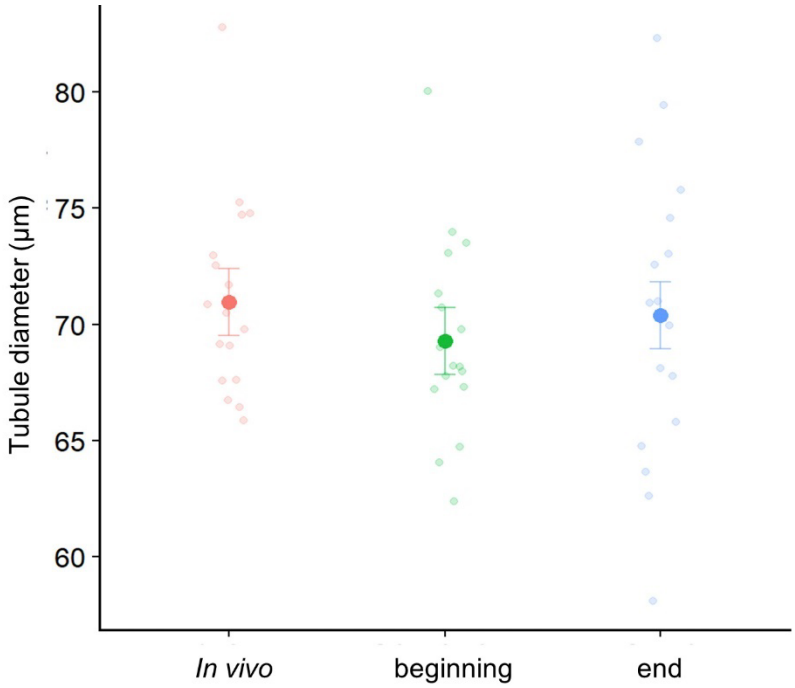

Figure 2.8. Manipulation of Malpighian tubules in the Ramsay assay did not affect their diameter. To exclude the possibility that manipulation during the assay affected tubule morphology, we measured the tubule's diameter *in vivo*, at the beginning, and at the end of the assay. The diameter was unaffected by the manipulation.

2.4.4 Net extrusion of Rhodamine B

We determined the concentration of Rhodamine B in each of the droplets collected from the Malpighian tubules exposed to the R60, V125 and V250 treatments at each time point. There was a significant interaction between treatment and time ($F_{4,73.19}$=15.19, p<.001, Figure 2.9A), indicating that the rhodamine B concentration changed over time depending on the treatment (Table 2.5). The concentration of rhodamine B in the droplets significantly increased during the incubation time in all the treatments (Table 2.5B, Figure 2.9A). In particular, the increase in rhodamine B concentration in the R60 treatment was more pronounced than in either the V125 or V250 treatments (Table 2.5C, Figure 2.9A). At each time point, the treatment significantly affected the rhodamine B concentration. Compared to the R60 treatment, the addition of verapamil in the V125 and V250 treatments reduced the rhodamine B concentration of the secreted droplets after 30, 60, and 90 minutes (Table 2.5D, Figure 2.9A). However, there was no significant difference in the rhodamine B concentration between the V125 and V250 treatments at any time point (Table 2.5D, Figure 2.9A).

Table 2.5. The outcomes of linear mixed effect model used to investigate the effect of incubation time and treatment on the rhodamine concentration (µM) of the droplets secreted. The model applied was (Rhodamine concentration ~ time * treatment + (1|locust) + (1+time|tubule)). (**A**) Summary of the mean values for each treatment at each time point. (**B**) Pairwise comparisons between time of incubation separately for each treatment. (**C**) Pairwise comparison of the interaction between time and treatment. D) Pairwise comparisons between treatments separately for each time of incubation.

A.	**Treatment**	**Time 30 min**	**Time 60 min**	**Time 90 min**
		Mean ± se	Mean ± se	Mean ± se
	R60	59.0 ± 7.3	217.9 ± 13.7	369.7 ± 22.4
	V125	9.0 ± 8.5	54.0 ± 15.8	103.4 ± 25.9
	V250	10.4 ± 8.5	74.4 ± 15.9	135.2 ± 25.9

B.	**Treatment**	**Time 60 vs time 30 min**		**Time 90 vs time 60 min**	
		estimate ± se	P-value	estimate ± se	P-value
	R60	158.9 ± 11.8	**<.001**	151.8 ± 11.8	**<.001**
	V125	45 ± 13.6	**0.004**	49.4 ± 13.6	**0.001**
	V250	64 ± 13.7	**<.001**	60.8 ± 13.7	**<.001**

C.	**Treatment**	**Time 60 vs time 30 min**		**Time 90 vs time 60 min**	
		estimate ± se	P-value	estimate ± se	P-value
	V125 vs R60	-113.9 ± 18	**<.001**	-102.4 ± 18	**<.001**
	V250 vs R60	-94.9 ± 18.1	**<.001**	-91 ± 18.1	**<.001**
	V250 vs V125	19 ± 19.3	0.588	11.4 ± 19.3	0.826

D.	**Treatment**	**Time 30 min**		**Time 60 min**		**Time 90 min**	
		estimate ± se	P-value	estimate ± se	P-value	estimate ± se	P-value
	V125 vs R60	-49.98 ± 11.2	**<.001**	-163.88 ± 20.9	**<.001**	-266.31 ± 34.2	**<.001**
	V250 vs R60	-48.59 ± 11.2	**<.001**	-143.49 ± 21.0	**<.001**	-234.53 ± 34.2	**<.001**
	V250 vs V125	1.39 ± 12.0	0.993	20.4 ± 22.5	0.637	31.78 ± 36.6	0.662

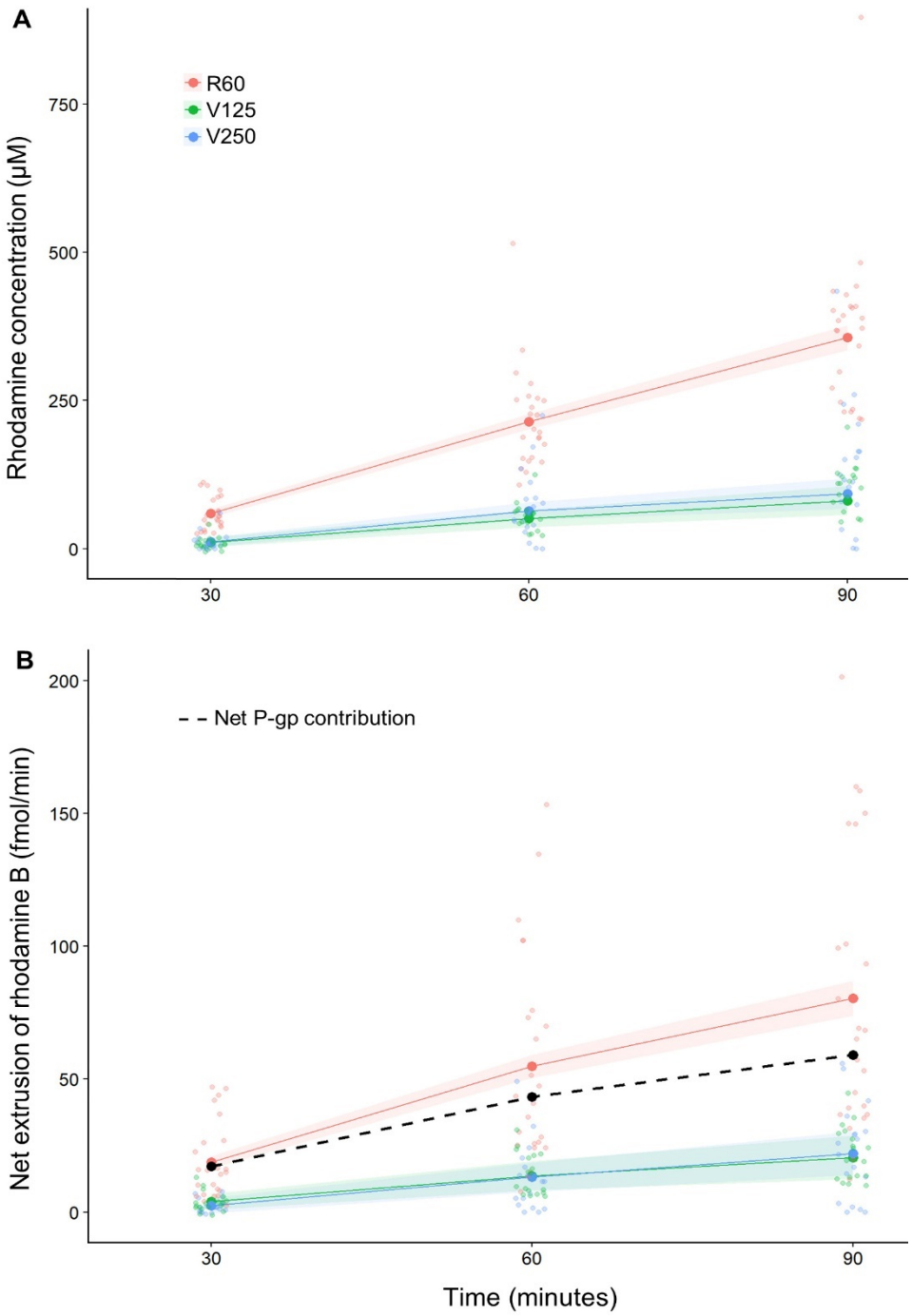

Figure 2.9. The mean rhodamine B concentration of the droplets secreted by the Malpighian tubules, and the mean net extrusion of rhodamine B increased over time in all the treatments. (A) The addition of verapamil (125 or 250 µM) reduced the rhodamine B concentration of the droplets secreted compared with the control treatment 60 µM rhodamine. (B) The addition of verapamil (125 µM or 250 µM) reduced the net extrusion of rhodamine B compared with the control treatment 60 µM rhodamine. The dashed line represents the net contribution of the P-glycoproteins obtained by subtracting the net extrusion of rhodamine in the V250 treatment from the R60. Each line with shaded region represents the mixed effect model fit with the standard error. Small circles represent the raw data.

We calculated the total number of moles of rhodamine B extruded by the Malpighian tubules per minute as the product of the secretion rate and the rhodamine B concentration of the droplets secreted, which we termed the net extrusion rate. There was an interaction between treatment, time and surface ($F_{4,74.59}$=2.56, p=0.046; Table 2.6, Figs 2.9B, 2.10A-C), indicating that the dependency of the net extrusion of rhodamine B upon tubule surface area was influenced by the incubation time and the treatment (Table 2.6A,E, Figure 2.10A-C). The net extrusion increased significantly between 30 and 60 minutes in all the treatments (Table 2.6B, Figure 2.9B). In contrast, between 60 and 90 minutes the net extrusion increased only for the tubules incubated in R60 and V250, whereas it remained steady for V125 treatments (Table 2.6B, Figure 2.9B). In particular, the net extrusion was more pronounced in the R60 treatment compared to V125 and V250 (Table 2.6C, Figure 2.9B). At each time point, the treatment affected the net extrusion of rhodamine B (Table 2.6D, Figure 2.9B). In comparison to the R60 treatment, the addition of verapamil in the V125 and V250 treatments significantly reduced the net extrusion of rhodamine B after 30, 60 and 90 minutes, however, there was no significant difference between the V125 and V250 treatments (Table 2.6D, Figure 2.9B).

Table 2.6. Outcomes of a linear mixed model to investigate the effect of incubation time and treatment on the net quantity of rhodamine extruded (fmol/min) in the droplets secreted per minute. The model applied was (Net extrusion of rhodamine ~ surface * time * treatment + (1| locust) + (1+time|tubule)). (A) Summary of the mean value for each treatment at each time point. (B) Pairwise comparisons between time of incubation separately for each treatment. (C) Pairwise comparison of the interaction between time and treatment. (D) Pairwise comparisons between treatments separately for each time of incubation. (E) Estimates of the influence of each unit of surface (mm^2) on the quantity of rhodamine extruded at each time point. (F,G) Outcomes of the linear mixed effects model to investigate the effect of the secretion rate (SR) on the quantity of rhodamine extruded in the droplets secreted. The model applied was (Net extrusion of rhodamine ~ secretion rate * treatment * time + (1| locust) + (1+time|tubule)). The means estimate the influence of each nL/min of secretion rate on the quantity of rhodamine extruded.

A.	**Treatment**	**Time 30 min**	**Time 60 min**	**Time 90 min**	**Total**
		Mean ± se	Mean ± se	Mean ± se	Mean
	R60	19.5 ± 2.5	58.9 ± 4.7	87.4 ± 7.0	165.8
	V125	3.7 ± 2.8	14.0 ± 5.3	21.5 ± 8.0	39.2
	V250	2.3 ± 2.9	13.5 ± 5.4	22.2 ± 8.0	38
	Net P-gp contribution	17.2 (88%)	45.4 (77%)	65.2 (75%)	127.8 (77%)

B.	**Treatment**	**Time 60 vs time 30 min**		**Time 90 vs time 60 min**	
		estimate ± se	P-value	estimate ± se	P-value
	R60	39.5 ± 3.1	**<.001**	28.5 ± 3.1	**<.001**
	V125	10.3 ± 3.5	**0.013**	7.5 ± 3.5	0.092
	V250	11.2 ± 3.6	**0.006**	8.7 ± 3.6	**0.046**

C.	**Treatment**	**Time 60 vs time 30 min**		**Time 90 vs time 60 min**	
		estimate ± se	P-value	estimate ± se	P-value
	V125 vs R60	-29.2 ± 4.7	**<.001**	-21.0 ± 4.7	**<.001**
	V250 vs R60	-28.2 ± 4.8	**<.001**	-19.8 ± 4.8	**<.001**
	V250 vs V125	1.0 ± 5.0	0.981	1.2 ± 5.0	0.971

D.	**Treatment**	**Time 30 min**		**Time 60 min**		**Time 90 min**	
		estimate ± se	P-value	estimate ± se	P-value	estimate ± se	P-value
	V125 vs R60	-15.8 ± 3.8	**<.001**	-44.9 ± 7.1	**<.001**	-65.9 ± 10.6	**<.001**
	V250 vs R60	-17.2 ± 3.8	**<.001**	-45.4 ± 7.1	**<.001**	-65.2 ± 10.7	**<.001**
	V250 vs V125	-1.4 ± 4	0.937	-0.4 ± 7.6	0.998	0.7 ± 11.3	0.998

E.	**Treatment**	**Time 30 min**		**Time 60 min**		**Time 90 min**	
		surf. trend ± se	P-value	surf. trend ± se	P-value	surf. trend ± se	P-value
	R60	4.1 ± 3.8	0.287	23.4 ± 7.0	**0.002**	40.2 ± 10.5	**<.001**
	V125	-1.1 ± 4.8	0.818	2.2 ± 8.9	0.803	5.2 ± 13.3	0.698
	V250	-1.1 ± 4.4	0.811	2.2 ± 8.2	0.793	0.9 ± 12.2	0.943

F.	Treatment	Time 30 min		Time 60 min		Time 90 min	
		SR trend ± se	P-value	SR trend ± se	P-value	SR trend ± se	P-value
	R60	63.1 ± 7.2	**<.001**	192.1 ± 11.0	**<.001**	254.4 ± 15.2	**<.001**
	V125	12.4 ± 7.4	0.749	30.4 ± 17.2	0.081	42.5 ± 34.3	0.219
	V250	-4.1 ± 5.2	0.429	53.4 ± 12.1	**<.001**	79.2 ± 22.5	**<.001**

G.	Treatment	Time 30 min		Time 60 min		Time 90 min	
		estimate ± se	P-value	estimate ± se	P-value	estimate ± se	P-value
	R60 vs V125	60.7 ± 10.3	**<.001**	161.7 ± 20.45	**<.001**	211.9 ± 37.5	**<.001**
	R60 vs V250	67.2 ± 8.8	**<.001**	138.6 ± 16.4	**<.001**	175.2 ± 27.2	**<.001**
	V125 vs V250	6.5 ± 9.0	0.754	-23.0 ± 21.1	0.522	-36.7 ± 41.1	0.645

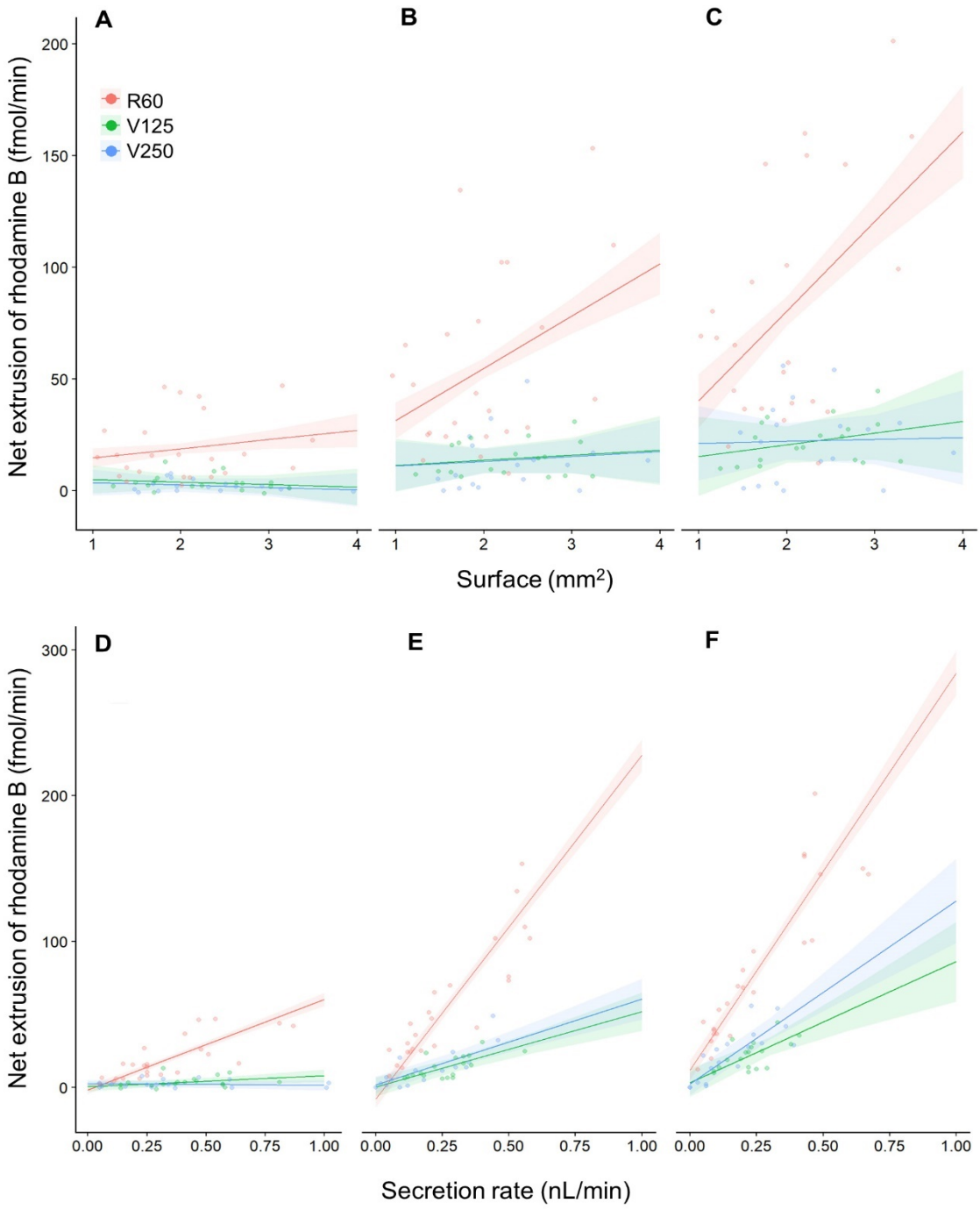

Figure 2.10. Tubule surface area and the fluid secretion rate positively influences the net extrusion of rhodamine B. (**A**) A plot of the surface area of the tubule exposed to the bath versus the net extrusion of rhodamine B for each of the three treatments after 30 minutes of incubation. (**B**) As in 'A' but after 60 minutes. (**C**) As in 'A' but after 90 minutes. (**D**) A plot of the fluid secretion rate versus the net extrusion of rhodamine B for each of the three treatments after 30 minutes of incubation. (**E**) As in 'D' but after 60 minutes. (**F**) As in 'D' but after 90 minutes. Small circles indicate the net extrusion of rhodamine B of individual tubules at a particular time point. Each line with the shaded area around represents the mixed effect model fit with the standard error.

