Evaluation of In2Care® mosquito stations for suppression of the Australian backyard mosquito, *Aedes notoscriptus*

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ABSTRACT

Aedes notoscriptus is a container-breeding mosquito endemic to Australia that vectors arboviruses and is suspected to transmit *Mycobacterium ulcerans*, the cause of Buruli ulcer. Localised control measures for this species are limited by its high dispersal capability. Here we evaluated the effectiveness of the In2Care® system in controlling *Ae. notoscriptus*. The In2Care® system suppresses mosquito populations via the entomopathogenic fungus, *Beauveria bassiana*, and the insect growth regulator pyriproxyfen, the latter of which is autodisseminated among larval habitats by contaminated mosquitoes. A field trial was conducted using 110 In2Care® stations in a 50,000 m^2 target area and the results were compared to four control areas that did not receive the In2Care® treatment. Efficacy was evaluated by comparing egg counts and measuring larvicidal impact in surrounding breeding sites. Laboratory experiments were conducted to validate the effect of *B. bassiana* on adult survival. Field results showed that six weeks after the In2Care® stations were deployed, treatment site ovitraps contained 43% fewer eggs than control site ovitraps, and 33% fewer eggs after ten weeks, indicating that the In2Care® system was able to reduce the population of *Ae. notoscriptus*. Population reduction remained evident for up to three weeks after In2Care® stations removal. Treatment site ovitraps had significantly fewer *Ae. notoscriptus* eclosing than control site ovitraps, confirming the pyriproxyfen autodissemination feature of the stations, and an average reduction of 50% in adult eclosion was achieved. Adult mosquitoes had a significantly shorter lifespan after

exposure to *B. bassiana*. The strategy has potential for suppressing *Ae. notoscriptus* populations.

1 INTRODUCTION

Mosquitoes of the genus *Aedes* continue to pose a significant threat to human and animal health. While much research has been focused on the yellow fever mosquito (*Ae. aegypti*) and the tiger mosquito (*Ae. albopictus*), other container-breeding and human-biting *Aedes* species have received less attention, limiting information about effective control measures. One such species is *Ae. notoscriptus*, a container-breeding mosquito that is broadly distributed in its native range in Australia and has become invasive in other regions, including the Torres Strait Islands, New Zealand, Papua New Guinea, New Caledonia, Indonesia (Dobrotworsky 1965, Lee et al. 1987, Sunahara and Mogi 2004), and the United States (specifically California) (Metzger et al. 2021). *Aedes notoscriptus* is a vector of human arboviruses (Doggett and Russell 1997), is suspected to play a significant role in the transmission of *Mycobacterium ulcerans* (the bacterium responsible for Buruli ulcer (BU)) (Wallace et al. 2017), and is also a primary vector of dog heartworm (Russell 1985). *Aedes notoscriptus* has greater dispersal capacity than other container-breeding *Aedes* (Watson et al. 2000, Trewin et al. 2019, Paris et al. 2023), making localised control efforts more challenging. In a recent pilot study in Victoria, Australia, gravid traps were applied to reduce the population size of *Ae. notoscriptus* on the Mornington Peninsula, with the objective to further explore the species' role in the transmission of Buruli ulcer (https://www.health.vic.gov.au/infectious-diseases/beating-buruli-in-victoria; Mee *et al.* unpublished). However, no measurable reduction in the number of *Ae. notoscriptus* eggs was observed, likely due to the species' capacity for long distance dispersal, which is evident from genetic data (Paris et al. 2023). In addition, the species breeds in many cryptic breeding sites (Trewin et al. 2019) in properties that cannot be easily accessed to apply larvicides.

One potential solution for controlling pests with high dispersal rates in an environment with an abundance of cryptic breeding sites is through autodissemination of insecticides by the target pest, a method that has been implemented in both agriculture (Ignoffo 1999) and public health settings (Cook et al. 2009). An example of this approach is the In2Care® system, an outdoor

contamination station designed to reduce local mosquito populations and thereby reduce the risk of disease transmission (https://www.in2care.org). The system lures gravid container breeding mosquitoes into the station and contaminates them with the insect juvenile hormone analogue and insect growth regulator, pyriproxyfen, which affects larvae and pupae (Unlu et al. 2020). Contaminated mosquitoes spread the insecticide to other breeding sites so that mosquito larvae are not only controlled inside the station, but also in other (often cryptic) breeding sites in the vicinity.

Pyriproxyfen is combined with the entomopathogenic fungus *Beauveria bassiana*. Entomopathogenic fungi are necrotrophic parasites (i.e. kill their host in order to support their own growth and complete their life cycle), potentially useful for biocontrol (Lacey et al. 2015). *Beauveria bassiana* infects a broad range of hosts (Ferron 1978) such as ants (Broome et al. 1976), termites (Culliney and Grace 2000), agricultural pests such as grasshoppers (Bidochka and Khachatourians 1991) and arthropod vectors of human diseases, including mosquitoes (Howard et al. 2010, Bukhari et al. 2011). Infected mosquitoes usually die of the infection after a few days, reducing the length of time available to transmit disease and removing them from the breeding population. The efficacy of the In2Care® system has previously been validated by experiments in some cases for *Ae. aegypti* and *Ae. albopictus* (Buckner et al. 2017, 2021, Khater et al. 2022), but not for other *Aedes* species.

In this study, we aimed to evaluate the potential of the In2Care® system in controlling a population of *Ae. notoscriptus* in Victoria, Australia. We conducted a field trial using 110 In2Care® stations in a designated area and compared temporal changes in mosquito numbers to those in four control areas that did not receive the In2Care® station treatment. The efficacy of the In2Care® system was evaluated by comparing egg counts between all sites, as well as by measuring the larvicidal impact achieved in surrounding mosquito breeding sites due to pyriproxyfen autodissemination. We also conducted laboratory experiments to validate the effect of *B. bassiana* on adult mosquito survival. The results provide insights into the effectiveness of the In2Care® system in suppressing populations of *Ae. notoscriptus* and suggest a promising new approach for controlling this species in locations where it has become invasive.

2 MATERIAL AND METHODS

2.1 Field experiments

2.1.1 Permits

This work was performed under APVMA Small-scale Trial Permit PER7250. Trials conducted to generate data relating to efficacy, residues, crop or animal safety or other scientific information outside the confines of a research facility where the size of the trial annually does not exceed the following: a total of 5 hectares nationally, with a maximum of 1 hectare in any one jurisdiction in the case of any food and/or fibre field crop (https://permits.apvma.gov.au/PER7250.PDF). The active ingredient In2Mix® sachets were imported under biosecurity permit 0006570560 from the Australian Government Department of Agriculture, Fisheries and Forestry. Permit conditions meant that the trial was limited to one treatment site, but we had access to several control sites.

2.1.2 Study sites

We assigned one treatment site and four control sites for this study: Eynesbury (In2Care® treatment) (Figure 1), Darley (C1), Aintree (C2), Diggers Rest (C3) and Wyndham Vale (C4). All sites were located north-west of Melbourne in Victoria, Australia and comprised ~80 residential houses over a total ~50,000 m^2 area. Previous surveillance has shown that all sites have established populations of *Ae. notoscriptus*. All sites represented residential areas with 1-2 storey houses on allotments of 572.42 m^2 to 958.83 m^2.

2.1.3 Mosquito monitoring

Mosquito population density surveillance was conducted using egg counts at control and In2Care® treatment from the 20th of January 2022 until the 21st of April 2022. A total of 150 ovitraps (30 per site) were used, each consisting of a 500 mL black plastic bucket half-filled with water and containing alfalfa pellets to attract gravid *Ae. notoscriptus*. A 15 cm long strip of red felt extending into the water provided a consistent oviposition substrate. Egg counts were taken at weekly intervals for two weeks prior to the deployment of In2Care® stations. After the deployment of In2Care® stations, eggs were collected fortnightly, followed by weekly

111 collections for two weeks after the intervention concluded. The same researcher performed all
112 egg counts under a microscope to ensure consistency. To prevent the ovitraps from becoming
113 functional breeding sites, all larvae and pupae present in the ovitraps were discarded at each
114 collection.

