Journal of *Marine Science and Engineering*

MDPI

Article

The Effect of Ultrasound Waves on the Pre-Settlement Behavior of Barnacle Cyprid Larvae

Rubens M. Lopes [1,*], Claudia Guimarães [1], Felipe M. Neves [1], Leandro T. De-La-Cruz [1], Gelaysi Moreno Vega [1], Damián Mizrahi [1] and Julio Cesar Adamowski [2]

[1] Departament of Biological Oceanography, Oceanography Institute, University of São Paulo, Praça do Oceanográfico 191, São Paulo 05508-120, Brazil; c.guimaraes@usp.br (C.G.); fmarcelneves@gmail.com (F.M.N.); leandro.ticlia@gmail.com (L.T.D.-L.-C.); gelaysi.vega@gmail.com (G.M.V.); damianmiz@yahoo.com (D.M.)

[2] Department of Mechatronics and Mechanical Systems Engineering, Polytechnic School, University of São Paulo, Av. Prof. Mello Moraes, 2231, São Paulo 05508-900, Brazil; jcadamow@usp.br

* Correspondence: rubens@usp.br; Tel.: +55-11-30918963

Abstract: Ultrasound waves have been employed to control marine biofouling but their effects on fouling organisms remain poorly understood. This study investigated the influence of ultrasound waves on barnacle (*Tetraclita stalactifera* cyprid larvae) pre-settlement behavior. Substrate inspection constituted most of the larval time budget, with a focus on the bottom surface rather than lateral or air–water interfaces. The frequency of substrate inspection decreased at 10 kPa when compared to higher acoustic pressures, while the time spent in the water column had an opposite trend. Various larval swimming modes were observed, including rotating, sinking, walking, and cruising, with rotating being dominant. Barnacle larvae exhibited higher speeds and less complex trajectories when subjected to ultrasound in comparison to controls. The impact of ultrasound waves on barnacle cyprid larvae behavior had a non-linear pattern, with lower acoustic pressure (10 kPa) inducing more effective substrate rejection than higher (15 and 20 kPa) intensities.

Keywords: barnacle larvae; meroplankton; behavior; ultrasound; biofouling

check for updates

Citation: Lopes, R.M.; Guimarães, C.; Neves, F.M.; De-La-Cruz, L.T.; Vega, G.M.; Mizrahi, D.; Adamowski, J.C. The Effect of Ultrasound Waves on the Pre-Settlement Behavior of Barnacle Cyprid Larvae. *J. Mar. Sci. Eng.* **2024**, *12*, 1364. https://doi.org/10.3390/jmse12081364

Academic Editor: Decheng Wan

Received: 16 July 2024
Revised: 5 August 2024
Accepted: 8 August 2024
Published: 11 August 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Marine biofouling, the undesirable accumulation of organisms on submerged surfaces [1], can result in significant economic and environmental impacts. For instance, the maritime industry faces substantial financial losses due to the increased fuel consumption required to counteract drag caused by organisms attached to ship hulls [2]. Moreover, offshore green energy facilities, such as wind, wave, and tidal technologies, encounter challenges with excessive biomass covering underwater structures [3].

Over four thousand species of marine macrofoulers have been identified, with common examples including algae, tubular worms, bryozoans, mussels, and barnacles [4,5]. Barnacle colonization is extensively reported to have significant biofouling impacts due to their size, rapid recruitment, and gregarious colonization [6].

Several methods have been proposed to control and minimize marine biofouling. Antifouling paints have been used on underwater surfaces since the 19th century and remain the primary antifouling strategy in the marine environment [7]. Although tributyltin (TBT) was the most effective chemical developed for biofouling control, it was banned by the International Maritime Organization in 2001 due to its high toxicity to nontarget organisms [8,9]. Copper-based paints, which are less toxic, are now the standard antifouling approach worldwide [10]. However, the search for alternative and more effective methods persists, including biomimetic materials, fouling release coatings (FRCs), and electric pulses, the latter being employed for controlling biofilm formation during the early stages of biofouling [11,12]. Reactive hull cleaning is employed to address biofouling (i.e., macrofouling) on

vessels in cases where preventive measures have proven ineffective, maintenance has been inadequate, or antifouling coatings have been poorly applied or became damaged [13]. More recently, ship hull grooming has been advocated as an effective proactive approach to maintain fouling control coatings in a smooth and fouling-free condition, minimizing the risk of transporting invasive species and avoiding excessive material discharge during cleaning [14,15].

Ultrasound waves have demonstrated high effectiveness as an antifouling technique in laboratory experiments with barnacle larvae [16,17], which has generated interest in their application in the marine environment. For example, [18] discovered that larval settlement rates decreased under low ultrasound frequencies, and the applied acoustic pressure also had an impact. Moreover, the pre-settlement behavior of barnacle cypris larvae has been extensively studied, including the tracking of their swimming behavior in both 2D and 3D [19,20]. Cypris behavior has been classified according to [21] into the categories of "wide search", "close search", "inspection", and "cementation".

However, the traditional categorization of larval behavior restricts the study to processes near the substrate, overlooking important aspects related to settlement. To address this, [20] conducted three dimensional observations of cyprid larvae, identifying five distinct movement patterns: "spiral", "swimming", "sinking", "rotation", and "walking". While the last two behaviors can occur in contact with the substrate, they are only observed following the preceding patterns directed towards them. Three-dimensional recordings offer a comprehensive understanding of both surface behavior and water column activity, enabling the reconstruction of trajectories and facilitating quantitative analysis, as demonstrated by [20]. There is a need to expand our knowledge and investigate additional aspects related to the effects of ultrasound on barnacle populations. The objective of this study is to observe and quantify potential changes in cyprid larvae pre-settlement behavior induced by ultrasound waves. Specifically, we aim to investigate the influence of ultrasound waves on the displacement patterns of barnacle larvae using three-dimensional trajectory analysis. By elucidating the relationship between ultrasound waves and barnacle larval displacement behavior, this research aims to contribute to a better understanding of the effects of ultrasound as a potential control method for marine biofouling organisms.

2. Materials and Methods

Adult barnacles of the species *Tetraclita stalactifera* (Lamarck, 1818) were collected from the Flamengo Inlet, Ubatuba, São Paulo, Brazil, in September–December 2018, and October–November 2019. This barnacle is a common and abundant intertidal species found along the coastlines of the Western Atlantic, especially in tropical and subtropical regions. In the laboratory, the adult barnacles were reared in running seawater at a temperature of 28 °C and a salinity of 36 PSU, following the protocol of [22]. The rearing conditions included natural light exposure (12:12 L/D) and the absence of food. After 1 or 2 days, the released nauplius larvae were manually attracted by white LED light, collected with a wide bore pipette, and placed in a container with 8 L of filtered seawater, maintaining the same environmental conditions as the adults. The nauplii larvae were fed every two days with 40 mL of a *Tetraselmis suecica* culture, with a density of approximately 5×10^5 cells mL^{-1}. Once the nauplii metamorphosed into cypris larvae (within approximately 8 days), they were collected and stored for 3 days in filtered seawater under the same environmental conditions as the adults before conducting the experiments.