The surface area positively influenced the net extrusion of rhodamine B in the tubules incubated for 60 and 90 minutes in the R60 treatment, while there was no correlation between surface area and net extrusion in V125 and V250 treatments (Table 2.6E, Figure 2.10A-C). In addition, we found that after 30 minutes the fluid secretion rate of the tubules incubated in R60 positively influenced the net extrusion of rhodamine B, while there was no significant effect in the V125 and V250 treatments (Table 2.6F, Figure 2.10D). After 60 and 90 minutes, the fluid secretion rate positively correlated with the net extrusion of rhodamine B in R60 and V250 treatments, but not V125 (Table 2.6F, Figure 2.10E,F). Moreover, the secretion rate of the tubules incubated in R60 showed a more pronounced effect on the net extrusion of rhodamine B than that of the tubules incubated in V125 and V250 (Table 2.6G, Figure 2.10D-F).

2.4.5 Net contribution of P-glycoproteins

A single Malpighian tubule with a mean surface area incubated in a solution of 60 µM rhodamine B, extruded rhodamine B with a rate of 19.5 ± 2.6 fmol/min in the first 30 minutes, 58.9 ± 4.7 fmol/min between 30 and 60 minutes and 87.4 ± 7.0 fmol/min between 60 and 90 minutes (Table 2.6A). The addition of verapamil significantly reduced the quantity of rhodamine B extruded but did not block it completely (Table 2.6A). The rhodamine B concentration of the droplets secreted did not differ between the V125 and V250 treatments (Table 2.5A). Therefore, we assumed that 125 µM verapamil was sufficient to inhibit all the P-glycoproteins that are verapamil sensitive and that passive diffusion and other types of pumps may contribute to extrusion of Rhodamine B. We subtracted the mean values of the net extrusion of rhodamine B obtained from the V250 treatment from the net extrusion of the R60 treatment to estimate the contribution of the P-glycoproteins alone. The average rates of rhodamine B extruded by the P-

glycoproteins were 17.2 fmol/min, 45.4 fmol/min and 65.2 fmol/min between 0-30, 30-60 and 60-90 minutes respectively (Table 2.6A, Figure 2.9B dashed line). In percentage, the P-glycoproteins are responsible for 88%, 77% and 75% (between 0-30, 30-60 and 60-90 minutes respectively) of the total extrusion of rhodamine B. Overall, P-glycoproteins account for the 77% of the total extrusion of rhodamine B, over the 90 minutes of incubation.

2.5 Discussion

Our aim was to determine whether P-glycoprotein transporters are involved in the removal of xenobiotic substances by Malpighian tubules from the haemolymph of desert locusts and, if so, how they perform physiologically. To this end, we developed an alternative method to liquid chromatography–mass spectrometry (Andersson et al., 2013), radiolabelled alkaloids (Gaertner et al., 1998) or confocal microscopy (Leader and O'Donnell, 2005; O'Donnell and Leader, 2006) based upon measuring dye concentration. A similar method has been used previously to investigate epithelial transport in tardigrades and desert locusts using chlorophenol red by imaging through the gut or tubules (Halberg and Møbjerg, 2012). By imaging extruded drops from Malpighian tubules, we assessed the performance of epithelial transporters more accurately than by imaging the lumen of the tubules. We used the P-glycoprotein substrate rhodamine B (Eytan et al., 1997) and the P-glycoprotein inhibitor verapamil (Tsuruo et al., 1981) to assay P-glycoprotein function through the colour of the droplets secreted by the Malpighian tubules. Using this strategy, we obtained evidence that desert locust Malpighian tubules express a P-glycoprotein transporter, that the fluid

secretion rate of these tubules is proportional to their surface area, and that the fluid secretion rate influences the net extrusion of rhodamine B.

2.5.1 A P-glycoprotein transporter extrudes xenobiotics in desert locust Malpighian tubules

Our conclusion that desert locust Malpighian tubules express P-glycoproteins is supported by two lines of evidence. Firstly, these tubules actively extrude the dye rhodamine B, a P-glycoprotein substrate (e.g. Eytan et al., 1997; Mayer et al., 2009), when it is present in the solution in which they are incubated. Rhodamine B has been widely used as a substrate for P-glycoproteins in cell culture and blood brain barrier models (e.g. Eytan et al., 1997; Mayer et al., 2009). Secondly, the addition of verapamil, a P-glycoprotein inhibitor (e.g. Andersson et al., 2014; Dermauw and Van Leeuwen, 2014; Hamada et al., 1987; Tsuruo et al., 1981), significantly reduced the extrusion of rhodamine B. The presence of P-glycoproteins in other tissues of the desert locusts also supports our conclusion that they are expressed in the Malpighian tubules; proteins with a comparable physiology and similar sequence to the human P-glycoprotein (ABCB1 gene) are expressed within the blood brain barrier of the locusts *S. gregaria* and *L. migratoria* (Al-Qadi et al., 2015; Andersson et al., 2014, 2013; Nielsen et al., 2011). Further support comes from comparison with other insects; P-glycoprotein transporters have been found in the Malpighian tubules of many other insects including the black field cricket (*Teleogryllus commodus)* (Leader and O'Donnell, 2005), tobacco hornworm (*Manduca sexta)* (Gaertner et al., 1998; Murray et al., 1994), fruit fly (*D. melanogaster*), kissing bug (*Rhodnius prolixus*), large milkweed bug (*Oncopeltus fasciatus*), yellow fever mosquito (*Aedes aegypti*), house cricket (*Acheta domesticus*), migratory locust (*Locusta*

migratoria), mealworm beetle (*Tenebrio molitor*), and the American cockroach (*Periplaneta americana)* (Rheault et al., 2006).

Rhodamine B extrusion was not blocked entirely by 125 µM verapamil or by double this concentration. Comparison of the extent of the reduction at both concentrations suggests that even at the lower verapamil concentration all the P-glycoproteins were inhibited. This suggests that approximately 77% of rhodamine B was transported by P-glycoproteins via a verapamil sensitive mechanism, whilst the remaining 23% was verapamil insensitive. Moreover, after 90 minutes the concentration of rhodamine B in the droplets secreted by the Malpighian tubules was higher than that of the bathing solution suggesting that rhodamine B transport in the presence of verapamil is not simply due to passive diffusion, but that other active transporters may be implicated. Several potential candidates for alternative active transporters exist. For example, in human cell lines (Calu-3), rhodamine B can interact with multiple organic cation transporters (OCT3, OCTN1,2) (Ugwu et al., 2016). Potentially, however, verapamil may be incapable of blocking rhodamine B transport completely allowing a small number of rhodamine B molecules to be extruded by the P-glycoprotein even in the presence of verapamil.

Verapamil inhibits P-glycoproteins and does not interact with other multidrug resistance proteins (Cole et al., 1989). This suggests that the effects of verapamil in our experiments are through its effects upon the activity of P-glycoproteins. The mechanistic basis of verapamil inhibition is unclear but the most widely accepted explanation is that P-glycoproteins extrude both verapamil and their substrate but that verapamil diffuses back across the lipid bilayer much faster than the substrate creating a futile cycle and thereby competing with the substrate transport (Eytan et al., 1996; Sharom, 1997).

Verapamil is, however, also a known L-type Ca^{2+} channel blocker (Abernethy and Schwartz, 1999). In *Drosophila*, L-type Ca^{2+} channels are expressed in the basolateral and apical membranes of the tubule principal cells, and are involved in the regulation of the fluid secretion (MacPherson et al., 2001). Indeed, an increase of the intracellular Ca^{2+} level mediates the effect of diuretic hormones (Paluzzi et al., 2013). Verapamil reduced the fluid secretion of *Drosophila* Malpighian tubules stimulated by the peptide agonist CAP2b and by the intracellular second messenger cGMP, but had no effect on unstimulated tubules (MacPherson et al., 2001). Our experiments cannot exclude the possibility that verapamil affects Ca^{2+} channels in locust Malpighian tubules by interfering with intracellular Ca^{2+}, and thereby has an indirect effect on Rhodamine B extrusion. However, because the fluid secretion rate did not differ between treatments, we suggest that the reduction of the net rhodamine B extrusion could be caused solely by verapamil inhibition of the P-glycoprotein activity, in agreement with previous studies (Andersson et al., 2014; Dermauw and Van Leeuwen, 2014; Hamada et al., 1987; Leader and O'Donnell, 2005). Thus, when considered within the context of the expression of P-glycoproteins in the blood brain barrier of locusts (Al-Qadi et al., 2015; Andersson et al., 2014, 2013; Nielsen et al., 2011) and the expression of P-glycoproteins in homologous Malpighian tubules in other insects species (see above), it seems highly likely that the tubules of gregarious desert locust express P-glycoproteins. Further pharmacological experiments using different P-glycoprotein substrates (e.g. digoxin, colchicine) and inhibitors (e.g. quinidine, vinblastine) would strengthen our findings (Kim, 2002; Srivalli and Lakshmi, 2012), and immunohistochemical and gene knockdown experiments will be necessary to prove definitively the presence of P-glycoproteins.

2.5.2 Malpighian tubule surface area and fluid secretion rate

We used linear mixed effect models to determine which physical feature of a tubule, its surface area, length or diameter has the greatest influence on fluid secretion rate. We found that the surface area of the Malpighian tubules positively influences their fluid secretion rate, and more accurately predicts the fluid secretion rate than tubule diameter or length. Some previous studies have reported that tubule length was linearly related to the fluid secretion rate (Beyenbach et al., 1993; Coast, 1988) but in our analysis length was consistently worse than surface area as a predictor. Even when the surface area (or length) has been accounted for in previous studies, this has involved dividing the fluid secretion rate by the surface area (or length) to obtain the fluid secretion rate per unit area (or unit length) (Bradley, 1983; Rheault et al., 2006). Although this approach is useful when comparing tubules of different sizes from different insect species, it fails to reveal the exact relationship between the surface area and the fluid secretion rate. Our statistical models demonstrate that interactions between factors such as surface area and fluid secretion rate depend upon the treatment applied. For example, our results show that the fluid secretion rate increases with the increase of the tubule surface in all the treatments apart from 250 µM verapamil, where the surface no longer influences the fluid secretion rate. Such interdependencies are unlikely to be detected or accounted for in simpler statistical analyses, causing such interdependencies to be ignored.

2.5.3 Reliability of isolated Malpighian tubule measurements

The decrease in fluid secretion rate that we found is comparable with other studies of isolated Malpighian tubules, where the fluid secretion rate of tubules incubated in saline decreased by 30% after 20 minutes (Coast et al., 1993) or over one hour (Proux et al.,

1988). In contrast, fluid secretion rates have been found steady over time in studies where the Malpighian tubules were left attached to the gut through the tracheae, and the whole preparation immersed in saline (James et al., 1993; Maddrell and Klunsuwan, 1973). One of the differences between the isolated tubule assay and the whole gut assay is the shorter portion of trachea in contact with the tubule immersed in the bathing solution. Therefore, the reduction of the fluid secretion rate in isolated tubules may be a consequence of a smaller amount of residual oxygen in the tracheae compared with the whole gut preparation. Additionally, the volume of the bathing solution in the isolated tubule assay is far smaller than in the whole gut preparation, which could lead to a quicker depletion of oxygen and/or other substances in the bathing solution. Replacing the bathing solution with fresh saline produced an increase in the fluid secretion rate, supporting this interpretation. Finally, there could be a decrease in the tubule diameter during the assay compared to the *in vivo* preparation. We can safely exclude this, however, because we found that the tubule diameter was unaffected by the assay and was similar to the tubule's diameter *in vivo*.

2.5.4 Determining the transepithelial transport of xenobiotics by P-glycoprotein transporters in locust Malpighian tubules

Transepithelial transport of xenobiotics by P-glycoproteins across the inner membrane of the Malpighian tubules can be determined from the net extrusion rate we measured. The disparity between the transepithelial transport and the net extrusion rate is due to the properties of rhodamine B. This lipophilic dye can passively permeate the lipid bilayer of liposomes, following the concentration gradient (Eytan et al., 1997). Likewise, in desert locust Malpighian tubules, rhodamine B can back diffuse into the bath solution when active transporters increase its luminal concentration creating a concentration

gradient. Consequently, the fluid secretion rate affects the net extrusion rate of rhodamine B because a decrease in the fluid secretion will increase the luminal concentration of the dye, allowing greater back diffusion whilst an increase in the fluid secretion rate will have the opposite effect (Maddrell et al., 1974). Therefore, if a dye, like rhodamine B, can back diffuse, its net extrusion will be proportional to the fluid secretion rate (Figure 2.11A-C), while if the dye cannot back diffuse there will be no correlation (Figure 2.11D-F). When the rhodamine B concentration in the lumen was lower or similar to the bath (60 μM) the net transport would be little affected by the secretion rate. Conversely, when the luminal rhodamine B concentration was higher than the bathing solution, part of the dye diffused back, so that the net extrusion was significantly influenced by the fluid secretion rate.

Previous studies demonstrated that Malpighian tubules can exhibit different degrees of passive permeability to different substances, depending on the species and on the properties (i.e. size, polarity) of the substrate used (e.g. dyes, alkaloid, drugs) (Maddrell et al., 1974). For example, tubules of the kissing bug (*R. prolixus*) have passive permeability to the alkaloid nicotine (Maddrell and Gardiner, 1976), but not to the dye indigo carmine (Maddrell et al., 1974), whereas tubules of the blowfly (*Calliphora erythrocephala*) are permeable to indigo carmine (Maddrell et al., 1974). Thus, when studying the effect of a substance on active transporters, it is important to take into account the fluid secretion rate because a change in the net extrusion rate of a substrate may be caused not only by a direct effect on the transporters, but also by an indirect consequence of a change in the fluid flow (O'Donnell and Leader, 2006). In our experiment, the fluid secretion rate did not differ between treatments, indicating that the reduction of net extrusion of rhodamine B following exposure to verapamil was

caused solely by inhibition of the P-glycoprotein. However, the fluid secretion rate decreased over time, which may produce an underestimation of the net transepithelial transport of rhodamine B.

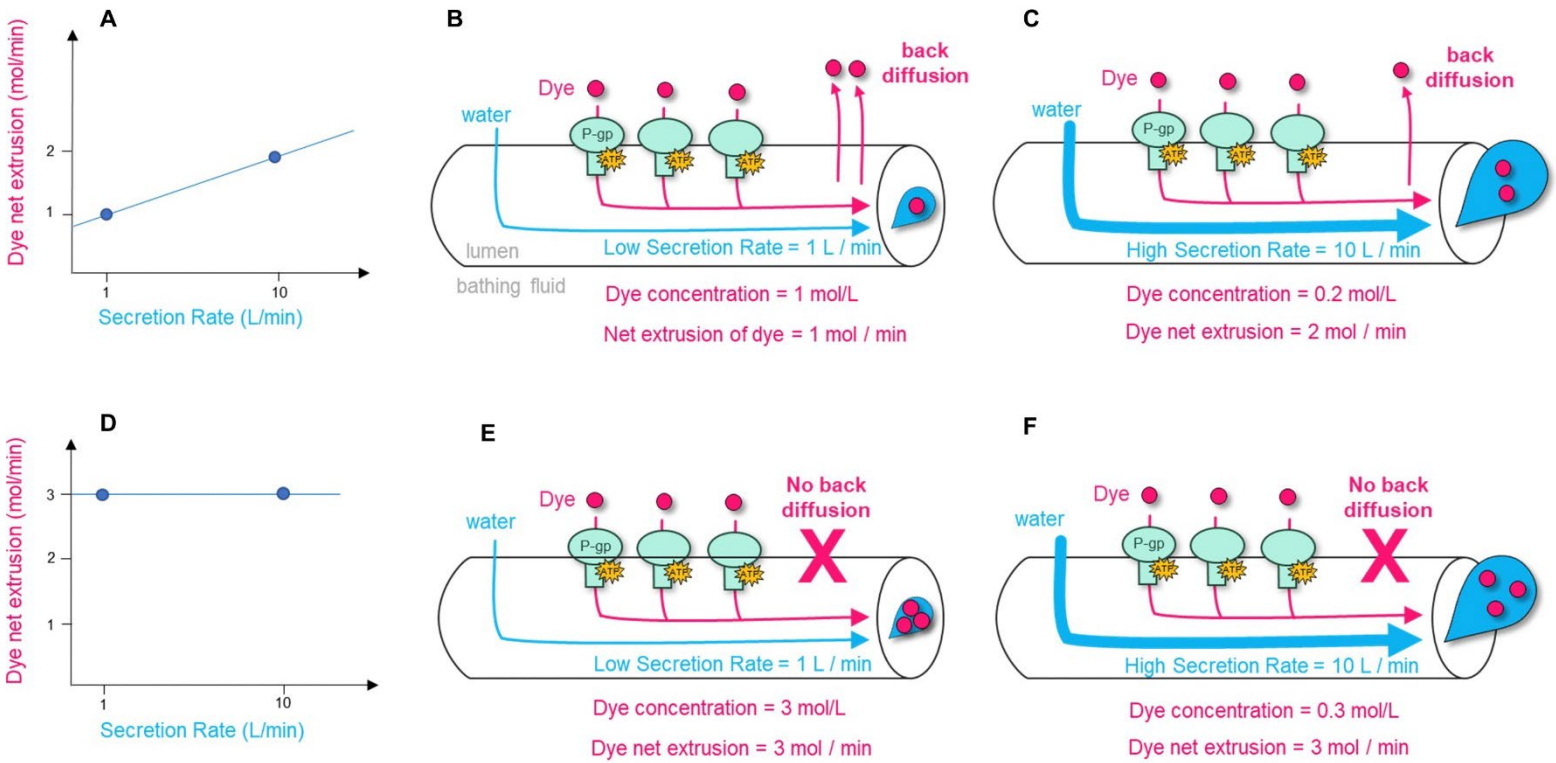

Figure 2.11. Schematic representation of the influence of the back diffusion on the net extrusion of a dye. (A) The net extrusion of a dye positively correlates with the fluid secretion rate when the dye can back diffuse from the lumen to the bathing fluid. (B) Indeed, at low fluid secretion rate, the dye concentration in the lumen rises, increasing the back diffusion and reducing the net transport of the dye. (C) Instead, at higher fluid secretion rates, the dye concentration in the lumen is diluted and the back diffusion is reduced, increasing the net transport of the dye. (D) If no back diffusion occurs, there is no correlation between the net transport of the dye and the fluid secretion rate. (E,F) At any rate of fluid secretion, the net transport of the dye remains constant, independently of the dye concentration in the lumen.

2.5.5 Implications for desert locust xenobiotic extrusion

Gregarious desert locusts feed on a broad variety of plants including species containing secondary metabolites such as atropine to become unpalatable to predators (Despland and Simpson, 2005a; Mainguet et al., 2000; Pener and Simpson, 2009; Sword et al., 2000). The expression of P-glycoproteins in the Malpighian tubules to extrude noxious substances may be an adaptation to cope with the ingestion of toxic plants. This may also be the reason for expression of P-glycoproteins on the blood brain barrier of desert locusts, which would prevent the uptake of hydrophobic substances in the central nervous system (Andersson et al., 2014). Yet the relationship between ingesting toxins and xenobiotic extrusion pathways in the Malpighian tubules is not straightforward; some species of Orthoptera, as well as Coleoptera, Lepidoptera, Heteroptera, Hymenoptera and Sternorrhyncha (Opitz and Müller, 2009), sequester toxins from the plants they ingest to deter predators. However, toxicity may also be conferred by gut contents, rather than through sequestration within bodily tissues. For example, the chemical defence of the spotted bird grasshopper, *Schistocerca emarginata* (=*lineata*), is mediated by the contents of toxic plant in its gut (Sword, 2001, 1999). This species is a congener of the desert locust, *S. gregaria*, which suggests that a similar strategy may be involved in the production of toxicity in this species. If this is the case, then the presence of toxins in the haemolymph may be a consequence of ingesting toxic plant material for storage within the gut. In such a scenario, xenobiotic extrusion pathways within the Malpighian tubules would then be essential for ensuring that toxins do not accumulate within the haemolymph to concentrations that would affect physiological processes.

