



Morphological assessment of free-spawned sperm in scleractinian coral: a relationship between cell morphology and motility

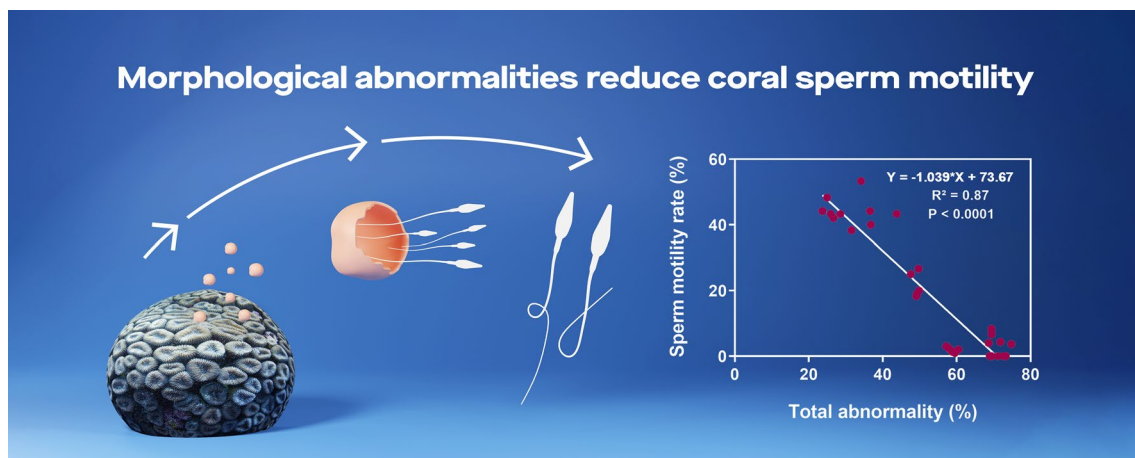
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Abstract

Although the quality of the spermatozoa is a key factor for reproductive success, there is few information describing the sperm quality for most marine invertebrates, especially corals. Given the high mortality rates and several threats currently faced by coral reefs, ways of assessing coral spermatozoa viability are needed. Thus, in this study we proposed a morphological classification for coral spermatozoa using *Mussismilia harttii* as model. In addition, we evaluated the relationship of morphology with the percentage and duration of motility. Spermatozoa were first classified into two main morphological categories: normal and abnormal. Then, we classified morphological abnormalities in the head and flagellum together with proposing subcategories that better described it. In general, $68.77 \pm 6.50\%$ of the spermatozoa had their whole structure intact, while $31.23 \pm 6.51\%$ had morphological abnormalities. Most abnormalities were in the flagellum (five types in total), while only two abnormalities were located in the head. Coiled flagellum and shortened flagellum were the most representative abnormalities, accounting for 24% of the total detected. We observed that reduction of sperm motility is directly related to increased morphological abnormalities and they are influenced by the cell ageing. Our results reinforce how the characterization of sperm morphology can be a practical and useful tool that can help to better understand the effects of multiple stressors on coral reproduction.

Graphical abstract



Keywords Coral gametes · *Mussismilia harttii* · Sperm abnormalities · Morphological classification · Sperm analysis · Reef conservation

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Introduction

Although spermatozoa are simple structure cells, their functions are specialized and crucial for sexual reproduction and the genetic variability of the species. Thus, to have a greater chance of successful fertilization, it is mandatory to have viable sperm, which is defined as the ability of spermatozoa to successfully fertilize an egg and, subsequently, to allow the development of a normal embryo (Kholodnyy et al. 2020).

Motility and morphology are essential parameters for sperm quality. However, some morphological abnormalities in spermatozoa can be generated during spermatogenesis. These morphological defects mean an abnormal spermatozoon morphogenesis process and lead to reduced fertilization capacity. In addition, they can indicate the individual's health status and reflect the response to physical, physiological, and environmental stressors (Menkveld et al. 2011).

Morphological abnormalities in the spermatozoa flagellum lead to motility impairments. For example, spermatozoa swim in a circular. In other cases, the flagellum displays oscillatory movements without bending forms. These impairments provoke a reduction of gamete interaction. Indeed, the higher the number of spermatozoa with abnormal morphology, the lower the chances of encountering the oocytes (Alavi et al. 2015). In addition to the primary abnormalities resulting from gametogenesis, spermatozoa are subject to damages of secondary origin after being released into the water. Such abnormalities can be caused by environmental contaminants or the cell ageing process (Kaya et al. 2014).

