Classification of the pre-settlement behaviour of barnacle cyprids

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Barnacle cyprids exhibit a complex swimming and exploratory behaviour on surfaces and settlement is a consequence of extensive surface probing and selection of suitable settlement sites. In this work, the behaviour of cyprids in their pre-settlement phase was studied by three-dimensional video stereoscopy. With this technique, three-dimensional trajectories were obtained that were quantitatively analysed. The velocity during vertical sinking of cyprids of *Balanus amphitrite* was used with a modified form of Stokes’ law to calculate their mean body density. Furthermore, a classification of the swimming patterns allowed the extension of existing models describing cyprid locomotion and swimming behaviour. The patterns were characterized with respect to their occurrence, transition between patterns and their velocity distribution, and motions were identified that led to surface contacts. This analysis provides a classification framework, which can assist future attempts to identify behavioural responses of cyprids to specific settlement cues.

1. Introduction

Barnacle cyprids are a model organism in marine biofouling research as they actively select a substratum for settlement and metamorphosis [1]. Because of their broad distribution in coastal and estuarine regions [2], and their prominence as a fouling species in warm waters worldwide [3], cyprids of *Balanus amphitrite* (=*Amphibalanus amphitrite*) [4] have been used extensively in field and laboratory research. Their successful rearing under laboratory conditions throughout the year, without any specific seasonal dependency, and unproblematic settlement in static water assays [5], contributed to their use in toxicity, settlement and behavioural studies. As a result, it has been shown that the behaviour of cyprids is not a random process, but a consequence of complex responses to a combination of environmental factors (e.g. settlement pheromones [6]) as well as various chemical (i.e. [7–10]) and physical [11,12] characteristics of the substratum [13]. An understanding of this behaviour is not only important to population and community dynamics [14], but also to the development of novel methods to interfere with and inhibit the settlement of cyprids [15]. The desire to understand the surface selection strategies of cyprids has led to development of two-dimensional video tracking methods to quantify cyprid motions [14,16–21] and novel surface-sensitive imaging techniques [22,23].

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two-dimensional tracking is that quantification is only reliable for motions within the projected plane. Three-dimensional tracking fills this gap, and quantitative behavioural parameters such as swimming distances, velocities and turn angles can accurately be determined for the intrinsically three-dimensional motion of cyprids [25].

This study aimed to provide a framework for the classification of the pre-settlement behaviour of cyprids based on the acquisition of three-dimensional trajectories. For this purpose, the stereoscopic set-up described in Maleschlijski et al. [26] was applied, and the general pre-settlement behaviour was analysed. As it is known from previous studies that different chemical surface terminations can induce different motions [10,14], patterns above a range of surface chemistries were acquired to ensure that the most common ones were included. The main motion patterns of the cyprids were quantified with respect to their velocities and their occurrence. As the aim of the study was simply to develop a classification framework, the behaviours associated with specific surface chemistries were not differentiated further. The velocities during the sinking phase were used to calculate the density of the cyprid body and to determine its change with increasing age of the larvae. Finally, the analysed data were used to define different groups of motion patterns and integrate them into a previously described cyprid behaviour model [27].