The P-glycoprotein xenobiotic extrusion pathway that we have characterised in desert locusts is likely to be highly effective in extruding xenobiotic compounds from haemolymph, especially when the number of Malpighian tubules within an individual locust is considered. However, it is important to consider that the locusts used for our experiments have experienced a diet free of toxins, such as atropine. P-glycoprotein expression can be modulated depending on the diet (Tapadia and Lakhotia, 2005); *Drosophila* larvae fed on colchicine increased the expression of the P-glycoprotein gene homologue mdr49 in the brain and gut. Consequently, adult gregarious desert locusts that have fed on a diet including plant toxins may have even stronger P-glycoprotein xenobiotic extrusion pathways (this hypothesis is tested in Chapter 3 of the thesis). In contrast to their gregarious counterparts, solitary desert locusts actively avoid plants containing atropine (Despland and Simpson, 2005a), and find odours associated with it aversive (Simões et al., 2013). Thus, Malpighian tubules of solitary desert locusts may express fewer P-glycoproteins than those of the gregarious phase, possibly due to their reduced exposure to secondary metabolites from their diet.

2.5.6 Implications for insect pest control

P-glycoproteins are implicated in the resistance of some insect pests to insecticides (Lanning et al., 1996) promoting the efflux of various xenobiotics thereby decreasing the intracellular drug accumulation. For instance, P-glycoproteins have been detected in a resistant strain of the pest cotton bollworm (*Helicoverpa armigera*) but not in a susceptible one (Srinivas et al., 2004). P-glycoproteins have also been implicated in the protection of the mitochondria from insecticide damage (Akbar et al., 2014). In Africa and Asia, applications of insecticides are carried out to try to control desert locust plagues (Van Huis, 2007). To increase the efficacy, we propose that a combination of P-

glycoprotein inhibitors and insecticide may act synergistically to increase the locust mortality, reducing the amount of insecticide used. Further investigation into the interaction between P-glycoproteins and different xenobiotics (i.e. insecticides, herbicides, miticides and secondary metabolites) may improve our understanding of the physiological effect of pesticides on insects, and subsequently lead to the development of more specific targeted insecticides.

3 The impact of phase polyphenism on extrusion and secretion by Malpighian tubules in the desert locust

3.1 Abstract

The desert locust (*Schistocerca gregaria*) exists as two extreme phenotypes with intermediate transiens forms. Solitarious locusts avoid eating potentially harmful compounds while gregarious ones broaden their diet including plants producing toxic alkaloid such as atropine, and remarkably, transiens locusts show active preference for toxic plants. We determined how phenotypic transformation and exposure to food containing atropine influence the Malpighian tubules performance of the desert locust. Using the P-glycoprotein substrate rhodamine B as a proxy for P-glycoprotein mediated extrusion of a toxin, we compared the tubule physiology of solitarious, transiens, and gregarious locusts fed on a diet containing atropine, and on an alkaloid-free diet. We found that (i) the net rhodamine extrusion per unit of surface area did not differ between solitarious, gregarious and transiens locusts reared on an alkaloid-free diet; (ii) the secretion rate positively correlated with the tubule surface area, was not different between solitary and gregarious locusts, and was not influenced by atropine; (iii) the affinity of P-glycoproteins to atropine was similar in gregarious and solitary locusts; (iv) exposure to the alkaloid diet increased the net rhodamine extrusion in gregarious and transiens locusts; (v) the affinity of P-glycoproteins to atropine was similar in gregarious locusts reared on alkaloid and alkaloid-free diet; and (vi) gregarious locusts reared on

the alkaloid diet decreased their net rhodamine extrusion after being switched to an alkaloid-free diet.

3.2 Introduction

The ongoing evolutionary arms race between plants and insects has led plants to evolve an arsenal of chemical compounds, known as secondary metabolites, as a defence against insect herbivores. These secondary metabolites can act as repellents or can induce toxicity, changing the behaviour of the herbivores that ingest them and, in some cases, leading to their death (Ibanez et al., 2012). To counter these defences, insect herbivores that ingest noxious compounds have evolved a variety of non-mutually exclusive mechanisms for coping with them. Such mechanisms include avoiding ingestion of noxious compounds, their enzymatic detoxification, conjugation, insensitivity to them at target sites, and their excretion (Després et al., 2007). Some insects even exploit noxious secondary metabolites to their advantage by sequestering toxins to deter their own predators (Nishida, 2002).

The feeding behaviours of insects, and consequently their exposure to noxious secondary metabolites, can change across their life history. The immature stages of holometabolous insects typically have different feeding habits and diets from the mature stage. For example, the larvae of the tobacco hornworm, *Manduca sexta*, feed on leaves of solanaceous plants containing alkaloids, while adults feed on floral nectar of various plants that is virtually toxin free (Gilmore, 1938). Hemimetabolous insects do not undergo such a dramatic change in development and growth, and the feeding habits of the nymphs typically reflect those of the adults. Nevertheless, in some hemimetabolous insects environmental cues can induce phenotypic and behavioural

changes that influence their feeding ecology. One such insect is the desert locust, *Schistocerca gregaria*.

In response to changes in local population density, the desert locust manifests two distinct phenotypes, the solitarious and gregarious phases, which differ considerably in ecology, physiology, colour, size and behaviour (Ahmed et al., 2005; Despland and Simpson, 2005a; Pener, 1991; Pener and Simpson, 2009; Simões et al., 2016; Uvarov, 1977). The transition (transiens phase) from one extreme phase to the other can occur within the lifetime of an individual (Uvarov and Zolotarevsky, 1929). Solitarious locusts are cryptic, live at low densities, are repelled by other locusts, and are relatively inactive. An increase in population density triggers solitarious locusts to shift to a swarming gregarious phase characterised by bright aposematic colouration, increased activity, and attraction to conspecifics (Ellis and Pearce, 1962; Roessingh et al., 1993; Simpson et al., 1999). During the shift from the solitarious to the gregarious phase, the locusts' foraging behaviour changes.

Desert locusts are generalist herbivores, but gregarious locusts consume a broader variety of plant species than do solitarious locusts. Conspicuous gregarious locusts incorporate toxic vegetation in their diet to acquire antipredator protection, regurgitating their gut content when disturbed (Sword, 2001). Conversely, solitary locusts are repelled by the presence of potentially harmful compounds in their diet (Despland and Simpson, 2005a; Simões et al., 2013). One of the preferred host plants of gregarious locusts, is the Egyptian henbane (*Hyoscyamus muticus*), which contains the toxic alkaloid atropine (hyoscyamine) (Popov et al., 1991). Food choice experiments show that solitary locusts avoid food containing atropine, but once they start their transition to the gregarious phase (transiens locusts), not only do they lose their

aversion to this compound, but they actively prefer feeding on food containing atropine (Despland and Simpson, 2005a,b; Simões et al., 2016).

Atropine is a toxic alkaloid that inhibits the muscarinic actions of acetylcholine (Pauling and Petcher, 1970), therefore, locusts, which have muscarinic receptors in their nervous system, must circumvent the potential deleterious effects of this noxious compound (Aguilar and Lunt, 1984; Trimmer and Berridge, 1985). Atropine and other alkaloids are substrates of the multidrug P-glycoprotein (P-gps, MDR1 or ABCB1), a member of the ABC transporter family (Joosen et al., 2016; Wright and Dantzler, 2004). In insects, P-glycoproteins are expressed in the gut membrane and the blood brain barrier to prevent the uptake of toxins and protect the nervous system, and in the Malpighian tubules to actively remove toxic substances from the haemolymph (Andersson et al., 2014, 2013; Dobler et al., 2015; Gaertner et al., 1998; O'Donnell, 2009; Petschenka et al., 2013). The expression of P-glycoproteins and other ABC transporters can be regulated by diet (Chahine and O'Donnell, 2009; Tapadia and Lakhotia, 2005). For example, in *Drosophila*, exposure to colchicine in the diet increases the expression of mdr49 (P-glycoprotein homologue gene) in the gut and in the brain (Tapadia and Lakhotia, 2005), and exposure to methotrexate increases the expression of several ABC transporters in the Malpighian tubules and in the gut (Chahine and O'Donnell, 2009).

P-glycoprotein transporters are expressed in the blood brain barrier and in the Malpighian tubules of the desert locust (Andersson et al., 2014; Rossi et al., 2019), raising the possibility that their expression may change according to the locust phase in response to their different feeding ecologies. We hypothesise that gregarious and transiens locusts that incorporate toxic plants in their diet may express more P-glycoprotein than solitary locusts that avoid them. If this is indeed the case, then

regulation of P-glycoprotein activity may be caused exclusively by the change in feeding habits and/or may reflect the phenotypic shift itself irrespective of the diet. We also hypothesized that, to cope with the ingested toxins, Malpighian tubules of gregarious and transiens locusts may have a higher baseline density of P-glycoproteins and/or their P-glycoproteins may have more affinity for atropine, compared to the solitarious locust tubules. To test these hypotheses, we used a modified Ramsay assay (Rossi et al., 2019) to compare the P-glycoprotein performance of Malpighian tubules of gregarious, transiens, and solitary locusts fed on a diet containing the alkaloid atropine or alkaloid-free diet. Our aim was to investigate: (i) whether the Malpighian tubules of the solitary locusts are less efficient in extruding toxins than those of gregarious locusts (ii) whether solitary, transiens, and gregarious locusts alter P-glycoprotein activity within their Malpighian tubules in response to the presence or absence of atropine in their diet; (iii) whether the phenotypic transformation itself alters P-glycoprotein activity irrespective of the presence of atropine in the diet.

3.3 Methods

3.3.1 Animals

Gregarious-phase adult desert locusts (*Schistocerca gregaria*; Forsskål, 1775) were purchased from Peregrine Livefoods (Essex, UK). Solitary-phase adult desert locusts were bred at the University of Leicester, where they were produced from gregarious stocks by isolation for three generations (Roessingh et al., 1993). Transiens locusts were obtained by crowding solitary locusts with gregarious ones for three days.

To compare the basal activity of P-glycoproteins solitarious and gregarious locusts were fed with organic lettuce and wheat germ *ad libitum*. To test the up and

down regulation of P-glycoproteins in response to long-term exposure to dietary atropine two groups of third instar gregarious locusts were raised until adults under two different diets. The alkaloid-free group were fed with a blended mix of 5 g wheat germ, 5 g cereal pellets (Su-bridge pet supplies Ltd) and 100 g fresh lettuce (5 g dry weight). For the alkaloid group, atropine was added to the diet at 2% dry weight. To test the short-term up regulation of P-glycoproteins in response to dietary atropine, solitarious, transiens and gregarious adult locusts were fed for three days on 2% atropine diet. An additional group of transiens locusts were fed on the alkaloid-free diet. The concentration of atropine in the diet was based on that of previous studies (Despland and Simpson, 2005a,b) and it is similar to the percentage found in Egyptian henbane, *Hyoscyamus muticus* (El-Shazly et al., 1997).

3.3.2 Saline and chemicals

The saline used was adapted from the Ringer solution of Maddrell and Klunsuwan (1973). A stock solution of rhodamine B was prepared in water and diluted to the final concentration of 60 µM in saline. Atropine was dissolved directly in saline to the final concentrations of 0.40 mM, 0.75 mM and 1.5 mM. All chemicals were purchased from Sigma-Aldrich (UK) or Fisher Scientific (UK).

3.3.3 Dissection and Ramsay assay

Locusts were cooled for 3 minutes in a freezer and restrained prior to dissection. The entire gut with the Malpighian tubules attached was removed from the body and placed onto an 8 cm Sylgard® 184 (Dow Corning, Midland, MI, USA) coated Petri dish, filled with saline (Maddrell and Klunsuwan, 1973; Rossi et al., 2019). Anterior Malpighian tubules were removed by cutting the proximal end approximately 5 mm from the gut and by

gently pulling the distal end to release them from the trachea attached to the gut. Each tubule was immediately moved into 30 µL drop of saline on a 5 cm Sylgard® coated Petri dish, covered with paraffin oil to prevent desiccation, and fixed with steel pins (Rossi et al., 2019). Each tubule was punctured near the proximal end to permit fluid secretion. After 30 minutes of equilibration, the saline solution was replaced with a 30 µL drop containing one of the following treatments: (i) rhodamine B 60 µM (R-only); (ii) rhodamine B 60 µM + atropine 0.40 mM (R+A); (iii) rhodamine B 60 µM + atropine 0.75 mM; or (iv) rhodamine B 60 µM + atropine 1.5 mM. The P-glycoprotein substrate rhodamine B (Eytan et al., 1997) was used as a substrate for P-glycoprotein-mediated extrusion. Atropine was used as a competitive inhibitor of P-glycoprotein-mediated extrusion (Joosen et al., 2016).

The tubules were incubated for 90 minutes, and every 30 minutes the droplets secreted were removed using a 10 µL pipette (Gilson Scientific UK, Dunstable, Bedfordshire, UK). We discarded the droplets secreted after 30 and 60 minutes, only analysing the droplets secreted at 90 minutes, to ensure that the tubules had fully flushed their contents. The droplets were photographed with a digital camera (Canon EOS 7D; Canon, Tokyo, Japan) mounted on a stereoscopic microscope (Nikon SMZ-U; Nikon Corp., Tokyo, Japan). The samples were kept in a custom dark box to prevent photobleaching of rhodamine B. Using the software ImageJ v.1.51p (Schneider et al., 2012) we measured the volume and the colour intensity of the photographed droplets to estimate their rhodamine B concentration from a known calibration curve (Rossi et al., 2019). At the end of the assay, the tubule was photographed, and its diameter and the length immersed in the treatment solution measured to calculate the surface area of the tubule in contact with the treatment solution.

3.3.4 Statistical Analysis

All statistical analyses were conducted in R version 3.4.1 (R Core Team, 2018). We fitted Linear Mixed Effect (LME) models by restricted maximum likelihood (REML) estimation (`lmer` function, package 'lme4' (Bates et al., 2015)). Significances of the fixed effects were determined using Satterthwaite's method for estimation of degrees of freedom (package 'lmerTest' (Kuznetsova et al., 2017)) and non-significant interactions (P>0.05) were removed. Estimated marginal means and pairwise comparisons were obtained using the 'lsmeans' package (Lenth and Lenth, 2018).

To test the effect of atropine concentration on the rhodamine B concentration (µM) of the droplets secreted, we fitted a LME model with atropine concentration (continuous (mM)) as a fixed effect. To investigate the effect of the locust phase and the incubation treatment on the fluid secretion rate (nL/min) we included surface area (continuous (mm^2)), locust phase (categorical; gregarious, solitary) and incubation treatment (categorical; R-only, R+A) as fixed effects. To investigate the effect of the locust phase and the incubation treatment on the net rhodamine extrusion per unit of surface area ($fmol/min \cdot mm^2$) we included locust phase and incubation treatment as fixed effects. To investigate the effect of diet and incubation treatment on the net rhodamine extrusion per unit of surface area, we included diet (categorical; alkaloid-free group vs alkaloid group) and incubation treatment as fixed effects. To investigate the effect of diet and incubation treatment on the net rhodamine extrusion per unit of surface area, we included diet (categorical; alkaloid-free group, alkaloid group, alkaloid-free group exposed to the diet containing atropine for one day (UP-1D), or seven days (UP-7D), alkaloid group fed for seven days on the alkaloid-free diet (DOWN-7D)) as fixed effect. To investigate the effect of diet and locust phase on the net rhodamine extrusion

per unit of surface area, we included diet (categorical; alkaloid-free, locusts exposed to the diet containing atropine for three days (UP-3D)) and locust phase (categorical; gregarious, transiens, solitary) as fixed effects. In each of the statistical models, we included individual locusts as random effects to account for the nested structure of the data. We transformed (log or square root) the data, where appropriate, to correct for non-homogeneity of the variance.

3.4 Results

3.4.1 Atropine is a competitive inhibitor of P-glycoproteins

We tested the effect of atropine on the rhodamine concentration of the droplets secreted by the Malpighian tubules of gregarious locusts. Three Malpighian tubules were removed from each locust and incubated in bath solutions containing 60 µM of Rhodamine with differing concentrations of atropine (0 mM, 0.40 mM, 0.75 mM, 1.5 mM) for 90 minutes (see Methods). There was a significant negative correlation between atropine concentration and the log of rhodamine concentration of the droplets secreted at 90 minutes (LMEM: coefficient estimate ± S.E. -1.30 ± 0.12, d.f.=48.94, t=-11.28, $p<0.001$; Figure 3.1, Table 3.1). Malpighian tubules of gregarious locusts decreased the rhodamine concentration extruded by 41%, 62%, and 86% when incubated in solutions containing atropine 0.40 mM, 0.75 mM, and 1.50 mM respectively (Table 3.1).

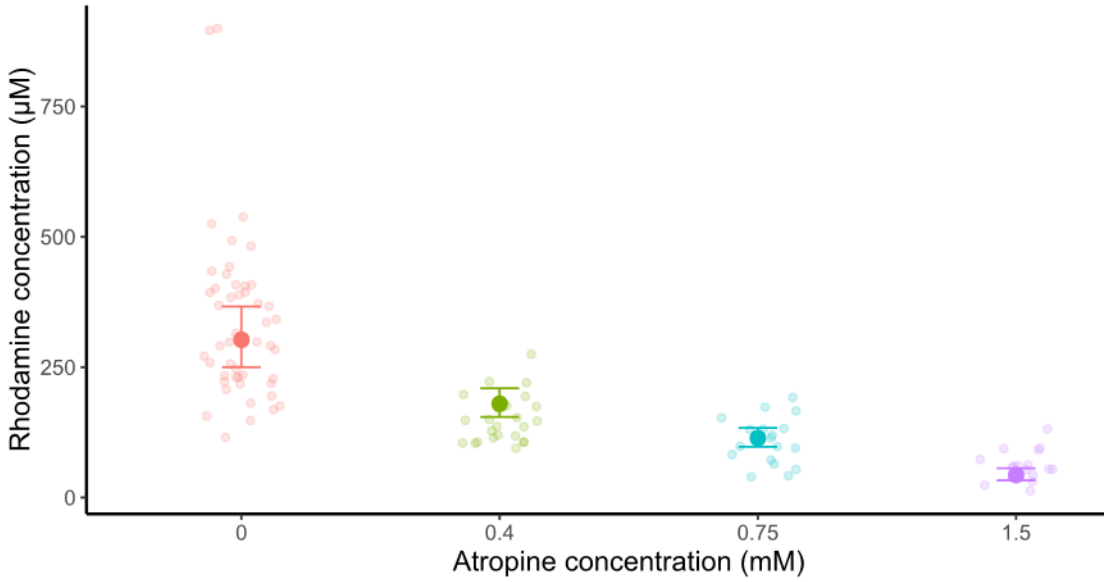

Figure 3.1. Atropine reduces the concentration of rhodamine in the droplets secreted. The rhodamine concentration of the droplets secreted decreased as the concentration of atropine in the incubation bath increased. The response variable is back-transformed to the original scale. Small circles represent the raw data, large filled circles represent means estimates fitted from LMEM, error bars represent the C.I.

Table 3.1. Atropine competitively inhibits the rhodamine extrusion. Estimated means ± s.e. (back transformed to the original scale) of rhodamine B concentration of the droplets secreted for increasing concentrations of atropine. The model applied was "log(Rho concentration) ~ Atropine concentration + (1|locust)"

Atropine concentration (mM)	Sample size	Rhodamine concentration (µM) Estimated means ± s.e.	Rhodamine concentration reduction (%)
0	N=16, n=48	302.64 ± 0.10	-
0.40	N=8, n=24	179.93 ± 0.08	41%
0.75	N=6, n=18	114.15 ± 0.08	62%
1.50	N=6, n=16	43.06 ± 0.14	86%

3.4.2 Malpighian tubules of solitarious and gregarious locusts respond similarly to atropine

To test whether the activity and affinity of P-glycoproteins to atropine was intrinsically higher in gregarious than in solitarious locusts, we reared solitary and gregarious locusts on alkaloid-free diet, and incubated their tubules in two different treatments: Saline containing 60 µM rhodamine B (R-only) or saline containing 60 µM rhodamine + 0.40 mM atropine (R+A). This concentration of atropine (0.40 mM) had previously been shown to decrease the rhodamine concentration of the droplets secreted by more than half in the gregarious locusts. Six tubules were removed from each of 9 gregarious locusts and 15 solitary locusts (see section 3.4.1). Of the six tubules, three were given the R-only treatment and three R+A treatment.