Although sperm quality is a critical factor for reproductive success, there is little information describing it for most marine invertebrates, especially corals (Lewis and Ford 2012). Furthermore, the relationship between factors that affect spermatozoa fertility, such as spermatozoa morphology, percentage and duration of motility, has not yet been studied in corals. Sperm morphology studies have difficulties standardizing and classifying morphological abnormalities (Shabtaie et al. 2016), and analyses from dissected mesenteries (see Steiner 1998) do not represent the released spermatozoa.

Coral reefs are currently threatened worldwide (Hughes et al. 2019). Therefore, ways of assessing coral spermatozoa viability are needed to evaluate individuals' reproductive performance and will be available for examining the effects of stressors and environmental contaminants on reproduction and progeny.

Thus, in this study we proposed a morphological classification for coral spawned spermatozoa using as a model *Mussismilia harttii*, a scleractinian hermaphroditic species

endemic to Brazilian reefs and one of the main builders of the Southwestern Atlantic reefs (Leão et al. 2016). In addition, we evaluated the relationship of morphology with percentage of motile spermatozoa and duration of motility.

Materials and methods

Colonies collection and permits

Hermaphroditic colonies of *Mussismilia harttii* ($n=40$) were collected at the Funil site at the Recife de Fora Marine protected area ($16^{\circ}24'31''S$; $038^{\circ}58'39''W$ —Bahia State, Brazil) under SISBIO licence N° 63368–1. Collections were performed in September 2019, two weeks prior to the predicted spawning period (Pires et al. 1999). Colonies were identified and placed in 1000-L semi-closed tanks connected to the ocean at the Coral Vivo Research Base. Local seawater temperature was 26.50 ± 0.25 °C. After the completion of the experiments, the colonies were returned back to the reef.

Collection of egg-sperm bundles and gametes separation

Experiment was performed over two nights during the spawning window period. Among the 40 colonies, 18 spawned on the first night and 11 on the second night. Each night, 100 egg-sperm bundles were randomly collected from the water surface using a Pasteur pipette and immediately transferred to 50-mL tubes (10 bundles per tube) containing 5 mL of seawater. After 10 min the egg-sperm bundles were naturally dissociated, with eggs floating on top of the tubes being removed and the remaining sperm concentrated was transferred to another 50-mL tube. It is important to mention that in *M. harttii*, when the egg-sperm bundle dissociates, the spermatozoa motility is initiated. Tubes (total = 10) containing the sperm samples were analysed individually for initial motility rate and then put together to compose a pool. The sperm pool was performed to prevent the colony's individual effects, since sperm concentration between *M. harttii* colonies vary significantly (Godoy, personal communication). Sperm concentration of the pooled semen ($10.57 \pm 2.11 \times 10^6$ cells per mL) was assessed using a hemocytometer under light microscopy. Subsequently, the pool was randomly distributed among experimental units, which were characterized by a closed tube containing 5 mL of pooled semen. There were five replicates per evaluation time (0, 5, and 10 h after spawning), totalizing 15 experimental units per night of experiment. The evaluation times were chosen based on previous studies on spermatozoa motility duration carried out by our group (Godoy et al. 2021), and the analyses were completed 10 h after spawning, which was the time when all cells were no longer motile. Throughout the experimental period, tubes containing the samples were kept into a glass aquarium

(80 L) at 26 ± 0.50 °C. Water temperature was controlled by an automatic system (Reef Angel Controller) using 15,000 W heaters and temperature recorders (HOBO U22-001 Data Logger). At each evaluation time, the sperm motility rate was evaluated, and aliquots of the sperm were fixed for analysis of spermatozoa morphology.

Sperm characterization throughout motility time

Sperm motility rate

Sperm motility rate was determined by placing (triplicate) a 10 μ L-aliquot from each.

experimental unit on a glass slide and visualizing the sample under light microscopy at a magnification of $100 \times (10 \times \text{objective lens and } 10 \times \text{ocular lens})$. The results were expressed as the percentage of motile spermatozoa exhibiting forward movement (Godoy et al. 2021).

Spermatozoa morphology assessment

To evaluate sperm morphology, 20- μ L aliquots of the sperm were fixed in microtubes (total = 10) containing 40 μ L of 4% buffered formal-saline solution (1606 mOsmol/kg) (Godoy et al. 2021). Afterwards, 20 μ L of each fixed sample were placed on a histological slide (total = 30 slides) and immediately stained with a 4 μ L-aliquot of 3% Bengal Rose dye and analysed under light microscopy at $100 \times$ objective with oil-immersion lens. Spermatozoa were firstly classified into two main morphological categories: normal and abnormal. Then, we classified the morphological abnormalities according to the damage observed in the head and flagellum together with proposing subcategories to better describe it. An average of 79 ± 17 cells per slide at each evaluation time were counted and the number of normal and abnormal spermatozoa was expressed as percentage of all observed cells. In addition, spermatozoa were analysed under a phase contrast field using an optical microscope (Zeiss Axio Scope.A1) ($1000 \times$ magnification) equipped with a camera (Zeiss AxioCam ERc 5 s). Digital images were taken using the Zeiss AxioVision LE software and head and flagellum length were measured (2 μ m scale) using the software ImageJ 1.1.4. The following equation was used to determine head size (HS):