115 **2.1.4 In2Care® station deployment, monitoring, and servicing**

116 We placed In2Care® stations in volunteer properties, as well as on nature strips and other
117 public land, with a density of approximately one station per 450 m^2 close to the manufacturer's
118 recommendation (one station per 400 m^2, https://www.In2Care.org). This resulted in a total
119 deployment of 110 stations as shown in Figure 1. The stations were strategically located in
120 shaded or semi-shaded areas that were expected to attract *Ae. notoscriptus*, such as areas with
121 vegetation or proximity to other breeding grounds. During the station deployment process, we
122 filled the water reservoir with approximately 4.7 litres of tap water before attaching the netting
123 containing the biocides to the floater and placing it onto the water surface. We aimed for a
124 homogeneous distribution of the biocides by shaking each In2Mix® sachet containing the
125 biocide powder, odour tablets, and netting well. The remaining biocide powder and odour
126 tablets were placed into the water reservoir before the lid was locked into place.

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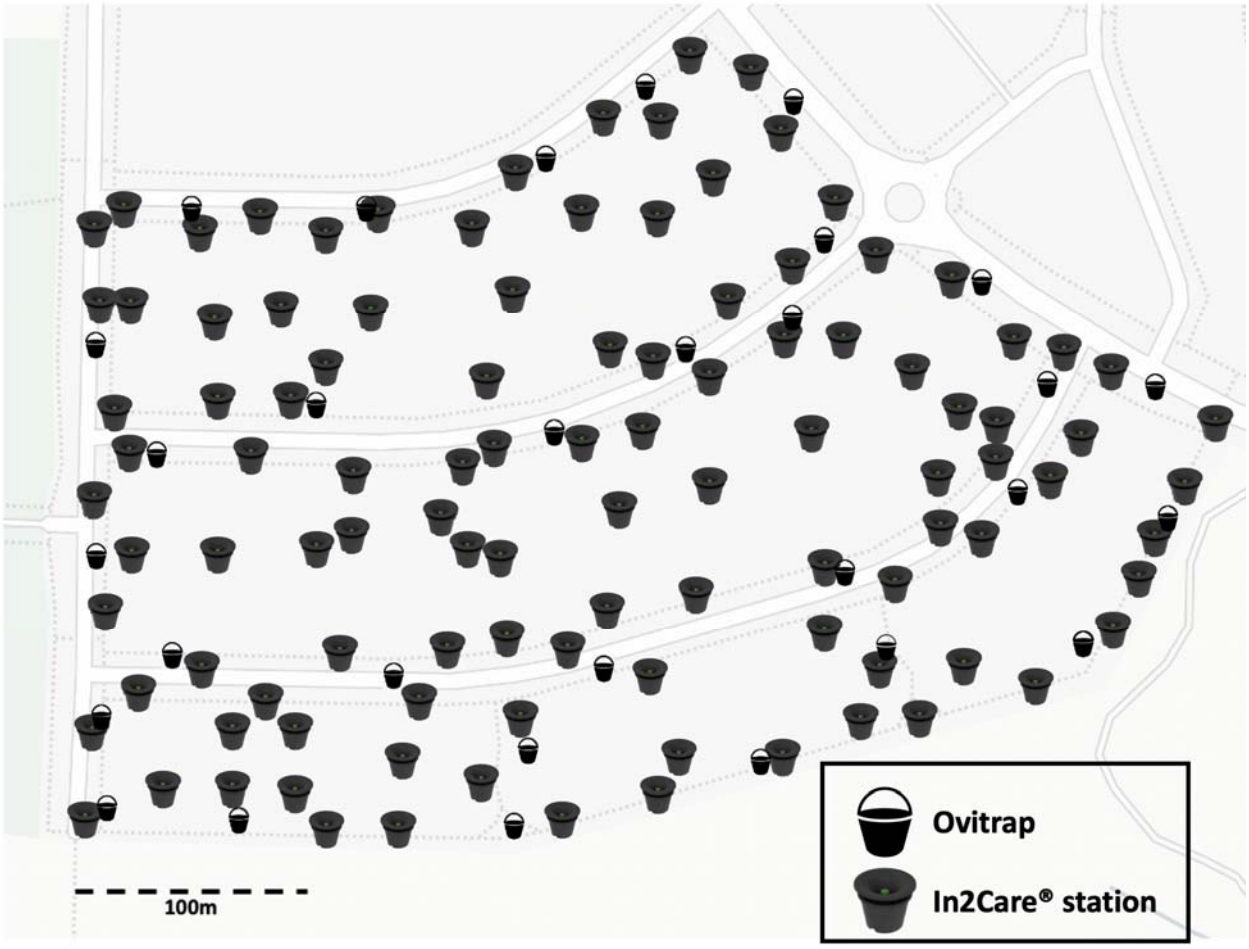


***Figure 1 In2Care® station and ovitrap placement in the treatment site*:** Stations were placed at a density of one station per 450 m^2, resulting in 110 stations deployed, represented by the In2Care® station symbol. Ovitraps (n=30) were placed on public land to monitor egg number before, during and after the intervention, shown by the bucket symbol (mean distance of ovitraps to stations = ~158 m).

Regular monitoring of the In2Care® stations was conducted on a fortnightly basis. The monitoring included checking for signs of disturbance, ensuring that the water level was sufficient, (i.e., filled more than halfway) and that the netting was still dry and intact. The presence of larvae and live and/or dead pupae in the stations was also recorded in order to validate the station's attractiveness to gravid female mosquitoes and to ensure that no mosquitoes developed into adults inside the stations. When recording larval and pupal counts, we did not discriminate between *Ae. notoscriptus* and other species that might have been

141 present in the stations. After four weeks, the biocides and odour tablets in the stations were
142 refreshed with In2Mix® as previously described.

143 2.2 Laboratory experiments

144 2.2.1 Mosquito rearing

145 All laboratory experiments in this study used mosquitoes from laboratory colonies of
146 *Ae. notoscriptus* and *Ae. aegypti*. These colonies were established from field collections made in
147 Cairns, Australia in 2019 for *Ae. aegypti*, and Brisbane, Australia in 2014 for *Ae. notoscriptus*.
148 The colonies were maintained in a temperature-controlled insectary at 26°C ± 1°C, with a 12-
149 hour photoperiod, as per the protocol outlined by Ross et al. (2017) for rearing of *Ae. aegypti*.
150 The mosquitoes were provided with constant access to a 10% sucrose solution, and were
151 housed in 27 cm^3 BugDorm-1® cages (MegaView Science Co., Ltd., Taichung City, Taiwan). Adult
152 females were blood-fed on a human volunteer's arm, once per generation, to initiate egg-laying
153 (with ethics approval from The University of Melbourne 0723847). The colonies were
154 maintained in replicate cages, each containing approximately 500 mosquitoes.

155 2.2.2 Laboratory validation of pyriproxyfen autodissemination

156 To determine whether pyriproxyfen was being disseminated from the In2Care® stations to
157 other breeding sites, we collected fortnightly water samples from 15 of the ovitraps monitoring
158 the treatment site. The traps were selected randomly after excluding ovitraps that did not
159 contain any eggs or larvae which ensured selected ovitraps had been visited by mosquitoes. A
160 total of 200 mL of water per trap was collected in a sealable plastic container which was then
161 placed into an individual zip-lock bag to avoid cross-contamination between samples. We also
162 collected 200 mL of water from five randomly selected In2Care® stations to serve as positive
163 controls. Finally, five 200 mL water samples were taken from randomly chosen ovitraps from
164 each control site as negative controls (20 samples total).

