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Chemoattractant-Mediated Preference of Non-Self Eggs in *Ciona robusta* Sperm

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Abstract. Self-fertilization in hermaphroditic species might or might not be advantageous based on the level of inbreeding or outbreeding depression and the opportunity to outcross. This study examined whether chemoattractants can influence selfing rates through changes in sperm swimming behavior in the hermaphroditic tunicate *Ciona robusta*. The first set of experiments tested sperm preference in a dichotomous choice chamber by allowing the sperm to choose between wells with no eggs and wells with eggs, while the second experiment gave sperm a choice between self eggs and non-self eggs from another *C. robusta* individual. We found that sperm were about 5 times more likely to be captured in wells with eggs than in empty wells ($P < 0.001$) and that they were about 1.6 times more likely to be captured in wells with non-self eggs than in those with self eggs ($P = 0.002$). Additionally, we found that although sperm were activated by water pretreated with eggs, there was no difference in sperm swimming speed and motility in water treated with pooled-egg water compared to self-egg-treated water ($P = 0.636$ and $P = 0.854$, respectively). Our results indicate that while chemoattractant identity does not affect the basic mechanics of sperm activation and thus fertilization ability, it can cause sperm to aggregate near non-self eggs in greater numbers. This may allow for sperm to fertilize non-self eggs in greater numbers when available while still retaining the ability to fertilize self eggs.

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Abbreviations: CASA, computer-assisted sperm analysis; SAAF, sperm-activating and attracting sulfate steroid.

Introduction

Hermaphroditic species have the potential to self-fertilize; however, in broadcast-spawning marine invertebrates, selfing is thought to be relatively rare (but see Jarne and Auld, 2006), and many species have evolved blocks to self-fertilization (*e.g.*, Sawada *et al.*, 2014). These blocks are thought to have evolved as a result of the combined costs of inbreeding depression and the probability that a self-fertilized gamete could have been fertilized by an outcrossed gamete (gamete discounting), outweighing the benefits of transmitting more self genes and assuring reproduction (Goodwillie *et al.*, 2005; Johnston *et al.*, 2009). Inbreeding depression caused by self-fertilization can appear at different stages of the life cycle and can include developmental abnormalities or depressed growth rates and small brood size relative to that of an outcrossed offspring (Beaumont and Budd, 1983; Charlesworth and Charlesworth, 1987; Hunter and Hughes, 1993). However, in cases where the costs of gamete discounting are low—that is, there is very little probability of encountering a non-self gamete, and the costs of gamete wastage are high—self-fertilization may be advantageous even with high inbreeding depression, because it would increase fitness to produce some offspring rather than none (Escobar *et al.*, 2011). This may explain why species that have self-incompatibility proteins can have versions that allow for self-fertilization to occur (Satou *et al.*, 2015) and why some populations that experience large fluctuations in population density can exhibit relatively high self-fertilization levels despite exhibiting high levels of inbreeding depression (Caputi *et al.*, 2015).

Mechanisms that allow sperm to discern self eggs from non-self eggs may allow for flexibility in mating systems, promoting outcrossing when non-self eggs are available but permitting self-fertilization to occur when no other options are available. One mechanism that may allow this flexibility is sperm swimming behavior in response to egg-produced chemoattractants. Sperm swimming behavior can influence

what sperm eggs have access to and can have an important effect on sperm-egg interactions (Yoshida *et al.*, 1993; Evans *et al.*, 2012; Yeates *et al.*, 2013). Chemoattractants are known to play an important role in fertilization by activating sperm and influencing their swimming behavior (Bolton and Havenhand, 1996; Yoshida *et al.*, 2002; Kaupp *et al.*, 2008; Hussain *et al.*, 2016). Sperm chemotaxis has been demonstrated in many species, including cnidarians (Miller, 1966, 1978, 1979), molluscs (Miller, 1977), urochordates (Miller, 1975, 1982), and echinoderms (Miller, 1985; Ward *et al.*, 1985). Recent work has suggested that some sperm may utilize chemoattractants to discriminate between eggs within a species and that this difference in response to eggs may be related to differences in compatibility and may lead to increased fertilization success (Evans *et al.*, 2012; Hussain *et al.*, 2016).