To ensure that the morphology of the Malpighian tubules of gregarious and solitary locusts was similar, we measured their length and diameter, and calculated their surface area. The diameter, length and surface area were similar between the two phases (Table 3.2). The fluid secretion rate of tubules positively correlates with their surface area (Figure 3.2, Table 3.3). There was also no difference in the fluid secretion rate of tubules taken from solitarious or gregarious locusts, and it was unaffected by the presence of atropine in the incubation bath (Figure 3.2, Table 3.3). We found no interaction between locust phase and treatment. Tubules incubated in R-only treatment extruded similar quantity of rhodamine B in both solitary and gregarious locusts, and the addition of atropine significantly decreased the net rhodamine extrusion in both phases (Figure 3.3, Table 3.4).

Table 3.2. Solitarious and gregarious locusts possess tubules with similar morphology. The length, diameter and surface area of solitary and gregarious tubules are similar. Each morphological trait response was fitted by a LMEM with locust ID as a random effect.

Morphological traits	Fixed effects	Estimate	s.e.	*t*	*p*
Length (mm)	Intercept (gregarious)	12.39	0.78	15.99	
	Solitarious phase	2.00	0.98	2.04	0.053
Diameter (μm)	Intercept (gregarious)	55.67	2.29	24.33	
	Solitarious phase	5.25	2.89	1.82	0.083
Surface (mm^2)	Intercept (gregarious)	2.18	0.24	9.11	
	Solitarious phase	0.62	0.30	2.03	0.054

Table 3.3. Outcomes of the model investigating the effect of surface area, treatment, and phase on the fluid secretion rate (nL/min) of Malpighian tubules. The fluid secretion rate positively correlates with the tubule surface area, and did not differ between solitary and gregarious locusts or between treatments. The model used was "Fluid secretion rate ~ surface + phase + treatment + (1|locust) + (1|date)".

Fixed effects	Estimate	s.e.	*t*	*p*
Intercept (gregarious, R-only)	0.083	0.081	1.021	
Surface	0.115	0.024	4.763	**<0.001**
Phase (solitary)	-0.110	0.064	-1.722	0.099
Incubation treatment (R+A)	-0.008	0.033	-0.243	0.808

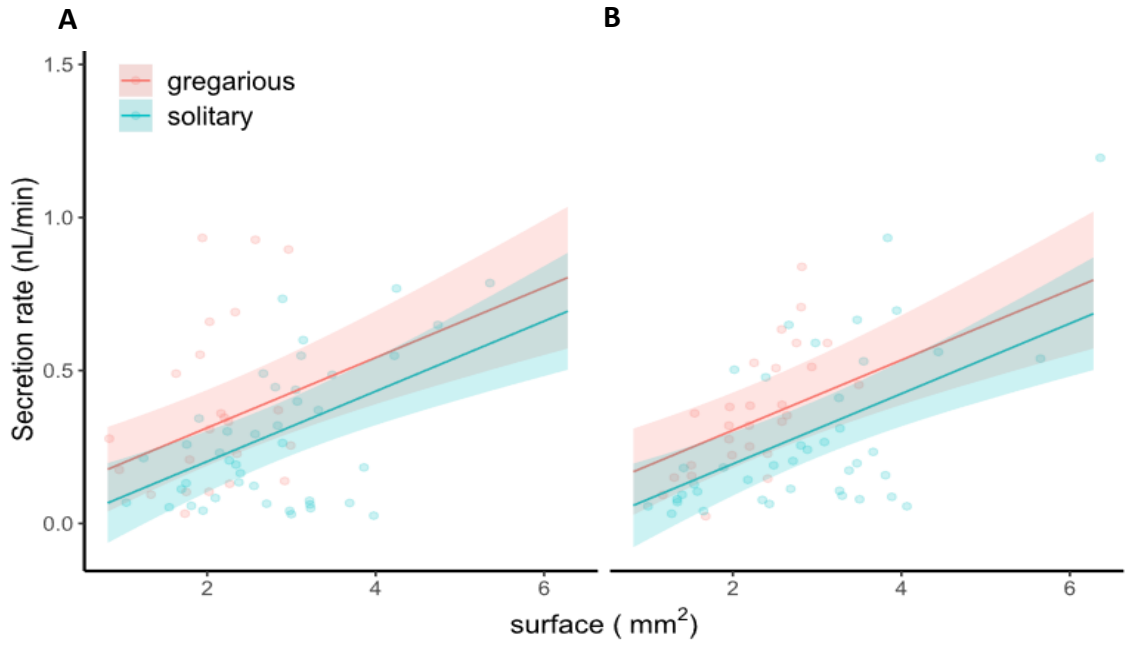

Figure 3.2. The fluid secretion rate of Malpighian tubules was not influenced by locust phase or by the presence of atropine. (A) The fluid secretion rate of tubules given the R-only treatment. (B) The fluid secretion rate of tubules given the R+A treatment. The fluid secretion rate increased with the surface area of tubules in both solitarious (blue) and gregarious (pink) locusts. Small circles represent the raw data, lines represent the regression line fitted from LMEM, shaded areas represent the 95% confidence interval.

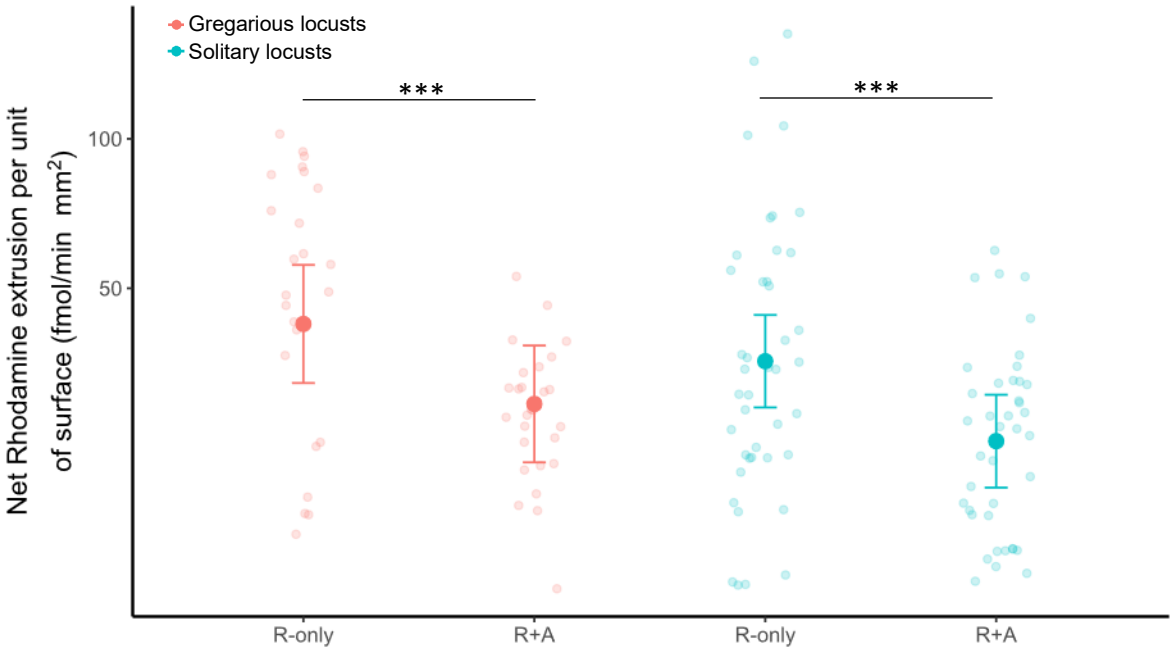

Figure 3.3. Net rhodamine extrusion was similar from the tubules of solitarious and gregarious locusts. The net rhodamine extrusion per unit of surface area was reduced by the addition of atropine in the incubation bath (R+A) compared to the R-only treatment in both solitarious (blue) and gregarious (pink) locusts. Small circles represent the raw data, large filled circles represent means estimates fitted from LMEM, error bars represent the 95% confidence interval. Y axis is in square root scale. (*** $p < 0.001$).

Table 3.4. Summary of the model investigating the effect of incubation treatment and locust phase on the net rhodamine extrusion per unit of surface area. The net rhodamine extrusion did not differ between solitarious and gregarious locusts, and was reduced by the application of atropine. The net rhodamine extrusion per unit of surface area (square root transformed) was fitted by a LMEM. The model applied was "sqrt(net rhodamine extrusion) ~ treatment + phase + (1|locust)".

Fixed effects	Estimate	s.e.	*t*	*p*
Intercept (gregarious, R-only)	6.371	0.591	10.788	
Incubation treatment (R+A)	-1.567	0.282	-5.553	**<0.001**
Phase (solitary)	-0.730	0.721	-1.012	0.323

3.4.3 P-glycoprotein activity is up and down regulated in response to dietary atropine

The lack of an intrinsic difference between gregarious and solitarious locusts in P-glycoprotein activity and affinity to atropine raised the possibility that long-term exposure to a diet containing atropine might increase the activity and affinity of P-glycoproteins for atropine in gregarious locusts. To this end, we reared two groups of third instar gregarious locusts under two different diets: an alkaloid-free diet (alkaloid-free group) and a diet containing atropine (alkaloid group). Two-week old adults were dissected and six tubules removed from each. From the six tubules, three were incubated in saline containing 60 µM rhodamine (R-only) and three in 60 µM rhodamine + 0.40 mM atropine (R+A). We found no interaction between locust diet and incubation treatment. The tubules of locusts fed on the alkaloid diet incubated in an R-only solution extruded significantly more rhodamine than did the tubules of locusts fed on the alkaloid-free diet (alkaloid group: 119.5 fmol/ min·mm^2, 95% CI = [92.2-154.8], alkaloid-free group: 27.7 fmol/ min·mm^2 [21.4-35.8]; Figure 3.4, Table 3.5). The presence of atropine (R+A) decreased the rhodamine extrusion in both the alkaloid and the alkaloid-free groups (alkaloid group: 39.5 fmol/ min·mm^2 [30.4-51.3], alkaloid-free group: 9.2 fmol/ min·mm^2 [7.1-11.9]; Figure 3.4, Table 3.5). This suggests that the locusts reared on the alkaloid diet increased their P-glycoprotein activity, and the affinity of P-glycoproteins to atropine was similar in gregarious locusts reared on alkaloid and alkaloid-free diet.

Subsequently, to test whether the Malpighian tubules could increase or decrease their P-glycoprotein activity during adulthood in the short-term, we exposed the alkaloid-free group to the diet containing atropine for either one day (UP-1D group) or

seven days (UP-7D group), while the alkaloid group was fed for 7 days on the alkaloid-free diet (DOWN-7D). Three tubules were removed from each locust and incubated in an R-only treatment. The net rhodamine extrusion of UP-1D was similar to the alkaloid-free group (UP-1D vs Alkaloid-free group; Figure 3.5, Table 3.6), but significantly increased after 7 days of atropine diet (UP-7D vs Alkaloid-free group; Figure 3.5, Table 3.6). Locusts fed for 7 days on the alkaloid-free diet exhibited a decrease in the net rhodamine extrusion compared to the alkaloid group (DOWN-7D vs Alkaloid group; Figure 3.5, Table 3.6). This suggests that adult gregarious locusts were able to increase and decrease their P-glycoprotein activity after only one week of exposure to a particular diet.

Table 3.5. Summary of the model investigating the effect of incubation treatment and diet on the net rhodamine extrusion per unit of surface area of tubules of gregarious locusts. The net rhodamine extrusion per unit of surface area (log transformed) was fitted by a LMEM. The model applied was "log(net rhodamine extrusion) ~ treatment + diet + (1|locust)".

Fixed effects	Estimate	s.e.	*t*	*p*
Intercept (alkaloid group, R-only)	4.7831	0.1322	36.192	
Incubation treatment (R+A)	-1.1063	0.1284	-8.617	**<0.001**
Diet (alkaloid-free group)	-1.462	0.1634	-8.948	**<0.001**

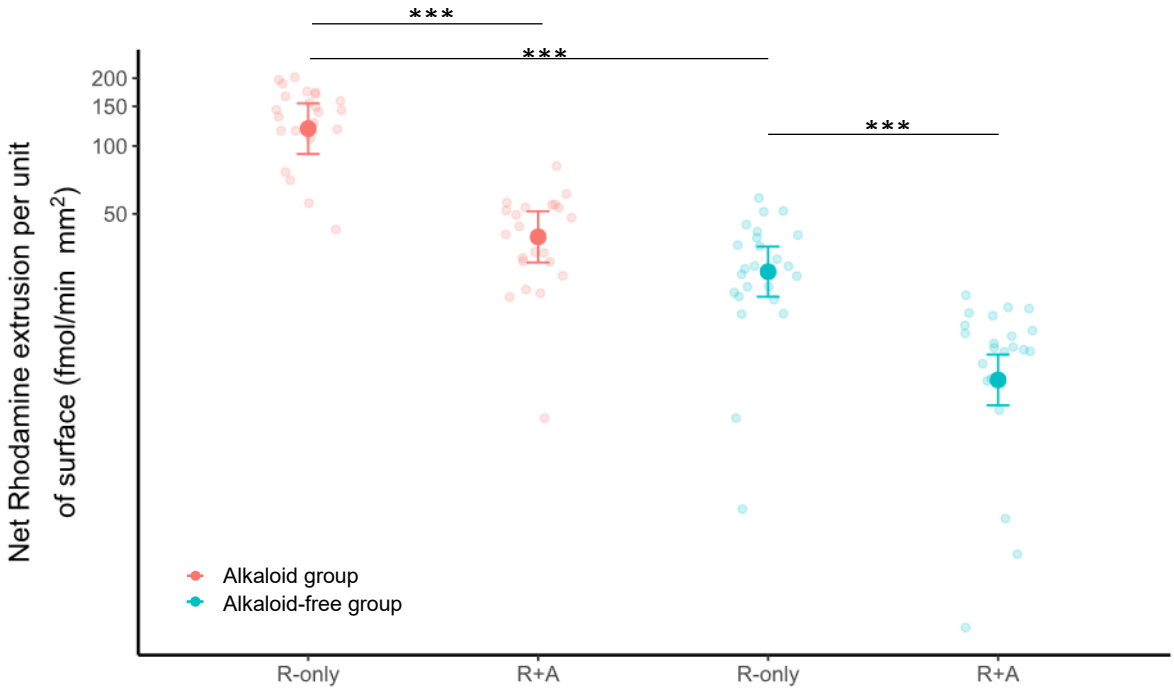

Figure 3.4. The presence of alkaloid in the diet increased the net rhodamine extrusion. The net rhodamine extrusion per unit of surface area was significantly higher in the droplets extruded by the tubules of locusts reared on the alkaloid diet compared to the alkaloid-free diet. The addition of atropine in the incubation treatment (R+A) significantly reduced the net rhodamine extrusion compared to the R-only treatment in both the alkaloid and alkaloid-free groups. Small circles represent the raw data, large filled circles represent means estimates fitted from LMEM, error bars represent the 95% confidence interval. Y axis is in log scale. (*** $p < 0.001$).

Table 3.6. Outcomes of the model investigating the effect of diet on the net rhodamine extrusion per unit of surface area of tubules of gregarious locusts. The net rhodamine extrusion per unit of surface area (log transformed) was fitted by a LMEM. The model applied was "log(net rhodamine extrusion) ~ diet + (1|locust)". The diet factor has 5 levels: locusts reared on atropine diet (alkaloid group), alkaloid group switched too alkaloid-free diet for 7 days (DOWN-7D), locusts reared on alkaloid-free diet (alkaloid-free group), alkaloid-free group switched to atropine diet either for one day (UP-1D) or seven days (UP-7D). (A) Summary of the model. (B) Pairwise comparisons between gregarious locusts fed on different diets. (C) Summary of the estimated mean values back-transformed to the original scale for each group.

A. Fixed effects	Estimate	s.e.	*t*	*p*
Intercept (alkaloid group)	4.849	0.174	27.797	
DOWN-7D	-0.500	0.247	-2.028	**0.050**
Alkaloid-free group	-1.591	0.246	-6.469	**<0.001**
UP-1D	-1.141	0.247	-4.625	**<0.001**
UP-7D	-0.287	0.256	-1.123	0.269

B. contrast			Estimate	s.e.	*t* ratio	*p*
UP-1D	vs	Alkaloid-free group	0.450	0.246	1.830	0.076
UP-7D	vs	Alkaloid-free group	1.304	0.255	5.119	**<0.001**
Alkaloid group	vs	Alkaloid-free group	1.591	0.246	6.469	**<0.001**
DOWN-7D	vs	Alkaloid group	-0.500	0.247	-2.028	**0.050**
Alkaloid group	vs	UP-7D	0.287	0.256	1.123	0.269

C. Diet groups	Sample size N	estimated means (fmol/ min·mm^2)	95% C.I.
Alkaloid group	8	127.6	90.7 - 179.7
DOWN-7D	8	77.4	55.0 - 108.9
Alkaloid-free group	8	26.0	18.5 - 36.5
UP-1D	8	40.8	29.0 - 57.4
UP-7D	7	95.8	66.4 - 138.1

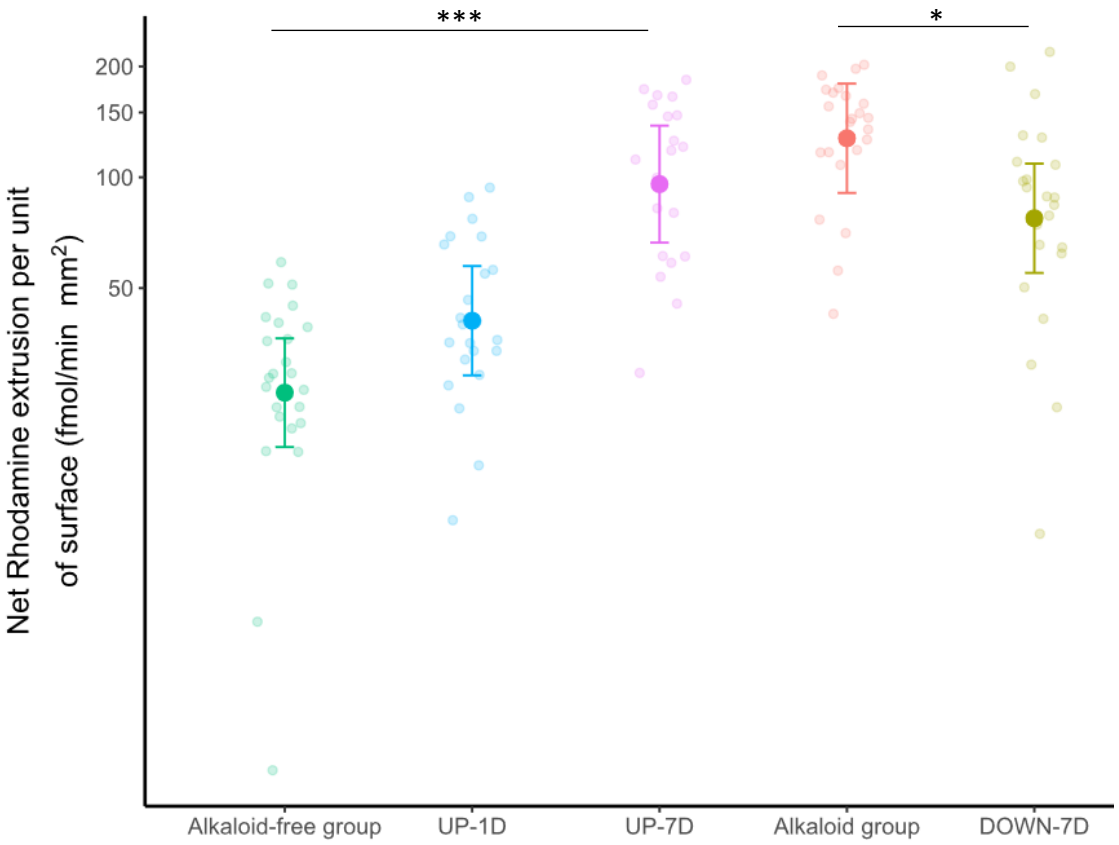

Figure 3.5. Adult gregarious locusts increased net rhodamine extrusion after exposure to the alkaloid diet, and decreased it after removal of the alkaloid. Locusts reared on alkaloid-free diet and subsequently switched to the alkaloid diet for seven days (UP-7D) showed an increased net rhodamine extrusion per unit of surface area, while locusts switched on the alkaloid diet for only one day (UP-1D) had a similar net rhodamine extrusion to the alkaloid-free group. Locusts reared on the alkaloid diet and subsequently switched to the alkaloid-free diet for 7 days (DOWN-7D) decreased their net rhodamine extrusion compared to the alkaloid diet group. Small circles represent the raw data, large filled circles represent means estimates fitted from LMEM, error bars represent the 95% confidence interval. Y axis is in log scale. (* p=0.05; *** p < 0.001).

