Ecological Informatics 29 (2015) 119–129

Contents lists available at ScienceDirect

Ecological Informatics

journal homepage: www.elsevier.com/locate/ecolinf

Inferring conflicting behavior of zebrafish (*Danio rerio*) in response to food and predator based on a self-organizing map (SOM) and intermittency test

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ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form 7 October 2014
Accepted 17 October 2014
Available online 6 November 2014

Keywords:
Distribution shape
Monitoring
Movement
Response behavior
Stimuli

ABSTRACT

We observed zebrafish (*Danio rerio*) movement continuously in response to conflicting stimuli (food and predator). Linear parameters (i.e., speed, acceleration, locomotory rate) were lower when food was provided to the arena, whereas angular parameters (i.e., turning rate) decreased in response to predator. Self-organizing map (SOM) trained with parameters revealed that stop duration, stop number, and *x*- and *y*-coordinates played major roles in determining variations in movement. Behavior patterns (e.g., rightward movement to food) were identifiable in different clusters in association with parameters responding to sources and order of stimuli. Distribution shapes obtained from the SOM were suitable for identifying responses of test organisms. Acceleration intermittency in movement data in different distribution shapes addressed data structure variation caused by sources and order of stimuli. The order of stimuli was influential in determining the behavioral states in the following courses of stimulus provision (i.e., addition of secondary stimulus and removal of the initial stimulus). Predator-first stimuli caused stronger response behaviors than food-first stimuli. Computational analysis of response behavior to stimuli could be an additional indicator of stress in animals.

1. Introduction

Animals in the wild often face a conflict between starvation and predation risk through their daily life events. Since starvation and predation both put strong selective forces on animals, their behaviors should be finely adapted to make the correct choice at the right moment, and the decision to approach food or escape would be critical for their survival in relation with surrounding conditions (Burns and Rodd, 2008). Behaviors under such conflicting situations have been empirically and theoretically investigated in numerous taxa (Lima, 1998a). Hunger often causes animals to accept a greater risk to obtain food (Lima, 1998b; Lima and Dill, 1990; Milinski, 1993). However, few studies have closely investigated how temporal patterns of risk influence the trade-off between foraging and anti-predator behavior (Ferrari et al., 2010). Accordingly, foraging attempts under different histories of risks should be tested. The risk allocation model (Lima and Bednekoff, 1999) expects that animals exposed to frequent risk should forage more actively during safe periods when compared to other animals that experience infrequent risk (Ferrari et al., 2009).

Numerous studies have focused on how feeding activity or speed changes under the risk of predation (Lima, 1998a). Almost all species exhibit decreased movement, increased refuging, or both in response to increased risk of predation. However, there are some exceptions (Houtman and Dill, 1994). For example, larval *Ambystoma* salamanders showed decreased movement, but only in the absence of a refuge; otherwise, movement increased in an effort to reach a refuge (Sih and Kats, 1991). However, few studies focusing on continuous behavioral changes have been conducted to date in conflicting situations during the course of stimuli provision. In the present study, we continuously tracked the position of individual zebrafish exposed to both food and predator in an aquarium.

Individual movement tracking has been reported with respect to monitoring stressors in the environment since the 1980s (Baganz et al., 1998; Chon et al., 2005; Kwak et al., 2002; Lawrence and Smith, 1989; Lemly and Smith, 1986; Stark, 1993). Various computational methods have been utilized to extract information from complex datasets describing response behaviors including correlation analysis (Dray et al., 2010; Liu et al., 2011a; Tobin and Bjørnstad, 2003), parameterization (e.g., fractal dimensions, permutation entropy) (Andrew et al., 2005; Weis and Weis, 1974), and data transform (e.g., Fourier transform, wavelets) (Chon et al., 2004; Kim et al., 2006). Informatics techniques including self-organizing maps (Chon et al., 2004; Liu

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http://dx.doi.org/10.1016/j.ecoinf.2014.10.004
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et al., 2011b; Park et al., 2005) and multi-layer perception (Kwak et al., 2002) have also been used to identify response behavior patterns following treatment with toxic chemicals. Additionally, the hidden Markov model has also been employed to address behavioral state changes with transition probability matrix (TPM) under stressful conditions (Liu et al., 2011b; Nguyen et al., 2011).

However, previous studies have primarily focused on behavioral changes in response to anthropogenic agents and have not extensively addressed the response to stress in conflict. Considering the urgency of feeding or escaping for survival of animal, we investigated whether conflict would cause changes in behavioral states that differed during the course of stimuli provision. Based on computational methods including the self-organizing map (SOM) and intermittency test, continuous movement was analyzed to characterize changes in response behaviors of zebra fish according to the source and order of stimuli.

2. Materials and methods

2.1. Test organisms

We used adult zebrafish (*Danio rerio*) as the test species. Zebrafish are considered as one of the most suitable vertebrate model organisms for various biological tests (Blaser and Gerlai, 2006; Fetcho and Liu, 1998; Levin et al., 2007). Due to vulnerability to chemical stress and availability of biological information (e.g., genomics), the species has high potential for use as an indicator of risk and behavior monitoring (Kato, 2004; Liu et al., 2011b; Park et al., 2005; Swain et al., 2004). Wild type *D. rerio* individuals were obtained from a local fish dealer as a stock population and reared for two weeks before observation (Blaser and Gerlai, 2006) at a temperature of 25 ± 1 °C and pH of 7.1 ± 0.3 under a light/dark cycle of 14/10 h (lights on at 7:00 h and off at 21:00 h). Two fluorescent lights (26 W) were placed approximately 50 cm above the container (Miller and Gerlai, 2007) for rearing and observation. Test organisms were randomly selected from the stock and placed individually in a glass aquarium (water volume; 400 mm × 200 mm × 100 mm) for observation. Other rearing and observation methods were conducted as described by Liu et al. (2011b).

The commercial food, Tetramin (Tetra ®), was used to visually stimulate feeding behavior of the test organisms. Zebrafish were fed Tetramin once a day in the rearing container; therefore, they recognized it as their food (Gerlai et al., 2009). In addition to food, a predator was introduced to the observation arena. Most predators of zebrafish in the wild are fish-eating fishes (Engeszer et al., 2007); therefore, in this study, we visually presented Oscar fish, Astronotus orbiculatus (approximately 12 cm in size) obtained from a local fish dealer.

2.2. Observation system

The movement tracks were recorded using an observation system consisting of an observation aquarium, camera, timer, video frame grabber card (Mor/2VD, Matrox Morphis®) and software for image recognition system (Kwak et al., 2002; Park et al., 2005). The 2-D movement track of the test organisms was scanned from the side view in two dimensions (Power, 1984) using a color CCD camera (Hitachi KP-D 20 BU®) during the observation period. We selected 0.25 s as the time frame based on our previous studies. Since our goal was to observe the overall changes in movement of fish in response to the chemical treatment, this time interval was sufficiently short for presenting the responses of test organisms with respect to spatial displacement (Chon et al., 2004; Liu et al., 2011b; Park et al., 2005). Sudden responses may also occur within 0.25 s when the organisms are exposed to stimuli, especially to predators. Extremely short term movement behaviors such as sudden retreat and shaking would require an extra resolution (e.g., close-up, 3D), including partial body movement; however, such an evaluation was beyond the scope of the present study. In order to examine intentional responses of fishes in a longer period based on currently available devices, we observed response behaviors ranging from 1–10 s in two dimensions (Blaser et al., 2010).

