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The role of development and duration of exposure to the embryotoxicity of diazinon

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Abstract

Medaka, *Oryzias latipes*, were used as a laboratory surrogate for species of concern to define the effects of diazinon exposure on teleost embryogenesis. Medaka embryos were placed in a static, non-renewal system and exposures initiated on days 1, 3, or 5 of development. Following initiation of exposure, replicates ($n = 5$) remained in diazinon for a total of 4 days or from the day of initiation to day 9 of development. This exposure scenario was designed to elucidate sensitive periods in development for diazinon-induced toxicity but also shows the effect of added exposure duration on the degree of toxicity. Embryos were observed daily and endpoints recorded included: edema formation, total hatch, mean day of hatch, percentage of larvae with swim bladder inflation, and total length of larvae on day 14, when observations were terminated. Diazinon exposure resulted in decreases in hatch success, swim bladder inflation and the total length of larvae. In addition, dose-response increases in the incidence of edemas of the pericardial sac and vitelline veins were recorded. As expected, severity of embryotoxicity was positively correlated with duration of exposure. While no developmental period was the most sensitive for all toxic effects, for certain endpoints the severity of effects was dependent on exposure timing. Total hatch was greatly affected in embryos exposed from day 1 until day 5 whereas edema was more prevalent in embryos exposed later in development. Finally, among endpoints recorded, total length of larvae was the most sensitive indicator of exposure with all exposure groups showing significant ($P < 0.05$) decreases in length at 5 ppm.

Keywords: Diazinon; Embryotoxicity; Developmental Sensitivity

1. Introduction

Medaka (*Oryzias latipes*), small aquarium fish native to Japan and Southeast Asia are an important laboratory model for experimental embryology (Hong et al., 1996; Ishikawa, 1996), molecular biology (Ozato et al., 1992; Murata et al., 1997) genotoxicity (Chen et al., 1996) and

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developmental toxicity (Marty et al., 1990; Villalobos et al., 1996). Precise staging of development and well established maintenance requirements are available for medaka (Kirchen and West, 1976; DeKoven et al., 1992; and Iwamatsu, 1994). In addition, their transparent chorion is advantageous for embryologic and developmental investigations, and over the past two decades, their sensitivity in controlled laboratory exposures has led to their increased use in toxicity investigations.

Diazinon, (*O*,*O* - diethyl - *O* - [2 - isopropyl - 4 - methyl-6-pyrimidyl] phosphorothioate), is a contact organophosphate (OP) insecticide. The organophosphorus pesticides were developed by chemical manipulation of nerve gases and further modifications have resulted in chemicals with greater species selectivity. OP compounds are useful as pesticides due to their ability to inhibit acetylcholinesterase, an enzyme responsible for inactivating the neurotransmitter acetylcholine (Ecobichon and Joy, 1994). Diazinon is used to control a variety of insects including aphids, beetles, scales and pillbugs in home gardens as well as a number of commercial crops. In addition, diazinon is applied to stone fruit orchards as a dormant spray. Following rain events, diazinon is flushed from residential sites and agricultural lands and carried downstream resulting in toxicant pulses in the Sacramento and San Joaquin Rivers, their delta and the Upper San Francisco Bay (Kuivila and Foe, 1995). A similar pattern of diazinon pulses has been recorded in a study of Mid-Atlantic surface waters (Ferrari et al., 1997). Ferrari et al., also identified diazinon as the most commonly detected pesticide in the examined surface waters.

Acute toxicity tests of adult fish using diazinon have shown that 96 Hr LC50 values vary by several orders of magnitude between species (Ferrando et al., 1991; Sakr and Gabr, 1992 and Alam and Maughan, 1993). While experience shows that early life stages of fishes are often the most sensitive to toxic effects (for a review see Weis and Weis, 1989), little is known about the toxicity of diazinon to fish during these developmental stages. This is important since during development sensitivity may change with some compounds showing higher sensitivity in embryos (Michibata et al., 1987; Marty et al., 1990) whereas others are more toxic to larvae (Fent and Meier, 1994; Gaikowski et al., 1996). Anguiano et al. (1994) and Takimoto et al. (1984) demonstrated that sensitivity to the acute toxic effects of OPs increased during embryo-larval development. With added development, embryo-larval medaka have lower LC50 values and have a greater degree of acetylcholinesterase inhibition in acute, 24 h exposures to diazinon (Hamm et al., unpublished observation, this laboratory). In addition, Takimoto et al. (1984) reported that timing of exposure of medaka embryos to sublethal concentrations of the OP fenitrothion resulted in significantly different degrees of mortality and hatch success.

The present study was performed to determine whether- and the extent to which- factors such as degree of development at initiation of exposure and duration of exposure affect diazinon-induced embryotoxicity in a well established fish model. Results are important because intermittent pulses of organophosphates have been recorded in riverine and estuarine habitats. Future investigations will target the question of repeated, intermittent exposures which likely characterize many field conditions (Kuivila and Foe, 1995).

2. Materials and methods

2.1. Chemicals

Diazinon, purity 99%, was purchased from Chem Services (Philadelphia, PA). The glycol-methacrylate embedding kit (JB-4) was purchased from Polysciences (Worrington, PA). Other reagents were purchased from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available.