165 To measure residual intervention effects after the In2Care® stations were removed from the
166 treatment site, we collected additional water samples from ovitraps in week 12 and 13 of the

167 trial. We collected ten water samples from In2Care® stations in week 10 to act as positive
168 controls for those additional samples.

169 All collected water samples were strained through fine mesh to remove any organisms or
170 organic material that might have been collected with the water. We used individual pieces of
171 mesh for each sample to avoid cross contamination between samples. We then added 25 L3
172 laboratory-reared *Ae. notoscriptus* larvae into each water sample and added two TetraMin®
173 tropical fish food tables (Tetra, Melle, Germany). The containers were placed into a 26°C
174 climate-controlled cabinet with a 12:12 light:dark cycle. All containers were checked daily to
175 ensure larvae had sufficient food, and any mosquito eclosing was recorded. We used the
176 number of free pupal exuviae as a proxy for successful adult eclosing.

177 **2.2.3 Laboratory validation of *B. bassiana* efficacy against *Ae. notoscriptus***

178 To validate the adulticidal effect of *B. bassiana* on adult *Ae. notoscriptus* and *Ae. aegypti,* we
179 performed survival assays in the laboratory. All mosquitoes used in the experiment were adults
180 seven days post-eclosion and were allowed to mate. We exposed 25 females to a fresh In2Mix®
181 netting (containing approximately 0.2 g of In2Mix® powder with 74.03% pyriproxyfen and 10%
182 *B. bassiana* spores) using the WHO standard forced exposure bioassay (The World Health
183 Organisation 2006). Eight replicates of 25 females were prepared for the treatment and the
184 control groups. We provided each replicate group with 10% sucrose solution. Individuals that
185 died within one hour post experiment set up were excluded from all analyses. Sucrose solution
186 was refreshed as needed. We scored survival daily until all individuals in each treatment cohort
187 died. Individuals were scored as dead if no movement occurred after being manipulated with
188 forceps. To test whether mosquitoes were still able to blood feed and produce eggs post
189 infection, we blood fed groups on day two post exposure. We recorded the proportion of
190 mosquitoes that successfully blood fed for each group and collected and counted eggs on day
191 four and five of the experiment. We repeated the same setup with In2Mix® nets collected from
192 In2Care® stations that were deployed in the field for four weeks.

193 We calculated the germination rate of spores used to infect the treatment groups to validate
194 the spore quality and to ensure consistent spore qualities across replicates. Germination rate

195 was also used to compare the quality of fresh In2Mix® with that of four-week-old nettings. To
196 estimate germination rate, we inoculated three Sabouraud Dextrose Agar (SDA) plates for each
197 used netting by rubbing a sterile cotton tip on the netting and transferring the spore sample
198 onto the plates. The plates were then sealed with Parafilm® "M" Laboratory Film (Amcor
199 Flexibles North America) and stored at 26°C for 24 hours. After incubation we identified
200 germinated and not germinated spores (300 spores total) on three randomly chosen areas of
201 each plate using a light microscope. The germination rate was calculated using the following
202 formula: [number of germinated spores] ÷ [total number of spores counted (germinated + not-
203 germinated)] x 100. We then averaged the calculated germination rate across the three
204 replicate plates.

205 **2.3 Statistical analyses**

206 All statistical analyses were performed in R Studio v1.14 (R Core Team 2021). We used one
207 sample t-tests to determine whether the average numbers of eggs per trap in the treatment
208 site differed from the control sites.

209 Contingency tests were used to test if the number of weeks since station deployment
210 influenced the number of larvae (alive) and pupae (alive and dead) in the In2Care® stations.

211 We tested whether the water samples collected from the treatment site ovitraps, the control
212 sites ovitraps and the In2Care® stations differed in the percentage of mosquitoes that
213 successfully eclosed by using Kruskal-Wallis tests (a Kologorov-Smirnov test revealed that the
214 data were not normally distributed). We ran a Mantel test to determine whether the
215 percentage of eclosed mosquitoes was correlated with the distance from the nearest In2Care®
216 station.

217 To investigate whether mosquitoes infected with *B. bassiana* had a shorter lifespan than
218 control groups, whether there were differences in survival between mosquitoes tested on fresh
219 or older nettings, and whether there were differences in survival between *Ae. notoscriptus* and
220 *Ae. aegypti*, we performed a Cox regression survival analysis using the 'survival' package in R
221 (Therneau and T. Lumley 2015). The variables 'species', 'treatment' and 'netting' were used as
222 explanatory variables, while the 'replicate' was added as a random factor.

We ran independent sample t-tests to investigate differences in the proportion of mosquitoes blood feeding between control groups and groups infected with *B. bassiana.*

3. RESULTS

3.1 Mosquito surveillance

The results of our mosquito population density surveillance, which was performed by counting eggs in ovitraps, revealed that the number of eggs was not obviously linked to rainfall events, but did gradually decrease as conditions became colder (Figure 2). The average number of *Ae. notoscriptus* eggs was higher in the control sites than the average value in the treatment site at weeks 8, 10, 12 and 13 of the trial (week 8 (week 6 post station deployment): $t = 7.39$, $df = 3$, $p = 0.003$; week 10 (week 8 post station deployment): $t = 7.03$, $df = 3$, $p = 0.003$; week 12 (week 10 post station deployment): $t = 8.4$, $df = 3$, $p = 0.002$; week 13 (week 11 post station deployment): $t = 3.49$, $df = 3$, $p = 0.019$).