$$HS = \frac{\text{larger diameter} + \text{smaller diameter}}{2}$$

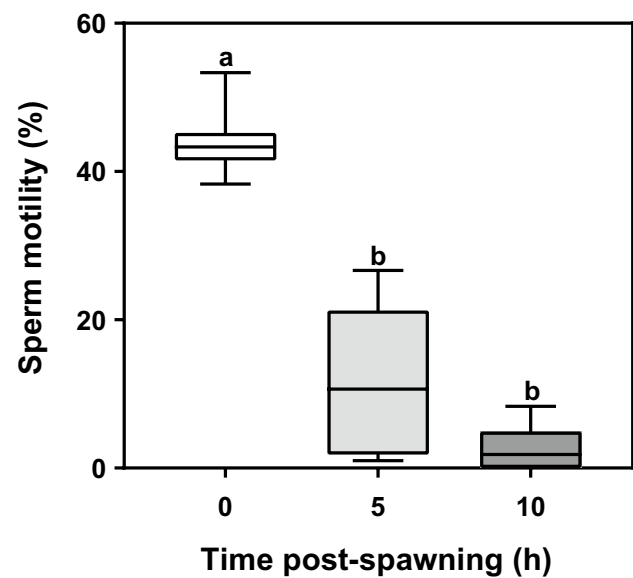


Fig. 1 Sperm motility rate of *Mussismilia harttii* over time post-spawning. All pairwise were compared by Dunn test. Distinctive letters indicate statistical difference over time ($p < 0.05$)

Statistical analyses

Normality and the homogeneity of variance of all data were first assessed using the Shapiro–Wilk and Bartlett’s test, respectively. For motility rate we used one-way analysis of variance (ANOVA) on Ranks (Kruskal–Wallis’s test) followed by Dunn test to identify the differences among evaluation times (0, 5 and 10 h) throughout spermatozoa motility duration. Total abnormality and normal spermatozoa data were transformed using the square root to reach the assumptions (normality and homoscedasticity) of parametric statistics. For all morphological classifications we used one-way analysis of variance (ANOVA) followed by Tukey’s test to identify the differences among evaluation times (0, 5 and 10 h) throughout spermatozoa motility duration. In addition, a simple linear regression was applied to test the relationship between sperm motility rate (dependent variable) and total abnormality, normal spermatozoa, coiled flagellum and shortened flagellum (independent variables). Non-parametric data (sperm motility) are presented as median, minimum and maximum, while parametric data are presented as mean \pm standard deviation and the level of significance was 5%.

Results

Sperm motility rate

Sperm motility was influenced by the duration of motility after spawning (Kruskal–Wallis, $p < 0.001$, Fig. 1), with a

reduction in the number of motile cells over time. Immediately after spawning sperm motility was $44.03 \pm 4.20\%$ (median = 43.33; minimum = 38.33 and maximum = 53.33), being more than 30 percentage points higher when compared to subsequent times (Dunn test; 0 h vs 5 h: $p = 0.0044$; 0 h vs 10 h: $p < 0.0001$), while there was no difference between the times 5 and 10 h after spawning (Dunn test; 5 h vs 10 h: $p = 0.6085$).

Morphological classification of coral spermatozoa

We proposed the following subcategories to classify the abnormalities observed in the head and flagellum: coiled

flagellum, shortened flagellum, bent flagellum, strongly coiled flagellum, free flagellum, free head, macrocephaly and agglutination of cells (Table 1 and Fig. 2).

In quantitative terms, $68.77 \pm 6.50\%$ of the spermatozoa were considered as normal cells while $31.23 \pm 6.51\%$ of the spermatozoa were structurally impaired. The main morphological abnormalities found for *M. harttii* spermatozoa (Fig. 3) were located in the flagellum ($97.50 \pm 1.92\%$ of the total quantified abnormalities), being coiled flagellum and shortened flagellum the most representative, since together accounted for $24.00 \pm 3.08\%$ of the total abnormalities observed (Fig. 4). Conversely, the damages to the head represented less than 1% of the total abnormal cells.

