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How osmolality, pH and egg presence may affect spermatozoa motility in Mussismilia harttii

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Abstract

Spermatozoa motility initiation have never been investigated in Brazilian corals. This study aimed to evaluate how osmolality, pH and egg presence may affect spermatozoa motility in Mussismilia harttii. Egg-sperm bundles were obtained during the spawning period and used for sperm motility analysis at different osmolality levels (1035, 905, 776 and 646 mOsm/Kg), pHs (from 7.0 to 8.0), cytoplasmic alkalinization and in the presence or absence of eggs. Spermatozoa motility was higher at osmolalities like that of seawater (1035 mOsm/Kg), reaching 97 ± 5.77%, but decreased at lower osmolalities, reaching 8 ± 0.58% at 776 mOsm/Kg and killing the cells at 646 mOsm/Kg. However, when spermatozoa were kept at 776 mOsm/Kg up to 24 h and then reactivated, they maintained their viability for 19 h with 45% of motile cells. The sperm motility was not affected by pHs and the presence of eggs. Our results suggest that osmolality could play a key role in the motility of M. harttii spermatozoa. These observations raise concerns within the context of climate change, since we observed that the sperm cells are dependent on the environment balance to be motile and be able to reach an egg.

Keywords: *Brazilian coral, coral gametes, osmolality, cytoplasmic alkalinization, motility*

Introduction

Corals support a rich environment and are essential to global ocean health. Scleractinian corals provide the reef complex habitats which serve as nurseries and shelters for about a quarter of all known marine species (Fisher et al. 2015). However, coral reefs have been severely impacted by natural and anthropogenic stressors such as diseases, predation, algae overgrowth, sedimentation, physical damage, pollution, and climate change (Hughes et al. 2019).

By investigating coral reproductive physiology, we can develop biotechnologies that aid in enhancing their ability to reproduce under changing environmental scenarios, contributing significantly to reef restoration efforts. It is known that hermaphroditic broadcast spawning is the dominant pattern of sexual reproduction among scleractinian corals, and most of them envelop their gametes with mucus which are expelled as small floating packages named bundles (Harrison 2011; Valente et al. 2023). The egg–sperm bundles have an important role in transporting gametes to the ocean surface, which minimizes sperm dilution, and increases the chance of spermatozoa and eggs from different colonies meeting (Harrison 2011).Contact with the external environment activates spermatozoa and this activation can be initiated by several ways. The process of sperm motility activation suggests the existence of a variety of signaling pathways. Specific differences between species have been investigated in relation to the sensitivity of spermatozoa to environmental osmotic pressure and ionic composition, processes of polarization/depolarization of the plasma membrane, involvement of signaling molecules and phosphorylation of flagellum and axoneme proteins (Kaupp et al. 2006; Morita et al. 2009; Kaupp and Strünker 2017).

The first studies of Brazilian coral species reproductive biology overcame many difficulties because of the lack of information about its distribution and specific biology which hamper the development of

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monitoring, management and recovery programs (Pires et al., 1999). Nowadays it is known that the cauliflower coral *Mussismilia harttii* (Verrill 1868) is a scleractinian species endemic to Brazilian reefs, one of the main builders of the Southwestern Atlantic reefs (Leão et al. 2016) and is currently classified as endangered (EN) (ICMBio 2018). Studies on the reproductive biology of corals of the genus *Mussismilia* are scarce and until recently, knowledge was restricted to morphological descriptions, without metabolic or physiological investigations (Pires et al. 1999; Neves and Pires 2002). The data available present information collected by histological examination from sexuality patterns, modes of reproduction, synchrony and spawning periods of the three species of the genus *Mussismilia* (Pires et al., 1999). Over the last few years, our team has expanded knowledge of this species' reproductive biology by investigating the ultrastructure details of egg-sperm bundles and single gametes (Valente et al. 2023), morphology and physiology of spermatozoa and morphology and physiology of oocytes. Our findings enlighten questions related to *Mussismilia* reproductive physiology. However, the aspects involved in the initiation of sperm motility in Brazilian corals have never been investigated. Knowing that *Mussismilia* spermatozoa becomes active shortly after the bundle dissociation, we postulate that osmolality may play a central role in the initiation of spermatozoa motility. Osmoconformers organisms, such as corals, use cellular volume regulation to react to osmotic changes. High sodium and chloride concentrations determine the osmolality of the extracellular space, and the osmolality of the intracellular space is determined by organic osmolytes and potassium. Also, it is known that major organic osmolytes have shown beneficial attributes such as protein and membrane stabilizing abilities (Podbielski et al., 2022). According to that we consider evaluating the role of osmolality and pH in the motility initiation of coral sperm cells. In order to test this hypothesis, we examined how osmolality, pH, cytoplasmic alkalinization and the presence and absence of eggs could affect the sperm of the reef-building coral *Mussismilia harttii*.