Questions about how individual differences in chemoattractant production may influence sperm behavior within a species have been examined, but it has proven difficult to parse out whether the increase in fertilization success observed is due to differences in gametic compatibility, increased activity exhibited by the sperm to certain eggs, or a combination of both (Evans *et al.*, 2012; Hussain *et al.*, 2016). And while differences in a sperm's ability to react to different females' chemoattractants have been examined in dioecious organisms (Evans *et al.*, 2012; Hussain *et al.*, 2016), there is a lack of studies examining this phenomenon in hermaphrodites (but see Kawamura *et al.*, 1987). In hermaphrodites, self-incompatibility produces differences in fertilization success that are similar in magnitude to among-species crosses, which could result in fairly strong selection pressures against self-fertilization when non-self eggs are available. Additionally, the evolution of mechanisms for sperm to recognize and avoid self eggs prior to attempted fusion may also be favored if sperm are damaged or permanently disabled from the self-egg rejection process (Saito *et al.*, 2012). If sperm can use chemoattractants to choose compatible eggs to swim toward in dioecious species (Evans *et al.*, 2012; Hussain *et al.*, 2016), then it is possible that sperm may also be able to distinguish between self eggs and non-self eggs based on the same factors in hermaphroditic species. This study aims to examine this possibility in the broadcast-spawning hermaphroditic tunicate *Ciona robusta*.

In *C. robusta*, eggs release a sperm-activating and attracting sulfate steroid (SAAF) from the vegetal pole, which can influence sperm directionality and speed (Yoshida *et al.*, 1993, 2002). *Ciona robusta* sperm behavior is dependent on the presence of chemoattractants, because they are almost completely nonmotile in the absence of chemoattractants but actively swim when exposed to them (Bolton and Havenhand, 1996). In addition, *C. robusta* have self-recognition proteins that minimize self-fertilization (Yamaguchi *et al.*, 2011; Sawada *et al.*, 2014). In cases where self-fertilization is successful, the resultant offspring tend to have decreased fitness through lower growth and survival rates (Murabe and Hoshi, 2002; Satou *et al.*, 2015). The importance of chemoattractants in sperm behavior and the negative fitness consequences for self-fertilization when non-self eggs are available make *C. robusta* a prime candidate for investigating whether sperm behavior can be modified based on chemoattractant identity.

Here we present the results of experiments conducted to determine how self-produced chemoattractants may differentially influence sperm behavior *via* sperm preference, speed, and motility. A chemoattractant gradient was created, and the sperm had the choice between (1) a chamber with no eggs or a chamber with eggs and (2) self eggs or eggs from another *C. robusta* individual. The first experiment tested the ability of the sperm to swim toward viable eggs and the ability of the dichotomous chamber to capture that choice, while the second experiment looked at whether the sperm would aggregate near self eggs or non-self eggs. While it is known that self-egg chemoattractants can activate sperm, that is, increase motility and velocity from its nonmotile state (Kawamura *et al.*, 1987), it is unknown whether the level of activation achieved is comparable to activation by non-self egg chemoattractants. Therefore, an additional experiment was performed that examined whether self egg-only chemoattractants reduced sperm velocity or motility when compared to sperm activated with chemoattractants from a population of eggs. Whether chemoattractant identity influenced sperm swimming speed or motility could ultimately determine which eggs the sperm would have access to for fertilization.

Materials and Methods

Gametes were removed from adult individuals of *Ciona robusta* Hoshino & Tokioka, 1967 collected from Quivera Basin in San Diego, California. Eggs were removed first from the gonoduct and rinsed with fresh seawater using a 60-μm mesh as precaution to remove any possible allosperm from the eggs. To ensure that allosperm was removed, a portion of the eggs was retained in order to assess whether self-fertilization had occurred, by visually inspecting eggs for cleavage after 65 minutes. Sperm were pipetted directly from the gonoduct, and undiluted sperm were stored on ice until utilized in an experiment. The egg concentration per milliliter per individual was estimated using the average egg count of three 25-μl subsamples from each individual. Sperm concentration was estimated using a hemocytometer.

Chemotaxis in a dichotomous chamber

A dichotomous chamber consisting of two wells connected by a shallow chamber made from thick acrylic plastic blocks was utilized to examine sperm choice (Fig. 1). The wells were 3 cm in depth and 1 cm in diameter, separated by a 2.5-cm-long depression that was 0.5 cm deep. The entire chamber held about 4 ml of seawater. Fluorescein dye was added to the chambers to examine diffusion and test for the

