SHORT COMMUNICATION

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

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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 quantify the impact of *Malpighamoeba* infection on fluid secretion and P-glycoprotein-dependent detoxification by Malpighian tubules of the desert locust using a modified Ramsay assay. Infected tubules have a greater surface area and a higher fluid secretion rate than uninfected tubules. Infection also impairs P-glycoprotein-dependent detoxification reducing the net extrusion of rhodamine B per unit of surface area. However, infected tubules' increased surface area and fluid secretion rate means they have similar total net extrusion per tubule to uninfected tubules. Increased fluid secretion rate of infected tubules likely subjects locusts to greater water stress and imposes increased energy costs. Coupled with reduced efficiency of P-glycoprotein detoxification, *Malpighamoeba* infection is likely to reduce insect survival within natural environments.

KEY WORDS: Osmoregulation, Excretion, ABC transporter, Xenobiotic, Honeybee.

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

38 products (such as nectar or pollen) because of the presence of secondary metabolites (Despres *et al*.,
39 2007). Moreover, feeding on plant tissues or their products can expose insects to xenobiotics such as
40 insecticides, fungicides or antibiotics that may act as toxins (Goulson *et al*., 2015). Consequently,
41 compromised tubule function may prevent extrusion of these xenobiotics increasing their effects on
42 insects.

43 Insect pathogens can interact with xenobiotics such as pesticides affecting survival (reviewed
44 in Goulson *et al*., 2015). Interactions between pathogens and xenobiotics, which have been studied
45 primarily in honeybees and bumblebees (e.g. Alaux *et al*., 2010; Vidau *et al*., 2011), suggest that
46 pesticide exposure can exacerbate the effects of pathogens. Moreover, pathogens may themselves
47 exacerbate the impacts of xenobiotics, possibly due to trade-offs between the activity of the immune
48 system and detoxification pathways (McMillan *et al*., 2018). In most cases, the mechanisms linking the
49 susceptibly of insects to xenobiotics with the presence of pathogens remain unknown, even in the case
50 of diseases of insect pollinators that provide commercially-important ecosystem services (reviewed in
51 Goulson *et al*., 2015). Consequently, identifying mechanisms by which pathogens could affect the
52 xenobiotic compound removal is important for understanding insect immune responses, detoxification
53 and health.

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

66 Here we compare Malpighian tubule performance of gregarious desert locusts infected with
67 *Malpighamoeba locustae* with that of their uninfected counterparts. We focussed on fluid secretion as
68 well as the removal of alkaloids and hydrophobic organic cations from haemolymph because this
69 requires active transport into the tubule lumen by P-glycoprotein (P-gp), a member of the ABC
70 transporter family (Wright and Dantzler, 2004). Desert locust Malpighian tubules express P-gp (Rossi
71 *et al*., 2019), likely in the brush border on the apical side of the tubule epithelium (Murray, 1996).
72 Consequently, the destruction of the brush border caused by *Malpighamoeba* infection may
73 compromise xenobiotic substance removal. To test this hypothesis, we used a modified Ramsay assay
74 (Rossi *et al*., 2019) to assess the performance of locust Malpighian tubules.

MATERIALS AND METHODS

Animals

Adult desert locusts (*Schistocerca gregaria*) were taken from a crowded colony maintained at the University of Leicester or were pruchased from Peregrine Livefoods (Essex, UK). Locusts were fed with organic lettuce, fresh wheat seedlings and wheat germ *ad libitum*.

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 (Supplemental Figure 1) (Rossi *et al*., 2019). Each tubule was punctured near the proximal end to allow the fluid secretion. The first droplet secreted immediately after puncturing was discarded. 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) (Supplemental Figure 2). Photobleaching of rhodamine B was prevented using a custom dark box. We measured the volume of each droplet, and estimated the rhodamine B concentration 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. 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 healthy locusts and nine locusts infected with *Malpighamoeba locustae*. Three Malpighian tubules were removed from each locust (Supplemental Figure 1). One infected tubule was damaged during the experiment and therefore excluded.

