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MOVEMENT BEHAVIOUR OF MEDAKA (*Oryzias latipes*) IN RESPONSE TO SUBLETHAL TREATMENTS OF DIAZINON AND CHOLINESTERASE ACTIVITY IN SEMI-NATURAL CONDITIONS

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Abstract. Behavioural changes of medaka (*Oryzias latipes*) treated with an anticholinesterase insecticide, diazinon (0.1 mg L^{-1}), were continuously observed for 4 days in semi-natural conditions. Although variations occurred in individual specimens, the movement tracks appeared differently with typical short-range movement with irregular turns and shaking after the treatments. Eight movement patterns frequently observed before and after the treatments were selected, and the variables characterising the movement patterns were compared quantitatively. The variables were clearly differentiated when the movement patterns were correspondingly matched before and after the treatments (e.g., vertical movements, horizontal movements, etc). Meander and stop duration were highly different among the selected movement patterns. Additionally, different degree of toxic response behaviours could also be detected by quantitative characterisation of the variables. Response behaviour was confirmed with toxicological experiments that show the decrease in the acetylcholine esterase activity in the head and body of specimens. Quantitative investigations on the variables of the movement tracks suggested the usefulness of response behaviour as a monitoring tool for environmental assessment.

Keywords: response behaviour, movement tracks, medaka, diazinon, cholinesterase activity

1. Introduction

Diazinon, an organophosphate insecticide, shows a high toxicity to organisms, especially fish and aquatic invertebrates, although it has relatively low toxic effects on mammals and humans (Smith, 1993; Gupta, 1994; Barabas, 1998). If the chemical is mismanaged and not accordingly exposed to aquatic ecosystems, it is highly likely that communities residing in the ecosystems would be disturbed.

The main toxic mechanism of diazinon is based on cholinesterase inhibition in organisms (Kozolvskaya and Mayer, 1984; Ferslew *et al.*, 1992). Accordingly acetylcholine esterase (AChE) measurements have been effectively used to express

the chemical's toxic effect on specimens (Galgani and Bocquene, 1990). There have been numerous assessments of toxicity of insecticides in this regard. However, not many accounts of research have been conducted on behavioural responses to the sublethal levels of toxic chemicals until recently.

Behavioural responses have been reported to be sensitive to sublethal exposures to various chemical pollutants (Lemly and Smith, 1986; Dutta *et al.*, 1992). Dutta *et al.* (1992) indicated that a behavioural bioassay might be more sensitive than other types of testing. Roast *et al.* (2000) reported disruptions in the swimming behaviour of the hyperbenthic mysid after a treatment of chlorpyrifos. Sublethal effects of toxic substances on crustaceans have also been observed with anti-sea lice formulations, azametiphos and cypermethrin (Abgrall *et al.*, 2000; Burridge *et al.*, 2000) and inorganic mercury (St-Amand *et al.*, 1999). Ibrahim *et al.* (1992) reported that sublethal concentrations of chloropyrifos induced a reduction in the production and hatching of eggs in snails. Phototactic behaviour of Daphnia was also observed to monitor the water quality, contaminated with heavy metals and pentachlorophenol (Michels *et al.*, 1999, 2000). Takiquchi *et al.* (2002) reported that *Paramecium* was repelled by a herbicide, 2,4-dichlorophenoxyacetic acid. Regarding fish, Moore and Waring (1996) demonstrated sublethal effects of diazinon on the olfactory system of the mature male Atlantic salmon parr. Gray *et al.* (1999) reported changes in the reproductive success of medaka after exposure to an environmental hormone, octylphenol. Recently, Oshima *et al.* (2003) observed suppression of sexual behaviour in male medaka exposed to estradiol.

These studies, however, have been mainly based on descriptions of a single or a combination of single observations. Not many studies have been directly conducted on the continuously observed data with quantitative analyses. Blübaum-gronau *et al.* (1994) videotaped the movement of swimming fish and developed a sensing system to statistically differentiate motility, the number of turns, swimming position, etc. Lorenz *et al.* (1995) similarly recorded continuous movement to characterise movement patterns to detect the effect of atrazine on fish. Lee and Lee (1996) reported that irregular movement of carp was first detected within 15 h of exposure to an acute level of diazinon. Baganz *et al.* (1998) videotaped movement of zebra fish and reported changes in motility after being treated with microcystin-LR, the cyanobacteria toxin. By continuously tracing with infrared-transmitters, temperature preference and thermal behaviour were investigated on juvenile cyprinids (Staaks, 1996) and sturgeon (Staaks *et al.*, 1999). In the research mentioned above, however, relatively simple aspects of movement are revealed with a limited number of variables such as speed and positions of specimens. Recently, studies on movement tracks of rats have been conducted on dynamic perspective (Tchernichovski *et al.*, 1998; Tchernichovsky and Benjamini, 1998), and statistical discrimination of motion (Drai *et al.*, 2000) have been investigated in exploration behaviour. Kwak *et al.* (2002) selected a limited number of movement patterns of medaka and used artificial neural networks to detect changes in the movement tracks after treatments of diazinon.

In this study we concentrated on how the two-dimensional movement tracks would be differentiated after the treatments by insecticide and would further be verified through changes in the variables characterising response behaviour. The physiological impact of diazinon was additionally investigated with the toxicological tests on acetylcholine esterase activity.

2. Materials and Methods

Medakas, the "*or*" strain originally developed from Bioscience Center, Nagoya University, were obtained from Toxicology Research Center, Korea Research Institute of Chemical Technology (KRICT; Taejeon, Korea) for testing. The stock populations were maintained in a glass tank, and were reared with artificial dry diet (Tetramin®) under the light regime of L10:D14 at a temperature of 25 ± 1 °C. Tap water in the test aquarium was sufficiently dechlorinated by adding $Na_2S_2O_3$ (0.3 mg per 10 L) as well as by bubbling air under sunlight for 2 or 3 days (Yamamoto, 1967).

Diazinon (DongYang Chemical®; O,O-diethyl O-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate, 93.9%) dissolved in dimethylsulfoxide (DMSO; 10 mg L^{-1}), was applied at the concentration of 0.1 mg L^{-1} directly into an aquarium in which a 6–12 month old individual adult medaka (*Oryzias latipes*) (body length 3.44 ± 0.19 cm; body weight 2.91 ± 0.05 mg; $n = 10$) resided. The level of LC_{50} for diazinon against medaka was 5 mg L^{-1} (Kim *et al.*, 1999). During the observational period, individual specimens (n = 15) were placed in a 9 L glass aquarium (volume of water: 45 cm × 20 cm × 10 cm), and their position was observed from the side view with a CCTV camera (Kukjae Electronics Co. Ltd.; IVC-841®) at 0.25 sec intervals continuously before (2 days) and after (2 days) the treatments of diazinon.

In order to maintain stable conditions for the monitoring system, disturbances to the observation aquarium were minimised. Aeration, water exchange and food were minimised for behavioural monitoring. Other environment factors in the observation systems were maintained to the same condition of rearing the stock population. Before monitoring behaviour with the image processor, the specimens were acclimated to the observation cage for 1–2 days.