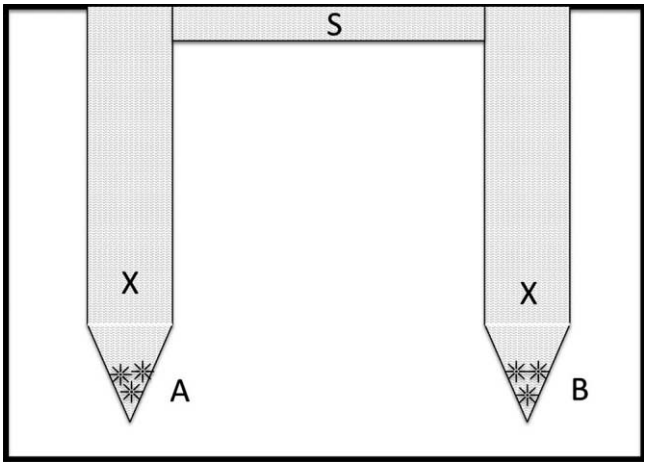

Figure 1. Diagram of the dichotomous chamber used in experiments. Sperm were placed in the center of the shallow groove (S), and after 15 minutes, each well was sampled (X), and the number of sperm recovered was recorded. When examining sperm choice in experiment 2, wells had either (A) self eggs or (B) non-self eggs.

presence of convection currents. The dye was observed to slowly diffuse to the wells from the center chamber, but no convection currents were observed. To preclude the possibility of unequal diffusion, potential biases due to collection artifacts, or a nonchemotactic directional swimming bias skewing the results, for all experiments individuals were tested twice, with the eggs switched to opposite wells for the second trial.

To determine the ability of *C. robusta* sperm to recognize and swim toward eggs in these chambers, one of the wells had eggs from a single female at a concentration of 300 eggs ml^{-1}, while the second well had no eggs. The eggs were allowed to sit in the chamber for 30 min prior to sperm addition to create a chemoattractant gradient.

Based on the initial sperm concentration per individual, dry sperm was added to the center depression after the 30-min gradient preparation period to create a diluted concentration of 10^7 sperm μl^{-1} based on the chamber's total volume. Approximately 300 μl of seawater was sampled from ~0.5 cm above the bottom of each well 10 min after sperm addition. Using a hemocytometer, the number of sperm found in a 2.5×10^{-4} μl subsample (the volume equivalent to the smallest squares in the hemocytometer) was counted, and the average of 4 such counts was recorded for each well. These averages were used in an ANOVA to determine whether there was a significant difference in the average number of sperm recovered in wells with eggs compared to wells without eggs. Egg identity was added as a random blocking factor for the two replicates. Between experiments, chambers were rinsed with hot, fresh water and allowed to dry for 48 hours or more to remove any lingering chemoattractants. Ten focal individuals were used for a total of 18 trials, as 2 individuals used did not have enough sperm to complete both replicates.

Sperm choice for self eggs or non-self eggs

To determine the ability of *C. robusta* sperm to recognize and choose non-self eggs, sperm from an individual were given the choice between their own eggs and eggs from a different *C. robusta* individual in a dichotomous choice chamber. Eggs from a non-self individual and those from that same individual were placed in different wells at a concentration of 300 eggs ml^{-1} and were allowed to sit for 60 min to establish a chemoattractant gradient prior to sperm addition. A longer wait time was utilized in these trials to ensure that enough of a gradient had built up for sperm to encounter both eggs' chemoattractants while still in the center depression.

After 60 min had elapsed, 20 μl of dry sperm were placed in the center depression of the chambers. The sperm were left for 15 min, after which a 300-μl sample was collected from each well. The number of sperm observed in a 0.004-μl volume of subsample (the volume equivalent to the medium-sized squares in the hemocytometer) was counted using a hemocytometer. The average from six counts per well was used in an ANCOVA to determine whether there were more sperm found in the well with non-self eggs compared to wells with self eggs. Initial sperm concentration was used as a covariate in the model to determine whether sperm concentration affected the number of sperm recovered, and sperm identity was added as a random variable to block by replicates. Initial sperm concentrations ranged from 2.67×10^6 to 1.93×10^7 sperm μl^{-1}. Two replicates were performed per individual, with the egg positioning switched between replicates to avoid any potential biases due to collection artifacts, uneven diffusion, or potential directional biases in sperm swimming unrelated to chemotaxis. Between experiments, chambers were rinsed with hot fresh water and allowed to dry for 48 hours or more to remove any lingering chemoattractants. Thirteen focal individuals were used, for a total of 26 trials.

