Analyzing movement behavior of zebrafish in different sized confined areas

Quach Kha Quang[1], Nguyen Quoc Anh[2]

[1]Office of Information and Communications, Dong Thap University, Cao Lanh, Viet Nam

[2]Office of International Affairs, Dong Thap University, Cao Lanh, Viet Nam

[*]Emails: qkquang@dthu.edu.vn

Abstract. Movement behavior of zebrafish (*Danio rerio*) was analysed according to different sizes of observation arena (four sizes: $5 \times 5 \text{ cm}^2$, $10 \times 10 \text{ cm}^2$, $15 \times 15 \text{ cm}^2$ and $30 \times 30 \text{ cm}^2$). The observation arena was separated into corner, boundary and central areas based on experimental data. The results showed that the shape of corner, boundary and central areas were accordingly different in different size of arena. The movement parameters (speeds, accelerations…) of individual were variable at different areas across different size. However, TPMs of moving between different areas in the observation are stable. These findings imply that there is a stereotypic inner state that maintains basic behaviors in animals. Information from this work would provide backgrounds of real-life process mechanism and would be useful for monitoring in response to environmental changes in practical aspect and be applicable to wide fields including pharmacological, neurological, and genetic fields.

Keywords: Confined areas, Monitoring, Movement behavior, Zebrafish (*Danio rerio*).

Classification numbers: 3.2.3, 4.8.2, 4.8.5.

1. INTRODUCTION

Response behavior of indicator animals has garnered a special attention in risk assessment in ecosystems, filling the gap between macro-scale (e.g., community structure) and micro-scale (e.g., molecular response) measurements [1, 2]. Behavioral monitoring could be conducted on the real time basis without much demanding observation efforts and facilities. Observation in small arena became increasingly important especially regarding assessment of behavioral responses to stimuli including toxic chemicals and drugs recently in experimental conditions [3, 4]. David Eilam [5] observed rodents on successive days under increasing, decreasing, or randomly changing arena size and found that locomotor behavior was adjusted to arena size by: (i) preserving the same level of activity, (ii) taking longer but less frequent trips in smaller arenas in contrast to taking more frequent yet shorter trips in the larger arenas, and (iii) moving in the entire space available for exploration in the smaller arenas in contrast to remaining along the walls of the open field in the larger arenas. The effect of testing order was minimal, probably being related to increased novelty under increasing arena size, as opposed to habituation under decreasing arena size, when parts of the same area were re-explored. To better understand fish

novelty behavior, Stewart *et al.* [6] exposed adult zebrafish to two different open field arenas for 30 min, assessing the amount and temporal patterning of their exploration. While (similar to rodents) zebrafish scale their locomotory activity depending on the size of the tank, the temporal patterning of their activity was independent of arena size. These observations strikingly parallel similar rodent behaviors, suggesting that spatio-temporal strategies of animal exploration may be evolutionarily conserved across vertebrate species. A similar impact of arena geometry was observed for travel away from the arena walls. Indeed, when the rats abandoned the arena walls to crosscut through the center of the arena, their center paths were circuitous in the round arena and relatively straight in the square arena. Osnat Yaski *et al.* [7] suggested that the shapes of these paths are exploited for the same spatial task: returning back to a familiar location in the unsighted environment.

The boundary zone, in which free movement would be minimally allowed, was considered important in describing behavior profiles of animals [8, 9]. The movement patterns at the boundary were investigated with Zebrafish (*Dario rerio*) in a small-size observation arena by defining one body length of fish (40 mm) in the horizontal direction (i.e., side walls) and one body height (20 mm) in the vertical direction (i.e., top or bottom) of aquarium [10]. Recently, Quach *et al.* [11] reported that behaviors of zebrafish were different according to areas observation arena. Intermittencies, defined as the probability distributions of the shadowing time during which data were above a pre-determined threshold, were different according to the center and boundary areas and between one- and two-individual groups. However, movement behaviors across in different sizes have not been specifically reported. In this study, we focused on how behavior would differentiate across different size of observation arena ($5 \times 5 \, \text{cm}^2$, $10 \times 10 \, \text{cm}^2$, $15 \times 15 \, \text{cm}^2$ and $30 \times 30 \, \text{cm}^2$) in relation to position specific behaviors at the boundary, corner and central areas from movement tracks.