2. Material and methods

2.1. Barnacle cyprids

Cyprids of *B. amphitrite* were cultured and harvested at the Newcastle University [28]. Following established procedures [17,19], the cyprids were shipped overnight in a cool-box (6°C) to Karlsruhe Institute of Technology, Germany, and the experiments were performed the following day. For the majority of experiments, the age of the cyprids at the time of recording was 3 days. For the experiments investigating the correlation between cyprid age and sinking velocity, additionally 4-, 8- and 12-day-old cyprids were used. These were taken out of the fridge when 3-days old and distributed equally over three different storage containers. The dead cyprids were collected and preserved in 70% ethanol. Prior to each experiment, cyprids were allowed to warm up for 60 min after storage at 6°C. Subsequently, SAM-coated quadrPERM culture vessels with a width of 75 mm and a height of 25 mm were filled with 18.75 ml fresh filtered (0.22 μm pore size) seawater and ~30 cyprids were added. The resulting depth of the water column was 10 mm. For each different chemically terminated surface, three replicates were used. Cyprids were allowed to equilibrate for an additional 5 min before the video recording was started. After the motions were recorded, a semi-automatic tracking algorithm was applied to extract trajectories from the videos. Therefore, the two-dimensional coordinates were recorded in both camera perspectives and merged into three-dimensional coordinates for each object of interest. To apply the epipolar transformation and to allow the extraction of quantitative information from the data points, both cameras were calibrated using a calibration procedure as described in Maleschlijski et al. [26]. Here, the z-values represented the distance to the surface, whereas the x- and y-values denoted the position in the surface plane. Connecting all three-dimensional coordinates of a single object of interest in time, produces a swimming trajectory from which quantitative parameters can be calculated. The field of view of the cameras was ~47 × 35 mm. Image analysis was restricted only to an area in the centre of the containers, in order to avoid effects occurring because of the interfacial regions close to the walls. Furthermore, the specifics of the geometry of the applied set-up did not allow the evaluation of cyprids attached or swimming close to the walls of the containers. Thus, the effective arena size used for analysis was ca 60% of the container size.

2.2. Stereoscopic three-dimensional tracking and measurement procedure

The stereo-camcorder setup consisted of two synchronized consumer camcorders as described recently [26]. The cameras imaged the surface of interest from the top at a relative angle to each other of ca 80° and a distance to the water surface of ca 40 cm. A low-intensity light source illuminated the scene from above. As the intention was to obtain as many different motions as possible, a collection of surface chemistries (based on SAMs) was included. These contained hydrophilic (–OH terminated), hydrophobic (–CH₃ terminated), positive (–NMᵉ⁺), negative (–COO⁻), zwitterionic (50% –NMᵉ⁺/50% –COO⁻), hydrated (EG₄OH, EG₅OH, EG₆OH) and PEG surfaces. The preparation of the SAMs followed earlier protocols [29,30]. Because the aim of this work was to provide a classification framework describing and extending current behavioural models, the patterns were not further differentiated with respect to surface chemistry. Prior to each experiment, cyprids were allowed to warm up for 60 min after storage at 6°C. Subsequently, SAM-coated quadrPERM culture vessels with a width of 75 mm and a height of 25 mm were filled with 18.75 ml fresh filtered (0.22 μm pore size) seawater and ~30 cyprids were added. The resulting depth of the water column was 10 mm. For each different chemically terminated surface, three replicates were used. Cyprids were allowed to equilibrate for an additional 5 min before the video recording was started. After the motions were recorded, a semi-automatic tracking algorithm was applied to extract trajectories from the videos. Therefore, the two-dimensional coordinates were recorded in both camera perspectives and merged into three-dimensional coordinates for each object of interest. To apply the epipolar transformation and to allow the extraction of quantitative information from the data points, both cameras were calibrated using a calibration procedure as described in Maleschlijski et al. [26]. Here, the z-values represented the distance to the surface, whereas the x- and y-values denoted the position in the surface plane. Connecting all three-dimensional coordinates of a single object of interest in time, produces a swimming trajectory from which quantitative parameters can be calculated. The field of view of the cameras was ~47 × 35 mm. Image analysis was restricted only to an area in the centre of the containers, in order to avoid effects occurring because of the interfacial regions close to the walls. Furthermore, the specifics of the geometry of the applied set-up did not allow the evaluation of cyprids attached or swimming close to the walls of the containers. Thus, the effective arena size used for analysis was ca 60% of the container size.

2.3. Data analysis

The total number of acquired trajectories was ~300 (generated by ~300 cyprids), each with a mean duration of ca 5 min. Manual classification of the patterns was performed and their occurrence in the dataset was calculated. Additionally, transitions between patterns were qualitatively analysed. Furthermore, the velocities of cyprids during each pattern were calculated and discussed. Because of the high frame rate (25 fps) used in this work, it was assured that the direction of the velocity vector coincided with the direction of the tangent vector of the trajectory. For this reason, the magnitude of the velocity vector is identical to the speed tangential to the trajectory.