Statistical Analysis

All statistical analyses were conducted in R version 3.4.1 (R Core Team, 2018). We fitted Linear Mixed Effect Models (LMEM) by restricted maximum likelihood (REML) estimation (`lmer` function, package 'lme4' (Bates *et al*., 2015)). To investigate the effect of the infection and incubation time on the fluid secretion rate (per tubule and per surface area), rhodamine B concentration and rhodamine B net extrusion (per tubule and per surface area), we analysed the interaction between health (categorical) and time (categorical). To account for the nested structure of data, we included the individual locust and date as random intercepts 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. Significances of the fixed effects were determined using Satterthwaite's method for estimation of degrees of freedom (`anova` function, package 'lmerTest' (Kuznetsova *et al*., 2017)) and non-

111 significant interactions (P>0.05) were removed. We retained all main effects even if they were not
112 statistically significant to avoid an increase in the type I error rate (Forstmeier and Schielzeth, 2011).
113 Estimated marginal means and standard errors (Table 1, Supplemental Table 1) were obtained using the
114 'emmeans' package in R.
115

116 RESULTS

117 Within the lumen of infected Malpighian tubules, the cysts of the protozoan *Malpighamoeba locustae*
118 (Amoebidae, Sarcodina) were visible (Figure 1A,B). The wall of infected tubules was thinner and less
119 clearly defined than that of uninfected tubules (Figure 1A–D). Infected tubules were significantly longer
120 and broader with a larger surface area than uninfected tubules (Supplemental Table 1A; Figure 1E–G).
121 The rhythmic movements of the tubules were also slower and less pronounced in infected compared
122 with uninfected locusts (Supplemental Video 1,2).
123 Fluid secretion rate was significantly higher in infected than in uninfected tubules, but showed
124 a similar decrease with time (Table 1A; Figure 2A). A greater surface area was exposed to the
125 incubation bath in infected compared to their uninfected counterparts (Supplemental Table 1B).
126 Nevertheless, the fluid secretion rate per surface area was near identical in infected and uninfected
127 tubules (Table 1B; Figure 2B). The P-glycoprotein substrate Rhodamine B (Eytan *et al*., 1997) was
128 actively transported into the lumen of the tubules (Figure 2C) and its concentration in the droplets
129 secreted increased significantly during incubation in both infected and uninfected tubules, but this
130 increase depended upon the infection status: the increase in rhodamine B concentration was more
131 pronounced in droplets collected from uninfected tubules than in droplets from infected tubules (Table
132 1C; Figure 2D; Supplemental Figure 2). The rhodamine B net extrusion rate per unit of surface area
133 (moles extruded / (time × area)) was higher in uninfected than in infected tubules; although it increased
134 during the incubation in both infected and uninfected tubules, this increase over time was more
135 pronounced in uninfected tubules (Table 1D; Figure 2E). Accounting for the larger surface area of
136 infected tubules, however, there was no significant difference in the total extrusion per tubule (Table
137 1E; Figure 2F).
138

139 DISCUSSION

140 By comparing xenobiotic detoxification in infected and uninfected gregarious desert locusts, we
141 determined how *Malpighamoeba* infection affects the performance of insect Malpighian tubules. In
142 doing so, our results directly quantify the impact of a common pathogen upon major components of
143 insect osmoregulation and xenobiotic detoxification, namely the Malpighian tubules and the P-
144 glycoproteins they express. Infected tubules were longer, wider, had a greater surface area, and showed
145 less pronounced and slower rhythmic movements than uninfected tubules (Supplemental Video 1,2).
146 We assessed two aspects of tubule performance; fluid secretion and net extrusion of rhodamine B, a

147 substrate for P-glycoproteins (Eytan *et al*., 1997). Infected tubules secreted more fluid per time than
148 uninfected ones. Conversely, the net extrusion of rhodamine B per surface area was reduced in infected
149 compared with uninfected tubules. The total net extrusion per tubule, however, was similar in both
150 infected and uninfected tubules.

151 The greater fluid secretion rate of the infected tubules may be a consequence of their greater
152 surface area. The tubule epithelium expresses transporters that move Na^+, K^+, and Cl^- ions into the
153 lumen followed by the passive movement of water (O'Donnell, 2008), so greater surface area may
154 permit increased ion and water movement. The transepithelial water movement is transcellular, through
155 aquaporin channels located both on the basolateral and apical epithelial membranes (O'Donnell, 2008).
156 The greater fluid secretion rate decreases the luminal concentration of rhodamine B and may increase
157 its net extrusion by preventing back diffusion in the bath solution. In uninfected tubules the fluid
158 secretion rate positively correlates with the tubule surface area, which in turn correlates with the net
159 extrusion of rhodamine B (Rossi *et al*., 2019). However, it is possible that loss of the brush border in
160 infected tubules compromises the septate junctions between epithelial cells (Beyenbach *et al*., 2010)
161 promoting water movement and increasing fluid secretion.