2.3. Experimental procedure

The placement of test organisms and two stimuli, food and predator, in experimental arenas is shown in Fig. 1. To avoid cross effects of the two stimuli in the same observation aquarium, we only exposed the test organisms to the stimuli visually. Food was wedged between two pieces of 4 cm × 4 cm transparent tape and attached to the right wall inside the arena, with the top of the food at the water surface (Fig. 1). The predator was placed in another aquarium that was in contact with the right side of the observation arena. To align the height of the predator, we set a plate 10 cm above the bottom of the predator's aquarium (Fig. 1). Consequently, the food and predator were visible by zebrafish in the observation arena, but inaccessible. Olfactory signals from food and the predator were not allowed in the arena.

In this study, the term “condition” was used to separate temporal order of the key stimulus (food or predator) provided to the test organisms. Under condition 1, food was provided as the initial stimulus, followed by predator as a secondary stimulus, whereas predator was given first followed by food under condition 2. The term “phase” was used to indicate sequential processes between treatments. Specifically, P1, P2, P3 and P4 referred to no stimulus, introduction of the initial stimulus, introduction of the secondary stimulus, and removal of the initial stimulus from the arena, respectively. Prior to testing, the organisms were acclimated to the observation system for 30 min (Gerlai et al., 2006). We began to record the movement of zebrafish starting with phase 1 separately under conditions 1 and 2. These different conditions were adopted to observe the short-term behavioral changes under foraging opportunity or predation risk. Previous studies indicated that animals show different behavioral patterns when they are safe or endangered (Ferrari et al., 2009). Based on preliminary tests, 10 min was selected as the observation period for each phase. A total of 30 individuals were observed for each condition, but 13 individuals with a low level of noise in time series data were selected for analysis. The movement segments (in 1 s) were selected when the percentage with an irregularly high speed (>200 mm/s either in the *x* coordinate or *y* coordinate) was less than 2%.

2.4. Computational analysis

2.4.1. Self-organizing map

A self-organizing map (SOM) consisting of two layers of input and output was used to conduct non-linear projection of data onto a space in two dimensions and provide a patterned map for input data (Kohonen, 2001). There is no universal rule to determine the size of

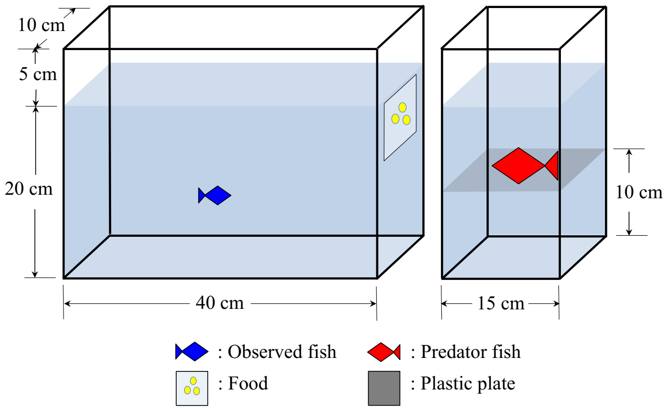

Fig. 1. Schematic diagram of observing zebrafish response behavior in response to conflicting stimuli when food and predator were provided in the observation arena.

the SOM (Lin and Chen, 2006). Vesanto et al. (2000) proposed that the number of map units could be approximately as $m = 5\sqrt{n}$, where n is the number of data samples. According to this proposal the number of nodes was 500 ($n = 10{,}000$) for each condition in this study. We set the vertical size of the SOM to be slightly longer than the horizontal size in order to show the highest variance in the input data along with the vertical axis while the following variance would be accordingly presented on the horizontal axis (Liu et al., 2011b). Finally the number of nodes used for training was 25 (vertical) × 20 (horizontal) in this study.

Based on preliminary studies and previous reports (Ji et al., 2007; Liu et al., 2011b), the following nine parameters were selected as input data for the SOM: x-coordinate (cm, mean x-coordinate during segment time), y-coordinate (cm, mean y-coordinate during segment time), speed (cm/s, distance between two consecutive coordinates divided by time), acceleration (cm/s^2, speed change divided by time), stop number (n, stop number during segment time), stop duration (s, total stop time during segment time), locomotory rate (cm/s, movement distance without stop time), turning rate (rad/s, angular change divided by time), and meander (rad/cm, angular change per movement distance). All parameters were scalar values. Similarly acceleration used in the intermittency test was scalar since the value was calculated from the difference between speeds. For each phase, 2500 segments (1 s) were randomly selected separately for conditions 1 and 2 (10,000 segments in total for each condition), and 9 parameters were used as variables. Initially, this dataset was used to train the entire dataset for conditions 1 and 2. In addition, the movement segments were separately trained in each phase for each condition. Since the total number of segments ranged 7735–7800 (595–600 segments multiplied by 13 individuals) in each phase, the number a little less than 10,000 was used for training for each phase in this study. A few data points (<5) were not recorded in different individuals and treated as missing data in some phases.

Distribution parameters were further used as input data for training of overall movement shapes. The mean values and standard deviation of x and y coordinates were calculated in different phases under different conditions and were used as input variables for the SOM training with 52 (13 replications × 4 phases) sample units. According to preliminary studies to identify grouping efficiently, 6 (vertical) × 4 (horizontal) nodes were used for training, slightly less than the recommended number (36) by Vesanto et al. (2000).

The distance, $d_j(t)$ at the j-th node on the SOM between weight at iteration time t and the input vector was calculated through learning processes:

$$d_j(t) = \sum_{i=0}^{p-1} \left[x_i - w_{ij}(t) \right]^2 \tag{1}$$

where x_i is the value of the i-th parameter, $w_{ij}(t)$ is the weight between the i-th variable and the j-th node on the SOM, and p is the number of the variable. The best matching neuron, which has the minimum distance, was selected as the winner. For the best matching neuron and its neighborhood neurons, the new weight vectors were updated as:

$$w_{ij}(t+1) = w_{ij}(t) + a(t)\left[x(t) - w_{ij}(t) \right] \tag{2}$$

where t is the iteration time and $a(t)$ is the learning rate. The learning process of the SOM was conducted using the SOM Toolbox (Vesanto et al., 2000) developed by the Laboratory of Information and Computer Science at the Helsinki University of Technology (http://www.cis.hut.fi/projects/somtoolbox/) in a Matlab environment (The Mathworks, R2009). The initialization and training processes were conducted following the suggestions made by the SOM Toolbox by allowing optimization of the algorithm. A detailed description regarding application of the SOM to ecological data has been provided by Park et al. (2003). To reveal the degree of association between the SOM units, Ward's linkage method (Ward, 1963; Wishart, 1969) was used to cluster the movement data according to the Euclidean distance. The linkage distances were rescaled at 0–100%.

2.4.2. Intermittency analysis

Intermittency is defined as the probability distribution of the shadowing time during which the data are consecutively higher than a threshold level (Fig. 2) (Do and Lai, 2004; Do et al., 2003; Hirsch et al., 1982; Sauer et al., 1976). The intermittency of time-series data in nonhyperbolic chaotic systems shows universal and non-universal features of scaling. At small shadowing time, there are universal algebraic scaling behavior with exponent $-3/2$ in log–log plot, however the intermittencies at large shadowing time become non-universal (Do et al., 2003; Hirsch et al., 1982). Intermittency has been reported in various fields, including coordination of muscular systems (Fernando and Lawrence, 1968; Gawthrop et al., 2011), chemical kinetics (De la Fuente et al., 1996; Pomeau et al., 1981), laser models (de Valcárcel et al., 1995), and fluid dynamics (Pomeau and Manneville, 1980).