Table 1 Morphological classification for coral spermatozoa with abnormalities detailed according to the damage observed in the head and flagellum

Spermatozoa classification	Description
Normal spermatozoa	Spermatozoa showing structural integrity, presenting $5.93 \pm 0.09 \mu\text{m}$ of total length, oval shaped head ($0.34 \pm 0.03 \mu\text{m}$) and a $5.60 \pm 0.08 \mu\text{m}$ flagellum
Coiled flagellum	Flagellum coils up once or flagellum twists up all over its length
Shortened flagellum	Spermatozoa with a normal shaped head but displaying an average reduction of 54% in flagellum length (ranging from 1.74 to 2.98 μm)
Bent flagellum	Flagellum with a U-shaped flexure
Strongly coiled flagellum	Flagellum with several turns on itself or around the head, forming a circle
Free flagellum	Spermatozoa with normal flagellum length, but with no head
Free head	Spermatozoa with a normal shaped head, but with no presence of flagellum
Macrocephaly	Sperm with a normal flagellum but showing a head 1.6 times greater in diameter (up to 0.60 μm) than normal cells
Agglutination of cells	Many spermatozoa with flagella entangled to each other, being impossible to individually distinguish them

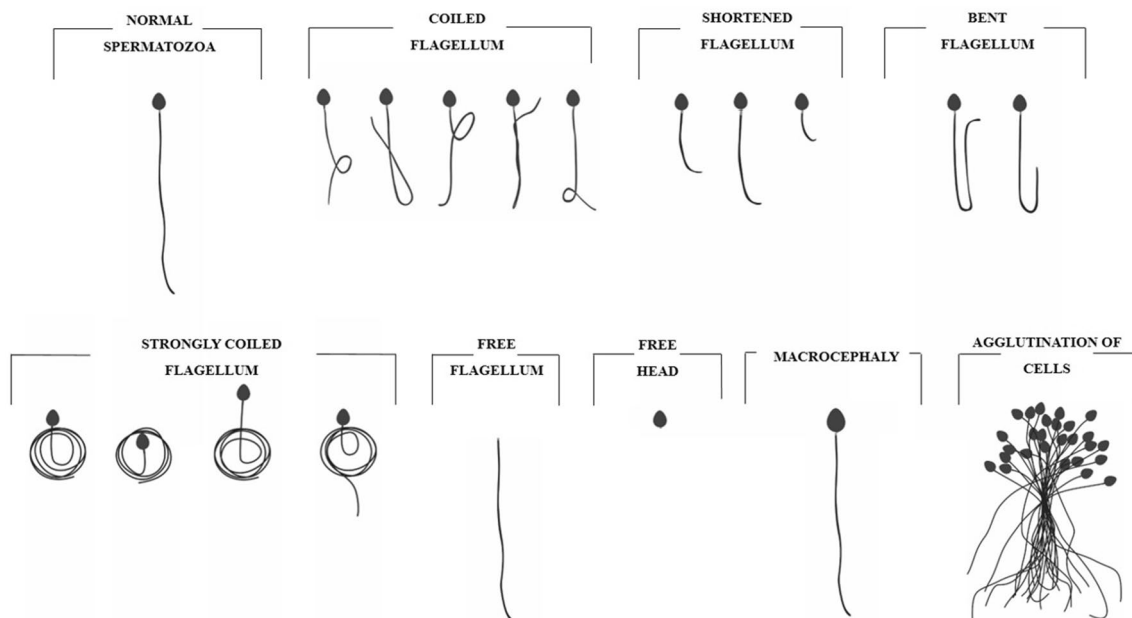


Fig. 2 Illustration designed to represent each morphological classification and abnormalities observed in the head and flagellum of coral spermatozoa

As time passed, it was observed that the number of morphologically normal cells was gradually decreasing from $68.77 \pm 6.50\%$ to $28.99 \pm 1.76\%$ (Tukey, $p < 0.0001$) (Fig. 5a), while the number of structurally impaired spermatozoa was increasing from $31.23 \pm 6.51\%$ to $71.01 \pm 2.25\%$ (Tukey, $p < 0.0001$) (Fig. 5b). Damages to the flagellum remained the most significant, mainly the coiled flagellum (Tukey, $p < 0.0001$) (Fig. 5c) and shortened flagellum (Tukey, $p < 0.0001$) (Fig. 5d).

Correlation between motility and spermatozoa morphology

We observed a strong positive linear relationship between sperm motility and the number of normal spermatozoa (Fig. 6a), where 86% of the highest sperm motilities were explained by higher percentage of normal spermatozoa ($R^2 = 0.86$, $p < 0.0001$). In contrast, a negative linear relationship was observed between sperm motility, total abnormalities ($R^2 = 0.87$, $p < 0.0001$) (Fig. 6b.), coiled flagellum ($R^2 = 0.67$, $p < 0.0001$) (Fig. 6c.) and shortened flagellum ($R^2 = 0.78$, $p < 0.0001$) (Fig. 6d.), where more than 60% of the reduction in sperm motility was explained by such morphological impairment.