162 Trans-tubular rhodamine B transport is predominantly dependent on P-glycoprotein
163 transporters in the brush border of the tubule epithelial cells (Murray, 1996; Ugwu *et al*., 2016). The
164 decreased net extrusion of rhodamine B per tubule surface area in infected tubules may therefore reflect
165 a reduced density of P-glycoproteins caused by the destruction of the brush border by the
166 *Malpighamoeba* trophozoites (King and Taylor, 1936; Liu, 1985). This reduced P-glycoprotein density
167 in infected tubules may be compensated by their greater total surface area compared to uninfected
168 tubules. The overall consequence of the reduced net extrusion coupled with the increased fluid secretion
169 rate is that the total net extrusion per tubule is similar in infected and uninfected tubules. This is
170 consistent with the finding that infection with *Malpighamoeba* does not increase the susceptibility of
171 grasshoppers (*Melanoplus sanguinipes*) to the insecticide cypermethrin, a P-glycoprotein substrate
172 (Hinks and Ewen, 1986; Sreeramulu *et al*., 2007).

173 Changes in feeding behaviour may also contribute to the ability of insects to compensate for
174 *Malpighamoeba* impairment of P-glycoprotein detoxification; infection reduces the amount of food that
175 locusts eat (M. Rossi pers. obs.; King and Taylor, 1936). Reducing food intake may reduce the exposure
176 of infected insects to noxious substances that their compromised detoxification pathways cannot
177 remove. This may be particularly important for generalist herbivores like desert locusts that feed on a
178 broad variety of plant species, many of which contain toxins (Bernays and Chapman, 1978).
179 Alternatively, reduced food intake in infected locusts may be driven by the activation of the immune
180 system. Both their immune system and their ability to detoxify noxious food substances rely on
181 pathways that involve the antioxidant glutathione (Habig *et al*., 1974; Stahlschmidt *et al*., 2015; Jeschke
182 *et al*., 2016). Consequently, competition for glutathione between these two physiological pathways
183 could lead to impaired detoxification when the immune system is already activated (McMillan *et al*.,

184 2018). Whether due to a direct reduction in feeding or as an indirect consequence of a trade-off with
185 the immune response, reduced food intake lowers the risk of ingesting toxins but also prevents locusts
186 acquiring water and nutrients.

187 A reduced food intake combined with an increased fluid secretion rate poses infected locusts a
188 difficult challenge. In adaptation to the dry and hot environment that they occupy, desert locusts must
189 balance their water secretion to avoid dehydration (Phillips, 1964a). The primary urine secreted by the
190 Malpighian tubules is isosmotic and the composition must be adjusted prior to excretion (O'Donnell,
191 2008). Most of the filtered water, ions and metabolites are reabsorbed in the anterior hindgut and in the
192 rectum (O'Donnell, 2008). The reabsorption of Na^+, K^+, Cl^- ions and hence water is an active process
193 consuming metabolic energy (Phillips, 1964a,b). To prevent desiccation, locusts infected with
194 *Malpighamoeba* would need to enhance the water reabsorption to counterbalance the increased fluid
195 secretion rate from the Malpighian tubules. Thus, infected locusts may be subjected to the increased
196 energy costs of reabsorbing the excess of water and ions transported into the lumen tubule. Thus,
197 *Malpighamoeba* infection may be highly debilitating for desert locusts in their natural environment,
198 imposing stresses in terms of water, ions, and energy even if they can avoid ingesting plant toxins.