3.4.4 Ingestion of atropine increased P-glycoprotein activity in the Malpighian tubules of gregarious and transiens but not solitary locusts

Transiens locusts, when compared to gregarious ones, show a preference for food containing atropine, whereas solitary locusts actively avoid it (Despland and Simpson, 2005a; Simões et al., 2013). We tested whether: (i) transiens locusts increase their P-glycoprotein activity to a greater extent than gregarious locusts when exposed to food containing atropine; and (ii) the phenotypic transformation induces an upregulation in the P-glycoproteins irrespective of the presence of the alkaloid in the diet, in preparation for the ingestion of toxic food.

Two groups of transiens locusts were fed for three days either on atropine diet (Transiens UP-3D) or alkaloid-free diet, and the net rhodamine extrusion per unit of surface area of their tubules were compared with those of gregarious locusts fed either on alkaloid-free diet or for three days on atropine diet (Gregarious UP-3D). Three tubules were removed from each locust and incubated in R-only treatment. We found no interaction between phase (gregarious, transiens) and diet (alkaloid-free, UP-3D; Table 3.7A), and the net rhodamine extrusion per unit of surface area of gregarious and transiens locusts fed on alkaloid-free diet was indistinguishable (Table 3.7B,C, Figure 3.6). Gregarious and transiens UP-3D locusts extruded significantly more rhodamine than gregarious and transiens locusts fed on an alkaloid-free diet (Table 3.7, Figure 3.6). Gregarious and transiens UP-3D extruded similar quantity of rhodamine (Table 3.7, Figure 3.6).

To compare the physiology of gregarious locust tubules (Gregarious UP-3D) with those of solitarious locusts when exposed to toxins, we fed solitarious locusts for three

days with atropine diet (Solitary UP-3D). However, solitarious locusts refused the food containing atropine and ate very little compared to gregarious and transiens locusts, likely causing to the lack of P-glycoprotein upregulation. Again, three tubules were removed from each locust and incubated in R-only treatment. We found a significant interaction between phase (gregarious, solitary) and diet (alkaloid-free, UP-3D; Table 3.7A). There was no difference in the net rhodamine extrusion per unit of surface area between solitarious locusts fed on alkaloid-free diet and solitary UP-3D (Table 3.7, Figure 3.6), and solitarious UP-3D tubules extruded significantly less rhodamine than gregarious UP-3D (Table 3.7, Figure 3.6).

Table 3.7. Outcomes of the model investigating the effect of diet on the net rhodamine extrusion per unit of surface area of tubules of gregarious, transiens, and solitary locusts. The net rhodamine extrusion per unit of surface area (log transformed) was fitted by a LMEM. The model applied was "log(net rhodamine extrusion) ~ phase * diet + (1|locust)". The factor phase has three levels (gregarious, transiens, solitary), while diet has two factors (alkaloid-free diet, UP-3D). (A) Summary of the model. (B) Pairwise comparisons between gregarious, transiens, and solitary locusts fed on alkaloid-free diet or atropine diet for three days (UP-3D). (C) Summary of the estimated mean values back-transformed to the original scale for each group.

A. Fixed effects	Estimate	s.e.	*t*	*p*
Intercept (gregarious alkaloid-free)	3.462	0.167	20.721	
phase: transiens (alkaloid-free)	0.080	0.287	0.279	0.781
phase: solitary (alkaloid-free)	-0.322	0.242	-1.330	0.189
diet: UP-3D (gregarious)	1.254	0.293	4.286	**<0.001**
phase × diet: transiens (UP-3D)	-0.450	0.454	-0.991	0.326
phase × diet: solitary (UP-3D)	-1.372	0.447	-3.068	**0.003**

B. contrast			Estimate	s.e.	*t* ratio	*p*
gregarious alkaloid-free	vs	transiens alkaloid-free	-0.080	0.287	-0.279	0.781
gregarious alkaloid-free	vs	gregarious UP-3D	-1.254	0.293	-4.285	**<0.001**
transiens alkaloid-free	vs	transiens UP-3D	-0.804	0.348	-2.314	**0.024**
gregarious UP-3D	vs	transiens UP-3D	0.370	0.352	1.051	0.298
solitary alkaloid-free	vs	solitary UP-3D	0.118	0.338	0.348	0.729
solitary UP-3D	vs	gregarious UP-3D	-1.694	0.376	-4.502	**<0.001**

C. Phase-Diet groups	Sample size	estimated means (fmol/ min·mm^2)	95% C.I.
gregarious alkaloid-free	16	31.9	23.0 - 44.2
transiens alkaloid-free	9	34.5	21.9 - 54.6
solitary alkaloid-free	15	23.1	16.4 - 32.6
gregarious UP-3D	8	111.8	69.8 - 179.1
transiens UP-3D	7	77.2	46.6 - 127.9
solitary UP-3D	6	20.6	11.7 - 36.2

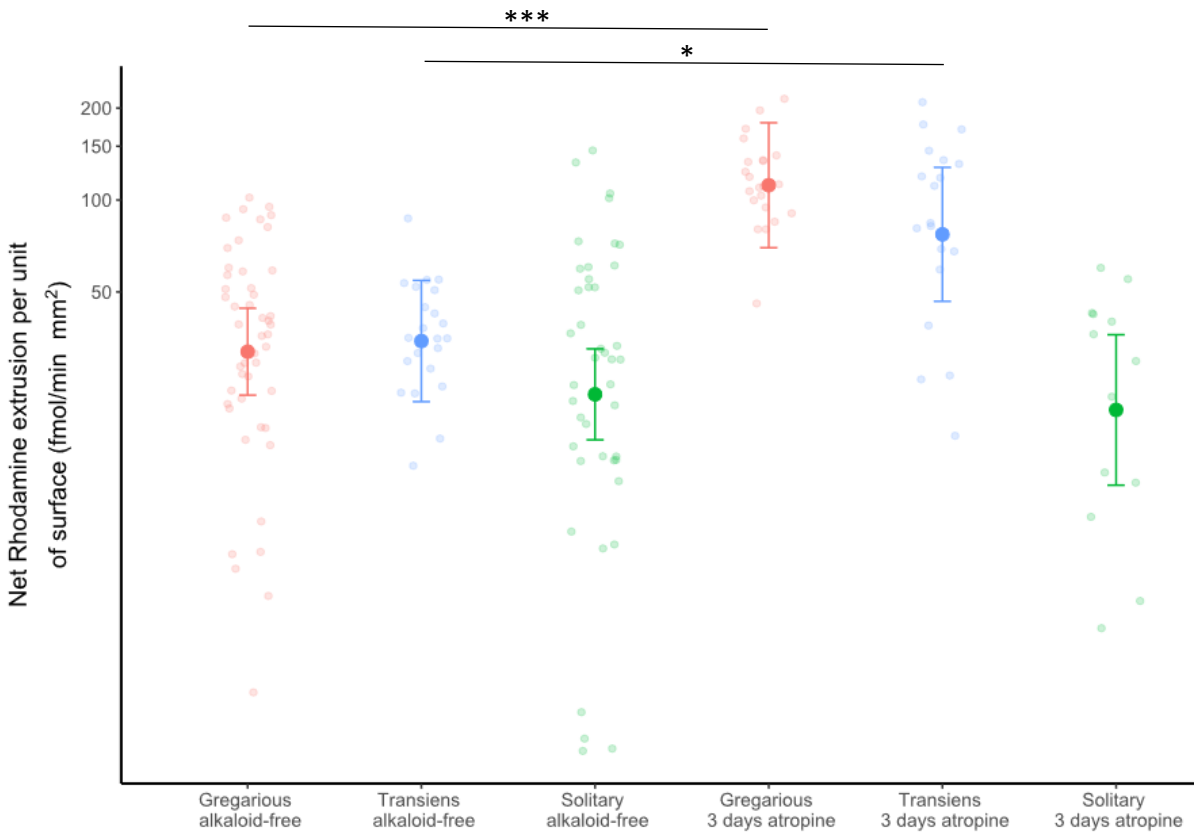

Figure 3.6. The tubule physiology of adult gregarious and transiens locust was similar. Gregarious, transiens and solitary locusts fed on alkaloid-free diet extruded a similar quantity of rhodamine per unit of surface area. After three days of exposure to the alkaloid diet, the transiens locusts increased their net rhodamine extrusion to the same extent as the the gregarious locusts. Conversely, the net rhodamine extrusion of solitary locusts exposed to the alkaloid diet was similar to the solitary ones fed on the alkaloid-free diet. Small circles represent the raw data, large filled circles represent means estimates fitted from LMEM, error bars represent the 95% confidence interval. Y axis is in log scale. (* $p<0.05$; *** $p < 0.001$).

3.5 Discussion

Many insect species are capable of generating polyphenisms, alternative developmental pathways and consequent phenotypes, in response to differing environmental conditions (Hardie and Lees, 2013; Nijhout, 1999). These alternative phenotypes have been shown to differ in their morphology and behaviour (Pener and Simpson, 2009; Simpson et al., 2011); differences that can be adaptive under differing environmental conditions (Shapiro, 1976; Simpson et al., 2011). Yet how alternative phenotypes differ in their physiology and whether any differences that exist are adaptive remains largely unknown. Our aim was to understand how the physiology of an organ system is affected by phenotypic plasticity and the occurrence of a polyphenism. To this end, we studied the desert locust (*Schistocerca gregaria*), which exists as two extreme phenotypes - solitarious and gregarious - with intermediate transiens forms (Uvarov and Zolotarevsky, 1929). Solitarious locusts avoid eating potentially harmful compounds whereas gregarious ones broaden their diet incorporating plants such as the Egyptian henbane (*Hyoscyamus muticus*), which produces the toxic alkaloid atropine (hyoscyamine). Locusts can make the transition from the solitarious to the gregarious phase within their lifetime, and whilst doing so these transiens locusts show active preference for toxic plants to become unpalatable to predators (Popov et al., 1991; Sword, 2001). Given these changes in feeding and the accompanying differences in exposure to toxins, we compared the function of the Malpighian tubules, the osmoregulatory and excretory organs of insects (O'Donnell, 2008).

We determined how phenotypic transformation and exposure to food containing the naturally occurring alkaloid atropine influence the physiological function of the Malpighian tubules of the desert locust, using the P-glycoprotein substrate

rhodamine B to calculate its net extrusion per unit of surface area as a proxy for P-glycoprotein mediated extrusion of a toxin. We showed that extrusion of the dye rhodamine B was inhibited by atropine. By comparing the tubule physiology of solitarious, transiens, and gregarious locusts fed on a diet containing atropine, and on an alkaloid-free diet, we showed that: (i) the net rhodamine extrusion per unit of surface area did not differ between solitarious, gregarious and transiens locusts reared on an alkaloid-free diet; (ii) the fluid secretion rate positively correlated with the tubule surface area, was not different between solitary and gregarious locusts, and was not influenced by atropine; (iii) the affinity of P-glycoproteins to atropine was similar in gregarious and solitary locusts; (iv) exposure to the alkaloid diet increased the net rhodamine extrusion in gregarious and transiens locusts; (v) the affinity of P-glycoproteins to atropine was similar in gregarious locusts reared on alkaloid and alkaloid-free diet; and (vi) gregarious locusts reared on the alkaloid diet decreased their net rhodamine extrusion after being switched to an alkaloid-free diet. To our knowledge, this is the first study comparing the physiology of locusts' Malpighian tubules in different phases, and investigating their xenobiotic extrusion plasticity in relation to their natural feeding ecology.

3.5.1 Methodological considerations

Our experimental approach involved the use of an isolated tubule preparation, ensuring that any observed differences can be attributed exclusively to the tubule physiology itself rather than indirect effects of other structures (Berridge, 1966; Maddrell, 1969; Ramsay, 1954). We used a modified Ramsay assay with rhodamine B to quantify P-glycoprotein transporter-mediated extrusion by Malpighian tubules. This is a reliable method because the specificity of rhodamine B as P-glycoprotein substrate in desert

locusts' tubules has being validated by the concomitant use of the P-glycoprotein inhibitor verapamil (Hamada et al., 1987; O'Donnell and Leader, 2006; Rossi et al., 2019). In the present work, to ensure ecological relevance, we used the naturally occurring P-glycoprotein competitive inhibitor atropine (Gaertner et al., 1998; Joosen et al., 2016; Maddrell and Gardiner, 1976) both to feed the locusts with a similar concentration to that found in the Egyptian henbane (El-Shazly et al., 1997), and to directly expose their tubules to it.

3.5.2 Gregarious, solitarious and transiens locusts reared on the alkaloid-free diet had similar P-glycoprotein activity

Although we hypothesised that the P-glycoprotein density would be intrinsically higher in gregarious and transiens than in solitarious locusts, we found that tubules incubated in rhodamine (R-only) had similar net extrusion per unit of surface area. Thus, the P-glycoprotein activity is similar in all the three phases when all are reared on the alkaloid-free diet. We also hypothesised that the isoform of P-glycoproteins expressed in gregarious locust tubules would have greater affinity for atropine when compared to the P-glycoproteins expressed in solitarious locust tubules. However, the presence of atropine (R+A treatment) decreased the quantity of rhodamine extruded to the same extent in both solitarious and gregarious locusts, suggesting that affinity of P-glycoproteins to atropine is similar in both phases. Alternatively, solitary and gregarious locusts might differ in other mechanisms that we did not consider in the present study. Tubules may diverge in the expression of other ABC transporters or detoxifying enzymes (Dermauw and Van Leeuwen, 2014; Yuan et al., 2018), or the gut or blood brain barrier may be more efficient in preventing the uptake of certain secondary metabolites in gregarious locusts (Petschenka et al., 2013; Scudder and Meredith, 1982).

The lack of difference in tubule physiology between distinct phenotypes that differ in their ecology, morphology and behaviour may at first seem surprising (Ahmed et al., 2005; Despland and Simpson, 2005a; Pener, 1991; Pener and Simpson, 2009; Simões et al., 2016; Uvarov, 1977). Some insects, independently of their diet, can intrinsically change their detoxification ability reflecting an ecological adaptation to different habits (Maddrell and Gardiner, 1976; Yuan et al., 2018). For example, the Malpighian tubules of the holometabolous insect tobacco hawkmoth (*Manduca sexta*) actively extrude nicotine even when fed on an alkaloid-free diet during the larval stage, but lose this ability in the adult (Maddrell and Gardiner, 1976). In contrast to *M. sexta* caterpillars that are specialised to feed on their host plant, *Nicotiana attenuata*, which is rich in nicotine, gregarious desert locusts are generalist phytophagous insects (Uvarov, 1977) that can choose whether to feed on toxic plants or not. Because the biosynthesis of additional P-glycoprotein is energetically costly, it is likely that gregarious locusts avoid excess expression unless consuming food containing toxins.

3.5.3 P-glycoprotein activity is up and down regulated in response to dietary atropine

Gregarious locusts reared on atropine diet or exposed for only three or seven days to the alkaloid, dramatically increased their net rhodamine extrusion compared to the locusts fed on the alkaloid-free diet. The process is reversible, and after the removal of the alkaloid from the diet, the tubules decreased their P-glycoprotein activity in one week, making them flexible to adapt quickly to new conditions. These results are consistent with previous findings in which the expression of P-glycoprotein transporters can be regulated in response to the amount of toxins ingested with the diet. For example, *Drosophila* increases the gene expression of ABC, MET and OATP transporters

in Malpighian tubules after exposure to Methotrexate (Chahine and O'Donnell, 2010, 2009), and increases the expression of a P-glycoprotein homologous gene in the brain and gut after exposure to colchicine (Tapadia and Lakhotia, 2005). Larvae of *Manduca sexta* down-regulate their ABC transporters when feeding on silenced *Nicotiana attenuata* plants compared to those feeding on wild type tobacco leaves (Govind et al., 2010). Interestingly, in locusts the downregulation process appears to be almost 4 times slower than the upregulation: locusts increased their net rhodamine extrusion by 79.9 fmol/min·mm^2 in three days (average increase per day: 26.6 fmol/min·mm^2), while it decreased by [-]50.2 fmol/mim·mm^2 in seven days (average decrease per day: [-]7.2 fmol/min·mm^2). We can use these data to produce a crude estimate of the half-life of locust P-glycoproteins. From the maximum net extrusion of the alkaloid group (127.6 fmol/min·mm2) and the basal extrusion of the alkaloid-free group (26 fmol/min·mm2), we can calculate a mid-point of 76.8 fmol/min·mm2, corresponding to 50% net extrusion decrease. The mean net extrusion of down-regulated locusts was 77.4 fmol/min·mm2, close to this mid-point. This might suggest that the half-life of locust P-glycoproteins is about seven days, a time span that is not far from the half-life of five days found in P-glycoproteins of rat hepatocytes (Yoshimura et al., 1989).

An alternative explanation for the increased net rhodamine extrusion observed is that the actual amount of P-glycoprotein does not change, but the transporter is activated to an increased extent by an unknown cellular control pathway. To distinguish between these two hypotheses, we could localise and quantify P-glycoproteins using western blotting and/or immunocytochemical experiments or we could quantify P-glycoprotein RNA using northern blotting and/or next-generation sequencing.

3.5.4 Ingestion of atropine increased P-glycoprotein activity in the Malpighian tubules of gregarious and transiens but not solitary locusts

The food avoidance behaviour that solitary locusts show towards toxic compounds (Despland and Simpson, 2005a; Simões et al., 2013) may reflect their inability to deal with toxic substances. To test the hypothesis that their tubules might be less efficient in upregulating P-glycoproteins upon exposure to atropine, we force fed solitary locusts for three days with atropine diet. However, the locusts rejected almost entirely the food provided and thus were exposed to little atropine, so it is perhaps unsurprising that their net rhodamine transport was similar to that of the locust on alkaloid-free diet. Yet after less than 24 hours of crowding, solitary locusts that have begun to gregarize (transiens) readily ate the atropine food provided (Despland and Simpson, 2005a), and after three days their tubules showed a P-glycoprotein upregulation similar to the gregarious locusts fed on atropine diet.

3.5.5 Conclusions

Phenotypic plasticity caused by gregarization occurs on multiple time scales (Ernst et al., 2015); some characteristics change within a lifetime or over several generations (Ernst et al., 2015; Islam et al., 1994), while behavioural changes (Despland and Simpson, 2005a; Roessingh et al., 1993; Simões et al., 2013) and detoxification mechanisms are induced in just a few hours. Gregarious and solitary locusts differ profoundly in many ways, including their physiology, behaviour and preferred diet (Pener and Simpson, 2009), but we found that they did not differ in their basal ability to extrude an alkaloid analogue. However, gregarious and transiens phases appear able to rapidly upregulate P-glycoprotein production when exposed to atropine. The rapidly inducible response

coupled with the slower catabolism of P-glycoproteins, ensures that locusts can produce and keep them only when they are needed, avoiding the cost of the production when they are not needed. The underlying reasons for the narrow diet of solitary locusts remain unclear. Whether they are incapable of upregulating P-glycoproteins or whether they simply avoid the cost of synthetizing detoxifying enzymes (Mainguet et al., 2000) and xenobiotic transporters need to be investigated with injection of secondary metabolites directly in their haemolymph.