The analog data captured by the camera were digitised by using a video-overlay board (Dooin Electronics Co., LTD.; OSCAR III®), and were sent to the image recognition system to the target specimens in spatial and time domain. The software for recognition of the individuals through image processing and other mathematical analyses were produced in cooperation with Neural network and Real World Applications Lab., Division of Computer Science and Engineering, Pusan National University.

According to our experience on behaviour of test animals and suggestions in a previous study on movement tracks (e.g., Schal *et al.*, 1983; Collins *et al.*, 1994), we chose 19 candidate variables: speed (average in movement distance of

the fish during the observation time; mm sec^{-1}), acceleration (average in velocity (in magnitude) differences during the observation time; mm sec^{-2}), stop duration (the total duration in which the specimen did not move a distance less than 1/30 of body length in one sampling time period (0.25 sec); sec), locomotory rate (the total path length divided by the cumulated duration of movement excluding the stop duration; mm sec^{-1}), turning rate (the sum of angle changes in radian in absolute values divided by the cumulated time duration of movement; rad sec^{-1}), meander (the total abstract angle changes divided by the total path length; rad mm^{-1}), position on y-coordinate in average (average in distance on y-coordinate measured from the surface during the observation time; mm), maximum distance on y-coordinate (mm), location on x-coordinate in average (average in distance on x-coordinate measured from the surface during the observation time; mm), maximum distance on x-coordinate (mm), maximum distance (maximum difference in locations during the observation time; min), angular speed (turn-right, turn-left, turn-backward; radian), angular acceleration (average of velocity differences in magnitude term during the observation time; rad/sec^{-2}), number of stops in 60 sec segment, number of backward movements, and number of movement in four directions (left, right, up and down; number).

After preliminary tests we chose eight variables to characterise the movement patterns, such as speed, acceleration, stop duration, locomotory rate, turning rate, meander, position on Y-coordinate (Y-position), and maximum distance in Y-coordinate (Y-max). The variables were selected to represent the overall picture of two dimensional movement tracks regarding linear and angular activity, and vertical positions. The first four variables, speed, acceleration, stop duration and locomotory rate, represented strength and weakness of linear activity of the tested specimens. The next two variables expressed the angular activity in the tested specimens' movement: tendency in directional change (turning rate) and the degree of curvature (meander). The last two variables, position on Y-position and Y-max, represented vertical movement of fish in stressful conditions.

It has been shown in preparatory experiments that 1 min sequence was in general suitable for expressing the tracks' pattern. We visually checked all the recorded movement data in 1 min sequence for 15 specimens, and selected eight typical movement patterns that are frequently and distinctively observed from the tested specimens before and after the treatments. Among the visually selected patterns, we randomly chose 10 segments for statistical analyses.

Acetylcholine esterase (AChE) assay was further conducted on *Oryzias latipes* after being exposed to diazinon at a concentration of 0.1 mg L^{-1} for 0.5, 1, 2, 3, 4, 6 and 12 h. The fish was anaesthetised by submersing in ice-cold water and immediately dissected into head and body. Tissues were homogenised (approximately 20 mg of tissue per ml of phosphate buffer (pH 8.0, 0.1 M)) in a Polytron homogeniser (Kinematica AG, Swiss; RT1200®). AChE activities in the head and body were separately assayed in 45 mM phosphate buffer (pH 8.0), using 0.56 mM

acetylthiocholine as substrate according to Ellman *et al.* (1961). Protein in the tissue samples was quantified according to Lowry's method using bovine serum albumin as the standard (Lowry *et al.*, 1951).

3. Results

3.1. Movement patterns

Although there were individual variations, the specimens before the treatments showed common characteristics in the movement tracks. The untreated specimens usually spanned a wide range of the aquarium and frequently crossed the aquarium in a diagonal shape. The tracks did not have clear break points and appeared to be relatively smooth and linear. One pattern frequently observed was the active, long movement (pattern A; Figure 1a). In this case the test specimens continuously

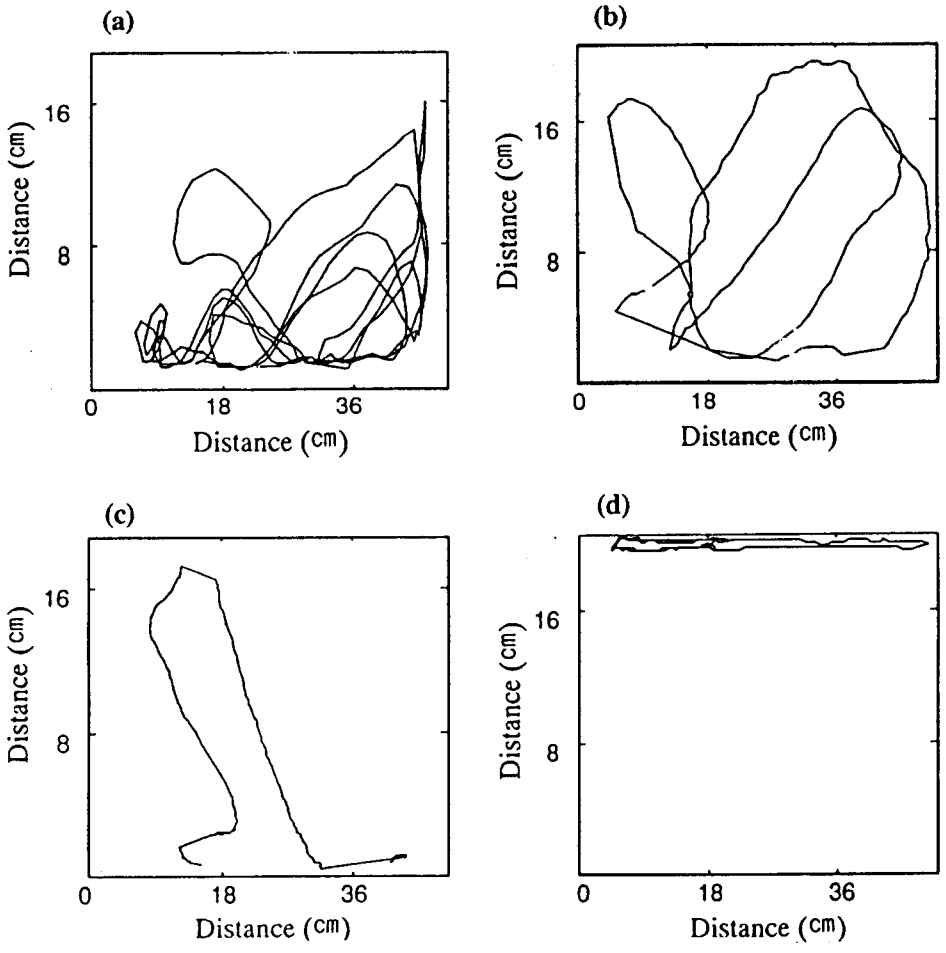


Figure 1. The movement tracks of medaka before the treatments of diazinon. (a) Long, active movement (pattern A), (b) movement with less activity (pattern B), (c) vertical movement (pattern C), and (d) surface movement (pattern D). Intervals in the *Y*-coordinate were enlarged to increase the resolution of vertical movement.

moved and tended to repeat the same routes: bottom-left to upper-right movement as shown in Figure 1a. These directional movements were occasionally observed in a reversed direction: bottom-right to upper-left movement.