Discussion

In this study, we proposed for the first time a morphological classification for coral spermatozoa. We showed that alterations, especially in the flagellum, have a direct relationship with sperm motility and depend on the duration of motility. Therefore, this morphological classification could be a valuable tool for evaluating the quality of coral sperm. Furthermore, microscopic observation is valuable to evaluate the reproductive performance of individuals as well as the effects of stressors and environmental contaminants on coral sperm. It is important to emphasize that our focus here is the mature spermatozoa spawned in the ocean water.

We first describe many types of abnormalities that can be generated either during spermatogenesis or after spawning. The abnormalities were diverse; macrocephaly, coiled flagellum, strongly coiled flagellum, bent flagellum, and free flagellum. Defective spermatozoa are naturally found during spermatogenesis, which are known as primary morphological anomalies (Czubaszek et al. 2019). Hagedorn et al. (2006) mentioned abnormal flagellum and agglutinated spermatozoon in the mushroom coral *Fungia scutaria*. However, these are morphological changes due to the cryopreservation preparation. Some factors such as genetics, animal health, nutritional status and environmental conditions can influence the process of gametogenesis, contributing to the appearance of cells with morphological abnormalities (Chemes 2018).

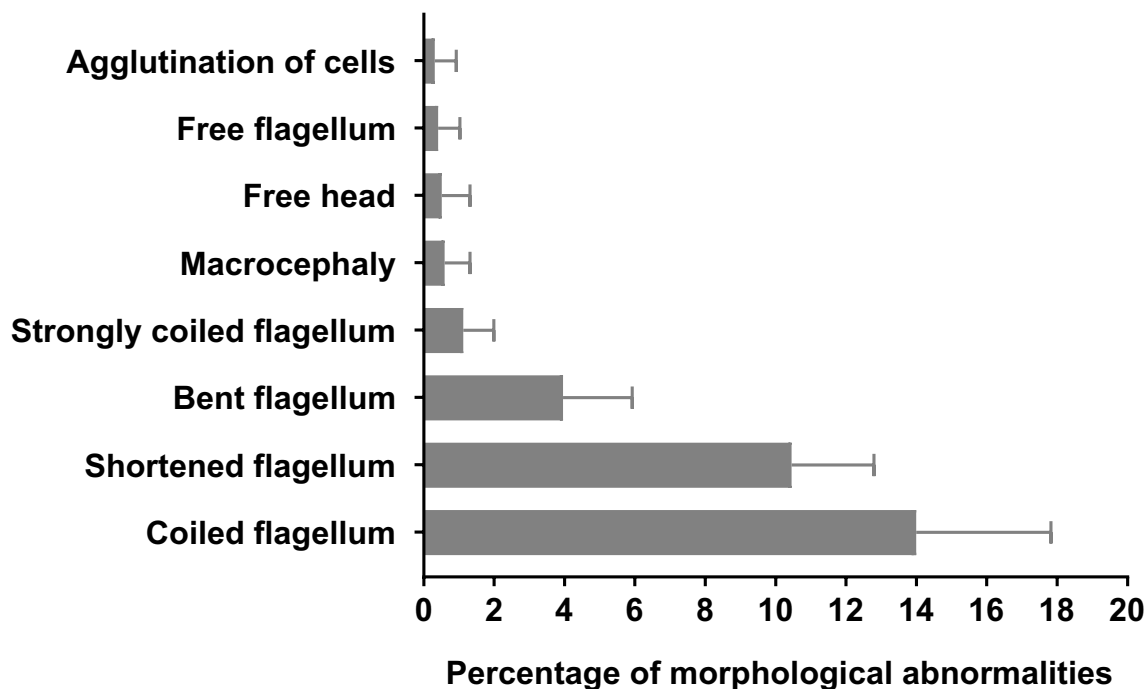


Fig. 3 Percentage of each morphological damage observed in *Mussismilia hartii* spermatozoa