Intermittency has been further applied to behavior and ecological studies. In ecology, flow intermittency regarding biodiversity determination in stream ecosystems has recently been investigated (Bogan et al., 2013; Datry et al., 2011). Harnos et al. (2000) analyzed scaling and intermittency in the temporal behavior of nesting gilts (Harnos et al., 2000). Mashanova et al. (2010) reported a truncated power law in aphid movement in addressing an alternate pattern of fast and slow phases. However, intermittency in response behavior of animals under chemical stress has not been extensively studied, especially under conflicting situations.

In this study, in order to reveal the structural properties of the movement data, we addressed the probability distributions of the shadowing time in time-series acceleration on fish observed in a confined area. We used the mean value of acceleration as the threshold after testing various levels of the threshold (Fig. 2) from 0.125 to 2 times the mean value. The shadowing times and their probability distributions were expressed on a logarithmic scale.

3. Results

3.1. Parameter estimation

To address behavioral changes across phases under each condition, parameter differences between the previous and next phases were measured during the course of stimulus provision (i.e., phase 1-2, phase 2-3, and phase 3-4) (Fig. 3). To present parameter differences, the values in current phase were subtracted from those in previous phase for each individual. The values in Fig. 3 are the secondary calculation obtained by difference between two scalar values. Minus value was obtained when the value in the previous phase was smaller than the value in the next phase. The averages of parameter differences over 13 individuals are presented in Fig. 3 with paired t-test ($n = 13$). Overall, stop duration, stop number, and y-coordinate changed substantially under both conditions (Fig. 3). Specifically, stop duration and stop number increased, whereas y-coordinate decreased in phase 1-2. Significant differences were observed in both conditions except for stop number in condition 2. The difference was especially high for the y-coordinate in condition 1 ($p < 0.001$) (Fig. 3). Speed, acceleration and locomotory rate showed substantial changes across phases, but the pattern was reversed in the two conditions. Specifically, an initial decrease occurred between phases 1 and 2 in condition 1, whereas increase occurred in condition 2. However, statistical significance was only observed in condition 1 ($p < 0.01$).

The values of the x-coordinate, turning rate and meander showed greater differences under condition 2, and all three variables decreased significantly in phase 1-2. It should be noted that the x-coordinate only decreased significantly in phase 2-3 ($p < 0.01$) under condition 1 (i.e., when predators were added as a secondary stimulus). The linear parameters in movement (speed, acceleration, and locomotory rate)

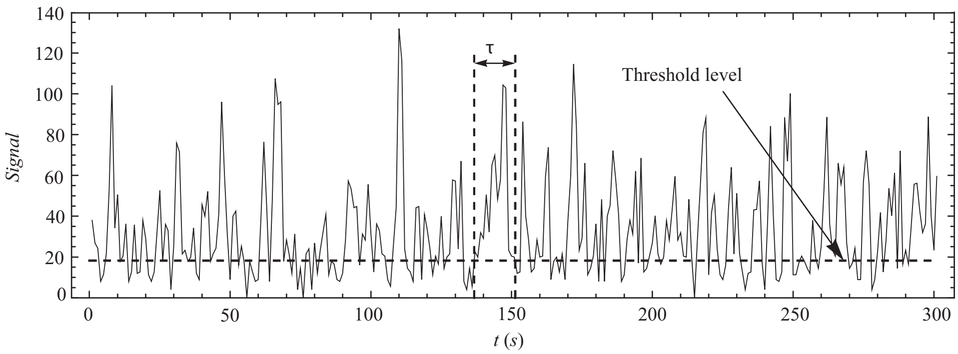

Fig. 2. The intermittency of a time series data. Given a time series data, the probability distribution function of τ is called the intermittency.

decreased when food was provided initially under condition 1, whereas angular parameters (turning rate and meander) decreased when predator was provided initially under condition 2 (Fig. 3). This trend was also observed when food or predator was added as secondary stimulus, but significant differences were not observed.

3.2. Training with SOM

Using parameters as input data (see Section 2.4.1), movement segments (1 s time interval) were trained with the SOM by combining all phases under both conditions. Six common groups were obtained in different phases under two conditions (Fig. 4a and d) according to clustering based on Ward's linkage method (Ward, 1963; Wishart, 1969). Component profiles visualized on the SOM showed overall vertical positions of stop duration in a similar configuration under both conditions, although orientation of the profiles was sometimes reversed on the map (i.e., the direction of the gradients was upside down) (e.g., Fig. 4c and f). Parameters related to position (y-coordinate), stop (stop number and stop duration), and direction change (turning rate and meander) played a key role in determining variance of the movement data, forming a gradient along the vertical axis on the map in both conditions. The x-coordinate was also important, forming a horizontal gradient on the map (Fig. 4c and f). Other parameters were locally aggregated and did not show outstanding gradients on the SOM. When the SOM was further trained in each phase separately, the component profiles showed similar configurations across phases under both conditions. Profiles of input parameters in different phases and conditions are given in the Supplementary file.

The six typical movement patterns were commonly identified across phases in both conditions (Fig. 5) according to clusters on the SOM as shown in Fig. 4a. Similarly the movement patterns were in accordance with the parameter profiles for the SOM trained for each phase separately (Figs. S1–S4). The movement patterns were also similarly associated with the SOM clustering for each phase in different conditions. These movements represented the following patterns (Fig. 5): pattern 1, rightward advancement; pattern 2, leftward advancement; pattern 3, right turn; pattern 4, left turn; pattern 5, stop; and pattern 6, stop and advancement. Considering the difficulty of direction identification immediately before (or after) being in a motionless state, "stop" and "advancement" are defined as the present movement direction sufficiently identifiable before (or after) the motionless state (i.e., a slightly longer duration). "Stop" was inclusively defined to cover both motionless and

