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ECOSYSTEMS

Presence of *Ascogregarina culicis* and *Ascogregarina* sp. in natural sympatric populations of *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae) in Argentina

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Abstract: *Aedes aegypti* is the main vector of the four arboviruses in America which have the greatest impact on human health. The introduction of *Aedes albopictus* in South America and Argentina acquires importance given the possibility that this species may be a new vector of arboviruses in this region. For this reason, the studies of the biology of their parasites, such as *Ascogregarina* spp., should be important for the knowledge of the invasive behavior of these vectors. We reported the finding of *Ascogregarina culicis* in *Aedes aegypti* and *Ascogregarina* sp. in *Ae. albopictus* populations in subtropical Argentina. The prevalence of parasitism by *A. culicis* in *Ae. aegypti* and *Ascogregrarina* sp. in *Ae. albopictus* was 34.81% (n = 464) and 37.23% (n = 70), respectively, differing between the seasons and habitats. The infection intensity caused by *A. culicis* and *Ascogregarina* sp. varied between 1 to 250 and 1 to 327 trophozoites respectively. *Ascogregarina culicis* was found throughout the all sampling period of *Ae. aegypti* (June 2016-April 2018). However the presence of *Ascogregarina* sp. in the midgut of *Ae. albopictus* was not recorded throughout the whole sampling period despite the presence of the host.

Key words: Ascogregarina, Culicidae, northeastern Argentina, South America, protozoa.

INTRODUCTION

Members of the genus *Ascogregarina* (Apicomplexa: Lecudinidae) are intracellular parasite protozoa of which nine species are parasites of mosquitoes (Culicidae). They are considered mostly non-pathogenic for their natural hosts but are harmful to others (Lantova & Volf 2014). *Ascogregarina culicis* (Ross) parasite the mosquito species *Aedes aegypti* Linnaeus, whereas *Aedes albopictus* (Skuse) is parasitized by *Ascogregarina taiwanensis* (Lien and Levine) (Lien & Levine 1980, Fukuda et al. 1997).

The mosquito larvae become infected when ingesting oocysts present in the water where they develop. Each oocyst consists of eight sporozoites that are released into the gut when they are ingested by the hosts, invading epithelial cells. Within the cells, the sporozoites become trophozoites and are released into the gut lumen when the epithelial cell is broken. There, they become attached to the gut epithelial cells and then, during the pupa stage, they migrate to the Malpighian tubules where they reproduce sexually forming oocysts in the adult mosquitoes. These oocysts are eventually released into the water during female oviposition or at the death of the infected adult mosquito in the habitat (Lantova & Volf 2014).

The competition through differential infection by *Ascogregrarina* is one of the hypotheses that would explain the phenomenon of displacement or competition between *Ae. aegypti* and *Ae. albopictus*. This hypothesis

is based on the differential pathogenicity or asymmetric effects caused by *A. taiwanensis* (a natural parasite of *Ae. albopictus*), that by infecting *Ae. aegypti*, it would cause a decrease its density. On the contrary, *A. culicis* would not infect *Ae. albopictus* (Munstermann & Wesson 1990, Blackmore et al. 1995, Juliano 1998, Reyes-Villanueva et al. 2001).

Both gregarines are similar morphologically, although they can be differentiated through trophozoites shape. This classification is based on host specificity and morphological characteristics (Reyes-Villanueva et al. 2001, Pereira et al. 2018). In addition, development of molecular tools also allow their identification (Morales et al. 2005).

Nowadays *Ae. aegypti* is the main vector of the four arboviruses in America which have the greatest impact on human health in the region: the yellow fever virus (YFV), dengue virus (DENV), chikungunya virus (CHYKV) and Zika virus (ZIKV) (Souza-Neto et al. 2019). The introduction of *Ae. albopictus* in Argentina (Rossi et al. 1999), a species native to Southeast Asia, leave open the possibility that this species might participate as a new vector in the transmission of arbovirosis in the northeastern region of the country. Different experiments have demonstrated that *Ae. albopictus* populations from Brazil and others American countries are highly competent at transmitting Dengue (DENV), Yellow fever (YFV) and Chikungunya (CHIKV) (Mitchell et al. 1987, Miller et al. 1989, Vega-Rúa et al. 2014).