Ultrasound was applied using a 36 mm diameter transducer with a frequency of 26 kHz, controlled by an electronic module with eight channels and software developed by our laboratory. The experimental setup is depicted in Figure 1, where a coupler gel (Shear Gel, Magnaflux, Glenview, USA) was applied between the transducer and the vial. The acoustic pressure was adjusted using the software and measured with a hydrophone (8103, Brüel & Kjær, São Paulo, Brasil) coupled to an oscilloscope (TBS 1102B, Tektronix, Beaverton, USA). Three acoustic pressures were tested: 10, 15, and 20 kPa, which fall within the range of pressures considered in the literature [6,18,23], in addition to a control treatment with

no transducer. The 3D optical system was constructed by combining two orthogonal 2D inline optical systems, with their respective focal planes intersecting at a defined point. This configuration allowed the simultaneous imaging of particles within the shared volume of interest from two different perspectives. Each 2D optical system consisted of an infrared LED and a high-resolution camera (Basler ac2040 25gm, Ahrensburg, Germany). The light beam generated by the LED passed through a collimating lens to illuminate a rectangular quartz vial used in the experiments, then reached a focusing lens positioned in front of the camera sensor. Digital image acquisition was performed using the inhouse LAPS Camera Recorder (LCR) software v.2.1, which provided a user-friendly interface for defining image acquisition parameters, camera settings, and automatic image triggering (Figure 1).

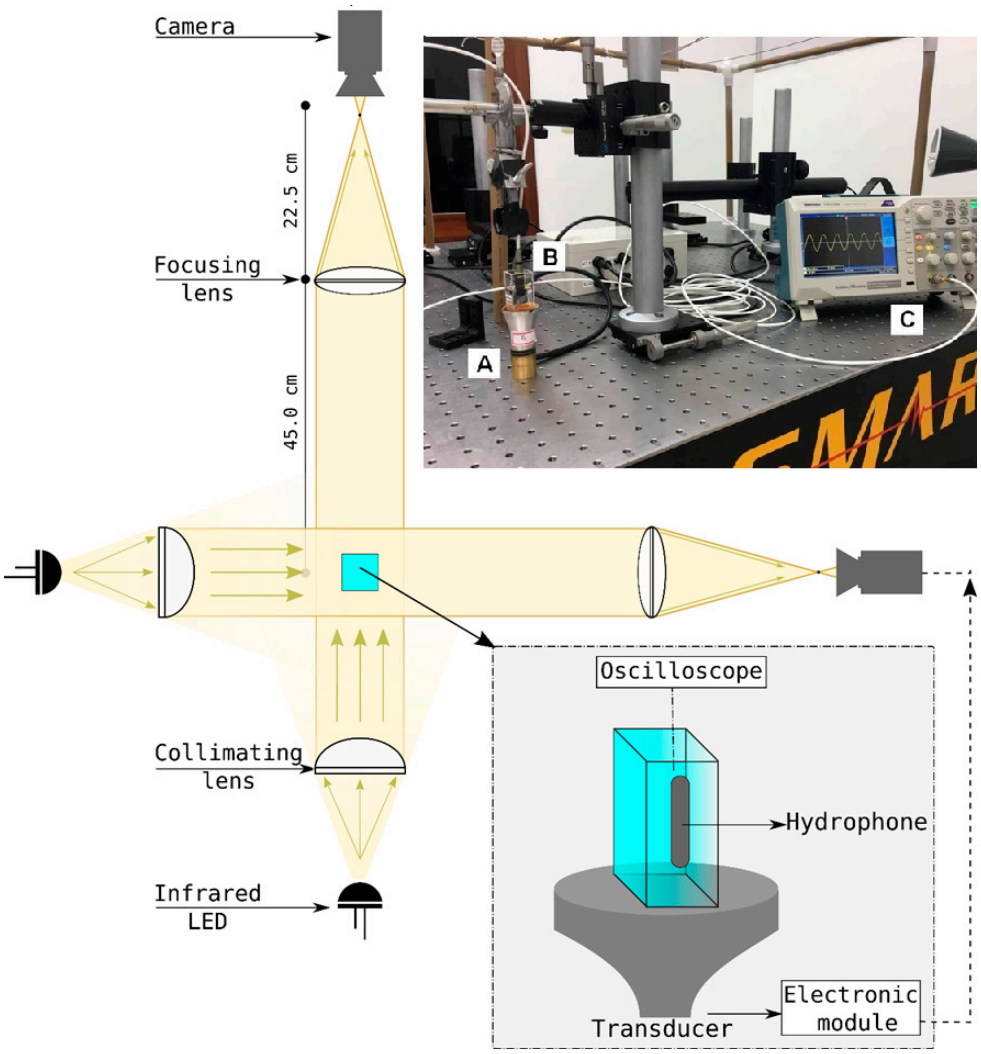

Figure 1. A scheme of the optical system and the ultrasound device used to evaluate the effects of ultrasound treatment on barnacle larvae. The inset shows the transducer (A), the quartz container with a hydrophone (B), and the oscilloscope (C) utilized to monitor the acoustic pressure.

Each trial was performed on a single cypris at a time (n = 20 cyprid larvae for each experimental treatment) under controlled conditions (26 °C, 12:12 L/D). A rectangular quartz vial (22 mm × 22 mm × 38 mm) filled with 5.5 mL of filtered seawater (36 PSU salinity, pore size 0.7 m, GF/F glass microfiber filter, Whatman) was used as the experimental container. The resulting water column depth in the vial was 22 mm. Each camera had a field of view of 18 × 22 mm, enabling observation of almost the entire area of the vial while excluding blind spots at the corner of the field of view. Prior to each recording, the larvae were acclimatized for ten minutes, with the ultrasound device turned off. Each recording

session with ultrasound on lasted 5 min. Trajectory extraction was performed using the in-house LAPS Plankton Detector (LPD) software v.2.1, which provided the 2D positioning and a time stamp of the recorded organisms along the video sequences. The 3D swimming trajectories were reconstructed by merging the 2D spatial and temporal information, after the extraction of the x, y, and z positions.

