Ascogregarina Taiwanensis Interfere in the Performance of Aedes Albopictus and in the Susceptibility of Aedes Aegypti to Temephos and Azadirachta Indica Oil

Josiane Somariva Prophiro
Unisul: Universidade do Sul de Santa Catarina
Thiago Nunes Pereira
UFRGS: Universidade Federal do Rio Grande do Sul
Joice Guilherme de Oliveira
Unisul: Universidade do Sul de Santa Catarina
Felipe Allan Silva da Costa
Unisul: Universidade do Sul de Santa Catarina
Harry Luiz Pilz Júnior (harry.pilz@ufrgs.br)
Universidade Federal do Rio Grande do Sul
Alessandra Bittencourt de Lemos
UFRGS: Universidade Federal do Rio Grande do Sul
Onilda Santos da Silva
UFRGS: Universidade Federal do Rio Grande do Sul
Mario Antônio Navarro da Silva
UFPR: Universidade Federal do Parana

Research

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Abstract

Background: *Aedes albopictus* and *Aedes aegypti* are mosquitoes commonly adapted to tropical and subtropical regions. These vectors can transmit different types of arboviruses causing a serious concern to public health. New alternatives for the vector/arboviruses control are emerging, and in this sense the protozoan *Ascogregarina taiwanensis* may present potential as a biological control agent against these mosquitoes.

Methods: To evaluate the effects of protozoan *A. taiwanensis*, mosquitoes were parasitized with a solution containing oocysts and evaluated to lifetime, fertility, fecundity for *Ae. albopictus* and for *Ae. aegypti* interaction with *Azadirachta indica* and Temephos.

Results: In this work it was possible to observe the protozoan morphology in mosquitoes *Ae. albopictus*, as well its negative influence on mortality, 73% and non-parasitized was 44%. The number of eggs oviposited by parasitized females of *Ae. albopictus* was lower (3,490) than for the non-parasitized females (5,586). In addition, the hatchability and/or viability of these eggs were also lower for the parasitized females (63%) than the non-parasitized ones (74%). For *Ae. aegypti* mosquitoes, a synergism between the use of *A. taiwanensis* associated with a chemical insecticide and a botanical insecticide was observed. The results demonstrate that when *Ae. aegypti* larvae was parasitized by *A. taiwanensis* and exposed to the oil of *Az. indica* or to the organophosphate Temephos present a greater mortality.

Conclusion: It was notable that *A. taiwanensis* can be a potential for biological control and adjuvant of insecticides. We also provide important information about the maintenance of *A. taiwanensis* in laboratory.

Background

Every year, around 17% of all infection diseases are caused by vector-borne diseases [1]. Among these vectors, the mosquitoes have playing the main role in the transmission of several arboviruses like dengue, Zika and chikungunya [2–6].

Aedes aegypti and *Aedes albopictus* are worldwide distributed [7] and have highly anthropophilic and opportunistic behavior [8, 9]. As competent vectors for several human arboviruses, these mosquito species are responsible for major public health concern.

Different methods for mosquito control have been suggested, and these methods can be classified as biological, genetic, environmental, mechanical and chemical [10]. Meanwhile, due to the problems surrounding arboviruses in recent years and the resistant selection of some mosquito populations through continuous insecticides use [11, 12], alternative methods for vector control must be thought. Studies have shown that the synergism between microorganisms and chemical insecticides can be useful when comparing to the exclusive insecticide use [13, 14]. In addition, several other methods of control including with microorganisms have been proposed in the last years, such as: growth regulators,

chitin synthesis inhibitors and behavior modifiers that can be influenced by virus, bacteria, fungi and protozoa [15–23].

Gregarines are protozoan that can naturally parasite a huge variety of insects [24]. Among these insects, some species of mosquito can harbor some gregarines bellowing to the genus *Ascogregarina* (Eugregarinida: Lecudinidae) [25]. In this way, [26] have proposed that gregarine parasite can interfere negatively in their biological host development and this influence depends on their environmental distribution.