As regard the *Ascogregarina* species, studies on this genus of protozoa in South America are scarce and most of them correspond to Brazil (Passos & Tadei 2008, Prophiro et al. 2017) and the temperate zone in Argentina (Vezzani & Wisnivesky 2006, Albicócco & Vezzani 2009). Likewise, researches in Argentina has focused on localities where *Ae. aegypti* is present, without the presence of *Ae. albopictus*.

The aim of the present study was to detect the presence of *Ascogregarina* in sympatric populations of *Ae. aegypti* and *Ae. albopictus* collected in different types of habitats, and in different seasons of the year, in Eldorado city, Misiones, Argentina, as well as, to obtain data on the prevalence and intensity of infection of these parasites.

MATERIALS AND METHODS

Study area

Eldorado city (26°24' S; 54°38' W, at 212 m) (Misiones province, northeastern Argentina) (Figure 1) is located in the Paranaense phytogeographic province, belonging to the Interior Atlantic Forest. It is characterized by three arboreal strata with lianas, epiphytes and hemiepiphytes, and an understory of ferns, herbaceous and bushy phanerophytes, including Bambuseae (Oyarzabal et al. 2018). The weather is warm and wet, subtropical without a marked dry season, with large thermal amplitude and abundant precipitations, which denotes its "continental" character and makes this region one of the wettest in the country. The average annual temperature is 22°C, with a maximum temperature of 38.5°C and a minimum of -5.4°C; up to 50 days with frost are recorded during the year. The average annual rainfall is 2017 mm; autumn (March to May) is the rainy season (563 mm) (Eibl et al. 1999, Manso Hernández et al. 2010).

Sample collection

From June 2016 to April 2018 larvae of species of *Aedes* were monthly collected, covering two warm periods (spring-summer-autumn) and two cold winter periods. Mosquito larvae were sampled in urban area, from artificial containers. The surveys were made in family homes, public places such as cemeteries, and

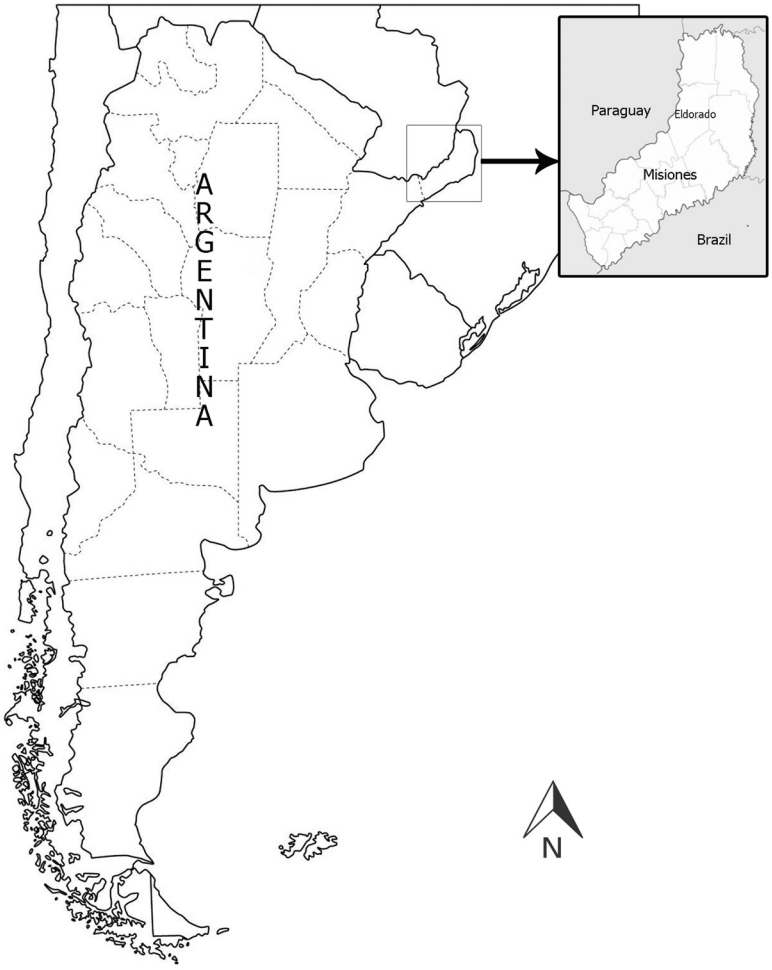

Figure 1. Location map in Argentina of the province of Misiones, collecting site: city of Eldorado.