2. MATERIALS AND METHODS

2.1. Test organisms

Zebrafish were obtained from a local fish dealer for stock population (300 individuals) and were reared for $2 - 4$ weeks before observation at a temperature of $25 \pm 1°C$ and pH of 7.1 ± 0.3 under a light/dark cycle of 14/10 h (light on at 7:00 and at 20:00) with two fluorescent lights (26 J/s) 50 cm above the rearing container [11]. Fishes were fed dry food (Nutron Hi − Fi, PRODAC) twice a day (once a day on weekends).

Males (ages: $5 \sim 6$ months; body lengths: $30 \sim 40$ mm) were randomly chosen from the stock population and were placed square-shaped area in different size: 5×5 cm^2, 10×10 cm^2, 15×15 cm^2, 30×30 cm^2, where water depth for all sizes was 5 cm [12]. Before observation, organisms were acclimated to the observation system for 30 minutes. Food and oxygen were not supplied to the area during the observation period while noise was minimized to simplify observation conditions. Other rearing and observation conditions were the same as those used to rear the stock population.

2.2. Observation and recording

The recording system consisted of an observation aquarium, camera (Logitech®VidTMHD), and PC computer (Intel® Core™ 2 Duo CPU E4500@ 2.20GHz). Software for tracking the motion of multiple individuals was developed at the Ecosystem and Behavior Laboratory at

Pusan National University ("Multrack"). The coordinates of each individual was continuously recorded at 30 frames per second from a top view in two dimensions [9, 11]. For each area size, we performed 20 replications; each replication was recorded in 40 minutes.

2.3. Determination of boundary and corner

$$f\left(x,\mu,\sigma\right)=\frac{1}{\sigma\sqrt{2\pi}}e^{-\frac{(x-\mu)^2}{2\sigma^2}} \tag{1}$$
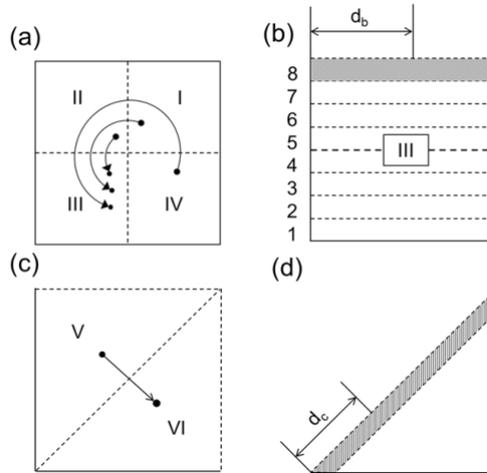
Figure 1. The folding of area space in order to accumulate position data of zebra fish in a confined area. The Roman numerals (I, II, III…) indicate the subareas.
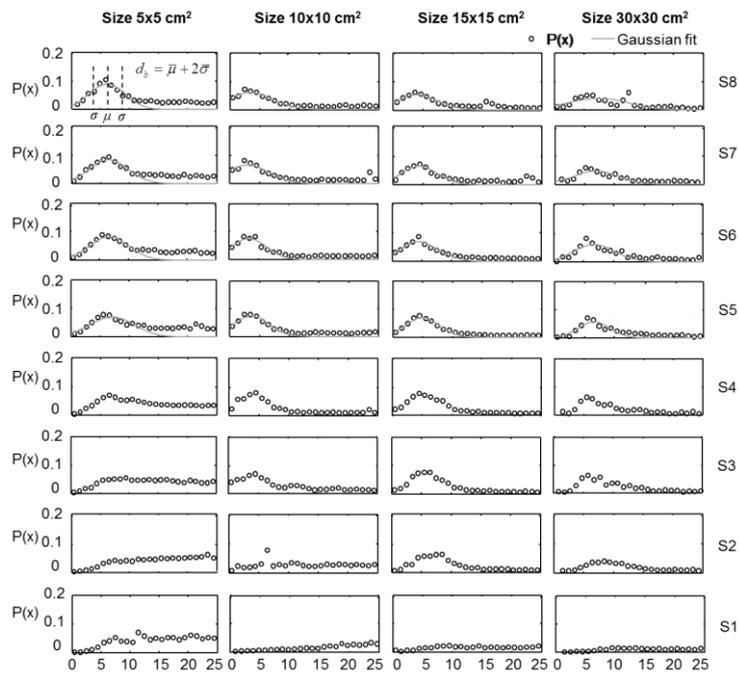
Figure 2. Distribution of points in each section poisoned from center to boundary in eight divisions with Gaussian fitting.