2.2. Stock preparation

Diazinon was weighed in a glass boat, transferred to a volumetric flask containing embryo rearing medium (ERM) (Kirchen and West, 1976) and its concentration was confirmed by gas chro-

matographic analysis (Aston and Seiber, 1996). Dilutions of the defined stock solution were used for tests described below.

2.3. Embryo collection and culture

Culture conditions for medaka were as described elsewhere (Marty et al., 1990). Briefly, broodstock were maintained in reverse osmosis, carbon filtered water reconditioned to United States Environmental Protection Agency moderately hard conditions (Horning and Weber, 1985) at 25°C under a 16L:8D photoperiod and fed a purified, casein-based diet (DeKoven et al., 1992) supplemented with brine shrimp nauplii to ensure continuous oogenesis. Egg clusters were separated into individual embryonated eggs by rolling them between finger tips and thereby breaking connecting filaments (Marty et al., 1990) and fertile, stage 11 embryos (Kirchen and West, 1976) were separated and pooled. After cleaning, embryos were placed in ERM, aerated and maintained at 25°C until exposure.

2.4. Embryo exposure

Embryos (5) were randomly assigned to individual 20 ml borosilicate vials (Fisher Scientific, Pittsburgh, PA) containing 2 ml of test solution and maintained under static, nonrenewal conditions previously shown to provide an adequate supply of oxygen and enabling normal development (Marty et al., 1990; Villalobos et al., 1996). Embryos within a given vial were exposed to ERM or to a solution of ERM and 1, 5, 7, 9, 13, 17, 22, or 26 ppm diazinon ($n = 5$ vials per concentration). Duration of development from fertilization to hatch is 10 days for medaka maintained at 25°C. To examine how the degree of embryonic development at initiation of exposure affects toxicity, exposures (4 day duration) were started at day 1 (stage 11, multicellular), day 3 (stage 23, initiation of heart beat), or day 5 (stage 29, advances in vasculature) of development. In a second exposure series, embryos were exposed from day 1 or day 3 until day 9. Following exposure, embryos were rinsed and transferred to ERM where they were allowed to develop and hatch. Both exposure series were terminated at day 14 of development by transferring larvae and remaining unhatched embryos to 10 vol. of the fixative 10% neutral buffered formalin. Termination on day 14 obviated the requirement for addition of an exogenous food.

2.5. Embryo observation

During exposure, embryos were observed a minimum of once per day for mortality or gross morphological alterations using a dissecting microscope. *Pericardial edema*, fluid accumulation in the pericardial sac, was identified by enlargement of the pericardial sac. Fluid accumulation along the vitelline veins was identified by the appearance of fluid filled spaces along veins creating the appearance of channels through the yolk and termed *yolk sac edema*. Curvatures of the vertebral column were identified in hatchlings and defined as *spinal deformities*. Embryos in the later stages of development were checked for hatching and when an individual had freed itself from the chorion the *day of hatch* was recorded. Following hatch, larvae were observed for *swim bladder inflation*, hatchlings were scored for partially or fully inflated swim bladders; swim bladder inflation was considered complete when the diameter of the swim bladder was approximately 2/3 the body width.

Following fixation, total lengths of larvae were determined using an ocular micrometer on a dissecting microscope. Larvae with curvatures of the spine were not included in the calculations for total length.

2.6. Histological analysis

Fixed embryos were manually dechorionated using fine tipped forceps, dehydrated in a graded series of ethanol, and infiltrated and embedded in the plastic resin, glycolmethacrylate (GMA). Four mm step sections were cut using a glass knife on a LKB historange microtome, mounted on glass slides and stained with hematoxylin and eosin. Following coverslipping, embryos were surveyed for histologic alteration.

2.7. Statistics

Levels of statistical significance were analyzed by ANOVA, followed by a Scheffé's F-test as a post hoc test comparing means between the different treatment groups. Differences were considered significant if $P < 0.05$.

3. Results

Characteristics of control embryo-larval medaka are provided in Tables 1 through 6. During the 14 day period of observation over 80% of control embryos hatched (Table 2) and developed fully-inflated swimbladders (Table 4). In addition no mortalities (Table 1), edema (Fig. 2A–Fig. 3E) or gross abnormalities (Table 6) were seen in controls.

While the purpose of these exposures was to examine sublethal effects, some mortality occurred (Table 1). No mortalities were seen before embryos began to hatch and mortality was most often recorded at the time of hatch. Most mortalities occurred when presumably weakened embryos partially hatched and appeared to be macerated by the undigested chorion. This possible relationship between hatching and mortality is demonstrated in embryos exposed from day 1 to 9 where there was increasing mortality between 17 and 22 ppm, when hatching was prevented by 26 ppm, there were no mortalities. In addition, some embryos exposed to 26 ppm from day 3 to 9 were able to hatch (Table 2) and 8/25 died as embryos (Table 1). Other than an apparent association with hatching, no clear correlation of mortality to timing or duration of exposure was noted.

The most apparent gross defect resulting from diazinon exposure was edema (Fig. 1A and B). both along vitelline veins (Fig. 1A) and then within the pericardial sac (Fig. 1B). Among all exposures, edema first appeared around vitelline veins at the margins of the yolk sac (yolk edema) and upon continued exposure, edematous fluid reached, filled and extended the pericardial sac (pericardial edema). The accumulation of fluid in the pericardial sac resulted in the formation of tubular hearts in embryos that had previously been observed with a more developed, chambered heart (Fig. 4).