Methods

Colony sampling and legal authorizations

Each year colonies of *Mussismilia harttii* (n = 40) were collected from around the Recife de Fora Municipal Marine Protected Area (16°24'31″S; 038°58'39″W), Bahia State, Brazil, three weeks before the species expected spawning period (Pires et al. 1999, 2016), in September 2019 and September 2021. These colonies were taken to the Research Base of the Coral Vivo Project and placed in circular 1,000 L open-flow tanks, with a constant renewal of seawater and under conditions of natural light and pH 7.82. After the end of the experiments, the colonies were returned to the reef. This research was approved by the Chico Mendes Institute for Biodiversity Conservation (ICMBio - SISBIO No. 63368-1) and by the Porto Seguro Municipal Environment Department (Authorization No. 01/2019).

Egg-sperm bundles collection and separation

During the spawning period, the water flow into the tanks was shut off and the bundles containing eggs and sperm were haphazardly collected from the surface of the tank using a Pasteur pipette. The bundles (n=40) were collected from four colonies (10 per colony), during the first and second night of spawning. The first group of bundles (n=10) were collected before dissociation, to avoid the contact between sperm and seawater, to keep the sperm cells immotile (Experiments 1 and 2). The second group of bundles (n=30) were collected and transferred to tubes containing10 mL of seawater (10 bundles each), for complete dissociation (~10 min), so the sperm cell was motile (Experiments 3 and 4). After bundle dissociation the eggs floated, and the spermatozoa remained at the bottom. The eggs were then removed with a Pasteur pipette and washed with 3 mL of seawater 10 times to remove any trace of spermatozoa, while the sperm cells concentrated at the bottom were transferred to another tube containing filtered seawater (FSW).

Experimental design

The sperm cells were evaluated according to different parameters (Fig. 1). The first one was the activation of motility through different osmolality solutions (Experiments 1 and 2). Afterwards, different tests were performed to evaluate the maintenance of sperm motility (Experiments 3 and 4).