The *Ascogregarina taiwanensis* has been frequently described as having different grades of pathogenicity to *Ae. albopictus* [26–28], and *Ascogregarina culicis* is considered a parasite for *Ae. aegypti* [26, 29, 30]. Regardless of specificity of these *Ascogregarina* species to *Aedes* mosquitoes, *A. taiwanensis* was already found parasitizing *Ae. aegypti* and *Ae. albopictus* in south Brazil (Prophiro *et al*. 2017).

Studies using *A. taiwanensis* as biological control and its influence in biological development of mosquitoes must be better understood, besides that the knowledge of laboratory maintenance of this protozoa is poorly known. So, after the encounter of *Ae. albopictus* and *Ae. aegypti* harbouring *A. taiwanensis* in south Brazil [31], we established this protozoan in laboratory conditions. So, we could evaluate the influence of this gregarine on some biological aspects of *Ae. albopictus*. Besides that, we induced their parasitism in *Ae. aegypti* in order to evaluated it susceptibility to insecticides after being infected. Such study could provide new information about the parasitism of *A. taiwanensis* in *Aedes* mosquitoes contributing for the studies in control of these vectors.

Material And Methods

Mosquito strains

Two mosquito strains were used in this study: *Ae. aegypti* (Rockefeller) and a non- parasitized *Ae. albopictus* collected in the field. Larvae were fed using pet food (Purina® Cat Chow®) 200 mg/mL, three times a week. Adult mosquitoes were reared under a 14 h light/10 h dark photoperiod, at 25°C in an incubator (132FC ELETROlab®). Honey solution (10% w/v) was continuously provided to adult males and females, while females were blood-fed on mice *Mus musculus* (Ethic Committee of Animals – 19843), twice a week in order to obtain eggs for the bioassays and colony development.

Ascogregarina taiwanensis **reared in laboratory**

Aedes albopictus larvae naturally harboring *A. taiwanensis* (GenBank, NCBC KM387708) were collected from traps (plastic pots and tires) in Tubarão/SC - Brazil in the year of 2014 and brought to the laboratory (Prophiro et al. 2017). The emerged adults were kept under controlled conditions as described below. After blood feeding, an artificial breading place containing 500 mL of water was offered to female for oviposition, and consequently where oocysts could be released, and posteriorly parasite new healthy

larvae. To become parasitized, these larvae were separated in two groups: one group with all larval stages together and one group with larvae separated by stage. The confirmation of *A. taiwanensis* infection in the new generations of mosquitoes was carried out based on morphology of parasites, according to Prophiro (2017). This new generation of *Ae. albopictus* harboring *A. taiwanensis* was used to infect a laboratory reared *Ae. aegypti* and a field collected *Ae. albopictus* in order to conduct the bioassays described below. This work was registered by Brazilian Genetic System SISGEN (A11AEC2).

Maintenance of Ascogregarina taiwanensis **in laboratory**

As cited above the oocysts of *A. taiwanensis* used for the infection of *Aedes* mosquitoes came from an adult colony of *Ae. albopictus* collected in the field naturally parasitized. A solution containing oocysts was produced using a similar method described in Beier and Craig (1985), where 100 parasitized adults were homogenized in 100 mL of filtered water, and posteriorly diluted in 3 liters of water containing *Ae. albopictus* and/or *Ae. aegypti* larvae to infect such populations. After 24 hours of infection, the larvae were transferred to other container containing filtered water, in order to avoid reinfection in different days.

Morphology of Ascogregarina taiwanensis **in different stages of vector Ae. albopictus**

After infection, a sample of 50 larvae (2nd, 3^{rd,} and 4th instars), 50 pupae and 50 adults (25 males and 25 females) of *Ae. albopictus* obtained through artificial transmission was dissected to confirm the presence of the protozoa and photographed in Microscopic photographs (OLYMPUS CX31-P and ZEISS STEMI 200 C).

Influence of Ascogregarina taiwanensis **on the performance of Aedes albopictus**

After obtaining the *Ae. albopictus* population harboring *A. taiwanensis* (15 females and 15 males) were separated in three cages (30 x 30 x 30 cm). The same was made for control group (without *A. taiwanensis)*, totaling six cages with 180 mosquitoes. Each group was treated with 10% honey solution.