A total of 82 trajectories was obtained. The control treatment showed no visible wall effect from the lateral side of the vial, as larvae spent less than 2% of the time within 2 mm of the wall. Therefore, any larval movement observed along the wall interfacial regions was considered a by-product of the ultrasonic irradiation. However, since *T. stalactifera* settlement was observed on lateral substrates, excluding larval movement along the wall could introduce bias into the data. To ensure statistically consistent swimming paths and mitigate scale-dependent issues in the metrics [24,25], the trajectories were divided into short paths of similar duration (10 s). Detailed descriptions of the observed behavioral motion patterns during substrate inspection and movement in the water column were provided, and their occurrence was subsequently quantified. The spatial locations of cyprid larvae during substrate inspection were calculated from the images, distinguishing between the surface, lateral, and bottom regions of the aquarium. Additionally, the speed and sinuosity of cyprid trajectories were quantified. The swimming speed (S) was estimated using the following equation:

$$S = dt \times p, \quad (1)$$

where dt is the displacement (cartesian displacement) between two successive video frames, and p is the frame rate used to record the data in this study (20 frames s^{-1}). The speed distributions were analyzed using an empirical cumulative distribution function (ECDF). The cumulative distribution function can be described by

$$FX\,(x) = P\,(X \leq x), \quad (2)$$

where $P\,(X \leq x)$ is the probability that the variable X exhibits a value less than or equal to x. The ECDF (Empirical Cumulative Distribution Function) was utilized to describe the cumulative occurrence of speed values within the dataset, ranging from 0 to 100%. This analysis allowed for a comprehensive understanding of the distribution of speed values and their relative frequencies within the dataset. The median values of instantaneous speeds were estimated for individual path segments in each treatment. Additionally, the proportion of occurrences where the speed was null and where it reached the 95th percentile was reported. The Net-to-Gross Displacement Ratio (NGDR), a metric of displacement complexity or trajectory sinuosity, was calculated for each 10 s path segment using the following formula [26]:

$$NGDR = ND/GD, \quad (3)$$

where ND represents the shortest distance between the starting point and the end point of the path segment, and GD is the actual distance traveled by the larvae within that specific path segment. NGDR values range from 0 to 1, with values closer to 1 indicating linear motion and values closer to 0 indicating a high degree of path tortuosity.

Statistical analyses were performed using R software version 3.6.0 [27]. Data visualization was conducted using the ggplot and yarr R packages [28]. Since the dataset exhibited a non-normal distribution (Shapiro–Wilk Test; [29]), non-parametric statistical tests were employed. The G-test of independence was used to compare the frequency of behavioral categories, motion patterns, and spatial locations [30]. The G-test of goodness-of-fit was applied for variables with two or more values, including post hoc pairwise comparisons. The RVAideMemoire R package [31] was utilized to implement both G-tests. The null hypothesis for the G-test of independence was that the relative proportions of one variable are the same as those of the second variable. For the G-test of goodness-of-fit, the null hypothesis was that the number of observations in each category is equal to that predicted by a hypothetical discrete uniform distribution. The Kruskal–Wallis (KW) test was used to compare speed and NGDR values among treatments. Post hoc Dunn's tests were conducted

using the dunn.test R package (Dinno, 2015) following the KW test. The statistical significance was considered at $p < 0.05$, and specific p-values were described when necessary. No correction for multiple comparisons was applied [32].

3. Results

Substrate inspection behavior included rotating and walking patterns observed in proximity to the bottom, sides, and surface of the observation vessel (Figure 2A,B). Rotating behavior involved circular or semicircular movements of the cyprid larvae around themselves while in contact with the substrate. Walking behavior exhibited high linearity, with the larvae moving in a straight-line manner but occasionally making turns. Sometimes, a brief vertical rising to the water column was also observed during walking.

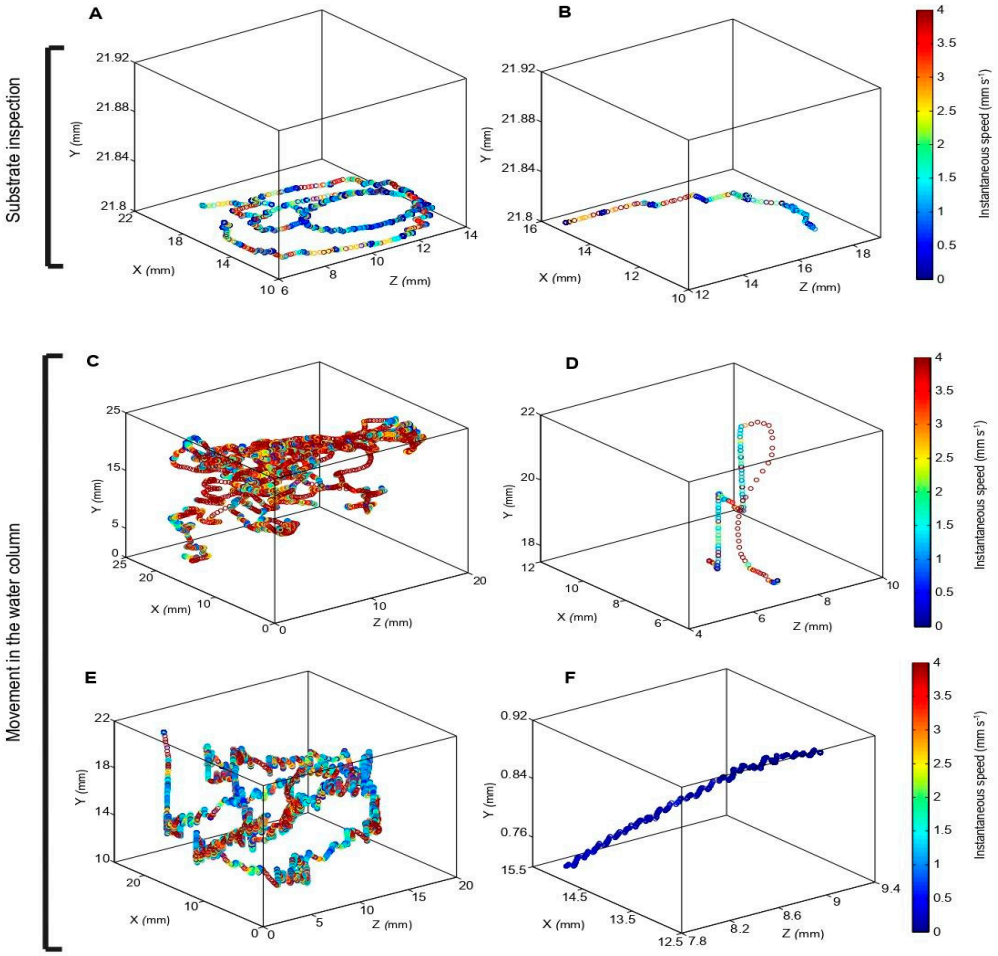

Figure 2. Examples of motion patterns observed for cyprid larvae during recording. Rotating (**A**), walking (**B**), swimming (**C**), sinking (**D**), swimming variation (**E**). and cruising (**F**).