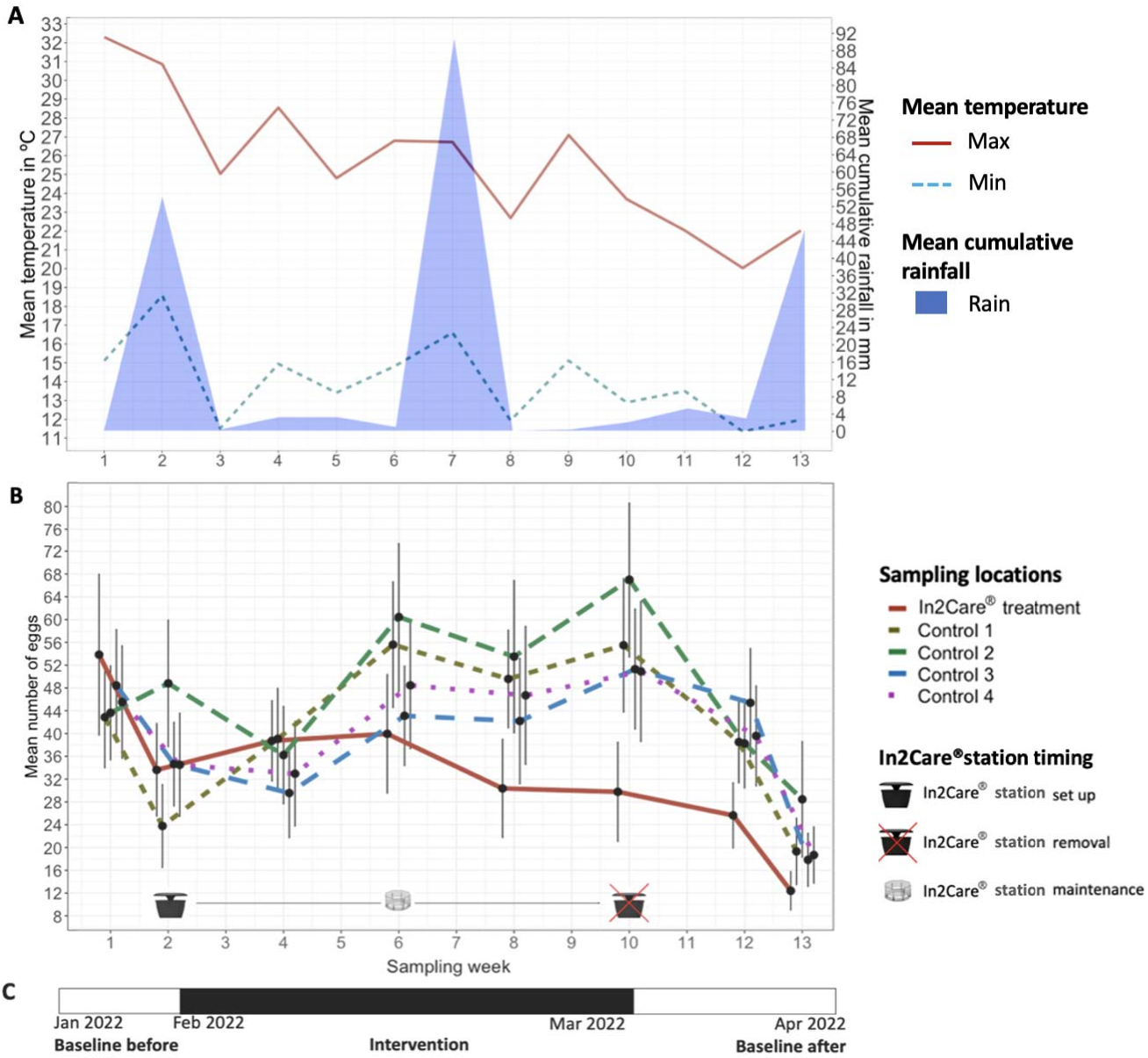


Figure 2 Egg counts and weather data during the In2Care® station trial: (A) *Weekly mean minimum and maximum temperature are shown as red solid and blue double dashed lines respectively. Mean cumulative rainfall is represented by the light blue area plot. Weather data were obtained from the Bureau of Meteorology (bom.gov.au) Melbourne Airport Observation Station.* ***(B)*** *Weekly mean egg counts in the treatment site are shown by the solid red line. Dashed green, brown, purple, and blue lines represent egg counts in individual control sites. Egg counts are averaged from n=30 ovitraps per site. Black error bars show standard errors per*

collection and site. The In2Care® station symbols indicate the timing of station set and removal. The In2Care® floater symbol indicates maintenance of stations in week 6 of the trial (4 weeks post-deployment).

Two weeks after the In2Care® stations were deployed, larvae were found in 53.64% (95% CI: 46.77% - 60.51%) of stations, indicating that most stations were visited. After four weeks 67.15% (95% CI: 58.22%, 76.32%) of stations were infested and infestation rates increased to 87.27% (95% CI: 80.91% - 91.28%) after week 6 since deployment, and 63.63% (95% CI: 53.60% - 71.68%) after week 8 (Figure 3). Over the entire monitoring period, only two stations were never visited. A contingency test showed that the weeks since initial deployment of the In2Care® stations significantly influences the number of stations that were positive for larvae ($X^2 = 9.67$, df = 3, $p = 0.022$).

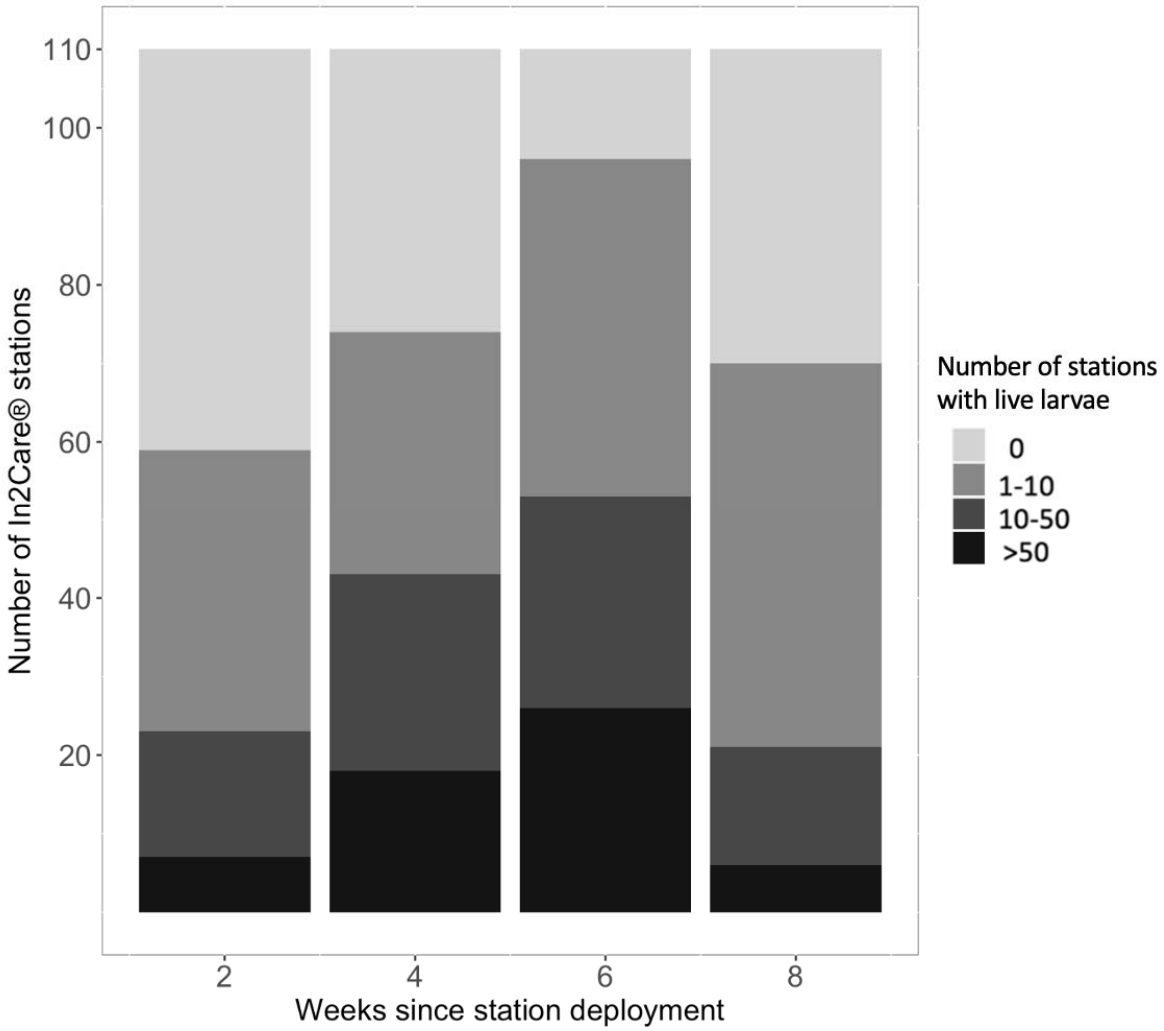


Figure 3 Number of In2Care® stations that presented live larvae since In2Care® stations were deployed: *Number of larvae are represented by different shades of grey, with lighter shades indicating fewer larvae and darker shades more larvae. n = 110 In2Care® stations per week. Fresh water and In2Mix® were added at 4 weeks.*

The numbers of live and dead pupae changed significantly across time (Contingency test: alive: $X^2 = 121.68$, df = 3, $P < 0.001$; dead: $X^2 = 177.82$, df = 3, $P < 0.001$). No In2Care® stations showed any pupae after being in the field for two weeks. After four weeks since In2Care® stations were deployed, 15.45% (95% CI: 14.96% - 15.95) of stations had live pupae in them. This increased to 81.82% (95% CI: 75.0% - 88.6%) after six weeks and 50% (95% CI: 40.43% - 59.57%) after eight

weeks since deployment. Dead pupae were observed in In2Care® stations after six (48.18% (95% CI: 38.4% - 57.9%.) and eight weeks (89.09% (95% CI: 85.04% - 93.14%) of deployment (Figure 4).