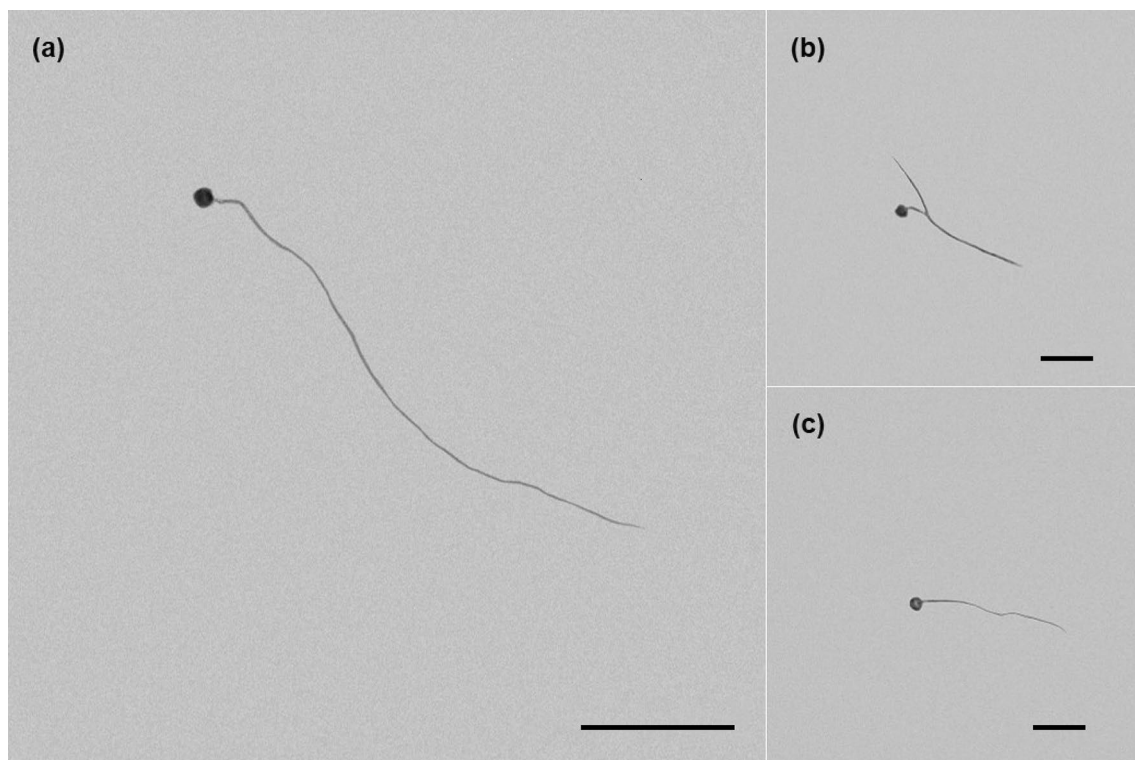


Fig. 4 Main morphological abnormalities found in spermatozoa of the South Atlantic coral *Mussismilia hartii*. **a** Normal spermatozoon. **b** Spermatozoon showing coiled flagellum. **(c)** Spermatozoon showing shortened flagellum. Black scale bar = 2 μ m

Spermatozoa abnormality could lead to reduced fertilization and decline of subsequent development. Abnormalities in the head of spermatozoa are associated with DNA damage, which can harm early embryonic development following fertilization (Kumaresan et al. 2020). In addition, anomalies in the flagellum decline sliding properly for the propagation of autonomous flagellar waves and, eventually, have an inadequate movement, which can compromise the functionality of the spermatozoon, i.e., its ability to move towards the egg and fertilize it (Cosson 2019). Thus, quantifying morphological abnormalities in coral spermatozoa could be available to assess fertilization ability (Kowalski and Cejko, 2019).

External factors such as contaminants and internal factors such as the cell metabolism generating reactive oxygen species (ROS) may influence the morphological changes in spermatozoa after spawning. Indeed, ROS inhibits axonemal phosphorylation for motility activation and causes morphological changes in the flagellum (Chianese and Pierantoni 2021).

In addition to all useful information already mentioned on the most varied forms of spermatozoa obtained during the morphological analysis, it is noteworthy that this is a relatively simple, inexpensive (it only needs dye, glass slide and light microscope) and fast technique. The morphological analysis is relevant for example, for toxicity and

environmental stressors assays, assisted reproduction techniques (in vitro fertilization) and during cryopreservation, as it can efficiently provide an accurate correlation to the fertilizing capacity of sperm (Roldan 2020). That is why this technique has been routinely applied in humans and farm animals as an important initial screening parameter to check the condition of fresh or cryopreserved semen (García-Vázquez et al., 2016).

Given the importance of this indicator, a threshold considered acceptable for morphological abnormalities was established for some animals (Oehninger et al. 2014). According to the Society for Theriogenology (USA), total spermatozoa morphological abnormalities should not exceed to 15% in ram and 20% in bull and swine (Ax et al. 2016), while for dogs a cutoff point between 30 and 40% of abnormalities is suggested as not to harm artificial insemination (Oehninger et al. 2014). However, there is no report in the literature regarding the acceptable limit of anomalies in sperm from aquatic organisms (Rodrigues et al. 2020), although some studies describe morphological damages between 15 to 30% for the black tiger shrimp (*Penaeus monodon*) (Leelatana-wit et al. 2014), and for some species of fish, percentages of abnormalities have ranged from 20 to 38% (Caldas and Godoy 2019; Fernandes et al. 2020; Rodrigues et al. 2020). Taking into consideration the reference values mentioned, the percentage of anomalies observed immediately after

