Formicine ants swallow their highly acidic poison for gut microbial selection and control

Simon Tragust[1†*], Claudia Herrmann[1], Jane Häfner[1], Ronja Braasch[1], Christina Tilgen[1], Maria Hoock[1], Margarita Artemis Milidakis[1], Roy Gross[2], Heike Feldhaar[1]

[1] Animal Ecology I, Bayreuth Center for Ecology and Environmental Research (BayCEER), University of Bayreuth, Universitätsstraße 30, 95447 Bayreuth, Germany

[2] Microbiology, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg

[†] Present address: General Zoology, Hoher Weg 8, Martin-Luther University, 06120 Halle (Saale), Germany

[*] Correspondence to: simon.tragust@zoologie.uni-halle.de

Abstract

Animals continuously encounter microorganisms that are essential for health or cause disease. They are thus challenged to control harmful microbes while allowing acquisition of beneficial microbes, a challenge that is likely especially important concerning microbes in food and in animals such as social insects that exchange food among colony members. Here we show that formicine ants actively swallow their antimicrobial, highly acidic poison gland secretions after feeding. The ensuing creation of an acidic environment in the stomach, the crop, improves individual survival in the face of pathogen contaminated food and limits disease transmission during mutual food exchange. At the same time, crop acidification selectively allows acquisition and colonization by known bacterial gut associates. The results of our study suggest that swallowing of acidic poison gland secretions acts as a microbial filter in formicine ants and indicate a potentially widespread but so far underappreciated dual role of antimicrobials in host-microbe interactions.

Introduction

Animals commonly harbor gut associated microbial communities (Engel and Moran, 2013, Moran et al., 2019). Patterns of recurring gut microbial communities have been described for many animal groups (Brune and Dietrich, 2015, Kwong et al., 2017, Ochman et al., 2010). The processes generating these patterns are however often not well understood. They might result from host filtering (Mazel et al., 2018), codiversification between gut associated microbes and their hosts (Moeller et al., 2016) or simply be the result of similar dietary preferences (Anderson et al., 2012, Hammer et al., 2017).

Food is an important environmental source of bacterial gut associates (Blum et al., 2013, Broderick and Lemaitre, 2012, David et al., 2014, Hammer et al., 2017, Perez-Cobas et al.,

2015) but also poses a challenge, the need to discriminate between harmful and beneficial microbes, as food may contain microbes that produce toxic chemicals or that are pathogenic (Burkepile et al., 2006, Demain and Fang, 2000, Janzen, 1977, Trienens et al., 2010). In social animals, control of harmful microbes in food while at the same time allowing the acquisition and transmission of beneficial microbes from and with food, is likely especially important. Eusocial Hymenoptera not only transport and store food in their stomach, the crop, but also distribute food to members of their colony via trophallaxis, i.e. the regurgitation of crop content from donor individuals to receiver individuals through mouth-to-mouth feeding (Gernat et al., 2018, Greenwald et al., 2018, LeBoeuf et al., 2016). While trophallaxis can facilitate the transmission of beneficial microbes, from an epidemiological perspective it can also entail significant costs, as it might open the door to unwanted microbial opportunists and pathogens that can take advantage of these transmission routes (Onchuru et al., 2018, Salem et al., 2015).

Here we investigate how formicine ants, specifically the Florida carpenter ant *Camponotus floridanus*, solve the challenge to control harmful microbes in their food while allowing acquisition and transmission of beneficial microbes from and with their food. Apart from specialized intracellular endosymbionts associated with the midgut in the ant tribe Camponotini (Degnan et al., 2004, Feldhaar et al., 2007, Russell et al., 2017, Williams and Wernegreen, 2015), formicine ant species have only low abundances of microbial associates in their gut lumen but carry members of the bacterial family Acetobacteracea as a major part of their gut microbiota (Brown and Wernegreen, 2016, Chua et al., 2018, He et al., 2011, Ivens et al., 2018). Some formicine gut associated Acetobacteracea show signs of genomic and metabolic adaptations to their host environment indicating coevolution (Brown and Wernegreen, 2019). But the recurrent presence of Acetobacteracea in the gut of formicine ants potentially also reflects direct transmission of bacteria among individuals, selective uptake on

61 the part of the ants, specific adaptation for colonizing ant guts on the part of the bacteria, or
62 some combination of all three (Engel and Moran, 2013).

63 Generally, the immune system together with physiochemical properties of the gut
64 environment maintains the homeostasis between gut associated microbes and the host (Chu
65 and Mazmanian, 2013, McFall-Ngai et al., 2013, Rakoff-Nahoum et al., 2004, Slack et al.,
66 2009, Watnick and Jugder, 2020, Xiao et al., 2019, see also Foster et al., 2017). Highly acidic
67 stomach lumens are ubiquitous in higher vertebrates, including amphibians, reptiles, birds and
68 mammals (Beasley et al., 2015, Koelz, 1992), while in insects, acidic regions have rarely been
69 described so far from midgut regions (Chapman, 2013, Holtof et al., 2019). However in both,
70 higher vertebrates, and the fruit fly *Drosophila melanogaster*, acidic gut compartments
71 together with the immune system serve microbial control and prevent infection by pathogens
72 (Giannella et al., 1972, Howden and Hunt, 1987, Martinsen et al., 2005, Overend et al., 2016,
73 Rakoff-Nahoum et al., 2004, Slack et al., 2009, Tennant et al., 2008, Watnick and Jugder,
74 2020). Formicine ant species possess a highly acidic poison gland secretion containing formic
75 acid that is foremost used as a defensive weapon but is also distributed to the environment of
76 these ants as an external immune defence trait (*sensu* Otti et al., 2014), to protect their
77 offspring and the nest and to limit disease spread within the society (see references in Tragust,
78 2016, Brütsch et al., 2017, Pull et al., 2018). Thereby, ants can take up poison gland
79 secretions from the acidopore, the opening of the poison gland at the gaster tip, into their
80 mouth during a specialized behaviour existing only in a subset of ant families among all
81 Hymenopterans (Basibuyuk and Quicke, 1999, Farish, 1972), termed acidopore grooming
82 (Tragust et al., 2013).

83 Here we first investigate whether poison gland substances are also swallowed during
84 acidopore grooming in *C. floridanus* and seven other formicine ant species from three genera
85 in a comparative survey through measurement of pH levels in the crop and midgut lumen,

experimental manipulation of poison gland access, and behavioural observations. In loss of poison gland function experiments, we then investigate whether analogous to acidic stomachs of higher vertebrates and acidic midgut regions in the fruit fly, swallowing of poison gland substances can serve gut microbial control and prevent bacterial pathogen infection and transmission. Finally, we explore whether swallowing of poison gland substances acts as a microbial filter that is permissible to gut colonization of bacteria from the family Acetobacteracea.

Results and Discussion

To reveal whether poison gland secretions are swallowed during acidopore grooming, we monitored acidity levels in the crop lumen of the Florida carpenter ant *Camponotus floridanus* at different time points after feeding them 10% honey water (pH = 5). We found that over time, the crop lumen became increasingly acidic, reaching highly acidic values 48h after feeding (median pH = 2; 95% CI: 1.5-3.4), whilst renewed access to food after 48h restored the pH to levels recorded after the first feeding trial (Fig. 1a; LMM, LR-test, $\chi^2 = 315.18$, df = 3, $P < 0.001$; Westfall corrected post-hoc comparisons: 0+4h vs. 48h+4h: $P = 0.317$, all other comparisons: $P < 0.001$). This acidification was limited to the crop and did not extend to the midgut (Fig. 1 – figure supplement 1; pH-measurements at four points along the midgut 24h after access to 10% honey-water; mean ± se; midgut position 1 = 5.08 ± 0.18, midgut position 2 = 5.28 ± 0.17, midgut position 3 = 5.43 ± 0.16, midgut position 4 = 5.31 ± 0.19). Prevention of acidopore grooming in *C. floridanus* ants for 24h after feeding resulted in a significantly diminished acidification of the crop lumen (Fig. 1b; LMM, LR-test, $\chi^2 = 44.68$, df = 1, $P < 0.001$), a result that was invariably obtained in a comparative survey across seven formicine ant species (genera: *Camponotus, Lasius* and *Formica*) (Fig. 1c; two-sided Wilcoxon rank sum tests, all comparisons: $P \leq 0.036$). This indicates that after feeding, crop lumens of

111 formicine ants are acidified through swallowing of poison gland secretions during acidopore
112 grooming. Although venomous animals often bear a cost of venom production and express
113 behavioural adaptations to limit venom expenditure (Casewell et al., 2013), *C. floridanus*
114 increases the frequency of acidopore grooming upon ingestion of food but also after ingestion
115 of water (Fig. 1 - figure supplement 2; GLMM, LR-test, $\chi^2 = 33.526$, df = 2, $P < 0.001$;
116 Westfall corrected post-hoc pairwise comparisons, water vs. 10% honey-water: $P = 0.634$,
117 unfed vs water and unfed vs 10% honey-water: $P < 0.001$). This suggests a prophylactic
118 acidification of crop lumens after fluid ingestion, irrespective of the fluids nutritional value.

119 To test whether crop lumen acidification serves microbial control and prevents infection by
120 pathogens, we prevented acidopore grooming in *C. floridanus* ants for 24h after feeding them
121 either honey water contaminated with *Serratia marcescens*, an insect pathogenic bacterium
122 (Grimont and Grimont, 2006), or non-contaminated honey water. We found that acidopore
123 access after pathogen ingestion increased the survival probability of ants (Fig. 2a). The
124 survival of ants prevented from acidopore grooming and fed with pathogen contaminated food
125 was significantly lower than that of non-prevented ants fed with the same food source, the
126 latter not differing in survival to similarly manipulated ants that were fed a non-contaminated
127 food source (COXME, LR-test, $\chi^2 = 20.95$, df = 3, $P = 0.0001$; Westfall corrected post-hoc
128 comparisons: FA - | *Serratia* presence + vs. all other ant groups: $P \leq 0.027$, all other
129 comparisons: $P \geq 0.061$). Food sanitation with antimicrobials that are either self-produced or
130 derived from the environment or symbiotic associations (Otti et al., 2014) is ubiquitous in
131 animals that provision food to their offspring or that store, cultivate, develop or live in food
132 (Cardoza et al., 2006, Currie et al., 1999, Herzner et al., 2013, Herzner and Strohm, 2007,
133 Joop et al., 2014, Milan et al., 2012, Mueller et al., 2005, Scott et al., 2008, Shukla et al.,
134 2018, Vander Wall, 1990, Vogel et al., 2017). The results of our study indicate that formicine
135 ants not only distribute acidic poison gland secretions to the environment as an external

136 immune defence trait (see references in Tragust, 2016, Brütsch et al., 2017, Pull et al., 2018),
137 but also use them to sanitize ingested food.

138 Crop lumen acidification in formicine ants upon ingestion of pathogen contaminated food
139 may not only improve individual survival but might also limit oral disease transmission
140 during food distribution via trophallaxis within a social insect society. To test an immune
141 functional role of crop lumen acidification during trophallaxis, we created two types of donor-
142 receiver ant pairs. Donor ants in both pairs were directly fed *S. marcescens* contaminated
143 food, while receiver ants obtained food only through trophallaxis with their respective donor
144 ants. Receiver ants in both pairs were precluded from crop acidification through blockage of
145 their acidopore opening, while donor ants were blocked in one pair but only sham blocked in
146 the other pair. We found that acidopore blockage *per se* had a significant negative effect on
147 the survival of donor as well as receiver ants (Fig. 2b; COXME, LR-test, $\chi^2 = 66.68$, df = 3, P
148 < 0.001). Importantly however, although receiver ants that obtained food from donors with
149 the ability to acidify their crop lumen died at a higher rate than their respective donor
150 counterparts (hazard ratio: 1.81; Westfall corrected post-hoc comparison: $P < 0.001$) they
151 were approximately only half as likely to die compared to receiver ants that obtained
152 pathogen contaminated food from blocked donors unable to acidify their crop lumen (hazard
153 ratio: 0.56; Westfall corrected post-hoc comparison: $P < 0.001$). Trophallactic behaviour
154 between the two donor-receiver ant pairs was not different (Fig. 2 – figure supplement 1;
155 LMM, LR-test, $\chi^2 = 1.23$, df = 1, $P = 0.268$). Although an antimicrobial activity of formicine
156 ant trophallactic fluids has been linked to the presence of proteins in previous studies
157 (Hamilton et al., 2011, LeBoeuf et al., 2016), the results of our study suggest a major role of
158 crop lumen acidification through the ingestion of poison gland substances. Prophylactic
159 acidification of the crop lumen after feeding in *C. floridanus* might therefore act as an
160 important barrier to disease spread within the colony and alleviate the cost of sharing

pathogen contaminated food (Onchuru et al., 2018, Salem et al., 2015). Together with other parasite defence traits in social insect societies (Cremer et al., 2007, Stroeymeyt et al., 2018), acidification of crop lumens likely effectively counteracts the generally increased risk of pathogen exposure and transmission associated with group-living (Alexander, 1974, Boomsma et al., 2005, Kappeler et al., 2015)

In addition to pathogen control, the acidification of the crop lumen might act as a chemical filter for gut associated microbial communities in formicine ants, similar to gut morphological structures that can act as mechanical filters in ants and other insects (Itoh et al., 2019, Lanan et al., 2016, Ohbayashi et al., 2015). To investigate the idea of a chemical filter, we tested the ability of the pathogenic bacterium *S. marcescens*, and the insect gut associated bacterium *Asaia* sp. (family Acetobacteracea) to withstand acidic environments *in vitro* and *in vivo*. Incubation of *S. marcescens* in 10% honey water acidified with formic acid for 2h resulted in a significantly reduced growth at pH 4 compared to 5, with zero growth at pH-levels less than 4 (Fig. 3 – figure supplement 1a; GLM, LR-test, $\chi^2 = 79.442$, df = 1, $P < 0.001$). Consistent with this, when fed to *C. floridanus*, *S. marcescens* presence decreased sharply over time in the crop (Fig. 3a; GLMM, LR-test, $\chi^2 = 220.78$, df = 4, $P < 0.001$) with the proportion of CFUs at 0.5h post-feeding relative to 0h in the crop diminishing from 48% (median, CI: 0-366%) to 0% at 4h (CI: 0-4%), 24h (CI: 0-2.7%), and 48h (CI: 0-21%) post-feeding. In addition, *S. marcescens* could only be detected at extremely low levels (median: 0%) in the midgut at 0h (CI: 0-5%), 0.5h (CI: 0-1%) and 24h (CI: 0-1%) post-feeding relative to 0h in the crop and not at all at 4h and 48h post-feeding (Fig. 3b; GLMM, LR-test, $\chi^2 = 1.044$, df = 2, $P = 0.593$). Taken together, *in vivo* and *in vitro* tests suggest that crop acidification results in a quick and effective reduction of *S. marcescens* viability in the crop thus preventing further transport to the midgut. The same results were obtained *in vivo* for *E. coli*, a bacterium that is not a gut associate of insects (Blount, 2015) (Fig. 3 – figure supplement 2; crop:

GLMM, LR-test, $\chi^2 = 156.74$, df = 4, $P < 0.001$; midgut: GLMM, LR-test, $\chi^2 = 14.898$, df = 3, $P = 0.002$). In contrast to *S. marcescens*, *Asaia* sp. was able to grow in 10% honey water acidified with formic acid to a pH of 3 for 2h in *in vitro* tests (Fig. 3 – figure supplement 1b; GLM, overall LR-test $\chi^2 = 21.179$, df = 2, $P < 0.001$; Westfall corrected post hoc comparisons: pH = 5 vs. pH = 4: $P = 0.234$, all other comparisons: $P < 0.001$). Moreover, in *in vivo* tests, *Asaia* sp. only gradually diminished over time in the crop (Fig. 3c; GLMM; LR-test, $\chi^2 = 124.01$, df = 4, $P < 0.001$) with 34% (median, CI: 3-85%) and 2% (CI: 0-7%) relative to 0h in the crop still detectable at 4h and 24h post-feeding, respectively. At the same time *Asaia* sp. steadily increased in the midgut (Fig. 3d; GLMM; LR-test, $\chi^2 = 59.94$, df = 3, $P < 0.001$) from its initial absence at 0h post-feeding to 2% (median, CI: 0-5%) relative to 0h in the crop at 48h post-feeding. This indicates that crop lumen acidification is permissible to gut colonization by *Asaia* sp.. Given the ubiquitous presence of crop lumen acidification in our comparative survey of formicine ant species (Fig. 1c) and the gut microbiota structuring properties of acidic gut compartments in humans (Imhann et al., 2016) and fruit flies (Overend et al., 2016), it is likely that host filtering (Mazel et al., 2018) through acidification of crop lumens can explain the recurrent presence of Acetobacteracea in the gut of formicine ants and the otherwise reduced microbial diversity and abundance of gut associated microbes (Brown and Wernegreen, 2016, Chua et al., 2018, Ivens et al., 2018). On the other hand, some formicine gut associated Acetobacteracea show signs of genomic and metabolic adaptations to their host environment (Brown and Wernegreen, 2019), indicating coevolution and potentially also mutual benefit, though this has not formally been established (see also Mushegian and Ebert, 2016). The creation of a challenging gut environment through the ingestion of poison gland substances that is easier to endure if colonizing microbes are mutualists agrees with the theoretical concept of screening, as opposed to signalling, as a means of partner choice in cross-kingdom mutualisms (Archetti et al., 2011a, Archetti et al., 2011b, Biedermann and Kaltenpoth, 2014, Scheuring and Yu, 2012). Experimental evidence for screening is so far

limited in insect-microbe associations (Innocent et al., 2018, Itoh et al., 2019, Ranger et al., 2018), but the results of our study provide support for the prediction that screening is more likely to evolve if a host's challenging environment is derived from defence traits against parasites (Archetti et al., 2011a, Archetti et al., 2011b). Altogether, our study provides evidence that the well-established cross talk between the immune system and gut associated microbes in vertebrates and invertebrates (Chu and Mazmanian, 2013, Rakoff-Nahoum et al., 2004, Slack et al., 2009, Watnick and Jugder, 2020, Xiao et al., 2019) holds for a broader range of immune defence traits (*sensu* Otti et al., 2014) and might be realized not only through signalling but also screening.

Conclusion

Overall our study provides evidence that swallowing of formic acid containing poison gland secretions acts as a chemical filter for microbial selection and control of gut associated microbes, protecting formicine ants from food borne bacterial pathogens and structuring gut associated microbial communities. In ants and other animals that lack acidic poison gland secretions, acids produced by other exocrine glands (Fernández-Marín et al., 2015, Yek and Mueller, 2011) or acidic derivatives produced by defensive symbionts (Florez et al., 2015) or other environmental bacteria (Ratzke and Gore, 2018) might provide functionally similar roles to acidic poison gland secretions, as indicated in bees (Palmer-Young et al., 2018) and termites (Inagaki and Matsuura, 2018). Antimicrobials as external immune defence traits (Otti et al., 2014) may generally not only serve pathogen protection and microbial control but may also act as microbial filters to manage host associated microbes, be it in food or the environment, and thus contribute to a host's ecological and evolutionary success. In the case of social species by alleviating the increased risk of pathogen exposure and transmission

236 associated with group living but allowing the acquisition and transmission of microbial

237 mutualists.

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Methods

Ant species and maintenance. Colonies of the carpenter ant *Camponotus floridanus* were collected in 2001 and 2003 in Florida, USA, housed in Fluon® (Whitford GmbH, Diez, Germany) coated plastic containers with plaster ground and maintained at a constant temperature of 25°C with 70% humidity and a 12h/12h light/dark cycle. They were given water *ad libitum* and were fed three times per week with honey water (1:1 tap water and commercial quality honey), cockroaches (*Blaptica dubia*) and an artificial diet (Bhatkar and Whitcomb, 1971). For comparison, workers of one other *Camponotus* species (*Camponotus maculatus*), collected close to Kibale Forest, Uganda in 2003 and housed under identical conditions as *Camponotus floridanus* were used. Additionally, six other formicine ant species, one *Lasius* and five *Formica* species (*Lasius fuliginosus*, *Formica cinerea, Formica cunicularia, Formica fuscocinerea, Formica pratensis* and *Formica rufibarbis*) were collected in Bayreuth, Germany in 2012 and 2018 and kept for approximately two weeks prior experimental use at 20°C, 70% humidity and a 14h/10h light/dark cycle.

Acidification of crop lumen and pH measurements. To determine whether formicine ants swallow poison gland secretions after feeding, we tracked changes in pH-levels of the crop lumen over time. Before use in experimental settings, cohorts of 50-100 ants were taken out of their natal colony (*C. floridanus*: n = 6 colonies) into small plastic containers lined with Fluon® and starved for 24-48h. Thereafter, ants were put singly into small petri dishes (Ø 55 mm) with damp filter paper covered bottom, given access to a droplet of 10% honey water (w/v) for 2h before removing the food source and measuring the pH of the crop lumen in *C. floridanus* after another 2h (group 0+4h: n = 60 workers), after 24h (group 0+24h: n = 59 workers) or 48h (group 0+48h: n = 52 workers). To assess the effect of renewed feeding, a separate group of *C. floridanus* ants were given access to 10% honey water 48h after the first feeding for 2h prior to measuring the pH of their crop lumen after another 2h (group 48h+4h:

n = 60 workers). To measure the pH, ants were first cold anesthetized on ice, then their gaster was cut off with a fine dissection scissor directly behind the petiole and leaking crop lumen (1-3µl) collected with a capillary (5µl Disposable Micro Pipettes, Blaubrand intraMARK, Brand, Wertheim). The collected crop lumen was then emptied on a pH sensitive paper to assess the pH (Hartenstein, Unitest pH 1-11). We also measured the pH of 10% honey water on pH sensitive paper, which gave invariably pH = 5. In addition, we measured the pH in the crop lumen and at four points in the lumen along the midgut (1st measurement directly behind proventriculus to 4th measurement one mm apical from insertion point of malpighian tubules) of *C. floridanus* workers that were fed 24 h prior to measurements with 10% honey-water. For these measurements worker guts were dissected as a whole and pH was measured in the crop (n = 2 workers from two colonies) and along the midgut (all midgut points n = 10, except point four with n = 9 workers from four different colonies) with a needle-shaped microelectrode for pH measurements (UNISENSE pH-meter; microelectrode with needle tip of 20µm diameter). In formicine ants, oral uptake of poison gland secretions into the mouth is performed via acidopore grooming (Tragust et al., 2013). During this behavior ants bend their gaster forward between the legs and the head down to meet the acidopore, the opening of the poison gland, at the gaster tip (Basibuyuk and Quicke, 1999, Farish, 1972). In an additional experiment we therefore compared the crop lumen pH of *C. floridanus* workers from four different colonies that were either prevented to reach their acidopore (FA- ants) or could reach their acidopore freely (FA+ ants). To do this, we again allowed single ants access to 10% honey water for 2h after a starvation period, before cold anesthetizing them briefly on ice and immobilizing FA- ants (n = 22 workers) in a pipetting tip, while FA+ ants (n = 23 workers) remained un-manipulated. After 24h we measured the pH of the crop lumen as before. To investigate whether crop lumen acidification is widespread among formicine ants, the latter experiment was repeated for six additional formicine ant species (FA- ants: n = 10 workers except for *Formica pratensis* with n = 21; FA+ ants: n = 10 workers except for *Formica*

290 *pratensis* with n=20; all ants: n = 1 colony) in the same fashion as described before with the
291 exception that apart from *Formica pratensis* the crop lumen was collected through the mouth
292 by gently pressing the ants' gaster. Crop lumen of *Formica pratensis* ants was collected in the
293 same fashion as crop lumen of *C. floridanus* ants.

294 **Bacterial strains and culture.** As model entomopathogenic bacterium *Serratia marcescens*
295 DSM12481 (DSMZ Braunschweig, Germany) was used. This bacterium is pathogenic in a
296 range of insects (Grimont and Grimont, 2006) and has been detected in formicine ants, i.e.
297 *Anoplolepis gracilipes* (Cooling et al., 2018) and *Camponotus floridanus* (Ratzka et al.,
298 2011). While often non-lethal within the digestive tract, *S. marcescens* can cross the insect gut
299 wall (Mirabito and Rosengaus, 2016, Nehme et al., 2007) and is highly virulent upon entry
300 into the hemocoel (Flyg et al., 1980), not least due to the production of bacterial toxins
301 (Hertle, 2005). As a model bacterial gut-associate of ants *Asaia* sp. strain SF2.1 (Favia et al.,
302 2007), was used. *Asaia* sp. belongs to the family Acetobacteracea, members of which often
303 thrive in sugar-rich environments (Mamlouk and Gullo, 2013), such as honey-dew that ants
304 like *C. floridanus* predominantly feed on. *Asaia* sp. is capable of cross-colonizing insects of
305 phylogenetically distant genera and orders (Crotti et al., 2009, Favia et al., 2007) and can be a
306 component of the gut associated microbial community of formicine ants (Chua et al., 2018,
307 Kautz et al., 2013a, Kautz et al., 2013b). In addition to *S. marcescens* and *Asaia sp.*,
308 *Escherichia coli* DSM6897 (DSMZ Braunschweig, Germany) was used as a model bacterium
309 that is not a gut-associate of insects. *E. coli* bacteria are a principal constituent of mammalian
310 gut associated microbial communities but are commonly also found in the environment
311 (Blount, 2015).

312 Bacterial stocks of *S. marcescens*, *Asaia* sp., and *E. coli* were kept in 25% glycerol at -80°C
313 until use. For use, bacteria were plated on agar plates (LB-medium: 10g Tryptone, 5g Yeast
314 extract, 20g Agar in 1L MilliQ-water, and GLY-medium: 25g Gycerol, 10g Yeast extract, 20g

Agar in 1L MilliQ-water with pH adjusted to 5.0, for *S. marcescens/E. coli* and *Asaia* sp. respectively), single colony forming units (CFUs) were picked after 24h (*S. marcescens/E. coli*) or 48h (*Asaia* sp.) of growth at 30°C and transferred to 5ml liquid medium (LB-medium and GLY-medium minus agar for *S. marcescens/E. coli* and *Asaia* sp. respectively) for an overnight culture (24h) at 30°C. The overnight culture was then pelleted by centrifugation at 3000g, the medium discarded and resolved in 10% (w/v) honey water to the respective working concentration for the experiments. The concentration of a typical overnight culture was determined for *S. marcescens* and *Asaia* sp. by plating part of the overnight culture on agar plates and counting CFUs after 24h or 48h of growth at 30°C, for *S. marcescens* and *Asaia* sp. respectively. This yielded a concentration of $1.865 * 10^9 \pm 5.63 * 10^7$ (mean ± sd) bacteria per ml for *S. marcescens* and $5.13 * 10^8 \pm 8.48 * 10^6$ (mean ± sd) bacteria for *Asaia* sp.

Survival experiments. In a first survival experiment we tested whether the ability to perform acidopore grooming within the first 24h after ingestion of pathogen contaminated food provides a survival benefit for individual *C. floridanus* ants. Ants from eight colonies were starved as described before and single workers in small petri dishes were then either given access to 5µl of *S. marcescens* contaminated 10% honey water ($9.33 * 10^9$ bacteria/ml; *Serratia*+ ants: n = 127) or uncontaminated 10% honey water (*Serratia*- ants: n = 135) for 2 min. Thereafter, all ants were cold anaesthetized and approximately half of the *Serratia*+ and the *Serratia*- ants (n = 65 and n = 69, respectively) immobilized in a pipetting tip, thus preventing acidopore grooming (FA- ants: n = 134) while the other half remained fully mobile (FA+ ants: n = 128). After 24h, FA- ants were freed from the pipetting tip to minimize stress. Mortality of the ants was monitored over 5 days (120h) every 12h.

In an additional survival experiment, we investigated whether the acidification of the crop lumen has the potential to limit oral disease transmission during trophallactic food transfer.