every urban location with potential habitats for these species (tyre repair workshops, mechanic workshops and scrapyards). The following variables were recorded for each larval habitat: pH, temperature, location of the container with regard to sunlight (sunlight, deep or partial shade) and type of container. At laboratory, the samples were placed in plastic trays and the fourth instar mosquito larvae were taxonomic identified based on characteristics outlined in Consoli & Oliveira (1994) for inmature Culicidae. Larvae from the first to third instars were reared to the fourth instar for identification.

Dissection of the larval digestive tract

The containers positive for *Aedes* spp. were classified into the following categories: positive for *Ae. aegypti*, positive for *Ae. albopictus*, and positive for both species. Three containers from each category were chosen at random and ten larvae of instars IV, were taken to perform dissections.

The dissections were performed under a stereoscopic microscope (Zeiss Stemi 2000-C; 1X) with a drop of physiological solution (NaCl 10%), using finely pointed entomology tweezers, holding the larva from the cephalic portion and stretching it from the postabdominal respiratory siphon. Once the digestive tract was extracted, a coverslip was placed over it, to observe under

a conventional light microscope (Olympus CX31) with 10X and 40X magnifications in search of trophozoites of *Ascogregarina* spp.

The trophozoites of *Ascogregarina* spp. were identified following the description provided by Reyes-Villanueva et al. (2001). Photographs were taken with an *INFINITY 1* camera, and the length and width of the gregarines were measured.

DNA extraction and amplification

DNA was extracted from parasitized gut of *Ae. aegypti* and *Ae. albopictus* using the Wizard Genomic Purification Kit (Promega). The integrity of the extracted DNA was checked on 1% agarose gel. PCR was performed as described by Morales et al. (2005), with the primers AU (5'-ACCGCCCGTCCGTTCAATCG-3´), AC (5' CACTTAGTGTTTTGTTTGATGTC 3'), and AT (5' GAGAAGCCGTCGTCAATACAGC 3').

The PCR mixture was performed in a total volume of 25µl containing 3 µl of genomic DNA of either *Ae. aegypti* or *Ae. albopictus*, 2.5 µl (10 pmol/ µl) of primers AC or AT, 2.5 µl (10 pmol/ µl) of primers AU, 2.5 µl of TAS 10X reaction buffers, 2.5 µl of dNTPs (10mM of each deoxynucleoside triphosphate), 0.2 µl of Taq polymerase (HIGHWAY 5U/µl), 3 µl of MgCl2 (3mM/µl), and 10.8 µl of water free of DNAses (Promega). The PCR conditions included an initial denaturation at 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, with a final extension of 10 min at 72°C (Prophiro et al. 2017). The amplified DNA fragments were visualized by electrophoresis using agarose gels at 2% supplemented with Gel Red (GenBiotech) under UV light. DNA was purified using the Agarose Gel DNA Extraction Kit (Roche). The genomic fragments were sent to Macrogen Inc. (Seoul, South Korea) for sequencing.

Data analysis

The spatial-temporal variation of *Ascogregarina* spp. was evaluated using quantitative descriptors of the parasites' populations according to Bush & Shostak's definitions of prevalence, the intensity of infection and mean intensity (MI) (Bush et al. 1997). The analysis was performed using the InfoStat /E version 2018 statistical software (Di Rienzo et al. 2018). To compare the seasonal prevalence of infection, the Pearson's Chi-square test was performed. Seasonal MI was compared using the Kruskall-Wallis test and a subsequent pairwise comparison test. In addition, we performed a Spearman's correlation test between the MI and the temperature of the water registered in the microhabitat.