3.5.6 A behavioural mechanism for regulating toxin extrusion by the Malpighian tubules following gregarisation

Locust polyphenism is an adaptation to the unpredictable and often arid environment they inhabit (Simões et al., 2016). When patches of vegetation are abundant and evenly distributed, locusts exist in the solitarious, cryptic phase and the low intraspecific competition enables them to feed selectively and avoid toxic plants (Despland and Simpson, 2005a; Simões et al., 2013), leading to a low level of P-glycoprotein activity in their tubules. Following a period of rain, the vegetation flourish and the population of solitarious locusts increases. However, the vegetation is quickly reduced to small patches, increasing intraspecific competition for food (Despland et al., 2000; Simpson et al., 1999; Uvarov, 1977). This forced crowding increases the tactile, visual and olfactory stimuli between conspecifics (Roessingh et al., 1998), inducing changes in gene expressions (Lucas et al., 2010) and in serotonin concentration (Rogers and Ott, 2015) that triggers the behavioural changes associated with gregarization (Anstey et al., 2009). During this process there is a behavioural switch, transiens locusts start eating toxic plants to acquire toxicity to predators (Simões et al., 2013; Sword, 2001). In response, the Malpighian tubules increase their P-glycoprotein activity to maintain the alkaloids'

concentration in the haemolymph under safe levels. Subsequent generations that emerge as gregarious locusts will be feeding on toxic plants throughout their life time, and will consequently express high levels of P-glycoproteins to cope with the ingested toxins.

4 *Malpighamoeba* infection compromises fluid secretion and P-glycoprotein detoxification in Malpighian tubules

4.1 Abstract

Malpighian tubules, analogous to vertebrate nephrons, play a key role in insect osmoregulation and detoxification. Tubules can become infected with a protozoan, *Malpighamoeba*, which damages their epithelial cells, potentially compromising their function. Here we used a modified Ramsay assay to quantify the impact of *Malpighamoeba* infection on fluid secretion and P-glycoprotein-dependent detoxification by desert locust Malpighian tubules. Infected tubules have a greater surface area and a higher fluid secretion rate than uninfected tubules. Infection also impairs P-glycoprotein-dependent detoxification by reducing the net rhodamine extrusion per surface area. However, due to the increased surface area and fluid secretion rate, infected tubules have similar total net extrusion per tubule to uninfected tubules. Increased fluid secretion rate of infected tubules likely exposes locusts to greater water stress and increased energy costs. Coupled with reduced efficiency of P-glycoprotein detoxification per surface area, *Malpighamoeba* infection is likely to reduce insect survival in natural environments.

4.2 Introduction

Osmoregulation and excretion are essential for the survival of animals through the homeostatic maintenance of the internal environment and the removal of harmful toxins that impair cellular processes. In insects the Malpighian tubules, which are analogous to the nephrons of the vertebrate kidney, play an important role in osmoregulation as well as in the removal of metabolic waste and xenobiotic substances (Maddrell and Gardiner, 1976). Consequently, compromised tubule function may disrupt osmoregulation and impair detoxification, preventing insects from maintaining their internal osmotic environment, and exposing their cells to the waste products of metabolism and to environmental toxins. The impact of such impairment may be particularly severe in those insects that are exposed to environmental toxins as a consequence of feeding directly on plant tissues or their products (such as nectar or pollen) because of the presence of secondary metabolites (Després et al., 2007). Moreover, feeding on plant tissues or their products can expose insects to xenobiotics such as insecticides, fungicides or antibiotics that may act as toxins (Goulson et al., 2015). Consequently, compromised tubule function may prevent extrusion of these xenobiotics, increasing their effects on insects.

Insect pathogens can interact with xenobiotics such as pesticides affecting survival (reviewed in Goulson et al. (2015)). Interactions between pathogens and xenobiotics, which have been studied primarily in honeybees and bumblebees (Manson et al., 2010; Richardson et al., 2015), suggest that pesticide exposure can exacerbate the effects of pathogens. Moreover, pathogens may themselves exacerbate the impacts of xenobiotics, possibly due to trade-offs between the activity of the immune system and detoxification pathways (McMillan et al., 2018). In most cases, the mechanisms linking

the susceptibility of insects to xenobiotics with the presence of pathogens remain unknown, even in the case of diseases of insect pollinators that provide commercially-important ecosystem services (reviewed in Goulson et al., (2015)). Consequently, identifying mechanisms by which pathogens could affect removal of xenobiotic compounds is important for understanding insect immune responses, detoxification and health.

In insects, including grasshoppers and honey bees, the Malpighian tubules can become infected by the protozoan *Malpighamoeba* (also known as *Malamoeba* or *Malameba*) (Bailey, 1968; Harry and Finlayson, 1976, 1975; King and Taylor, 1936; Liu, 1985a,b; Taylor and King, 1937). This protozoan develops and multiplies primarily in the Malpighian tubules before cysts pass into the gut and spread to uninfected individuals through faeces and food contamination (Harry and Finlayson, 1976; King and Taylor, 1936). Infected tubules appear swollen and cloudy, and their lumen is packed with cysts (Harry and Finlayson, 1975; King and Taylor, 1936). As the disease progresses, tubule diameter increases in conjunction with the thinning of the epithelium and the destruction of the brush border of the epithelial cells (King and Taylor, 1936; Liu, 1985a). Yet, despite the initial characterisation of *Malpighamoeba* infection over 80 years ago (King and Taylor, 1936; Taylor and King, 1937) and descriptions of the disease progression (Bailey, 1968; Harry and Finlayson, 1975; Liu, 1985a,b), the effect upon the physiology of Malpighian tubules remains unknown.

Here we compare Malpighian tubule performance of gregarious desert locusts infected with *Malpighamoeba locustae* with that of their uninfected counterparts. We focussed on fluid secretion as well as the removal of hydrophobic organic cations from haemolymph because this requires active transport into the tubule lumen by various

types of carriers belonging to the ABC transporter family, including P-glycoprotein (Wright and Dantzler, 2004). P-glycoproteins are xenobiotic transporters found in many tissues throughout the animal kingdom, generally acting to reduce exposure to harmful compounds. Here we use the P-glycoprotein substrate rhodamine B (Eytan et al., 1997) as a proxy for a xenobiotic, to quantify P-glycoprotein activity in Malpighian tubules of the desert locust (Rossi et al., 2019). P-glycoproteins (Rossi et al., 2019) are likely expressed in the brush border on the apical side of the tubule epithelium (Murray, 1996), consequently, the destruction of the brush border caused by *Malpighamoeba* infection may compromise xenobiotic substance removal. The infection may also compromise fluid secretion, by damaging the primary and secondary active transporters that move K^+, Na^+ and Cl^- ions into the lumen to create an osmotic gradient that drives the water into the tubule (O'Donnell, 2008). To test these hypotheses, we used a modified Ramsay assay (Rossi et al., 2019) to assess the performance of locust Malpighian tubules.

4.3 Materials and methods

4.3.1 Animals

Adult desert locusts (*Schistocerca gregaria,* Forsskål, 1775) infected with *M. locustae* were taken from a crowded colony maintained at the University of Leicester, whereas adult uninfected locusts were purchased from Peregrine Livefoods (Essex, UK). The presence/absence of the infection was visually assessed. To our knowledge none of the locusts had been treated with anti-parasitic sulphonamides. Locusts were fed with organic lettuce, fresh wheat seedlings and wheat germ *ad libitum*.

4.3.2 Malpighian tubules, saline and Ramsay assay

Malpighian tubules were removed by cutting the proximal end at ~5 mm from the gut and moved immediately into a 30 µL drop of saline on a 5 cm Sylgard® coated Petri dish, covered with paraffin oil, and fixed with steel pins (Rossi et al., 2019) (Figure 4.1A-D). Isolated tubules ensure that any observed differences in function can be attributed to a direct effect of the infection on the tubules themselves, rather than indirect effects acting at higher levels of organisation (Berridge, 1966; Maddrell, 2004; Ramsay, 1954) e.g. at the level of hormonal control. Each tubule was punctured near the proximal end to allow fluid secretion, and it was allowed to equilibrate for 30 minutes. Subsequently, tubules were incubated in saline containing 60 µM rhodamine B, and secreted droplets were removed every 30 minutes using a 10 µL pipettor and photographed with a digital camera (Canon EOS 7D; Canon, Tokyo, Japan) mounted on a stereoscopic microscope (Nikon SMZ-U; Nikon Corp., Tokyo, Japan) (Figure 4.1E). Photobleaching of rhodamine was prevented using a custom dark box. We measured the volume of the droplet secreted by each tubule between 60 and 90 minutes of incubation, and estimated the rhodamine concentration in this droplet from the colour intensity using a calibration curve (Rossi et al., 2019).

At the end of the assay, we measured tubule diameter and length, and the length of tubule in contact with the treatment bath (Table 4.1). From these measurements, we calculated the surface area of the tubule and the surface area in contact with the treatment bath. The sample consisted of eight uninfected locusts and nine locusts infected with *Malpighamoeba locustae*. Three Malpighian tubules were removed from each locust and incubated in rhodamine B. One infected tubule was damaged during the experiment and therefore excluded from further analysis.

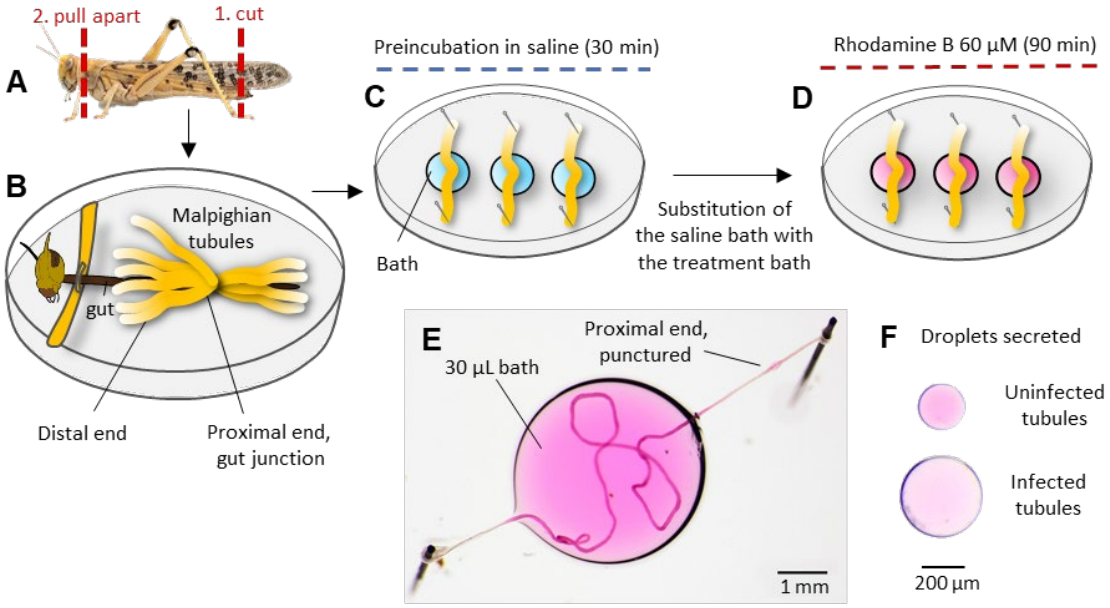

Figure 4.1. Rhodamine B extrusion assay of Malpighian tubule function. A) Locust dissection. **B)** Locust dissected with Malpighian tubules exposed. **C)** Three tubules removed from each locust and placed in a saline bath. The proximal end of each tubule was punctured with a sharpened capillary tube to allow the luminal fluid to be secreted. **D)** After 30 minutes of preincubation, the saline was removed from the bath wells and replaced with 60 µM rhodamine B in saline; we discarded the droplets secreted after 30 and 60 minutes, analysing only those secreted at 90 minutes. **E)** An example of a tubule incubated in the treatment bath containing rhodamine. The proximal end of the tubule is punctured to allow fluid secretion. **F)** Examples of droplets secreted after 90 minutes of incubation by uninfected (above) and infected (below) Malpighian tubules. These droplets were removed from the puncture site in the proximal end and then placed onto a Petri dish for photography.

4.3.3 Statistical Analysis

All statistical analyses were conducted in R version 3.4.1 (R Core Team, 2018). The distributions of the dependent variables analysed had a theoretical lower bound of zero and showed clear heteroscedasticity and positive skewness. To account for these distributional properties, we fitted Generalized Linear Mixed Model (GLMM) with Gamma error distributions and “inverse” or “identity” link functions accordingly to the distribution of the data (glmer function, package ‘lme4’ (Bates et al., 2015). Adequacy of the model fits was assessed from diagnostic plots of the standardised residuals (quantile-quantile and residuals over fitted). We investigated: (i) the effect of surface and infection on the fluid secretion rate; (ii) the effect of fluid secretion rate and infection on rhodamine concentration; and (iii) the effect of fluid secretion rate and infection on the net rhodamine extrusion. The predictors were scaled to SD=1, and centred on mean=0, while the dependent variables were scaled to SD=1 without re-centring. To quantify these effects, we selected the most parsimonious model from candidate models of different complexity using the Bayesian information criterion (Schwarz, 1978) (BIC) (Tables 2-4). To account for the nested structure of data, we included the individual locust as random intercepts in the model.

4.4 Results

We compared the morphology of the Malpighian tubules of uninfected desert locusts with those infected by the protozoan *Malpighamoeba locustae* (Amoebidae, Sarcodina) (Figure 4.2). Whilst uninfected Malpighian tubules are transparent with a distinct lumen (Figure 4.2A,B), those of infected locusts were filled with *M. locustae* cysts and had a thinner and less clearly defined wall than that of uninfected tubules (Figure 4.2C,D). The

rhythmic movements of the tubules were also slower and less pronounced in infected compared with uninfected locusts. We measured the length and diameter of Malpighian tubules in both uninfected and infected locusts (Figure 4.3A,B). Infected tubules were significantly longer and broader with a larger outer surface area than uninfected tubules (Table 4.1; Figure 4.3A–C). We calculated the surface area of uninfected and infected tubules from their length and diameter. A greater surface area was exposed to the incubation bath in infected compared to uninfected tubules (Table 4.1).

Table 4.1. Summary of the statistical models to compare morphological differences between uninfected and infected tubules (effect of infection). Infected tubules are longer and broader with a larger surface area than uninfected tubules. The surface area exposed to the incubation bath was greater in infected tubules than uninfected ones. Each morphological trait response was fitted by a GLMM with gamma error distribution and identity link function. Locust ID was included as random effect.

Morphological traits	Fixed effects	Estimate	s.e.	*t*	*p*	
Length (mm)	Intercept (uninfected)	21.09	0.67	31.35		
	Effect of infection	9.84	1.09	9.00	**<0.001**	***
Diameter (µm)	Intercept (uninfected)	56.36	7.36	7.66		
	Effect of infection	48.88	8.81	5.55	**<0.001**	***
Surface (mm^2)	Intercept (uninfected)	3.87	0.70	5.52		
	Effect of infection	6.17	0.87	7.05	**<0.001**	***
Surface in bath (mm^2)	Intercept (uninfected)	2.16	0.26	8.31		
	Effect of infection	4.51	0.50	9.08	**<0.001**	***

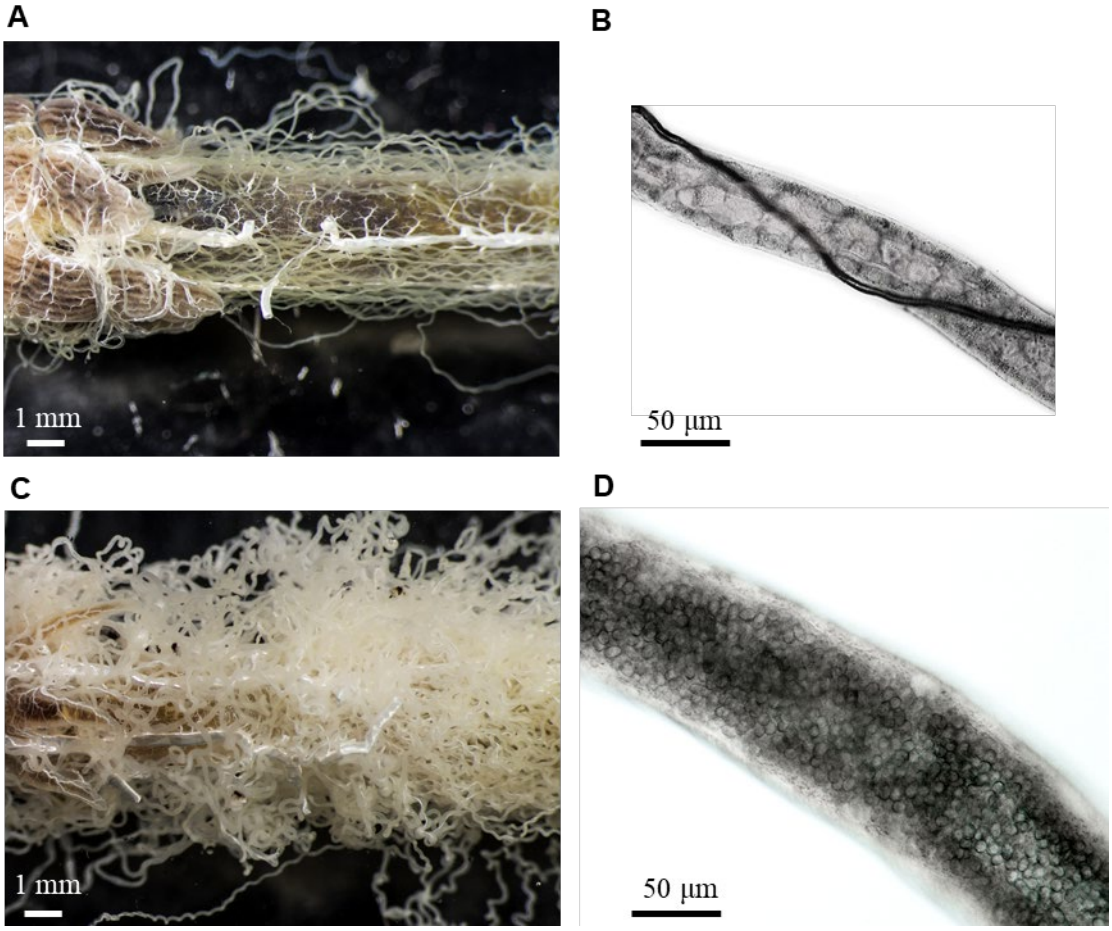

Figure 4.2. The Malpighian tubules of Malpighamoeba-infected locusts differ from those of uninfected locusts in appearance. (A) Uninfected tubules are thinner and more transparent. (B) The lumen of uninfected tubules is free from cysts. The dark structure running along the length of the tubule in this image is a tracheal branch. (C) Infected tubules are swollen and cloudy. (D) The lumen of infected tubules is filled with cysts.

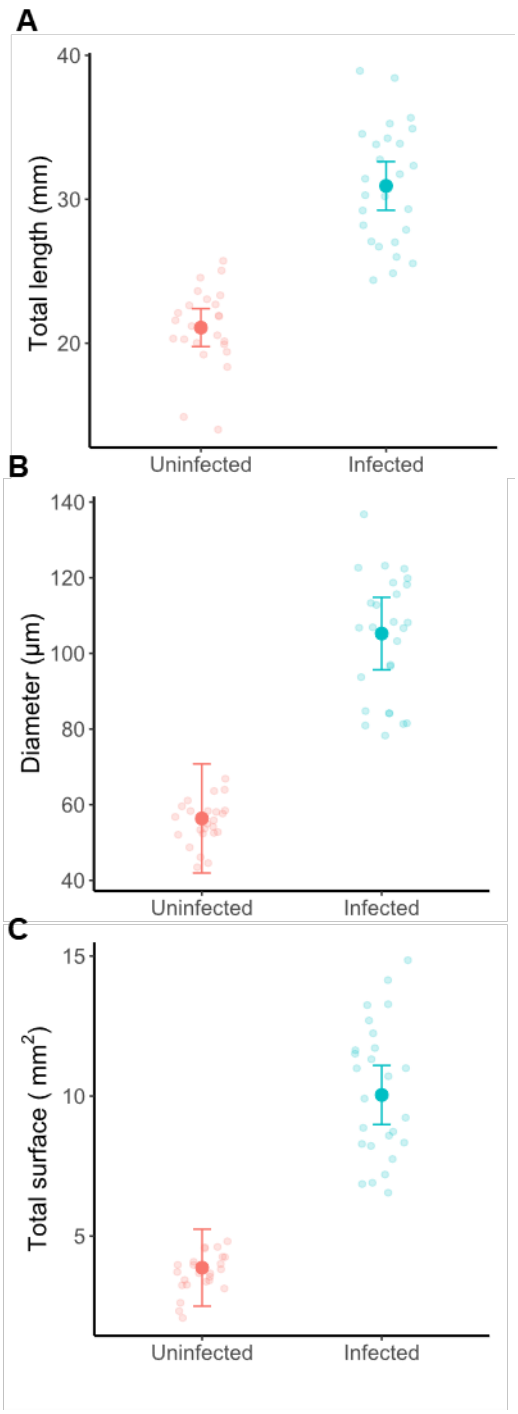

Figure 4.3. The Malpighian tubules of Malpighamoeba-infected locusts differ from those of uninfected locusts in length, diameter and surface area. Infected locusts had longer (A) and wider (B) tubules than uninfected locusts. (C) Consequently, the surface area of infected tubules was greater than that of uninfected tubules. Large filled circles with error bars represent mean estimates from GLMM fits with the 95% confidence interval. Small circles represent the raw data.