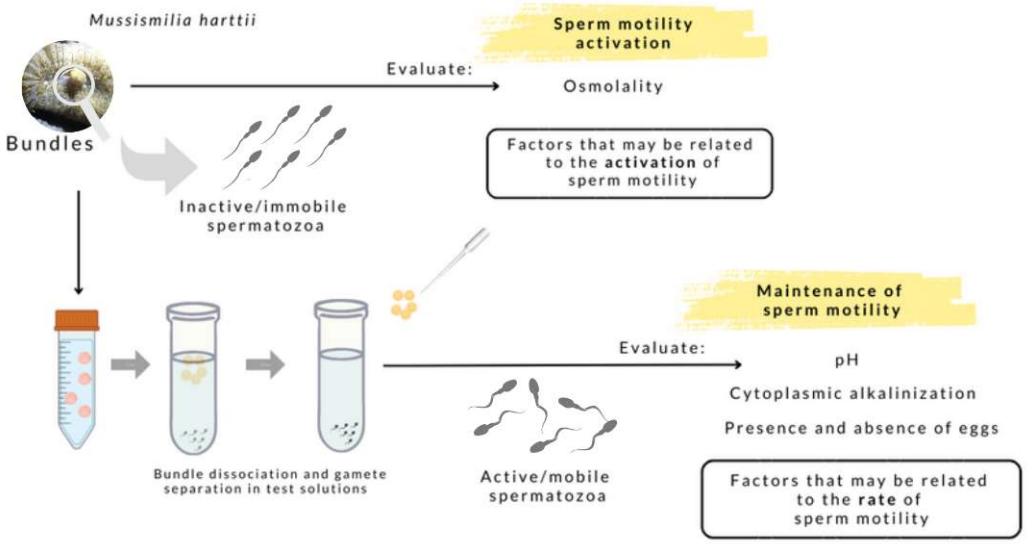

Fig. 1: Summary of experimental design and main goals.

Experiment 1: The effect of osmolality on sperm motility activation

The first experiment aimed to test whether the osmolality of seawater influences coral sperm motility activation. This experiment was carried out over two different nights. On each night 10 bundles were collected from four colonies, totaling 20 bundles (11.66 ± 1.27 x 106 cells/ml). A pool was created with these 10 bundles to carry out the tests. Local seawater with an osmolality of 1035 mOsm/kg was the base for making the three tested solutions with the following osmolalities: 905, 776 and 646 mOsm/kg. NaOH (1.0 M) or HCl (1.0 M) solutions were used, when necessary, to adjust the pH of all tested solutions to 7.8 using a portable pHmeter (LAQUAtwin, HORIBA). To retrieve the

immotile sperm, intact egg-sperm bundles were placed individually on glass slides and opened using tweezers and a scalpel, avoiding any contact of the gametes with the seawater, to prevent sperm activation. The sperm cells were immotile inside the bundle, then became activated when in contact with seawater after bundle dissociation (see Valente et al., 2023).Then aliquots (4 µL) of spermatozoa were collected in triplicate (n=30) and transferred to microtubes containing 50 µl of the different solutions with test osmolalities. After 5 minutes incubation at room temperature (23°C), 10 µl were retrieved from each solution for motility and sperm membrane integrity analysis.

Experiment 2: Using osmolality for preservation of sperm viability

After identifying in experiment 1 that it was possible to maintain viable sperm cells, although immotile, at an osmolality of 776 mOsm/kg, the second experiment was designed. Considering that keeping sperm cells immotile but viable is important to extend their lifespan *in vitro*, we decided to evaluate for how long it would be possible to keep the sperm cells at 776 mOsm/kg and reactivate the motility by exposing them to local seawater (1035 mOsm/kg). Spermatozoa were maintained in 50 µL of the 776 mOsm/Kg solution (triplicate) and incubated for 0, 15, 19 and 24 h at room temperature. Three samples (2 µL) were evaluated at each time, on two different nights, totaling 24 samples analyzed. Sperm reactivation was performed with 12 µL of FSW and motility was assessed under 40 X light microscopy immediately after reactivation (Nikon Eclipse e100). The average sperm concentration used in the experiment was 11.66 ± 1.27 x 10^6 cells/ml.

Experiment 3: Artificial seawater (ASW) as a standard base solution and evaluation of the pH effect on sperm motility

Before testing the effect of pH on the cells, we tested the ASW as solubilization medium for the spermatozoa. Once working, using ASW would bring the advantage of no need for natural seawater collection, and facilitate the execution of experiments and manipulation of gametes in laboratories far from

the coast. The ASW was composed of430 mmol l^{-1} NaCl, 10 mmol l^{-1} $CaCl_2$, 9 mmol l^{-1}KCl, 23 mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} $MgSO_4$ and 10 mmol l^{-1}Hepes-NaOH.The spermatozoa samples used were from 10 bundles collected each night (n=20), from four colonies, submitted to complete dissociation. For the ASW test, a 4 µL-aliquot of sperm cells suspension was diluted on 50 µl of ASW (triplicate).The control group was composed of a 4 µL sperm cell suspension diluted on natural seawater (NSW) (triplicate). After 5 min at room temperature the sperm motility was evaluated under optical microscope 40 X (triplicates) (Nikon Eclipse e100).