Changes in swimming behavior based on self versus *non-self chemoattractants*

To determine whether sperm velocity or motility was different based on chemoattractant identity, videos of sperm activated in self- and pooled-egg water were analyzed using a computer-assisted sperm analysis (CASA) program in Image J (ver. 1.43, Schneider *et al.*, 2012). Egg water was obtained by filtering out eggs that had soaked in seawater for over an hour, using a 60-μm mesh. Because we were interested in examining the differences in sperm behavior when activated by self egg chemoattractants *versus* any other chemoattractants from the population, we used pooled-egg water to reduce potential variance that might arise due to differences in chemoattractant production among individuals. To create the pooled sample of egg water, an equal amount of the egg water from four individuals was combined. These four individuals were filmed as a block, such that each individual's sperm was

filmed with only its own egg water, as well as the pooled-egg water that consisted of itself plus the other three individuals in the block.

For each of the 32 individuals utilized, sperm were videoed at a concentration of 10^5 sperm μl^{-1}, with 3 videos taken of sperm activated in self egg water and 3 in pooled-egg water, for a total of 6 videos. Sperm were videoed at 80 fps using a Fuji Finepix HS30 (Minato, Tokyo). For each video, 15 seconds were analyzed using CASA (ImageJ, ver. 1.43; Schneider *et al.*, 2012), and the curvilinear velocity and percent motility were recorded. An ANCOVA was used to find whether there were significantly faster swimming speeds in the non-self egg water over the self egg water. An ANCOVA was also performed on percent motility to determine whether there was a difference in the percent of sperm activated by self *versus* non-self chemoattractants. For both models, egg concentration was added as a covariate in order to account for possible differences in chemoattractant concentration; for the pooled-egg water, the average egg concentration of the four individuals in the pool was used. Additionally, sperm identity was added as a random variable to block by individual and identify differences in sperm behavior among males.

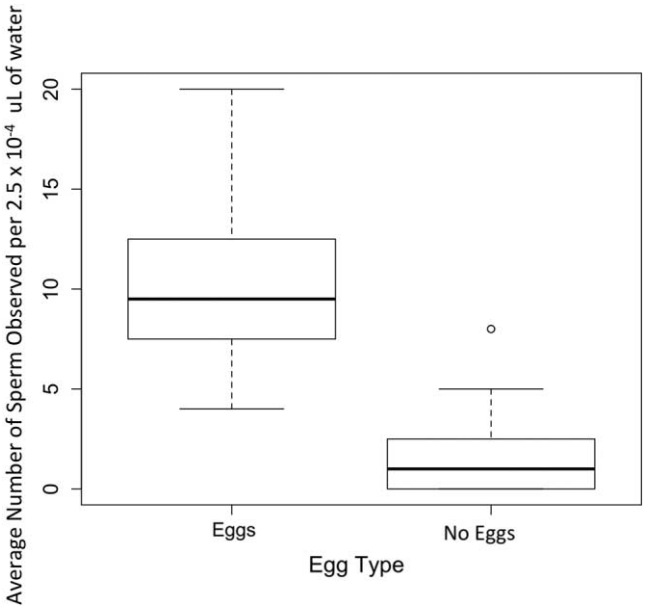

Figure 2. Boxplot of the average number of sperm counted in samples of 2.5×10^{-4} μl of water taken from each well of the dichotomous chamber. Boxes represent the upper and lower quartiles, while the lines represent the median values. Whiskers encompass 95% confidence intervals. Wells contained either eggs or no eggs.

Results

The spermatozoa from *Ciona robusta* had a clear preference toward chambers that contained eggs rather than those that were empty ($P < 0.001$; Table 1). On average, 10.2 ± 4.3 sperm per 2.5×10^{-4} μl (±SD) were recovered from wells with eggs, while 2.1 ± 2.7 sperm per 2.5×10^{-4} μl were recovered from wells without eggs (Fig. 2). Egg identity also affected how many sperm were recovered ($P < 0.001$; Table 1).

There was also a significant increase in the number of sperm recovered from wells with non-self eggs when compared to wells that contained self eggs ($P = 0.002$; Table 1). From non-self wells, on average, 5.23 ± 4.53 sperm per 0.004 μl were recovered, while 3.10 ± 2.53 sperm per 0.004 μl were recovered from wells that contained self eggs, resulting in an increase of 1.6× sperm recovered in non-self wells (Fig. 3). Initial sperm concentration and sperm ID were also found to affect the amount of sperm recovered from the wells ($P = 0.011$ and $P < 0.001$, respectively; Table 1).