One function of the Malpighian tubules is to maintain osmotic balance through secretion of water and ions during diuresis (O'Donnell, 2008). To test whether infection with *M. locustae* affects the fluid secretion rate of tubules, we quantified the relationship between fluid secretion rate and surface area for uninfected or infected tubules (Figure 4.4). We selected the most parsimonious model from four candidate models with decreasing complexity (Table 4.2A). We found that surface area positively influences the fluid secretion rate independently of the health status of tubules (Table 4.2B, Figure 4.4). Thus, for a given unit of surface area tubules possess similar secretion rates, and consequently infected locusts with distended tubules tend to secrete more fluid (1.019 nl/min, 95% CI = [0.65, 1.39]) than uninfected locusts with smaller tubules (0.50 nl/min [0.13, 0.88]).

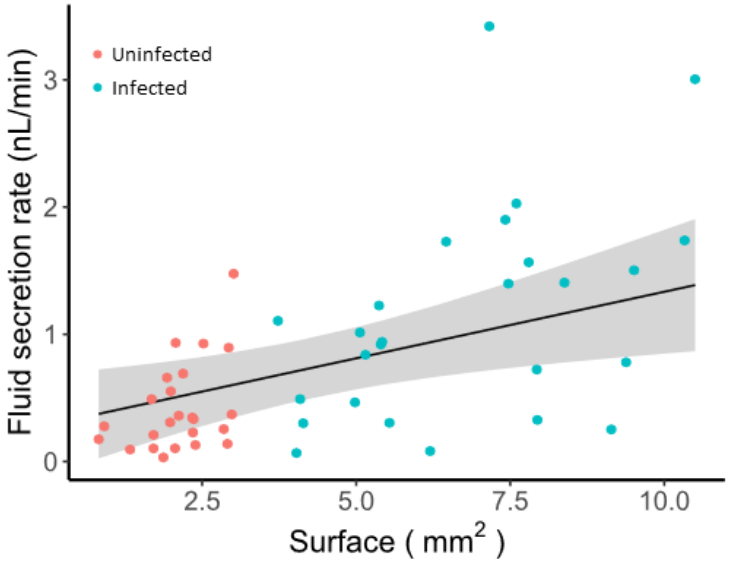

Figure 4.4. *Malpighamoeba* infection affects the fluid secretion rate of Malpighian tubules. Tubule surface area positively correlates with fluid secretion rate, therefore infected tubules with larger surface tend to secrete more fluid than the smaller uninfected tubules.

Table 4.2. Summary of the statistical models to investigate the effect of surface and health status on the fluid secretion rate. A) Comparison of statistical models predicting the fluid secretion rate (SR; scaled to S.D.=1) in relation to tubule surface area (scaled to S.D.=1 and mean centred on 0) and/or health status (uninfected, infected). The SR response was fitted by a GLMM with gamma error distribution and identity link function. Locust ID was included as random effect. According to the BIC, surface area alone explains the observed secretion rate (model in bold). **B**) Summary of the statistical model predicting SR in relation to surface area.

A.

Candidate models	df	BIC
`SR ~ surface * health status`	6	54.614
`SR ~ surface + health status`	5	56.135
`SR ~ surface`	**4**	**52.254**
`SR ~ health status`	4	55.046

B.

Fixed effects	Estimate	s.e.	*t*	*p*	
Intercept	0.699	0.112	6.26		
surface	0.267	0.096	2.77	**0.00566**	**

In addition to fluid secretion, Malpighian tubules also contribute to the extrusion of metabolic waste and xenobiotics from the haemolymph (O'Donnell, 2008). The altered morphology (Figs. 2,3) and increased fluid secretion (Figure 4.4) may affect the ability of infected Malpighian tubules to extrude xenobiotics. To test this, we quantified the extrusion of the P-glycoprotein substrate rhodamine B (Eytan et al., 1997) during tubule incubation. Rhodamine B was transported into the tubule lumen (Figure 4.5A), and its concentration in the droplets secreted by tubules was higher in uninfected than infected tubules (uninfected: 211.54 µM [127.35, 624.32]; infected: 77.12 µM [61.97, 102.08]; Table 4.3, Figure 4.5B). The rhodamine concentration in droplets was independent of the secretion rate, so that the lower rhodamine concentration in droplets secreted by the infected tubules cannot be attributed to their higher secretion rates and the consequent dilution (Table 4.3).

The net rhodamine extrusion rate (moles of rhodamine extruded per minute) increased with the fluid secretion rate, but this increase was less pronounced in infected tubules than in uninfected ones (Table 4.4A,B, Figure 4.5C). Consequently, for a given fluid secretion rate, infected tubules extrude less rhodamine. At the mean tubule fluid secretion rate of 0.79 nl/min, the mean net extrusion rate of uninfected tubules was 191.29 fmol/min [153.92, 228.65], whereas the mean net extrusion rate of infected tubules was less than half of this (70.75 fmol/min [46.36, 95.15]). However, the tendency of infected tubules to have a greater fluid secretion rate (uninfected, mean: 0.50 nl/min; infected, mean: 1.019 nl/min) counteracts, at least partially, the reduced net extrusion at any given fluid secretion rate, so that the total amount of rhodamine extruded by a typical infected tubule (89.58 fmol/min [65.92, 113.25] and a typical uninfected tubule (136.67 fmol/min [108.57, 164.77]) are broadly comparable (Figure 4.5C, black stars). Importantly, comparable net extrusion in infected tubules thus requires greatly increased fluid secretion rates. The reduction in rhodamine extrusion by infected tubules in comparison to uninfected tubules suggests decreased extrusion per unit surface area. We quantified the net rhodamine extrusion per unit surface area and found that it was indeed substantially reduced in infected tubules (13.53 fmol/min·mm^2, [10.36, 19.51]) compared with uninfected tubules (41.72 fmol/min·mm^2 [24.56, 138.64]; t=3.97, p<0.001; Figure 4.5D).

Table 4.3. Summary of the statistical models to investigate the effect of fluid secretion rate and health status on the rhodamine concentration of the droplets secreted. A) Comparison of statistical models predicting the rhodamine concentration (scaled to S.D.=1) in relation to the fluid secretion rate (SR; scaled to S.D.=1 and mean centred on 0) and/or health status (uninfected, infected). The rhodamine concentration response was fitted by a GLMM with gamma error distribution and inverse link function. Locust ID was included as random effect. According to the BIC, health status alone explains the observed rhodamine concentration (model in bold). **B**) Summary of the statistical model predicting the rhodamine concentration in relation to health status.

A.

Candidate models	df	BIC
`Rhodamine concentration ~ SR * health status`	6	25.215
`Rhodamine concentration ~ SR + health status`	5	21.86
`Rhodamine concentration ~ SR`	4	25.912
`Rhodamine concentration ~ health status`	**4**	**21.767**

B.

Fixed effects	Estimate	s.e.	*t*	*p*	
Intercept (uninfected)	1.190	0.402	2.964		
health status	2.075	0.548	3.784	**<0.001**	***

Table 4.4. Summary of the statistical models to investigate the effect of fluid secretion rate and health status on the net rhodamine transport. **A**) Comparison of statistical models predicting the net rhodamine transport (scaled to S.D.=1) in relation to the fluid secretion rate (SR; scaled to S.D.=1 and mean centred on 0) and/or health status (uninfected, infected). The net rhodamine transport response was fitted by a GLMM with gamma error distribution and identity link function. Locust ID was included as random effect. According to the BIC, the interaction between SR and health status best explains the net rhodamine transport (model in bold). **B**) Summary of the statistical model predicting the net rhodamine transport in relation to SR and health status.

A.

Candidate models	df	BIC
`Net rhodamine transport ~ SR * health status`	**6**	**12.943**
`Net rhodamine transport ~ SR + health status`	5	28.363
`Net rhodamine transport ~ SR`	4	33.871
`Net rhodamine transport ~ health status`	4	67.018

B.

Fixed effects	Estimate	s.e.	*t*	*p*	
Intercept (uninfected)	1.507	0.150	10.032		
SR	1.098	0.165	6.648	**<0.001**	***
Health status (at mean SR)	-0.950	0.179	-5.316	**<0.001**	***
SR × health status	-0.746	0.175	-4.269	**<0.001**	***

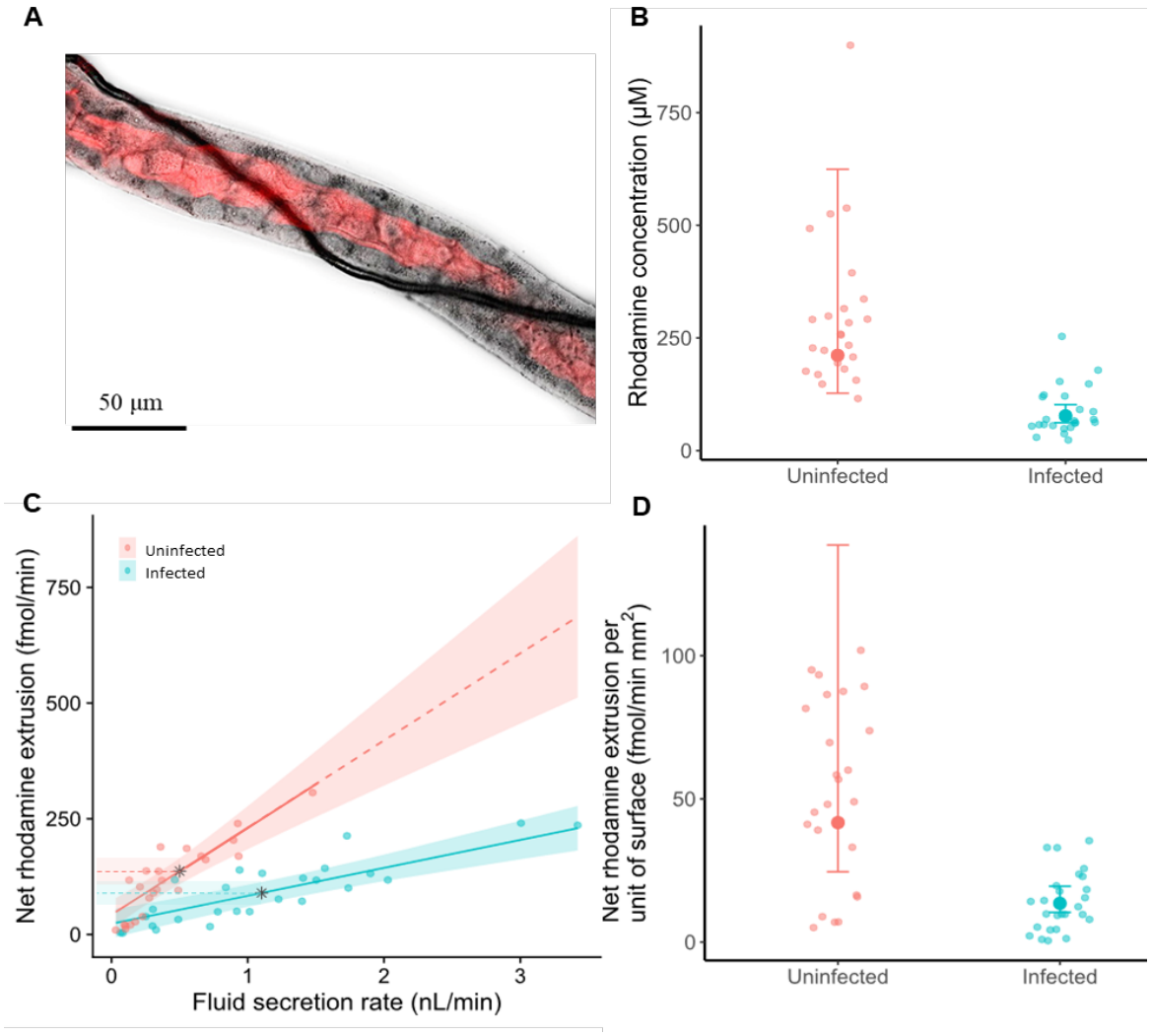

Figure 4.5. ***Malpighamoeba*** **infection affects rhodamine B extrusion by Malpighian tubules. A**) The uninfected tubule shown in **Figure 4.1**B transporting rhodamine B. **B**) Uninfected tubules secreted more concentrated droplets than the infected tubules. **C**) The secretion rate positively correlates with the net extrusion rate of rhodamine B, but the steepness is more accentuated in the uninfected tubules compared to the infected ones. Black stars represent the mean fluid secretion rate for uninfected and infected tubules. **D**) The net rhodamine extrusion rate per unit surface area is higher in uninfected than infected tubules. Small circles represent the raw data, large filled circles represent means estimates, and lines represent the regression line fitted from GLMM. Shaded areas and error bars represent the 95% confidence interval. All the values are back transformed to the original scale

4.5 Discussion

We determined how infection by the protozoan *Malpighamoeba locustae* affects the performance of insect Malpighian tubules using gregarious desert locusts (*Schistocerca gregaria*) as a model system. We compared the performance of infected and uninfected tubules in terms of secretion and xenobiotic extrusion. By doing so, our results directly quantify the impact of a common pathogen upon major components of insect osmoregulation and xenobiotic excretion, namely the Malpighian tubules and the P-glycoproteins they express. Infected tubules differed from uninfected tubules in morphology being longer, wider, and possessing a greater surface area. The infected tubules also differed in behaviour from uninfected tubules showing less pronounced and slower rhythmic movements. We assessed two key aspects of the physiological performance of tubules; their fluid secretion and net extrusion of rhodamine B, which is a P-glycoprotein substrate (Eytan et al., 1997). Our analysis showed that tubules with a larger surface area secrete more fluid per unit time. Therefore, as a consequence of their greater surface area, infected tubules secrete more fluid per unit time than uninfected tubules. Despite this increased secretion, however, the net extrusion of rhodamine per unit surface area is far lower in infected compared with uninfected tubules.

Fluid secretion from tubules is powered by the V-ATPase, which creates an electrochemical gradient that drives Na^+, K^+, and Cl^- ion movement into the lumen followed by the passive water movement (O'Donnell, 2008). The V-ATPase is expressed within the apical brush border of the tubule epithelium (Maddrell and O'Donnell, 1992; Wieczorek et al., 2009). The *Malpighamoeba* trophozoites destroy the apical brush border of Malpighian tubules (King and Taylor, 1936; Liu, 1985a), raising the possibility that the V-ATPase is reduced. The greater fluid secretion rate of the infected compared

to the uninfected tubules is therefore at first quite surprising. However, the reduction of the brush border and consequent change in the composition of cellular cytoplasm may produce ionic gradients that permit continued transcellular water movement, which may be sufficient to produce similar rates of fluid secretion rate per unit of surface area. Alternatively or additionally, the thinning of the epithelium or the damage of the brush border in infected tubules may compromise the septate junctions, which exist between epithelial cells (Garrett et al., 1988), promoting water movement through a paracellular pathway.

Trans-tubular rhodamine transport is predominantly dependent on P-glycoprotein transporters in the brush border of tubule epithelial cells (Murray, 1996), though they may be present on the basal side (Gaertner et al., 1998). The destruction of the brush border by the *Malpighamoeba* trophozoites (King and Taylor, 1936; Liu, 1985a) likely impairs P-glycoprotein transport. However, the greater fluid secretion rate of infected tubules will decrease luminal rhodamine B concentration in comparison to uninfected tubules, reducing back diffusion (Maddrell et al., 1974; Rossi et al., 2019) and thereby increasing its net rhodamine B extrusion. Nevertheless, infected tubules extrude less rhodamine B for a given rate of fluid secretion and less rhodamine B per unit surface area than do uninfected ones. Infected tubules do extrude rhodamine B, which may be via diffusion down a concentration gradient or by relying on other organic cation transporters, as suggested by studies of human cell lines (Ugwu et al., 2016).

The overall consequence of the reduced net extrusion per unit of surface area coupled with the increased fluid secretion rate is that the total net extrusion per tubule is similar in infected and uninfected tubules. This is consistent with the finding that infection with *Malpighamoeba* does not increase the susceptibility of grasshoppers

(*Melanoplus sanguinipes*) to the insecticide cypermethrin, a P-glycoprotein substrate, within one hour of application (Hinks and Ewen, 1986; Sreeramulu et al., 2007). However, tubules of heavily infested locusts present globular melanised encapsulations that can fuse together and to adjacent tissues forming tissue masses that can no longer contribute to excretion (Harry and Finlayson, 1975; King and Taylor, 1936) (Figure 4.6). Therefore, it is possible that these encapsulations reduce the number of functional tubules, further compromising locust excretion.

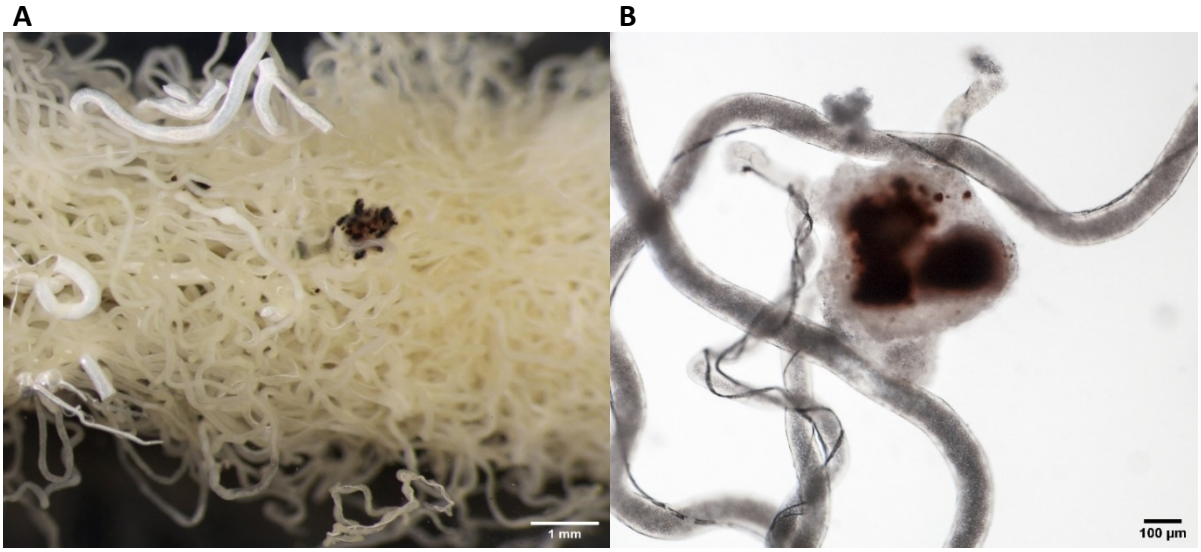

Figure 4.6. Locusts infected with *Malpighamoeba locustae* contain globular melanised encapsulations. A) Malpighian tubules fused together with melanised encapsulations. **B)** Isolated encapsulation.

