ZOOPLANKTON ECOLOGY

Effects of animal density, volume, and the use of 2D/3D recording on behavioral studies of copepods

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Abstract Studies on the behavior of copepods require both an appropriate experimental design and the means to perform objectively verifiable numerical analysis. Despite the growing number of publications on copepod behavior, it has been difficult to compare these studies. In this study, we studied two species of copepods, *Eurytemora affinis* and *Pseudodiaptomus annandalei*, and employed recently developed scaling and non-scaling methodology to investigate the effects of density and volume on the swimming behavior of individual organisms in still water. We also compared the results of two- and three-dimensional projections of the swimming tracks. A combination of scale-dependent and scale-independent analysis was found to characterize a number of behavioral observations very effectively. We discovered that (i) density has no effect except to increase the time spent in the swimming state of "breaking", (ii) smaller volumes resulted in more complex trajectories, and larger volumes, like density, increased the time spent in the swimming state "breaking", and (iii) three-dimensional projections gave a more accurate estimation of speed and the time spent cruising. When only a vertical 2D projection was used, "cruising" could be confused with "sinking". These results indicate that both experimental conditions and the selection of 2D or 3D projection have important implications regarding the study of copepod behavior. The development of standardized procedures with which to compare the observations made in different studies is an issue of particular urgency.

Keywords Swimming behavior · Analysis · Methodology · Calanoid copepod · *Eurytemora affinis* · *Pseudodiaptomus annandalei*

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Introduction

The study of animal behavior requires that animals be observed alive, preferably within their natural environment, or at the very least, in an environment that

does not interfere with their natural patterns of behavior. Most forms of plankton, including Calanoid copepods, are microscopic and transparent (Strickler & Hwang, 1999), making any observation of their behavior particularly difficult. For this reason, most behavioral studies on copepods have been performed in laboratories, with a few notable exceptions (see Davis et al., 1996 for field observations of large copepods). Even in such manageable environments, analyzing the behavior of copepods can be technically challenging. These animals are difficult to observe, due to their speed (up to 500 body length s^{-1}) (Strickler, 1975) and small size (1–10 mm in length). In addition, researchers must also take into account a number of facts specific to copepods, such as the influence of light and an awareness of boundaries on their behavior (Strickler & Hwang, 1999). For these reasons, all researchers investigating the swimming behavior of copepods have attempted to provide sufficient spatial and temporal resolution, to account for the side wall effect and positive phototaxis. None of these studies, however, have considered the effects of volume or density while filming (Table 1). It remains unclear whether these variables influence the behavior of copepods, and inter-study comparisons have yet to produce reliable results.

Population density influences the life history traits of copepods. High density may induce stress factors limiting the success of egg production and hatching (Medina & Barata, 2004; Peck & Holste, 2006; VanderLugt & Lenz, 2008), although this phenomenon may be species-specific or strain-specific (Jepsen et al., 2007; Camus & Zeng, 2009). Density has also been associated with mortality in *Calanus finmarchicus* (Ohman & Hirche, 2001) and *Acartia sijiensis* (Camus & Zeng, 2009), but not for *Acartia tonsa* (Medina & Barata, 2004; Jepsen et al., 2007). Very low densities may limit the ability of individual organisms to find mates, thereby reducing the fecundity of the population (Kiørboe, 2006) and restricting the scope of mate choice (Kokko & Rankin, 2006). Little is known, however, about the effect of density on the swimming behavior of copepods.

Another variable is the volume of space to be filmed. Determining the ideal volume for filming is a compromise between several factors, including the ability to maintain focus on individual organisms and the size of the container; the smaller the species is, the smaller the container must be (Kiørboe, 2007).

Table 1 Volume and density used for a non-exhaustive list of diverse investigations on copepod swimming behavior

Reference	Volume (l)	Density (ind l^{-1})
Bagøien & Kiørboe (2005a)	1.000	40–60
Bagøien & Kiørboe (2005b)	1.000	40–60
Buskey et al. (1996)	8.000	25
Choi & Kimmerer (2008)	0.002	1000
	0.030	66.7
	0.125	16
	0.250	8
	0.500	4
	1.000	2
	2.000	1
	4.000	0.5
	8.000	0.25
	16.00	0.125
Doall et al. (1998)	1.500	10–23
Doall et al. (2002)	1.000	10–20
Dur et al. (2010)	0.125	160
Goetze (2008)	1.000	40
Kiørboe (2007)	0.125	80
Kiørboe (2008)	1.000	15–25
	0.512	7.7–12.8
Mazzocchi & Paffenhöfer (1999)	3.000	3
Michalec et al. (2010)	0.125	80
Nihongi et al. (2004)	1.000	40
Paffenhöfer & Mazzocchi (2002)	1.000	7–10
Saiz (1994)	2.460	8.13
Saiz & Alcaraz (1992)	0.900	100
Schmitt & Seuront (2001)	3.375	0.29
Schmitt et al. (2006)	1.125	46
Seuront et al. (2004a, b)	0.400	n.a.*
Seuront (2006)	2.000	25
Seuront & Vincent (2008)	2.000	0.5
Tiselius (1992)	0.550	41
Tiselius & Jonsson (1990)	9.000	0.1
Titelman (2001)	31.25	30–300
Titelman & Kiørboe (2003)	0.250	1,000–5,000
	5.000	1,000–5,000
Tsuda & Miller (1998)	250.0	0.08–0.12
Uttieri et al. (2007)	1.000	40
Van Leeuwen & Maly (1991)	0.250	4
VanDuren & Videler (1995)	1.000	8–10
Waggett & Buskey (2007a)	0.070	7
Waggett & Buskey (2007b)	0.340	147

There was strong variability in the volume and the density

n.a. non available

When focusing on swimming behavior, the ability to process a sufficient number of trajectories is an important issue (Seuront et al., 2004d), and the more individuals being observed, the more trajectories are involved. However, density should not be too high for extraction programs to provide good data. Various volumes are required when studying the behavior of various species (Table 1). Despite the importance of this issue, no information is currently available concerning the influence of container volume on the behavior of the organisms held within it.

Density and volume are closely related; however, a critical understanding of the effects of each is crucial to improving the quality of results obtained in behavioral experiments and to provide insight regarding the comparison of results obtained in different experimental settings.

Another challenge encountered by scientists wishing to compare results from behavioral experiments with those of previous research is selecting the projection system to be used. Analysis of the movement patterns of copepods through a volume of space preferentially requires a system capable of recording three-dimensional (3D) data. Until recently, this was not a trivial problem, and has resulted in very few investigators being able to record the swimming paths of copepods in 3D, with most researchers resorting to recording only in two dimensions (2D). Such observational inconsistencies have resulted in difficulties in areas such as the comparison of speed. Although two-dimensional estimation provides true values for sinking velocity, real cruise and loop speeds are underestimated (Kiørboe & Bagøien, 2005). Extrapolation to higher dimensions (from 2D to 3D) requires isotropy with regard to the swimming path (Seuront et al., 2004a; Kiørboe & Bagøien, 2005). Although copepods move in three dimensions, no a priori factors insure the isotropy of their paths. Anisotropy in the swimming of copepods can be attributed to gravity (Strickler, 1982), food exploitation strategies (Leising & Franks, 2000; Leising, 2001; Seuront & Lagadeuc, 2001), and prey switching behavior (Kiørboe et al., 1996; Capparoy et al., 1998). Moreover, isotropy and anisotropy are species-specific (Tiselius & Jonsson, 1990). Nonetheless, isotropy is required to extrapolate 2D data to 3D behavior, consequently restricting the reliability of data extrapolated to a higher dimension.