199 *Malpighamoeba* infections are not limited to locusts and grasshoppers, occurring in other
200 insects including honeybees (Bailey, 1968). Although the exact details of *Malpighamoeba* infection
201 likely depend upon the specific host and pathogen species combination, honeybee Malpighian tubules
202 infected with *Malpighamoeba mellificae* show striking similarities to locust tubules infected with
203 *Malpighamoeba locustae*. In both cases, the lumen of the tubules is swollen and packed with cysts, the
204 epithelium thins, and the brush border of the epithelial cells is destroyed (King and Taylor, 1936; Bailey,
205 1968; Harry and Finlayson, 1975; Liu, 1985). Such similarities in tubule pathology despite the
206 separation of the insect hosts by approximately 380 million years (Misof *et al*., 2014) suggests that
207 *Malpighamoeba* infection may have similar consequences in other insects susceptible to infection.
208 Moreover, infection in honeybees and other insects may similarly compromise detoxification of
209 xenobiotic compounds and increase water and ion loss. Even though xenobiotic compounds may be
210 removed from insect haemolymph by infected tubules, our results show a substantial increase in fluid
211 secretion by infected tubules, which implies greater energy costs for reabsorption in the insect hind gut.
212 Such impacts are highly injurious and can lead to the premature death of the insect.
213

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216 **Data accessibility**
217 All supporting data can be found in the electronic supplementary material, tables.

218 **Authors' contributions**

M.R. and J.E.N. designed the experiments and data analysis and wrote the paper. M.R. performed the experiments and data analysis. S.R.O. provided the infected locusts, advised on data analysis and contributed to writing the manuscript.

Competing interests

The authors declare no competing or financial interests.

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Ethics statement

There are no ethical considerations.

Supplementary information

Supplementary information available online.

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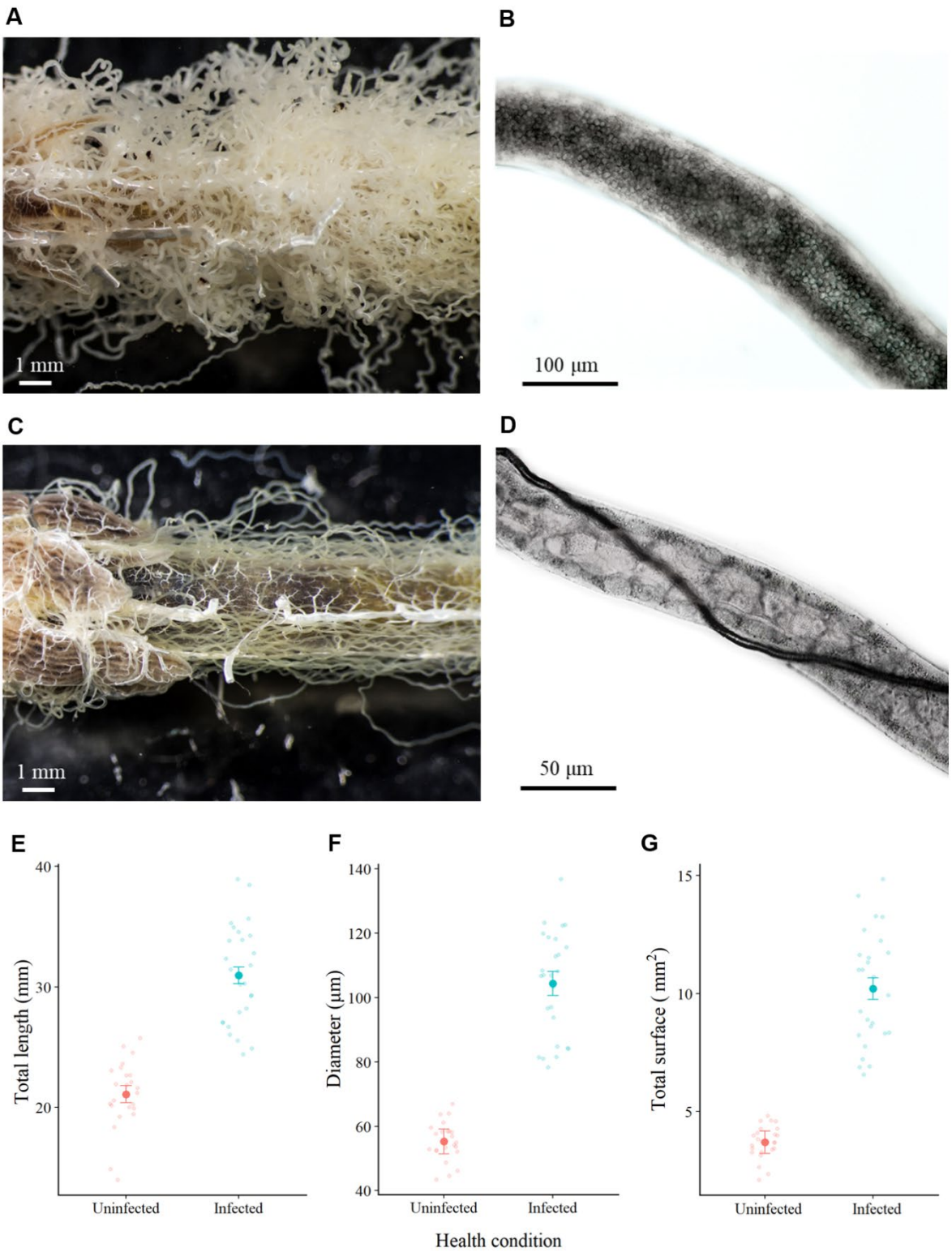