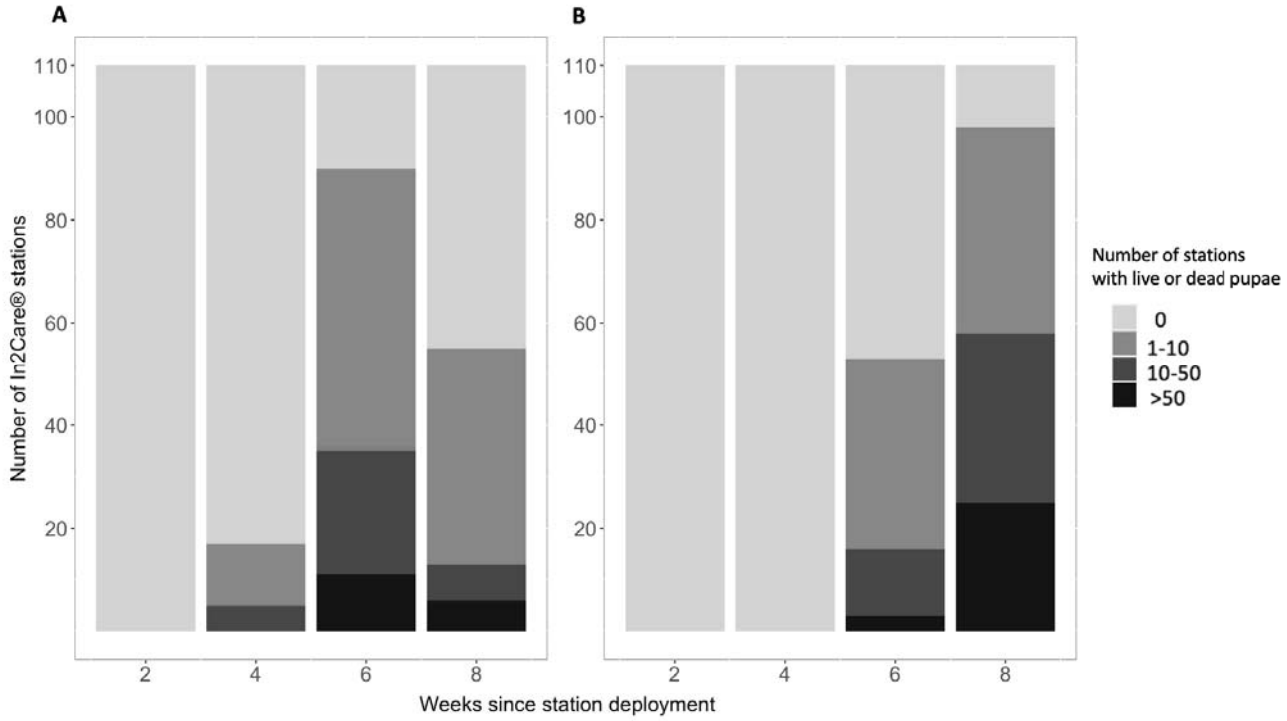
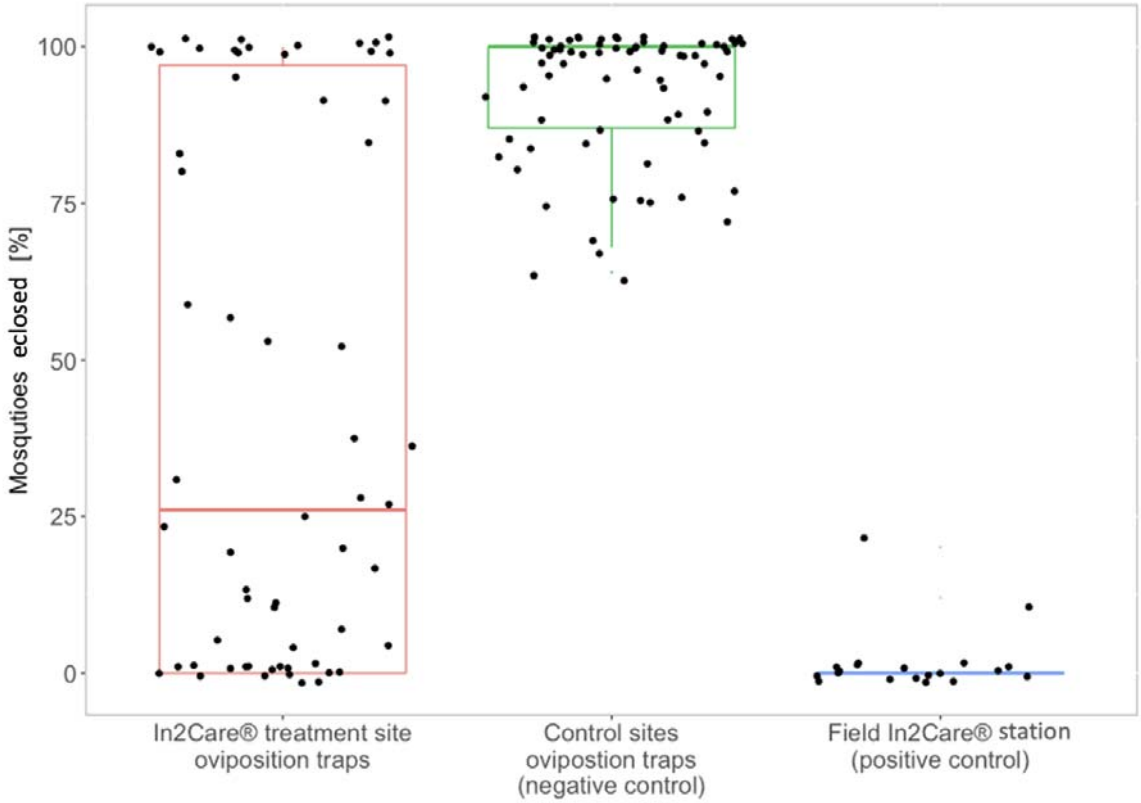


Figure 4 Number of In2Care® stations that presented live pupae (A) and dead pupae (B) since In2Care® stations were deployed: *Number of pupae are represented by different shades of grey, with lighter shades indicating fewer pupae and darker shades more pupae. n = 110 In2Care® stations per week.*

3.2 Autodissemination

Significantly more mosquitoes successfully eclosed from pupae if water samples were collected in the control sites when compared with the samples collected from ovitraps in the In2Care® treatment site (Kruskal-Wallis tests, $X^2 = -41.043$, df = 2, $p < 0.001$) and from samples directly taken from the In2Care® stations ($X^2 = 82.926$, df = 2, $p < 0.001$). Eclosion of mosquitoes from pupae in water samples collected from ovitraps from the treatment site were also significantly higher compared to samples collected from In2Care® stations and showed also a significant difference ($X^2 = 41.925$, df = 2, $p < 0.001$) (Figure 5). This indicates that pyriproxyfen was

280 autodisseminated from the In2Care® stations to the ovitraps. Baseline adult eclosing rates in
281 the control site samples was on average 92%, compared to 42% in the intervention site.



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283 ***Figure 5 Percentage of eclosed mosquitoes from water samples collected during the In2Care®***
284 ***field trial.*** *Eclosed mosquitoes from all collections combined. The red box represents water*
285 *samples that were collected from ovitraps in the In2Care® treatment site (n=60). The green box*
286 *represents water samples collected from control sites (n=64) and the blue box shows water*
287 *samples collected from field In2Care® stations (n=20).*

288 Prolonged autodissemination effects were observed. Water samples collected from ovitraps
289 two weeks after the In2Care® stations were removed from the treatment site showed that
290 *Ae. notoscriptus* larvae had a significantly reduced percentage of eclosed adults compared to
291 samples collected from control sites (Kruskal Wallis test: X^2= 15.83, df = 2, $p < 0.001$). Positive
292 control samples (from In2Care® stations) showed 100% eclosion inhibition, which was
293 significantly higher than the average 55% adult emergence inhibition in the treatment site

294 ovitraps for week 12 and 13 combined (Kruskal Wallis test: X^2= 26.380, df = 2, $p < 0.001$) (Figure
295 6), which also reflects the larvicidal effect of the In2Care® stations.