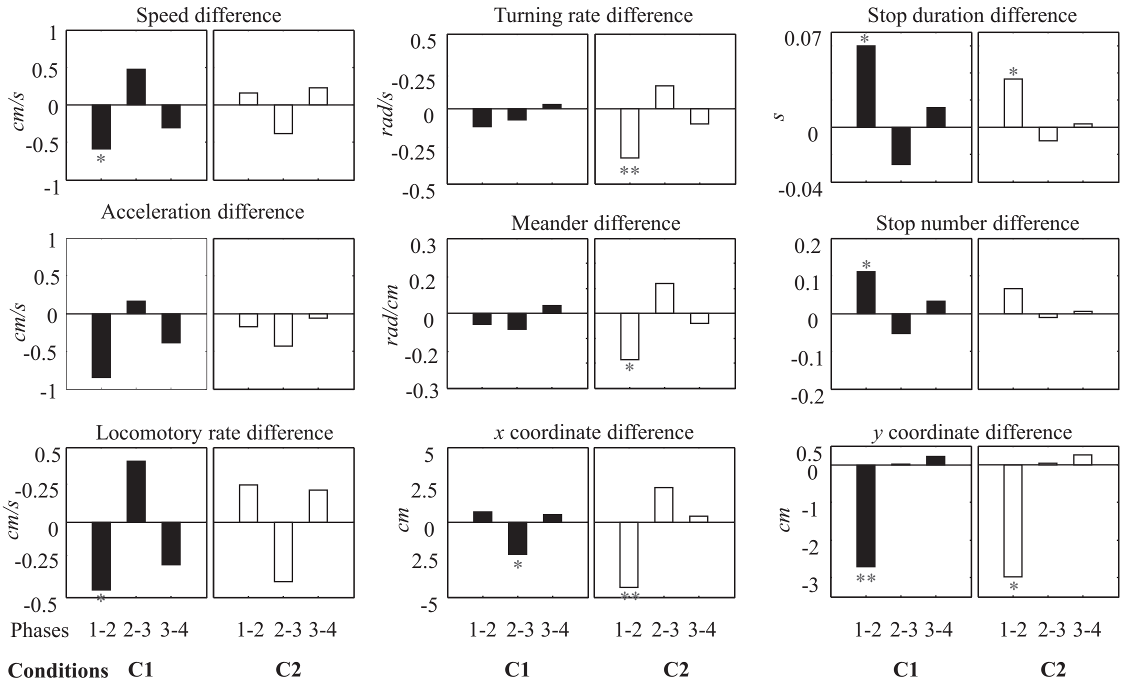

Fig. 3. Difference of parameters (mean values) between two phases during the course of stimuli provision in two conditions (C1: condition 1, and C2: condition 2) (1-2: phase 1 to phase 2; 2-3: phase 2 to phase 3; 3-4 phase 3 to phase 4, and * and ** indicate significance ($p < 0.01$) and high significance ($p < 0.001$) according to paired t-test).

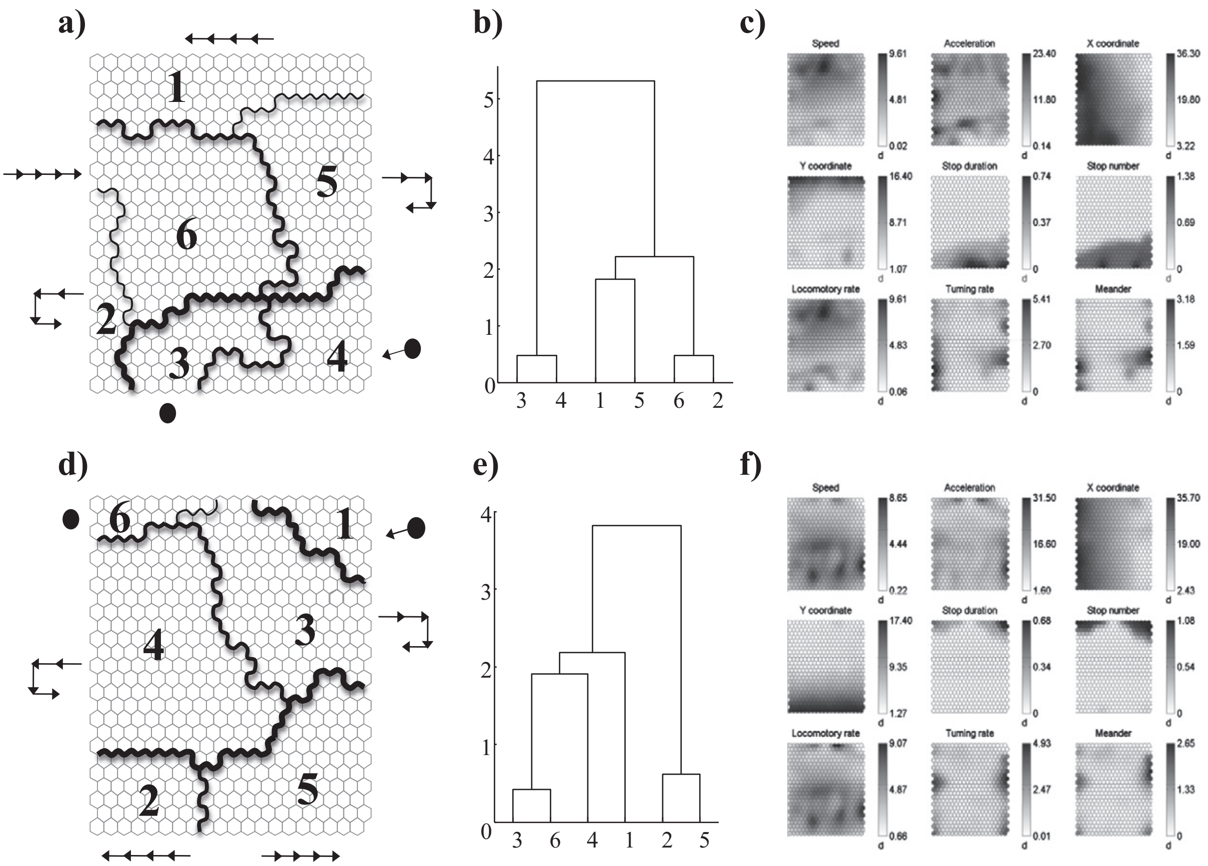

Fig. 4. Clustering based on the trained SOM showing clusters with corresponding movement patterns for condition 1 (a) and condition 2 (d), dendrogram for condition 1 (b) and condition 2 (e), and component map for condition 1 (c) and condition 2 (f).

immediate initiation of movement (i.e., the overall maximum movement less than 2 cm per second from 0 cm per second), whereas “advancement” was defined as the stage when movement direction could be clearly identified (i.e., overall maximum distance longer than 2 cm per second). Accordingly, the movement pattern matched the profiles of parameters. Pattern 1 showing right advancement matched high speed

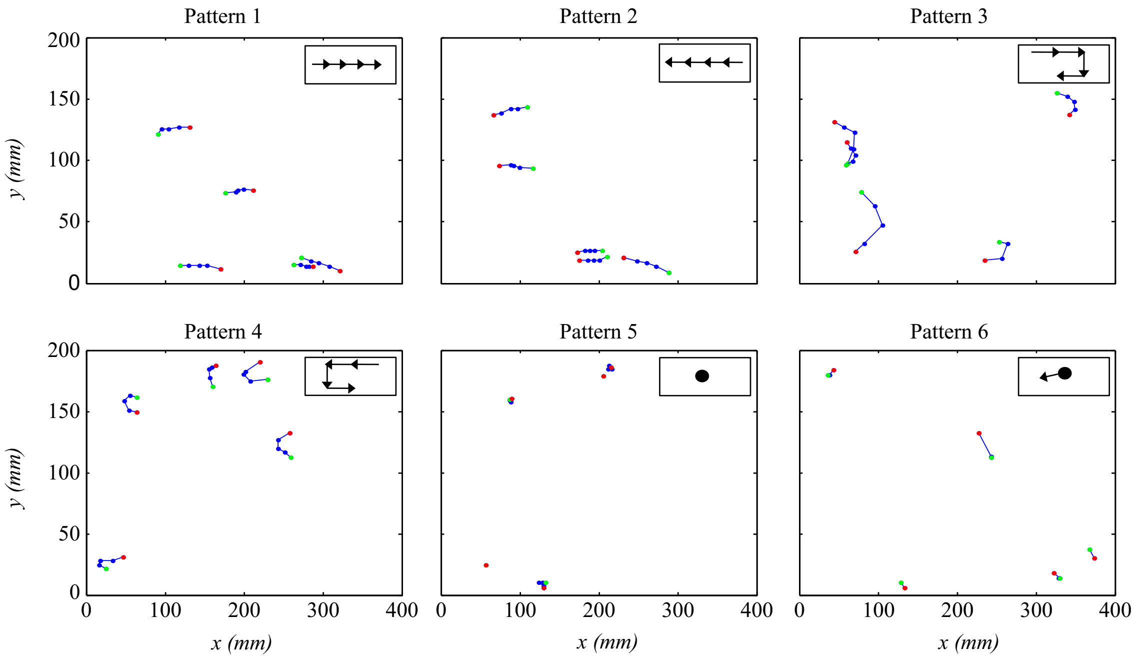

Fig. 5. Movement patterns corresponding to different clusters on the SOM (Fig. 4). Pattern 1: rightward advancement, pattern 2: leftward advancement, pattern 3: right turn, pattern 4: left turn, pattern 5: stop, and pattern 6: stop and advancement (starting point; green dot, and stopping point; red dot). (Insets are schematic patterns.)

