

# Effects of pH on Excystation of *Gregarina cuneata* and *Gregarina polymorpha* (Eugregarinida: Gregarinidae)<sup>1</sup>

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**ABSTRACT.** The mechanisms that signal gregarine excystation are unknown. Previous authors have demonstrated that gregarine sporozoites excyst from their surrounding oocyst in response to stimuli contained in host digestive fluids, but the role of host intestinal pH in this signaling system has not been investigated. In this study, an in vitro assay is used to quantify the effects of 3 pH levels (6.1, 7.0, 8.0) on the excystation of two gregarine species, *Gregarina cuneata* and *Gregarina polymorpha*. Both gregarine species excyst at all three pH levels, but there are significant within and among species differences in excystation rate and cumulative excystation over time. *Gregarina cuneata* excysts more rapidly at pH 6.0 and *G. polymorpha* excysts more rapidly at pH 8.0. Cumulative excystation is maximized at pH 6.0 for *G. cuneata* and at pH 7.0 for *G. polymorpha*. Hydrogen ion mediated excystation may lead to the formation of foci for subsequent establishment or migration and may play a role in parasite site specificity.

**Supplementary key words.** Apicomplexa, excystation, life cycle, oocyst, parasite, parasitism, physiological ecology, Septatina, Sporozoa.

**E**UGREGARINIDA comprise approximately 1,656 protistan species whose members are all obligate parasites of invertebrates [16]. The majority of eugregarine taxa belong to the suborders Aseptatina and Septatina [16]. Complete life cycles are not known for all members of these suborders, but known life cycles are direct and utilize resistant oocysts to transmit infective sporozoites [6]. When dormant oocysts are ingested by a suitable host, sporozoites activate, excyst in the host gut lumen and infect the mesenteric epithelium [14]. Only those oocysts that excyst in response to the host gut environment contribute to the survival of the population as a whole. By chance or selection, excystation signaling mechanisms cull a very few progeny to constitute the next generation. These mechanisms may play a role in host-specificity and thus speciation and radiation within Eugregarinida. They certainly play a role in site-selection, dictating initial sites for sporozoite establishment or migration within a host.

The mechanisms that signal gregarine excystation are unknown. Various authors [2, 26, 27] have postulated that sporozoites excyst in response to host "digestive juices." Several authors [11-13, 20, 21] have published anecdotal reports of sporozoites excysting from oocysts in fresh preparations of host gut fluid. Hoshide et al. [10] developed an in vitro protocol in which oocysts were incubated in an extract of host gut tissue homogenate. In their study, oocysts of *Gregarina blattarum* placed in an extract of gut tissues taken from *Blattella germanica* (the German cockroach [Dictyoptera: Blattellidae]) excysted approximately 5-10 h later [10]. If the techniques of Hoshide et al. [10] can be translated to other gregarine systems, they can be used to identify host physiological factors that signal gregarine excystation.

*Gregarina cuneata* and *Gregarina polymorpha* (Apicomplexa: Eugregarinida) are parasites of larval *Tenebrio molitor* (the yellow mealworm [Coleoptera: Tenebrionidae]). These parasites are common in laboratory colonies [e.g., 3-5, 8, 17, 18, 22-25] and wild populations [7, 9, 17] of *T. molitor*. Göhre [5] examined the pH of *G. cuneata* plasma, *G. polymorpha* plasma, and the mesenteric lumen of *T. molitor* larvae. He concluded that the pH of gregarine plasma was identical in both parasite species, but reported an increasing pH gradient along the length of the larval midgut [5]. Although Göhre [5] drew no such conclusion, other authors [e.g. 19] have concluded that pH signals gregarine excystation and dictates gregarine site-selection within larval *T.*

*molitor*. These conclusions have not been tested; in fact, no study has addressed the effects of pH on sporozoite excystation or site-selection by *G. cuneata* or *G. polymorpha*.

We have adapted the techniques of Hoshide et al. [10] and used them to study the effects of pH on sporozoite activation and excystation in *G. cuneata* and *G. polymorpha*. We quantify the effects of pH on the in vitro excystation rates of *G. cuneata* and *G. polymorpha* and discuss the implications for site-specificity within the Eugregarinida. Apicomplexan terminology used here is consistent with that of Levine [15].

## MATERIALS AND METHODS

**Host and parasite culture.** Parasite species were maintained in vivo using host insect colonies. *G. polymorpha* and *G. cuneata* were isolated from research colonies of *T. molitor* established using subcultures of mealworm beetle colonies maintained by the School of Biological Sciences, University of Nebraska, Lincoln, Nebraska. Mealworm beetles were reared at 25°C in plastic sweater boxes with a wheat bran substrate; potato slices and water were added weekly to provide moisture.