After oviposition of females the eggs were counted and conditioned in a climatized room. Three weeks after oviposition, the eggs were placed in plastic trays with water and food for stimulating larvae hatching. The development through larvae to the adult stage were monitored daily and the longevity of these mosquitoes was monitored every 48h. This experiment was carried out three times at different days.

Susceptibility of Aedes aegypti **to insecticides when parasitized with** Ascogregarina taiwanensis

In these bioassay two insecticides were used: Temephos technical grade 96% lot #SZBD128XV manufactured by the laboratory "Fluka Analytical", St. Louis, MO 63103 – USA, and *Azadiracta indica* (Neem oil) lot 44796-04 manufactured by the laboratory "Handa Fine Chemicals", West Sussex - USA. Both insecticides were calibrated with *Ae. aegypti* Rockefeller strain.

For the bioassays third instar late and early fourth instar larvae of *Ae. aegypti* were used in two groups: non-parasitized (control group) and parasitized with *A. taiwanensis*. Three replicates of 15 larvae, totaling 45 larvae/concentration + 15 control larvae were exposed to six different concentrations of Temephos (0.009–0.024 ppm) or *Az. indica* oil (14–169 ppm) in 100 mL of solution. A total of 90 larvae for each product and population were exposed to solvent ethanol and Tween 80 (polysorbate) as control. Larval mortality was verified after 24h of exposure. Moribund larvae and unable to reach the surface of the water when touched with a needle were considered dead (WHO, 1981). The surviving larvae were discarded, and the bioassays were reproduced three times on different days for each product.

Statistical analysis

Kruskal-Wallis non-parametric test (KW) was used to detect differences in the treatments in relation to the different generations and strains. When the differences were detected, the Multiple Comparisons test was applied through the STATISTICA 7.0 program, with significance level $P < 0.05$. The Probit GW-Basic program was used to determine lethal concentrations. Two-way ANOVA of GraphPad Prism 5.03 was used to analyze the results, with a significance level of 5%. A t test was used to compared differences between oviposition (parasitized and non-parasitized) and in viability of these eggs.

Results

Aedes albopictus **infection by** Ascogregarina taiwanensis

The morphology of trophozoites usually had appearance of comma or was rounded. The average size of this protozoan was: second instar (58.5 μm), third instar (77 μm) and the fourth instar (168.1 μm). The location of the trophozoites was normally observed at the end of midgut, next to the Malpighian tubule. The parasites could be observed in second instar larvae (Fig. 1A and B), third instar (Fig. 1C and D), fourth instar (Fig. 1E and F), and pupal stage (Fig. 1G and H). The presence of the gametocytes in the adults was also observed (Fig. 1I). Due to the small size of the first instar larvae of *Ae. albopictus*, only in 2nd, 3^{rd,} and 4th instars *A. taiwanensis* could be observed.

When larvae of the second group were separated by stage and exposed to infection by oocysts resulting from the macerate of parasitized adult of *Ae. albopictus*, the second and third instar demonstrated greater potentiality of being parasitized, showing 100% of infection. The fourth instar larvae did not show any trophozoite in their digestive system. May be that this larva stage does not provide a viable time to the development of oocysts into trophozoites, because in a short time (about 48 hours) turns into pupa.

Influence of Ascogregarina taiwanensis **on the performance of** Aedes albopictus

The population of *Ae. albopictus* parasitized by *A. taiwanensis* showed shorter period of longevity when compared the non-parasitized population (Fig. 2). Significant differences were observed in mortality among the parasitized and non-parasitized population (independent of sex) ($KW = 12.25$, $gl = 1$, $P < 0.05$,

X^2 = 6.13, *P* = 0.0005). No significant differences were observed in mortality over the days analyzed, both in the parasitized and non-parasitized populations (*P* = 0.41 and *P* = 0.47, respectively).

The number of eggs oviposited by parasitized females of *Ae. albopictus* was lower than for the non-parasitized females, however there is no significant differences between them (Table 1). In addition, the hatchability and/or viability of these eggs were also lower for the parasitized females than the non-parasitized ones (Table 1). Significant differences were observed in egg viability of the parasitized and non-parasitized populations (*p* = 0.0143). These results are like those obtained by Comiskey *et al*. (1999), were *Ae. albopictus* parasitized by *Ascogregarina* sp., presented a decrease in the reproductive capacity of females, even with high nutrient conditions.