Other patterns were also observed in the 1 min observation period and the patterns were, in general, differentiated according to the degree of activity and positions of the tested specimens. In addition to the active, long movement (pattern A), the test specimens were occasionally in the phase of less-activity (pattern B; Figure 1b). The specimens crossed the aquarium in a diagonal shape, spanning a wide area of the aquarium, but not with fast and repetitive movements in a higher degree as shown in pattern A (Figure 1a).

Sometimes the fish showed even less activity with the slow up–down movements, covering vertically only a limited area of the aquarium (pattern C; Figure 1c). Frequently the test individual stayed on the surface area of water and moved back and forth in the horizontal position repeatedly (pattern D; Figure 1d).

When the fish was affected by diazinon at a concentration of 0.1 mg L^{-1}, typical characteristics such as small-scale shaking movements were more frequently observed on the movement tracks. The treated specimens were, in general, less active, and pattern A shown in the untreated specimens (Figure 1a) was much less observed after the treatments. One typical pattern after the treatments was the vertical and horizontal movement in the observation aquarium (pattern E; Figure 2a). The vertical and horizontal movements were separately observed in pattern E. The test specimens moved vertically for a while, and the vertical movement was followed by repetition of the horizontal movement. These vertical and horizontal movements were repeated during the observation period in a variable number of times. While the specimens moved either vertically or horizontally, the tracks were frequently interspersed with irregular shaking patterns.

Another typical pattern displayed by the treated specimens in the observation aquarium was the sequential, vertical–horizontal–vertical movement (pattern F; Figure 2b). In contrast to pattern E, after the fish vertically moved up to the surface it continuously made a horizontal movement to cross the observation aquarium. Then the specimen moved down vertically along the other side of the observation aquarium. The sequence of vertical–horizontal–vertical movement was continuously repeated during the observation period. Similarly to pattern E, however, the movement tracks were frequently interspersed with irregular shaking patterns (Figure 2b).

A slow vertical movement was also frequently observed in the treated specimens (pattern G; Figure 2c). The shaking movement, however, appeared to be stronger in the vertical movement. The fish additionally showed sharper angle changes, being contrasted with the untreated specimen's round angle turns in the vertical movement in pattern C (Figure 1c). In each vertical movement, the shaking pattern was more strongly observed during the upward climb than during the downward movement.

The treated specimens were also observed to stay at the surface area for a longer time (pattern H; Figure 2d). The shaking movement intermittently appeared on

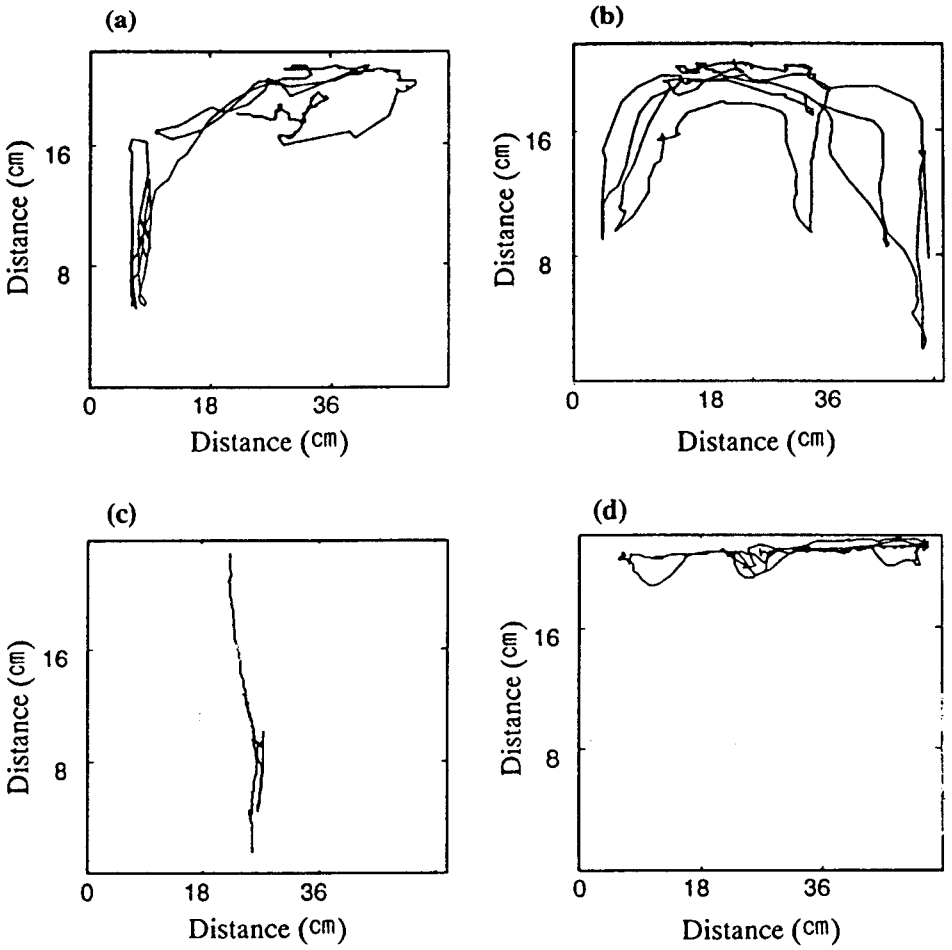


Figure 2. The movement tracks of medaka after the treatments of diazinon. (a) Interrupted vertical–horizontal movement (pattern E), (b) continuous vertical–horizontal vertical movement with less activity (pattern F), (c) vertical movement with shaking (pattern G), and (d) surface movement with shaking (pattern H). Intervals in the *Y*-coordinate were enlarged to increase the resolution of vertical movement.

the movement tracks, and the up–down movements in a limited length were also more frequently observed. The surface movements before (pattern D; Figure 1d) and after (pattern H; Figure 2d) the treatments were clearly contrasted when the movement tracks were observed with the time progress as shown in Figure 3 (*Y*-coordinate; time). While the simple back-and-forth movements were observed with the untreated specimens at the surface in pattern D (Figure 3a), the intermittent 'shaking' movements frequently appeared on the contour of the back-and-forth movements of the treated specimens in pattern H (Figure 3b).