1082

Behaviors according to boundary, corner and center, in the arena, we checked distribution shapes of fish positions during the observation time. In order to have sufficient amount of data for distribution, we suppose distribution pattern of position data of zebrafish would be symmetric, and the tracking data were accumulated for combining the subareas as shown in Figure 1. Initially the area was folded two times to produce four subareas, I, II, III, and IV (the arrows shown in Figure 1a). The areas of I, II, and IV were overlapped on the area III. The position data in quarters I, II and IV were rotated counterclockwise around the center point with rotation angles π, $\pi/2$ and $3\pi/2$ respectively, and data on these quarters were superimposed on data existed in the quarter III. The folded area was subsequently divided into 8 evenly-spaced strips from bottom to top in the area III (Figure 1b). Consequently the strip 1 presented the area closest to the boundary whereas the strip 8 is nearest to the middle zone. The data distributions along the horizontal direction the 8 strips were fitted to with Gaussian distribution function (Figure 2).

Where μ and σ are the mean and standard deviation of distribution, respectively. The parameters μ and σ were calculated separately for each section. The boundary was determined by average of the four-upper sections plus two times of standard deviation, that is $d_b = \bar{\mu} + 2\bar{\sigma}$.

In order to define the corner zone, the overlapped data in the quarter III were further divided into two triangle parts (V and VI in Figures 1c and 1d). The data in triangle V were superimposed on the triangle VI by assuming symmetry along the diagonal line of quarter III (Figure 1c): if $y \geq x$, $(x, y) \rightarrow (y, x)$. Then, all data points in the area were accumulated in the triangle VI as shown in Figure 1d. Finally the data distribution was fitted again to Gaussian distribution function to determine distance from the corner along the diagonal line as

$$d_c = \frac{\mu + 2\sigma}{\sqrt{2}} \quad .$$

The defined areas of center, boundary and corner are presented in Figure 3a. The size of center area was variable depending upon area size, larger with the increase in area size (Figure 3b).