296 We also tested whether the results differed between the week 12 and week 13 water
297 collections to determine how long the residual effect of pyriproxyfen lasted in ovitraps. We
298 found a significant difference in eclosed adult between the treatment site samples and samples
299 from control sites (Kruskal Wallis test: X^2 = 8.71, df = 2, p = 0.030) when collected two weeks
300 after the removal of the In2Care® stations (week 12). The reductions in adult emergence
301 inhibition were not significantly different in samples collected three weeks after station
302 removal (Kruskal Wallis test: X^2 = 7.92, df = 2, p = 0.260) although eclosion in controls was lower
303 at this time (Figure 6). Baseline adult eclosing rates in the control site samples was on average
304 87%, compared to 55% in the intervention site.

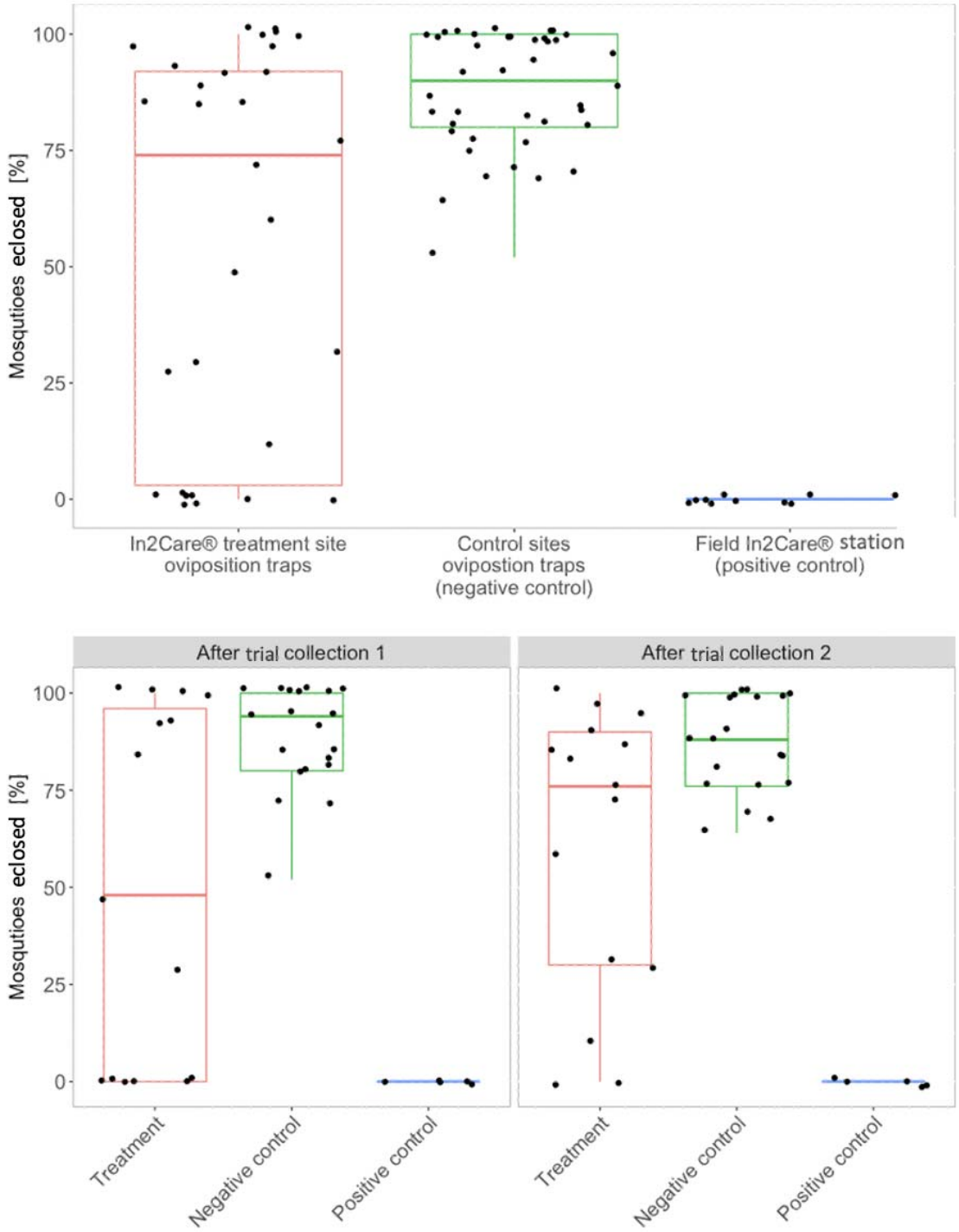


Figure 6 Percentage of eclosed mosquitoes from water samples collected after the In2Care® field trial. (Above) *Both collections combined for week 12 and 13 (2 and 3 weeks after removal of In2Care® Stations from the treatment site). Red boxes represent water samples that were collected from ovitraps in the In2Care® treatment site (n=30). Green boxes represent water*

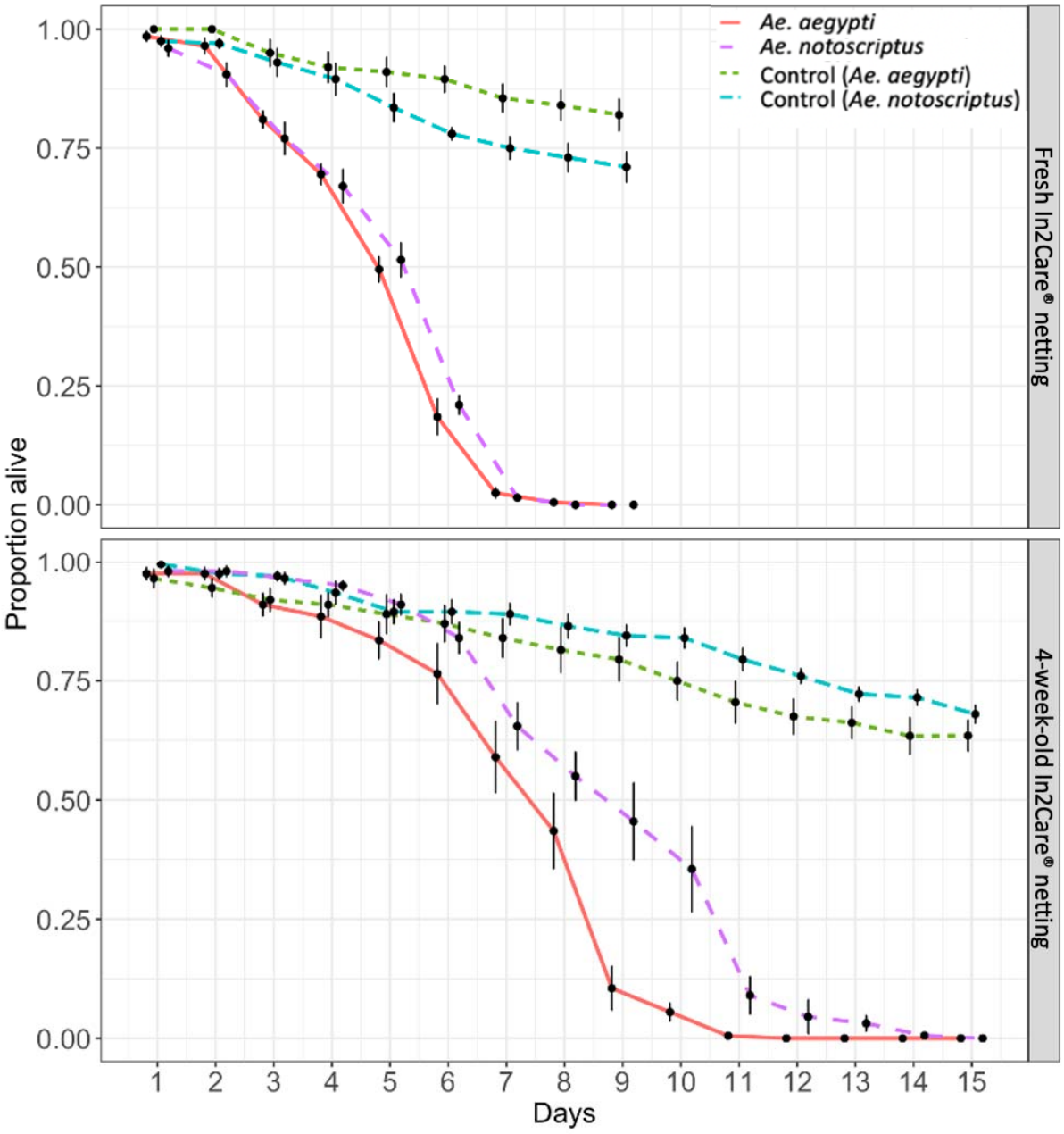
samples collected from control sites (n=40) and blue boxes show water samples collected as positive control from field In2Care® stations in week 10 (n=10). Error bars indicate standard error.