3.2. Characterisation of Movement Tracks

The variables appeared differently depending upon the movement patterns (Figure 4). For the longer, active movement (pattern A; Figure 1a), speed, acceleration, and *Y*-position were in the highest range while stop duration and meander

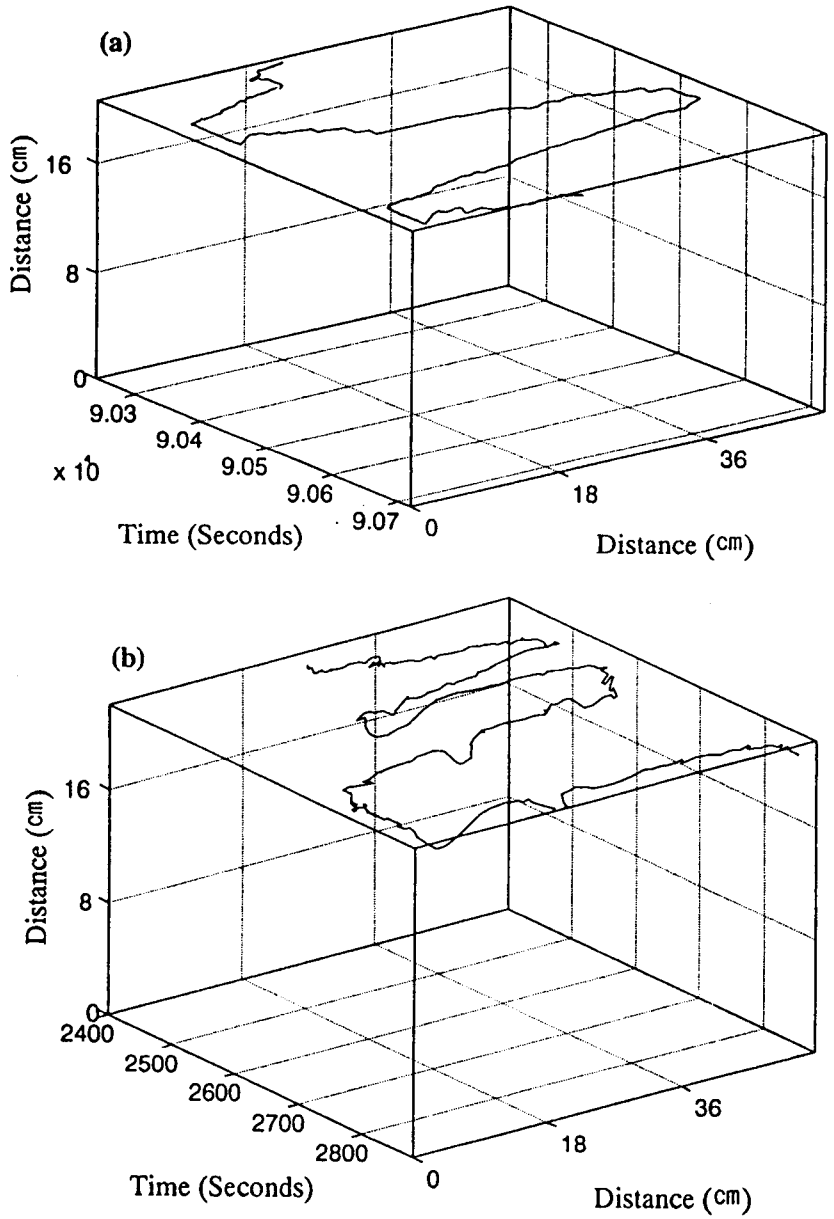


Figure 3. Comparison of the surface movements of medaka before and after the treatments of diazinon as time progressed (Y-coordinate). (a) Before treatment (pattern D), and (b) after treatment (pattern H). Intervals in the Y-coordinate were enlarged to increase the resolution of vertical movement.

were in the lowest range. For the less active movement before the treatments (pattern B; Figure 1b), speed, acceleration and locomotory rate were lower than for pattern A. Stop duration, meander and turning rate were in similar range in both patterns. The up–down movement (pattern C; Figure 1c) was also contrasted with the long, active movement of pattern A: turning rate and meander appeared to be higher, while speed, acceleration and locomotory rate were lower in pattern C.

For the surface movement (pattern D; Figure 1d), the variables were generally contrasted with the typical active movement, pattern A, by showing lower levels of speed, acceleration, Y-position and turning rate (Figure 4). Only meander was