The DNA sequences were visualized and edited with the Chromas free software version 2.6.5. (http://www.technelysium.com.au). Bioinformatic analysis was performed with the BLASTn tool available in the NCBI platform (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/).

RESULTS

Molecular detection and morphological description of trophozoites of *Ascogregarina* spp. in larvae of *Aedes aegypti* and *Ae. albopictus*

The trophozoites of *A. culicis* inside and outside the midgut of fourth-instar larva of *Ae. aegypti* were observed. These infected larvae had normal external color, behavior and appearance. The size of the trophozoites was variable, with an average of 82.22 µm (±21.69) in length and 28.27 µm (±9.61) in width, n= 49. The shape looks like comma; the anterior part, consisting of protomerite and epimerite, is wider than the conical part corresponding to the deutomerite (Figure 2a). In *Ae. albopictus,* trophozoites

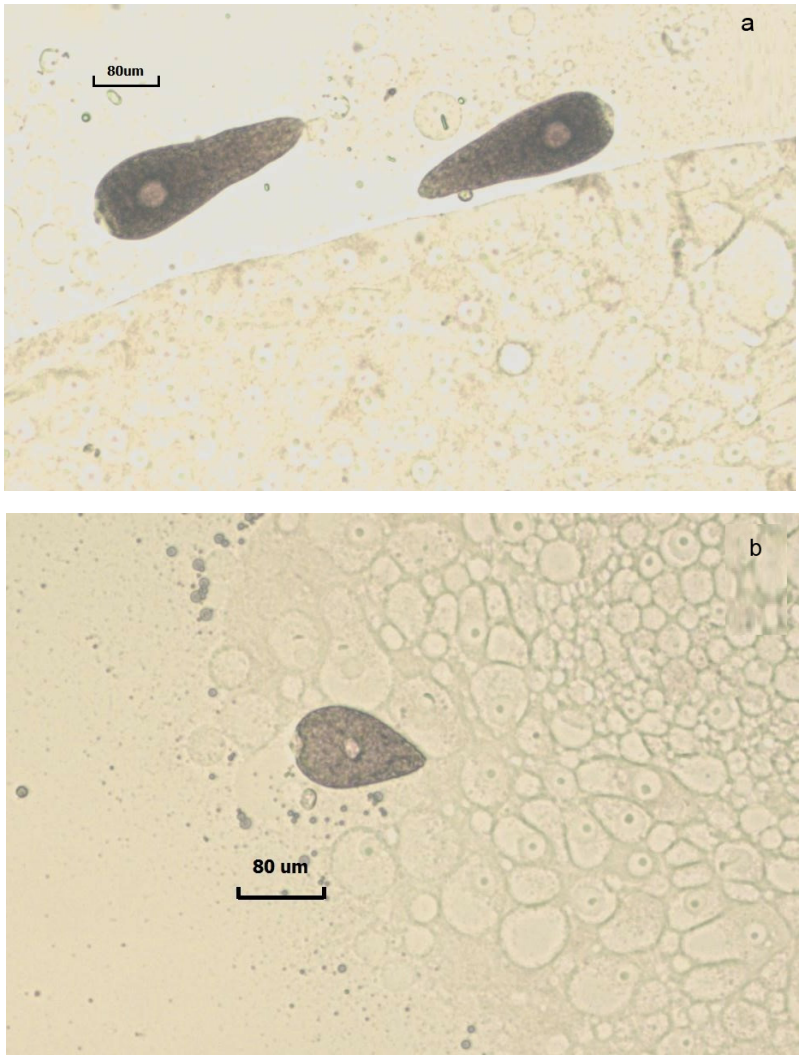

Figure 2. Trophozoites in the mid-gut of the larvae, (a) *Ascogregarina culicis* in *Aedes aegypti* (b) *Ascogregarina* sp. in *Aedes albopictus* (bar=80 µm).

of *Ascogregarina* sp. had a much shorter deutomerite, ending more abruptly than in *A. culicis*, with a length of 81.60 µm (± 20.4) and a width of 40.04 µm (± 13.24), n=75 (Figure 2b).