For the analysis of the velocity distributions, an empirical cumulative distribution function (ECDF) was calculated. The general equation for calculation of the cumulative distribution function (CDF) is

\[ F_X(x) = P(X \leq x), \]

with \( P(X \leq x) \) being the probability that the test variable \( X \) would exhibit a value less than or equal to \( x \) [31]. With the empirical CDF, the cumulated occurrence (0–100%) of the values of the test variable within a given dataset is described. For the estimation of the ECDF of the velocity in this work, first the histogram of velocity values for a given pattern, obtained from all replicates, was plotted. Subsequently, the histogram was used to calculate the ECDF by applying the following equation [32]

\[ F_{ECDF}[x] = \frac{100}{n} \sum_{i=1}^{n} I[X_i \leq x], \]

where \( I[X_i \leq x] = \begin{cases} 1 & \text{if } X_i \leq x \\ 0 & \text{otherwise} \end{cases} \).
D. wcyprid body. In this case, the equation for the sinking velocity
translations in this paper, we assumed an ellipsoidal shape of the
which apply to ellipsoidal and cylindrical particles. For the calcula-
the cyprid (longest, intermediate and smallest axial diameters
non-spherical cyprid),
diameter of a sphere having the same volume and weight as the
cyprids, we obtain
Re-writing equation (2.1) in order to calculate the density of
with
E
where
D
is the measure of the shape of the cyprid [34], defined as
2.4. Modified Stokes’ law
For the calculation of the body density of cyprids, a modified
form of Stokes’ equation was used as described by Komar et al.
[33]. The modification is necessary as Stokes’ law strictly applies
only to spherical objects. The authors proved that objects with
shapes that deviate from spheres show different sinking speeds. Komar et al. have derived semi-empirical equations, which apply to ellipsoidal and cylindrical particles. For the calcula-
tions in this paper, we assumed an ellipsoidal shape of the
cyprid body. In this case, the equation for the sinking velocity
v_s (also settling rate) of cyprids was

\[
v_s = \frac{1}{18} \cdot \frac{1}{\mu} \cdot (\rho_{cyprid} - \rho_{fluid}) \cdot g \cdot D^2 \cdot E^{0.380}.
\]  

(2.1)

Re-writing equation (2.1) in order to calculate the density of
cyprids, we obtain

\[
\rho_{cyprid} = \frac{18 \cdot \mu \cdot v_s}{g \cdot D^2 \cdot E^{0.380} + \rho_{fluid}}.
\]  

(2.2)

with E being the measure of the shape of the cyprid [34], defined as

\[
E = D_n \cdot \left[ \frac{D_l^2 + D_i^2 + D_s^2}{3} \right] \cdot \frac{1}{2},
\]  

(2.3)

where D_n denotes the ‘nominal’ diameter of the cyprid (the
diameter of a sphere having the same volume and weight as the
non-spherical cyprid), D_l, D_i and D_s are the dimensions of
the cyprid (longest, intermediate and smallest axial diameters
of the ellipsoid [33]), \( \mu \) the viscosity of seawater (1.071 g m \(^{-1}\) s \(^{-1}\)), \( \rho_{fluid} \) the density of seawater (1.023 g cm \(^{-3}\)) for the experimental
conditions (temperature of seawater 20\(^\circ\)C and salinity 34\%w) and
\( g \) the gravitational acceleration (9.81 m s \(^{-2}\)).

3. Results and discussion

3.1. Motion patterns
Stereoscopic tracking was applied to record trajectories of
cyprids. For the experiments, a range of different surface
chemistries was used to account for as many surface proper-
ties as possible and to minimize the risk of missing obvious
and important motion patterns. Figure 1 summarizes the
typical motion patterns that were detected in our experi-
ments. The differentiation of the patterns was done by
visual analysis of the trajectories and manual segmentation
whereby a new pattern was defined and assigned to every
uniquely observed shape in the trajectory. The patterns
were grouped into two main categories depending on their
position in relation to the substrate surface and only patterns
originating from cyprids which were active in the observation
window were considered. **Spiralling**, **swimming** and **sinking**
(patterns figure 1a–c) were included in the group of patterns
used for locomotion and occasional surface contacts.