To this end *C. floridanus* ants from seven colonies were again starved, divided randomly in two groups (donor and receiver ants, each n = 322) and their gaster marked with one of two colours (Edding 751). Additionally, to prevent uptake of poison gland secretion, the acidopore opening of all receiver ants (receiver FA-) and half of the donor ants (donor FA-) was sealed with superglue, while the other half of the donor ants were sham treated (donor FA+) with a droplet of superglue on their gaster (Tragust et al., 2013). We then paired these ants into two different donor-receiver ant pairs. Pairs with both donor and receiver ants having their acidopore sealed (donor FA- | receiver FA-) and pairs with only receiver ants having their acidopore sealed (donor FA+ | receiver FA-). Six hours after pairing, donor ants from both pairs were isolated and given access to 5µl of *S. marcescens* contaminated 10% honey water ($1.865 * 10^9$ bacteria/ml) for 12h. Thereafter donor ants were again paired with the respective receiver ants for 12 h and all pairs filmed for the first 30min. (Logitech webcam c910). These videos were then analyzed for the duration of trophallaxis events donor-receiver ant pairs engaged in. After this first feeding round, donor ants were fed in the same fashion, i.e. isolation for 12h with access to *S. marcescens* contaminated 10% honey water, every 48h, while they were maintained with the respective receiver ants for the rest of the time. This experimental design ensured that receiver ants were fed only through the respective donor ants with pathogen contaminated food. Survival of both, donor and receiver ants, was monitored daily for a total of 12 days.

Bacterial growth assays. We tested the ability of *S. marcescens* and *Asaia* sp. to withstand acidic environments in the crop *in vitro* and *in vivo*, as well as their ability and the ability of *E. coli* to pass from the crop to the midgut *in vivo*. In ants, gut morphological structures, i.e. the infrabuccal pocket, an invagination of the hypopharynx in the oral cavity (Eisner and Happ, 1962), and the proventriculus, a valve that mechanically restricts passage of fluids from the crop to the midgut (Eisner and Wilson, 1952), consecutively filter solid particles down to

365 2µm (Lanan et al., 2016) which would allow *S. marcescens* (Ø: 0.5-0.8µm, length: 0.9-2µm,
366 Grimont and Grimont, 2006), *Asaia* sp. (Ø: 0.4-1µm, length: 0.8-2.5µm, (Komagata et al.,
367 2014), and *E. coli* (length: 1µm, width: 0.35µm, Blount, 2015) to pass. For the *in vitro* tests
368 we incubated a diluted bacterial overnight culture (10^5 and 10^4 CFU/ml for *S. marcescens* and
369 *Asaia* sp., respectively) in 10% honey water (pH = 5) and in 10% honey water acidified with
370 commercial formic acid to a pH of 4, 3 or 2 for 2h at room temperature (*S. marcescens*: n = 15
371 for all pH-levels, except pH = 4 with n = 13; *Asaia* sp.: n = 10). Then we plated 100µl of the
372 bacterial solutions on agar-medium (LB-medium and GLY-medium for *S. marcescens* and
373 *Asaia* sp., respectively) and incubated them at 30°C for 24h (*S. marcescens*) or 48h (*Asaia*
374 sp.) before counting the number of formed CFUs. For the *in vivo* tests *C. floridanus* ants from
375 five (*Asaia* sp.), four (*E. coli*) or from six colonies (*S. marcescens*) were starved as before and
376 then individually given access to 5µl of bacteria contaminated 10% honey water (*Asaia* sp.
377 and *E. coli*: $1 * 10^7$ CFU/ml, *S. marcescens*: $1 * 10^6$ CFU/ml) for 2 min. To assess the number
378 of CFUs in the digestive tract, i.e. the crop and the midgut, ants were dissected either directly
379 after feeding (0h; *S. marcescens*: n = 60 workers; *Asaia* sp. and *E. coli*: n = 15 each), 0.5h (*S.*
380 *marcescens*: n = 60; *Asaia* sp. and *E. coli*: n = 15 each), 4h (*S. marcescens*: n = 60; *Asaia* sp.
381 and *E. coli*: n = 15 each), 24h (*S. marcescens*: n = 53; *Asaia* sp. and *E. coli*: n = 15 each) or
382 48h (*S. marcescens*: n = 19; *Asaia* sp. and *E. coli*: n = 15 each) after feeding. These timepoints
383 were chosen according to literature describing peak passage of food from the crop to the
384 midgut within 20h after food consumption in ants (Gösswald and Kloft, 1960, Howard and
385 Tschinkel, 1981). For dissection, ants were cold anesthetized, the gaster opened and the whole
386 gut detached. The crop and the midgut were then separated from the digestive tract, placed in
387 a reaction tube, mechanically crushed with a sterile pestle and dissolved in 100µl (*Asaia* sp.
388 and *E. coli*) or 150µl (*S. marcescens*) phosphate buffered saline (PBS-buffer: 8.74g NaCl,
389 1.78g Na_2HPO_4,$2H_2O$ in 1L MilliQ-water adjusted to a pH of 6.5). The resulting solutions
390 were then thoroughly mixed, 100µl streaked on agar-medium (LB-medium and GLY-medium

for *S. marcescens*/*E.coli* and *Asaia* sp., respectively) and incubated at 30°C for 24h (*S. marcescens* and *E. coli*) or 48h (*Asaia* sp.), before counting the number of formed CFUs. No other bacteria (e.g. resident microbes) were growing on the agar plates which agrees with the very low number of cultivable resident bacteria present in the midgut of *C. floridanus* (Stoll and Gross, unpublished results).

Statistical analyses. All statistical analyses were performed with the R statistical programming language (version 3.6.1, R Core Team, 2019). All (zero-inflated) General(ized) linear and mixed models and Cox mixed-effects models were compared to null (intercept only) or reduced models (for those with multiple predictors) using Likelihood Ratio (LR) tests to assess the significance of predictors. Pairwise comparisons between factor levels of a significant predictor were performed using pairwise post-hoc tests adjusting the family-wise error rate according to the method of Westfall (package "multcomp", Bretz et al., 2011). We checked necessary model assumptions of (zero-inflated) General(ised) linear and mixed models using model diagnostic tests and plots implemented in the package "DHARMa" (Hartig, 2019). Acidification of the crop lumen (log transformed pH to normalize data) and midgut lumen in *C. floridanus* was analyzed using linear mixed models (LMM, package "lme4", Bates et al., 2015) including time since feeding (four levels: 0+4h, 0+24h, 0+48h, 48h+4h; Fig. 1a), ant manipulation (two levels: FA+ and FA-, i.e. ants with and without acidopore access; Fig. 1b) or digestive tract part (four levels: crop, midgut position 1, midgut position 2, midgut position 3, midgut position 4; Fig.1 – supplementary figure 1) as predictors and natal colony as a random effect. Due to non-normality and heteroscedasticity, the acidification of crop lumen in the seven formicine ant species other than *C. floridanus* (Fig. 1c) was analysed using per species Wilcoxon Rank Sum tests with ant manipulation (FA+ and FA-) as predictor. The frequency of acidopore grooming in *C. floridanus* upon feeding different types of fluids was analyzed using Generalized linear mixed models (GLMM,

package "lme4", Bates et al., 2015) with negative binomial errors and type of fluid (three levels: unfed, water-fed or 10% honey water fed) as predictor and natal colony as random effect (Fig. 1 – supplementary figure 2).

Survival data were analysed with Cox mixed effects models (COXME, package "coxme", Therneau, 2019). For the survival of individual ants (Fig. 2a), ant treatment (four levels: *Serratia*- | FA-, *Serratia*- | FA+, *Serratia*+ | FA-, *Serratia*+ | FA+) was added as a predictor and the three "blocks", in which the experiment was run and the colony, ants originated from, were included as two random intercept effects. For the survival of donor-receiver ant pairs (Fig. 2b), ant treatment (four levels: donor FA+, donor FA-, receiver FA+, receiver FA-) was included as a predictor and the three "blocks", in which the experiment was run, the colony, ants originated from, and petri dish, in which donor and receiver ants were paired, were included as three random intercept effects. Survival of receiver ants was right censored if the corresponding donor ant died at the next feeding bout (right censoring of both donor and receiver ants in one pair upon death of one of the ants yielded statistically the same result: COXME, overall LR $\chi^2 = 60.202$, df = 3, $P < 0.001$; post-hoc comparisons: receiver FA- vs donor FA-: $P = 0.388$, all other comparisons: $P < 0.001$). The duration of trophallaxis events (square-root transformed to normalize data) between donor-receiver ant pairs was analysed using a linear mixed model with ant pair type (two levels: donor FA+ | receiver FA- and donor FA- | receiver FA-) as predictor and the three "blocks", in which the experiment was run and the colony ants originated from as random effect (Fig. 2 - supplementary figure 1).

Bacterial growth *in vitro* was analysed separately for *Asaia sp.* and *S. marcescens* using Generalized linear models (GLM) with negative binomial errors and pH as predictor, excluding pH levels with zero bacterial growth due to complete data separation (Fig. 3 - supplementary figure 1). Relative values shown in Fig. 3 -supplementary figure 1 were calculated by dividing single CFU numbers through the mean of CFU numbers at pH 5.

441 Bacterial growth *in vivo* within the digestive tract of *C. floridanus* over time was analysed
442 separately for the crop and midgut for both *Asaia sp.* and *S. marcescens* (Fig. 3) and for *E.*
443 *coli* (Fig. 3 – figure supplement 2). Zero-inflated generalized linear mixed models with
444 negative binomial errors (package "glmmTMB", Brooks et al., 2017) were used to model
445 CFU number, with time after feeding as fixed predictor and ant colony as random effect,
446 except for *E. coli* growth in the crop were colony was included as fixed factor as the model
447 did not converge with colony as a random factor. Time points with zero bacterial growth were
448 again excluded from the model. Relative values shown in Fig. 3 and Fig. 3 – supplementary
449 figure 2 and reported in the main text were calculated by dividing single CFU numbers
450 through the mean of CFU numbers at timepoint 0h in the crop.

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Author contributions

S.T. and H.F. conceived the experiments. S.T. and M.A.M. performed the survival assays and the behavioral observations. C. H., C. T., R. B. and J. H. measured crop lumen acidification. H. F. measured pH in the midgut. M. H. and C. H. performed *in vivo* bacterial growth measurements. C. T. performed the *in vitro* bacterial growth measurements. S.T. analyzed the data and prepared the manuscript. S.T., C.H., J.H., R.B., C.T., M.H., M.A.M., R.G. and H.F. edited the manuscript.

Competing interests

The authors declare no competing interests.

Data and code availability

The authors declare that all data supporting the findings of this study and that all code required to reproduce the analyses and figures of this study are available within the article and its supplementary information and will be made publicly available at a digital repository upon acceptance.

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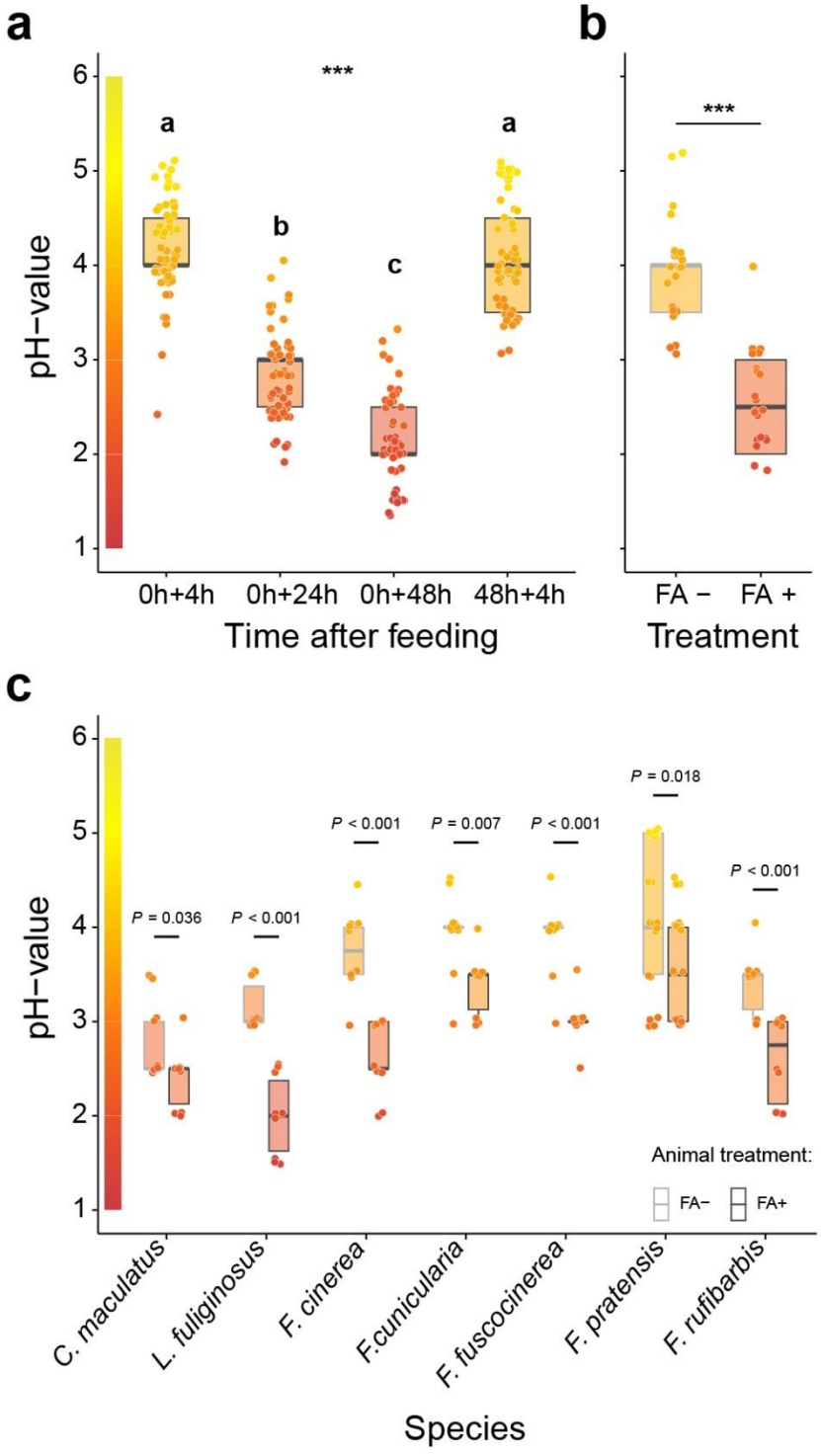


Fig. 1. Acidification of crop lumen through swallowing of acidic poison gland secretions. **a**, pH of crop lumens at 4h, 24h and 48h after feeding *C. floridanus* ants 10% honey water (pH = 5) at 0h and at 4h after re-feeding ants at 48h (LMM, LR-test, $\chi^2 = 315.18$, df = 3, *** $P < 0.001$, same letters indicate $P = 0.317$ and different letters indicate $P < 0.001$ in Westfall corrected post hoc comparisons). **b**, pH of crop lumens in *C. floridanus* ants that were either

prevented to ingest formic acid containing poison gland secretions (FA-) or not (FA+) for 24h after feeding (LMM, LR-test, $\chi^2 = 44.68$, df = 1, $^{***}P < 0.001$). **c**, pH-value of crop lumens 24h after feeding in seven formicine ant species that were either prevented to ingest formic acid containing poison gland secretions (FA-) or not (FA+). Wilcoxon rank sum tests (two-sided). Lines and shaded boxes show median and interquartile range; points show all data. Colours in shaded boxes and points correspond to universal indicator pH colours. Border of shaded boxes represents animal treatment (light grey: prevention of poison ingestion, FA-; dark grey: poison ingestion not prevented, FA+).