and locomotion as shown in cluster 4 in Fig. 4a, for instance. Overall, linear advancement movement (patterns 1 and 2) were in association with linear parameters (speed, and locomotory rate) whereas directional changes (patterns 3 and 4) matched angular parameters (turning rate and meander). Stop patterns (patterns 5 and 6) were further in accordance with stop-related parameters (stop duration and stop number) (Figs. 4a and 5). This association between movement patterns and the SOM grouping was also observed for each phase when the SOM was performed separately for each phase in two conditions although slight variations were observed in some phases (Figs. S1–S4).

Even though the profiles of parameters were similar across phases, the frequencies of movement segments varied according to the source and order of stimuli provision. Fig. 6 shows the frequencies of different movement segments in different clusters across different phases according to Tukey test ($n = 13$). Frequencies were obtained as the number of segments observed in each cluster divided by the total number of segments in each phase. Even though the profiles of parameters were similar (Figs. 4 and S1–S4), the frequencies of movement segments belonging to different clusters on the SOM varied according to phases and conditions. Overall, pattern 1 was dominant under condition 1, whereas pattern 2 appeared to be more abundant than other patterns under condition 2. These findings indicated an effect of stimulus provision. When food was provided on the right side of the aquarium under condition 1, more rightward advancement (i.e., toward food) was observed. In contrast, more leftward advancement away from stimulus was presented when the predator was provided to the arena under condition 2.

Overall, pattern 1 was significantly higher in phase 3 than in other phases under condition 1 ($p < 0.05$). It was noteworthy that the rightward advancement (pattern 1; toward stimuli) was significantly abundant when food and predator were both present in phase 3. These rightward advancing movements were abundantly found when test organisms were away from the stimuli (i.e., left area of the arena), and seldom observed when close to stimuli. The rightward advancement significantly decreased after food was removed in phase 4 compared with phase 3 ($p < 0.05$). Under condition 2, the pattern 1 also significantly increased in phase 3 when food was added as the secondary stimulus compared with phase 2 ($p < 0.05$). When predator was removed in phase 4 in condition 2, the frequency remained in the same level without statistical significance although the frequency tended to decrease slightly compared with phase 3. The appearance of pattern 1 indicated that test fish showed directional intention of rightward advancement (toward food) when food was located together with predator.

Other minor patterns varied according to phases and conditions, although not as greatly as patterns 1 and 2. Pattern 4 appeared to decrease consistently after phase 2 under condition 1, whereas no significant change was observed under condition 2. Under condition 2, pattern 3 significantly increased in phase 4 ($p < 0.05$). Pattern 6 was notably high in phase 3 relative to phase 2 ($p < 0.05$) although the frequency was not high. These findings indicated that frequency of behavior pattern was variable by reflecting the effect of conflicting stimuli according to the source and order. Detailed variations in frequencies of behavioral aspects will be reported elsewhere.

3.3. Positional patterns and intermittency

For further understanding of fish movement, not only the movement patterns, we analyzed the positional patterns of a fish during experiment. To present the positional distribution of zebra fish movement directly, SOM was also trained with the mean and variance of *x*- and *y*-coordinates within the observation arena across phases. Four clusters were commonly obtained in each phase under conditions 1 and 2 (Figs. 7 and 8). Based on visual judgment, distribution shapes in different clusters were defined under different phases and conditions. Overall, four basic shapes were initially identified as wide (W), surface (S), boundary (B), bottom (O), and corner close to stimulus (C). Subsequently, more complex shapes were expressed by combining the symbols. For instance, SB and WB indicated a combination of "S and B" and "W and B", respectively, with the first letter indicating higher abundance. In cases in which two patterns contributed equally to movement

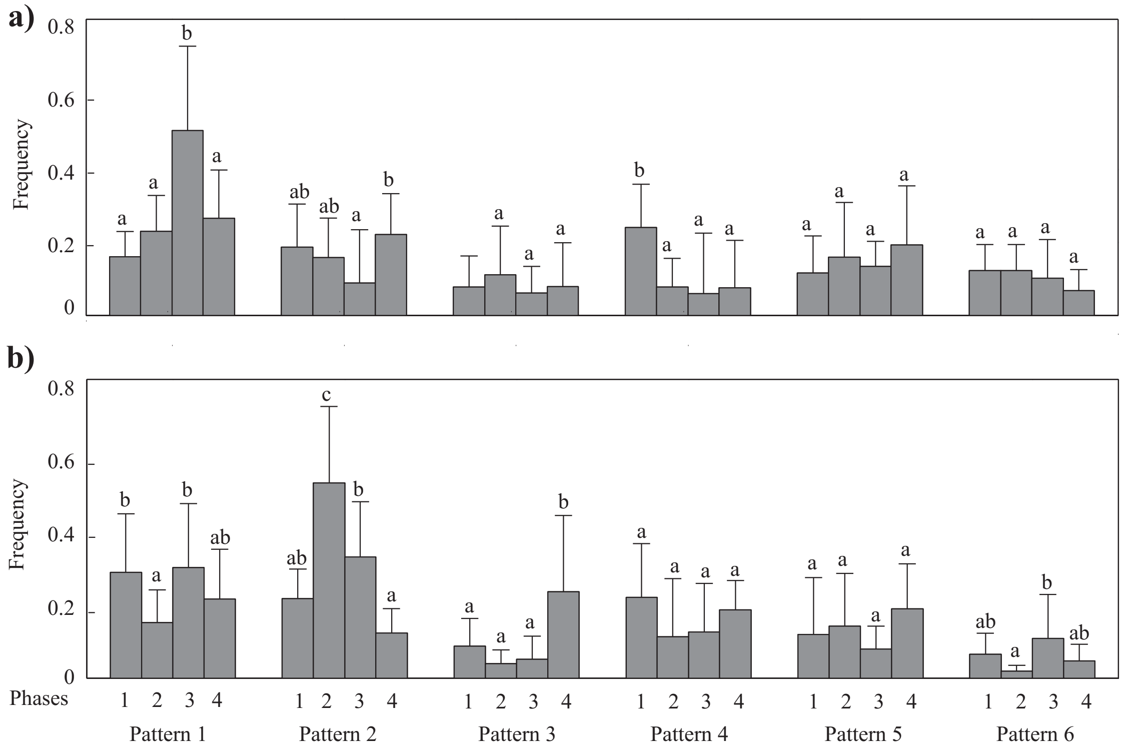

Fig. 6. Frequency of movement patterns observed in different phases in conditions 1 and 2. (Different alphabets indicate significance ($p < 0.05$) among different movement patterns within each phase according to Tukey test.)