The general pattern that was most frequently observed for
moving across the water volume over wider distances was
**swimming** (figure 1b). The movements were generally charac-
terized by smooth sections, including parts with fast
unidirectional locomotion or gentle turns and the values for
the velocities were typically broadly distributed.
A special form of the swimming pattern was spiralling (figure 1a), which included pseudo-helical movements. This pattern occurred in the water volume, and the axis of the helical rotation was found to change rapidly, which altered the swimming direction. Lagersson et al. [27] presented the biomechanical explanation of swimming, during which, one antennule is maximally extended by the larvae, probably involved in determining the swimming direction, whereas the thoracopods beat in a burst-like mode, functioning as a motor for the movement. In spiralling a similar biomechanical process might be involved. It can be speculated that by extending the antennule outwards, the cyprid changed the centre of mass of its body and a rotational moment occurred.

The beating of the thoracopods has a metachronous rhythm [27] and as soon as they stop moving, the larva starts to sink in the water column, driven by gravity. Although this movement pattern seemed to have a passive character, it was often observed and we referred to it as sinking (figure 1c). According to our observation, this was the main pattern that led to a contact with the horizontally positioned substrates below the cyprids.

The movement patterns in close proximity to the substrate were termed rotating and walking (figure 1d,e). The resolution of our set-up allowed only 'macroscopic' observations, i.e. single steps were not resolved. Thus, we assigned all cyprids to the walking motion if movements on the surfaces had a low velocity profile and high linearity (cyprids moved in a straight-line manner). Walking contained the patterns ‘wide search’ and ‘close search’ identified earlier by Lagersson et al. [27].

The pattern rotating was perceived as a special form of spiralling, which extended to the substrate interface and involved circular movements at intermediate velocities. Surface interaction at similar velocities has been reported previously for highly hydrated surfaces (polyethylene glycol) [26], hydrophilic surfaces and also for zwitterionic hydrated surfaces [10].

3.2. Occurrence and transitions of the motion patterns

In order to quantify the occurrence of the different patterns shown in figure 1, their distribution was analysed in a dataset containing ca 300 trajectories, each with a mean duration of 5 min. For this purpose, counts were made of how often each pattern occurred in each trajectory. These occurrence values were plotted in a pie diagram, to visualize the relative distribution of the patterns in the dataset. The results are shown in figure 2a. As pointed out above, only cyprids that were active during the observation period were considered for this analysis. Therefore, the resting pattern (15% occurrence) described only the inactive parts of the trajectories and did not provide any quantitative information on cyprids that remained inactive throughout the whole recording time. The reason not to include such cyprids in the analysis was that the resolution of the set-up was not sufficient to discriminate resting from settling larvae. From figure 2a, it can be noted that the swimming pattern was most frequently observed. This pattern occurred around one-third of the time (31%) and allowed cyprids to freely explore a broad perimeter and to move across the water volume. The counterpart of swimming, sinking, also occurred frequently (26%). Generally, the passive patterns, resting and sinking, amounted to 41% of total behaviours observed. Their total occurrence was slightly lower than the sum of occurrences for swimming, spiralling, walking and rotating—the active patterns (57%). The overall occurrence of the surface patterns remained less than 17%, of which rotating was less frequently (4%) observed compared with walking (13%).