Size	5x5 cm²	10x10 cm²	15x15 cm²	30x30 cm²
Corner	20.8 mm	21.9 mm	19.9 mm	26.6 mm
Boundary	13.4 mm	10.1 mm	10.8 mm	14.9 mm
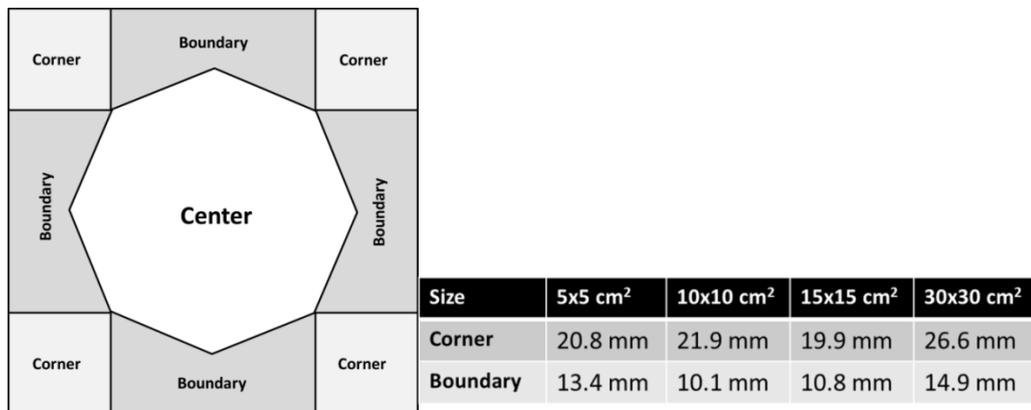
Figure 3. (a) Visualization of center, boundary and corner, (b) values of the corner and the boundary of four sizes obtained by fitting the accumulated distribution data with the Gaussian function.

The center area was outstandingly small with the minimum size 5×5 cm². Hereafter the surface area (e.g., 5×5 cm²) is used to present the size of the area since the depth (5 cm) is equal to all sizes. However, the size of corner area was somewhat conservative, ranging invariably from 19.9 mm to 20.8 mm with the smaller sizes 5×5 cm², 10×10 cm² and 15×15

cm^2. At the maximum size, 30×30 cm^2, however, the corner size somewhat increased to 26.6 mm. The boundary size was variable, being divided into two groups: smaller in the intermediate size showing 10.1 mm and 10.8 mm, and larger in the maximum or minimum size with 13.4 mm and 14.9 mm, respectively. It was noteworthy that the boundary for the minimum size 5×5 cm^2, was relatively broader, comparing with that for intermediate area size, 10×10 cm^2 (10.1 mm) and 15×15 cm^2 (10.8 mm) (Figure 3b). The observation suggested that areas in a confined area caused specific behaviors of zebra fish.

3. RESULTS

3.1. The spatial distribution of movement
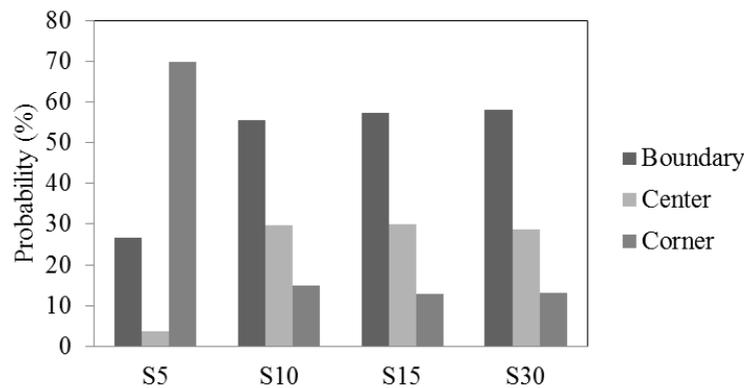
Figure 4. Probability of individuals stay at boundary, corner and center areas in different size: 5×5 cm^2 (S5), 10×10 cm^2 (S10), 15×15 cm^2 (S15) and 30×30 cm^2 (S30) of aquarium.

The probabilities of positions at different areas are shown in Figure 4 across different area size (5×5 cm^2, 10×10 cm^2, 15×15 cm^2 and 30×30 cm^2). In minimal size 5×5 cm^2 the probabilities were differentiated from other larger size areas. The individuals in the minimum size tended to stay in the corner area longer (approximately 70 % of observation time). With the larger sizes (size 10×10 cm^2, 15×15 cm^2 and 30×30 cm^2), behaviors were similar. Individuals stayed at boundary zone for a substantial proportion (approximately 55 - 58 %) of the observation period while at the corner zone they stayed for the shortest time period (approximately 12 - 14 %). Overall probability at the boundary was substantially higher than those at the areas except the smallest size.