One final limitation regarding data comparison is associated with the variety of temporal scales available for observation and the fact that common metrics [i.e., speed and Net-to-gross displacement ratio (NGDR)] require different values depending on the scale at which they are measured (Seuront et al., 2004b). Several articles have demonstrated the unreliability of such scale-dependent metrics, highlighting the advantages of a scale-independent framework in characterizing the swimming behavior of plankton (e.g., Schmitt & Seuront, 2001; Seuront et al., 2004a, c; Uttieri et al., 2004, 2005).

Recent studies have shown differences in swimming behavior between females (ovigerous and non-ovigerous) and males of two euryhaline calanoid copepods: the temperate species *Eurytemora affinis* (Michalec et al., 2010) and the tropical species *Pseudodiaptomus annandalei* (Dur et al., 2010). These studies showed that males are more active than females and activity of males can consequently be used to compare the effects of various factors.

This study aims to facilitate comparisons between existing behavioral studies, by quantifying the effects of specific factors. The first two experiments investigated whether the swimming behavior of *Eurytemora affinis* (Poppe, 1880) males is influenced either by the density of individuals in the container or by the volume in which they were placed. We believed that each characteristic related to the environment (vessel size and density) has a singular impact on movement of calanoid copepods and the space they inhabit. For this reason, we employed two experimental designs to avoid the confounding effects of each factor. Finally, we compared two- and three-dimensional data on *P. annandalei* (Sewell, 1919) males, by following several individual organisms over time using high-resolution video equipment. We focused on basic swimming behavior using a fairly large set of trajectories displayed by the individuals as the basis for our investigation. To compare treatments, standard scale-dependant metrics were first used to allow comparisons with previous studies employing similar temporal resolutions. Scale-independent metrics were selected for their capacity to provide quantitative estimates of the complexity of trajectories (Schmitt & Seuront, 2001; Seuront et al., 2004c). Lastly, we used the method developed by Schmitt et al. (2006), which has been applied by other authors (Moison et al., 2009; Dur et al., 2010; Michalec et al., 2010), to study the transition between different swimming states. The results of this study have

implications regarding the methodologies adopted to study the behavior of copepods.

Materials and methods

Studied species

Individuals of *E. affinis* were sorted from samples of zooplankton collected from the Seine estuary, France, at the edge of Tancarville Bridge (49°28′23″ N 0°27′51″ E). Although several samplings were necessary, they were conducted in the same period of the year (in autumn) during ebb tide. Consistent conditioning procedures were maintained to avoid additional bias. Live copepods were transported from the sampling point to the laboratory in isothermal containers filled with aerated water within a few hours of capture. Once in the laboratory, individuals were placed in a tank (350 l) and maintained at salinity 25 and 8°C (temperature close to that of the sampling site) under a 12L/12D light cycle. The conditions of temperature and salinity provided a good survival rate with minimal reproduction (Devreker et al., 2007, 2009), thereby preserving sample integrity. Copepods were fed a mono-algal diet of *Rhodomonas marina* from laboratory culture, at the same time every day and 8 h prior to, but not during, the recording.

Pseudodiaptomus annandalei adults were obtained from surface tows using a similar WP2 plankton net off of the Danshuei estuary, Taiwan, at the edge of Chong Yang Bridge (25°05′02″ N 121°30′10″ E) during ebb tide in March 2008. Similar procedures to that used for *E. affinis* were employed to transport the individuals back to the laboratory. The *P. annandalei* individuals were then sorted from the containers, transferred into an aquarium (>1 l), and placed in an incubator. Individuals were maintained at salinity 15 and 25°C, under a 12L/12D light cycle. They were fed *Isochrysis galbana* cultured in the laboratory following the same procedure as that used for *E. affinis*. These conditions were selected to provide optimal survivability and development (Golez et al., 2004; Chen et al., 2006).

We opted to work with a single sex to reduce variability, selecting males, because they are generally more active than females (Kiørboe & Bagøien, 2005; Dur et al., 2010; Michalec et al., 2010). Only mature, normal, and active individuals were used for the experiments. *E. affinis* males have a mean prosome length averaging 0.85 mm in the autumn (Souissi, unpub. data). *P. annandalei* males also exhibit mean prosome lengths of 0.85 mm (Beyrend-Dur, unpublished data). Under a dissecting microscope, males were isolated from the culture and placed in a 1-l aquarium, 1–2 days prior to their use for video experiments. During this period, the copepods were held in similar conditions to that of the dark room and fed a surplus concentration of microalgae (*R. marina* for *E. affinis* and *I. galbana* for *P. annandalei*) to insure *ab libitum* food supply.

Filming conditions

The motility patterns of males were analyzed through the examination of video recordings. Four replicated video recordings were conducted with a different group of males for each tested condition, to compensate for variations among individuals (Seuront et al., 2004d). For each replication, individuals were placed in transparent acrylic vessels. The size of the vessels differed according to the investigation. Three main investigations were conducted.

Our first set of investigations focused on the influence of population density within the vessel. Therefore, the volume of the vessel was kept constant (i.e., 0.125 l; $5 \times 5 \times 6$ cm) and the number of individuals was varied (Supplementary material—Appendix 1). Considering the size of the studied animals, the size of the vessel ($5 \times 5 \times 6$ cm) had to be sufficiently large to avoid the side wall effect. The aquarium was lit from the bottom using a 4×7 infra-red (I.R.) LED array (emitting at 880 nm).

We then focused on the effect of volume on the movement of the organisms. Density was maintained at a constant level (i.e., 40 ind l^{-1}) and the volume was modified (Supplementary material—Appendix 1). Three different volumes were tested: small (0.125 l), intermediate (1,000 l; $10 \times 10 \times 11$ cm), and large (3.375 l; $15 \times 15 \times 16$ cm). We used lighting conditions similar to those used for the investigation into the effects of density.

For each investigation, the vessels were filled with brackish water at a salinity of 25 (0.45-μm filtered sea water and deionized water). To prevent turbulence from external factors, glass plates were used to enclose the vessels and experiments were conducted in a dark room dedicated to behavioral studies. A

stable temperature was maintained throughout the experiments.

The final investigation was a comparison of the results obtained by a 3D projection of the trajectories with those of 2D projections taken on the three planes (i.e., *xy*, *xz*, *yz*). Comparisons were conducted on a set of 4 replicated video recordings with constant volume and density (0.125 1 and 80 ind l^{-1}). We used lighting conditions similar to those used in previous experiments (i.e., I.R. light); however, in this experiment the lights were placed above the aquarium. Due to technical constraints, these experiments were limited to the subtropical species *P. annandalei*, instead of *E. affinis*. For the four replicated video, ten males were placed in a vessel filled with brackish water at salinity of 15.

For each investigation, individual animals were acclimated for 15 min before swimming activity was recorded for 30 min. During this period, animals were not fed. Identical conditions were used for both species.

Video techniques

The video equipment and its configuration varied slightly depending on species. For experiments on *E. affinis*, we placed a camcorder (Sony-DCR100—1/25 Hz) overlooking the entire volume of the aquarium from the side.