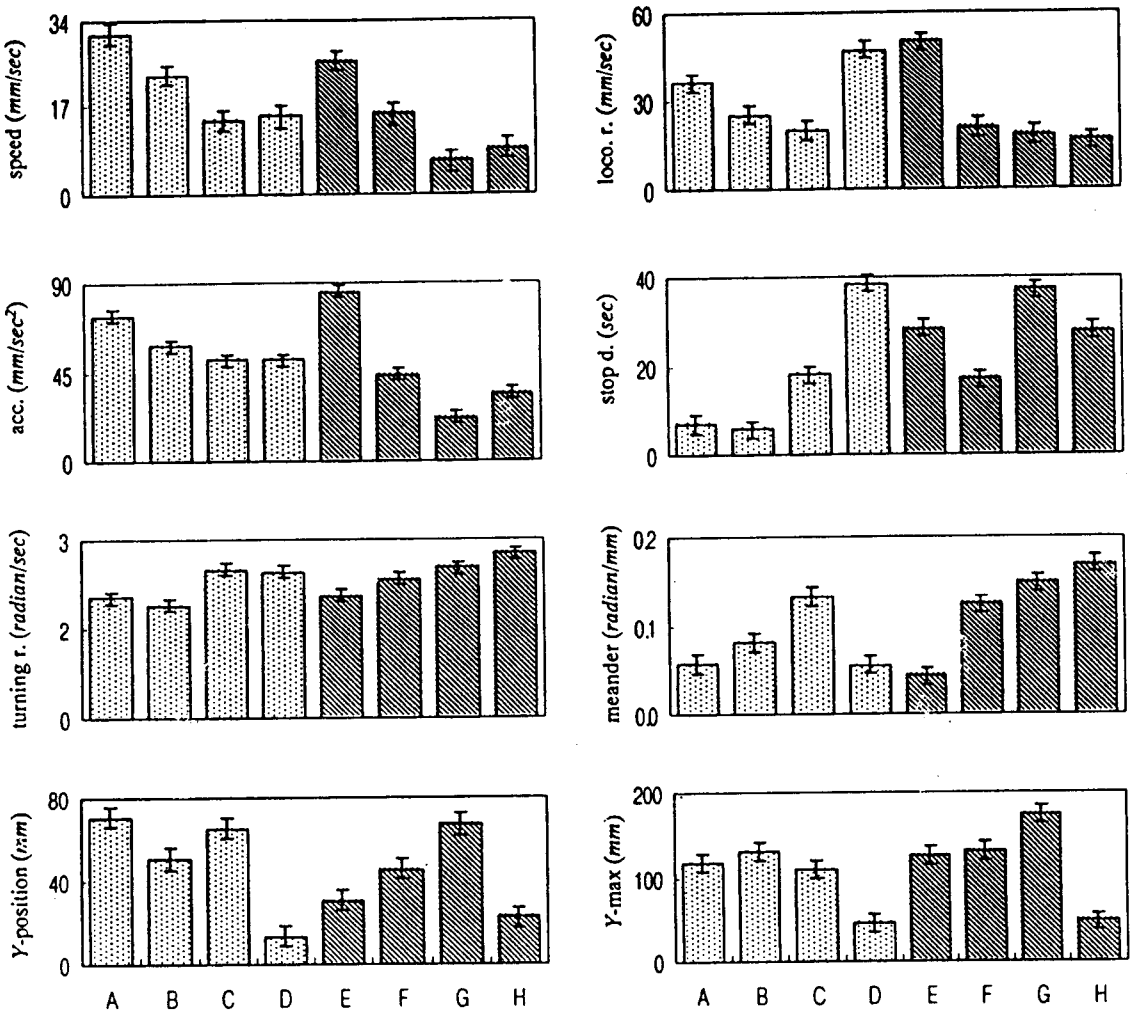


Figure 4. Variables characterising the movement tracks of medaka among different patterns before and after the treatments of diazinon ($n = 10$ for each parameter for each pattern).

similar in the lowest range for both patterns. Pattern D was also comparable with the vertical movement, pattern C. Meander and Y-position were lower in pattern D, while stop duration and locomotory rate were lower in pattern C. For both patterns speed and acceleration were similarly low while turning rate was high in common. As expected, Y-max and Y-position showed the lowest values in pattern D, the horizontal movement along the surface area.

The variables of the movement tracks appeared differently after the treatments. In general, speed was decreased in the movement patterns observed after the treatments. Pattern E (Figure 2a), the separate horizontal and vertical movement, was comparable with pattern A (Figure 1a) before the treatments. While speed was lower in pattern E, acceleration and locomotory rate were relatively higher compared with those in pattern A (Figure 4). Additionally, stop duration was higher in pattern E. This indicated that, in pattern E, the specimens rested more frequently but were stronger in activation of new movement. Overall, it was observed that pattern E was less affected by the insecticide, showing the highest values of speed,

acceleration and locomotory rate among the patterns observed after the treatments (Figure 4).

The variables of pattern F, the continuous vertical–horizontal–vertical movement (Figure 2b), were highly contrasted with the variables of pattern E. Speed, acceleration, locomotory rate and stop duration were distinctively lower, while meander was higher in pattern F. Consequently pattern F appeared to be more strongly affected by the insecticide than pattern E.

In the vertical movement after the treatments (pattern G; Figure 2c), speed, acceleration and locomotory rates were in the lowest range, while stop duration and Y-max were in the highest range (Figure 4). This indicated that pattern G represented a strong intoxication of the specimens. Pattern G was contrasted with pattern C, the vertical movement frequently observed before the treatments (Figure 1c). Speed and acceleration were lower while stop duration was higher in pattern G. The surface movement after the treatments (pattern H; Figure 2d) was also contrasted with the surface movement before the treatments, pattern D (Figure 1d). In pattern H, locomotory rate, speed, acceleration and stop duration were distinctively decreased while meander was increased.

Although the variables were contrasted among the movement patterns, overall differences in the variables were not clearly addressed before and after the treatments. According to the nested ANOVA, all the variables were not significantly different 'before' and 'after' the treatments (group; Table I). In contrast, all the variables among the movement patterns 'within the treatment' (subgroup) were

TABLE I

Nested analysis of variance (ANOVA) on the variables characterising different movement patterns of medeka before and after the treatments of diazinon ($n = 10$ for each parameter for each pattern)

	Treatments (nested)			
	Between treatments[a] (group)		Among patterns[b] (subgroup)	
Parameters	F	P[c]	F	P
Speed (mm sec $^{-1}$)	3.724	$0.1 < P < 0.2$	5.147	<0.001
Acceleration (mm sec^{-2})	2.143	$0.2 < P < 0.5$	3.915	<0.001
Locomotory rate (mm sec^{-1})	0.997	>0.5	7.739	<0.001
Stop duration (sec)	4.253	$0.1 < P < 0.2$	5.549	<0.001
Turning rate (radian sec^{-1})	2.627	$0.2 < P < 0.5$	0.550	>0.50
Meander (radian mm^{-1})	4.336	$0.1 < P < 0.2$	4.002	<0.001
Y-position (mm)	0.842	>0.5	4.110	<0.001
Y-max (mm)	0.984	>0.5	11.705	<0.001

[a] H_0: No difference between treatments (group), $F_{0.05}(2), 1, 18 = 5.98$.
[b] H_0: No difference among patterns (subgroup), $F_{0.05}(2), 18, 60 = 1.98$.
[c] Number of significant digits: 3.

significantly different except the turning rate. This confirmed that the movement patterns in subgroup were highly diverse: the variables' deviations were higher and were difficult to be differentiated between groups, 'before' and 'after' the treatments. According to F values, differences were mostly high on Y-max and locomotory rate (Table I).

Within each subgroup of 'before' and 'after' treatment, we investigated how the variables were differentiated in different movement patterns. Tukey test was conducted to check statistical differences for each variable (Table II). After the treatments, all variables were significantly different without exception. Exceptionally high F values were obtained on acceleration, locomotory rate and Y-max. Turning rate, however, showed relatively little difference among the patterns.

Before the treatments, the majority of the variables were also significantly different among the patterns except turning rate and acceleration (Table II). Y-position, stop duration and Y-max showed distinctively high F values. Speed, for instance, was significantly different among the patterns A, B and C before treatments, and was also differentiated clearly among the patterns E, F and H at high values after the treatments. Acceleration was characteristically different before and after the treatments. While acceleration was not different in the different movement patterns before the treatments, it was significantly different among all the movement patterns after the treatments (Table II). This demonstrated that the acceleration (i.e., changes in speed) would be critical in differentiating movement patterns after the treatments.

The surface movement D showed the highest locomotory rate, being significantly different from the active movement A (Table II). This indicated that test specimens move quickly once they start to move at the surface of the aquarium. For the surface movement pattern H after the treatment, however, locomotory rate showed the lowest value. As expected, stop duration was higher in patterns D and C before the treatments. After the treatments, the vertical movement G showed the highest value in stop duration. In contrast to pattern D, stop duration was relatively lower in the horizontal movement pattern H after the treatments.

Turning rate appeared not to be different among the movement patterns in general except the surface movement H after the treatments (Table II). In contrast, meander was more clearly differentiated compared with turning rate, especially before the treatments. Y-position was different at low levels among the movement patterns D, B and A before the treatments, while the differences were shown between the patterns F and G at high levels after the treatments. Maximum Y-position was different among all the patterns before the treatments. After the treatments, differences were partially observed between pattern F and pattern G at high values, and between pattern H and pattern E at low values.