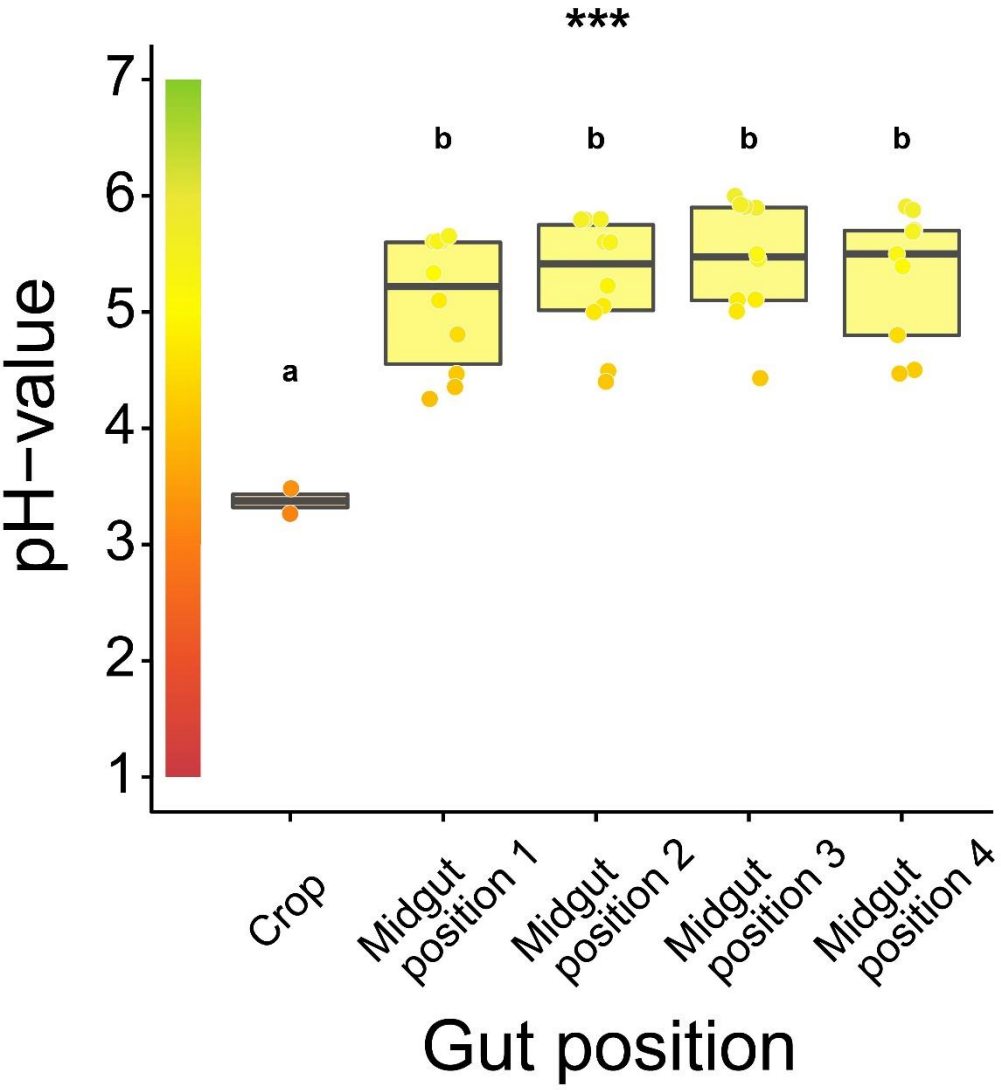
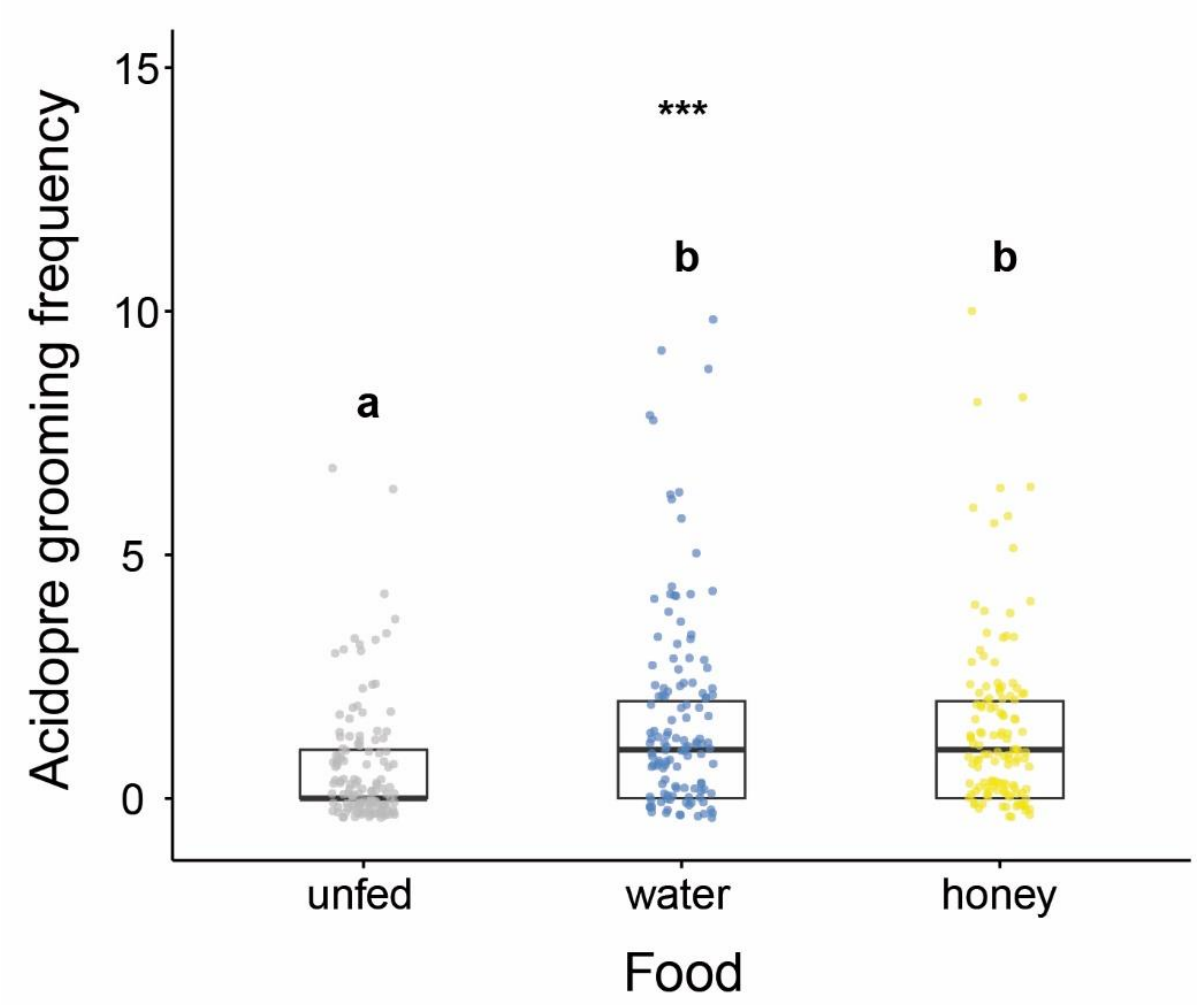


Fig. 1 – figure supplement 1: Acidification along the gastrointestinal tract of *C. floridanus*. pH-measurements 24h after access to 10% honey-water in the crop (N = 2) and directly after the proventriculus at four points along the midgut (N = 10 except position 4 with N = 9) (LMM, LR-test, χ^2=22.152, df=4, *** P <0.001, same letters indicate $P \geq 0.443$ and different letters indicate $P < 0.001$ in Westfall corrected post hoc comparisons).



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840 **Fig. 1 – figure supplement 2: Acidopore grooming frequency of *C. floridanus*.** Frequency
841 of acidopore grooming within 30 min. after fluid ingestion (water or 10% honey water)
842 compared to ants that did not receive any fluid (unfed) (GLMM, LR-test, χ^2=33.526, df=2, ***
843 *P* <0.001, same letters indicate *P* = 0.634 and different letters indicate *P* < 0.001 in Westfall
844 corrected post hoc comparisons).

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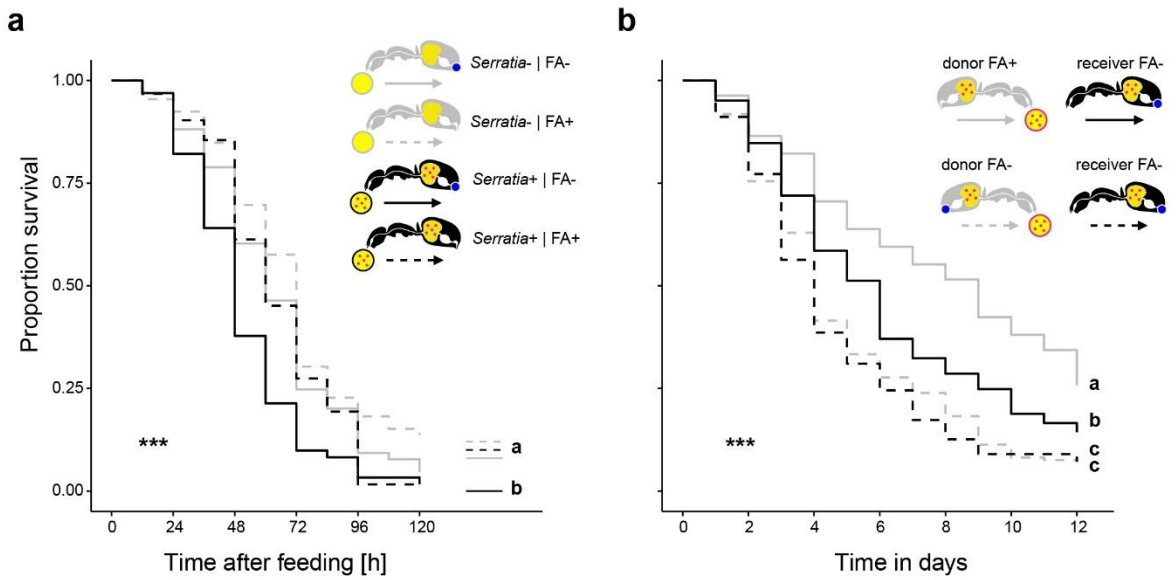


Fig. 2. Survival after ingestion of pathogen contaminated food. a, Survival of individual ants that were either prevented to ingest formic acid containing poison gland secretions (FA-; ant outlines with blue dot) or not (FA+) after feeding on either honey water contaminated with *Serratia marcescens* (*Serratia*+, yellow circle with pink dots and black ant outlines) or non-contaminated honey water (*Serratia*-) (COXME, LR-test, $\chi^2 = 20.95$, df=3, $^{***}P = 0.0001$, same letters indicate $P \geq 0.061$ and different letters indicate $P \leq 0.027$ in Westfall corrected post hoc comparisons). **b**, Survival of donor ants (light grey ant outlines) that were directly fed with pathogen contaminated food (yellow circle with pink dots in insert) and were either prevented to ingest formic acid containing poison gland secretions (FA-; ant outlines with blue dot) or not (FA+) and survival of receiver ants (black ant outlines) that received pathogen contaminated food only through trophallaxis with donor ants and were always prevented to ingest formic acid containing poison gland secretions (FA-) (COXME, LR-test, $\chi^2 = 66.68$, df = 3, $^{***}P < 0.001$, same letters indicate $P = 0.309$ and different letters indicate $P \leq 0.002$ in Westfall corrected post hoc comparisons).

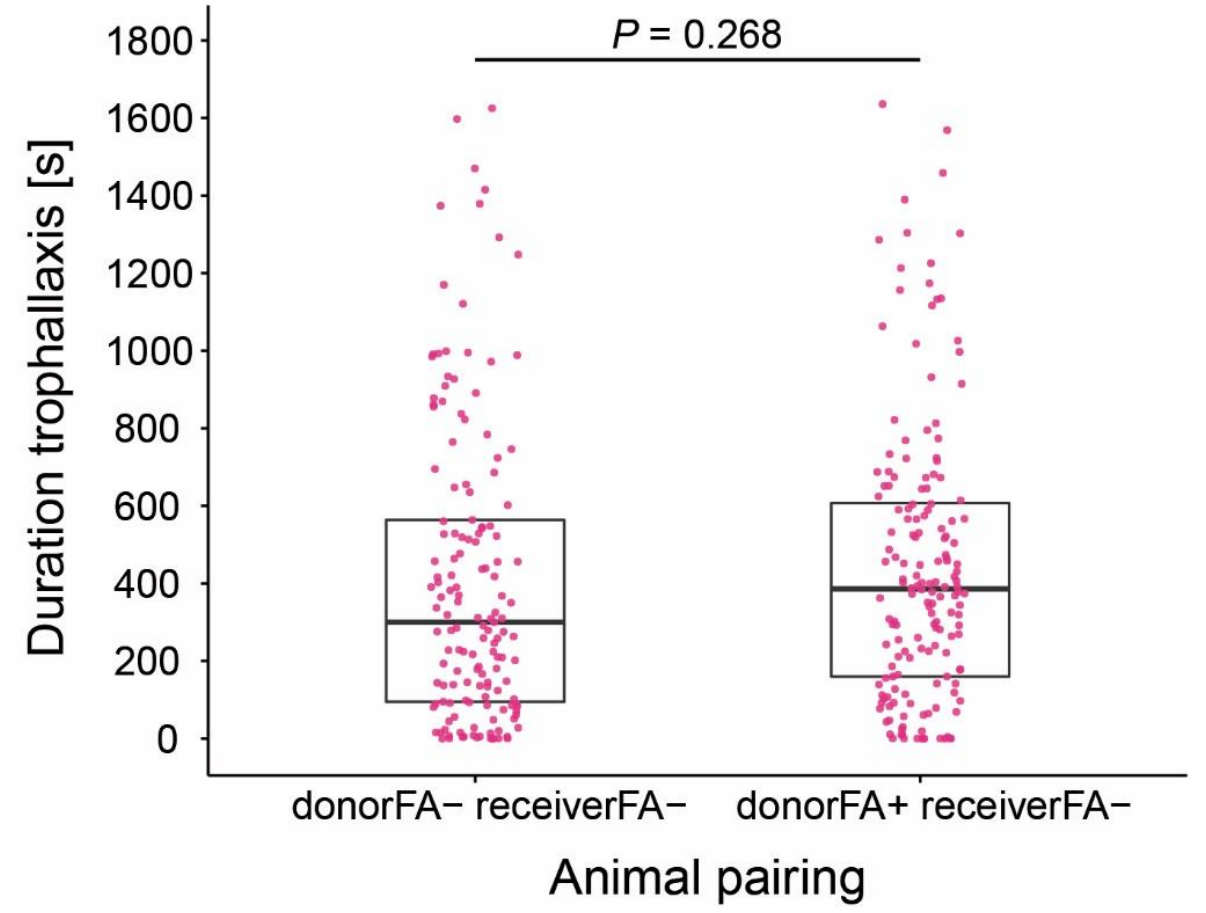


Fig. 2 – figure supplement 1: Duration of trophallaxis in donor-receiver ant pairs. Total duration of trophallaxis events within 30 min. of the first bout of food exchange between donor-receiver ant-pairs (LMM, LR-test, $\chi^2 = 1.23$, df = 1, $P = 0.268$). Donor ants in both pairs were directly fed with *Serratia marcescens* contaminated 10% honey water and were either prevented to ingest formic acid containing poison gland secretions (FA-) or not (FA+), while receiver ants received pathogen contaminated food only through trophallaxis with the respective donor ants and were always prevented to ingest formic acid containing poison gland secretions (FA-).