Table 1
– Number of eggs laid by females of *Aedes albopictus* ant their viability comparing groups parasitized and non- parasitized with *Ascogregarina taiwanensis.* Numbers are showed by the median, the upper and lower limit.

	Number of eggs	***p***	**Hatched eggs**	***p***
Parasitized	1.134 (1.089–1.267)	0.148	721 (634–852)	< 0.05
Non-parasitized	1.765 (1.189–1.932)		1.343 (1.332–1.478)	

Susceptibility of Aedes aegypti **to insecticides when parasitized with** Ascogregarina taiwanensis

In our bioassays there was higher larval mortality of *Ae. aegypti* after exposure to *Az. indica* oil, when this vector was parasitized by *A. taiwanensis* (Fig. 3A). In the presence of the parasite, the LC_{50} was 0.815 mg/L whereas in the non-parasitized group the LC_{50} was 1,812 mg/L. The results showed that there was a significant difference between the values of mortality comparing the parasitized and non-parasitized group (*P* < 0.001). There was no mortality in the control groups (polysorbate and water).

For Temephos treatment, a higher mortality was also observed where there was synergism between the protozoan *A. taiwanensis* and Temephos (Fig. 3B). In the presence of the parasite the LC_{50} was 0.025 mg/L whereas without the parasite the LC_{50} was 0.063 mg/L. The results showed that there was a significant difference between the values of mortality comparing the parasitized and non-parasitized group (*P* < 0.001). There was no mortality in the control groups (ethanol and water).

Discussion

In this work we demonstrate that when *Ae. aegypti* larvae was parasitized by *A. taiwanensis* and exposed to the oil of *Az. indica* or to the organophosphate Temephos induce a higher mortality. Mosquitos' mortality (parasitized males and females) was 73%, while mortality of the non-parasitized was 44%. These results are like those reported by [32, 33], which obtained reduction in the longevity for *Ae. aegypti* parasitized by *A. culicis* and *Ochlerotatus triseriatus* parasitized by *Ascogregarina barretti*. These authors also observed prolongation of the larval stage and reduction of adult size for both species of mosquitoes

[32, 33]. [34], observed that *Ae. albopictus* parasitized by *Ascogregarina* sp. presented higher mortality of immature stages when larvae were under nutrient. These morphological observations in all protozoa stages were like that found by Lien and Levin (1980).

These results are like those obtained by Comiskey *et al*. (1999), were *Ae. albopictus* parasitized by *Ascogregarina* sp., presented a decrease in the reproductive capacity of females, even with high nutrient conditions. The synergism between microorganisms and insecticides inducing higher mortality was also reported by [14]. This author verified that when larvae of *Ae. aegypti* were exposed to *Az. indica* and the fungus *Metarhizium anisopliae* (5×10^5 conidia/mL) presented higher mortality. Similarly, [13] observed that when *Bacillus thuringiensis* var. *israelensis* is used with Temephos in *Ae. aegypti*, a 90% greater larval mortality is obtained in the first hour of exposure, compared to the group treated with Temephos alone.

The compounds of *Az. indica* have several forms of action, which may act in an antiparasitic, antihelmintic, antimicrobial and other forms [35, 36]. In the present work, the higher mortality of parasitized *Ae. aegypti* when exposed to *Az. indica* may be related to antiparasitic action. According to [37], extreme variations of physiological conditions in association with parasitic infection can cause necrosis in the cells, resulting in direct damage to the plasma membranes of the host. Thus, we can suggest that if there was an antiparasitic action of *Az. indica* on the gregarine facilitated the insecticidal activity of this oil on the larvae.

Although the *A. indica* concentration is higher than Temephos in the dosage values, it is noteworthy that there are reports that the survival of *Ae. aegypti* exposed to more than 0.02 mg/L of Temephos indicates the possibility of resistance among the population tested (Brown, 1986; Denham *et al*. 2015; Arslan *et al*. 2015).