**Collection and surface sterilization of gregarine gametocysts.** Gregarine gametocysts were collected from insect feces obtained by isolating groups of host animals. One hundred to 200 *T. molitor* larvae were isolated over night in a plastic shoebox with a damp paper towel and their feces were examined the following morning. Gametocysts were removed from fecal material and soaked in a neutral saline solution to soften and remove adherent frass. Gametocysts were immersed for 5 min in 70 PPM Methyl Paraben (p-hydroxybenzoic acid, methyl ester: distilled water) to insure surface sterilization, sorted according to parasite species [3], and pipetted to 6-mm circles of black construction paper. Paper circles holding gametocysts were placed in the bottom of 40-mm plastic petri dishes that were in turn placed in a plastic shoebox lined with 4 cm of damp sand to maintain humidity. Gametocysts incubated at 25°C matured and dehisced within 72 h, releasing chains of infective oocysts.

**Preparation of host gut tissue extracts.** In vitro excystation studies were conducted using extracts of host gut tissue homogenates prepared as follows. Larval *T. molitor* were dissected in neutral buffered saline. The mesenteron was removed from each animal and stored on ice in neutral buffered saline until approximately 1 ml of tissue had been collected. This volume was equivalent to the mesenteric tissue mass of approximately 20 mealworm larvae. The collected tissue was macerated in 70 µl of phosphate buffered saline (Belton and Grundfest's [1] *T. molitor* muscle saline without sucrose) for 1 min with a tissue grinder. The resulting homogenate was cleared of large cellular debris by centrifugation for 5 min at ca. 3,180 g. Supernatant

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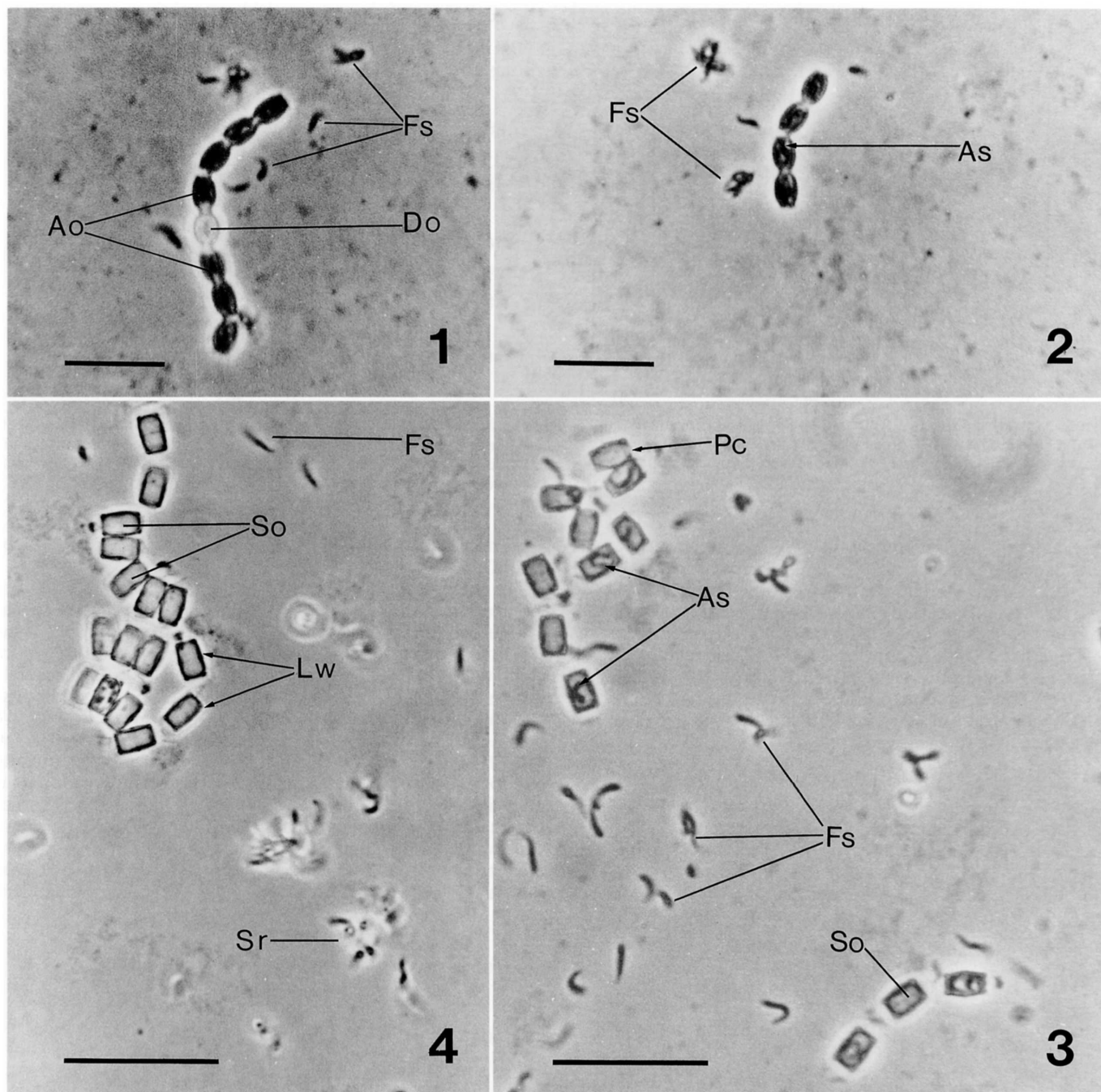