3.1. Timing of initiation of exposure

3.1.1. Edema formation

Day of initiation affected incidence of yolk sac edema when duration of exposure was limited to 4 days. Edema incidence was higher when exposure was initiated later in development; however, as is shown below, effects such as decreased hatch were of greater magnitude in those embryos exposed

Table 1
Mortality in early life stage medaka exposed to diazinon[a]

Diazinon concentration time (mg/l)	Time of embryo exposure				
	Day 1–5	Days 3–7	Days 5–9	Days 1–9	Days 3–9
0	0	0	0	0	0
1	0	1	0	0	0
5	1	0	0	0	0
7	3	0	0	1	0
9	1	0	0	1	0
13	0	0	0	1	0
17	1[b]	0	0	1[b]	1[b]
22	4	2	3	4[b]	3[b]
26	2	5[c]	1	0	113

[a] Values are the numbers of embryo mortalities per 25 embryos exposed.
[b] Indicates larval mortality of 1.
[c] Indicates larval mortality of 2.

Table 2
Total hatch of embryos exposed to diazinon

Diazinon concentration (mg/l)	Time of embryo exposure*				
	Day 1–5	Days 3–7	Days 5–9	Days 1–9	Days 3–9
0	100 ± 0	100 ± 0	88 ± 7.99	100 ± 0	96 ± 3.99
1	100 ± 0	96 ± 3.99	80 ± 19.96	100 ± 0	100 ± 0
5	96 ± 3.99	100 ± 0	96 ± 3.99	96 ± 3.99	100 ± 0
7	88 ± 7.99	96 ± 3.99	100 ± 0	92 ± 4.89	100 ± 0
9	96 ± 3.99	100 ± 0	100 ± 0	92 ± 4.89	96 ± 3.99
13	96 ± 3.99	100 ± 0	100 ± 0	92 ± 7.99	100 ± 0
17	88 ± 7.99^{a,b}	100 ± 0	92 ± 7.99^{a,b}	68 ± 18.51**^{a}	88 ± 4.89^{a,b}
22	80 ± 10.94**^{b}	88 ± 4.89**^{b}	84 ± 7.47^{b}	24 ± 9.78**^{a}	72 ± 4.89**^{b}
26	16 ± 7.47**^{a}	76 ± 7.47**^{b}	100 ± 0^{c}	0 ± 0**^{a}	60 ± 6.31**^{b}

* Superscripted letters indicate values which represent the total number of embryos that hatched. Numbers are the mean ± S.E.M. expressed as percentages for five replicates of five embryos.

** Indicates value significantly different from control ($P<0.05$). For a given diazinon concentration, groups that are not followed by the same superscripted letter are statistically different ($P<0.05$).

early in development. Among embryos exposed for 4 days, yolk edema occurred in embryos exposed from day 1 to 5 at 22 and 26 ppm (Fig. 2A) but also occurred at 17 ppm in embryos exposed day 3 to 7 and as low as 13 ppm following exposure from day 5 to 9 (Fig. 2B and C). In addition, when exposure was initiated later in development, the incidence of edema was greater. However, interestingly, regardless of when exposure was initiated, yolk edemas were first observed on day 5 (Fig. 2A–E) and their highest incidence occurred between days 7 and 8. When exposures were terminated, and final evaluation made (day 14) yolk edemas were no longer apparent.

During days 7 and 8, when yolk sac edemas were most prevalent, pericardial edemas were first recorded (Fig. 3A–E). A comparison of the time course of yolk edema, Fig. 2A–E, to the time course of pericardial edema, Fig. 3A–E, demonstrates that as the incidence of yolk edema decreased, pericardial edemas increased. Embryos exposed from day 5 to 9 (Fig. 3C) showed a higher incidence of pericardial edema than when exposure was initiated earlier in development (Fig. 3A and B). Interestingly, regardless of when exposure was initiated, the incidence of pericardial edema was highest on day 10 and began to decline thereafter (Fig. 3A–E); except for the highest dose with longest exposure, as seen in Fig. 3D.

3.1.2. Hatch success

A comparison of groups exposed to 26 ppm for 4 days shows that total hatch by the end of day 14 was decreased to a greater extent when exposure was initiated early in development (Table 2). At 26 ppm, all embryos exposed from day 5 to 9 hatched but, in contrast, only 16% of embryos exposed from day 1 to 5 hatched. Effects of diazinon on the mean day of hatch are presented in Table 3. Effects were difficult to discern because initiation of exposure on day 5 appeared to delay hatching of control, 1 ppm, and 5 ppm groups until approximately day 13, whereas embryos exposed at these levels of diazinon in other exposure scenarios hatched in an average of 11 days. This delay obscured effects of exposure. However, in a comparison of embryos exposed to 22 ppm beginning on day 3 to embryos with exposures initiated on day 1, a delay in hatching was seen.

3.1.3. Swim bladder inflation

These are presented in Table 4. In the exposure initiated on day 5, control embryos showed a reduction in swim bladder inflation versus other controls. However, 85% of control embryos in this group fully inflated swim bladders. Similarly, in 1 ppm and 5 ppm treatments, swim bladder inflation was reduced but this may reflect the fact that hatching was delayed in this group. At concentrations of 7 ppm or above, average day of hatch for embryos exposed from days 5 to 9 was similar to other exposures (Table 3). Examination of diazinon concentrations greater than or equal to 9 ppm revealed dose-response decreases in swim bladder inflation for all groups exposed for 4 days. Interestingly, it appeared that initiation of exposure at different points during development did not alter the effect of diazinon on swim bladder inflation (Table 4). Depicted in Fig. 4 is a control larva (top) with full swim bladder inflation and an individual exposed to 26 ppm from day 5 to 9 showing an absence of swim bladder inflation.

