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Planarians in Toxicology, Standardization of a Rapid Neurobehavioral Toxicity Test Using Phenol in a Crossover Study

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Planarians are free-living flatworms which are important to the aquatic ecology of unpolluted streams (Hyman 1951; Kenk 1976). These 2 cm long animals are very sensitive to low concentrations of environmental toxicants (Kostelecky 1988) and have been used in Europe for decades to detect pollutants in water (Kolkwitz and Marson 1908; Steinmann and Breslau 1913; Kenk 1976). Planarians have a sensitive nervous system with synapses (McConnell 1965) and a true brain (Best and Noel 1969). Consequently, they show complicated social behavior such as a crowding effect on fissioning (Best et al. 1974) and on sexual reproduction (Jenkins 1974; Kostelecky et al. 1989). However, the behavioral patterns of planarians exposed to a toxicant have not been described, and thus forms the subject of this paper.

MATERIALS AND METHODS

Dugesia dorotocephala (Carolina Biological Supply, Gladstone, Oregon) were maintained in the laboratory at 19 C in synthetic media (Kostelecky et al. 1989). Animals were fed once-weekly for 3 hr on beef liver and then placed in fresh media. Standard tests (Kostelecky 1988) were carried out at 21-22 C by adding five planarians to media containing a selected concentration of phenol.

The standard five animal test was not sufficient for the statistical analyses needed to characterize the effects of concentration and time on the behavioral response patterns. Consequently, a crossover study using 25 animals in each of four groups in a 4 x 4 Latin square design (Cochran and Cox 1957) was carried out. Groups were columns, periods were rows, and concentrations (1, 22, 43, 64 mg/L) were treatments. Behavioral responses were recorded at 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, and 60 min of exposure. Between periods, animals were maintained for 48 hr in clean media. The complete crossover experiment was repeated three times. Several distinct behavioral responses were scored (Table 1).

Data from the standard tests and crossover experiments were analyzed using Fourier analysis and multivariate repeated measures profile analysis (Morrison 1976) using concentration as the grouping variable. Results from each endpoint in the crossover study were analyzed as a Latin square without carryover effects (Cochran and Cox 1957). Crossover data were also analyzed using K-means cluster analysis (Hartigan 1975) and stepwise linear regression.

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Table 1. Behavioral Responses of Planarians by Response Group

Response		Description
		LOCOMOTIVE
(1)	Restlessness	Forward movement usually faster than normal; head raised off glide surface; frequent position switches.
(2)	Hyperkinesia	"Inch-worming"; glide not smooth; almost always forward movement; few/none abrupt direction changes.
(3)	Swims upside down	
		MORPHOLOGICAL
(4)	Spiraling	Corkscrew-shaped movements. Animal twists around cranio-caudal axis.
(5)	Head/nose twist	Tip of nose curls up or down.
(6)	Shape change	Loss of normal body shape, e.g., extreme elongation or dumbbell shape.
(7)	Ornamentation	Edges of animal become crenelated.
(8)	Banana curl or coil	Horseshoe shape, head brought closer to tail either distal or ventral.
		NEUROLOGICAL
(9)	Convulsions	Violent twitching; no forward motion; rapid coiling or twisting in quick, thrashing motion.
(10)	Nervous signs	Abrupt direction changes and/or lack of coordination.
		MORBIDITY
		epidermis are discharged).
(11)	Labored movement	Animal is stationary or moving slowly but moves normally when poked.
(12)	Depression	Animal barely moves when poked, stationary otherwise.
(13)	Unconsciousness	Animal does not move when poked but responds when placed in clean media.
(14)	Death	Does not move when poked or revive in clean media; autolyses rapidly.
		PROTECTIVE
(15)	Pharynx protrusion	Pharynx extends outside the body.
(16)	Vomiting	Pharynx discharge.
(17)	Mucus covering body	Covered with mucus (rhabdite cells in
(18)	Lesions	Loss of structural integrity.

Responses were grouped as **Loc**omotive [(1)-(3)], **Morp**hological [(4)-(8)], **Neuro**logical [(9)-(10)], **Morb**idity [(11)-(14)], and **Prot**ective [(15)-(18)]. Times were pooled into the three groups of 1-5 min, 10-30 min, and 40-60 minutes. Stepwise linear regression analysis was used to develop the relationship between the cumulative number of responses for a response category,

the observation time, and concentration.

RESULTS AND DISCUSSION

For the first crossover, multivariate profile analysis and Fourier analysis of the means showed that some responses were affected by concentration and time. Responses such as restlessness (1) and spiraling (4) occurred within 5 minutes at 1 mg/L. Other responses, including head/nose twist (5), shape change (6), nervous signs (10) and labored movement (11) increased with time and concentration. Similar analyses were carried out separately with the two subsequent trials and with pooled data from the three trials. Responses (13), (14) and (17) were not observed and responses (3), (9), (15) and (16) occurred infrequently (mean $<<1.0$). Dropping these from the cluster analysis, four exposure/time groups were formed using K-means clustering (Hartigan 1975) of the summed responses from the three crossovers (Table 2). Subsequent single linkage Euclidean distance cluster analysis of the columns in Table 2 gave four response clusters: I (1) restlessness, (2) hyperkinesia; II (4) spiraling, (5) head/nose twist; III (6) shape change; IV (11) labored movement.