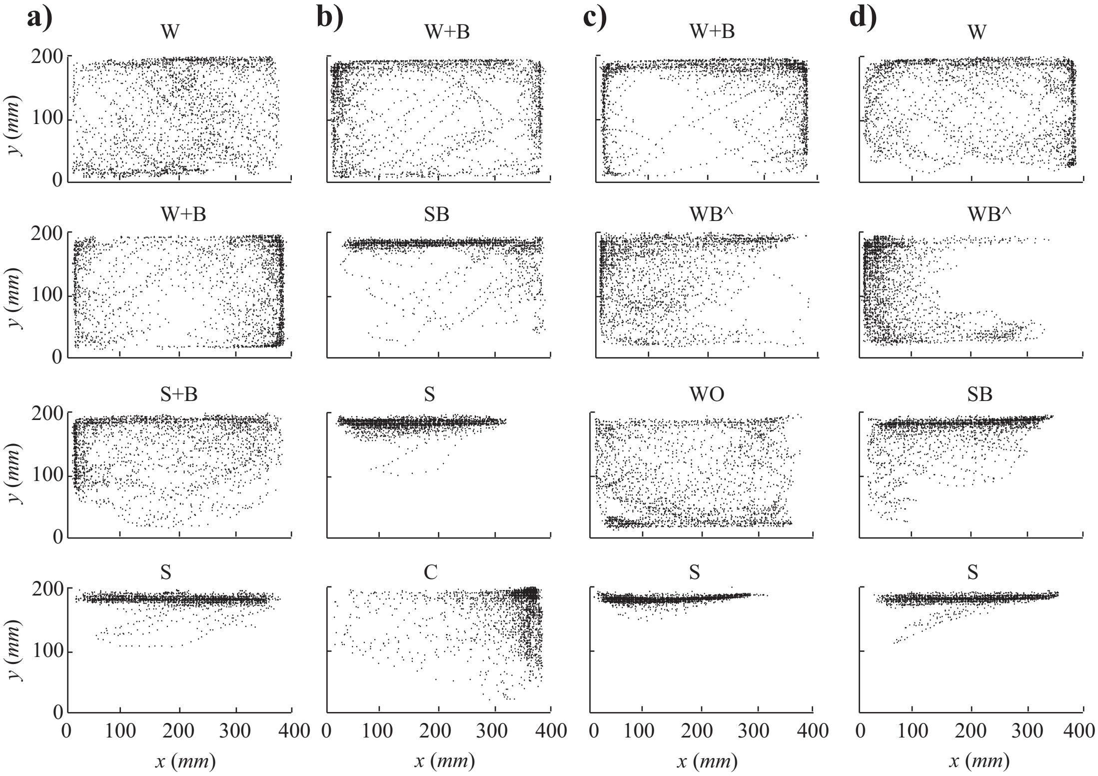

Fig. 7. Representative movement tracks for each cluster on the SOM when trained with parameters for positional data as input in condition 1 for phase 1 (a), phase 2 (b), phase 3 (c), and phase 4 (d). (See text for explaining abbreviations.)

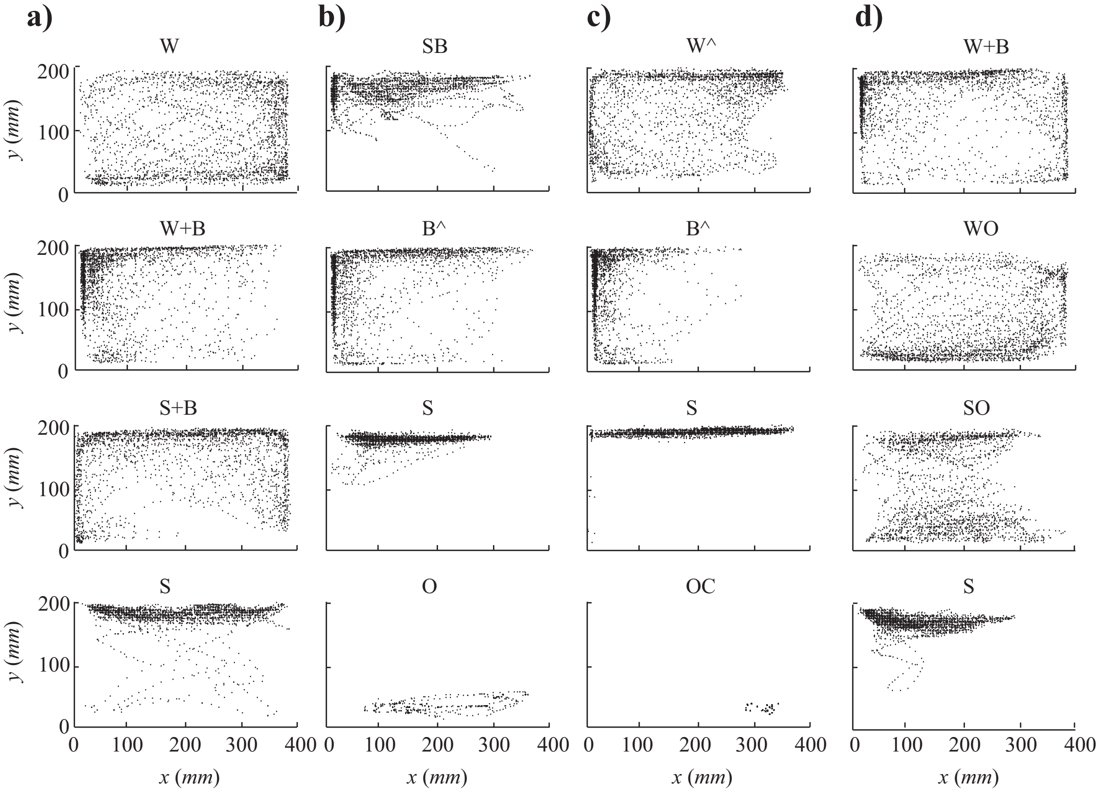

Fig. 8. Representative movement tracks for each cluster on the SOM when trained with parameters for positional data as input in condition 2 for phase 1 (a), phase 2 (b), phase 3 (c), and phase 4 (d). (See text for explaining abbreviations.)

patterns, the symbol "+" was used (e.g., W + B). In addition, the symbol "^" was added when the positions were located away from the stimulus position (i.e., lower values on the *x*-coordinate in the arena), such as B^. Figs. 7 and 8 showed typical distributional shapes according to the SOM under conditions 1 and 2. In phase 1, the distribution shapes were commonly classified for both conditions, including surface positions (S), surface plus boundary positions (SB), wide area (W), and wide area plus boundary (W + B).

In phase 2 under condition 1 (initial provision of food), overall patterns did not change greatly from the patterns observed in phase 1, with S, SB, and W + B commonly being observed (Fig. 7). However, the pattern of corner positions close to the stimulus, C, was also observed. This was understandable since food was provided to the corner of the arena in this phase (Fig. 1). When the secondary stimulus, predator, was added to the arena in phase 3 under condition 1, distribution patterns changed substantially (Fig. 7). More patterns were found in relation to the wide area, showing wide and boundary positions (W + B), wide area with boundary away from stimuli (WB^), and wide area with bottom positions (WO). It should be noted that bottom positions were newly observed in this phase (Fig. 7). Upon removal of food from the two stimuli (predator only remaining) in phase 4, wide area (W) and wide area with boundary (away from stimulus) (WB^) remained, and surface positions were diversified, including surface movement (S) and surface movement with boundary (SB) (Fig. 7).