Movement in the water column consisted of swimming, sinking, and cruising behaviors (Figure 2C–F). Cruising is a previously undescribed displacement pattern, here recorded only for the 20 kPa acoustic treatment. It was characterized by unidirectional movement in almost a straight line, including intermittent upward and downward movements across the water column. Cruising behavior was rare (found in less than 1% of time) and will not be considered in subsequent quantitative analysis.

Swimming behavior exhibited a mix of straight and curved swimming paths, with varying speeds and occasional contact with the water surface. Some swimming path segments displayed a spiraling motion due to helical movement, comparable to the description by [20]. However, due to the difficulty of visually distinguishing between spiraling and

swimming, they were considered as swimming behavior in this study. It is worth mentioning that swimming behavior occasionally showed variations, possibly influenced by the ultrasonic irradiation (Figure 2).

This variation was characterized by intermittent series of upward and downward movements across the water column, possibly resulting from intense beating of the thoracopods. Sinking behavior involved a passive falling motion of the cyprid larvae driven by gravity, causing them to descend from the water column and reach the bottom substrate.

Among all the treatments, substrate inspection was the most frequent behavioral category compared to displacement in the water column (G-test, $p < 0.001$; Figure 3A). The frequency of substrate inspection was similar for the control, 15 kPa, and 20 kPa treatments (~84%), but lower for the 10 kPa treatment (66.9%). Displacement in the water column was more frequent at 10 kPa (44%), compared to the control, 15 kPa, and 20 kPa treatments, which had lower frequencies (Figure 3A). Regarding the spatial location of the larvae during substrate inspection (Figure 3B), a significantly higher occurrence was observed at the bottom of the vial for the control, 10 kPa, and 15 kPa treatments (G-test, $p < 0.001$). In contrast, at 20 kPa, cyprid larvae did not show a preference for any specific spatial location, with a similar frequency distribution on the surface, bottom, and lateral sides of the vial (G-test, $p = 0.32$). However, the frequency of spatial location for each treatment was higher at the bottom. Substrate inspection at the lateral sides of the aquarium was only observed at 20 kPa (~34%; Figure 3A).

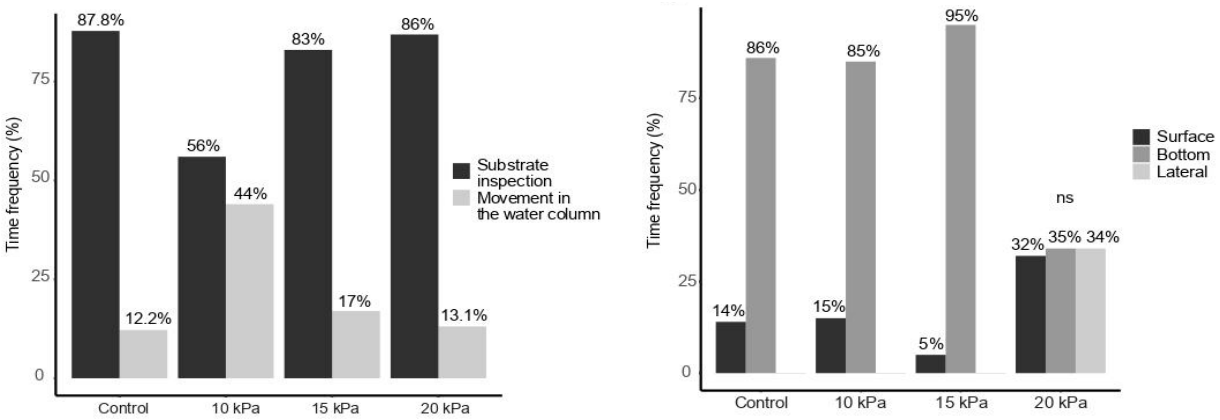

Figure 3. The time budget frequency of the behavioral categories considered (excluding inspection and movement in the water column) for cyprid larvae (**a**). The time budget frequency of spatial location (surface, bottom, lateral) during substrate inspection (**b**). Almost all comparisons were statistically significant (G-test; $p < 0.001$); exceptions were signaled by ns (i.e., non-significant).

The statistical analysis (G-test) of behavioral patterns within each treatment revealed that all pairwise comparisons were different from each other ($p < 0.01$; Figure 4A), except for walking and resting at 10 kPa, and swimming and sinking at 20 kPa. The most frequently observed behavior was rotating (~40.3%), followed by walking (~27.45%), swimming (~13%), resting (~10.6%), and sinking (~8.5%). Comparing each motion pattern across the treatments, rotating, walking, swimming, and resting were all significantly different from each other (G-test, $p < 0.01$; Figure 4C,E,F). However, rotating was not significantly different between 10 kPa and 20 kPa, and sinking was not significantly different between the control and 10 kPa, as well as between 10 and 15 kPa (Figure 4C,E,F).