Although the overall differences were not observed collectively in the variables before and after the treatments, variables were more clearly differentiated when the movement patterns were correspondingly matched before and after the treatments (Table III). For patterns A and E, which showed the highest activity before and after

TABLE II

Comparison of the variables characterising different movement patterns of medaka by analysis of variance (ANOVA) and Tukey test separately conducted before and after the treatments of diazinon ($n = 10$ for each parameter for each pattern)

	Before treatment			After treatment		
Parameters	F[a]	P[b]	Comparison of parameters[c] (Tukey test, $\alpha = 0.05$)	F	P	Comparison of parameters (Tukey test, $\alpha = 0.05$)
Speed (mm sec^{-1})	11.777	<0.001	C = D ≠ B ≠ A	35.419	<0.001	G = H ≠ F ≠ E
Acceleration (mm sec^{-2})	2.022	$0.2 < P < 0.5$	C = D = B = A	81.804	<0.001	G ≠ H ≠ F ≠ E
Locomotory rate (mm sec^{-1})	13.441	<0.001	C = B ≠ A ≠ D	76.563	<0.001	H = G = F ≠ E
Stop duration (sec)	29.852	<0.001	B = A ≠ C ≠ D	9.406	<0.001	F ≠ H = E ≠ G
Turning rate (radian sec^{-1})	1.374	>0.5	B = A = D = C	3.646	$0.02 < P < 0.05$	E = F = G ≠ H
Meander (radian mm^{-1})	11.285	<0.001	D = A ≠ B ≠ C	16.386	<0.001	E ≠ F = G ≠ H
Y-position (mm)	30.086	<0.001	D ≠ B ≠ C = A	7.869	<0.001	H = E = F ≠ G
Y-max (mm)	29.714	<0.001	D ≠ C ≠ A ≠ B	53.816	<0.001	H ≠ E = F ≠ G

[a] $F_{0.05}(2), 3, 36 = 3.52$.

[b] Number of significant digits: 3.

[c] Patterns were listed in the increasing order from left to right.

TABLE III

Comparison of the variables of the movement tracks of medaka between corresponding patterns before and after the treatments of diazinon ($n = 10$ for each parameter for each pattern)

Patterns		Speed	Acceleration	Locomotory rate	Stop duration	Turning rate	Meander	*Y*-position	*Y*-max
					Before and after treatment				
A ↔ E	*t*[a]	2.227	1.644	4.138	6.903	0.123	4.158	5.485	1.111
	P[b]	$0.02 < P < 0.05$	$0.1 < P < 0.2$	<0.001	<0.001	>0.5	<0.001	<0.001	$0.2 < P < 0.5$
A ↔ F	*t*	5.441	3.844	5.062	2.452	1.182	3.477	2.530	1.297
	P	<0.001	$0.001 < P < 0.002$	<0.001	$0.02 < P < 0.05$	$0.05 < P < 0.1$	$0.002 < P < 0.005$	$0.02 < P < 0.05$	$0.2 < P < 0.5$
B ↔ E	*t*	0.925	2.763	6.991	13.458	1.128	5.735	3.706	0.572
	P	$0.2 < P < 0.5$	$0.01 < P < 0.02$	<0.001	<0.001	$0.2 < P < 0.5$	<0.001	$0.001 < P < 0.002$	>0.5
B ↔ F	*t*	2.019	1.441	1.277	3.580	1.912	2.188	0.602	0.023
	P	$0.05 < P < 0.1$	$0.1 < P < 0.2$	$0.2 < P < 0.5$	$0.002 < P < 0.005$	$0.05 < P < 0.1$	$0.02 < P < 0.05$	>0.5	>0.5
C ↔ G	*t*	4.461	3.649	0.698	5.437	0.100	0.609	0.152	9.005
	P	<0.001	$0.001 < P < 0.002$	$0.2 < P < 0.5$	<0.001	>0.5	$0.2 < P < 0.5$	>0.5	<0.001
D ↔ H	*t*	2.093	3.493	5.757	2.009	0.694	7.792	2.442	0.158
	P	$0.05 < P < 0.1$	$0.002 < P < 0.005$	0.001	$0.05 < P < 0.1$	>0.5	<0.001	$0.02 < P < 0.05$	>0.5
					Within treatment				
E ↔ F	*t*	3.942	8.553	9.554	3.772	1.370	4.183	0.581	1.822
	P	<0.001	<0.001	<0.001	$0.001 < P < 0.002$	$0.1 < P < 0.5$	<0.001	>0.5	$0.05 < P < 0.1$

[a] *t*-values in absolute; $t_{0.05(2),18} = 2.101$.

[b] Number of significant digits: 3.

the treatments, respectively, stop duration, *Y*-position, meander and locomotory rate were highly different. Speed was also significantly high in pattern A. When pattern A was compared with pattern F, which showed a stronger response than pattern E (e.g., Figure 4), speed, locomotory rate and meander were highly different. Acceleration was additionally lower in pattern F (Table III). The less active movement B before the treatments was also comparable with the active response pattern E after the treatments. Stop duration, locomotory rate, meander and *Y*-positions were clearly contrasted while speed, turning rate and *Y*-max were not significantly different. When pattern B was compared with pattern F, only stop duration and meander were significantly different.

The differences in variables were also observed on the corresponding vertical movements between patterns C and G. *Y*-max, stop duration, speed and acceleration were highly different between two patterns. Patterns D and H, respectively for the surface movements before and after the treatments, were different in meander, locomotion rate, acceleration and *Y*-position (Table III).

Especially in patterns E and F, which showed different degree of response to the treatments (Figures 2 and 4, and Table II), the difference in the movement patterns was more elaborated for each parameter in Table III. Speed, acceleration, locomotory rate, meander and stop duration were distinctively lower in pattern F.

3.3. Occurrence of response behaviour

Although time occurrence of response behaviour was variable in individual specimens, there was consistency in the initiation of response behaviour. The affected movement behaviour appeared mostly 3–6 h after the treatments of the chemical. Table IV shows the frequency of observations of 'treated movement patterns' in each 10-min duration in 1-h period. The frequency observed in 1-h period was recorded during the total observation period (10 h) after the treatments for each specimen. Since variations existed in response behaviour and it was difficult to classify clearly all the input segments of the movement tracks by visual judgment, the frequency for the treated patterns shown in Table IV was counted conservatively only when any response behaviour (Figure 2) was observed consistently longer than 70% of the 10 min observation time. The variations in response behaviour were observed among different specimens. Among the 15 individuals observed, 13 specimens clearly showed the patterns of the treatments during 10 h observation period. The on-set of symptomatic behaviour ranged from 2:10 (h:min) to 6:50 after the treatments (Table IV). The frequency for the response behaviour rapidly increased in 3–4 h after the treatments. Six specimens showed the movements patterns of the treatments at this time period while only one individual showed response behaviour in the 2–3 h period. The frequency peaked in 6–7 h, and nine specimens produced the treated patterns. The occurrence of the treated patterns decreased afterwards.