For *P. annandalei*, we placed two orthogonally oriented, black and white, I.R. sensitive CCD cameras (JVC-TK 250U—1/30 Hz) each equipped with a 105-mm Nikon lens overlooking the entire volume of the aquarium from different sides. These cameras were each linked to a camcorder (Sony DCR-SR100—1/30 Hz) serving as a recorder.

The pre- and post-observational image processing was similar for both species. At the beginning of each filming session, a reference grid was recorded to measure the scale of distance. Filming sessions were digitalized and imported into Adobe® Premiere® Pro 2.0 video editing software. The sessions were divided into 5-min sequences to allow the software (Labtrack v.2.1, Bioras, Kvistgård, Denmark) to run more smoothly during the extraction of two-dimensional coordinates representing swimming tracks. Only swimming paths in which individuals were swimming at least three body lengths away from the walls were considered. In addition, all paths had to be away from the surface, or from the bottom.

Three-dimensional swimming patterns of *P. annandalei* individuals were obtained by combining information from the 2D views, to enable the computation of 3D coordinates.

For each experiment, a minimum of 37 trajectories were extracted (the lowest density) (Supplementary material—Appendix 2), and the mean length of all trajectories was approximately 1 min.

Quantifying swimming behavior

Swimming paths can be characterized according to a variety of measures. Despite their scale-dependence, we initially opted for common measures, to provide a frame of reference within which readers could make comparisons with previous studies using similar temporal resolutions.

Swimming speed

The distance d_t (mm) between two points was computed from the *x* and *y* coordinates in 2D space and *x*, *y*, and *z* coordinates in 3D space, respectively, as:

$$d_t = \sqrt{(x_{t+1} - x_t)^2 + (y_{t+1} - y_t)^2} \tag{1}$$

$$d_t = \sqrt{(x_{t+1} - x_t)^2 + (y_{t+1} - y_t)^2 + (z_{t+1} - z_t)^2} \tag{2}$$

where (x_t, y_t, z_t) and $(x_{t+1}, y_{t+1}, z_{t+1})$ are the positions of the copepod at time t and $t + 1$, respectively. The swimming speed V_t (mm s^{-1}) over consecutive tracking intervals was estimated as:

$$V_t = d_t \times p \tag{3}$$

where p is the frame rate of the camera ($P = 25$ and 30 frames s^{-1} for, respectively, *E. affinis* and *P. annandalei*). Average speeds and their SD values were computed over the duration of each individual track.

Net-to-gross displacement ratio

Net-to-gross displacement ratio was computed at the smallest available scale (1/30 s) for each individual track, according to Buskey (1984):

$$NGDR = ND/GD \tag{4}$$

where ND and GD are the net and gross displacements of the copepod, corresponding to the shortest

distance between the start and end points of the trajectory and the actual distance travelled by the copepod, respectively. By definition, NGDR lies between 0 and 1, providing a means to quantify the relative linearity of swimming paths of copepods, with a smaller NGDR (closer to 0) implying a trajectory with higher degree of curve.

Multifractal properties of the copepod displacement

Multifractal analysis has been described in detail in previous research (Schmitt & Seuront, 2001; Seuront et al., 2004c) and recently used in a study by Dur et al. (2010). Briefly, multifractal analysis consists of studying the qth-order structure functions (Monin & Yaglom, 1975) corresponding to the qth-order moments of the norm of displacement: $\Delta X_\tau = X(t + \tau) - X(t)$, based on the hypothesis that the moment of order of the displacement $q > 0$ depends only on the time increment τ. In the case of scaling:

$$\langle \|\Delta X_\tau\|^q \rangle \propto \tau^{\zeta(q)} \tag{5}$$

where $\zeta(q)$ is the exponent function, which is useful for characterizing random walk statistics, for ballistic motion, $\zeta(q) = q$; whereas $\zeta(q) = q/2$ for Brownian motion. In the case where $\zeta(q)$ is non-linear and concave, the motion is considered multifractal (Schmitt & Seuront, 2001). The appropriate range of scale over which the exponents $\zeta(q)$ were estimated was selected according to R^2-SSR criterion (see Seuront et al., 2004c). The exponent function $\zeta(q)$ was considered a scale-independent descriptor regarding the complexity of copepod behavior. It characterized in a scale-invariant manner: (i) the exploration of the volume by the copepod, and (ii) for large and small displacements (i.e., high and low q values, respectively), the means by which this exploration is optimized.

Symbolic analysis

The complex behavior of males was described quantitatively as a sequence of discrete states. We divided the swimming behavior into four states expressed as symbols: (i) sinking (S), when a copepod is not swimming and sinks slowly due to a density slightly greater than that of the surrounding fluid; (ii) breaking (B), when the copepod uses various means to avoid sinking; (iii) cruising (C); and

(iv) jumping (J). This decomposition was based mainly on instantaneous velocity, V_t, resulting in the symbolic series $\delta(t)$ and conducted as follows: if $V_t < 1$ mm s^{-1} then $\delta(t) = $ B; if $1 < V_t < 8$ mm s^{-1} and the individual is moving straight down, then $\delta(t) = $ S; if $1 < V_t < 20$ mm s^{-1} then $\delta(t) = $ C; and if $V_t > 20$ mm s^{-1} then $\delta(t) = $ J.

Subsequently we observed the relative duration in each state and the distribution of time intervals spent in the three main states (here B, C, and S). The symbolic sequence was investigated through: (i) computation of the probability density of the time spent in a given state (A_i) to test the memory of the symbol sequence (see Dur et al., 2010 for details), when such an event occurred, (ii) the conditional probability transition, i.e., the probability (denoted Q_{ij}) of progressing to state A_j when leaving state A_i, with $j \neq i$ (Schmitt et al., 2006; Moison et al., 2009; Dur et al., 2010; Michalec et al., 2010).

Statistical analysis

As the average swimming speed in each trajectory and NGDRs did not have normal distribution (Kolmogorov–Smirnov test, $P < 0.01$), we performed non-parametric statistical analysis, by comparing each condition using the Kruskal–Wallis (KW) test. To identify homogeneous subsets of means, multiple comparisons were conducted using pairwise comparison with Wilcoxon–Mann–Whitney (WMW) test, making corrections to determine whether the post hoc tests were significant according to the Tukey–Kramer method (TK test). To compare 2D and 3D data pairwise, we used the Wilcoxon signed ranks (WSR) test due to the linking of the samples. Probability density functions (pdfs) were compared pairwise using the Kolmogorov–Smirnov two-sample (KS2) test. All statistical analysis was performed using MATLAB V6.1 software (Mathworks Inc.).