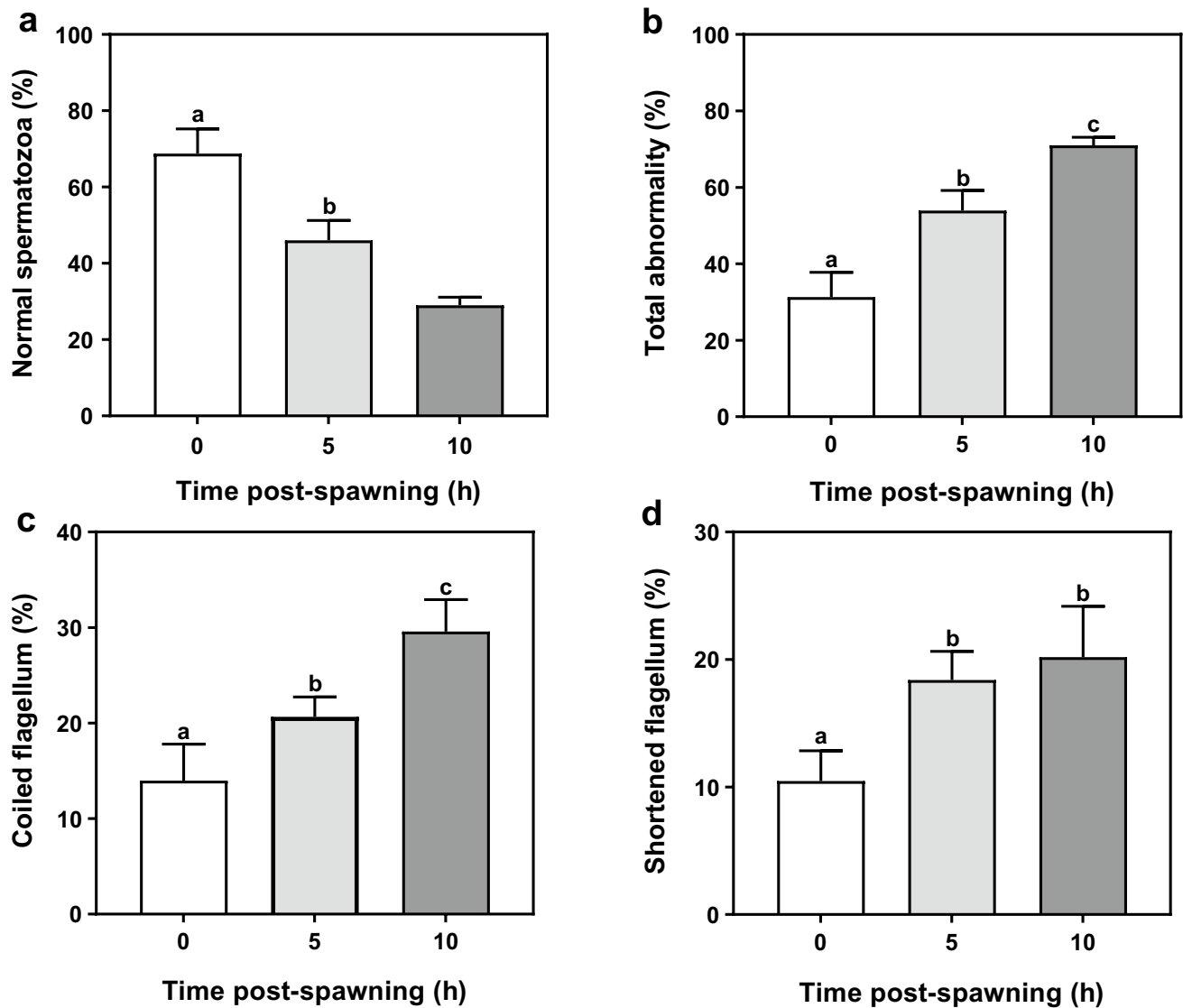


Fig. 5 Sperm morphology of the South Atlantic coral *Mussismilia hartii* over time post-spawning. **a** Normal spermatozoa. **b** Total morphological abnormalities. **c** Spermatozoa with coiled flagellum. **d**

Spermatozoa with shortened flagellum. All pairwise were compared by Tukey test. Distinctive letters indicate statistical difference over time ($p < 0.05$)

spawning of *M. hartii* is within the ones considered acceptable for sperm populations, since it was observed that 31% of the total counted cells had some type of morphological damage.

Our results reinforce how the characterization of sperm morphology can be a practical and useful tool, and the association of this analysis with other indicators can help having a better understanding of the effects of multiple stressors in coral reefs.