Afterwards, to assess the pH effect, bundles (n=10 each night) were collected on a 50-mL tube with NSW from the tank (triplicate), and after their dissolution sperm cell samples were submitted to the test. Therefore, the spermatozoa were already motile for that experiment since they were in contact with seawater. The ASW was used for the different pH solutions preparation and the pH was adjusted to a range between 7 and 8 using solutions of 1.0 M NaOH or 1.0 M HCl. A portable pHmeter (LAQUAtwin, HORIBA) was used for pH determination. The sperm motility was evaluated (2 µL aliquot) after 5 minutes incubation at room temperature (23°C) under optical microscope40 X in triplicates (Nikon Eclipse e100).

Experiment 4: The effect of cytoplasmic alkalinization on sperm motility

The ammonium chloride (NH_4Cl, 20 mmol L^{-1}) was used to increase the intracellular pH (pH_i) to investigate the possible effect of cytoplasmic alkalinization on motile sperm (Morita et al. 2006). For that experiment the solutions used were artificial seawater (ASW) with pH 7,8; and ASW with ammonium chloride pH 7.39 (NH_4Cl, 20 mmol L^{-1}); ASW with oocytes; and ASW conditioned with oocytes with ammonium chloride pH 7.39 (NH_4Cl, 20 mmol L^{-1}) (based on Morita et al. 2006).For the solutions conditioned with oocytes, 3 oocytes from different colonies were placed on a microtube containing 800 µL of ASW or ASW+NH_4Cl and added 100 µL of sperm suspension. The sperm samples used were from 20 bundles completely dissociated. The sperm motility was immediately evaluated under optical microscope 40 X (triplicates) (Nikon Eclipse e100).

Evaluation of sperm motility and membrane integrity

Sperm motility was determined by analyzing triplicates of 10 µL aliquots from each experimental unit on a glass slide and visualizing the sample under light microscopy at 40X magnification. The results were expressed as the percentage of motile spermatozoa exhibiting progressive movement (Godoy et al. 2021).

The sperm membrane integrity was assessed using eosin and nigrosin vital staining. On one end of a coverslip, 2 µL of 5% eosin (Sigma-Aldrich), 2 µL of 10% nigrosin (Sigma Aldrich), and 2 µL of the sample were spread linearly. After the sample was mixed with the two stains, a smear was carried out and the coverslip was let dry out and then analyzed 100 sperm cells per sample under an optical microscope (Nikon Eclipse e100) using a 100Xmagnification. The spermatozoa stained in either red or pink were considered with damaged membrane, while those that were not stained were considered with intact membrane. We used three replicas per treatment and data are expressed as the percentage of cells with an intact membrane.

Statistical analysis

The normality of the data was assessed using the Shapiro-Wilk test. The osmolality experiment was evaluated over time (0, 15, 19 and 24 hours) using ANOVA one-way for motility with Tukey post hoc test. For membrane integrity it was applied Kruskal-Wallis test with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. For before and after motility reactivation on each time point it was applied ANOVA two-way with Tukey post hoc test. Sperm motility among pH varying from 7.0 to 8.0 was evaluated using ANOVA one-way followed by Holm Sidak test. Evaluations between NSW and ASW and between ASW and ASW + NH_4Cl were performed using ANOVA one-way followed by Tukey's post hoc test. The effect of egg's presence using ASW and ASW + NH_4Cl was evaluated using ANOVA one-way followed by Tukey's post hoc test. All experiments were carried out in triplicate and repeated three times. The analyses were carried out using GraphPad Prism 9.0 software, the data are presented as mean ± standard deviation and the level of significance used was 5%.