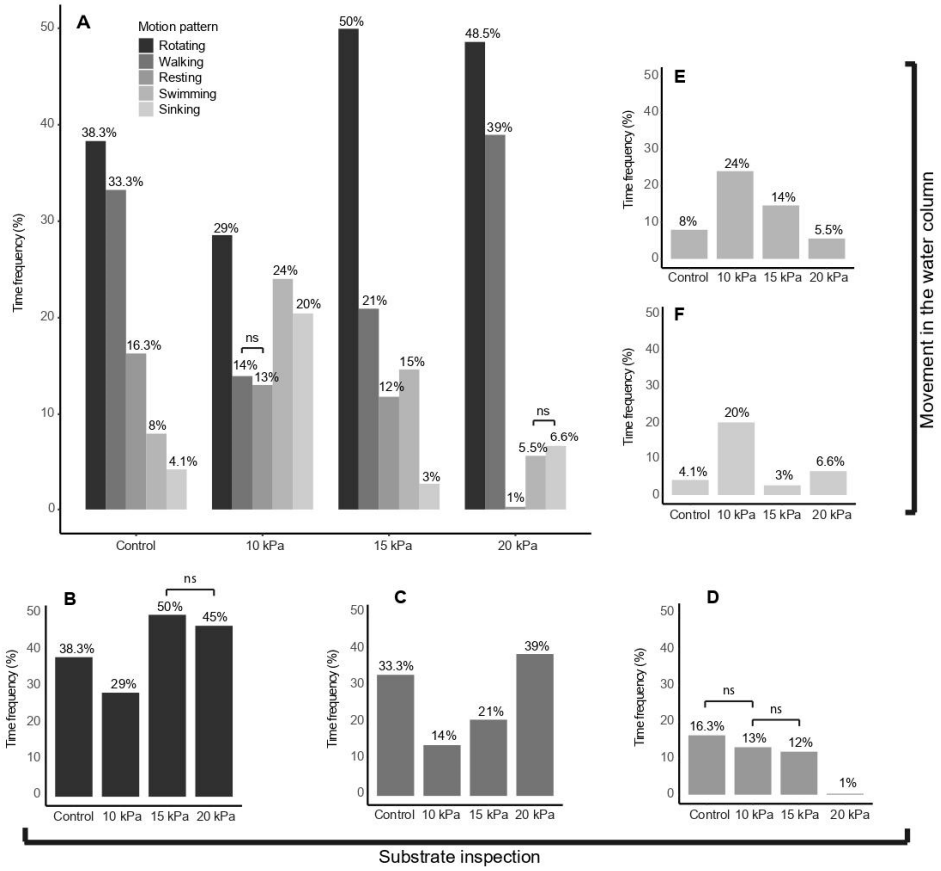

Figure 4. The time budget frequency of the motion patterns of barnacle cyprid larvae within the treatments (**A**) and among them (**B**–**F**). Almost all comparisons were statistically significant (G-test; $p < 0.001$); exceptions were signaled by ns (i.e., non-significant).

Speed values showed significant differences among the treatments. The ECDFs illustrated distinct patterns, with the speed at 10 kPa differing markedly from the other treatments for all behaviors and during substrate inspection (Figure 5A). The ECDFs showed broader speed distributions in the water column for all treatments compared to the control (Figure 5A). Considering all behaviors, speed values at 10 and 15 kPa were statistically higher compared to the control and 20 kPa (KW, $p < 0.001$; Figure 5B). During substrate inspection at the surface, speed values at 10 kPa were significantly higher compared to all other treatments, followed by 15 kPa, the control, and 20 kPa (KW, $p < 0.001$; Figure 5B). The controls had significantly higher speed values compared to the remaining treatments, which did not differ statistically from each other (KW, $p < 0.001$; Figure 5B). Speed values equal to 0 mm s^{-1} (indicating inactivity) were more frequent during substrate inspection (~38.5%) than in the water column (~23.5%) (Figure 5C). The instantaneous speed values at which the ECDFs reached 95% are shown in Figure 5D. Speed values ranging from 0 to 2.28 mm s^{-1} represented 95% of all velocities for all behaviors, with the highest speed values observed at 15 kPa, followed by the control, 20 kPa and 10 kPa. During the substrate inspection and water column displacement, maximum speeds reached 2.8 and 3.7 mm s^{-1}, respectively, with the highest speed observed in the control, followed by 15, 20, and 10 kPa in both cases.

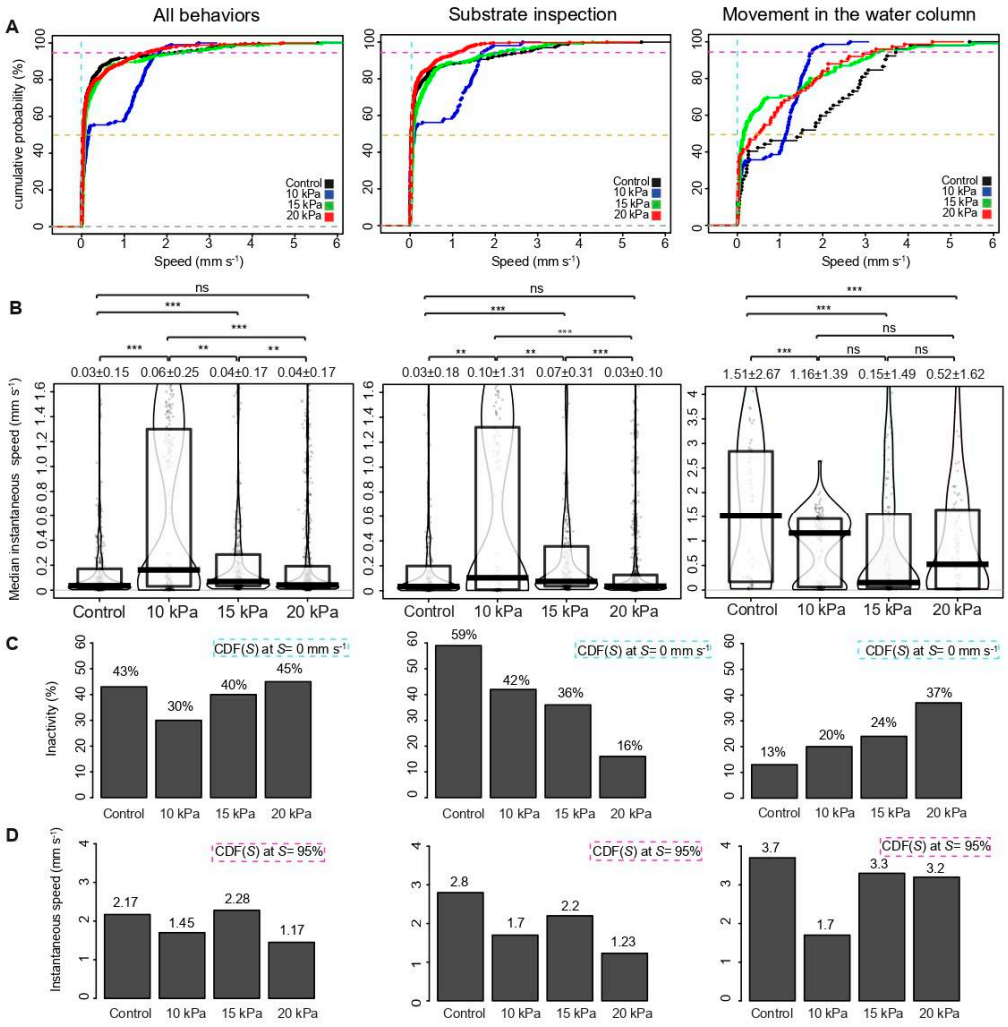

Figure 5. Empirical cumulative distribution functions of the displacement speed of barnacle cyprids for the different treatments (control, 10 kPa, 15 kPa, 20 kPa) (**A**). Light yellow dashed lines mark the overall median speed, light blue dashed lines mark the speed at 0 mm s^{-1}, and the light purple dashed line marks where the lines reach 95% of the cumulative probability. The distribution of the data, density plot, overall median speed for each treatment, and interquartile range for each treatment considered are presented (**B**). The occurrence (proportion) where the speed is 0 mm s^{-1} (**C**), as well as where it reaches the 95th percentile (**D**) is reported. Statistical differences (Kruskal–Wallis test) are signaled by ** and *** (*p* values less than 0.01 and 0.001, respectively); non-significant values were signaled by ns.