TABLE IV

The frequency of the characteristic movement tracks of medaka, which had been affected by the treatments of diazinon (0.1 mg L^{-1}) as time progressed (The movement tracks of medaka were observed discontinuously at intervals of 10 min for 10 h)

No. specimens	Time of initial response (h:min)	Observation time (h)										Sum
		0–1	1–2	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	
1	3:20				2		3	4	2		3	14
2	4:10					2	1	2	3	6		14
3	4:40					1	4		1			6
4	6:50							1	1			2
5	5:40						2	1		1		4
6	3:10				4							4
7	2:10			2	2		4	4				12
8	4:40					1		1				2
9	3:20				4	1	1					6
10	4:50					2	4	4	3			13
11	4:20					1	2	4	1			8
12	3:30				2	4	5	6	6	1		24
13	3:50				2	1						3
14	–											–
15	–											–
Total no. observation				2	16	13	26	27	17	8	3	112
No. responding specimens				1	6	8	9	9	7	3	1	

3.4. ACTIVITY OF ACETYLCHOLINE ESTERASE

Figure 5 shows toxicological test for the decrease in acetylcholine esterase activity when medaka was exposed to diazinon at 0.1 mg L^{-1}. Diazinon inhibited the enzyme activities both in the head and body with similar pattern. In 30 min after the treatment, AChE activity in the head rapidly decreased from 179 ± 29 to 121 ± 32 nM substrate hydrolysed min^{-1} mg^{-1} protein, and the enzyme activities in the body from 573 ± 38 to 264 ± 54 nM substrate hydrolysed min^{-1} mg^{-1} protein. Between 1 and 4 h after the treatment, the acetylcholine esterase activity remained relatively stable. After this period, however, the enzyme activity appeared to decrease again, 4 h in the head and 3 h in the body after the treatments. In the period of 3–4 h, the response behaviour clearly occurred in the tested specimens. This was in accord with the appearance of the high frequency of the affected response behaviour in this period (Table IV). The level of cholinesterase activity at the corresponding

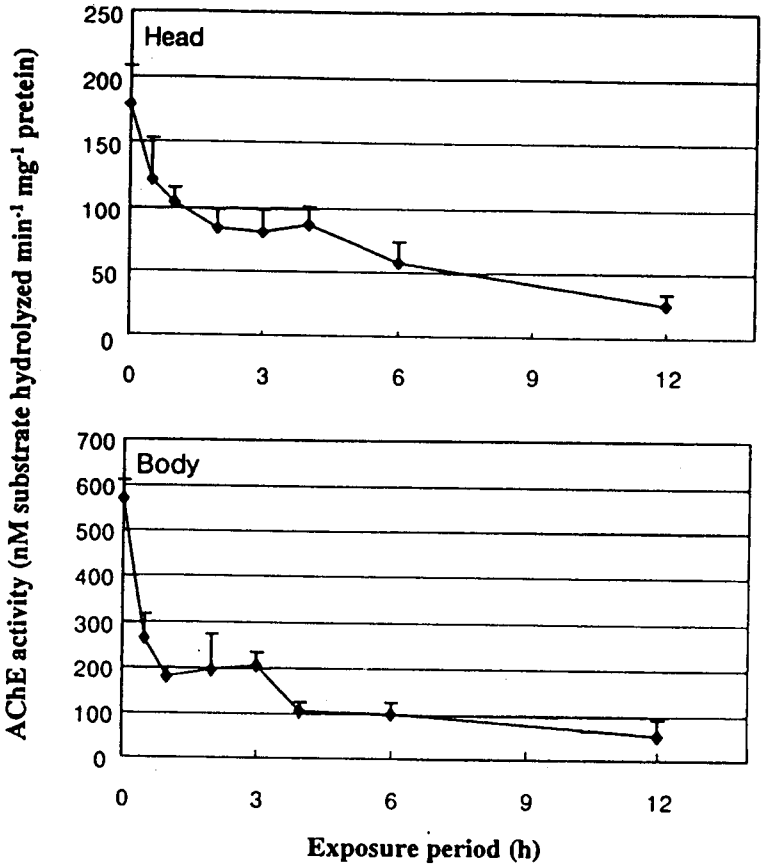


Figure 5. Change in acetylcholine esterase activities of medaka fish after being exposed to diazinon (0.1 mg L^{-1}).

time was 87 ± 13 nM substrate hydrolysed min^{-1} mg^{-1} protein in the head and 102 ± 20 nM substrate hydrolysed min^{-1} mg^{-1} protein in the body. This indicated that the decrease in AChE activities by 51–82% was required to cause the clear characteristic responsive behaviour of the fish treated with diazinon. In the early phase of decrease in AChE activity in 1 h as shown in Figure 5, however, it was difficult to detect the response pattern in locomotion at the tested concentration of diazinon at 0.1 mg L^{-1}.

4. Discussion and Conclusions

This study demonstrated that behavioural differences could be effectively detected in response to diazinon through analyses of the continuously observed movement tracks. The decrease in the cholinesterase activity (Figure 5) indicated that the movement tracks reflected disturbed status of the nervous system in the tested specimens after the treatments, and that the behavioural response of specimens against treatment of diazinon at low concentraton had neuro-physiological background.

The variables of the movement tracks characteristically represented the different patterns as shown in Tables I–III. While overall differences in variables were not

observed before and after the treatments (Table I), the differences were effectively revealed when the corresponding patterns were directly compared on 'one-to-one' basis before and after the treatments (Table III). This indicated that behavioural changes could be more clearly addressed between corresponding movement patterns. For example, pattern G, the vertical movement after the treatments with shaking (Figure 2c), was clearly contrasted with pattern C, the vertical movement before the treatments (Figure 1c; Table III). Similarly, the surface movement H after the treatments (Figure 2d) was also characteristically different from the movement pattern at the surface movement D before the treatments (Figure 1d). It also seems reasonable that the movements before the treatments such as patterns A and B could be projected to patterns E and F after the treatments. Differences in variables were demonstrated when the patterns were correspondingly matched (Table III). This indicated that detection of toxic behaviour would be more conveniently conducted if corresponding behaviours were elucidated in different variables.

The movement patterns further revealed different degree of chemical impacts. Pattern E indicated less disturbance compared with pattern F (Figure 4). As stated above, pattern F reflected stronger response to the insecticide, and this was confirmed by statistical differences in variables: speed, acceleration, locomotory rate and stop duration were distinctively lower while meander was higher in pattern F (Table III). Additionally, this suggested that different degree of response behaviours could be detected through quantitative characterisation of the variables.

When the corresponding movement patterns were matched, meander and stop duration were in general most highly different among the selected patterns, followed by locomotory rate, acceleration and Y-position (Table III). This contrasted with the results for comparing the whole movement patterns (Table I), where no differences in the variables were observed between the movement patterns before and after the treatments.

With a few exceptions, all F values were increased after the treatments (Table II), indicating the greater impact of the insecticide on causing variability of movement behaviour. This suggested that the spectrum of response behaviour was more 'scattered' after the specimens were affected by the insecticide, and consequently complex response behaviour was produced after the treatments. Acceleration deserves an attention in differentiating response behaviours in this regard (Table II). The variable was statistically different among all the movement patterns after the treatments, while it was in the similar range in different movement patterns before the treatments (Table II). Further physiological and behavioural study is required in the future to reveal the mechanism of causing different levels of acceleration in response behaviours.