According to [38] new methods for *Aedes* vector control aimed at reducing the use of chemical insecticides should be urgently prioritized. Thus, we believe that integrated and interleaved control may also reduce the pressure on the selection of individuals who are resistant to routinely used chemical insecticides. The results obtained, indicate that *A. taiwanensis* negatively influences its host, in this case both *Ae. albopictus* as *Ae. aegypti*. In this way, we believe that this gregarine has potential for biological control of vectors.

The synergism between microorganisms and insecticides inducing higher mortality was also reported by [14]. This author verified that when larvae of *Ae. aegypti* were exposed to *Az. indica* and the fungus *Metarhizium anisopliae* (5×10^5 conidia/mL) presented higher mortality. Similarly, Andrande and Modolo (1991) observed that when *Bacillus thuringiensis* var. *israelensis* is used with Temephos in *Ae. aegypti*, a 90% greater larval mortality is obtained in the first hour of exposure, compared to the group treated with Temephos alone. Interestingly, it has also been reported that *Ae. albopictus* infected with Ascogregarina reduces its competitiveness in the habitat with different larvae such as *Ae. triseriatus* [39]. In addition, it

has already been shown that the propitious infection by Ascogregarinas can impact the *Ae. Albopicuts* microbiota. [40].

The compounds of *Az. indica* have several forms of action, which may act in an antiparasitic, antihelmintic, antimicrobial and other forms [35, 36]. In the present work, the higher mortality of parasitized *Ae. aegypti* when exposed to *Az. indica* may be related to antiparasitic action. According to Golstein and Kroemer (2007), extreme variations of physiological conditions in association with parasitic infection can cause necrosis in the cells, resulting in direct damage to the plasma membranes of the host. Thus, we can suggest that if there was an antiparasitic action of *Az. indica* on the gregarine facilitated the insecticidal activity of this oil on the larvae. Although the *A. indica* concentration is higher than Temephos in the dosage values, it is noteworthy that there are reports that the survival of *Ae. aegypti* exposed to more than 0.02 mg/L of Temephos indicates the possibility of resistance among the population tested [41–43]

According to Guirado and Bicudo, (2009) new methods for *Aedes* vector control aimed at reducing the use of chemical insecticides should be urgently prioritized. Thus, we believe that integrated and interleaved control may also reduce the pressure on the selection of individuals who are resistant to routinely used chemical insecticides. The results obtained, indicate that *A. taiwanensis* negatively influences its host, in this case both *Ae. albopictus* as *Ae. aegypti*. In this way, we believe that this gregarine has potential for biological control of vectors.

Conclusions

In this work we demonstrate the parasitism capacity of *Ascogregarina taiwanensis* in *Aedes albopictus* and show its impact on the different stages of development of the mosquito, we show the decrease in its longevity, quantity of eggs and hatching. We also show that larvae of *Aedes aegypti* parsitated with the protozoan, have a synergistic effect with Temephos and *Azadiracta indica* oil, increasing mortality and decreasing their lethal concentration. We believe that this is another indication of the use of new biological agents for vector control and that its use can open new fields for research and development of tools for its integrated control.

Abbreviations

LC lethal concentration

Declarations

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Ethics approval and consent to participate

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Consent for publication

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Availability of data and material

Not applicable

Competing interests

The authors declare that there is no conflict of interest

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Authors' contributions

JSP and MANS conceived the experiments; TNP, JGO, FASC and JSP conducted the experiments; JSP, OSS, HLPJ, ABL analyzed the results; JSP, OSS, HLPJ and ABL wrote the paper. All authors read and approved the final manuscript.