The Net-to-Gross Displacement Ratio (NGDR) calculated for short-duration segments did not differ among the treatments for all behavioral patterns (KW, $p = 0.62$; Figure 6A), and the substrate inspection (KW, $p = 0.7$; Figure 6B). However, NGDR values for the water column were significantly higher at 15 and 10 kPa compared to other treatments (KW, $p < 0.01$; Figure 6C).

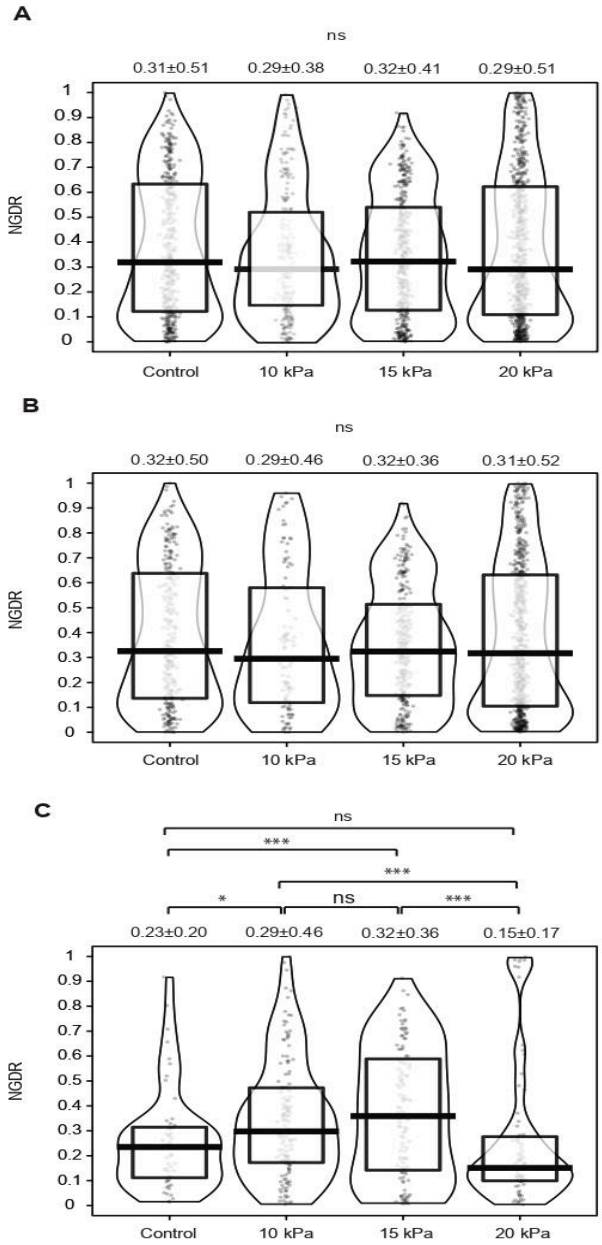

Figure 6. NGDR values for barnacle cyprid larvae considering both behavioral categories (i.e., all motion patterns) (**A**), only substrate inspection (**B**), and only movement in the water column (**C**). The distribution of the data, density plot, overall median speed, and interquartile range for each treatment considered are shown. Statistical differences (Kruskal–Wallis test) are signaled by *, and *** (p values less than 0.05, and 0.001, respectively); non-significant values were signaled by ns.

4. Discussion

The motion patterns observed in *T. stalactifera* resembled those reported in *Amphibalanus amphitrite* [20,33]. The walking behavior is a combination of the wide search and close search patterns identified by [34] for cyprid larvae of *A. amphitrite*. The findings presented in this study demonstrate that the impact of ultrasound on barnacle larvae varies depending on the applied pressure. Specifically, the 10 kPa treatment resulted in more pronounced behavioral changes compared to both the control group and the higher acoustic pressure treatments of 15 and 20 kPa. Across all treatments, surface inspection was the most frequently observed behavior, except for the 10 kPa treatment, which led to a higher time allocation in the water column. Surface exploration events predominantly occurred at the bottom of the vial, except for the 20 kPa treatment, which exhibited a nearly equal

distribution among the surface, bottom, and lateral surfaces. Moreover, no significant differences in velocity and NGDR within the substrate were observed among the different treatments, except once again for the 10 kPa treatment.

Compared to the ultrasound treatments, the control group larvae displayed lower NGDR values and speed. This behavior aligns with the 'close searching' stage of substrate exploration by cyprid larvae, where they confine their search behavior, including speed and NGDR, to a smaller area upon detecting an acceptable surface for settlement [21,33,35]. However, the response of cyprid larvae in the absence of ultrasound emission differed from [20], who reported swimming and sinking as the most common displacement patterns. In our study, rotating and walking were more frequent. As mentioned, these surface-related movements characterized by low velocities indicate a detailed exploration of the substrate. Such differences in larval response may be attributed to the temperature conditions prior to the experiments. Unlike [20,36], our larvae were not refrigerated before the experiments, which may have resulted in increased surface exploratory behavior due to higher metabolic rates and depleted energetic reserves. This is consistent with the 'desperate larva hypothesis' [37–39].

In the high-pressure treatments (15 and 20 kPa), cyprid larvae allotted more time inspecting the substrate than swimming in the water column, a behavior more closely resembling the control than the 10 kPa treatment. This was accompanied by the reduction in step length and increased step duration, which are indicative of a thorough examination of the substrate [6]. Conversely, the 10 kPa treatment caused the larvae to remain in the water column for a longer duration. These divergent responses within the same ultrasound application method suggest the involvement of different underlying mechanisms depending on the applied pressure.

Low-pressure ultrasound waves are more susceptible to attenuation due to acoustic absorption as they propagate through the water medium, resulting in a shorter effective range compared to high-pressure waves. Consequently, we suggest that when barnacle larvae are exposed to low-pressure ultrasound waves (10 kPa, in this case), they experience a more concentrated and intense exposure over a smaller area close to the substrate. This localized exposure may trigger stronger physiological and behavioral escape responses in the larvae compared to high (15 and 20 kPa) acoustic pressures, which would provide no "escape route" because the entire water column of our small experimental vessel was affected by pressure waves. In such a scenario, cyprid larvae would have no choice but to attempt to settle regardless of substrate quality.