Under condition 2, when the predator was added to the arena in phase 2, distribution shapes changed substantially relative to phase 1. Boundary positions away from stimulus (B^) and bottom position (O) were newly found (Fig. 8). However, distributions related to wide area, W, were not observed in this phase. With the addition of secondary stimulus (food) in phase 3, wide distributions away from stimulus W^ appeared newly (Fig. 8). Boundary distributions away from stimulus B^ and the bottom pattern observed in phase 2 were similarly observed near the corner (OC) in phase 3, but only a few data points were observed in this case. After the predator was removed from two stimuli in phase 4 under condition 2, surface distribution with bottom (SO) appeared. It is worth noting that bottom positions, SO and WO, were observed, even though food only remained in the observation arena.

The distribution patterns were further differentiated in response to the order of stimuli provision. When both stimuli were present in phase 3, differences in positional shapes were observed under different conditions. Under condition 1, wide patterns were more abundant, whereas the positions away from stimulus (predator and food in this case) were found (i.e., W^, B^) under condition 2. The bottom pattern OC was also found in condition 2. However, a trace of the bottom pattern was still found under condition 1 with WO. The patterns in phase 4 also reflected the previous phase somewhat, with an additional tendency for surface patterns under both conditions. It is worth noting that the pattern of WB that included the area near the stimulus (predator in this case) was still observed under condition 1. However, the bottom movement was also found under condition 2 (e.g., WO, SO). Additionally, the distribution shapes presented individual specificity to the source of stimuli. Overall, the results indicated that distribution shapes were formed according to the source and order of stimuli. Predator produced stronger responses, and the stress effect remained in the subsequent courses of stimuli provision (i.e., addition of secondary stimulus and removal of initial stimulus). Additional studies considering diversified spatial distribution shapes according to individuals will be reported elsewhere.

Intermittency on speed was measured using the movement data clustered by the SOM. However, speed showed no clear difference among phases under different conditions (data not shown). In contrast, intermittency on acceleration was characteristic and variable according to the source and order of stimuli. Intermittency generally showed a linear shape with respect to the shadowing time (*x*-coordinate) in association with the logarithm of abundance (*y*-coordinate) on the semi-log plot (Fig. 9). Regression analysis was applied to the data up to 2 s (data points of early part from 1 to 8 ticks in Fig. 9) considering that fish may not continuously accelerate longer than 2 s according to preliminary data analysis. Consequently some slopes (matching early points) appeared to be lower than the whole data points (e.g. phases 2 and 3 in condition 2 in Fig. 9). Statistical significance of the slopes of the regression equations (Zar, 2009) was more frequently observed under condition 2 than condition 1 (see Appendix A for slope values and statistical significance). Overall, distribution shapes related to surface distribution (e.g., S) were differentiated from shapes related to wide distribution (e.g., W) under both conditions.

Specifically in phase 1, W-related patterns showed steeper slopes (absolute value) and were separable from S-related patterns under both conditions. In phase 2, intermittency was differentiated between the two conditions for slopes according to statistical results. Under condition 1, no significance was observed among the slopes for different clusters. However, under condition 2, the slope for W was sharply separated from that of other clusters, indicating a stronger impact of predator in wide distribution. In phase 3, when both stimuli were present, variability was strongly demonstrated under condition 2: slopes for all clusters were significantly different (Fig. 9, Appendix A). However, the flatter slope for B^ observed in this case was not reliable due to the limited number of observation points. Under condition 1, S was significantly different from other clusters in phase 3. In phase 4, a significantly higher difference was observed under condition 1, separating all distribution shapes, WB^, S and W. Under condition 2, S was separated from other clusters. Overall intermittency on acceleration addressed variation in response behaviors to conflicting stimuli. It was also worth noting that the order of stimuli was influential in determining intermittency.

4. Discussion

Previous studies reported different behaviors in response to foods and predators (Houtman and Dill, 1994; Lima, 1998b; Sih and Kats, 1991). However, overall activity was mainly reported in a static manner in most cases. In this study, temporal changes in movement patterns were characterized according to physical expressions (i.e., variation in parameters during the course of stimuli provision) in response to stimuli. Notably, the linear parameters (i.e., speed, locomotory rate) decreased when food was added, whereas the angular parameters (i.e., turning rate, meander) were lower when predator was added (Fig. 3). This trend was commonly observed when the stimulus was provided as either the initial or secondary stimulus, although no statistical significance was observed for the case of secondary stimulus (Fig. 3). These observations indicate that response behaviors states under conflicting condition could be characterized by parameters.

Variability of other parameters was also suitable for presenting additional behavioral states. Observation of the maximum increase in stop duration and stop number in phase 2 under both conditions, for instance, supported the existence of stop-related activity responding to stimuli under both conditions (Fig. 3). These findings are in accordance with those of previous studies reporting decreased movement in the presence of predators (e.g., Lima, 1998b). Further study would be required to check parameter changes when the presence or absence of refuge is critical (Sih and Kats, 1991). In the present study, it was noteworthy that, when fish were exposed to predators, the option for more directional change decreased whereas variability in the linear advancement movement was limited in response to food. However, the reason why the linear and angular parameters and stop-related parameters were differentiated in accordance with sequential order of stimuli provision is currently unknown. More biological and behavioral investigations to study physiological and behavioral processes should be conducted in the future. Nevertheless, the present study confirmed that animal response behavior is unique in response to conflicting stimuli (i.e., favorable and unfavorable) provided concurrently, reflecting the fact that conflicting situations of hunger versus predation represent

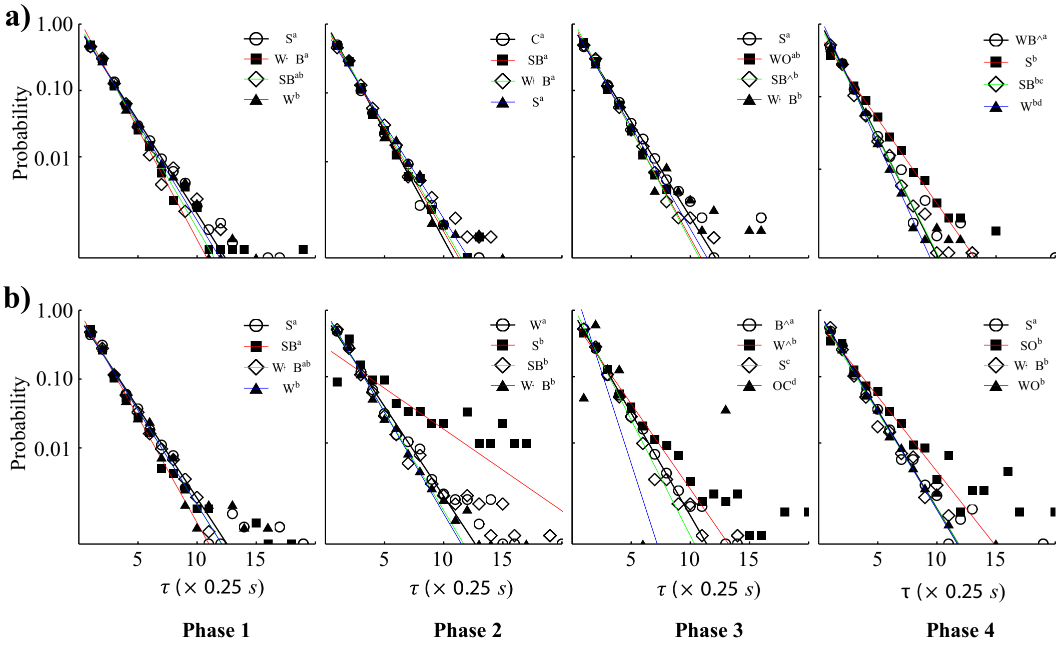

Fig. 9. Intermittency of acceleration applied to the positional data (Figs. 7 and 8) clustered by the SOM in conditions 1 and 2. Regression lines fitting to each cluster are shown with statistical significance (see text and Appendix A for explaining statistical tests).