As shown in figure 2a, behaviour was dominated by patterns in the water column (68%), whereas surface exploration was observed 32% of the time. Although not further quantified, we noted a general trend that older cyprids (8 days or more) tended to exhibit more surface patterns. This trend is in good agreement with the ‘desperate larva hypothesis’ which states that older non-feeding larvae tend to decrease selectivity on settlement sites, as discussed by Elkin et al. [35]. It is worth mentioning that spiralling occurred only 11% of the time. Besides the active movements, sinking was an important component of the locomotion of cyprids. As shown in figure 1, during sinking, the movements were vertical (in this set-up alignment) with a relatively constant and low speed. The sinking phases were followed by active swimming, during which the cyprid swam upwards, followed by a new sinking period. The observed constant alternation between swimming and sinking (31% and 26%, respectively; figure 2a) agrees with previous investigations which discussed that this might be an energy efficient way of locomotion under turbulent conditions [21,36,37].
of low velocities, the differed markedly from ming values. It can be noted that the ECDFs of cal ECDFs which resulted after integration of the histogram walking (figure 1) for better comparison, figure 3 velocities of the patterns, histograms were calculated on the region was 0–3.1 mm s

Afterwards, the cyprid accelerated and returned at a velocity of $1.6 \text{ mm s}^{-1}$, which was higher for the surface patterns (e.g. $\approx 32\%$ for walking) and lower for the volume patterns (5% for swimming and 12% for spiralling). Additionally, the velocity values at which the ECDFs reached 95% are shown in figure 3c. For the swimming pattern, values between 0 and 4.1 mm s$^{-1}$ represented 95% of all velocities. For spiralling, the region was $0–3.1 \text{ mm s}^{-1}$. In the case of walking, 95% of the velocity values were between 0 and 1 mm s$^{-1}$ and for rotating the region were between 0 and 1.7 mm s$^{-1}$. The narrower distribution of velocities of the surface patterns was coupled to a higher occurrence of low velocities and smaller values for the 95% EDCF. In turn, the volume motions showed a broader distribution stretched towards larger values, with fewer slow velocities and higher 95% EDCF numbers. This analysis indicated a slower motion on the surface, presumably used by the cyprids for sensing and inspecting the interface.

In addition, the linearity of the patterns was investigated. For this purpose, the trajectories were divided into 1.5 s long segments and the term linearity described the fraction of absolute spatial displacement and the effective swimming distance between the first and the last point of an analysed segment [38]. The patterns with low linearity were rotating (0.15 ± 0.06) and spiralling (0.17 ± 0.03). As these patterns mainly showed circular and helical movements, total swimming distances were large, whereas the effective displacement from the starting position was limited. A high linearity was observed for the walking pattern (0.43 ± 0.08). Although cyprids move at rather slow velocities, bipedal walking was unidirectional. The linearity of swimming (0.33 ± 0.19) was high as well, probably because the pattern was used mainly for locomotion across the water column. The high standard deviation of the values of the swimming linearity is attributed to the fact that the pattern involved parts that were not only directed and linear, but also with more convoluted swimming.

3.3. Quantitative characterization of sinking

Figure 4a shows a representative three-dimensional trajectory, which includes several sinking phases, one of them highlighted by a black circle. During these vertical movements, the z-position of the swimming cyprid decreased gradually and it sank at a speed of $\approx 1.6 \text{ mm s}^{-1}$ (indicated by the blue colour). Between the sinking phases, the cyprid swam actively and returned to its initial z-position. During sinking, it changed its z-position from ca 3 mm to less than 1 mm above the substratum. This sinking phase lasted $\approx 2$ s at an average velocity of $\approx 1.6 \text{ mm s}^{-1}$. Afterwards, the cyprid accelerated and returned at a velocity of $\approx 7.8 \text{ mm s}^{-1}$ to its initial swimming depth. Subsequently, a new sinking period was initiated. It is worth mentioning that at the end of the sinking period, the cyprid seemed to actively