3.3. Linear speed and acceleration

The average speeds of zebrafish varied across area and size of observation area (Figure 5, gray bar). The average speeds were overall higher in size 15×15 cm^2 showing the highest value (50.43 ± 8.94 mm/s), comparing with either smaller size 5×5 cm^2 (32.41 ± 5.97 mm/s) and 10×10 cm^2 (31.60 ± 5.30 mm/s) or larger size 30×30 cm^2 (33.58 ± 10.11 mm/s). Statistical significances were differently observed as showed between different sizes (P < 0.05). The average accelerations of zebrafish were shown in Fig. 5 (black bar) and somewhat different from speed. Comparing with smaller sizes (5×5 cm^2 (290.54 ± 66.87 mm/s^2), 10×10 cm^2 (205.14 ± 58.33 mm/s^2) and 15×15 cm^2 (387.06 ± 126.13 mm/s^2)), the average acceleration of

size $30 \times 30 \text{cm}^2$ was outstandingly higher in the largest size (622.18 ± 295.52 mm/s^2) (P < 0.05). It is noteworthy that speed was highest in the size of 15×15 cm^2 whereas acceleration was highest in the size of 30×30 cm^2. These reveal that the movement behaviors of zebrafish were effected by different sizes of arenas, in the larger size they move actively than smaller sizes
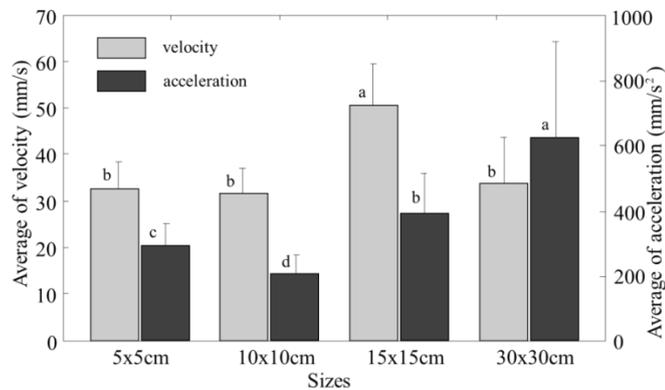
Figure 5. Average of speed (gray shape) and acceleration (black shape) of Zebrafish in different size (Vertical bars indicating standard deviation). Different alphabets indicate statistical significances difference among different size, separately in velocity and acceleration (Tukey test: P < 0.01).

In this study site specific behaviors were investigated in relation with area and size in observation arena. Behaviors of individuals were observed according to boundary, corner and center, in the arena. Average speed in the boundary zones and center zones separated significantly between larger sizes and smaller sizes, but at the corner zones average speed not seem to differ between different sizes (Figure 6). In large size, 15×15 cm^2 and 30×30 cm^2 difference was observed whereas not much difference was found at small size of arena. The speed in the center in 15×15 cm^2 (49.54 ± 9.78 mm/s) was higher than in 30×30 cm^2 (28.31 ± 9.45 mm/s) (Tukey test, P < 0.01). Regarding to areas, speed was overall high in the boundary followed by the center and corner. Variability among different size was larger at the boundary and center whereas not much variability was found at small size of area.
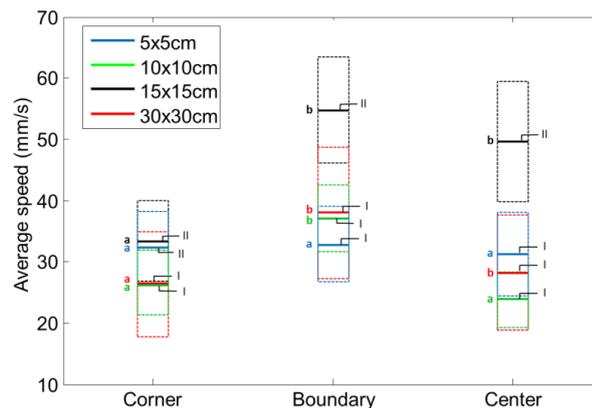
Figure 6. Average speed accompanying to different areas and different size. Different colors indicate different sizes. Solid lines showed average speed values. Dash line indicates standard deviation of speed. Alphabets indicated statistical significances between different areas (Tukey test, P < 0.05). Roman number indicated statistical significances between different sizes (Tukey test, P < 0.05).