Results

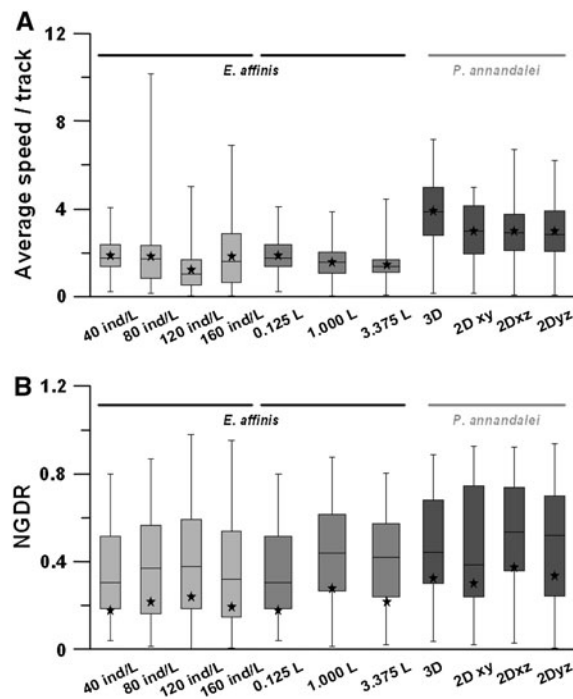Traditional scale-dependent metrics

Effect of density

The smallest value for average speed (i.e., 1.23 mm s^{-1}) was obtained from the data collected

at 120 ind l^{-1}; whereas the highest value (i.e., 1.87 mm s^{-1}) was obtained from the data collected at 40 ind l^{-1} (Fig. 1). The speed maxima were higher than the mean values, between two and nine times the mean value. Among all treatments, the fastest path, with an average speed of 10.15 mm s^{-1}, was recorded at a copepod density of 80 ind l^{-1}. The standard deviation for each density ranged between 0.82 and 1.42 mm s^{-1}. Males travelled, on average, at statistically similar speeds (KW test, $P > 0.01$ followed by TK post hoc test) at 40, 80, and 160 ind l^{-1}. The mean value computed at a density of 120 ind l^{-1} was nevertheless significantly lower than that at other densities (KW tests, $P < 0.01$ followed by TK test).

The instantaneous swimming speeds (i.e., computed at the lowest resolution, 1/30 s) of males in densities of 40, 80, 120, and 160 ind l^{-1} ranged

between (0.0 and 75.0 mm s^{-1}), (0.0 and 60.1 mm s^{-1}), (0.0 and 78.3 mm s^{-1}), and (0.0 and 84.3 mm s^{-1}), respectively. No significant difference was found regarding the frequency of individual swimming speeds ranging between 4 and 30 mm s^{-1} (KS2 test, $P > 0.01$, Fig. 2A). However, significant differences (KS2 test, $P < 0.01$) were found between the frequency of instantaneous swimming speed bounded between 1 and 4 mm s^{-1} and between 30 and 60 mm s^{-1}. A significantly higher proportion of swimming speeds between 1 and 4 mm s^{-1} was observed at a density of 120 ind l^{-1}, while a higher proportion of swimming speed bounded between 30 and 60 mm s^{-1} was found at a density of 80 ind l^{-1}, compared to other conditions.

The NGDRs of male were statistically similar (KW test, $P > 0.01$) under all conditions of density (Fig. 1B). The NGDRs estimated in all conditions ranged between 0.01 and 0.98, averaging 0.37 ± 0.23 ($\bar{x} \pm$ SD). The distribution of NGDR values, bounded between 0.0 and 0.8, did not differ significantly at various densities (KS2 test, $P > 0.05$, Fig. 2B). Nonetheless, a greater proportion of NGDRs above 0.8 were found under all density conditions compared to the lowest density (Fig. 2B).

Effect of volume

An increase in volume resulted in a decrease in mean path speed (Fig 1A) with the lowest value of 1.43 mm s^{-1} for individuals placed in a 3.375-l container. However, no significant difference was found between the smallest and intermediate volume (WMW test, $P > 0.01$ confirmed with TK post hoc test). The average speed per trajectory was nevertheless significantly lower in the larger volume (WMW test, $P < 0.01$ confirmed with TK post hoc test). The faster path, with an average speed of 4.43 mm s^{-1}, was recorded in the larger volume. The speed maxima were only two or three times greater than the mean value. The standard deviation among all volumes ranged between 0.54 and 0.82 mm s^{-1}.

The instantaneous swimming speed for the small, intermediate, and large volumes ranged from 0.0 to 75.0, 0.0 to 80.6, and 0.0 to 79.0 mm s^{-1}, respectively. No significant difference between the three volumes was found for the total range of swimming speed (KS2 test, $P > 0.01$). Nevertheless, higher proportions of instantaneous speed bounded between

Fig. 1 Box and Whisker plot of average speed per track (A) and net-to-gross ratio (B) in the different experimental conditions tested on E. affinis and the different projections of P. annandalei's swimming tracks. The centre horizontal line in the box is the median, the top and the bottom of the box are the 1st and 3rd quartiles, and the extremities of the whiskers are the 5th and 95th percentiles. Mean values for average speed per trajectory and NGDR are also presented (black stars)

Fig. 2 Probability density function of the individual's speed computed at the first scale, $t = 1/30$ s (**A**, **C**, **E**) and frequency distribution of NGDRs (**B**, **D**, **F**). These two metrics' pdf are provided for the experiment on density effect on *E. affinis* (**A**, **B**), the effect of volume on *E. affinis* (**C**, **D**), and the comparison between 3D and 2D data of *P. annandalei* swimming tracks (**E**, **F**)
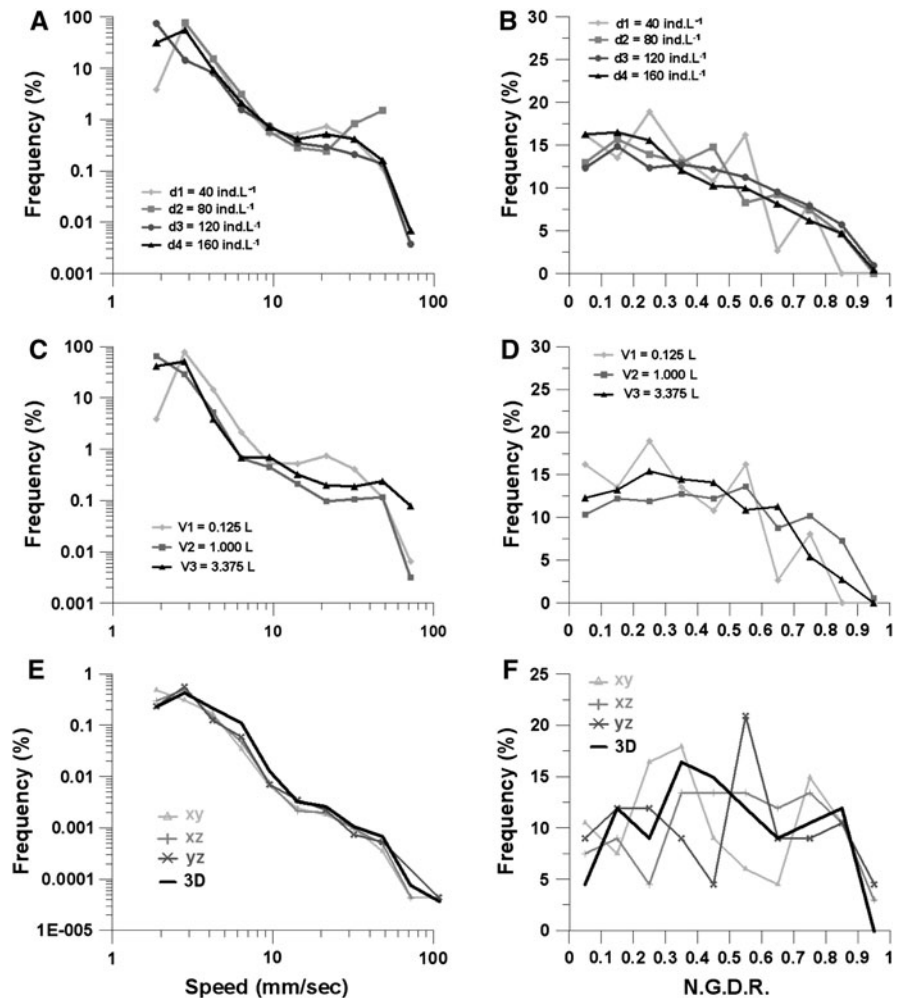
10.0 and 50.0 mm s^{-1} were found for individuals in the smallest volume (KS2 test, $P > 0.01$, Fig. 2C).