Fig. 1–4. Excystation of two species of *Gregarina*. 1. Activated and dormant oocysts and free sporozoites of *G. polymorpha*. 2. Free sporozoites and activated oocysts enclosing discernible sporozoites of *G. polymorpha*. 3. Free and encysted sporozoites of *G. cuneata*. Polar canals and opaque lateral walls distinguish spent oocysts from dormant oocysts (cf., Fig. 1, 4). 4. Spent oocysts and free sporozoites of *G. cuneata*. Free sporozoites sometimes adhere caudally to form rosettes. Ao, activated oocyst; As, activated sporozoite; Do, dormant oocyst; Fs, free (excysted) sporozoite; Lw, lateral wall of spent oocyst; Pc, polar canal; So, spent oocyst; Sr, sporozoite rosette. Bar = 10  $\mu$ m.

fluid was collected and filtered through a 0.45  $\mu$ m acetate filter, producing an extract free of cellular debris and microorganisms. Phosphate buffered salines were used to prepare host gut tissue extracts of pH 6.1, 7.0, and 8.0. ColorpHast® indicator strips (resolution  $\pm$  pH 0.3 [EM Science, Gibbstown, New Jersey]) were used to confirm the pH of prepared extracts.

**Comparative excystation studies.** For *in vitro* excystation studies, about 3  $\mu$ l of host tissue extract was placed on a 10  $\times$

10 mm clean glass coverslip and oocysts were added. Each preparation contained at least 1,000 oocysts. A wet mount was prepared and sealed with paraffin oil. The preparation was examined under phase microscopy for evidence of sporozoite activation and excystation. Preparations using neutral buffered saline in lieu of gut extract served as controls.

Three studies were conducted to determine the effect of pH level (6.1, 7.0, 8.0) on the activation and excystation of *G.*

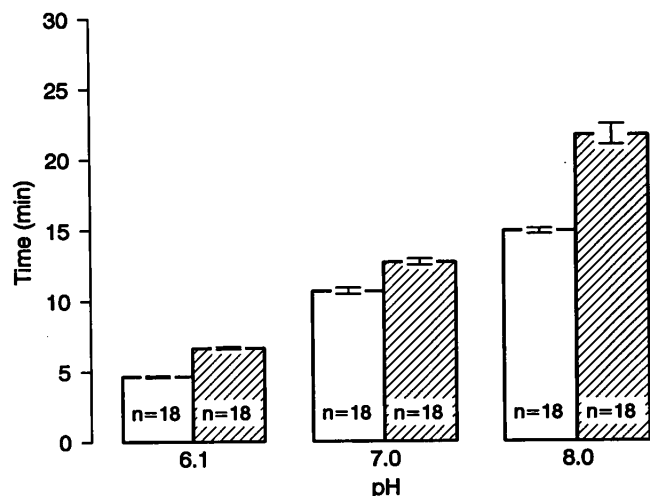


Fig. 5. Mean elapsed time (min) to first observed excystation (open bars) and 50% excystation (shaded bars) of *G. cuneata* oocysts incubated at 3 pH levels. Error bars signify standard error. n, replicate populations tested.