3.2. Transition probability matrix (TPM)

Probabilities of moving between different areas in the observation arena are showed by TPMs (Figure 7a). Generally TPMs were similar and probabilities on the diagonal line were substantially high across different arena size, indicating high probability of remaining the same pattern. However, slight differentiation in probabilities was also observed according to arena size. As the size increased, the probabilities in the peripheral area tended to increase. As the size decreased asymmetry in probabilities in the peripheral increased, showing maximum in $5 \times 5 \text{cm}^2$, and indicating higher chance of changing to different patterns.
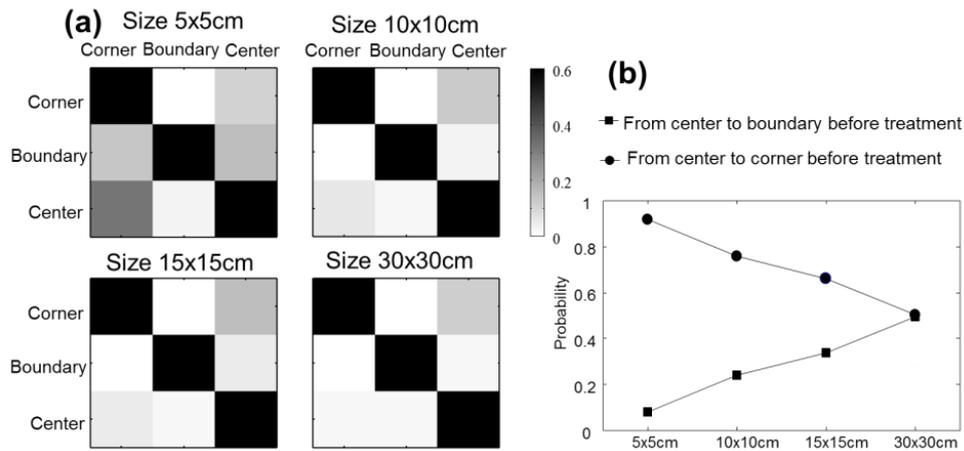
Figure 7. (a) Transition probability from across different areas in observation arena in different size and (b) probability of moving from center to either boundary or corner in different arena size.

The transition probability from center to corner area was markedly higher but the transition probability from corner to center area was lower. With the maximum size, $30 \times 30 \text{cm}^2$ (Figure 7a) diagonal lines more clearly appeared. Only the probability "corner to center" was somewhat higher, which is understandable with large size of arena, meaning higher chance of moving from corner to center.

As the arena size increased, transition probability from center to boundary increased linearly, whereas the probability from center to corner decreased in a similar manner (Figure 7b). In the minimum arena size probabilities from center to corner was substantially high, which is understandable since corner area is broad in the minimum size. In the arena size $30 \times 30 \text{ cm}^2$, however, transition probabilities from "center to corner" and "center to boundary" were similar. This indicated that, in the size $30 \times 30 \text{ cm}^2$, individuals had an even chance of choosing corner and boundary from the center. This further suggested that test organisms may prefer to stay in the corner as the size increased, considering that corner area is substantially narrower comparing with the boundary area in the maximum size of arena.

4. CONCLUSIONS

The movement behaviors of zebrafish were affected by different sizes of arenas, in the larger size they move actively than smaller size. This study also showed that fish behavior was more sensitive at the boundary and corner areas as size small. The behavioral parameters

1086

indicated that the arena size had a significant effect on behavioral states of Zebrafish. Parameters describing the movement of individuals were variable and TPMs of moving between different areas in the observation are stable. These findings imply that there is a stereotypic inner state that maintains basic behaviors in animals.