Understanding these differential impacts is crucial for evaluating the potential ecological consequences of ultrasound exposure on barnacle populations and for informing responsible management practices in marine environments. To gain further understanding of the effects of ultrasound on barnacle larvae, we recommend additional experimental work addressing different cyprid stages, container sizes, and ultrasound configurations. Specifically, investigating the responses of young and late stage cyprids to ultrasound exposure would provide insights into the sensitivity of different developmental stages to this stressor. The data reported here concern mid-stage cyprids (3-day old larvae), which may react differently compared to recently metamorphosed individuals. For instance, [40] reported that late-stage (7-day old) cyprid larvae displayed a marked reduced discrimination in their exploration of surfaces. Moreover, varying the size of the containers used in the experiments would allow for the examination of potential container effects on larval behavior and settlement, which was not possible to investigate during our experiments. Finally, exploring different ultrasound configurations, such as frequency and pressure variations, would enable a more detailed understanding of how specific ultrasound characteristics influence larval behavior. The informative studies by Guo and collaborators [6,18,23] and the present results may provide valuable insights into the design of future experiments to enhance our understanding of the effects of ultrasound exposure on biofouling organisms, including settling barnacle larvae.

5. Conclusions

Ultrasonic waves, as an antifouling technique to control and minimize the marine biofouling of barnacle larvae, have demonstrated high effectiveness in laboratory tests, generating interest in their application in the marine environment. The swimming and pre-settlement behavior of adult barnacles of the species *Tetraclita stalactifera*, under the influence of three acoustic pressure values, showed swimming patterns in the form of rotation, sinking, walking, and cruising, with rotation being dominant. Cruise is a displacement pattern observed by us during the experiments, recorded only for the acoustic treatment with a pressure of 20 kPa, appearing less than 1% of the time. The substrate inspection frequency was the most frequent behavior when compared in relation to the displacement throughout the water column, being lower in the 10 kPa treatment, while the residence time in the water column showed an opposite trend. During substrate inspection and water column displacement, barnacle larvae exhibited different speed values between treatments, with broader speed distributions and less complex trajectories when subjected to ultrasound compared to controls. The impact of ultrasonic waves on the swimming behavior of barnacle cyprid larvae, when considering all trajectories, showed a non-linear pattern with more effective substrate rejection when using lower intensity waves (10 kPa). By evaluating how barnacle cyprid larvae respond to different intensities of ultrasonic waves, as well as their availability for settlement or movement within the water column, it becomes possible to predict and better understand the effects of ultrasound as a potential method for the control of marine biofouling.

Author Contributions: Formal analysis, investigation, visualization, original draft preparation, R.M.L., C.G., F.M.N., and L.T.D.-L.-C.; supervision, resources, project administration, funding acquisition, R.M.L. and J.C.A.; methodology, software, validation, data curation, R.M.L., C.G., F.M.N., L.T.D.-L.-C., D.M., and J.C.A.; writing—review and editing, R.M.L., C.G., F.M.N., D.M., and G.M.V. All authors have read and agreed to the published version of the manuscript.

Funding: This study was part of the project "Research and implementation of technologies for sun coral detection/monitoring and biofouling prevention", a cooperation agreement between USP and PETROBRAS 0050.0100341.16.9 regulated by the RDI investment clauses of the Brazilian Agency of Petroleum, Natural Gas and Biofuels (ANP Resolution 05/2015). Damián Mizrahi received a FAPESP (Fundação de Apoio à Pesquisa do Estado de São Paulo) postdoctoral fellowship (2017/04904-2) during the final stages of manuscript preparation. Rubens M. Lopes is a CNPq Research Fellow (315033/2021-5).

Institutional Review Board Statement: Ethical review and approval were waived for this study, due to its focus on invertebrate larvae.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Acknowledgments: We acknowledge the Chico Mendes Institute for Biodiversity Conservation (ICMBio) for the collecting permit Sisbio # 33988 granted to Rubens M. Lopes.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Lin, S.; Bi, H.; Weinell, C.E.; Dam-Johansen, K. Submerged surfaces exposed to marine biofouling—Experimental investigation of cleaning parameters effectiveness. *Prog. Org. Coat.* **2022**, *172*, 107097. [CrossRef]
2. Schultz, M.; Bendick, J.A.; Holm, E.R.; Hertel, W.M. Economic impact of biofouling on a naval surface ship. *Biofouling* **2011**, *27*, 87–98. [CrossRef]
3. Vinagre, P.A.; Simas, T.; Cruz, E.; Pinori, E.; Svenson, J. Marine biofouling: A European database for the marine renewable energy sector. *J. Mar. Sci. Eng.* **2020**, *8*, 495. [CrossRef]
4. Bressy, C.; Lejars, M. Marine fouling: An overview. *J. Ocean Technol.* **2014**, *9*, 19–28.