Results

Experiment 1 showed that at 1035 mOsm/Kg 97 ± 5.77% of the sperm cells were motile, displaying 90 ± 5.20% of membrane integrity (Figure 2A and 2B). Considering that this first experimental group was kept on the natural seawater osmolality, it was expected to find such high motility and membrane integrity results. However, when the osmolality was lowered to 905 mOsm/Kg the motility dropped to 63 ± 5,77% although 98% ± 2 of the sperm cells had an intact membrane (Figure 2A and 2B).When the spermatozoa were subjected to 776 mOsm/Kg, the motility decreased to 8 ± 0.58%, which means 92% of the sperm were immobile. However, these sperm cells had a high rate of membrane integrity (98 ± 1%), demonstrating that they were still alive. At 646 mOsm/Kg all the spermatozoa were immobile and presented a decrease in the membrane integrity (68 ± 10.69%) (Fig. 2A and 2B).

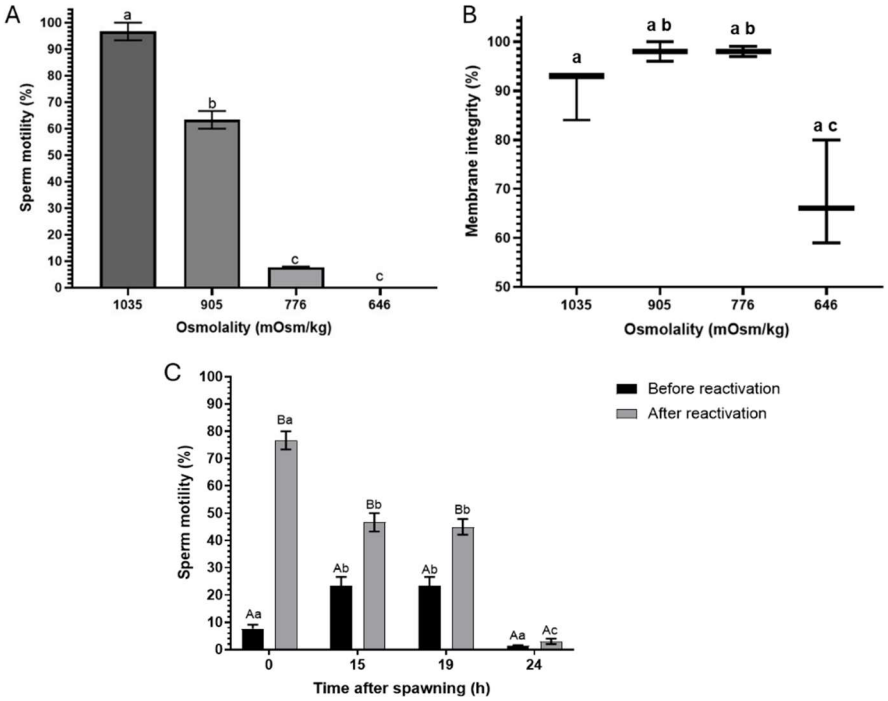

Fig. 2: The effect of osmolality on sperm motility of *Mussismiliaharttii*. A - Motility suppression of spermatozoa according to the decrease osmolality. Different lowercase letters indicate a statistical difference (F (3, 8) = 381.3 P<0.0001); B - Membrane integrity of spermatozoa according to the decrease osmolality. Different lowercase letters indicate a statistical difference (P=0.0012); C - Spermatozoa motility evaluation kept in 776 mOsm/Kg for 24 hours. Motility reactivation with seawater at 1035 mOsm/Kg. Distinctive capital letters and lowercase letters indicate a statistical difference (F (3,6) = 49.18, P = 0.0001).

In Experiment 2 we corroborate the data from Experiment 1 regarding the intact membrane of sperm cells at 776 mOsm/Kg by reactivating their motility with natural seawater (1035 mOsm/Kg) after 0, 15, 19 and 24 hours to evaluated the presence of motile cells. It is important to consider that although the motility showed a significant decrease after 15 h of incubation compared to the initial assessment time, it remained stable up to 19 h (Fig. 2C). This result demonstrated that it is possible to preserve *Mussismilia harttii* sperm cells motility and viability for 19 h (45 ± 5% motile cells) without the need of any special solutions, only by decreasing the seawater osmolality.