3.1.4. Total length

Length of larvae proved the most responsive measure of exposure with significant ($P < 0.05$) decreases at 5 ppm in all exposure groups. While length was a sensitive measure of effect, comparison of embryos exposed for a total of 4 days did not reveal any trends that would suggest a sensitive period of development (Table 5).

3.2. Duration of exposure

3.2.1. Edema formation

Incidence of yolk edema increased with added duration of exposure. Embryos exposed from days 1 to 9 developed edema at a lower concentration (9 ppm) than did other exposure groups. Despite incidence of yolk edema as high as 100% in embryos exposed from day 1 to 9 at 26 ppm, this condition was absent when exposures were terminated.

As with yolk edema, the highest incidence of pericardial edema occurred in embryos exposed from day 1 to 9 and in contrast to embryos exposed for a shorter duration, this group showed no recovery from pericardial edema at 26 ppm diazinon (Fig. 3D). Despite exposure durations of 6 or 4 days, respectively, little difference existed in the incidence of pericardial edema between embryos exposed from day 3 to 7 or day 5 to 9. As with embryos exposed for 4 days, incidence of pericardial edema peaked at day 10.

3.2.2. Hatch success

Total hatch decreased in response to the length of exposure such that embryos exposed continually from day 1 to 9 showed the greatest effect.

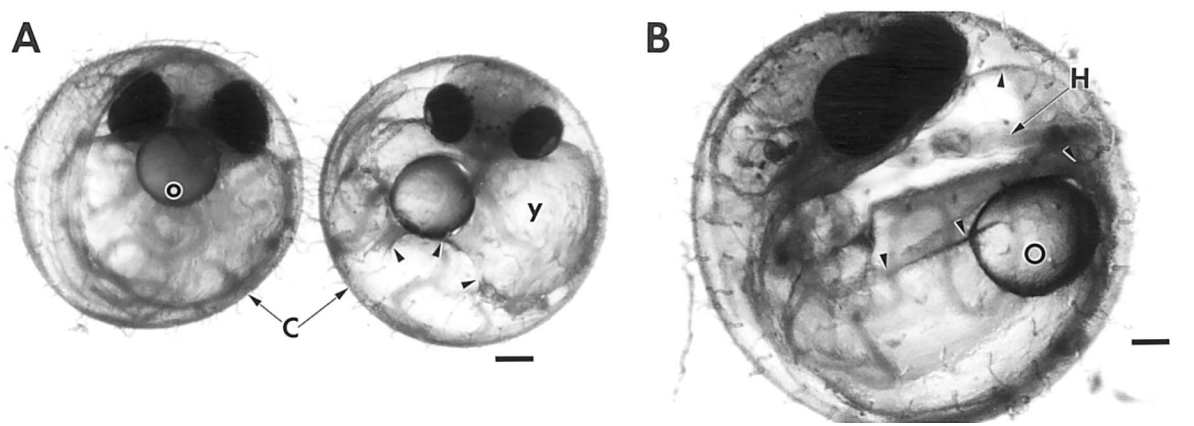
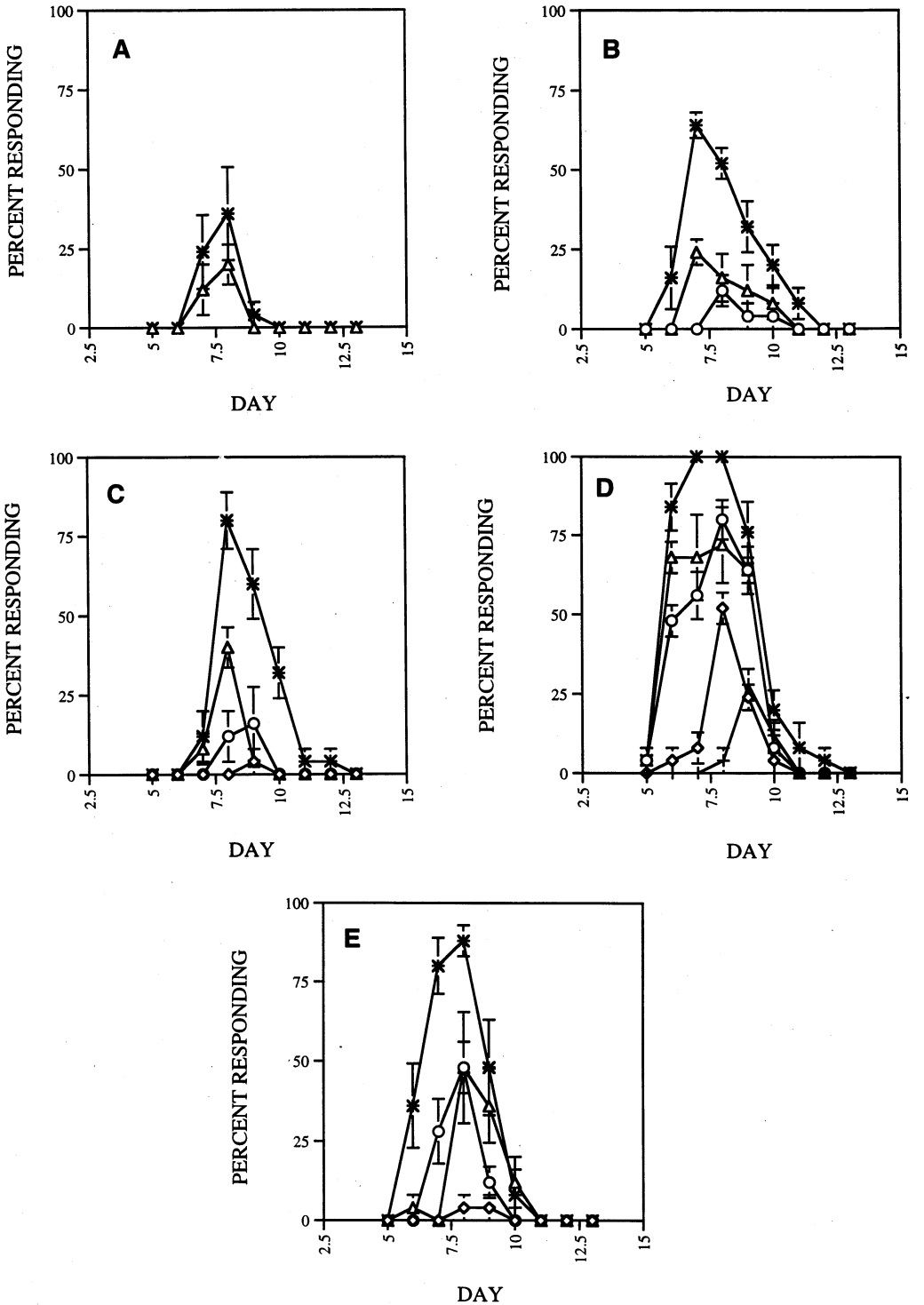