Figure 3. (a) Empirical cumulative distribution functions of the velocity for the different motion patterns. The orange dashed line marks the velocity of 0 mm s$^{-1}$, and the green dashed line marks the 95% cumulative probability. (b) Occurrence of velocity values of 0 mm s$^{-1}$ (orange dashed line in (a)) for each of the patterns and (c) velocities of the different patterns at which the empirical cumulative distribution function reaches 95% (green dashed line in (a)).
at ca mean. The dashed line denotes the density of seawater at 20°C measured mean sinking velocity (red) and resulting body density (black) of cyprids of different ages and dead ones. Error bars represent the standard error of the Gaussian function characterized the sinking motion. Additionally, the ‘model’ of the sinking velocity from segmented data (grey line) is composed of a Gaussian contribution and an exponential decay (denoted by grey lines). The parameters (μ, σ²) = (−0.71 mm s⁻¹, 0.39 mm s⁻²) of the Gaussian function characterized the sinking motion. The fit on the left side (black line) is composed of a Gaussian contribution and an exponential decay (denoted by grey lines). The parameters (μ, σ²) = (−1.23 mm s⁻¹, 0.36 mm s⁻²) of the Gaussian function characterized the sinking motion. Additionally, the ‘model’ of the sinking velocity from segmented data (c) is shown in red. (e) Change in mean sinking velocity (red) and resulting body density (black) of cyprids of different ages and dead ones. Error bars represent the standard error of the mean. The dashed line denotes the density of seawater at 20°C.

To statistically investigate the sinking velocity, 28 parts of swimming trajectories showing only the sinking motion were manually segmented and analysed. For a motion to be classified as a ‘sinking’ pattern, no locomotion in the x- and y-direction for at least 15 sequential frames was detected and at the same time the change in z position was negative. The histogram of the velocity in z-direction is shown in figure 4c with a Gaussian fit possessing a mean value of −1.11 mm s⁻¹ and a standard deviation of 0.39 mm s⁻¹.

This information was compared with the velocity histogram of a complete dataset (300 trajectories) of swimming cyprids (figure 4d). Positive values on the x-axis represented upward swimming, negative values a downward motion. Because of the frequent occurrence of resting phases (e.g. at the water/solid interface), a strong contribution was usually detected in the histogram at vz ≈ 0 mm s⁻¹, as visible from the complete histogram shown in the inset. From the magnified view of the histogram (main panel in figure 4d), it can be seen that positive values occurred up to 3.75 mm s⁻¹, whereas the values on the negative side fell to only ≈−2 mm s⁻¹. This ‘asymmetry’ of the values on the x-axis around vz = 0 represented different speeds when swimming towards and away from the substrate respectively. It indicated that cyprids were more active when they had to counteract the gravitational force while moving up, compared with a downward swimming or sinking motion. A second characteristic of the histogram shown in figure 4d was the enhanced occurrence of vz-values close to −1.25 mm s⁻¹. The data on the left side were fitted using a mixed model of a Gaussian function and an exponential background. The resulting fit is shown with a black line in figure 4d (μ = −1.23 mm s⁻¹, σ² = 0.357 mm s⁻²). For comparison, the Gaussian fit of the segmented sinking phases from figure 4c is also added in the histogram (red curve). The velocity of 1.23 mm s⁻¹ as well as the form of the Gaussian of both fits were very similar and indicated that this peak could be assigned to the sinking phases.

Beyond characterizing the sinking motion, the information contained in the trajectories can be further used to extract physical properties of the barnacle cyprids. As the motion during the sinking phase contained almost no x and y components, it can be anticipated that sinking was only determined by the gravitational force and the counteracting hydrodynamic Stokes’ force. For such a sinking motion, the dimensions for the cyprids are important. Several reports determined the dimensions of cyprids of B. amphitrite, for example [2,39,40] to be ra ≈ 300 μm and rg ≈ 125 μm. For
the third dimension of the cyprid body, no reference value could be found in the literature, and this was assumed to be \( r_z \approx 60 \mu \text{m} \) based on our own microscopy observations. For the application of Stokes’ law, the cyprid was described as an ellipsoid, and the three radii were transferred into dimension parameters as \( D_n = 262 \mu \text{m} \) (nominal diameter) and \( E = 0.314 \) (shape factor; equations are given in the Material and methods section).