(Below) *Collections two and three weeks after removal of In2Care® Stations from the treatment site separately (In2Care® treatment site n=15; control sites n=20; In2care® stations n=5). Error bars indicate standard error.*

There was no correlation between the proximity of the ovitraps to the closest In2Care® station and to all In2Care® stations (mean distance = ~158 m; longest distance = ~307 m; shortest distance = ~4 m) and the percent of successful eclosing of mosquitoes from collected water samples (Mantel test: $r = -0.05$, $p = 0.983$; Figure S1)

3.3 Efficacy of *B. bassiana* on *Ae. notoscriptus* and *Ae. aegypti*

The results of the Cox-regression analysis indicated that there was a significant effect of the species tested (*Ae. notoscriptus* or *Ae. aegypti)* ($z = -3.53$, $df = 3$, $p = 0.001$), the age of the In2Care® netting (fresh or 4-weeks-old) ($z = -59.49$, $df = 3$, $p < 0.001$), and the treatment (infected or control) ($z = 48.15$, $df = 3$, $p < 0.001$) on survival. On average, survival of *Ae. aegypti* was 9% lower compared to *Ae. notoscriptus* (HR = 0.91 (0.87 – 0.96), $p < 0.001$). Additionally, both species had a four-times higher chance of dying if infected with In2Care® netting compared to uninfected control groups (HR = 4 (3.8 - 4.1), $p < 0.001$). Furthermore, if either species was infected using fresh In2Care® nettings, average survival was reduced by 88% compared to older nettings (HR = 0.12 (0.11 – 0.13), $p < 0.001$) (see Figure 7).



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334 ***Figure 7 Survival of adult* Aedes notoscriptus *and* Aedes aegypti *after exposure to fresh***
335 ***In2Care® netting (above) and nettings that were in the field for four weeks (below).*** *Solid lines*
336 *represent the survival of the treatment groups with* Ae. notoscriptus *in magenta and* Ae. aegypti
337 *in red. Dashed lines show the survival of control groups with* Ae. notoscriptus *in blue and* Ae.
338 aegypti *in green. Data shows averaged proportion of survival from n = 8 replicates per group*
339 *and species. Error bars indicate standard error.*

We calculated an average *Beauveria* spore germination rate of 93.41% (95% CI: 91.18% - 97.79) for the fresh In2Mix® samples with no replicate being lower than 90%. The four-week-old nettings presented an average germination rate of 53.74% (95% CI: 49.92% - 57.71%).

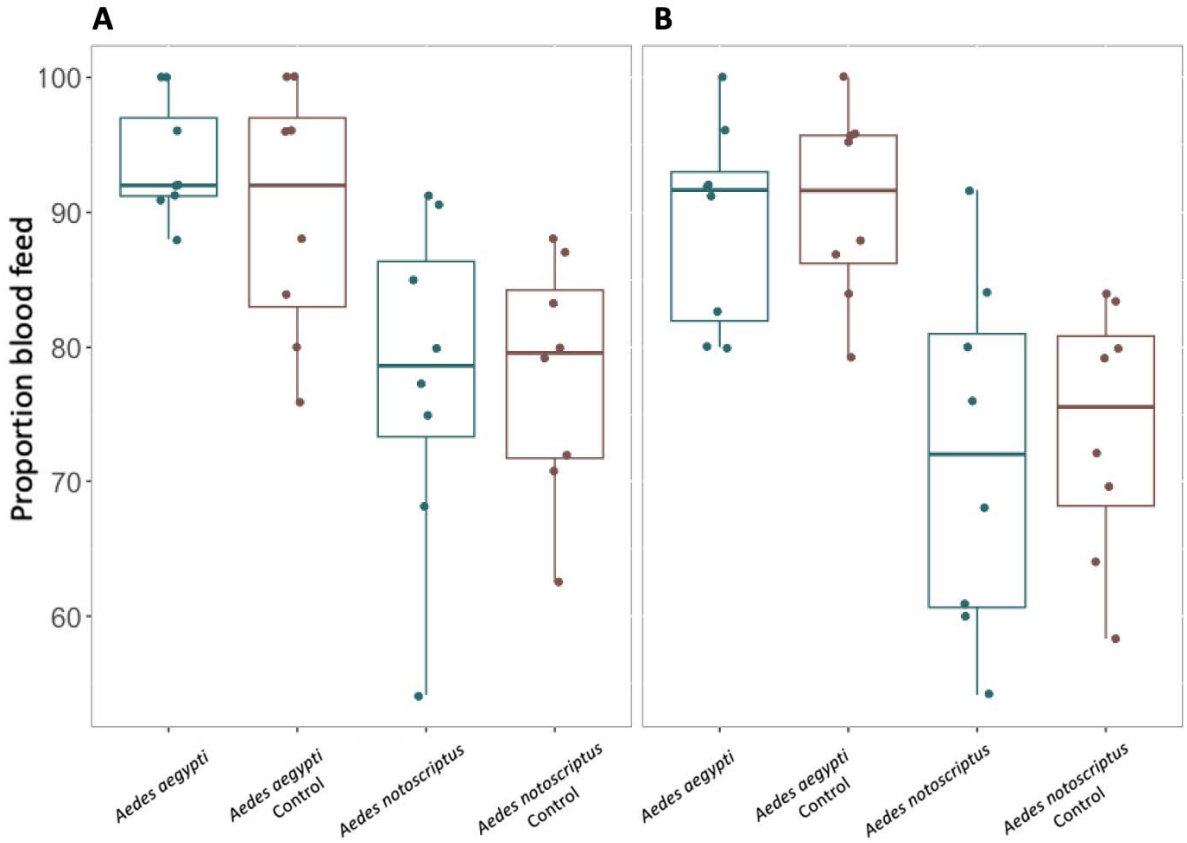

Figure 8 Proportion of mosquitoes that blood feed after* B. bassiana *infection. (A) Fresh In2Care® netting (B) 4-weeks-old In2Care® netting. *Blue boxes indicate the proportion that blood feed of the treatment groups, red boxes indicate the proportion that blood feed in the control groups. Data is averaged from n = 8 replicates per group and species. Error bars show standard error.*

We did not find a significant difference in the proportion of females that blood fed, between the infected and control groups (t-test: fresh In2Care® mix: *Ae. notoscriptus:* t = -0.03, df = 7, p = 0.98; *Ae. aegypti:* t = -0.01, df = 7, p = 0.41; 4-weeks old In2Care® mix: *Ae. notoscriptus:* t = 0.88, df = 7, p = 0.79; *Ae. aegypti:* t = 0-.34, df = 7, p = 0.74) (Figure 8). We did not observe any oviposition by any of the infected groups, while control groups laid eggs.