CRediT authorship contribution statement: Quach Kha Quang: Methodology, investigation, formal analysis, writing-review & editing*; Nguyen Quoc Anh:* Methodology, analysis.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Brodin T., Nordling J., Lagesson A., Klaminder J., Hellström G., Christensen B., and Fick J. - Environmental relevant levels of a benzodiazepine (oxazepam) alters important behavioral traits in a common planktivorous fish, (Rutilus rutilus), Journal of Toxicology and Environmental Health, Part A **80** (16-18) (2017) 963-970. https://doi.org/10.1080/15287394.2017.1352214

2. Saaristo M., Brodin T., Balshine S., Bertram M. G., Brooks B. W., Ehlman S. M., McCallum E. S., Sih A., Sundin J., Wong B. B., and Arnold K. E. - Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife, Proceedings of the Royal Society B **285** (1885) (2018) 20181297. https://doi.org/10.1016/j.ecoenv.2018.01.057

3. Araujo-Silva H., Pinheiro-da-Silva J., Silva P. F., and Luchiari A. C. - Individual differences in response to alcohol exposure in zebrafish (Danio rerio), PLoS One **13** (6) (2018) e0198856. https://doi.org/10.1371/journal.pone.0198856.

4. Severo E. S., Marins A. T., Cerezer C., Costa D., Nunes M., Prestes Zanella R., and Loro V. L. - Ecological risk of pesticide contamination in a Brazilian river located near a rural area: a study of biomarkers using zebrafish embryos, Ecotoxicology and environmental safety **190** (2020) 110071. https://doi.org/10.1016/j.ecoenv.2019.110071

5. Eilam D. - Open-field behavior withstands drastic changes in area size, Behavioural Brain Research **142** (1-2) (2003) 53-62. https://doi.org/10.1016/S0166-4328 (02)00382-0.

6. Stewart A. M., Gaikwad S., Kyzar E., and Kalueff A. V. - Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test, Brain research **1451** (2012) 44-52. https://doi.org/10.1016/j.brainres.2012.02.064

7. Yaski O., Portugali J., and Eilam D - Arena geometry and path shape: When rats travel in straight or in circuitous paths?, Behavioural brain research **225** (2) (2011) 449-454. https://doi.org/10.1016/j.bbr.2011.07.055

8. Jeanson R., Blanco S., Fournier R., Deneubourg J. L., Fourcassié V., and Theraulaz G. - A model of animal movements in a bounded space, Journal of Theoretical Biology **225** (2003a) 443-451. https://doi.org/10.1016/S0022-5193(03)00277-7.

9. Braun C. D., Gaube P., Afonso P., Fontes J., Skomal G. B., and Thorrold S. R. - Assimilating electronic tagging, oceanographic modelling, and fisheries data to estimate movements and connectivity of swordfish in the North Atlantic, ICES Journal of Marine Science **76** (7) (2019), 2305-2317. https://doi.org/10.1093/icesjms/fsz106

10. Liu Y., Chon T. S., and Lee S. H. - Analysis of behavioral changes of zebrafish (Danio rerio) in response to formaldehyde using Self-organizing map and a hidden Markov model, Ecological Modelling **222** (14) (2011) 2191-2201. https://doi.org/10.1016/j.ecolmodel.2011.02.010.

11. Quach Q. K., Chon T. S., Kim H., and Nguyen T. V. - One and two-individual movements of fish after chemical exposure, Journal of the Korean Physical Society **63** (1) (2013) 18-27. https://doi.org/10.3938/jkps.63.18.

12. Katz Y., Tunstrøm K., Ioannou C. C., Huepe C., and Couzin I. D. - Inferring the structure and dynamics of interactions in schooling fish, Proceedings of the National Academy of Sciences **108** (46) (2011) 18720-18725. https://doi.org/10.1073/pnas.1107583108.