Using the measured value for the sinking velocity of 3-day-old cyprids of \( \bar{w}_3 = 1.23 \text{ mm s}^{-1} \) (with \( \sigma_{\bar{w}_3} = \pm 0.36 \text{ mm s}^{-1} \); figure 4d), the modified form of Stokes’ law for elliptical objects \( \rho_{\text{cyprid}} = \frac{18 \cdot \mu \cdot \bar{w}}{g \cdot D_n^2 \cdot E^{0.80}} + \rho_{\text{fluid}} \),

\[
(3.1)
\]

With the experimentally determined values and the dimension parameters \( D_n \) and \( E \), the cyprid density \( \rho_{\text{cyprid}} = \frac{18 \cdot \mu \cdot \bar{w}}{g \cdot D_n^2 \cdot E^{0.80}} + \rho_{\text{fluid}} \),

\[
(3.2)
\]

\( \) (with \( \sigma_{\rho_{\text{cyprid}}} = \pm 0.01587 \text{ g cm}^{-3} \)) was calculated. These obtained values can be compared with literature reports on the weight of cyprids. The dry-body mass of cyprids of *Semaebalanus balanoides* (= *Balanus balanoides*) has been reported to be \( m_{\text{cyprid}} \approx 38 \mu \text{g} \) [41] which corresponds to a wet weight of \( m_{\text{cyprid}} \approx 126 \mu \text{g} \) considering a general percentage of water in the body of zooplankton of \( \alpha 70% \) [42,43]. The dimensions for *B. balanoides* cyprids were shown by Crisp et al. [44] to be \( r_x \approx 663 \mu \text{m} \) and \( r_y \approx 346 \mu \text{m} \). Although these values were measured for cyprids in Arctic waters (74° N), Pineda et al. [45] demonstrated that they can also be found for cyprids from warmer regions (41° N). For the third dimension, no literature data were available, and we used our own microscopy data and observed an \( r_z \approx 90 \mu \text{m} \). With these values in an ellipsoid model to describe the shape of the cyprid body, the density was calculated as

\[
\rho_{\text{cyprid}} = \frac{m_{\text{cyprid}}}{V_{\text{cyprid}}} = \frac{3 \cdot m_{\text{cyprid}}}{4 \cdot \pi \cdot r_x \cdot r_y^2 \cdot r_z} = 1.45 \text{ g cm}^{-3}.
\]

(3.3)

Although this value contained several major approximations and overestimated the water content, the density was just slightly higher than that obtained from the sinking analysis for *B. amphitrite*. Interestingly, several other marine organisms have densities in the same range as our measurements, e.g. *Cerastoderma edule* larvae \( \rho = 1.1 \text{ g cm}^{-3} \) [46], *Calanus finmarchicus* \( \rho = 1.08 \text{ g cm}^{-3} \) [47]. This further supports our conclusion that the sinking analysis, according to Stokes’ law, yields an accurate density value.