354 **4. DISCUSSION**

355 Our experiments demonstrated the efficacy of the In2Care® system for control of the container
356 breeding mosquito *Ae. notoscriptus*. We found a reduction in egg numbers in the treatment site
357 after In2Care® stations had been deployed for six weeks with the effect persisting until three
358 weeks after station removal. The autodissemination results indicate that the In2Care® stations
359 were visited by mosquitoes which then successfully contaminated surrounding ovitraps with
360 pyriproxyfen and that the disseminated larvicide persisted at lethal levels for at least two weeks
361 after the In2Care® stations were removed. Additionally, we found that the fungal pathogen
362 *B. bassiana*, which is incorporated in the In2Care® system, significantly decreased the lifespan
363 of mosquitoes in a laboratory setting.

364 The In2Care® stations were successful in attracting mosquitoes as shown by the high proportion
365 of stations that housed larvae (Figure 3). After two weeks of deployment, more than half of the
366 stations contained larvae, which continued to increase over following weeks, before dropping
367 somewhat to 63% after eight weeks. This decrease may reflect a reduction in the local *Ae.*
368 *notoscriptus* population, particularly given that traps had been serviced at the 4-week mark
369 when attraction levels should have been reset. A lower number of new eggs laid in the stations,
370 as well as continued development of existing larvae to the pupal stage, would also have
371 contributed to a decrease in larval numbers. This is further supported by an increase in the
372 number of pupae in the stations after four weeks (Figure 4). The reduction in live pupae is
373 caused by pyriproxyfen-induced mortality, which was observed in about half of the stations at
374 six weeks post-deployment and 87% of stations after eight weeks of deployment. Pupal
375 mortality might have started earlier than detected as larvae in the stations might have fed on
376 dead pupae. These findings also suggest that the In2Care® stations function as an 'egg dump'
377 that contributes to the overall reduction of the mosquito population, as eggs laid in the stations
378 do not mature into adult mosquitoes.

379 Mosquito population sizes naturally fluctuate with rainfall and temperature (Bomblies 2012),
380 with rainfall triggering egg hatching and higher temperatures accelerating development. We
381 found that the general trend of egg counts in all sites followed these weather parameters

382 (Figure 2). However, after six weeks of the In2Care® stations being in place, we observed a
383 significant reduction in egg counts in the treatment site (Figure 2). The decrease in egg numbers
384 at the treatment site cannot be attributed to weather factors, as all control sites displayed
385 increasing egg numbers, while the treatment site showed a decline (Figure 2).

386 Our experiments on water collected from ovitraps at the treatment site confirm the effective
387 autodissemination of pyriproxyfen. The results reveal a significant average decrease of about
388 50% in eclosing of adults in water samples from the treatment site compared to control sites
389 (Figure 5). This finding suggests that mosquitoes carried pyriproxyfen from the In2Care®
390 stations to ovitraps and likely to other surrounding breeding sites. Female *Aedes* mosquitoes
391 tend to distribute their eggs across multiple breeding sites instead of laying them in one place,
392 which helps reduce the risk of losing all offspring if the chosen breeding site proves to be
393 unfavourable (Colton et al. 2003). This strategy of 'skip oviposition' favours the
394 autodissemination of pyriproxyfen, as it increases the number of breeding sites that
395 mosquitoes use. The autodisseminated pyriproxyfen likely contributed to the decrease in adult
396 mosquitoes in the treatment site, with a cumulative effect over multiple generations.
397 Importantly, this positive impact persisted for two weeks even after the removal of the
398 In2Care® stations from the treatment site (Figure 6).

399 The potential for mosquitoes to distribute pyriproxyfen to other parts of the ecosystem where
400 it would influence other insect systems should be considered before widespread application of
401 the technology is implemented, as some lab studies have shown pyriproxyfen transfer by male
402 mosquitoes (artificially dosed with high quantities of pyriproxyfen) to bees during nectar
403 feeding (Kancharlapalli et al. 2021). In2Care® Stations, however, specifically target gravid
404 female *Aedes* mosquitoes that only disseminate pyriproxyfen to water bodies during egg-laying,
405 and these will be less likely to visit flowers for sugar feeding at that stage. Moreover,
406 pyriproxyfen has already been registered and approved globally (including Australia) for
407 widescale agricultural applications given its acceptable risk for non-target organisms and
408 pollinators. If studies confirm these effects are found to be minimal or less damaging than the
409 effects of broadscale mosquito control methods, then implementation of the In2Care® system

410 will be of benefit in reducing the vectoring capacity of *Ae. notoscriptus* and other container
411 breeding mosquitoes.

412 We validated the adulticide effect of *B. bassiana* on *Ae. notoscriptus* and compared them to
413 *Ae. aegypti*, using both fresh In2Mix® and nettings that had been in the field for four weeks.
414 Both species had a 75% higher chance of reduced survival if infected with In2Mix®-treated
415 netting compared to control groups, and using fresh In2Care® nettings significantly reduced the
416 average survival rate of both species by 88% compared to older nettings (Figure 7). These
417 results show that older nettings still infect and kill adult *Aedes* mosquitoes, though their
418 lifespan increases by approximately three to six days, which indicated that the nettings should
419 be refreshed after four to six weeks, as recommended by the manufacturer. Our laboratory
420 investigations suggest that *B. bassiana* used in the In2Care® technology has the potential to be
421 an effective tool in controlling mosquito populations and has most likely contributed to the
422 positive results in our field intervention.

423 Our results are consistent with studies investigating the effect of the In2Care® system on
424 *Ae. aegypti, Ae. albopictus* and *Culex quinquefasciatus* (Buckner *et al.* 2017; Buckner *et al.* 2021;
425 Khater *et al.* 2022; Su et al. 2021) and suggest that the In2Care® stations successfully kill larvae
426 of container breeding mosquitoes by spreading pyriproxyfen to surrounding breeding sites. The
427 In2Care® system, in conjunction with source reduction and other integrated vector
428 management tools, has potential to provide sustained vector control and reduce transmission
429 of *Aedes*-borne arboviruses, parasites, and mechanically transmitted bacteria. Benefits of the
430 In2Care® system to public health may extend beyond the reduction of mosquito vectors.
431 Broadscale insecticide application may be obviated in an In2Care®-treated area, thereby
432 reducing public exposure as well as off-target environmental impacts.

433 Though the results present a promising demonstration of the potential for the In2Care® system
434 for *Ae. notoscriptus* control, there may be some challenges in implementing the approach in
435 other areas. In our trial, the treatment and control sites were relatively isolated, reducing the
436 potential for migration of mosquitoes back into the treated area. Implementation of the
437 strategy in less isolated areas may require the treatment of larger areas. Our study took place

438 in a relatively new developed area, where most houses are occupied and there are few empty
439 blocks or construction sites, making it easier to place traps. We did identify a few mosquito
440 breeding sources (mostly bird baths or drip trays of pot plants), but the area is generally well
441 maintained, reducing the likely incidence of cryptic breeding sites. In areas with a low
442 occupation rate of houses, there may be an increased risk of overflowing gutters and containers
443 in backyards that can act as breeding sites. It is worth noting that in a study utilising the
444 In2Care® system conducted in Hawaii (Brisco et al. 2023), a failure to find a reduction in
445 mosquito numbers was partly attributed to numerous cryptic breeding sites in properties that
446 did not participate in the intervention (in addition to an inadequate number of In2Care®
447 stations being deployed), and source reduction measures may be required in some contexts.

448 **5. CONCLUSION**

449 The In2Care® system was found to be effective in controlling a population of the container
450 breeding mosquito *Ae. notoscriptus* in Victoria, Australia. The system demonstrated a reduction
451 in egg numbers in the treatment site after six weeks of deployment. The autodissemination
452 results indicate that the In2Care® stations were visited by mosquitoes which then successfully
453 contaminated ovitraps with pyriproxyfen, and the larvicidal effect persisted for at least three
454 weeks after the In2Care® stations were removed. The experiments validated the adulticide
455 effect of the fungal pathogen *B. bassiana* on *Ae. notoscriptus* and *Ae. aegypti* and suggest that
456 it has the potential to be an effective tool in controlling mosquito populations.

457

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