The NGDRs obtained in the three volumes were not significantly different (KW test, $P > 0.01$, Fig. 1B). The NGDRs in 1 l ranged between 0.01 and 0.93, averaging 0.43 ± 0.24 ($\bar{x}$ ± SD). The NGDRs in the largest volume were slightly smaller, but not different from that obtained in 1 l (WMW test, $P > 0.01$ confirmed with TK post hoc test), ranging between 0.001 and 0.890, and averaging 0.38 ± 0.22 ($\bar{x}$ ± SD). Trajectories recorded in the smallest volume had NGDR values averaging 0.35 ± 0.22 ($\bar{x}$ ± SD) and ranging between 0.01 and 0.79. A larger proportion of NGDR was found below 0.3 in the smallest volume, which may be associated with the lower average value obtained for that volume. This finding was reinforced by the larger

proportion of NGDRs found above 0.6 in intermediate and larger volumes.

2D/3D comparison

The average speed per trajectory estimated using the 3D data set was significantly higher than those estimated using the 2D data set (WSR test, $P < 0.01$, Fig. 1A), averaging, respectively, 3.88 ± 1.47 and 2.96 ± 1.25 mm s^{-1} ($\bar{x}$ ± SD). The fastest average trajectory speed was estimated at 6.69 and 6.21 mm s^{-1} on the two vertical 2D projections, and reached 7.15 mm s^{-1} in the 3D projection. The instantaneous swimming speeds of males in 2D and 3D exhibited similar ranges of values between 0.00 and 117.4 mm s^{-1} (Fig. 2E). The similarity in the frequency of instantaneous speed measurements

between the two types of projection could not be rejected (KS2 test, $P > 0.01$).

The NGDRs estimated using the 3D projection ranged between 0.03 and 0.88, averaging 0.47 ± 0.24 ($\bar{x} \pm$ SD). When estimated using the 2D projection, the range of NGDRs was larger (Fig. 1B) between 0.01 and 0.94 and averaging 0.48 ± 0.26 ($\bar{x} \pm$ SD). Similarities in NGDR values between the different projections could not be rejected (KW test, $P > 0.01$). Moreover, the distribution of NGDR values could not be differentiated between projections (Fig. 2F, KS2 test, $P > 0.01$).

Multifractal approach

Effect of density

For each condition of density, the scaling of several moments was observed for durations between 0.1 and 2 s at the lowest density; and up to 3 s for higher densities (not shown). Scaling exponents estimated as the least-square power-law slope were non-linear and convex for each density (Fig. 3A), revealing the multifractal nature of *E. affinis* displacement. Comparing densities, scaling exponent values were similar until the fourth order, where the results obtained for 160 ind l^{-1} began separating from the others. For higher order values, scaling exponent at this density tends even more toward Brownian motion compared to other conditions.

Effect of volume

Scaling of several moments was observed for each volume condition for times between 0.1 and 2 s (not shown). For each volume tested, scaling exponent functions, $\zeta(q)$, were non-linear and convex (Fig. 3B). For each condition, the departure from linear (q) was very clear for moments larger than 1.5. Comparing volume treatments, the scaling exponent values are similar until the second order. Over this value, the scaling exponent obtained for the smallest volume started to separate from others tending even more toward Brownian motion.

2D/3D comparison

For the 3D projection, the scaling was verified for a range of scales over one decade, for times between

0.1 and 2 s. The resulting $\zeta(q)$ functions were clearly non-linear and convex (Fig. 3C) compared to the linear behavior expected in the case of normal diffusion ($\zeta(q) = q$). This highlights the multifractal nature of the swimming paths of *P. annandalei*. The departure from linearity was very clear only for moments larger than 2. The multifractal properties of the swimming path of *P. annandalei* were very similar in two and three dimensions (Fig. 3C). Consequently, the swimming behavior of *P. annandalei*'s males can be considered isotropic.

Discretization of speed into symbolic sequences

Effect of density

The relative occurrence of the different swimming states (Fig. 4A) did not differ significantly among conditions of density (KS2 test, $P > 0.01$). A subtle shift in the occurrence of each swimming state was nevertheless observed between the lowest density and other conditions. Males spent more time sinking and cruising at the lowest density than at higher densities. The successive durations spent in each state exhibited intermittent patterns for each density (not shown), resulting in various residence time probabilities (Fig. 5A–C). For all swimming states and all conditions related to density, a clear power-law trend was observed with the form: $p(T) \sim At^{-c}$. The slope (c) was significantly different (Friedman test, $P < 0.05$) between each swimming state (Table 2). However, density did not have a significant effect on swimming behavior (Friedman test, $P > 0.05$). The average value of c for all densities was 2.2, 3.2, and 3.3 for breaking, cruising, and sinking, respectively. Such power-tails indicated many occurrences of long durations spent in a particular state, with larger values occurring much more frequently than expected, in the case of Gaussian or exponential (Poisson) distribution. Moreover, the power-law model was a better fit to the data than any exponential model, indicating the presence of memory in the symbolic time series.

Effect of volume

The relative occurrence of the different swimming states (Fig. 4B) did not significantly differ among conditions of volume (KS2 test, $P > 0.01$). Subtle but not significant shifts in the occurrence of each