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Figures

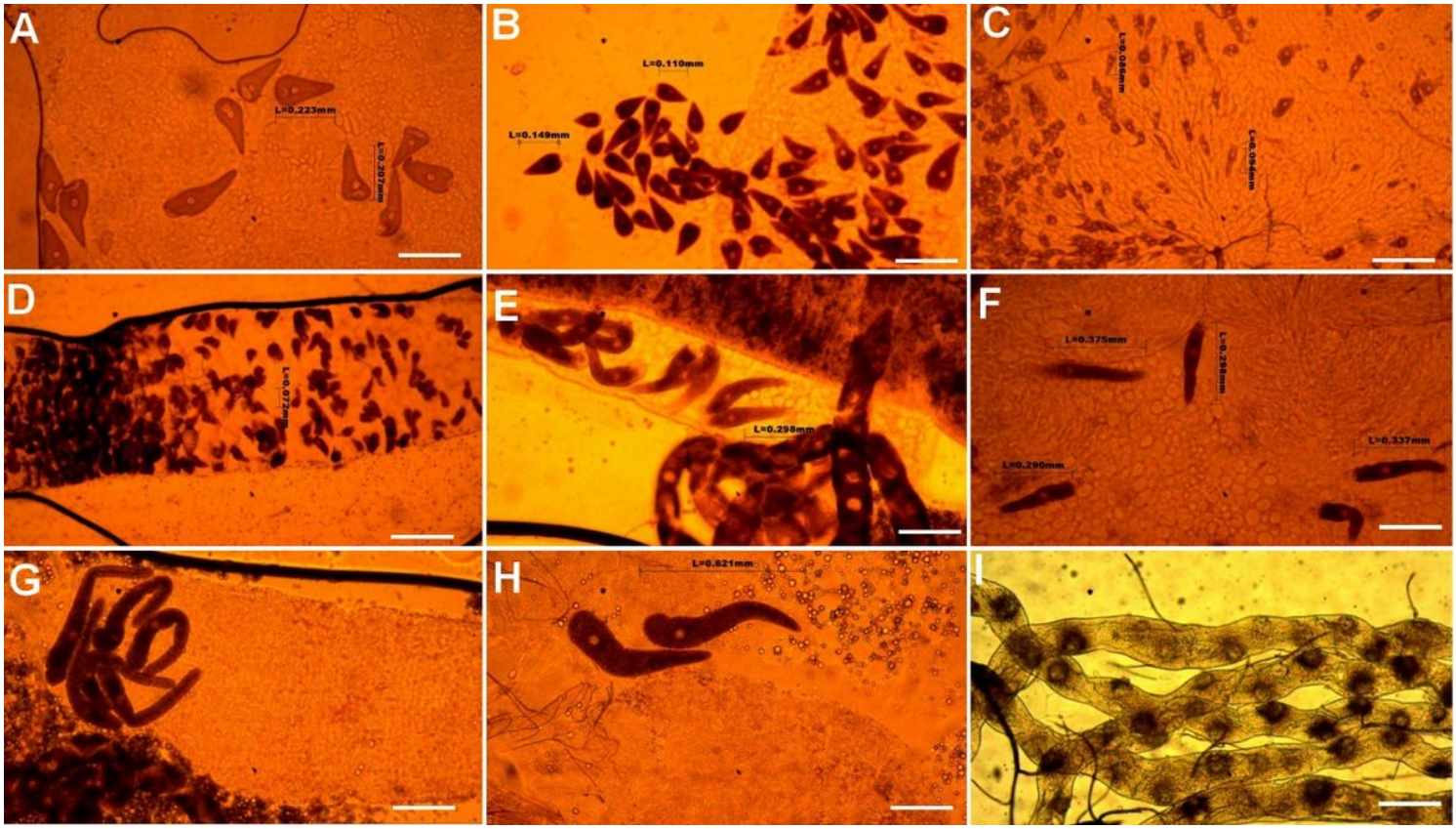

Figure 1

Ascogregarina taiwanensis in different stages of Aedes albopictus. (A and B) Digestive tract of 2nd instar larvae parasitized with trophozoites; (C and D) Digestive tract of 3rd instar larvae parasitized with trophozoites; (E and F) Digestive tract of 4th instar larvae parasitized with trophozoites; (G and H) Digestive tract of pupae parasitized with trophozoites; (I) Digestive tract (Malpighi tubules) of the adult stage infected by trophozoites and/or gametocytes. The samples were photographed through the VMS3 program 5. All scale bars represent 200 μm.