There was no significant change in sperm motility when sperm was activated by self-egg water when compared to pooled-egg water ($P = 0.636$; Table 2), nor was there a difference in sperm swimming speed ($P = 0.854$; Table 2). Egg concentration ranged from 906 to 4850 eggs ml^{-1} in the preparation of egg water, but this variation did not significantly influence sperm swimming speed or motility ($P = 0.752$ and $P = 0.268$, respectively; Table 2). There was a significant difference in both sperm swimming speed and motility based on individual identity ($P < 0.001$ and $P < 0.001$, respectively; Table 2).

Table 1

Results of ANOVA and ANCOVA for the number of sperm recovered from the wells of the dichotomous chamber experiments

Experiment	Source	df	SS	MS	*F*	*P*-value
Eggs *vs.* no eggs	Treatment	1	520	520	94.186	<**0.001**
	Egg ID (block)	8	256.7	32.1	5.812	<**0.001**
	Residuals	22	121.5	5.5		
Self eggs *vs.* non-self eggs	Treatment	1	76.7	76.73	11.114	**0.002**
	Sperm concentration	1	49.2	49.22	7.128	**0.011**
	Sperm ID (block)	11	411.5	37.41	5.418	<**0.001**
	Residuals	38	262.4	6.9		

df, degrees of freedom; MS, mean square; SS, sum of squares.

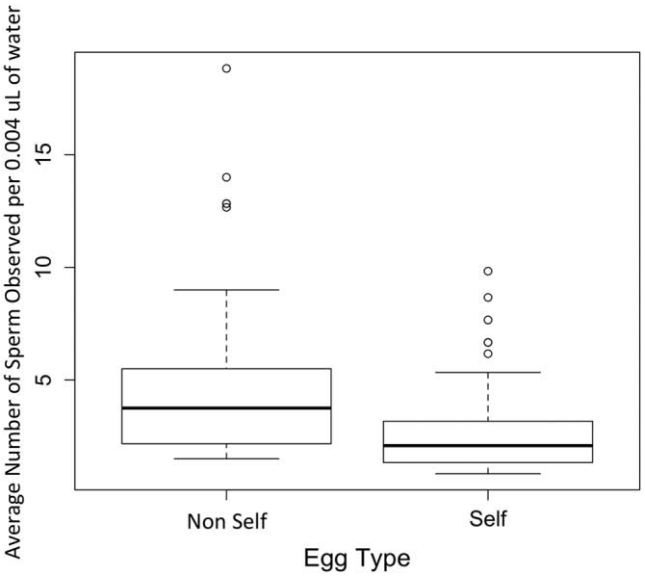

Figure 3. Boxplot of the average number of sperm counted in samples of 0.004 μl of water recovered from each well of the dichotomous chamber. Boxes represent the upper and lower quartiles, while the lines represent the median values. Whiskers encompass 95% confidence intervals. Wells contained either self eggs or non-self eggs.

Discussion

Our results suggest that sperm were able to sense and follow egg chemoattractants, as evidenced by the recovery of almost five times as many sperm from wells with eggs than from wells without eggs. This is not surprising given the noted ability of *Ciona robusta* sperm to be activated and attracted to chemoattractants produced by their eggs (Millar, 1982; Yoshida *et al.*, 1993). However, we found that sperm were recovered at a higher number (almost 1.6 times as many sperm) from wells with non-self eggs than those wells that had eggs from the same individual. This is the first evidence that sperm may be able to distinguish between self and non-self eggs and that they will aggregate more toward non-self eggs when given the choice.

It is possible that significantly fewer sperm were recovered from wells with self eggs because those sperm were removed from the water column by attachment with self eggs; but this seems unlikely, as the attachment rate between self eggs and sperm would have to be almost twice as fast as attachment between sperm and non-self eggs, and fertilization mechanics suggest that this would not occur unless collision rates were increased (Styan, 1998). Given that sperm swimming speed seemed similar between sperm activated by self and non-self chemoattractants, it seems unlikely that collision rates would be higher for self eggs. Additionally, self sperm can be detached from the egg in *C. robusta* (Yamada *et al.*, 2009; Yamaguchi *et al.*, 2011; Saito *et al.*, 2012). This all suggests that increased attachment to self-eggs is an unlikely reason why fewer sperm were recovered from self-egg wells.