Fig. 3 Structure function scaling exponent $\zeta(q)$ for the different tested density (**A**) and volume (**B**) conditions on *E. affinis* and the different projections of *P. annandalei*'s swimming tracks (**C**) compared to results expected in the case of linear motion (*q, dashed line*) and Brownian motion (*q*/2, *dotted line*)
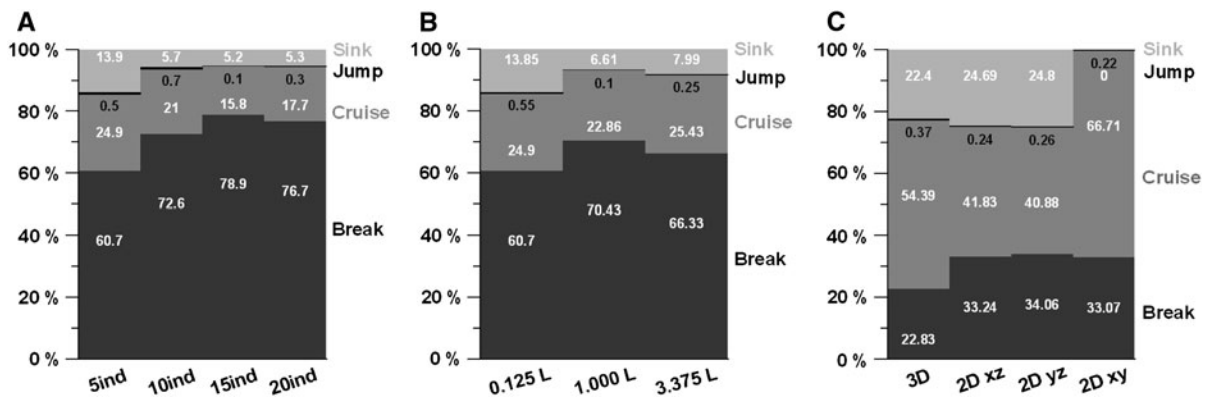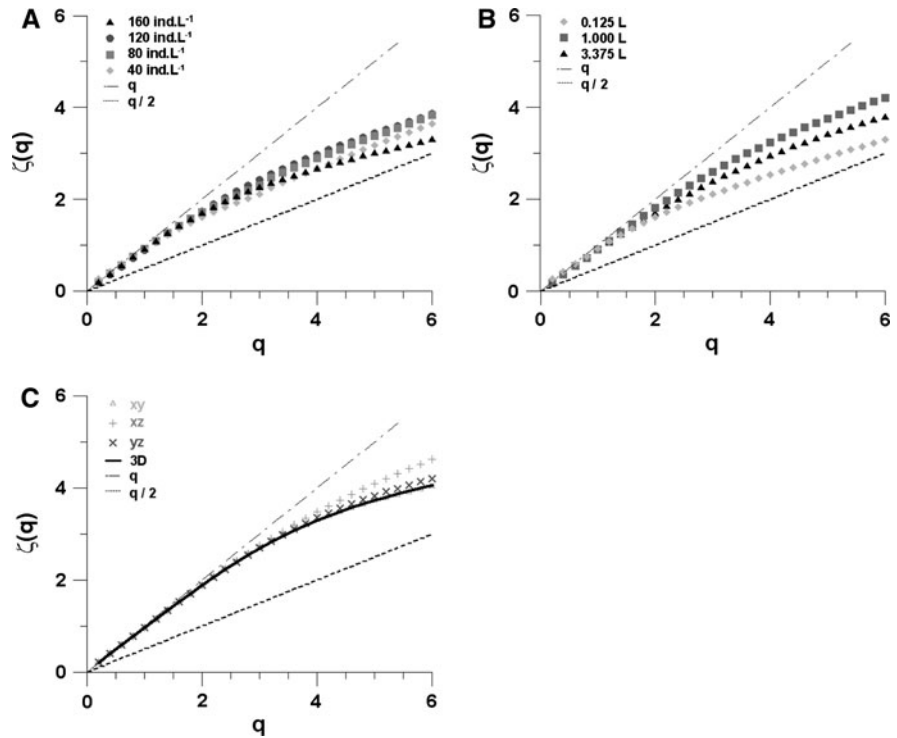
Fig. 4 Relative occurrence (in %) of swimming states break, cruise, jump, and sink for each density tested on *E. affinis* (**A**), each volume tested on *E. affinis* (**B**), and the comparison between 2D and 3D projection of *P. annandalei*'s swimming tracks (**C**)

swimming state were observed between smaller volumes and the two larger ones. Males spent less time sinking in larger volumes and spent more time in the breaking state. We also observed intermittent patterns in the series of successive durations in each state for the three volume conditions, resulting in variations in the probability related to residence time (Fig. 5D–F). For all swimming states and in all tested volumes, a clear power-law trend was observed in the form: $p(T) \sim At^{-c}$. The slopes (*c*) were neither significantly different between each swimming state (Friedman test, $P > 0.05$, Table 2) or between volumes (Friedman test, $P > 0.05$, Table 2). The average values of *c* for each volume were 2.7, 3.9, and 3.7 for breaking, cruising, and sinking, respectively. Again, the power model was a better fit to the data than any exponential model, indicating the presence of memory in the symbolic time series.
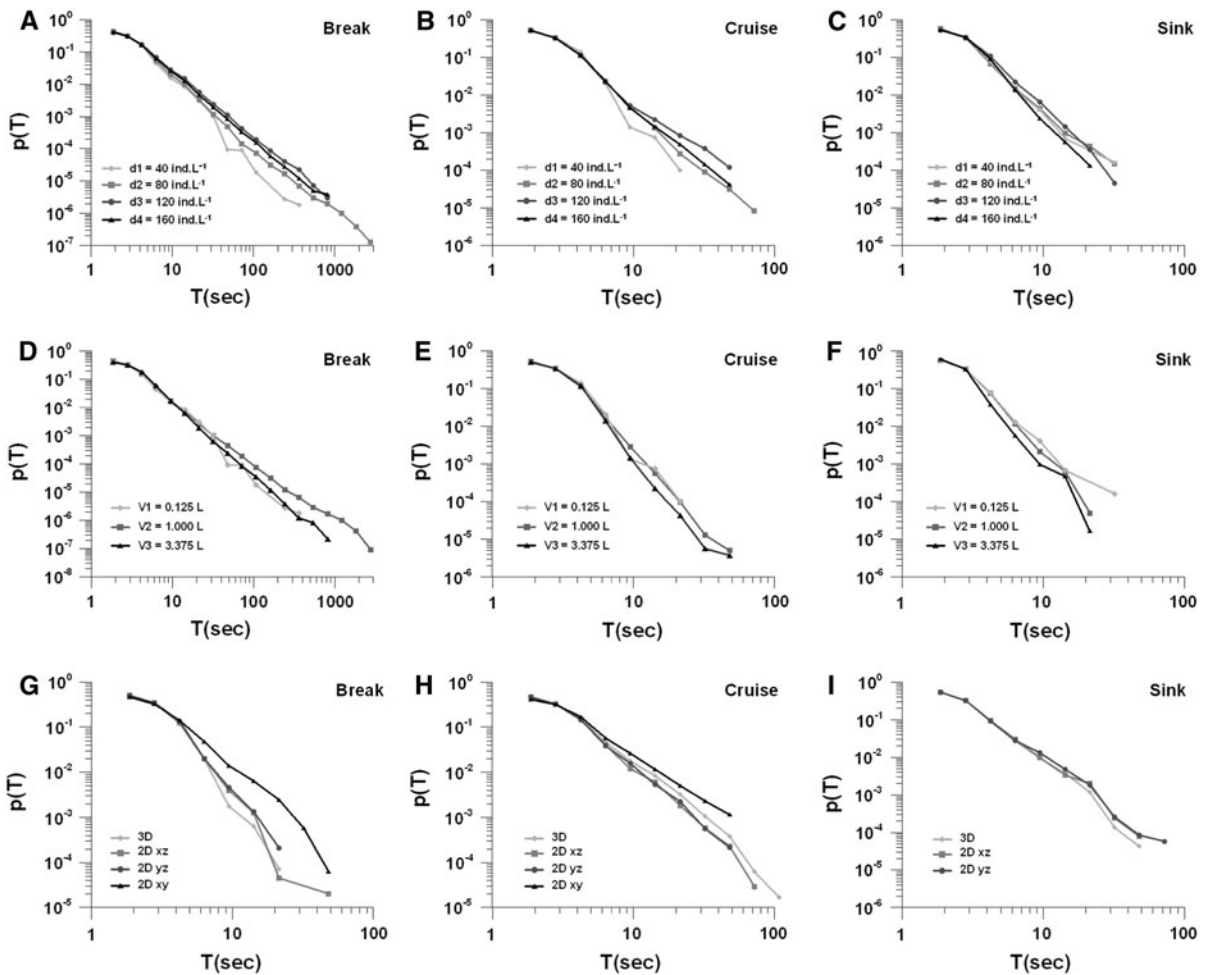
Fig. 5 Log–log plots of the probability densities of residence times in the breaking state (**A**, **D**, **G**), the cruising state (**B**, **E**, **H**), and the sinking state (**C**, **F**, **I**), as estimated from the experimental data of *E. affinis* displacement for the density conditions (**A–C**) and the volume conditions (**D–F**) and the comparison between 2D and 3D data of *P. annandalei* displacement (**G–I**). For graphical reason, the *x*-axes have different ranges between swimming states and studied species

2D/3D comparison

The proportion of the swimming states did not differ when considering either 3D or 2D vertical planes (KS2 test, $P > 0.01$, Fig. 4C). Males spent more than 2/5 of their time cruising and the rest of the time pausing, sinking, and jumping in decreasing proportions. As expected, the time spent in sinking was null on the horizontal plane (i.e., *xy*). The proportion of time spent in that state was distributed between breaking and cruising on that plane. While the increase in the proportion of breaking state was easily understandable, the state of cruising was linked to the partition procedure of the behavior. Even a small displacement on the horizontal plane of 0.1 mm resulted in a speed of 2.7 mm s^{-1}, and such displacements are subsequently classified as cruising.