After the tests of osmolality with natural seawater (NSW) we decided to test the artificial seawater (ASW) to identify if it would be possible to use it as a diluent for other experiments. The motility of *M. harttii* spermatozoa exposed to ASW was 90 ± 5.77% and did not differ significantly from those kept in NSW (87 ± 9.43%)(P= 0.4063).For this reason, ASW was used in Experiment 3 as base for the different

pH solutions tested. The results from Experiment 3 demonstrated no significant difference in motility among the different ASW pH solutions, showing that the pH between 7 and 8 was not able to promote changes in sperm motility in *M. harttii* (Fig. 3A).

The ASW was also used as a base solution in Experiment 4. The incubation of sperm cells in ASW/eggs solution did not result in any significant change in spermatozoa motility compared to the ASW without eggs group (89±6.01% and 94±5.27%, respectively) (Fig. 3B). Regardless of the presence or absence of eggs, the addition of NH_4Clpromoted a significant decrease in spermatozoa motility (12 ± 7.5% and 16 ± 8.20%, respectively) (Fig. 3B).

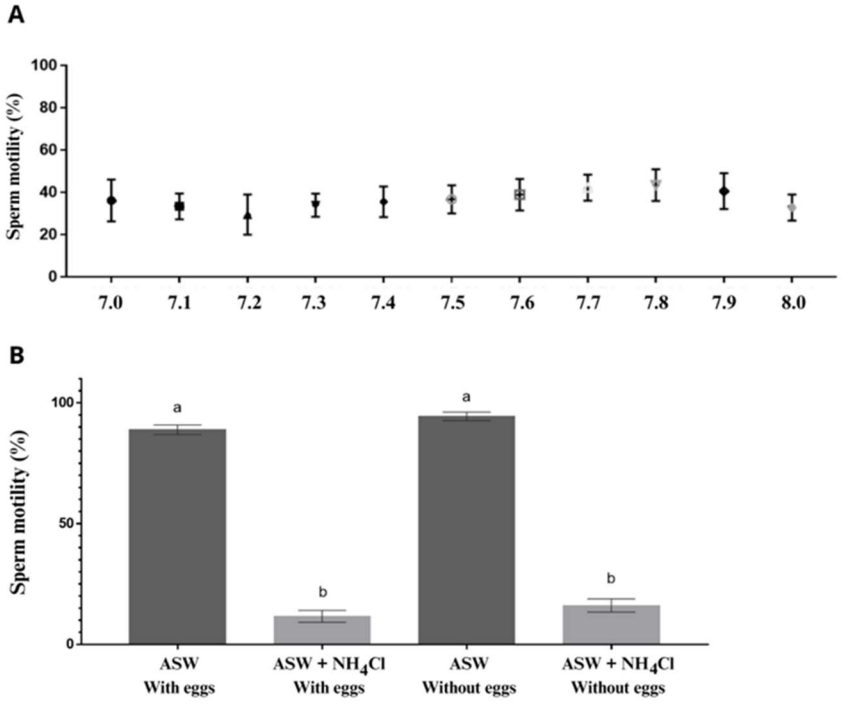

Fig. 3. Effect of seawater pH on motile sperm of *Mussismiliaharttii*. A – Motile spermatozoa kept at room temperature (23°C) in artificial seawater (ASW) in different pHs range. Data among pHs were analyzed using one-way ANOVA, F (3.117,24.93) = 2.874, P= 0.0546. B – Sperm motility evaluation according to the exposition to eggs or NH_4Cl in ASW. Data among groups were analyzed using one-way ANOVA, F (2.504, 20.03) = 249.4, P< 0.0001. Different letters indicate significant differences (P< 0.01). Error bars represent standard deviations.