Changes in feeding behaviour may also contribute to the ability of locusts to compensate for *Malpighamoeba* impairment of P-glycoprotein detoxification; infection reduces the amount of food that locusts ingest (King and Taylor, 1936) (M. Rossi pers. obs.). Reducing food intake may reduce the exposure of infected insects to noxious substances that their compromised detoxification pathways cannot remove without substantial water loss into the gut. This may be particularly important for generalist herbivores like desert locusts that feed on a broad variety of plant species, many of which contain toxins (Bernays and Chapman, 1978). Alternatively, reduced food intake in infected locusts may be driven by the activation of the immune system (McMillan et al., 2018). Both their immune system and their ability to detoxify noxious food substances rely on pathways that involve the antioxidant glutathione (Habig et al., 1974; Jeschke et al., 2016; Stahlschmidt et al., 2015). Consequently, competition for glutathione between these two physiological pathways could lead to impaired detoxification when the immune system is already activated (McMillan et al., 2018). Whether due to a direct reduction in feeding or as an indirect consequence of a trade-off with the immune response, reduced food intake lowers the risk of ingesting toxins but also prevents locusts acquiring water and nutrients.

A reduced food intake combined with an increased fluid secretion rate poses infected locusts a difficult challenge. In adaptation to the dry and hot environment that they occupy, desert locusts must balance their water secretion to avoid dehydration (Phillips, 1964a). Diuretic and antidiuretic hormones play an important role in the control of the tubular fluid secretion (Phillips, 1981). However, *Malpighamoeba* infection may damage the hormone receptors, decreasing the ability of the insect to respond to the circulating hormones (Proux, 1991). The primary urine secreted by the

Malpighian tubules is isosmotic to the haemolymph, and the composition must be adjusted prior to excretion (O'Donnell, 2008). Most of the filtered water, ions and metabolites are reabsorbed in the anterior hindgut and in the rectum (O'Donnell, 2008). The reabsorption of Na^+, K^+, Cl^- ions and hence water is an active process consuming metabolic energy (Phillips, 1964a,b). To prevent desiccation, locusts infected with *Malpighamoeba* would need to enhance water reabsorption to counterbalance the increased fluid secretion rate of the Malpighian tubules. So, infected locusts may be subjected to the increased energy costs of reabsorbing the excess of water and ions transported into the lumen tubule. Thus, *Malpighamoeba* infection may be highly debilitating for desert locusts in their natural environment, imposing stresses in terms of water, ions, and energy even if they can avoid ingesting plant toxins.

Malpighamoeba infections are not limited to locusts and grasshoppers, occurring in other insects including honeybees (Bailey, 1968). Although the exact details of *Malpighamoeba* infection likely depend upon the specific host and pathogen species combination, honeybee Malpighian tubules infected with *Malpighamoeba mellificae* show striking similarities to locust tubules infected with *Malpighamoeba locustae*. In both cases, the lumen of the tubules is swollen and packed with cysts, the epithelium thins, and the brush border of the epithelial cells is destroyed (Bailey, 1968; Harry and Finlayson, 1975; King and Taylor, 1936; Liu, 1985a). Such similarities in tubule pathology despite the separation of the insect hosts by approximately 380 million years (Misof et al., 2014) suggests that *Malpighamoeba* infection may have similar consequences in other insects susceptible to infection. Moreover, infection in honeybees and other insects may similarly compromise detoxification of xenobiotic compounds and increase water and ion loss. Even though xenobiotic compounds may be removed from insect

haemolymph by infected tubules, our results show a substantial increase in fluid secretion by infected tubules, which implies greater energy costs for reabsorption in the insect hind gut. Such impacts are highly injurious and can lead to the premature death of the insect (Venter, 1966).

5 General discussion

5.1 Contributions of the thesis

Overall this thesis makes novel contributions to the understanding of the physiology of desert locust Malpighian tubules in the context of ecology, phenotypic plasticity and pathology.

5.1.1 Methodological contribution

In the second chapter, we developed a simpler and cheaper method based upon measuring dye concentration to investigate the presence and physiology of P-glycoprotein in Malpighian tubules of *Schistocerca gregaria*, as an alternative to confocal microscopy (Leader and O'Donnell, 2005; O'Donnell and Leader, 2006), liquid chromatography–mass spectrometry (Andersson et al., 2013), or radiolabelled alkaloids (Gaertner et al., 1998). By combining a modified Ramsay assay with the application of the P-glycoprotein substrate rhodamine B (Eytan et al., 1997) and the P-glycoprotein inhibitor verapamil (Tsuruo et al., 1981), we estimated the contribution of P-glycoproteins to the toxin extrusion by measuring the size and colour of the droplets secreted by isolated tubules. By measuring the size of the droplets we calculated the fluid secretion rate, the colour of the droplets in relation to their diameter provided the rhodamine concentration, and the combination of the fluid secretion and rhodamine concentration provided an estimate of the net rhodamine extrusion, which we used as proxy for the presence of P-glycoprotein.

Halberg and Møbjerg (2012) used a similar method to investigate epithelial transport in tardigrades and desert locusts using chlorophenol red by imaging through

the gut or tubules. Leader and O'Donnell (2005) also attempted to quantify the fluorescent dye transport in tubules of the cricket *Teleogryllus commodus* and *Drosophila*, but they found it impractical because of the presence of concretions in the tubule cells that interfered with the luminal fluorescent dye. Consequently, they opted to collect the droplets secreted by the tubules into rectangular glass capillary tube to measure the concentration of the fluorescent dye with confocal laser microscopy. By avoiding the need for confocal microscopy, our protocol provides an easier method to assess the net dye transport using a digital camera, and avoiding the interference of the tubule cells.

P-glycoproteins and other ABC transporters play a key role in multidrug resistance in cancerous cells in humans (Eckford and Sharom, 2009; Gottesman, 2002) by increasing the extrusion of chemotherapeutic drugs at almost the same speed they enter in the cells, leading to failure of these chemotherapies (Schinkel and Jonker, 2003). In addition, P-glycoproteins expressed on the human blood brain barrier prevent the entry of other therapeutic drugs into the brain (Schinkel, 1999). Intensive efforts have been undertaken to understand the interaction of pharmacological drugs and P-glycoproteins, and several methods such as *in vitro* vesicles, *in silico* models, and cultured cells assays have been used (Andersson et al., 2013; Eytan et al., 1997, 1996; Goodwin and Clark, 2005). More recently the locusts *S. gregaria* and *L. migratoria* have been proposed as invertebrate models for the human blood brain barrier to screen early drug discovery (Al-Qadi et al., 2015; Andersson et al., 2013; Nielsen et al., 2011). The dissected insect brain is incubated in solutions containing candidate drugs, and by using liquid chromatography-mass spectrometry to quantify the amount of drug permeated into the brain it is possible to discriminate between P-glycoprotein substrates and non-

substrates (Al-Qadi et al., 2015; Andersson et al., 2013; Nielsen et al., 2011). Because we found that *S. gregaria* expresses P-glycoproteins on their Malpighian tubules, we suggest that our method may provide an inexpensive alternative, suitable for screening the affinity of new drugs to P-glycoproteins.

5.1.2 Feeding ecology and polyphenism

In the third chapter, using the method developed in the previous chapter, we investigated the impact of phenotypic plasticity upon the locust's Malpighian tubules in a natural ecological context, with a focus on the desert locust's phase polyphenism and the associated change in feeding habits. In contrast to solitarious locusts that reject toxic plants, gregarious locusts readily include them in their diet, while transiens stage locusts actively show a preference for them (Despland and Simpson, 2005a; Simões et al., 2013). Therefore, we investigated whether the Malpighian tubules differ in their ability to extrude toxic substances between phases, and whether the differences are induced solely by the phase shift, or whether they are induced by the simultaneous change in feeding habits. To discriminate the effects of phase and food, we fed solitarious, transiens and gregarious phases either on an alkaloid-free diet or a diet containing the alkaloid atropine, a P-glycoprotein competitive inhibitor (Gaertner et al., 1998; Joosen et al., 2016; Maddrell and Gardiner, 1976). To ensure our treatments retained an ecological relevance, we used a concentration of atropine that is similar to that found in the Egyptian henbane, one of the host-plants of gregarious and transiens locusts in natural conditions (El-Shazly et al., 1997; Popov et al., 1991).

We found that net rhodamine extrusion is similar in solitarious, transiens and gregarious locusts fed on the alkaloid-free diet, suggesting that the phase shift does not itself cause up- or downregulation of P-glycoprotein activity in the tubules.

Consequently, the basal level of toxin excretion is similar between locusts in different phases. Subsequently, to compare the ability of the three phases to increase P-glycoprotein activity after exposure to atropine, we fed solitarious, transiens and gregarious locusts with a diet containing atropine for three days. Our hypothesis was that the Malpighian tubules of solitarious locusts are less effective in upregulating the P-glycoprotein transporters than transiens and gregarious locusts. As reported in the literature (Despland and Simpson, 2005a; Simões et al., 2013), solitarious locusts rejected the food provided almost entirely, and therefore their P-glycoprotein activity was no different from solitarious locusts fed on an alkaloid-free diet. However, after less than 24 hours of crowding, solitary locusts that had begun to gregarize (transiens) readily ate the atropine-contaminated food. Gregarious and transiens locusts exposed for three days to a diet containing atropine, showed a significant increase in P-glycoprotein activity, allowing the insects to cope with a potentially noxious diet by excreting toxins more rapidly.

We also found that the process is reversible; gregarious locusts reared on a diet containing atropine downregulated P-glycoprotein transporter activity in their Malpighian tubules after being switched for seven days to an alkaloid-free diet, emphasising their phenotypic flexibility to adapt swiftly to new environmental conditions. To our knowledge, this is the first study comparing the physiology of locusts' Malpighian tubules in the three different phenotypic phases, and investigating plasticity in xenobiotic extrusion in relation to their natural feeding ecology.

It is known that density dependant phenotypic plasticity involves many different changes taking place over multiple time scales (Ernst et al., 2015). For example, muscle morphology and brain structure change over several generations, and coloration is

modified within a lifetime (Ernst et al., 2015; Islam et al., 1994; Ott and Rogers, 2010), whereas behavioural changes relying on short-term neuronal plasticity and regulation of toxin excretion relying on the Malpighian tubule plasticity occur in just a few hours or days (Despland and Simpson, 2005a; Roessingh et al., 1993; Rogers and Ott, 2015; Simões et al., 2013). Solitarious cryptic locusts are found when patches of vegetation are evenly distributed and intraspecific competition is low. In this context, the locusts can feed selectively on specific plants, rejecting those containing toxic substances (Despland and Simpson, 2005a; Simões et al., 2013), thereby avoiding the costs associated with xenobiotic detoxification, including upregulation of detoxifying enzymes and P-glycoproteins. However, after a period of rainfall vegetation thrives and the population of solitarious locusts quickly increases, leading to increased intraspecific competition for food as the vegetation is reduced to small sporadic patches (Despland et al., 2000; Simpson et al., 1999; Uvarov, 1977). The resulting crowding increases the visual, tactile and olfactory stimuli between conspecifics that in turn causes a cascade of genetic and hormonal processes associated with the gregarization (Roessingh et al., 1998; Lucas et al., 2010; Rogers and Ott, 2015; Anstey et al., 2009). Transiens locusts in transition to the gregarious phase change their feeding behaviour, showing a preference for plants containing toxic compounds, to become unpalatable to predators (Simões et al., 2013; Sword, 2001). In response to this environmental change, the Malpighian tubules upregulate their P-glycoproteins, likely to maintain the concentration of toxin ingested under safe levels.

5.1.3 Pathology

In the fourth chapter, we investigated how the parasitic infection of the protozoan *Malpighamoeba locustae* influences the phenotypic plasticity and physiological performance of the locust's Malpighian tubules. Previous studies have shown that *M. locustae* damages the cellular structure of the tubules, in particular the apical border of the cells (Harry and Finlayson, 1976, 1975; King and Taylor, 1936; Taylor and King, 1937), but to our knowledge no physiological study of infected tubules has been performed before. We found that infected tubules are longer and wider than uninfected ones. We also found a higher fluid secretion rate in infected tubules compared to uninfected ones, as the result of their greater surface area. This result may at first seems surprising because the infection destroys the apical border where the mitochondria and the primary proton-motive V-ATPase are located (see Figure 1.2). How can the tubules maintain this high secretion rate with the likely reduced production of ATP and the lack of the proton motive force? We hypothesise that the thinning of the epithelium caused by the infection may also compromise the septate junctions between the cells (Garrett et al., 1988), which would allow water to flow through a paracellular pathway. Alternatively or additionally, the cytoplasm is in direct contact with the lumen of the tubule because the epithelial cell is no longer a sealed compartment, allowing ions to move through the cotransporter Na^+:K^+:$2Cl^-$ from the haemolymph directly into the lumen even without the V-ATPase. Water may follow these ions osmotically. Our result may explain the dysentery observed in honey bees infected with *M. mellificae* (Fyg, 1964), a protozoan belonging to the same genus of *M. locustae*, that similarly damage the brush border of the honey bee tubule's cells (Liu, 1985a,b) (see heading 1.4.1.4).

In addition, we found that infected tubules had a lower net rhodamine extrusion per unit of surface area compared to those of uninfected locusts, suggesting that the P-glycoprotein density (i.e. number of P-glycoprotein per unit of surface area) may be reduced by the damage to the brush border. Although the net rhodamine extrusion per unit of surface area was reduced, the increased fluid secretion rate of the infected tubules reduced the rhodamine back diffusion from the lumen to the haemolymph (Maddrell et al., 1974; Rossi et al., 2019), leading to a total net extrusion per tubule that was similar in infected and uninfected tubules. However, in heavily infested locusts the tubules can fuse together and to nearby tissues, forming globular melanised encapsulations (Figure 4.6) that can no longer contribute to xenobiotic extrusion (Harry and Finlayson, 1975; King and Taylor, 1936). Hence, it is likely that the number of functional tubules is reduced, further compromising locust excretion.

5.2 Open questions and future work

5.2.1 Screening of pesticides

We propose that the protocol we developed on desert locusts' Malpighian tubules could be extended to other insects and may, for example, be employed in toxicological studies. Using various dyes that are substrates of different ABC transporters, we could investigate the interaction of pesticides with different xenobiotic transporters. Understanding which ABC transporters are expressed by the Malpighian tubules of both pest and beneficial insects could lead to the development of novel insecticides that control a particular pest, but with the reduced negative side-effect of damaging pollinators.

At the moment, the regulatory system for new pesticides is based on the exposure of test insects to a single chemical at a time, ignoring the reality that non-target insects such as honeybees and bumblebees are routinely exposed to a combination of pesticides and medications applied by beekeepers to the hives. For example, a meta-analysis by Sanchez-Bayo and Goka (2014) found 161 different pesticides in honeybee hives from around the world, with food stores in individual colonies often containing complex mixtures of pesticides. Bees may cope with exposure to sublethal doses of single compounds, but the simultaneous exposure to two or more chemicals may have a synergistic effect that is greater than the sum of the effects of the single compounds (Guseman et al., 2016; Hawthorne and Dively, 2011; Johnson et al., 2009; Pilling and Jepson, 1993; Sanchez-Bayo and Goka, 2014). For example, demethylation-inhibiting fungicides (DMI) appear to block detoxification mechanisms, via interference with the cytochrome P450 enzymes, rendering insecticides effectively more toxic (Colin and Belzunces, 1992; Iwasa et al., 2004). As a result, interest has grown in understanding the potential for synergisms or antagonisms between the insecticide residuals found in pollen and nectar, and fungicides, miticides and antibiotics that are applied in honey bee hives. One possible route for interactions between pesticides is via their effects on P-glycoproteins, hence we propose that our bioassay could be used to screen different pesticides in combination with other chemicals or secondary metabolites commonly found in nectar and pollen, to assess potential synergisms that may impair the fitness of pollinators.

Several compounds can modulate the activity of P-glycoproteins acting as inducers, activators, or inhibitors. Inducers increase the transporter expression, whereas activators act faster than inducers by changing the transporter conformation

so that the substrate can bind on an additional binding site (Silva et al., 2015; Sterz et al., 2009; Vilas-Boas et al., 2013). Therefore, it would be interesting to perform *in vitro* and *in vivo* assays to test whether the administration of these compounds may increase the resistance of bees to pesticides. On the other hand, because P-glycoproteins are implicated in the resistance of some pests to insecticides (Lanning et al., 1996; Srinivas et al., 2004), one could investigate whether it is possible to increase the efficacy of insecticides by combining P-glycoprotein inhibitors and insecticides that may act synergistically to increase the pest mortality.

5.2.2 Polyphenism and self-medication

In the third chapter we have demonstrated that transiens and gregarious locusts can upregulate the P-glycoproteins on their tubules after exposure to a diet containing alkaloids. However, an unsolved question remains; what are the underlying reasons for the narrow diet of solitary locusts? Are they incapable of upregulating P-glycoproteins or are they avoiding the cost of synthetizing detoxifying enzymes (Mainguet et al., 2000) and xenobiotic transporters? Because solitarious locusts refuse to eat food containing atropine, to overcome this issue we could inject a solution containing atropine directly in their haemolymph. This will provide a definitive answer to our question as to whether they are capable of regulating the activity of P-glycoproteins.

Another open question is whether the density-dependent phenotypes evolved not only in response to intraspecific food competition and increased predation risk, but also in response to the increased probability of becoming infected by a pathogen (Cullen et al., 2017; Simpson and Sword, 2008; Wilson and Cotter, 2009) (see heading 1.3). Increased population density and contact between individuals would be expected to result in enhanced transmission of pathogens. During the gregarization, insects may

thus invest in disease resistance by enhancing their immune defence (Reeson et al., 1998; Wilson and Cotter, 2009), or through the ingestion of plants containing allelochemicals as a prophylactic measure to prevent the infection, or as a therapeutic measure to reduce the load of infection (Abbott, 2014; de Roode and Hunter, 2019). For example, bumblebees seem to exploit nectar containing alkaloids to reduce the infection of the gut protozoan, *Crithidia bombi* (Baracchi et al., 2015; Manson et al., 2010; Richardson et al., 2015). Although the mechanism is not yet clear, Manson et al. (2010) suggested that secondary metabolites ingested in nectar may increase the bee's extrusion rate, effectively flushing the protozoan from the gut. Because grasshoppers infected with *M. locustae* have been found more often in laboratory cultures than in nature (Henry, 1968; King and Taylor, 1936; Taylor and King, 1937), it may be that grasshoppers in the field have evolved mechanisms to counteract this infection that are not available to them in captivity. We propose that the ingestion of toxic plants, in addition to provide protection against predators, may be prophylactic or curative in transiens and gregarious locusts.

To test whether alkaloids are prophylactic, we could inoculate *M. locustae* on gregarious locusts either fed with an alkaloid-free diet or with a diet containing a range of alkaloids found in their host plants, and subsequently compare the load of cysts in their tubules. In addition, we could investigate whether the alkaloids can be therapeutic, by feeding infected gregarious locusts with an alkaloid-free or an alkaloid diet. After a few days of exposure to the diets, we could compare the load of infection in their tubules, and also test whether the tubules of the locusts fed on the alkaloid diet increase their fluid secretion rate compared to those fed on the alkaloid-free diet. Fitness traits

such as longevity and fecundity could also readily be recorded to quantify the overall effects of the interaction between pathogen exposure and diet.

However, locusts heavily infected with *M. locustae* may be unable to cope with an increased amount of alkaloids in their diets because the expression of P-glycoprotein per unit of surface area is lower in infected tubules than uninfected ones (see chapter 4). Are infected tubules still able to upregulate their P-glycoproteins after exposure to alkaloids? Does the ability of upregulating P-glycoprotein depend on the extent of the damage of the brush border? To investigate these questions, we could feed locusts with different degree of infection on a diet containing alkaloid and measure the P-glycoprotein expression after a few days of exposure to the alkaloids.

5.2.3 Hydration and *Malpighamoeba* infection

In the fourth chapter, we investigated the effect of the protozoan *M. locustae* infection on the performance of the locust Malpighian tubules. In our *ex vivo* experiment, we found that infected tubules have higher fluid secretion rate than those uninfected. However, the situation in *in vivo* insects remain unclear. Locusts may reabsorb part of the fluid in the ileum and rectum, increasing their energetic costs, or the fluid may be expelled with the faeces, exposing locusts to the risk of dehydration. To answer this question, we could collect faeces from infected and uninfected locusts, and compare their mass and water content. If the water content is similar, the increased primary urine produced by the tubules has been reabsorbed in the hindgut, whereas if the water content in the faeces of the infected locusts is greater than those of uninfected tubules, the infection is likely to cause dehydration.

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