5. Callow, M.E.; Callow, J.E. Marine biofouling: A sticky problem. *Biologist* **2002**, *49*, 10–14.
6. Guo, S.; Lee, H.; Chaw, K.; Miklas, J.; Teo, S.; Dickinson, G.; Birch, W.; Khoo, B. Effect of ultrasound on cyprids and juvenile barnacles. *Biofouling* **2011**, *27*, 185–192. [CrossRef] [PubMed]
7. Yebra, D.; Kiil, S.; Dam-Johansen, K. Antifouling technology—Past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Prog. Org. Coat* **2004**, *50*, 75–104. [CrossRef]
8. Dafforn, K.A.; Lewis, J.A.; Johnston, E.L. Antifouling strategies: History and regulation, ecological impacts and mitigation. *Mar. Pollut. Bull.* **2011**, *62*, 453–465. [CrossRef]
9. Champ, M.A. Economic and environmental impacts on ports and harbors from the convention to ban harmful marine anti-fouling systems. *Mar. Pollut. Bull.* **2003**, *46*, 935–940. [CrossRef]
10. Paz-Villarraga, C.A.; Castro, Í.B.; Fillmann, G. Biocides in antifouling paint formulations currently registered for use. *Environ. Sci. Pollut. Res.* **2022**, *29*, 30090–30101. [CrossRef]
11. Bazaka, K.; Crawford, R.J.; Ivanova, E.P. Do bacteria differentiate between degrees of nanoscale surface roughness? *Biotechnol. J.* **2011**, *6*, 1103–1114. [CrossRef] [PubMed]
12. Sultana, S.; Babauta, J.; Beyenal, H. Electrochemical biofilm control: A review. *Biofouling* **2015**, *31*, 745–758. [CrossRef] [PubMed]
13. Scianni, C.; Georgiades, E. Vessel in-water cleaning or treatment: Identification of environmental risks and science needs for evidence-based decision making. *Front. Mar. Sci.* **2019**, *6*, 467. [CrossRef]
14. Tribou, M.; Swain, G. Grooming using rotating brushes as a proactive method to control ship hull fouling. *Biofouling* **2015**, *31*, 309–319. [CrossRef] [PubMed]
15. Swain, G.; Erdogan, C.; Foy, L.; Gardner, H.; Harper, M.; Hearin, J.; Hunsucker, K.Z.; Hunsucker, J.T.; Lieberman, K.; Nanney, M.; et al. Proactive in-water ship hull grooming as a method to reduce the environmental footprint of ships. *Front. Mar. Sci.* **2022**, *8*, 808549. [CrossRef]
16. Kitamura, H.; Takahashi, K.; Kanamaru, D. Inhibitory effect of ultrasonic waves on the larval settlement of the barnacle, *Balanus amphitrite* in the laboratory. *Mar. Fouling* **1995**, *12*, 9–13. [CrossRef]
17. Seth, N.; Chakravarty, P.; Khandeparker, L.; Anil, A.; Pandit, A. Quantification of the energy required for the destruction of *Balanus amphitrite* larva by ultrasonic treatment. *J. Mar. Biol. Assoc. U. K.* **2010**, *90*, 1475–1482. [CrossRef]
18. Guo, S.; Lee, H.P.; Khoo, B.C. Inhibitory effect of ultrasound on barnacle (*Amphibalanus amphitrite*) cyprid settlement. *J. Exp. Mar. Biol. Ecol.* **2011**, *409*, 253–258. [CrossRef]
19. Alsaab, A.; Aldred, N.; Clare, A.S. Automated tracking and classification of the settlement behaviour of barnacle cyprids. *J. R. Soc. Interface* **2017**, *14*, 20160957. [CrossRef]
20. Maleschlijski, S.; Bauer, S.; Aldred, N.; Clare, A.S.; Rosenhahn, A. Classification of the pre-settlement behaviour of barnacle cyprids. *J. R. Soc. Interface* **2015**, *12*, 20141104. [CrossRef]
21. Crisp, D.J. Settlement responses in marine organisms. In *Adaptation to Environment: Essays on the Physiology of Marine Animals*; Newell, R., Ed.; Butterworth-Heinemann: Oxford, UK, 1976; pp. 83–124.
22. Rittschof, D.; Branscomb, E.S.; Costlow, J.D. Settlement and behavior in relation to flow and surface in larval barnacles, *Balanus amphitrite* Darwin. *J. Exp. Mar. Biol. Ecol.* **1984**, *82*, 131–146. [CrossRef]
23. Guo, S.; Lee, H.; Teo, S.; Khoo, B. Inhibition of barnacle cyprid settlement using low frequency and intensity ultrasound. *Biofouling* **2012**, *28*, 131–141. [CrossRef] [PubMed]
24. Seuront, L.; Hwang, J.S.; Tseng, L.C.; Schmitt, F.; Souissi, S.; Wong, C.K. Individual variability in the swimming behavior of the sub-tropical copepod *Oncaea venusta* (Copepoda: Poecilostomatoida). *Mar. Ecol. Prog. Ser.* **2004**, *283*, 199–217. [CrossRef]
25. Visser, A.; Kiørboe, T. Plankton motility patterns and encounter rates. *Oecologia* **2006**, *148*, 538–546. [CrossRef] [PubMed]
26. Buskey, E.J. Swimming pattern as an indicator of the roles of copepod sensory systems in the recognition of food. *Mar. Biol.* **1984**, *79*, 165–175. [CrossRef]
27. R Development Core Team. R: A Language and Environment for Statistical Computing, 2011. Available online: https://www.R-project.org/ (accessed on 6 March 2023).
28. Wickham, H. Getting started with ggplot2. In *Elegant Graphics for Data Analysis*; Springer: Cham, Switzerland, 2016; pp. 11–31.
29. Shapiro, S.S.; Wilk, M.B. An analysis of variance test for normality (complete samples). *Biometrika* **1965**, *52*, 591–611. [CrossRef]
30. Sokal, R.R.; Rohlf, F.J. Taxonomic congruence in the Leptopodomorpha re-examined. *Syst. Zool.* **1981**, *30*, 309–325. [CrossRef]
31. Hervé, M. RVAideMemoire: Diverse Basic Statistical and Graphical Functions, 2015. Available online: https://cran.r-project.org/web/packages/RVAideMemoire/index.html (accessed on 12 September 2022).
32. Saville, D.J. Multiple comparison procedures: The practical solution. *Am. Stat.* **1990**, *44*, 174–180. [CrossRef]
33. Aldred, N.; Alsaab, A.; Clare, A.S. Quantitative analysis of the complete larval settlement process confirms Crisp's model of surface selectivity by barnacles. *Proc. R. Soc. B Biol. Sci.* **2018**, *285*, 20171957. [CrossRef] [PubMed]
34. Lagersson, N.C.; Høeg, J. Settlement behavior and antennulary biomechanics in cypris larvae of *Balanus amphitrite* (Crustacea: Thecostraca: Cirripedia). *Mar. Biol.* **2002**, *141*, 513–526.
35. Aldred, N.; Li, G.; Gao, Y.; Clare, A.; Jiang, S. Modulation of barnacle (*Balanus amphitrite* Darwin) cyprid settlement behavior by sulfobetaine and carboxybetaine methacrylate polymer coatings. *Biofouling* **2010**, *26*, 673–683. [CrossRef] [PubMed]
36. Maleschlijski, S.; Bauer, S.; di Fino, A.; Sendra, G.H.; Clare, A.S.; Rosenhahn, A. Barnacle cyprid motility and distribution in the water column as an indicator of the settlement-inhibiting potential of nontoxic antifouling chemistries. *Biofouling* **2014**, *30*, 1055–1065. [CrossRef] [PubMed]

37. Toonen, R.J.; Pawlik, J.R. Foundations of gregariousness. *Nature* **1994**, *370*, 511–512. [CrossRef]
38. Marshall, D.; Keough, M. Variation in the dispersal potential of non-feeding invertebrate larvae: The desperate larva hypothesis and larval size. *Mar. Ecol. Prog. Ser.* **2003**, *255*, 145–153. [CrossRef]
39. Elkin, C.; Marshall, D. Desperate larvae: Influence of deferred costs and habitat requirements on habitat selection. *Mar. Ecol. Prog. Ser.* **2007**, *335*, 143–153. [CrossRef]
40. Chaw, K.; Birch, W. Quantifying the exploratory behaviour of *Amphibalanus amphitrite* cyprids. *Biofouling* **2009**, *25*, 611–619. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.