a typical situation that animals have to face frequently and respond to promptly. However, it is important to note that this experiment was conducted under limited conditions in which food and predator were only provided visually within a limited area in this study. Test organisms could not eat food or be caught by the predator; accordingly, further experiments should be conducted under natural conditions to confirm the results of the present study.

We also demonstrated information extraction by SOM. SOM training was useful for characterizing complex behavioral data to provide overall views regarding response behaviors (Ji et al., 2007; Liu et al., 2011b; Zhang et al., 2012). Although the movement segments were accordingly classified (i.e., governing role of stop duration, stop number and *x*- and *y*-coordinates) (Fig. 4), the profiles of the parameters were basically similar across phases (Figs. S1–S4). The SOM was suitable for showing the invariable structural properties in complex behavior responding to stimuli in this study. Frequencies of movement segments in different clusters, however, provided additional information on characterizing response behaviors, being differentiated according to phases and conditions (Fig. 6). The actual pattern (e.g., rightward advancement shown in Fig. 5) could be identified in association with the profile of parameters in this case. For example, patterns 1 and 2 were more commonly observed in the clusters responding to high speed and acceleration (Figs. 4 and 5). The rightward advancement was characteristically observed when food was located together with predator in the arena (Fig. 6). The patterns related to directions changing, right and left turns (patterns 3 and 4, respectively), were also associated with a high turning rate and meander. These results indicated that specific movement patterns were identifiable in response to stimuli.

Some recent papers extended original SOM for different types of data (Hsu et al., 2002, Hsu et al., 2011; Kiang, 2001). The flexibility of SOM training was also demonstrated in this paper. SOMs were trained with different input data: parameter training to find conserving property in response behaviors in determining important parameters (e.g., stop number, stop duration) (Fig. 4), and position data training to extract variable movement patterns (Figs. 8 and 9). Whereas the common characters in parameters were found in addressing response behaviors, the positional parameters could be additionally trained to present variability in distribution shapes across phases and conditions (Figs. 7 and 8). The SOM networks could be further developed to accommodate both regularity and variability in the data concurrently within one model. In order to deal with complex data, various methods in SOMs were proposed by maximizing their self-organizing property (Chon, 2011) including hierarchical SOM (e.g., Carpinteiro et al., 2007), temporal data patterning (e.g., Hammer et al., 2004; Strickert and Hammer, 2005), growing SOM (e.g., Bauer and Villmann, 1997), and modular SOM (Furukawa, 2005). In the future SOMs flexible in data extraction stated above could be further utilized to trace common and variable characters of time-series data efficiently.

Acceleration was critical in showing differences in intermittency (Fig. 9), indicating that “change in speed”, rather than “speed”, is important in expressing behavioral data structure. The patterns related to S and W could be readily differentiable according to acceleration intermittency. Overall, the slopes for S had a low absolute value, indicating that speed will be variable during surface movement. Accordingly, changes in speed (i.e., high value in acceleration) would be more likely during surface movement. We also found that the order of stimulus was important in determining behavioral state in the following course of stimulus provision. Intermittency was further differentiable in phase 4 when the initial stimulus was removed. Initial provision of predator had a greater influence on test organisms during the process of secondary stimulus provision and removal of initial stimulus (Fig. 9). These findings indicated that some memory effect (e.g., Turner et al., 2006) exists in response to stimuli. However, we did not investigate the time-series response of behavior patterns or quantitatively measure how the initial impact persisted; accordingly, further studies should be conducted to evaluate these factors.

Additionally, boundary conditions were not considered in this study. Since the arena is small and the movement range of the test organisms would be longer in nature, the boundary effects also need to be considered. As shown in Figs. 7 and 8, boundary shapes were frequently observed. However, in this study, we dealt with the boundary shapes as separate patterns. In the future, behavior processes near boundary areas should be more carefully investigated since site-specific behaviors were reported in boundaries and corners (Quach et al., 2013).

5. Conclusions

The continuous movement data of zebrafish were useful for elucidating response behaviors in conflicting situation, and behaviors could be effectively characterized according to different computational methods. Behavioral states in response to conflicting stimuli (food and predator) were addressed by parameters, SOM, and intermittency tests. Parameters could be differentiated according to the source of stimuli. Linear parameters such as speed, acceleration and locomotory rate decreased when food was added to the arena, whereas angular parameters such as turning rate and meander were lower when test organisms were exposed to predator. According to SOM, parameters related to position, stop and direction change, played a key role in determining variance of the movement data, and frequencies in movement patterns were differentiated according to phases and conditions (e.g., more abundant rightward (toward stimulus) movement after exposure to food). Intermittency on acceleration effectively differentiated the effect of stimuli provision, separating distribution shapes such as surface (S) and wide distribution (W). The impact of predator-first introduction was stronger than that of food-first introduction, revealing the effect of source and order of stimuli. Behaviors responding to stimuli would be useful for monitoring stress under natural conditions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ecoinf.2014.10.004.

Acknowledgments

This work was supported by the Convergence Research Grant funded by Pusan National University (PNU, Convergence Research Grant) (PNU-2012-0071-0001).

Appendix A

Estimates of the slopes based on regression analysis applied to the intermittency of acceleration in positional patterns (Figs. 7 and 8) in each phase in conditions 1 and 2. Alphabets indicate statistical significance ($p < 0.05$) (see text for symbol explanation).

	Phases	Slope values (Patterns)			
Condition 1	1	-0.6719^{a} (S)	-0.6920^{a} (W + B)	-0.7130^{ab} (SB)	-0.7382^{b} (W)
	2	-0.6719^{a} (C)	-0.6894^{a} (SB)	-0.7046^{a} (W + B)	-0.7205^{a} (S)
	3	-0.6789^{a} (S)	-0.7159^{ab} (WO)	-0.7392^{b} (SB^)	-0.7417^{b} (W + B)
	4	-0.5998^{a} (WB^)	-0.7259^{b} (S)	-0.7274^{bc} (SB)	-0.7680^{bd} (W)
Condition 2	1	-0.6517^{a} (S)	-0.6695^{a} (SB)	-0.6700^{ab} (W + B)	-0.7164^{b} (W)
	2	-0.5176^{a} (W)	-0.6675^{b} (S)	-0.7065^{b} (SB)	-0.7132^{b} (W + B)
	3	-0.6475^{a} (B^)	-0.7174^{b} (W^)	-0.7705^{c} (S)	-1.0936^{d} (OC)
	4	-0.5951^{a} (S)	-0.6976^{b} (SO)	-0.7015^{b} (W + B)	-0.7078^{b} (WO)

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