Fig. 1. (A,B) Medaka embryos at day 8. Control is on the left and an embryo exposed to 26 ppm from days 1 to 8 is on the right. Note the displacement of yolk (Y) by yolk edema (denoted by the arrows). Other structures labeled include oil droplet (O) and the chorions (C) of the embryos. Bar = 0.20 mm. Fig. 1B depicts a medaka embryo at day 10 of development exposed to 26 ppm from days 1 to 9. Note the enlarged pericardial sac (denoted by arrows) and the tube-like heart (H). Oil droplet (O). Bar = 0.14 mm.



ig. 2. (A–E) Time course and incidence of yolk sac edemas in embryos exposed days 1–5 (A), days 3–7 (B), days 5–9 (C), days –9 (D), and days 3–9 (E). Values represent replicate means expressed as the percentage of embryos exhibiting yolk sac dema ± S.E.M. ($n = 5$). Diazinon concentrations are represented by the following symbols: + (9 ppm), ◇ (13 ppm), ○ (17 ppm),

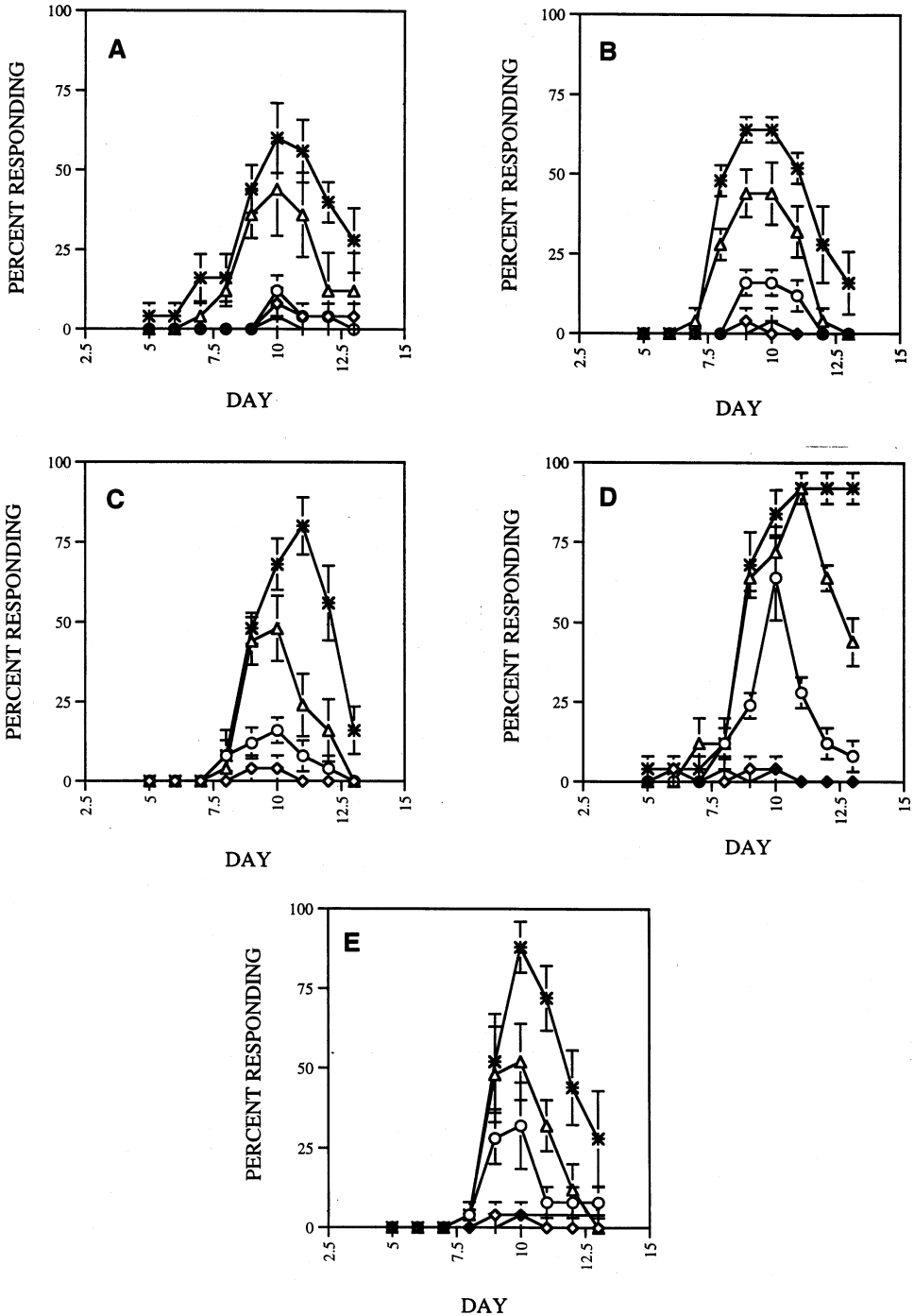


Fig. 3. (A–E) Time course and incidence of pericardial edemas in embryos exposed days 1–5 (A), days 3–7 (B), days 5–9 (C), days 1–9 (D), and days 3–9 (E). Values represent replicate means expressed as the percentage of embryos exhibiting pericardial edema ± S.E.M. ($n = 5$). Diazinon concentrations are represented by the following symbols: + (9 ppm), ◇ (13 ppm), ○ (17 ppm), △ (22 ppm) and * (26 ppm).

Table 3
Mean day of hatch of embryos exposed to diazinon

Diazinon concentration time (mg/l)	Time of embryo exposure*				
	Days 1–5	Days 3–7	Days 5–9	Days 1–9	Days 3–9
0	11.5 ± 0.2a	11.0 ± 0.2[a]	13.2 ± 0.1[b]	11.5 ± 0.1[a]	11.0 ± 0.2[a]
1	10.9 ± 0.2**,[a]	11.0 ± 0.2[a]	12.2 ± 0.2**,[b]	11.0 ± 0.1**,[a]	10.6 ± 0.1**,[a]
5	11.2 ± 0.1[a]	10.9 ± 0.3[a]	13.0 ± 0.2[b]	10.9 ± 0.1**,[a]	10.8 ± 0.1[a]
7	10.9 ± 0.2**,[a,b]	11.0 ± 0.2[a,d]	11.9 ± 0.2**,[c]	10.8 ± 0.1**,[a,b]	10.5 ± 0.1**,[b]