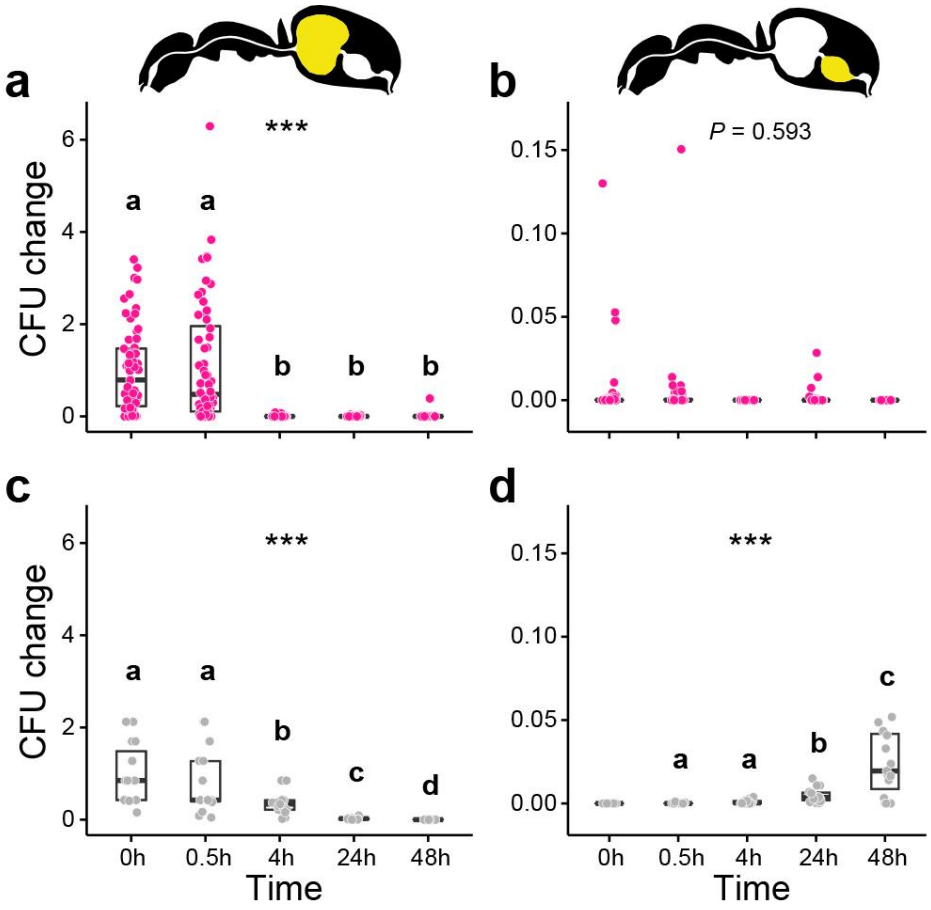
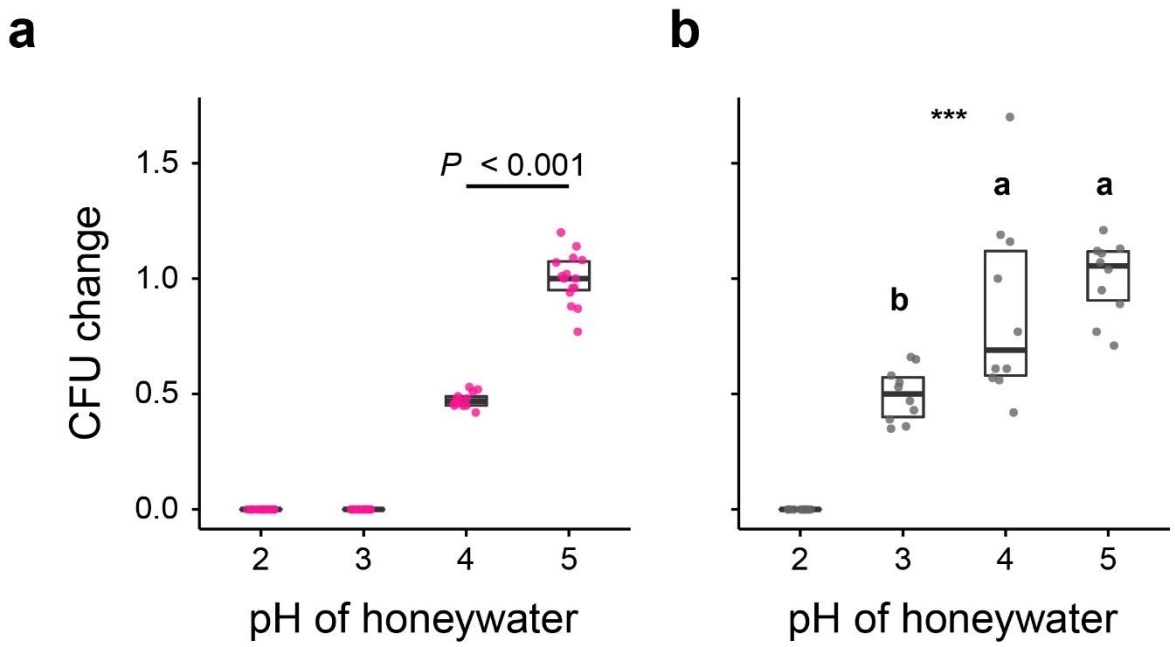


Fig. 3. Bacterial passage through the digestive tract of *C. floridanus* over time. Change in the number of colony forming units (CFUs) in the crop (**a,c**) and midgut (**b,d**) part of the digestive tract (yellow colour in insert) relative to 0h in the crop at 0h, 0.5h, 4h, 24h, and 48h after feeding ants 10% honey water contaminated with *Serratia marcescens* (**a,b**) or *Asaia* sp. (**c,d**). **a**, Change of *S. marcescens* in the crop (GLMM, LR-test, $\chi^2 = 220.78$, df = 4, $^{***}P$ <0.001, same letters indicate $P \geq 0.623$ and different letters indicate $P < 0.001$ in Westfall corrected post hoc comparisons). **b**, Change of *S. marcescens* in the midgut (GLMM, LR-test, $\chi^2 = 1.044$, df = 2, $P = 0.593$). **c**, Change of *Asaia* sp. in the crop (GLMM; LR-test, $\chi^2 = 124.01$, df = 4, $^{***}P < 0.001$, same letters indicate $P = 0.488$ and different letters indicate $P \leq 0.013$ in Westfall corrected post hoc comparisons). **d**, Change of *Asaia* sp. in the midgut (GLMM; LR-test, $\chi^2 = 59.94$, df = 3, $^{***}P < 0.001$, same letters indicate $P = 0.116$ and different letters indicate $P \leq 0.005$ in Westfall corrected post hoc comparisons).



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886 **Fig. 3 – figure supplement 1: Bacterial growth *in vitro*.** Change in the number of CFUs
887 relative to pH 5 after incubation of *Serratia marcescens* (**a**) and *Asaia* sp. (**b**) in 10% honey
888 water (pH = 5) or in 10% honey water acidified with commercial formic acid to a pH of 4, 3
889 or 2 for 2h (*S. marcescens*: GLM, LR-test, $\chi^2 = 79.442$, df = 1, $P < 0.001$; *Asaia* sp.: GLM,
890 LR-test $\chi^2 = 21.179$, df = 2, $P < 0.001$, same letters indicate $P = 0.234$, and different letters
891 indicate $P < 0.001$ in Westfall corrected post hoc comparisons).

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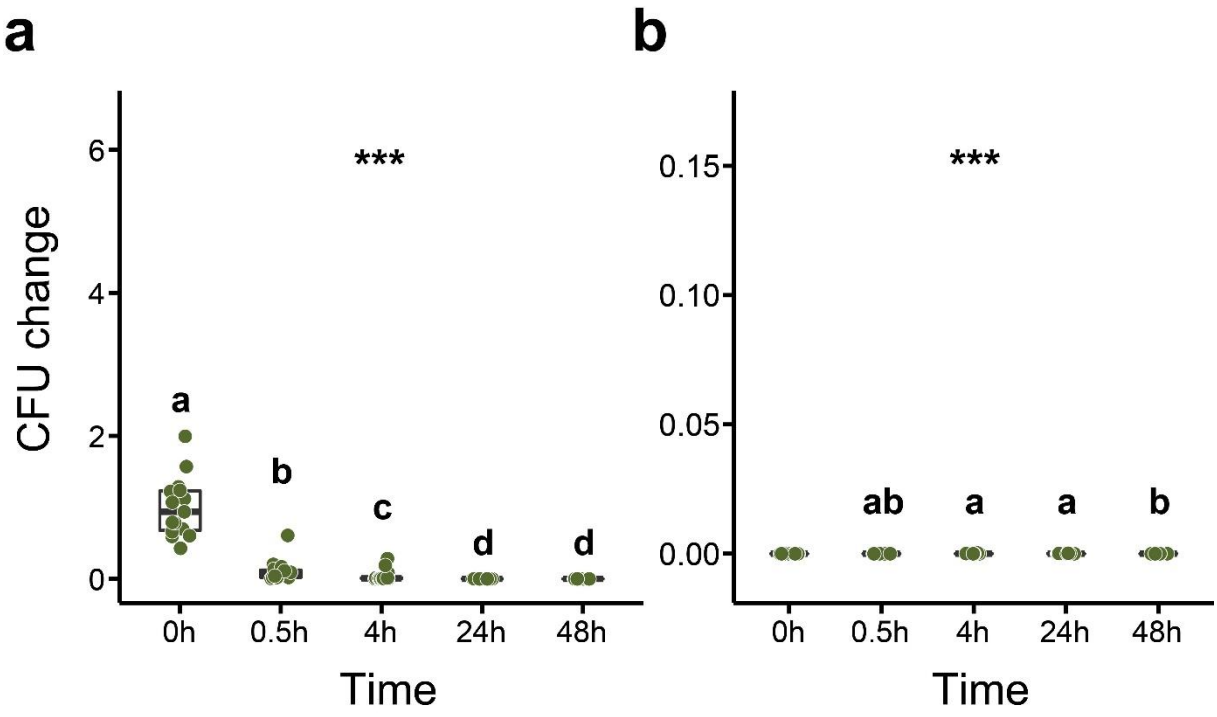
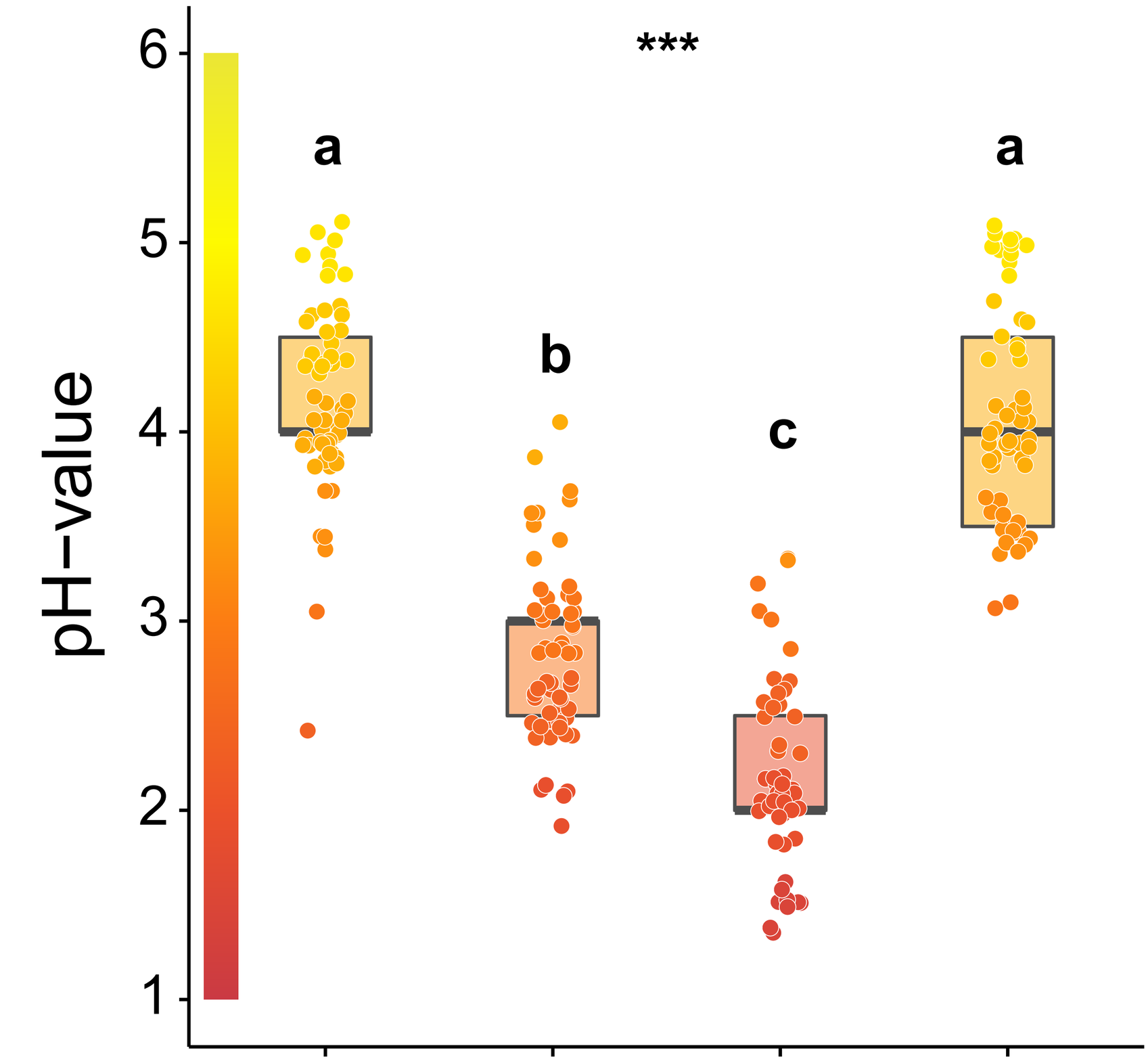