Table 2: Crossover Cumulative Responses by Cluster

		Responses[1]					
Cluster[1]	Conc[2]	1	2	4	5	6	11
I	1A	41	3	3	0	1	139
	1B	4	0	1	9	6	183
	2A	176	10	11	2	1	103
	2B	28	2	11	15	4	249
	3A	276	116	20	29	29	199
	3B	66	46	19	77	81	204
II	1C	0	0	2	16	2	430
	2C	3	1	10	23	12	563
III	4A	317	445	73	64	520	179
IV	3C	17	14	9	272	326	575
	4B	15	159	32	218	662	416
	4C	1	49	3	272	525	462

[1]Cluster I=(1) Restlessness+(2) Hyperkinesia, Cluster II=(4) Spiraling+(5) Head/nose twist, Cluster III=(6) Shape Change, Cluster IV=(11) Labored movement.
[2]1=1 mg/L, 2=22 mg/L, 3=43 mg/L, 4=64 mg/L. A=1-5 min, B=10-30 min, C=40-60 min.

Table 2 does not make clear the temporal and concentration relationships for individual responses. Therefore, the pooled response counts in each category (**Loc, Morp, Neuro, Morb, Prot**) were regressed against concentration, time group and concentration x time group. Few animals had protective responses so models could not be developed for the **Prot** category. The combined data from the three crossovers were similarly analyzed. A regressor identifying the crossover trial was not significant ($P > 0.05$) for any response category.

The number of responses in a category increased asymptotically with time and concentration. The models in Table 3 show that for a given response category, similar regressors were important in each crossover trial. Thus, **Loc** was related to concentration and concentration x time in each trial. The signs of the terms in the **Loc** models show that the number of locomotive responses increased with concentration but that the rate of increase was lower at higher concentrations. **Morp** was related to concentration x time in the three trials and to time in trials 2 and 3 and 1+2+3. The number of **Morp** responses tended to decrease with time, but this decrease was slower at higher concentrations. **Neuro** was related to concentration in the three trials and overall. Time was significant in two trials and overall. The results suggest a marginally significant (concentration x time) interaction since this term was significant in only one trial and for the pooled data. **Morb** was strongly related to concentration and concentration x time. These responses were only weakly related to time since this was not significant for one trial or for the pooled data. Although the number of morphological responses tended to decrease with time, the rate at which morphological responses were produced increased with concentration.

Table 3. Regression Models for Response Categories

Response	Conc.	Time	Conc. x Time	R	Trial[1]
Locomotive	8.3	--	-2.32	0.92	1
	30.5	--	-10.7	0.89	2
	2.5	--	-8.9	0.88	3
	21.3	--	-7.3	0.82	1+2+3
Morpologic	--	--	6.6	0.82	1
	--	-16.6	12.3	0.95	2
	--	-11.7	10.1	0.91	3
	--	-12.6	10.7	0.86	1+2+3
Neurologic	2.6	-1.7	--	0.80	1
	5.2	--	-1.7	0.85	2
	2.6	-1.9	--	0.78	3
	3.6	-1.5	-0.59	0.80	1+2+3
Morbidity	17.2	--	--	0.88	1
	-5.7	6.7	5.4	0.97	2
	-10.1	9.7	6.1	0.98	3
	--	--	5.8	0.81	1+2+3

[1]The crossover experiment was repeated three times. Rows in each response category are the linear regression coefficients for the given experiment.

The dose/time clusters (i.e., rows) in Tables 2 and 3 have the following descriptions. Responses in clusters I and II initially increased with concentration and exposure duration, attained an asymptote and then decreased (i.e., curve was bell-shaped). Responses in cluster III increased with concentration but tended to decrease with exposure duration. Responses in cluster IV occurred at all concentrations and became more pronounced as exposure increased.

The crossover study confirmed several observations made in tests of waste

samples using five planarians/concentration. First, some responses are neither time or concentration dependent. Responses in this category include restlessness and hyperkinesia which occur frequently and often immediately, or convulsions, pharynx protrusion and vomiting which occur occasionally and generally at higher concentrations and longer exposures. Second, responses such as shape change and head/nose twist appear to be primarily related to concentration and not exposure duration. Third, responses such as labored movement are evident over the entire toxic concentration range, but become more pronounced as exposure duration increases. Consequently, these cross-over studies provide quantitative substantiation for, and clarification of the 1-hr planarian toxicity screening assay proposed by Kostelecky (1988).

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