Disregarding the horizontal plane, the pdf of residence time was similar between 2D and 3D projections for the three swimming states (Fig. 5G–I). However, on the horizontal plane, a higher proportion of long residence times was observed for breaking and cruising. For all swimming states under each of the projections, a clear power-law trend was observed in the form: $p(T) \sim At^{-c}$. Slope (*c*) was not significantly different between each swimming state (Friedman test,

Table 2 Values of A and c parameters of the power-law of the form $p(t) = At^{-c}$ used to fit the probability density function of the residence time in each swimming state for the different tested conditions on *E. affinis* and the different projection of 3D trajectories of *P. annandalei*

Species	Variable	Value	Break	Cruise	Sink
E. affinis	Density	40 ind l^{-1}	1.53–2.6–0.98	2.51–3.7–0.96	1.67–3.1–0.98
		80 ind l^{-1}	0.75–2.1–0.96	1.94–3.2–0.99	1.62–3.1–0.99
		120 ind l^{-1}	0.89–2.0–0.99	1.32–2.7–0.99	2.20–3.3–0.98
		160 ind l^{-1}	0.88–2.0–0.99	1.78–3.1–0.99	2.32–3.6–0.98
		0.125 l	1.53–2.6–0.98	2.51–3.7–0.96	1.67–3.1–0.98
E. affinis	Volume	1.000 l	0.69–2.1–0.99	2.72–3.9–0.98	2.60–3.9–0.98
		3.375 l	1.42–2.5–0.99	2.89–4.1–0.98	2.77–4.2–0.97
P. annandalei	2D/3D	2D xy	2.16–2.8–0.98	0.95–2.0–0.99	/–/–/
		2D xz	2.92–3.7–0.96	1.95–2.8–0.99	1.85–2.9–0.99
		2D yz	2.98–3.7–0.99	1.68–2.6–0.99	1.68–2.7–0.99
		3D	3.68–4.3–0.99	1.98–2.6–0.99	2.21–3.1–0.99

Data are provided per swimming state as follows: $(A - c - r^2)$

$P > 0.05$, Table 2) or between the 3D and vertical 2D projections (Friedman test, $P > 0.05$, Table 2), averaging 3.9, 2.7, and 2.9 for breaking, cruising, and sinking, respectively. As for the two previous experiments, the presence of memory in the symbolic time series was revealed in almost all states and conditions, and the power model fitting the data better than any exponential model.

Analysis of symbolic dynamics

Regardless of density, vessel volume, and projection, the pdf of residence time in each state exhibited a power-law trend, suggesting a non-Markovian model (i.e., a degree of memory remained in the sequence). We, therefore, focused on the probability of going to state A_2 when leaving state A_1 (Table 3).

Effect of density

The importance of swimming activity in the characterization of the behavior of male *E. affinis* was observed in all density conditions. Indeed, the adults mostly cruised, regardless of their initial state. An important proportion was also directed toward the breaking state from the states of swimming and sinking in each condition of density. The probability of transitioning toward the jumping state was minimized in all conditions. Cruising was the strongly preferred transition state after jumping in the lowest

density, with a probability of 90% (Table 3). This pattern changed at 80 ind l^{-1} where males switched to breaking in higher proportions (i.e., 69%). However, it returned to higher proportions for the cruising state at higher densities (Table 3). The shift from breaking tended toward swimming and sinking for all densities, but slightly less toward swimming at the lowest density.

Effect of volume

In the smallest volume, the behavior was characterized by the importance of cruising (Table 3). Males returned consistently to cruising, regardless of the initial state: breaking (60%), sinking (62%), and jumping (90%). As the volume increased, the transition toward cruising from the jumping and cruising state decreased in favor of a transition toward breaking. Moreover, the transition from cruising to breaking rose with an increase in volume (69, 78, and 82% for 0.125, 1, and 3.375 l, respectively).

2D/3D comparison

The behavior of *P. annandalei* is characterized by the importance of cruising, which was the main activity, regardless of the initial state. A large proportion was also directed toward the breaking state from the state of cruising. The probability of transitioning toward the jumping state was the smallest, regardless of the

Table 3 Conditional transition probability in each state between t and $t + 1$ for the four different swimming states of *E. affinis* among the three tested volumes, and of *P. annandalei* for the different projections

Variable/species	Value	A_1 state	A_2 state			
			Break	Cruise	Sink	Jump
Density/*E. affinis*	40 ind 1^{-1}	Break	–	0.598	0.392	0.010
		Cruise	0.688	–	0.294	0.017
		Sink	0.372	0.624	–	0.004
		Jump	0.080	0.904	0.015	–
	80 ind 1^{-1}	Break	–	0.758	0.207	0.034
		Cruise	0.779	–	0.211	0.010
		Sink	0.477	0.520	–	0.030
		Jump	0.693	0.290	0.017	–
	120 ind 1^{-1}	Break	–	0.733	0.258	0.009
		Cruise	0.690	–	0.304	0.005
		Sink	0.486	0.509	–	0.004
		Jump	0.454	0.495	0.051	–
	160 ind 1^{-1}	Break	–	0.753	0.234	0.013
		Cruise	0.716	–	0.272	0.012
		Sink	0.458	0.538	–	0.004
		Jump	0.278	0.691	0.031	–
Volume/*E. affinis*	0.125 1	Break	–	0.598	0.392	0.010
		Cruise	0.688	–	0.294	0.017
		Sink	0.372	0.624	–	0.004
		Jump	0.080	0.904	0.015	–
	1.000 1	Break	–	0.795	0.199	0.005
		Cruise	0.783	–	0.215	0.001
		Sink	0.443	0.556	–	0.001
		Jump	0.524	0.408	0.067	–
	3.375 1	Break	–	0.758	0.233	0.009
		Cruise	0.810	–	0.181	0.003
		Sink	0.560	0.439	–	0.004
		Jump	0.575	0.357	0.068	–
2D/3D/*P. annandalei*	2D *xy*	Break	–	0.998	–	0.001
		Cruise	0.987	–	–	0.012
		Sink	–	–	–	–
		Jump	0.029	0.970	–	–
	2D *xz*	Break	–	0.565	0.433	0.001
		Cruise	0.551	–	0.442	0.010
		Sink	0.494	0.502	–	0.003
		Jump	0.055	0.833	0.111	–
	2D *yz*	Break	–	0.546	0.452	0.001
		Cruise	0.553	–	0.438	0.009
		Sink	0.524	0.472	–	0.003
		Jump	0.026	0.857	0.117	–
	3D	Break	–	0.595	0.403	0.001
		Cruise	0.408	–	0.579	0.013
		Sink	0.333	0.663	–	0.004
		Jump	0.038	0.877	0.085	–

The table should be read from the left to right (e.g., for a density of 40 ind 1^{-1} an individual in the break state at time t will switch to the Cruise state with a proportion of 0.6 at the following time step $t + 1$)

initial state. Although we did not consider the sinking state from the horizontal plane, the patterns were similar regardless of the projection. The results differed only slightly between different projections when considering departure from the sinking state. The results using 3D data show that cruising was the preferred transition state after sinking, with a proportion of approximately 60% compared to 40% for the switch toward breaking (Table 3). The latter was observed in higher proportions when considering vertical two-dimensional planes (Table 3), giving a more or less equal switch from sinking to cruising and sinking to breaking.