It is important to note that although NH_4Cl is known to produce mildly acidic solutions, the solution with ASW did not become acid, keeping its pH from 7.2 to 7.3. Therefore, the observed changes in sperm motility probably were related to some interference at the cellular level and not a result of changes in the solution parameters.

Discussion

Despite significant variations in reproductive traits and activation cues among different taxa, the downstream signaling pathways responsible for activating sperm motility appear to be highly conserved in the few investigated taxa of marine invertebrates' sea urchins and, more recently, corals (Speer et al. 2021; Glass et al. 2023).According to Valente et al. (2023) the spermatozoa of *Mussismilia harttii* remain inactive, without flagellar movement, while inside the bundle. This observation suggests that a microenvironment is created inside the bundle, with different characteristics from the external environment, and one of the parameters that could differ is osmolality. When the bundle is dissociated and the spermatozoa are exposed to the external environment, they become actively motile. Osmolality has been pointed out as an important

factor for initiation of sperm motility in many aquatic species (and, in fact, our results showed that the motility of *Mussismilia harttii* spermatozoa was strongly influenced by the seawater osmolality.

Sperm cells kept in 776 mOsm/Kg were able to become motile again after reactivation with normal seawater. In that condition, it was possible to keep the spermatozoa alive for 19 h and recover motility in almost 50% of the sperm cells. Usually, the spermatozoa of *Mussismilia harttii* lost their motility after 16 h of spawning, when sperm cells were kept in normal osmolality seawater (Godoy, pers comm). Here, the motility and intact membrane results after reactivation demonstrated that it was possible to paralyze the sperm cells without killing them, saving energy and extending their viability, a strategy that could be used for short-term sperm preservation, without the need to add any substance or use low temperature.

ASW can be used as a substitute for NSW, and its use brings the advantages of not having to collect seawater, avoiding the possibility of having contaminants to the system and avoiding collect water samples with variation in salinity, which is common in coastal regions, promoting harmful changes on the experimental systems. In addition, it allows the execution of experiments and manipulation of gametes by institutions that are located far from coastal areas, or to act in an emergency. Moreover, it contributes to the standardization of procedures in scientific experiments to ensure consistency and comparability between results, as we did at the pH test.

We expected that it would be possible to observe some interference on motility of *Mussismilia harttii* spermatozoa between the range of pH tested. Several studies suggest that the alkalization of coral sperm cytosol promotes motility (Morita et al. 2006; Speer et al. 2021; Glass et al. 2023). The cytosolic alkalinization of spermatozoa stimulates soluble acetyl cholinesterase (sAC) and activates sperm motility via soluble adenylyl cyclase (cAMP) and protein kinase A (PKA) that leads to a phosphorylation of axonemal proteins controlling motility. This pathway has been shown to be conserved between sea urchins and the broadcast spawning hermaphroditic coral *Montipora capitata* (Speer et al. 2021) and the gonochoric *Astrangia poculata* (Glass et al. 2023). It led to the discovery that sAC is the central signaling node and that the downstream signaling pathways that activate sperm motility are highly conserved in a diversity of cnidarian species with divergent sexual systems, as well as between phyla (Glass et al. 2023). Additionally, the CatSper Ca^{2+}channel, a principal calcium channel found in sea urchins and conserved in mammalian and coral sperms, responds to both depolarization and elevated pH. This response generates Ca^{2+}influx signals that alter the sperm flagellar waveform (Speer et al. 2021). However, in this study the pH test showed that the spermatozoa from *Mussismilia harttii* were able to survive between a pH range from 7.02 to 8, without loss or improvement of motility.