9	11.2 ± 0.2[a,c]	11.5 ± 0.2[c]	12.6 ± 0.2**,[b]	11.0 ± 0.1**,[a]	10.9 ± 0.1[a]
13	10.9 ± 0.2**,[c]	11.4 ± 0.2[a,d]	12.2 ± 0.1**,[b]	11.5 ± 0.2[a]	11.2. ± 0.2[a,c]
17	12.7 ± 0.2**,[b]	11.6 ± 0.2[a,c]	12.4 ± 0.2**,[b]	11.9 ± 0.2**,[a]	11.3 ± 0.2[a]
22	11.9 ± 0.2[b,d]	12.5 ± 0.2**,[a,c]	12.9 ± 0.2[a]	13.2 ± 0.4**,[a]	12.2 ± 0.1**,[b,c]
26	13.8 ± 0.3*,[b]	12.7 ± 0.1*,[a]	12.8 ± 0.2[a]	DNH	13.0 ± 0.2**,[a]

* Superscripted letters indicate the values which represent the mean day of hatch. Numbers are the mean ± S.E.M. expressed as percentages for all embryos that hatched in five replicates.

** Indicates a value significantly different from control ($P<0.05$). For a given diazinon concentration, groups that are not followed by the same superscripted letter are statistically different ($P<0.05$). DNH = did not hatch.

Among embryos exposed to 26 ppm diazinon, none hatched when exposed from day 1 to 9 whereas all embryos exposed from day 5 to 9 hatched (Table 2).

3.2.3. Swim bladder inflation

In terms of swim bladder inflation, the length of exposure seemed to be more critical than when the exposure was initiated. In contrast to exposures conducted for 4 days which did not show any differences due to day of initiation, sensitivity of embryos exposed for varying duration showed sensitivity of day 1 to 9 > day 3 to 9 > day 5–9 (Table 4).

3.2.4. Total length

Diazinon (5 ppm) was the lowest concentration that produced a decrease in total length. At this same concentration, a significant difference ($P<0.05$) existed between embryos exposed day 1–9 and those exposed day 5–9. With increasing diazinon concentrations, the embryos exposed day 1 to 9 had significantly shorter total length so that at 22 ppm, they were approx. 10% shorter than other exposure groups and 20% shorter than controls (Table 5).