Fig. 3 – figure supplement 2: Passage of *E. coli* through the digestive tract of *C. floridanus* over time. Change in the number of colony forming units (CFUs) in the crop (**a**) and midgut (**b**) part of the digestive tract relative to 0h in the crop at 0h, 0.5h, 4h, 24h, and 48h after feeding ants 10% honey water contaminated with *Escherichia coli*. **a**, Change of *E. coli* in the crop (GLMM, LR-test, $\chi^2 = 156.74$, df = 4, *** $P < 0.001$, same letters indicate $P = 0.979$ and different letters indicate $P < 0.025$ in Westfall corrected post hoc comparisons). **b**, Change of *E. coli* in the midgut (GLMM, LR-test, $\chi^2 = 14.898$, df = 3, *** $P = 0.002$, same letters indicate $P \geq 0.629$ and different letters indicate $P \leq 0.038$ in Westfall corrected post hoc comparisons).

a



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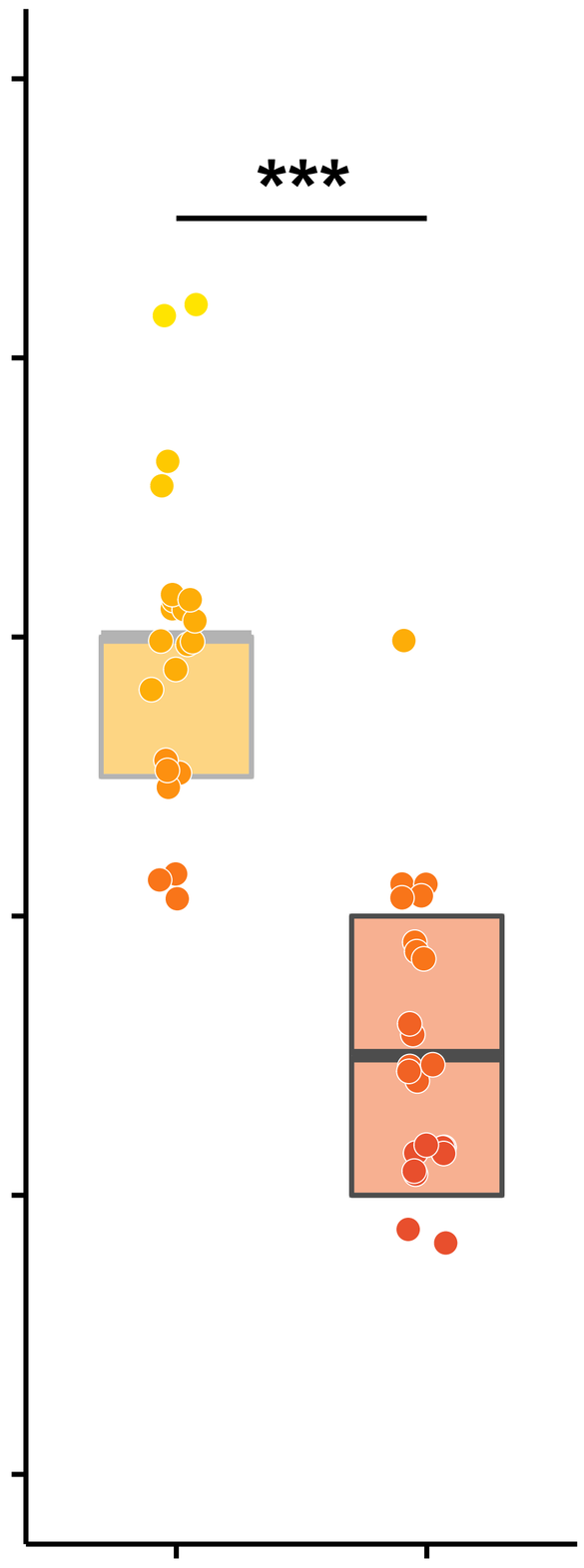

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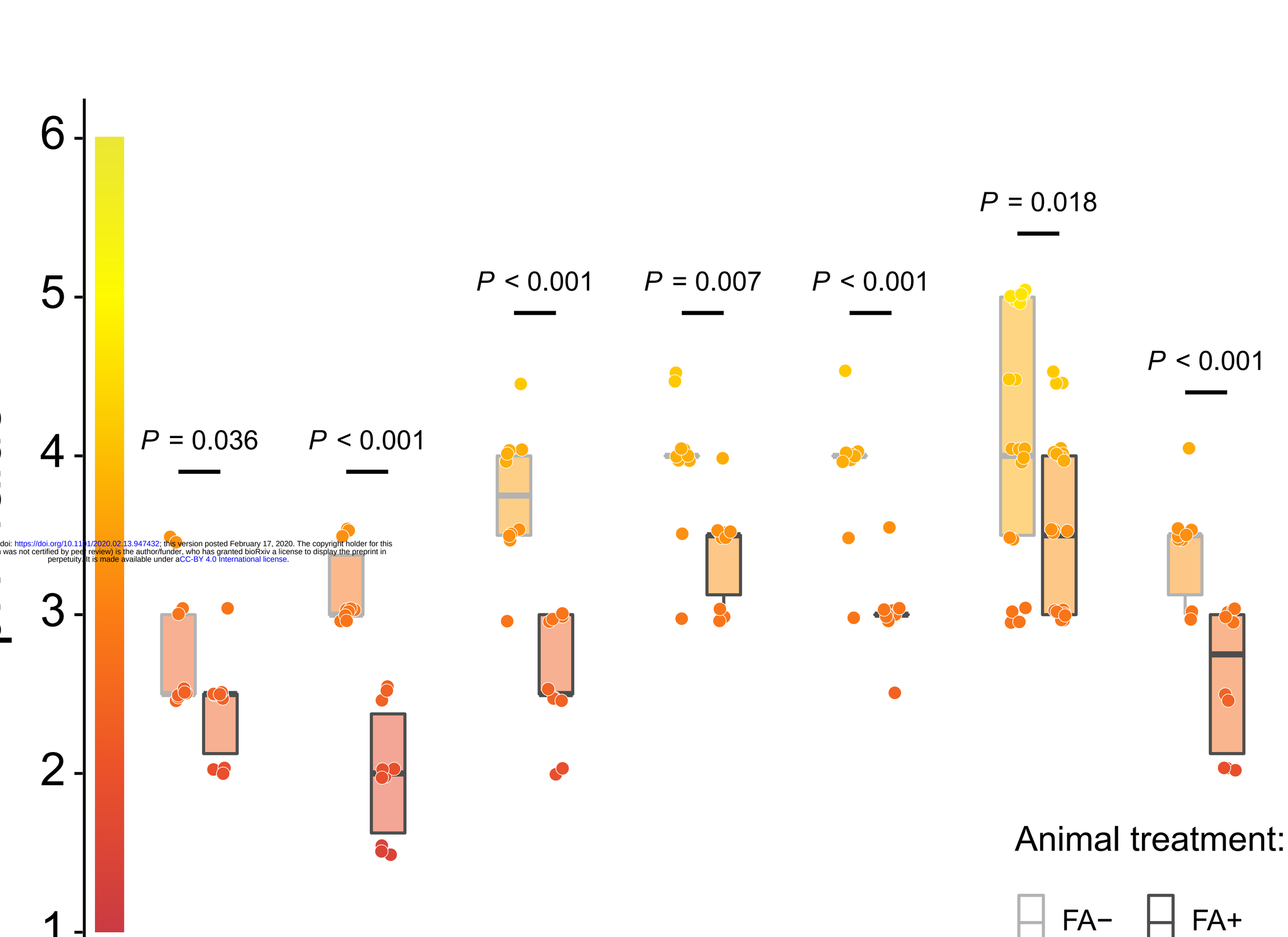