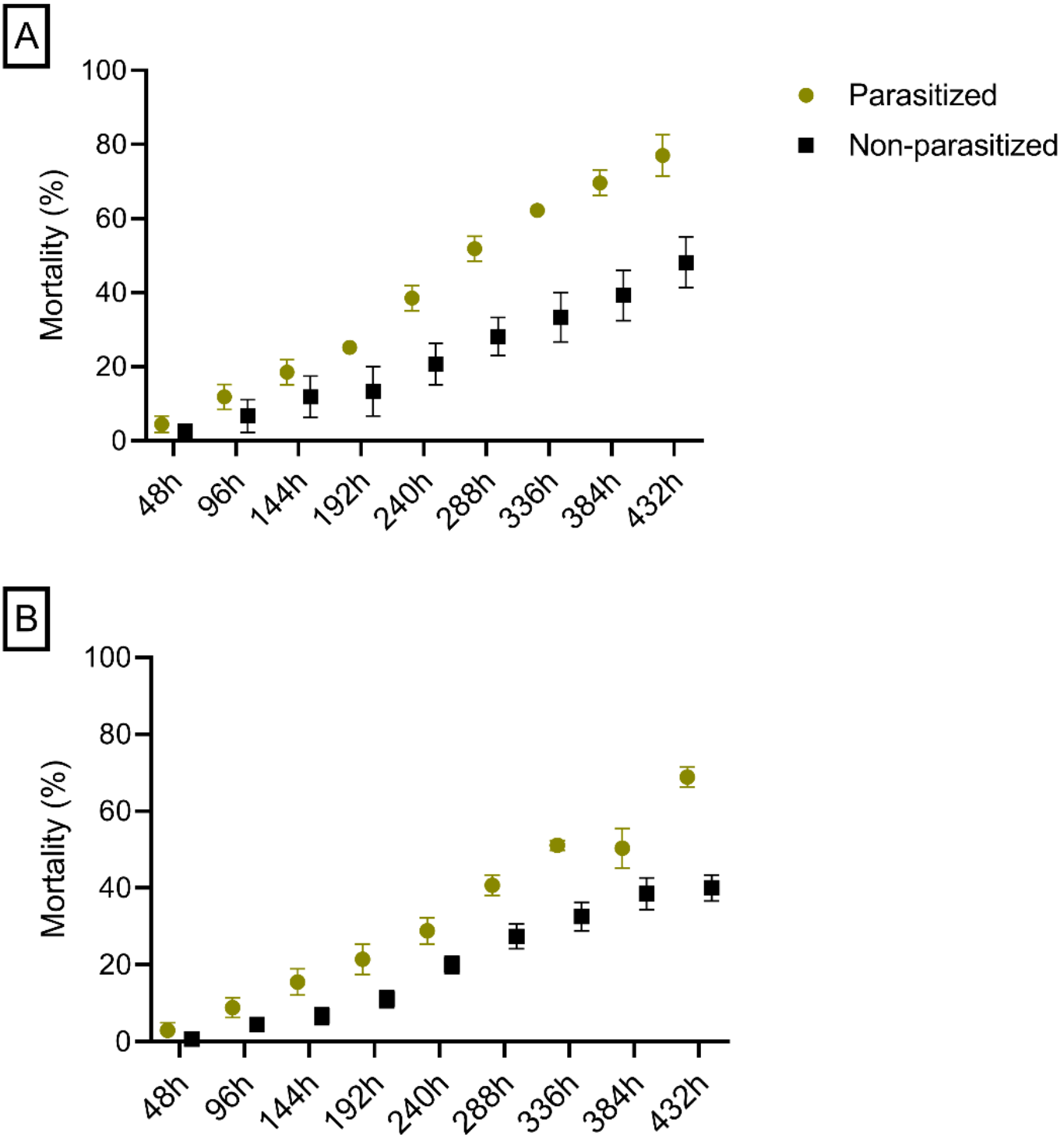

Figure 2

Mortality of Aedes albopictus parasitized with Ascogregarina taiwanensis. Accumulated mortality of Aedes albopictus parasitized and non-parasitized with Ascogregarina taiwanensis, between 48 and 423 hours. Males (A) and Females (B). Bars represent 95% CI.