*cuneata* and *G. polymorpha* sporozoites. Individual studies addressed each of the following benchmark events: time elapsed until the first excystation event ( $TE_1$ ), time elapsed until 50% of the oocysts had excysted ( $TE_{50}$ ), and the percentage of oocysts excysted 30 min post-inoculation ( $ER_{30}$ ). Each parasite species/pH combination was replicated 18 times and a total of 108 preparations were observed. In the  $TE_{50}$  and  $ER_{30}$  studies, 50 oocysts within a random field of view were observed at the beginning of each time interval to determine the percentage of excysted oocysts (percent excystation).

### RESULTS

In all experiments and replications, no excystation was observed in neutral buffered saline or neutral phosphate buffer controls. Excystation was observed in all host tissue homogenate extracts (Fig. 1–4). Although free sporozoites survived in paraffin-oil sealed coverglass preparations for over 30 h, motility

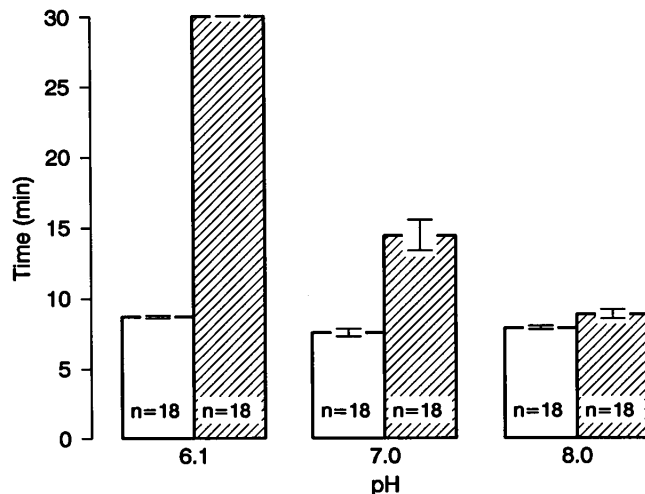


Fig. 7. Mean elapsed time (min) to first observed excystation (open bars) and 50% excystation (shaded bars) of *G. polymorpha* oocysts incubated at 3 pH levels. Error bars signify standard error. n, replicate populations tested.

diminished quickly and bacterial contamination increased rapidly after 3 h.

The visual appearance of individual oocysts changed during excystation. Dormant oocysts were refractive in water preparations and during initial incubation in gut extract preparations (Fig. 1). Oocysts became dark and opaque as sporozoites were activated (Fig. 1, 2). Individual sporozoites were clearly visible during this phase (Fig. 2). Following activation, sporozoites exited the oocyst through polar canals (Fig. 3). Weak progressive locomotion was observed in free sporozoites (Fig. 1–4), although they frequently became caudally ensnared, forming sporozoite clumps or rosettes (Fig. 4). Spent oocysts became transparent after liberating their enclosed sporozoites, but thick sections of the lateral walls remained opaque (Fig. 3–4). These dark lateral walls were readily apparent and were used to distinguish spent oocysts from dormant oocysts. Some of the oocysts observed in this study contained activated sporozoites (they turned dark

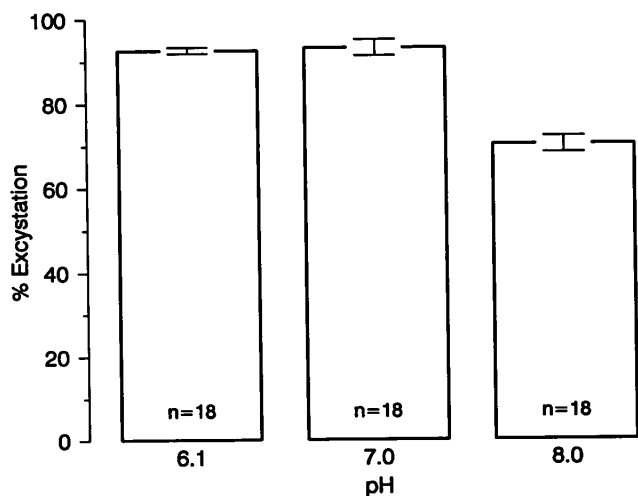


Fig. 6. Mean percentage of *G. cuneata* oocysts excysted after 30 min incubation at 3 pH levels. Error bars signify standard error. n, replicate populations tested.