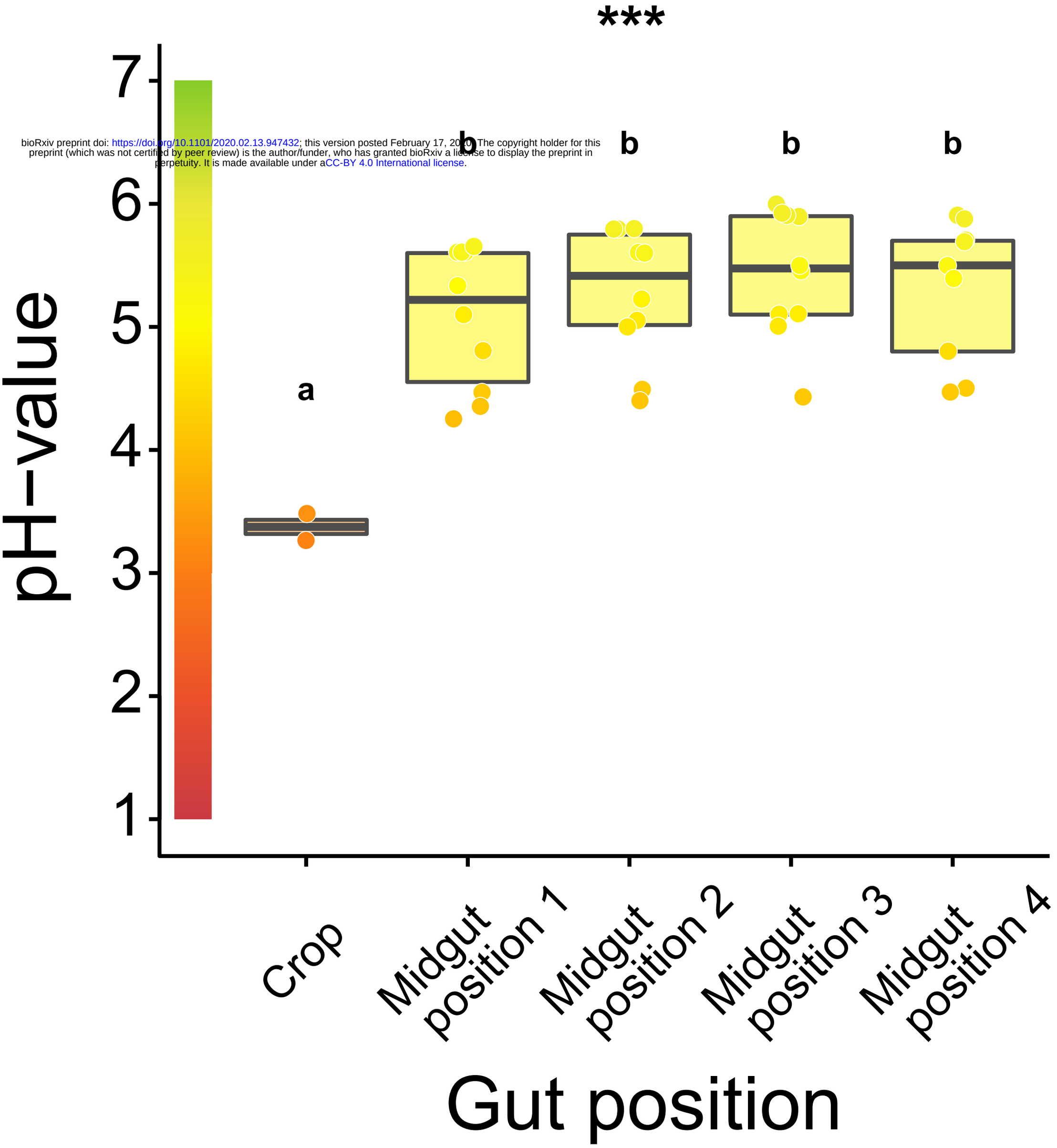

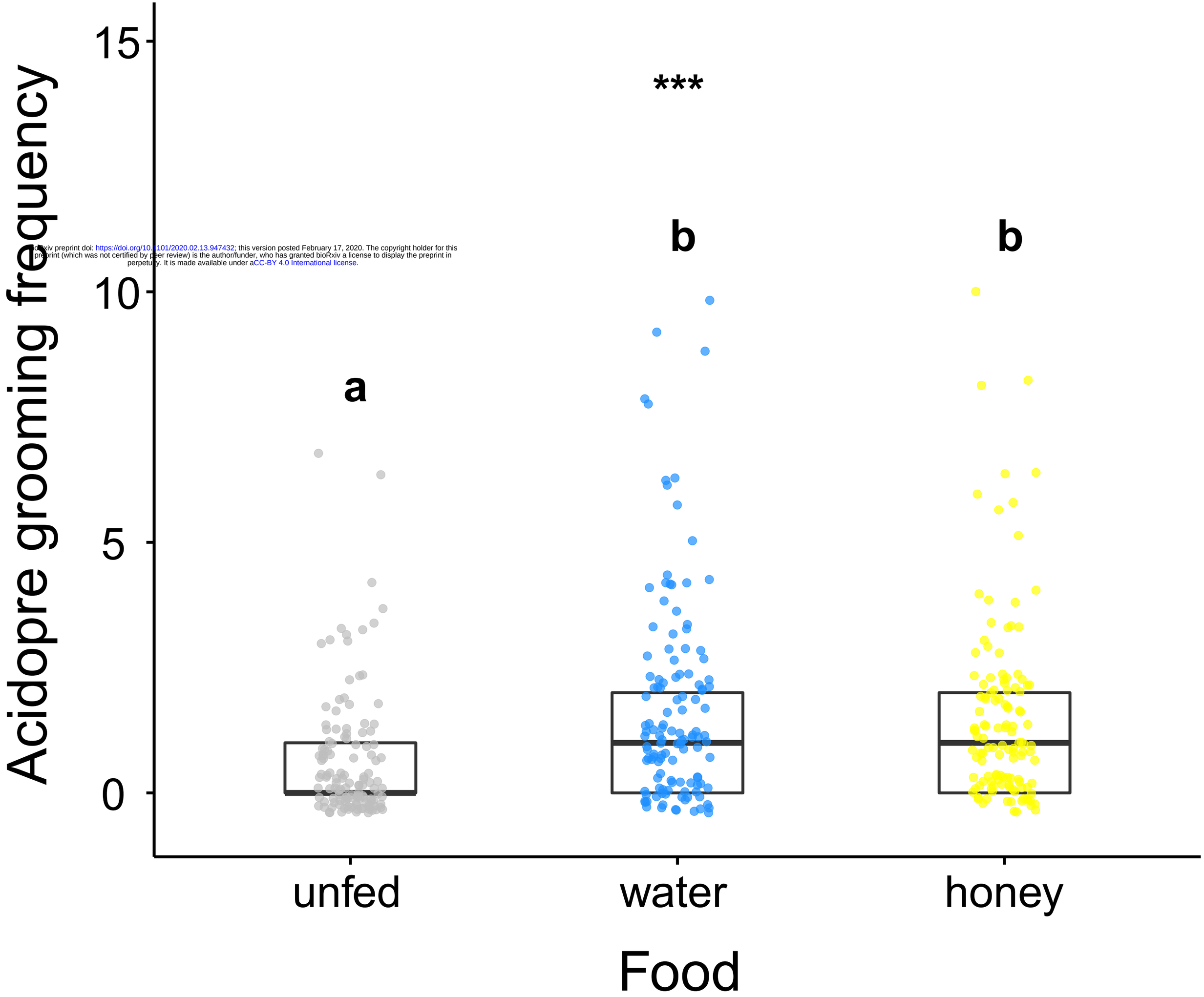

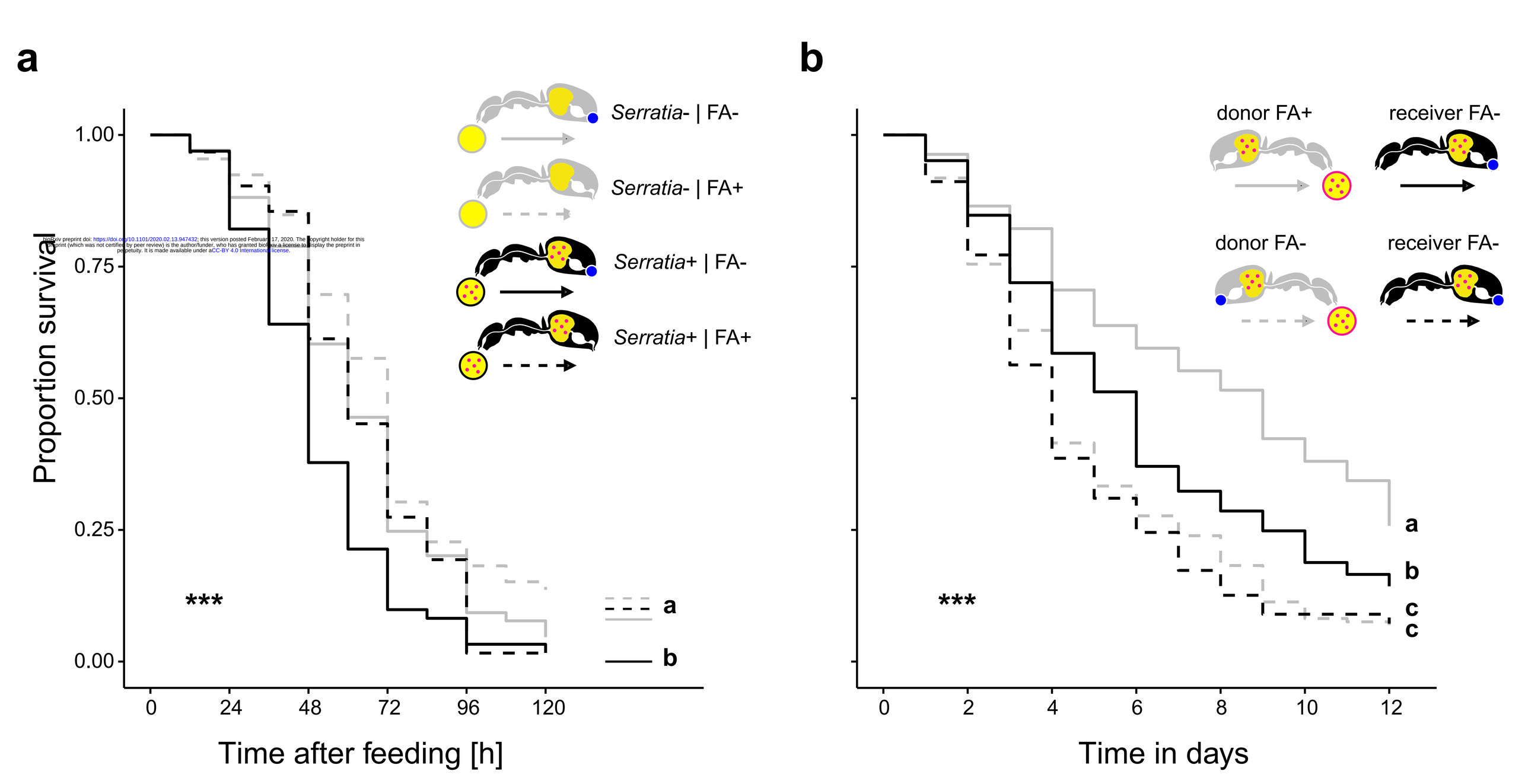

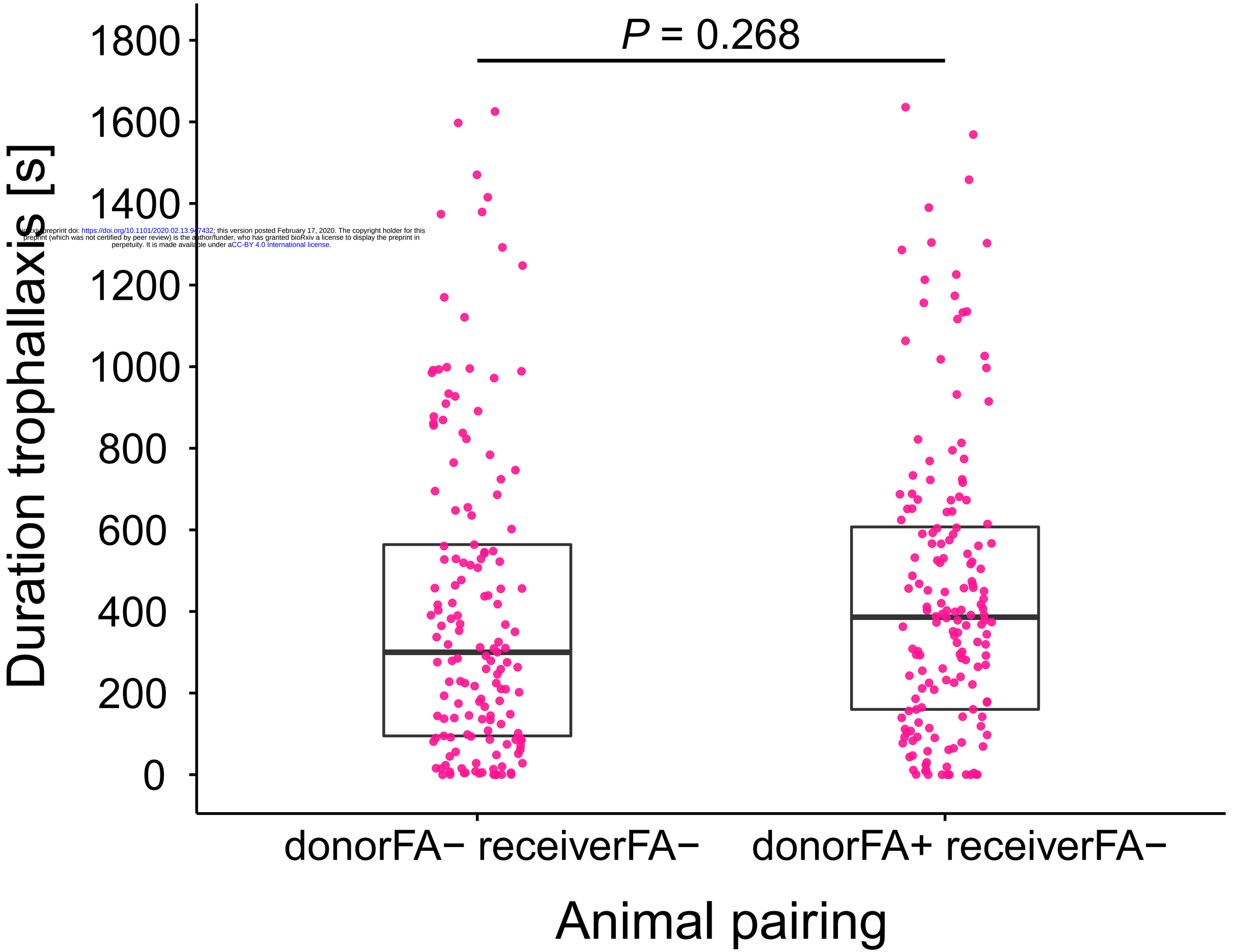

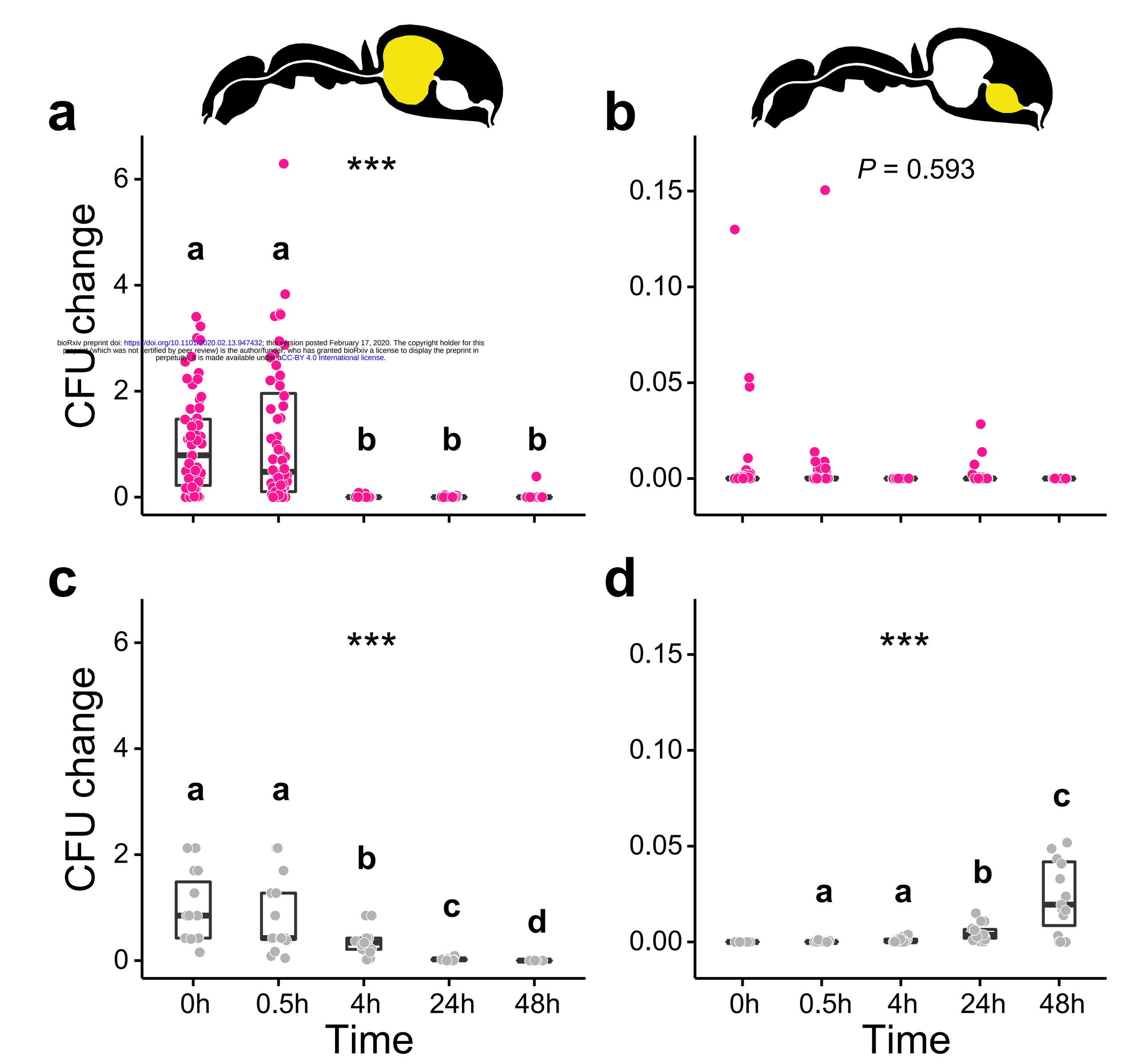

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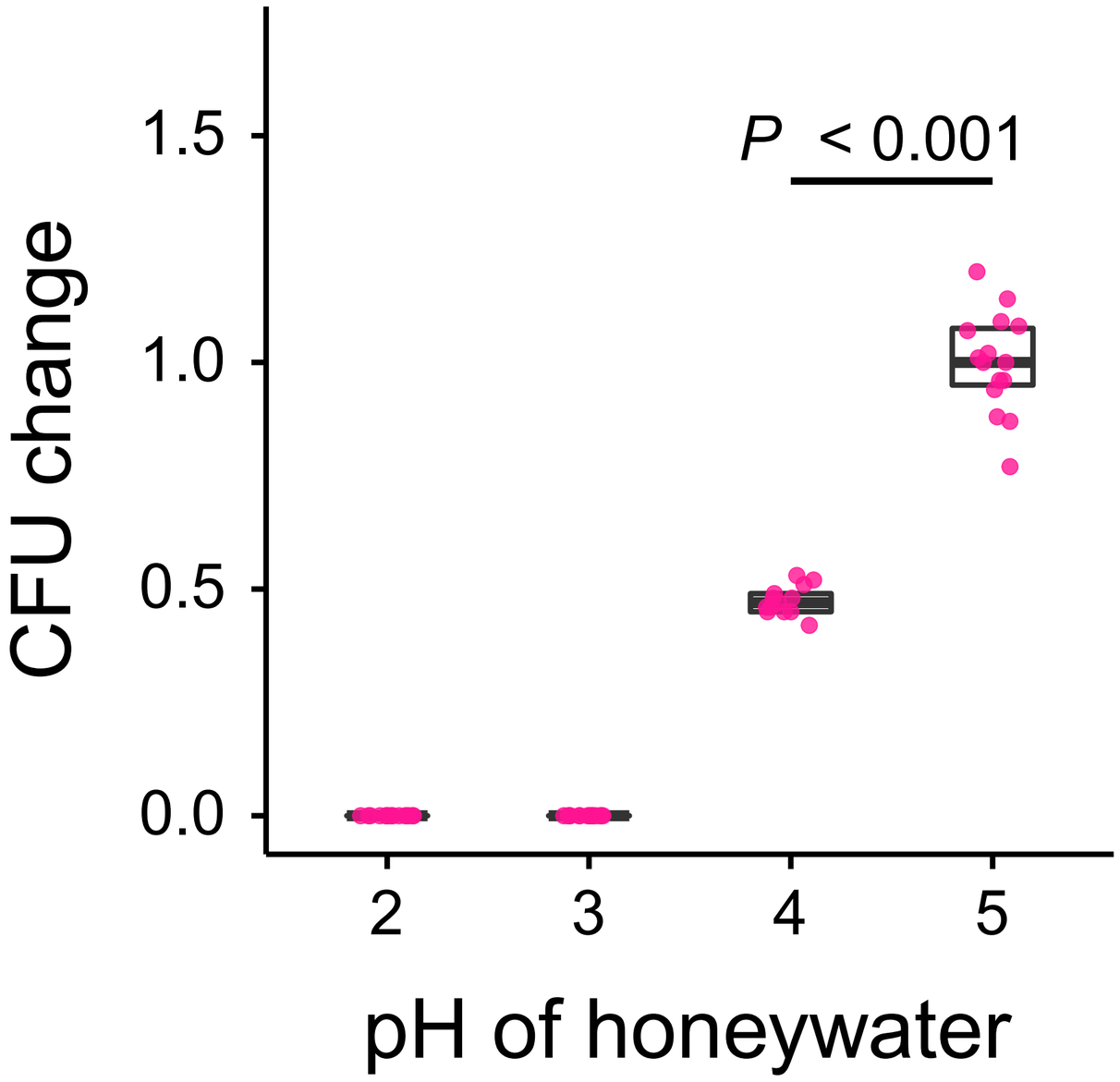