The morphological classification of *M. hartii* spermatozoa can serve as a model for studies to determine acceptable limits of abnormalities in coral sperm and help in the quality evaluation of spawned gametes. This knowledge will allow identifying if environmental impacts that the reefs have been suffering alter these biological characteristics in coral gametes. In addition, they may open opportunities for a series of biotechnological applications, such as gamete cryopreservation, as it will allow the selection of the best sperm characteristics to optimize fertilization and assist in species conservation.

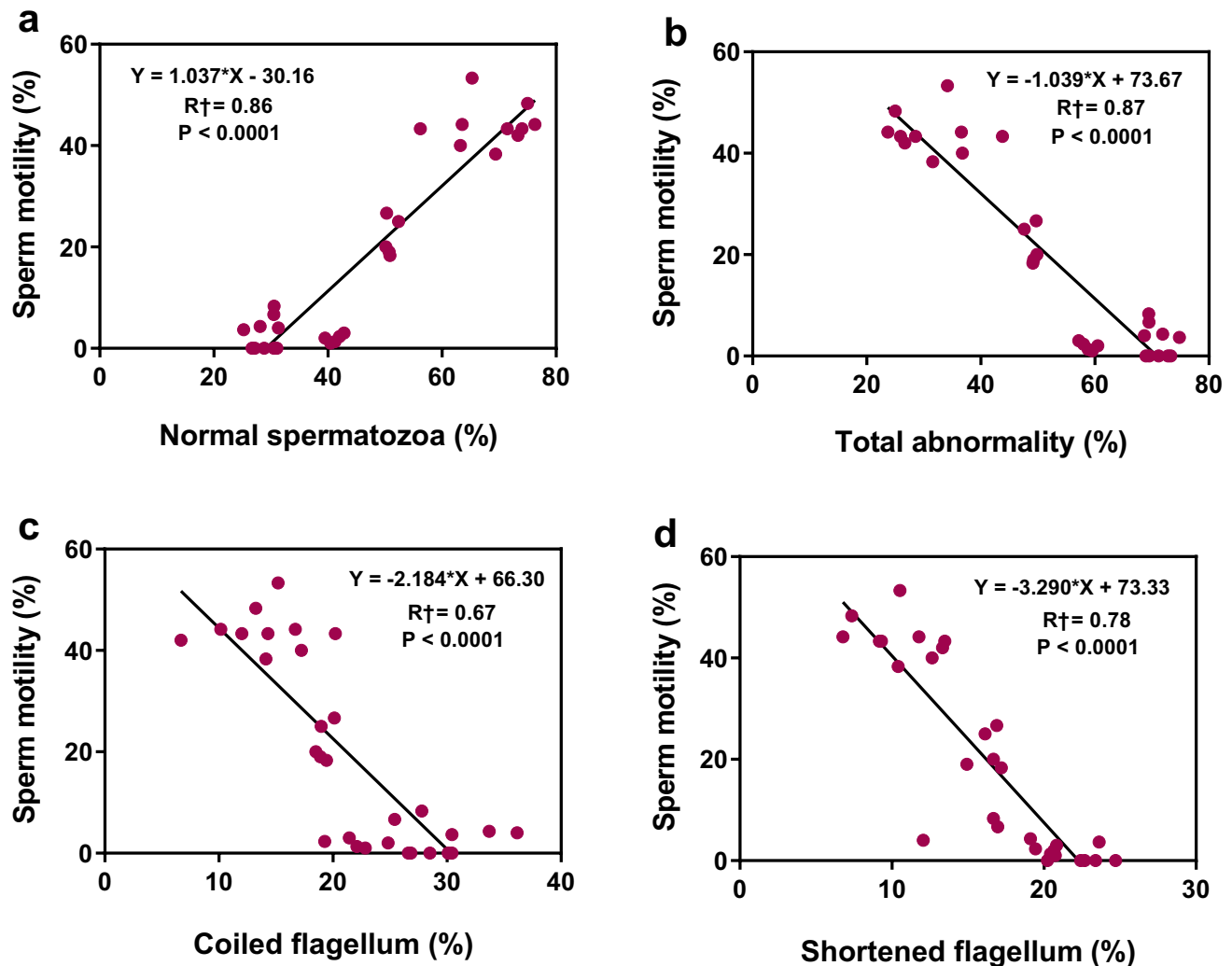


Fig. 6 Simple linear regressions showing the relationship between sperm motility and spermatozoa morphological abnormalities in *Mussismilia harttii*. **a** Normal spermatozoa x Sperm motility. **b** Total

morphological abnormalities x Sperm motility. **c** Spermatozoa with coiled flagellum x Sperm motility. **d** Spermatozoa with shortened flagellum x Sperm motility

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Author contributions AA, LG and YP designed the study; AA, LG, YP and CP performed the experiments; AA and WV performed morphological and morphometric analysis and graphic illustration; and all authors contributed to the manuscript.

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

Declarations

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 experi-

mental 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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