As it is known from literature that settlement success [48] and lipid content [49] change with age of the cyprids larvae, the question arose if the body density also changes. As the cyprids are non-feeding larvae, energy consumption during swimming and surface exploration might alter their density. Figure 4e (red curve) shows the mean sinking velocity of cyprids of different ages as well as of dead ones (the full velocity histograms can be found in the electronic supplementary material, figure S2). It can be seen that the absolute mean sinking velocity of the 3-day-old cyprids of \( 1.23 \text{ mm s}^{-1} \) decreased slightly for 4- (1.13 mm s\(^{-1}\)) and 8-day olds (1.03 \text{ mm s}^{-1} ), although the changes were within the error bars. After 12 days, the value was found to be considerably smaller (0.75 \text{ mm s}^{-1} ), and the lowest value was observed for the dead cyprids (0.46 mm s\(^{-1}\)). If equation (3.1) is used to calculate the cyprid body density with the obtained sinking velocity values, it becomes obvious that the density values also decreased over time (figure 4c, in black) and the body density of 3-day-old larvae being \( \rho = 1.08 \text{ g cm}^{-3} \) became \( \rho = 1.06 \text{ g cm}^{-3} \) for the 12-day old and \( \rho = 1.04 \text{ g cm}^{-3} \) for the dead cyprids, thus approaching the density value of the seawater under the experimental conditions (1.03 \text{ g cm}^{-3} \) for temperature of 20°C). At present, we can only speculate why the body density decreased. It is known that lipid cells are used by cyprids as energy reserves and the neutral lipid TAG (triacylglycerol, primary used for growth, behaviour and metamorphosis [49–51]) has been reported to account for \( \alpha 25\% \) of the total body weight of cyprids of *B. balanoides* [41]. Miron et al. [52] investigated the normalized quantity of TAG in cyprids of *B. amphitrite* of different ages and found that it decreased by \( \alpha 25\% \) between 1-day and 12-day-old larvae. In addition, increasing age of the cyprids leads to a decrease in a vitelin-like storage protein [48]. The drop in the sinking velocity between 3- and 12-day-old cyprids in our measurements was \( \alpha 33\% \), which would correlate with the drop in both, TAG and vitelinated storage protein content. This or related physiological changes during ageing of the cyprids could be responsible for the observed decrease in larvae density. The fact that the sinking velocity of dead cyprids was even lower suggested that in addition to the body density, physiological phenomena (e.g. stretching of thoracopods or antennules, etc.) or active osmoregulation could also contribute to the decreased values.

3.4. Summary

To summarize, we presented a three-dimensional characterization of the swimming movements of barnacle cyprids under laboratory conditions. Different surface chemistries were considered, to stimulate a large variety of patterns. Figure 5 summarizes the collection based on two classifiers—cyprid position with respect to the substratum and velocity. The general categories presented in this work extend previous reports by Lagersson et al. [27] based on the availability of the three-dimensional information of the set-up used here. According to Lagersson et al. [27], cyprid settlement behaviour can be divided into five different categories: swimming, substratum exploration, wide search, close search and finally permanent attachment. Because the resolution of our data does not allow the distinction of the single surface inspection categories, for the classification presented in this work, they have been combined into surface exploration, whereas the new categories—spiralling, sinking and resting have been added.
The relative occurrence of each of the different patterns, as well as transitions between them, were investigated. Most of the time, motile cyprids showed active patterns (60% of the time), and in the remaining time resting was observed. In general, it was found that the volume movements showed higher velocities, whereas the surface patterns were slower. One special swimming pattern was the ‘sinking’ motion. Evaluation of the general histogram of the velocity perpendicular to the surface revealed that velocities away from the substrate surface were higher than velocities towards that surface. Furthermore, it could be shown that the sinking velocity decreased for older cyprids, probably as a consequence of the decreasing body density values. An empirical sinking value of $\approx 1.23 \text{ mm s}^{-1}$, obtained for 3-day-old cyprids, was used together with a modified version of Stokes’ equation to calculate the density of the cyprid body to be $1.07768 \text{ g cm}^{-3}$, which is in good agreement with literature data on other barnacle cyprids and marine organisms. With increasing age, the density of the cyprids was found to be reduced. Probably, a reduction of the lipid deposits during ageing was responsible for most of the change in density.

While our study adds to the understanding of motion and behaviour of barnacle larvae, the motility analysis is inevitably a snapshot in time for several batches of cyprids in a certain physiological state, bred under laboratory conditions. For the future, it would be interesting to extend the work to cyprids subjected to chemical and morphological surface cues, external factors and stimuli (e.g. temperature and pH), compare the surface exploration of cyprids of different ages, and to compare the results with the motility of wild cyprids in the ocean. In addition, a more detailed analysis of the behaviour of cyprids on surfaces at higher spatial resolution is anticipated. The application of the presented classification framework will facilitate a better understanding of the motility of cyprids and how behaviour is involved in surface selection. Such analysis could help to identify surface cues that moderate or counteract settlement. Applying this knowledge is meant to support the development of a new generation of specially designed anti-biofouling coatings to interfere with the settlement process.

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