The sequence obtained from *Ae. aegypti* corresponding to 200 pb (access number GenBank MK684162) was aligned and revealing an identity of 100% with other sequences of *A. culicis*. The sequence obtained from midgut of *Ae. albopictus* (180 pb) was not sufficient to confirm its identity that is why we named as *Ascogregarina* sp. Anyway, based on the morphology of the trophozoites and the host specificity, this could potentially be *A. taiwanensis*.

Prevalence and intensity of infection of *A. culicis* and *Ascogregarina* sp. in different habitats and seasons

During the sampling period, 1333 larvae of *Ae. aegypti* and 188 larvae of *Ae. albopictus* collected from different sites in the city of Eldorado were dissected. The total prevalence of infection was 34.81% (n=464) and 37.23 % (n= 70), for *A. culicis* and *Ascogregarina* sp., respectively.

Ascogregarina culicis was found throughout the sampling period, and seasonal prevalence

showed statistically significant differences (Pearson's Chi-square =84.59; p< 0.0001). The highest prevalence of *A. culicis* was observed in spring 2017, both in microhabitats located to sunlight (41.79%) and deep or partial shade (42.68); and in autumn 2018, in habitats located to sunlight (55.00%) and deep or partial shade (52.77%). The greatest abundance of immature of *Ae. aegypti* was in summer 2017/2018. The intensity of infection of *A. culicis* in larvae of *Ae. aegypti* varied from 1 to 250 trophozoites. Although significant difference between the seasons was not detected (H = 16.87; p = 0.0315), the highest MI was found in spring 2016 and autumn 2017 (Table I).

The presence of *Ascogregarina* sp. in the midgut of *Ae. albopictus* was not recorded throughout the whole sampling period despite the presence of the host. The differences between seasonal prevalence were significant (Pearson's Chi-square = 29.36; p= 0.0003), with the highest prevalence in spring 2016 (60.00%) and summer 2018 (90.00%), in both cases the habitats were located at partial or deep shade; the greatest abundances of *Ae. albopictus* were collected in summer 2018. The intensity of infection varied from 1 to 327 trophozoites per larva. The highest MI was observed in autumn 2018, although the differences were not statistically significant between the seasons (H=6; p>0.9999) (Table II).

The highest number of trophozoites for both parasites was observed in autumn and spring, when the mean air temperatures varied between 18.66 ° C and 25.62 ° C, respectively.

When comparing the prevalence of infection among different habitats, statistically significant differences were found for *A. culicis* and *Ascogregarina* sp. (Pearson's Chi-square = 144.97 and 33.13; p < 0.0001 respectively). The tire was the container with the highest prevalence for both protozoa. On the other hand, the comparison between the average intensity in the different habitats did not showed any significant differences for the two parasites (H = 7.83; p = 0.4503 and H = 6.76; p = 0.1493, respectively).

Micro-environmental habitat conditions

In the habitats with the presence of *A. culicis* and *Ascogregarina* sp., the average water temperature was 22.86° C (SD 6.02) and 22.85°

Table I. Seasonal infection rates and number of trophozoites of *Ascogregarina culicis* in natural populations of *Aedes aegypti*.

	Sun			Shade	
Season	**N° *Ae. aegypti***	**N° trophozoites(MI)[1]**	**% infected**	**N° trophozoites[1]**	**% infected**
Autumn 16	1178	4.9 (0)	24.39	-	-
Winter 16	629	12.56 (11.89)	18.32	55.50 (0)	42.10
Spring 16	2127	30.22 (16.1)	23.53	18.57 (14.84)	41.17
Summer 16/17	3171	23.16 (14.37)	37.12	22.5 (12.35)	16.76
Autumn 17	2361	51.67 (23.1)	28.71	26.03 (36.3)	26.44
Winter 17	1035	8.06 (9.98)	31.25	13.75 (0)	18.18
Spring 17	4007	37.27 (25.35)	41.79	15.7 (14.28)	42.68
Summer 17/18	4411	13.42 (16.83)	1.66	11.44 (10.31)	25.27
Autumn 18	1138	30.88 (6.9)	55.00	60.13 (48.83)	52.77

[1]**Mean±SD.**

C (SD 4.07), respectively. The water temperature and the average number of trophozoites of *A. culicis* presented a negative correlation, but not significant (rs = -0.11; p = 0.3608). In the case of *Ascogregarina* sp. the correlation was not significant (rs=0.1; p = 0.7518).