3.2.5. Other effects of diazinon exposure

One type of spinal deformity was seen in some diazinon exposed individuals. Following hatch, affected larvae showed dorsal displacement of the spine such that the caudal-most portion of the tail region was directed toward the head (Fig. 5). Of the 22 larvae with this deformity, 16 were exposed to 26 ppm diazinon. Among embryos exposed beginning on day 1, either day 1 to 9 or day 1 to 5, only four larvae developed spinal deformities (Table 6). Since hatch success in embryos exposed to 26 ppm was limited to 16% in exposures day 1–5 and 0% in day 1–9, it is unclear if these embryos had a higher incidence of deformities than recorded.

Histological examination of embryos focused on retina (Hamm et al., 1998) but other alterations primarily in hatching gland were seen. Control embryos on day 7 of development showed numerous granules within cells of the hatching gland (Fig. 6A). In contrast, embryos exposed to 26 ppm beginning on day 1 lacked granules of hatching gland cells and extensive vacuolization (Fig. 6B). Further examination, showed that granules containing the hatching enzyme develop between days 5 and 6 in the control

embryos but fail to develop in diazinon exposed medaka. Other gross morphologic changes described above were not examined by histology.

4. Discussion

Edema in developing medaka was a prominent result of diazinon with the greatest effects when exposure was initiated in the late embryonic period. Edema has proven to be a prevalent feature of vertebrate embryotoxicity in general and earlier work from this and other laboratories suggests this is true for fishes as well. Edema was seen in medaka after exposure to *N*-nitroso compounds (Marty et al., 1990), thiobencarb (Villalobos et al., 2000) or products of incomplete combustion of trichloroethylene (Villalobos et al., 1996). The organism in which edema formed and the compound to which it was exposed are given below. These include: fathead minnow and toluene (Devlin et al., 1985); frog and *N*-phenyl-α-naphthylamine (Greenhouse, 1976); medaka (Wisk and Cooper, 1990a and Wisk and Cooper, 1990b), zebrafish (Henry et al., 1997), or chick and TCDD (Cheung et al., 1981).

Since edema has proven to be such a common response, the definition of sensitive developmental stages at which exposure leads to edema would aid in determining the mechanism for this condition. For example, some enzyme systems may be absent early during development and if biotransformation reactions are not present, changes would be attributable to the parent compound and not a metabolite. Marty et al. (1990) exposed three age groups of medaka embryos to a 2 h pulse of *N*-nitroso-*N*-methylurea to define age dependent toxicity and reported increased pericardial edema when embryos were exposed at early organogenesis rather than at later embryonic stages. Takimoto et al. (1984) demonstrated that medaka exposed between days 0 to 3 of development to fenitrothion, another organophosphate compound, had a similar incidence of circulatory defects (95.8%) as those exposed from days 0 to 8 (100%) but exposure between days 3 and 6 or 5 to 8, resulted in a significantly lower incidence (10 and 15%, respectively). No stage proved more susceptible to pericardial edema brought about by carbaryl (Solomon and Weis, 1979).

Unlike the above reports, diazinon exposures resulted in a greater incidence of edema if exposure was initiated late in development. This may be explained by the fact that diazinon must be bioactivated to its more toxic metabolite and a greater bioactivation of diazinon has been demon-

Table 4
Percentage of embryos with full swim bladder inflation

Diazinon concentration (mg/l)	Time of embryo exposure*				
	Days 1–5	Days 3–7	Days 5–9	Days 1–9	Days 3–9
0	96 ± 3.99	96 ± 3.99	84 ± 9.78	96 ± 3.99	88 ± 7.99
1	96 ± 3.99	96 ± 3.99	80 ± 19.96	96 ± 3.99	96 ± 3.99
5	96 ± 3.99^{a}	100 ± 0^{a}	68 ± 4.89^{b}	92 ± 4.89^{a}	92 ± 4.89^{a}
7	68 ± 7.99**,^{b}	88 ± 7.99^{a}	100 ± 0^{a}	84 ± 3.99^{a,b}	88 ± 4.89^{a}
9	84 ± 11.64^{c}	84 ± 7.47^{c}	76 ± 9.78^{b,c}	44 ± 9.78**,^{a}	56 ± 7.47**,^{a,b}
13	48 ± 7.99**,^{b}	60 ± 6.31**,^{b}	56 ± 11.64*,^{b}	8 ± 4.89*,^{a}	24 ± 3.99**,^{a}
17	8 ± 7.99**	16 ± 3.99*	16 ± 3.99**	4 ± 3.99**	8 ± 4.89**
22	0 ± 0*	0 ± 0*	4 ± 3.99**	0 ± 0**	0 ± 0**
26	0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0**	0 ± 0**

* Superscripted letters indicate values which represent the percentage of larvae with complete swim bladder inflation. Numbers are the mean ± S.E.M. for five replicates of five embryos.

** Indicates value significantly different from control ($P<0.05$). For a given diazinon concentration, groups that are not followed by the same superscripted letter are statistically different ($P<0.05$).