Interestingly, while we found that there was a difference in sperm aggregation based on egg identity, we found no difference in the swimming mechanics as measured in this study. If a complete block to self-fertilization evolved, it seems logical that sperm activation should not occur unless in the presence of a non-self egg, given that once activated, sperm lifespan is considerably shortened (Bolton and Havenhand, 1996; Levitan, 2000). Evidence from among-species comparisons suggests that different processes may govern sperm activation and attraction, as sperm can be activated but not attracted to some eggs from different species (Yoshida *et al.*, 2013). Our results suggest that this may be the case as well, as chemoattractant identity did not affect sperm activation. Others have found that self eggs can activate allosperm (Kawamura *et al.*, 1987), but we found that the degree of activation as measured by percent motility and curvilinear velocity was the same whether sperm were exposed to self-egg water or pooled-egg water. Instead, we found there was a significant difference based on sperm identity, suggesting that some individuals possess sperm that are less motile or swim slower when exposed to any chemoattractant, regardless of its source.

It is possible that swimming behavior, rather than overall speed, is different depending on the chemoattractant presented

Table 2

Results of ANCOVAs for sperm swimming speed and motility when exposed to either self- or pooled-egg water

Experiment	Source	df	SS	MS	*F*	*P*-value
Sperm swimming speed (VCL)	Treatment	1	8	7.53	0.225	0.636
	Egg concentration	1	3	3.36	0.1	0.752
	Sperm ID (block)	30	5619	187.3	5.6	<**0.001**
	Residuals	152	5084	33.45		
Sperm motility (%)	Treatment	1	0	0.00046	0.034	0.854
	Egg concentration	1	0.017	0.01674	1.235	0.268
	Sperm ID (block)	30	5.516	0.18388	13.563	<**0.001**
	Residuals	152	2.061	0.01356		

df, degrees of freedom; MS, mean square; SS, sum of squares; VCL, curvilinear velocity.

to the sperm. Because we filmed sperm in a monotonic environment and indirectly assessed swimming behavior in the dichotomous chambers, it is unclear which behavioral mechanism may be responsible for causing the difference in aggregation; videos of sperm movement using a point-source-created chemoattractant gradient would be necessary to directly assess differences in sperm swimming behaviors. By using videos of point-source chemoattractant gradients, studies have shown that differences in the sperm's ability to orient using chemoattractants can result in differences in sperm aggregation around presumably compatible eggs within a species (Evans *et al.*, 2012; Hussain *et al.*, 2016, 2017). It is possible that non-self egg chemoattractants elicit a stronger bias in sperm movement toward non-self eggs or induce sperm to directly orient toward non-self eggs.

What is clear is that the ability of self chemoattractants to activate self sperm can allow for self-fertilization to occur, but when given the choice, sperm will aggregate in greater numbers toward non-self eggs than self eggs. How sperm are able to distinguish between self and non-self eggs, and whether a genetic or functional linkage between chemoattractants and allorecognition proteins exists, still needs to be elucidated. *Ciona robusta* possess allorecognition proteins that are highly variable and are responsible for rejecting self sperm (Yamada *et al.*, 2009; Yamaguchi *et al.*, 2011). If the basis for the genetic variation in allorecognition proteins is translated into a chemical signal that sperm can distinguish prior to encountering eggs, either *via* pleiotropy or by the proteins themselves being shed into the water to be detected by sperm, sperm would be able to distinguish between eggs. This also could be feasible if more than one chemoattractant is produced, providing sperm with slightly different chemoattractant signatures for each individual's eggs, which the sperm can then use to differentiate between them. Hussain *et al.* (2017) found that there were multiple chemoattractants produced by sea urchin (*Lytechinus pictus*); and while not directly compared, their data suggest that there may be differences in the rank order of the amount of each attractant produced. If true, this could provide a way for sperm to distinguish between egg sources, because each female would produce a slightly different blend of chemoattractants.

Being able to distinguish between self and non-self eggs in *C. robusta* can be advantageous because of the selection pressures to avoid self-fertilization when non-self eggs are available (Murabe and Hoshi, 2002) and the tendency for sperm to be rendered immotile during rejection by self eggs after attachment (Yamada *et al.*, 2009; Yamaguchi *et al.*, 2011; Saito *et al.*, 2012). Similarly, given the large fluctuations in population size that some *C. robusta* populations can experience, a total inability to self-fertilize may not be advantageous either (Caputi *et al.*, 2015). Our work suggests that *C. robusta* sperm can activate in the presence of both self and non-self chemoattractants so that they can attempt to fertilize any egg they encounter, but sperm also will aggregate around non-self eggs that can increase their reproductive success when non-self eggs are available. This suggests that *C. robusta* have the ability to be flexible in their mating system, based on the interplay of the relative strengths between selection pressures such as sperm limitation, gamete discounting, and inbreeding depression on self-fertilization.

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