DISCUSSION

Our observations provide information regarding the parasites of *Ae. albopictus* and *Ae. aegypti* in sympatric populations from Argentina. We reported the finding of *Ascogregarina culicis* in *Aedes aegypti* and *Ascogregarina* sp. in *Ae. albopictus* populations in subtropical Argentina for the first time.

Researches related to the morphology of trophozoites has revealed size differences between geographical isolations and depend largely of the host in both species (Garcia et al. 1994, Reyes-Villanueva et al. 2001). Our study found that the size of trophozoites of *A. culicis* is smaller than that reported by Reyes-Villanueva et al. (2001) for a strain from Florida (USA). Dellapé et al. (2005) reported *Ae. aegypti* infected with *A. culicis* for the first time in Buenos Aires, Argentina; they founded smaller trophozoites than those reported in the present study (59.3 µm long and 12.1 µm wide). Then, Albicócco & Vezzani (2009) recorded the size of *A. culicis* as being 76.88-116.56 µm long and 11.6-12.4 µm wide. Lien & Levine (1980) described *A. culicis* for the first time in isolations from Taiwan, corresponding to 170 µm long and 26 µm wide in the front area. The morphology of trophozoites of *Ascogregarina* sp. found in *Ae. albopictus* is similar to that described for *A. taiwanensis* by Passos & Tadei (2008) for isolation from Brazil. However, is smaller than that described by Lien & Levin (1980) (234 µm long and 32 µm wide) for an isolation from Taiwan. This demonstrates the difficulty of relying on the size and morphology of trophozoites for identification of gregarine species, thus it is necessary to corroborate with molecular approach. In this study *A. culicis* was molecularly identified but it not was possible for the gregarine infecting *Ae. albopictus*. It could potentially be *A. taiwanensis* but accurate molecular identification is necessary to confirm our assumptions based on morphology and host specificity.

Table II. Seasonal infection rates and number of trophozoites of *Ascogregarina* sp. in natural populations of *Aedes albopictus*.

	Sun			Shade	
Season	**N° *Ae. albopictus***	**N° trophozoites[1]**	**% infected**	**N° trophozoites[1]**	**% infected**
Autumn 16	76	1.0 (0)	25.00	-	0.00
Winter 16	472	-	0.00	-	0.00
Spring 16	215	-	0.00	50.0 (0)	60.00
Summer 16/17	407	8.00 (0)	50.00	42.64 (48.94)	40.74
Autumn 17	661	12.18 (5.44)	36.66	50.93 (64.04)	39.39
Winter 17	128	-	0.00	-	0.00
Spring 17	300	5.20 (7.26)	62.50	-	0.00
Summer 17/18	1101	-	0.00	8.78 (6.36)	90.00
Autumn 18	190	123.00(91.27)	52.63	26.00 (47.48)	42.10

[1]**Mean±SD.**

The percentage of infection of *A. culicis* infecting larvae of *Ae. aegypti* found in the present study (34.81%) was lower than that recorded in Brazil (78%–95%) and in the USA (50%–70%) (Passos & Tadei 2008, Blackmore et al. 1995). However, this value was higher in relation to previous reports for larvae collected in a cemetery (19.9%) and females (21.2%) from populations of *Ae. aegypti* in a temperate region in Argentina (Vezzani & Wisnivesky 2006, Albicócco & Vezzani 2009). The infection of *Ae. albopictus* by *Ascogregarina* sp. found in the present study was lower (37.23 %) than those reported for Manaus (Brazil) and Florida (USA), 21%–39.5% and 68%–100%, respectively for *Ascogregarina taiwanensis* (Passos & Tadei 2008, Prophiro et al. 2017).