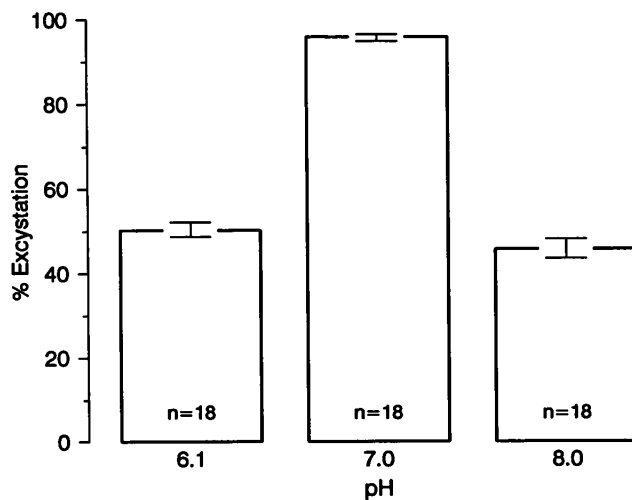


Fig. 8. Mean percentage of *G. polymorpha* oocysts excysted after 30 min incubation at 3 pH levels. Error bars signify standard error. n, replicate populations tested.

and opaque) but they did not excyst (polar caps remained in place and no liberated sporozoites were observed). Successful excystation, biologically, results in sporozoite liberation. For the purposes of this study, fully spent oocysts and oocysts in the process of liberating sporozoites were considered to be "excysted." All other oocysts were considered to be "unexcysted," regardless of the activation state of their enclosed sporozoites.

Mean  $TE_1$  values for *G. cuneata* were 4.6 ( $\pm 0.5$  SD), 10.7 ( $\pm 1.8$  SD), and 14.9 ( $\pm 1.6$  SD) min at pH 6.1, 7.0, and 8.0, respectively (Fig. 5). Among the 3 pH levels tested, there was a significant difference in the time elapsed before initial excystation was observed (ANOVA:  $F = 235.9$ ,  $P = 1.6 \times 10^{-26}$ ,  $\alpha = 0.05_{2,51}$ ). Mean  $TE_{50}$  values for *G. cuneata* were 6.6 ( $\pm 0.6$  SD), 12.7 ( $\pm 1.8$  SD), and 21.7 ( $\pm 6.1$  SD) min at pH 6.1, 7.0, and 8.0, respectively (Fig. 5). Among the 3 pH levels tested, there was a significant difference in the time elapsed before 50% excystation was observed (ANOVA:  $F = 76.1$ ,  $P = 4.9 \times 10^{-16}$ ,  $\alpha = 0.05_{2,51}$ ). Mean  $ER_{30}$  values for *G. cuneata* were 92.4% ( $\pm 6.1$  SD), 93.1% ( $\pm 16.7$  SD), and 70.2% ( $\pm 16.7$  SD) at pH 6.1, 7.0, and 8.0, respectively (Fig. 6). Among the 3 pH levels tested, there was a significant difference in the percentage of oocysts excysting after 30 min incubation (ANOVA:  $F = 15.4$ ,  $P = 5.9 \times 10^{-6}$ ,  $\alpha = 0.05_{2,51}$ ).

Mean  $TE_1$  values for *G. polymorpha* were 8.6 ( $\pm 0.8$  SD), 7.2 ( $\pm 2.0$  SD), and 7.8 ( $\pm 1.0$  SD) min at pH 6.1, 7.0, and 8.0, respectively (Fig. 7). Among the 3 pH levels tested, there was a significant difference in the time elapsed before initial excystation was observed (ANOVA:  $F = 4.7$ ,  $P = 0.01$ ,  $\alpha = 0.05_{2,51}$ ). Mean  $TE_{50}$  values for *G. polymorpha* were 30.0 ( $\pm 0.0$  SD), 14.4 ( $\pm 9.3$  SD), and 8.8 ( $\pm 2.9$  SD) min at pH 6.1, 7.0, and 8.0, respectively (Fig. 7). (These trials were limited to 30 min. Although percent excystation exceeded 40% in most cases, 50% excystation was not observed in any of the 18 *G. polymorpha* trials at pH 6.1.) Among the 3 pH levels tested, there was a significant difference in the time elapsed before 50% excystation was observed (ANOVA:  $F = 68.8$ ,  $P = 3.3 \times 10^{-15}$ ,  $\alpha = 0.05_{2,51}$ ). Mean  $ER_{30}$  values for *G. polymorpha* were 50.1% ( $\pm 14.57$  SD), 95.8% ( $\pm 6.7$  SD), and 45.5% ( $\pm 19.3$  SD) at pH 6.1, 7.0, and 8.0, respectively (Fig. 8). Among the 3 pH levels tested, there was a significant difference in the percentage of oocysts excysting after 30 min incubation (ANOVA:  $F = 66.2$ ,  $P = 6.8 \times 10^{-15}$ ,  $\alpha = 0.05_{2,51}$ ).