Figure 1. The Malpighian tubules of Malpighamoeba-infected locusts differ from those of uninfected locusts in appearance, size, and surface area. (A) Infected tubules are swollen and cloudy. (B) The lumen of infected tubules is filled with cysts. (C) Uninfected tubules are thinner and more transparent. (D) The lumen of uninfected tubules is free from cysts. A tracheole (dark line) wraps around the uninfected tubule. Infected locusts had longer (E) and wider (F) tubules than uninfected locusts. (G) Consequently, the surface area of infected tubules was

greater than that of uninfected tubules. Large filled circles with error bars represent mean estimates from mixed effect model fits with the standard error. Small circles represent the raw data.

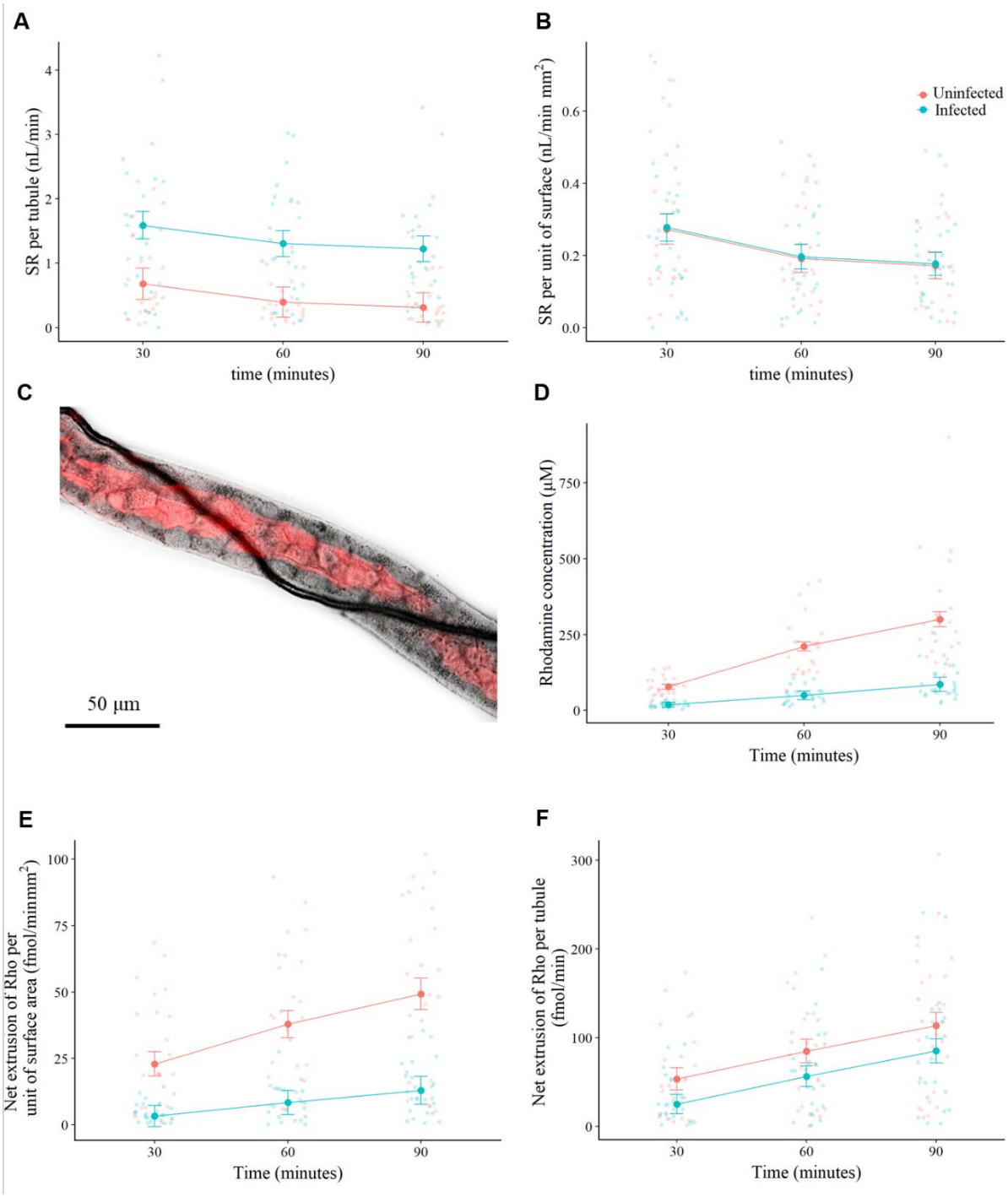

Figure 2. Malpighamoeba infection affects the fluid secretion rate and rhodamine B extrusion of Malpighian tubules. Infected tubules secreted more fluid than the uninfected ones (A), whereas the secretion rate (SR) per unit of surface area did not differ between infected and uninfected Malpighian tubules (B). (C) The uninfected tubule shown in 1D transporting rhodamine B. (D) The rhodamine B concentration of the droplets secreted increased over time. Uninfected tubules secreted more concentrated droplets than the infected tubules. (E,F) The net extrusion rate of rhodamine B increased over time. The net extrusion rate per unit of surface area was higher in the healthy tubules than in the infected ones (E), however,