Ascogregarina culicis was detected throughout the whole sampling period. It is relevant to highlight the presence of *Ae. aegypti* during the whole year in the study area (Stein 2018), which would enable a more established infection and a higher prevalence of the parasite. The periods of higher prevalence of *Ascogregarina culicis* corresponded to higher mosquito abundance in the study area. The meteorological variables influence the abundance of the mosquito vector, which would influence the abundance of the parasite (A.C. Alonso, unpublished data). This correspondence between the both abundances, host and parasites, is also reported in the temperate region in Argentina, with seasonal patterns proper of those latitudes (Albicócco & Vezzani 2009).

The intensity and prevalence of infection of *A. culicis* and *A. taiwanensis* vary considerably between different regions around the world and even within the same geographical region, as was shown by Passos & Tadei (2008) in the Amazon region in Brazil. In Eldorado city, Argentina, the intensity of infection of *A. culicis* varied between 1 to 250 trophozoites per larva, lower than that recorded in Manaus, Brazil, which varied between 1 to 582 trophozoites per larva. For *Ascogregarina* sp. the intensity of infection varied between 1 to 327 trophozoites per larva, similar to that registered by Passos & Tadei for *A. taiwanensis* (2008).

In the present study, the total MI of *A. culicis* was significantly higher than that reported by Albicócco & Vezzani (2009) (28.94, SD 35.78). This could be related to the continuous presence of the host throughout the year in the subtropical region, whereas in the temperate region, the host is only present as the egg stage in winter and the parasite must to persist in the containers as oocysts.

Our MI values agree with others reported for warm zones. Blackmore et al. (1995) found values varying between 1 and 486 trophozoites in Florida (USA), with MI values of 52.5 parasites per larva for *Ae. aegypti* and 33.5 parasites per larva for *Ae. albopictus*.

In addition, the habitats with a greater intensity of infection of both gregarines were those located at shade, protected from sunlight and lower water temperatures, mainly founded in houses and tire workshop.

The tires were the habitats with the higher intensity of infection, probably because these habitats are more protected from sunlight, so it would not reach high temperatures to affect the viability of the oocysts; in addition, the total evaporation of water is not a common event in the tires, so oocysts are not exposed to desiccation.

In coincidence with our findings, Albicócco & Vezzani (2009) also found the highest prevalence of infection in containers protected from sunlight. Oocysts exposed to high temperatures show a decrease in their viability (McCray et al. 1970). However, in the present study variation in the intensity of parasitism did not correlate

significantly with the water temperature in the containers.

Parasitism by this protozoan are opened new questions about the possible role of modeler of interspecific competition between *Ae. albopictus* and *Ae. aegypti*. Our observations constituting a possible explanation about the restricted distribution of the *Aedes albopictus*, 20 years after its first finding in Argentina (Lizuain et al. 2019). However, further studies from the effects of gregarines on the coexistence of both *Aedes* species in this region are needed.

Different studies conducted in other regions of the world, have shown that the infection of *A. taiwanensis* in *Ae. albopictus* can lengthen larval development, diminish the adult size and affect female fertility (Aliabadi & Juliano 2002). We believe that more studies should be done to see if there is a relationship between the low abundance of *Aedes albopictus* with respect to *Aedes aegypti* (1/10 - 4/10) registered in the study area (Stein 2018) and the intensity of infection by this protozoan. Despite the continuous presence of the host, the parasite was no monthly detected in the present study and considering that twenty years have passed since the first detection of *Ae. albopictus* in Argentina, makes us postulate whether or not this species has managed to overcome the parasite escape phase proposed by Aliabadi & Juliano (2002). In this escape phase, the species would not be highly parasitized by the protozoan in a new distribution area, which could represent a certain inter- and intra-population reproductive advantage that would increase its abundance. However, the lower abundance of *Ae. albopictus* in relation to *Ae. aegypti* recorded in Eldorado city, and its restricted distribution in Argentina, would indicate that, up to the present, this mechanism of reproductive advantage has not been enough to enable *Ae. albopictus* to become an invasive species, as it has been considered in other regions of America.

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MS and MVM designed mosquito collection; ACA collected mosquito samples, identified mosquito samples and dissected the larvae; all authors draft the manuscript.