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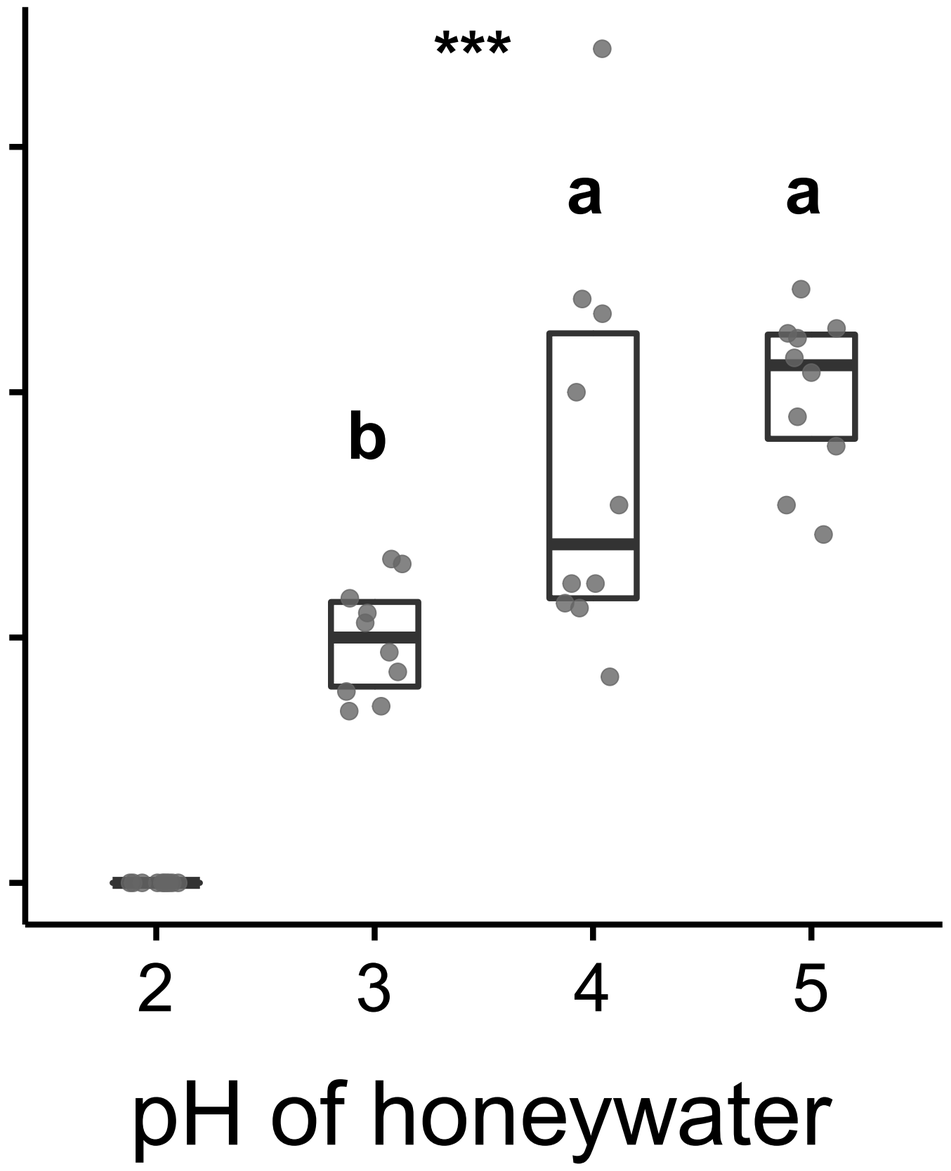
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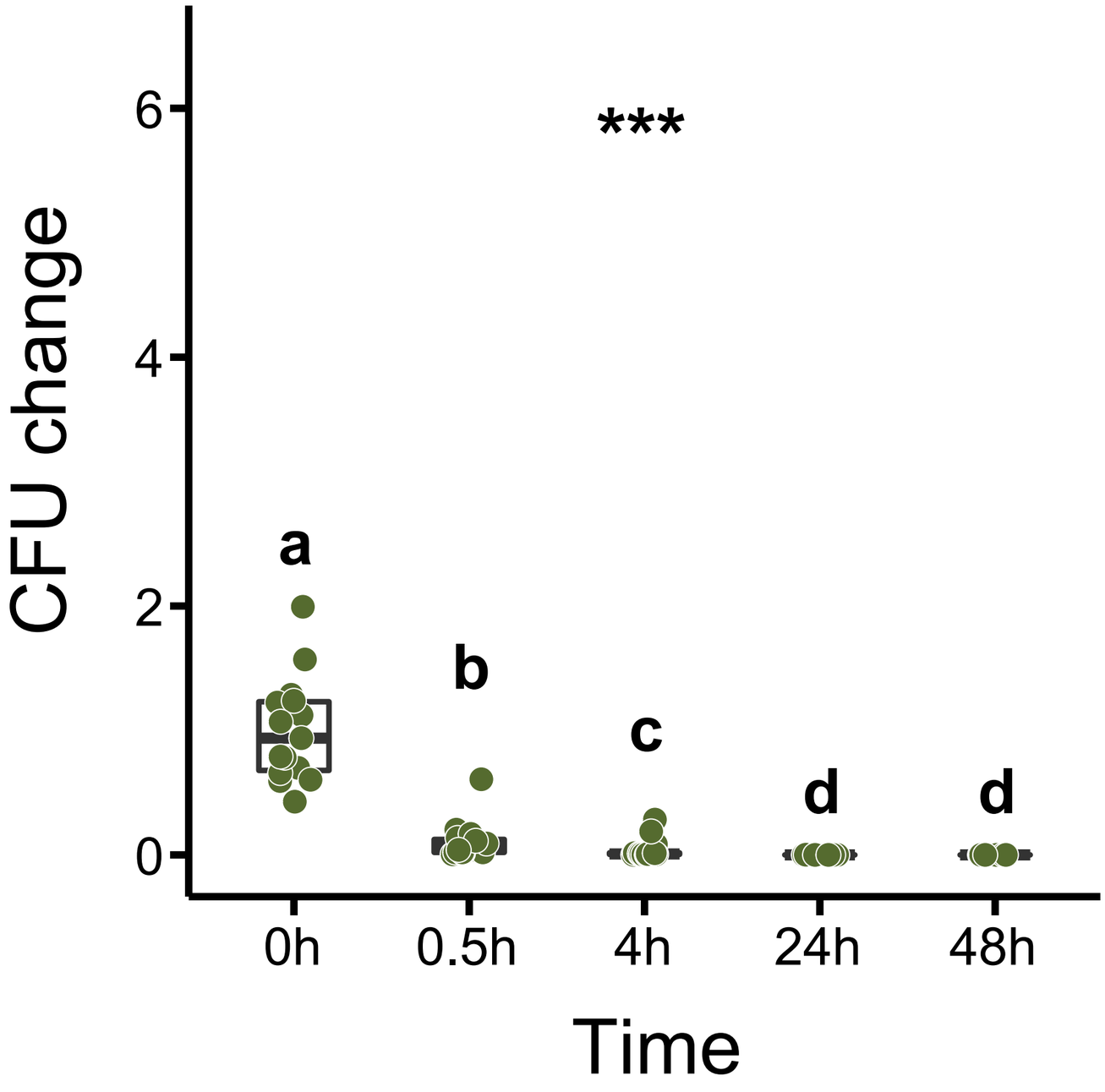
pH of honeywater

a


CFU change
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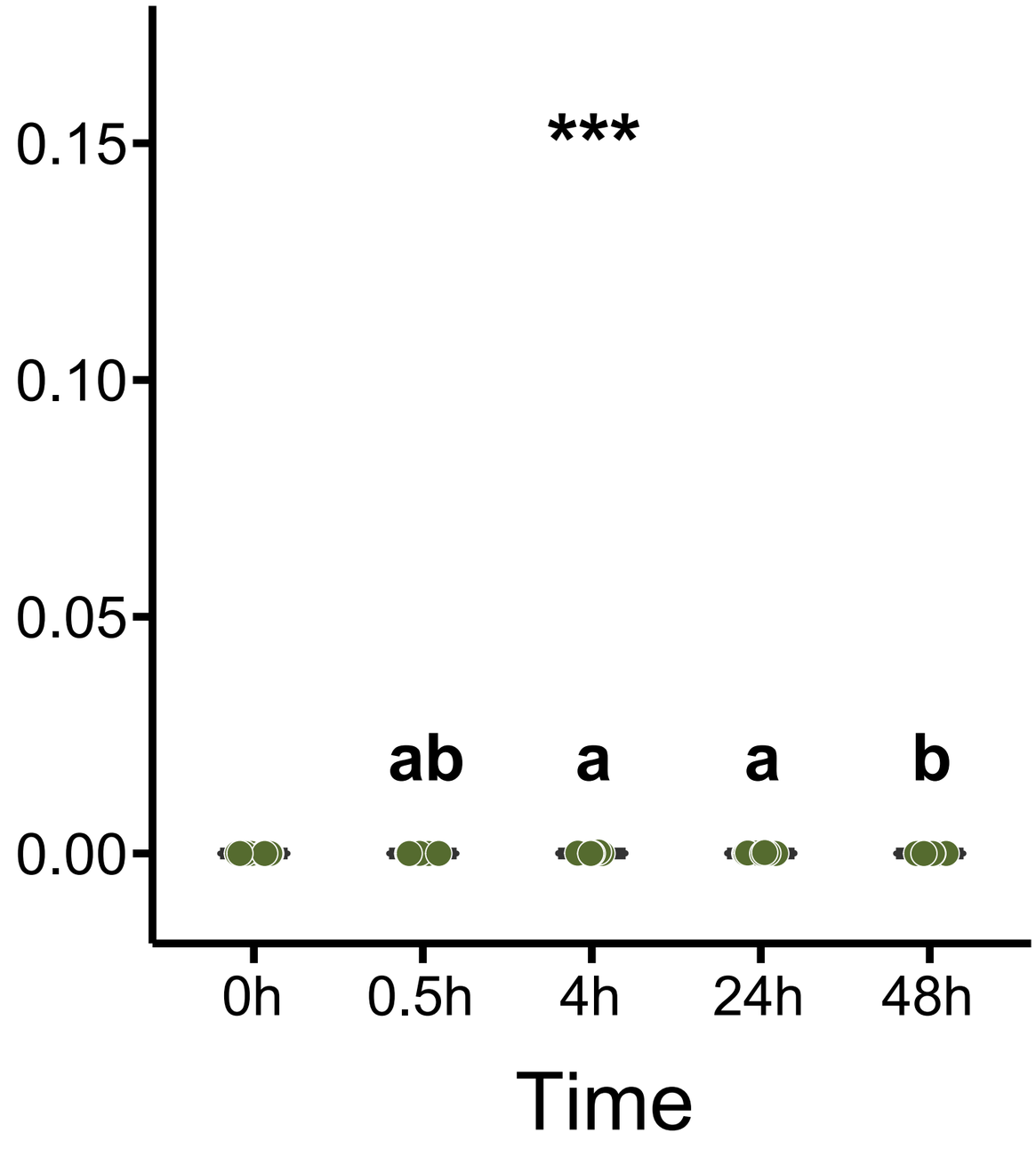
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