considering the whole surface area, the net rhodamine B extrusion rate per tubule was similar in healthy and infected tubules (F). Large filled circles with error bars represent means estimates from mixed effect model fits with the standard error. Small circles represent the raw data.

333 **Table 1.** ANOVA results and estimated marginal means of fluid secretion rate per tubule (A) and per unit of surface area (B); rhodamine B
334 concentration of the droplet secreted (C), and net extrusion of rhodamine B per unit of surface area (D) and per tubule (E) for infected and
335 uninfected tubules at each time point of observation.

			Type II ANOVA (Satterthwaite's method)					Group means ± s.e. (model estimates)		
			num. df	den. df	F value	p		30 min	60 min	90 min
A	Fluid secretion rate per tubule (nL/min)	Time:	2	53.05	8.32	**<.001**	Uninfected	0.65 ± 0.30	0.36 ± 0.29	0.28 ± 0.29
		Health:	1	11.67	9.11	**0.011**	Infected	1.62 ± 0.25	1.34 ± 0.24	1.26 ± 0.24
B	Fluid secretion rate per unit of surface (nL/min mm^2)	Time:	2	53.91	14.49	**<.001**	Uninfected	0.27 ± 0.05	0.19 ± 0.05	0.17 ± 0.05
		Health:	1	10.33	0.07	0.803	Infected	0.28 ± 0.05	0.20 ± 0.04	0.18 ± 0.04
C	Rhodamine concentration (μM)	Time x Health:	2	62.03	16.21	**<.001**	Uninfected	77.6 ± 11.0	210.0 ± 16.7	299.9 ± 26.0
							Infected	19.2 ± 9.4	50.1 ± 15.3	86.7 ± 24.7
D	Net rhodamine extrusion per unit of surface (fmol/min mm^2)	Time x Health:	2	52.47	8.13	**<.001**	Uninfected	23.0 ± 5.4	38.0 ± 5.8	49.4 ± 6.5
							Infected	3.0 ± 4.5	8.1 ± 5.0	12.6 ± 5.7
E	Net extrusion of rhodamine per tubule (fmol/min)	Time:	2	51.72	31.62	**<.001**	Uninfected	53.7 ± 14.8	85.1 ± 15.6	113.7 ± 17.1
		Health:	1	12.78	3.04	0.105	Infected	24.6 ± 12.3	55.9 ± 13.3	84.5 ± 15.0

336