It is known that the ammonium chloride (NH_4Cl) has been used in several studies to increase the internal pH (pH_i) of spermatozoon and thus activate motility in the absence of eggs (Morita et al. 2006; Morita et al. 2009; Speer et al. 2021; Glass et al. 2023). Additionally, the pH_i is important for sperm motility and cytosolic alkalinization is responsible for the onset of sperm motility. Given that the motility of *M. harttii* spermatozoa is activated shortly after exposure to seawater, it is reasonable to postulate that the environmental factor responsible for sperm initiation in this species would likely have already induced alterations in pH_i and the concurrent regulation of intracellular Ca^{2+}concentration within the spermatozoon. When we tested the increase in pH_i using NH_4Cl, our expectation was changes related to the balance among intracellular alkalinization, sAC-cAMP-PKA signaling and calcium levels, consequently leading to changes in sperm motility. However, NH_4Cl inhibited sperm motility, as it did in the European eel, *Anguilla Anguilla* (Vílchez et al. 2017).

It is known that for most invertebrates the presence of substances released from the eggs will contribute to cytoplasmic alkalinization. Peptide or proteinaceous sperm-activating factors have been identified among the substances released by the eggs of jellyfish, *Hippopodius hippopus* (Cosson et al. 1986), and sperm-activating peptides (SAPs) have been identified in egg jelly from sea urchins (Suzuki and Yoshino 1992) and starfish, *Asterias amurensis* (Nishigaki et al. 1996).Some *Acropora* species showed alkalinization of sperm cytosol induced by sperm-activating factors released from eggs initiating motility (Morita et al. 2006, 2009). In contrast, the sperm-activating factors released by the eggs from coral *Montipora digitata* (Coll et al. 1994) and ascidians *Ciona intestinalis* and *Ciona savignyi* (Yoshida et al. 2002) are small organic compounds: unsaturated fatty alcohols and sulfate-conjugated hydroxysteroids, respectively. Considering what is known to date regarding the spermatozoa of a few coral species, they are immobile in seawater, and activated when they are close to the eggs, as occurs in the species *Montipora digitata* (Coll et al. 1994), *Lobophytumcrassum* (Coll et al. 1995), *Acropora digitifera, A. gemmifera* and *A. tenuis* (Morita et al. 2006), we decided to test if the sperm cells will be stimulated by their presence. According to our observations, the presence of eggs on the ASW with or without NH_4Cl did not improve sperm motility in *M. harttii*. It was possible to see that the harmful effect of the NH_4Cl was stronger than the possible beneficial effect of the eggs.

Conclusion

It is still unclear how the signaling pathway for motility activation is initiated. In this case, the pattern we saw in *M. harttii* seems to be like other marine species, where the hypertonicity of the surrounding environment triggers sperm motility, as in some fish. Other promising factors should also be considered, such as responses to different levels of inorganic carbon, such as bicarbonate, which could lead to cAMP production. Our results showed that changes in osmolality do indeed inhibit the spermatozoa motility in *M. harttii*. Additionally, maintaining spermatozoa at 776 mOsm/Kg can prolong their lifespan, making it easier to handle the sperm samples in laboratory experiments and enabling their transportation between research and conservation centers. Furthermore, our findings suggest that modifications in intracellular pH can lead to significant changes in *M. harttii* sperm motility. These observations raise concerns within the context of contemporary climate change scenarios, including ocean warming and acidification.

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Author contributions

Cláudia Kelly Fernandes da Cruz– Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft
Nayara Oliveira Cruz– Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing
Andrea Giannotti Galuppo– Conceptualization, Formal analysis , Methodology, Validation, Writing – review & editing
Amanda Pereira Amaral– Conceptualization, Data curation, Investigation, Methodology
Yugo Moraes Pastrana– Conceptualization, Data curation, Investigation, Methodology
Ivan Cunha Bustamante-Filho– Investigation, Methodology
Leandro Godoy– Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing

Data availability

The data generated and analyzed during this study are available from the corresponding author upon request.

Compliance with ethical standards

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval

The authors declare that all applicable international, national and/or institutional guidelines for sampling, care, and experimental use of animals for the study have been followed, all and necessary approvals by the Chico Mendes Institute for Biodiversity Conservation – ICMBio (SISBIO N° 63368-1) have been obtained.

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