Discussion

Understanding the relative importance and consequences of experimental conditions is important when designing a protocol or trying to compare results among studies carried out under different conditions.

Our results first showed that variation in density had little effect on the swimming behavior of *E. affinis* males. We found no significant relationship between copepod density and swimming speed or the complexity of trajectory (NGDR), as previously observed by Buskey et al. (1996) who found no correlation between the density of copepods in the center of a swarm and individual behavior. Furthermore, when placed in vessels of similar size, copepods appeared to use nearly the same amount of space indicated by the structure function, regardless of density, until a threshold value was exceeded. The long displacements of *E. affinis* males were more confined to the conditions of highest density, possibly suggesting that the expansion of the exploration area may be constrained by spaces of higher density. Limitations to the movement of *E. affinis* males could be related to physical barriers created by the presence of other individuals in their paths. The constraining effect of density on movement and the use of space has been documented in other animals such as chickens (Estevez & Christman, 2006), cattle (Kondo et al., 1989), deer (Pollard & Littlejohn, 1996), and sheep (Arnold & Maller, 1985). In our study, this restriction occurred even at a density of 160 ind l^{-1}, which was well below the standard of density in the field and in aquaculture (Cheng et al., 2002). Indeed,

in the Seine estuary, *E. affinis* populations (all developmental stages) often reach densities above 500 ind l^{-1} during the spring and summer (Mouny & Dauvin, 2002; Devreker et al., 2008). Therefore, further investigation of high densities would be needed to confirm such patterns. The study of higher densities would require superior video and optical techniques to track such high number of trajectories at the same time. At a constant density, the likelihood of encountering an obstacle (in the form of another copepod) as an individual moves would be similar, regardless of the size of the vessel, which should equate to a null response to confinement. However, the introduction of copepods into smaller volumes would result in an increase in the complexity of long displacements. Both the scale-dependant metric (i.e., NGDR) and the scale-independent metric (i.e., structure function) highlighted this in our study. These results may be caused by different mechanisms. When more space is available, a copepod may be able to expand its search area to minimize competition for resources, or may have more opportunities to explore and look for resources within a larger volume. However, neither scale-dependent nor scale-independent metrics differed when in our comparisons between vessels larger than 1 l. This suggests that copepods adapt their space use patterns to the size of the vessel within certain limits. An increase in behavioral complexity in small volume may also be a reflection of a higher level of activity related to higher temperature (24 vs. 20°C for the other conditions), a factor known to influence the swimming behavior of crustaceans (Lindström & Fortelius, 2001). Results from symbolic analysis indicate a higher level of activity in experimental conditions 40 ind l^{-1}/0.125 l. In this condition, a greater proportion of copepods exhibited cruising and sinking activities. The proportion of swimming and transitional behavior between states was similar among all densities as well as between intermediate and large volumes.

Our findings suggest that both vessel size and density may have different but equally important influence on behavior. In contrast, the effect of increasing population size was less evident for *E. affinis* males, at least under the conditions in this particular study. Investigations on the characteristics of temporal motion patterns (Tiselius & Jonsson, 1990) and the changes induced by environmental

conditions contribute to a better understanding of the perception that copepods have of their environment.

Improvement of experimental design

Similar protocols combining experimental design and numerical analysis should be used to study behavioral sequences intra- and inter-individuals, both within and between species if comparisons are to be objective. First and foremost is a need to establish reference conditions for the investigation and characterization of the behavior of copepods of similar size (~ 1 mm). The intermediate volume used in these experiments provided sufficient space for the copepods to move around at the lowest density (i.e., 40 ind l^{-1}). However, density in the field varies with the seasons (Uye & Kano, 1998; David et al., 2005) as does the behavior of copepods (Moison, 2009). The observed behavior should only be associated with the field behavior of an individual at a given time. An important issue in experimental design is the selection of 2D or 3D observation. The multifractal analysis revealed a lack of clearly defined differential modes of swimming behavior of *P. annandalei* males in the horizontal and vertical dimensions, with the exception of sinking behavior. The same results were obtained for *Temora longicornis* females (Seuront et al., 2004c). Given the isotropic conditions, we could compare the average 3D velocity with that estimated for two dimensions. This gave a 24% higher 3D value, similar to the values estimated by Kiørboe & Bagøien (2005) making it possible to extrapolate 2D to 3D values simply by increasing the former by 22% or $\sqrt{3/2}$ (a value derived from the fact that a 3D increment is estimated from 3 components and a 2D increment from 2). Nevertheless, even if isotropy is respected for males, it will not necessarily be the same for females considering the factors of sex dependence (VanDuren & Videler, 1996; Dur et al., 2010; Michalec et al., 2010) and stage dependence (VanDuren & Videler, 1995) in swimming behavior. For a more comprehensive and reliable estimation of average speed, three-dimensional data are, therefore, preferred. However, 2D horizontal projections provide a good estimate of the speed probability function which can be used for highlighting the effects of factors such as salinity on swimming behavior (Michalec et al., 2010), and even differences between

reproductive stages (Dur et al., 2010). Finally, the characterization of behavior intended for integration in a model should be conducted on 3D data. Indeed, the results from our symbolic analysis carried out on 2D projections underestimated the importance of the cruising state.

Conclusion

In summary, the goal of this study was to separate, to the greatest extent possible, the relative effects of density and vessel size on movement and use of space. Our results showed that movement patterns in the temperate estuarine species are independent of vessel size and density to a limited degree. Copepods usually explore the entire volume. Second, we compared the results of 2D and 3D observational techniques used for swimming copepods and concluded that, whenever possible, 3D is preferable. 3D observation provides more information on aspects of swimming behavior such as isotropic conditions; however, 3D observation requires a more complex setup. Provided that a suitable high number of trajectories are recorded, 2D observation provides statistically reliable information on velocity. Therefore, 2D is sufficient for routine investigations regarding the effects of given factors.

Our results contribute to the body of knowledge concerning the behavior of copepods, making an important contribution to future researchers desiring to interpret and compare results from previous studies. The experimental parameters and processes we developed here fulfill the requirements for experimental consistency and repeatability. With the huge diversity of copepod species, it would not be surprising to discover that different species are influenced differently by the experimental conditions. In addition, density and volume are not the only possible sources of errors. The duration of acclimation, the use of copepods from culture or from the field, and the type of light source may also induce changes in copepod behavior and, therefore, need to be thoroughly investigated. Clearly these investigations highlight the need for a common protocol to conduct the inter-species comparisons required to elucidate the key factors driving the huge diversity of behavior displayed among species.

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