## DISCUSSION

Host intestinal pH has demonstrable within-species effects on the excystation of *G. cuneata* and *G. polymorpha*. In general, excystation of *G. cuneata* appears to be inhibited by an increase in pH level (Fig. 5). *Gregarina cuneata* begins to excyst and reaches 50% excystation rapidly at pH 6.1. Increases in pH delay initial excystation and increase the time lag between inoculation and excystation of 50% of a sample population. Over 90% of *G. cuneata* oocysts excyst within 30 min when incubated at pH 6.1 and 7.0, but excystation rates are reduced to about 70% in populations incubated at pH 8.0 (Fig. 6). A statistically significant difference in the timing of initial excystation was observed among experimental populations of *G. polymorpha* incubated at three pH levels (Fig. 7). However, this difference may have no biological significance: the range of elapsed times to initial excystation was narrower for *G. polymorpha* populations than for *G. cuneata* (cf., Fig. 5, 7). Hydrogen ion potential does affect other *G. polymorpha* excystation parameters. Experimental populations of *G. polymorpha* incubated at pH 8.0 reach 50% excystation twice as fast as populations incubated at pH 7.0 and at least 3 times faster than populations incubated at pH 6.1 (Fig. 7). Over 95% of *G. polymorpha* oocysts excyst within 30 min

when incubated at pH 7.0, but average excystation rates are reduced to about 50% in populations incubated at pH 6.1 or 8.0 (Fig. 8).

*G. cuneata* and *G. polymorpha* do not share a common relationship between host intestinal pH and excystation. There are comparative differences. There is a direct functional correlation between pH level and the excystation rate of *G. cuneata* (Fig. 5). Over the pH range of 6.1–8.0, excystation rate is maximized at pH 6.1. In contrast, the functional correlation is indirect for *G. polymorpha* and excystation rate is maximized at pH 8.0 (Fig. 7). Cumulative excystation over time partially mitigates the inhibitory effect of high pH on the excystation rate of *G. cuneata*. Percent excystation is depressed at high pH, but there is little difference in total excystation across lower pH levels (Fig. 6). The relationship between excystation and pH level is more complex for *G. polymorpha*. Although pH 8.0 appears to maximize *G. polymorpha* excystation rate, cumulative excystation is significantly higher at pH 7.0 than at the other two levels tested (cf., Fig. 7, 8). Excystation rate and cumulative excystation over time are both maximized at the same pH level for *G. cuneata*; but for *G. polymorpha*, these responses are maximized at different pH levels. Both gregarine species excyst at all 3 pH levels. This indicates that excystation signaling mechanisms are, to some extent, pH tolerant. But, different pH levels elicit different responses within and among gregarine species, suggesting that there is an optimal pH level that maximizes excystation. The data demonstrate that the pH optimum for excystation varies among gregarine species, but the pH level that maximizes excystation rate does not always maximize cumulative excystation.

Göhre [5] quantified the pH of *G. cuneata* plasma, *G. polymorpha* plasma and the mesenteric lumen of *T. molitor* larvae. He reported an increasing luminal pH gradient in *T. molitor*, ranging from pH 4.4, just posterior of the proventriculus, to 8.2 at the junction of the malpighian tubules and the pylorus [5]. He also noted the differential site specificity of *G. cuneata* and *G. polymorpha* along the length of the host intestine. Göhre [5] did not explicitly attribute gregarine site specificity to host intestinal pH level but, the apparent correlation between host intestinal pH and gregarine site specificity led some authors to assert that pH alone was the causative agent in parasite distribution [19]. The data presented here do not link gregarine site-specificity to host intestinal pH. These data do demonstrate that the excystation responses of *G. cuneata* and *G. polymorpha* are pH sensitive and variable among parasite species. At low pH, *G. cuneata* populations excyst more rapidly and attain higher cumulative excystation than populations of *G. polymorpha*. Conversely, *G. polymorpha* populations excyst more rapidly and attain higher cumulative excystation than populations of *G. cuneata* when both species are incubated at high pH. These differential responses suggest that pH may play some role in gregarine site-specificity. When gregarines excyst in the host intestine, they establish a nascent focus for subsequent establishment or migration. Thus, differences in excystation signaling among gregarine species that lead to specific excystation foci may play a regulatory role in gregarine site-specificity.

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