As shown in Tables I–III, one parameter could not be used as a sole indicator for detecting difference in the movement patterns. This implied that the patterns should be characterised in a multivariate fashion. Considering that the image processors designed for detecting the movement tracks in a sequence produce voluminous

amount of data, it is not a simple task to classify continuously and objectively each input segment of the movement tracks on real-time basis. Development of automatic pattern recognition techniques for patterning the complex data of the movement tracks is needed in the future, and this will be discussed elsewhere.

Individual variability in response behaviour was a problem in patterning the movement tracks. The movement behaviour was variable among the tested specimens, and variability was additionally observed in behaviour patterns within one specimen as time progressed. Besides the movement patterns shown in Figures 1 and 2, numerous other movement tracks were suspected to represent other movement patterns before or after the treatments. With more sophisticated classification methods, these movement tracks could be further identified. Additionally, the period of acclimation might be related to producing variability in movement behaviours, and more consideration should be given in this regard in the future. As shown in Table IV, the specimen No. 14 and No. 15 did not show response behaviours. This may be due to variation in sensitivity of individual specimens against toxic treatment, since a low level of insecticide was applied to the specimens and expression of abnormal behaviour would not be deterministic at low concentrations. However, some internal (e.g., physiological stress) or external (e.g., environmental instability) disturbances may be still relevant to insensitivity of the specimens to the treatments, and further toxicological and behavioural study is needed in the future.

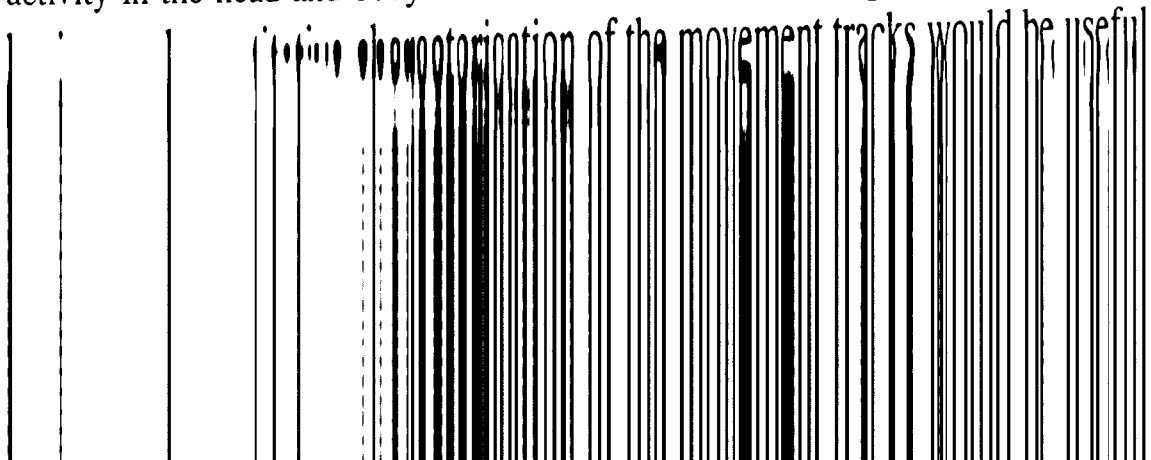
Food and oxygen were not provided to test specimens during the acclimation and observation period. If the acclimation and observation periods were prolonged, hunger and oxygen deficiency would be stressful to the test specimens. In our previous experiments, in general, however, the behaviours specifically caused by experimental conditions were not clearly observed. For instance, the medaka specimens did not show distinctive breathing behaviour or loss of activity in response to deficiency in oxygen during the observation period. Although the test specimens consistently showed frequent movement in the near surface of the test aquarium both before and after the treatment in accordance with the fact that the medaka is well known as a typical surface swimmer (Yamamoto, 1967), the surface movement D (Figure 3a) did not show breathing behaviour clearly. The surface movement H after the treatments (Figure 3b) was characteristically dominated by intoxicated behaviour of shaking, and breathing behaviour was neither apparent in this case. Since medaka is the inhabitant of stagnant waters or slowly streaming waters rather than rapidly flowing rivers, they do not need much oxygen. If the fishes are not unduly crowed, aeration is unnecessary (Yamamoto, 1967). In Korea it has been generally noticed that the fish species lives in shallow lakes, agricultural irrigation channels or rice pads, and that medaka usually inhabits in the water that the dissolved oxygen concentration is about 5 mg L^{-1} . The dissolved oxygen concentration in the water used in the experiment was saturated through continuous aeration for several days before to use in the experiment and maintained about 7 mg L^{-1} at the end of experiment. Therefore we considered that the concentration of dissolved oxygen did not influence the changes in movement patterns after the

treatment of diazinon. Similarly, feeding behaviour was not specifically increased. In general, those stressful conditions tended to change behaviours of medakas after 7–10 days in our experimental conditions.

In toxicological tests, the cholinesterase activity was initially decreased in 30 min, and was stable 1–4 h after the treatments of the chemical (Figure 5). In the early period (1–3 h), however, it was difficult to detect response behaviour based on the observer's judgments, as previously mentioned (Table IV). The affected movement tracks more clearly appeared after acetylcholine esterase activity was further decreased to the substantially low level below 100 nM substrate hydrolysed min^{-1} mg^{-1} protein. In a few specimens, although they showed short segments of movement tracks similar to the patterns shown in Figures 2, those were somewhat sporadically observed and were not sufficiently continuous to be counted as the obvious characteristic movement patterns after the treatment. In the present time it is unknown how the affected behaviour would appear under a certain threshold of acetylcholine esterase activity. It seems that acetylcholine should be accumulated to a certain level in the head and/or body prior to causing behavioural changes sufficiently.

The inhibition of cholinesterase activity is persistent after the treatment of the insecticide, and the low levels of AChE activity were continuously observed for 6–12 h (Figure 5). However, the frequency of responsive behaviour peaked 6–7 h after the treatment and subsequently decreased to low level from 8–10 h after the treatment (Table IV). This indicated that there exists either desensitisation process or some other compensatory pathways to adaptively resume the normal behaviour in the context of physiological homeostasis of the treated specimens. However, further verifications through interdisciplinary neuro-behavioural studies are needed in this regard in the future.

In conclusion, although relatively large variations occurred in individual behaviours, some characteristic patterns in the movement tracks, repetitive small-scale shakings and irregular turns could be identified in the treated specimens with diazinon at sublethal treatments. The differences in the variables representing the movement tracks could be efficiently elucidated by comparing the corresponding patterns of the movement tracks before and after the treatment. The appearance of response behaviour in 4–5 h after the treatments of diazinon was in accord with the toxicological test with a significantly reduced level of acetylcholine esterase activity in the head and body at this time. Identification of specific response be-



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