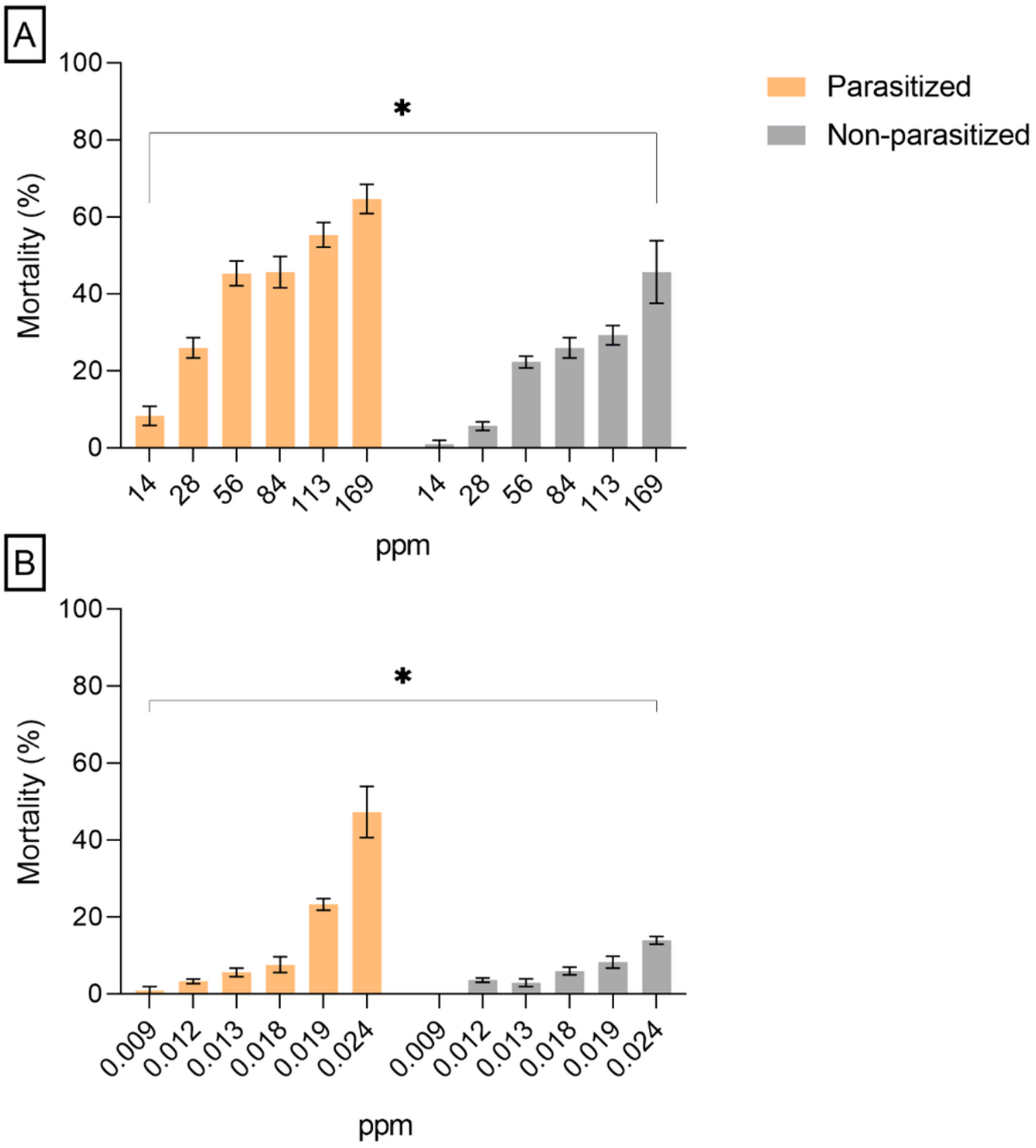

Figure 3

Mortality of Aedes aegypti parasitized with Ascogrgarina taiwanensis. Comparison of the susceptibility rate to Azadirachta indica oil (A) and Temephos (B), in two groups of Aedes aegypti parasitized and non-parasitized with Ascogrgarina taiwanensis.

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