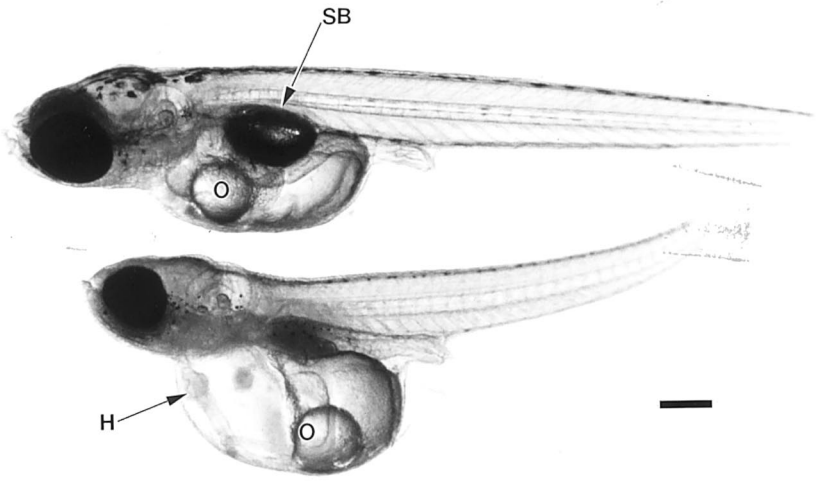


Fig. 4. Medaka larvae: control on top with inflated swimbladder (SB), larvae on bottom was exposed to 26 ppm from days 5 to 9. Note the enlarged pericardial sac and the tube-like heart (H). Oil droplet (O). Bar = 0.24 mm.

strated with added development (unpublished observations this laboratory). Sensitivity could be reflective of developmental changes in the circulatory system. Under our conditions of maintenance and culture, heart contraction is initiated on the third day of embryonic life and onset of blood circulation is detected between days 3 and 4. If the circulatory system was exposed at, or just prior to onset of muscular contraction, increased hydrostatic pressure could have led to greater incidence of edema. Additionally, regardless of timing of exposure initiation, yolk sac edema developed only after day 7 to 8 when associated vitelline veins became sinuous. After yolk sac edema formed, a shift of location to pericardial edema was seen in all exposure groups. Such shifts followed exposure of medaka embryos to incomplete combustion products of trichloroethylene (Villalobos et al., 1996). It is important to note that regardless of where the edema forms, the initial appearance of fluid accumulation involves the endothelium at that site. These observations suggest that onset of edema formation is linked to developmental events in the endothelium of large veins and pericardial cavity.

When zebrafish embryos were exposed to TCDD, pericardial edema preceded development of yolk edema (Henry et al., 1997). This apparent difference in progression of edema, if real, may enable investigations of possible species differences in factors leading to this condition and to an analysis of variations between toxicities elicited by various compounds. Wisk and Cooper (1990a) examined toxicity of TCDD using medaka embryos and reported pericardial edema with hemorrhage but made no mention of edema of the adjacent yolk sac. Later (Wisk and Cooper, 1990b), these same workers exposed medaka embryos to other polychlorinated aromatic hydrocarbons and again found pericardial edema without yolk sac edema. In contrast, lake trout (*Salvelinus namaycush*) and rainbow trout (*Salmo gairdneri*, Richardson; now *Oncorhynchus mykiss*) developed blue-sac disease, a subcutaneous edema of the yolk sac, but are apparently resistant to pericardial edema since few individuals developed this condition (Helder, 1981; Spitsbergen et al., 1991). From the above studies, factors determining the site of edema remain unclear but appear to be a combination of variations due to species and to the compound under test. More sensitive and precise measures of how and when the endothelium is affected by toxicant exposure should facilitate our understanding of the early events and progression of this form of edema.

An important element in understanding the toxicity of diazinon is its requirement for bioactiva-

Table 5
Total length of larvae exposed to diazinon

Diazinon concentration	Time of embryo exposure*				
	Days 1–5	Days 3–7	Days 5–9	Days 1–9	Days 3–9
0	4.56 ± 0.05	4.55 ± 0.0	54.55 ± 0.0	44.55 ± 0.0	54.54 ± 0.06
1	4.49 ± 0.04	4.54 ± 0.04	4.52 ± 0.05	4.53 ± 0.03	4.56 ± 0.05
5	$4.44 \pm 0.04^{**,b}$	$4.35 \pm 0.03^{**,a,b}$	$4.39 \pm 0.05^{**,b}$	$4.27 \pm 0.03^{**,a}$	$4.36 \pm 0.04^{*,a,b}$
7	$4.34 \pm 0.05^{**,c}$	$4.18 \pm 0.04^{**,b}$	$4.28 \pm 0.04^{**,a,b,c}$	$4.22 \pm 0.05^{*,a,b}$	$4.30 \pm 0.03^{*,a,c}$
9	$4.29 \pm 0.03^{**,b}$	$4.20 \pm 0.03^{**,b}$	$4.21 \pm 0.03^{**,b}$	$4.04 \pm 0.05^{*,a}$	$4.30 \pm 0.05^{**,b}$
13	$4.22 \pm 0.03^{**,c}$	$4.06 \pm 0.03^{**,d}$	$4.20 \pm 0.04^{**,b,c}$	$3.88 \pm 0.06^{*,a}$	$4.10 \pm 0.04^{*,b,d}$
17	$3.96 \pm 0.06^{**,a,c}$	$3.94 \pm 0.03^{**,a,c}$	$4.02 \pm 0.04^{**,b,c}$	$3.82 \pm 0.08^{*,a}$	$4.13 \pm 0.05^{*,b}$
22	$4.00 \pm 0.09^{**,b}$	$3.90 \pm 0.05^{**,a,b}$	$4.04 \pm 0.09^{**,b}$	$3.63 \pm 0.16^{*,a}$	$4.07 \pm 0.05^{*,b}$
26	$3.77 \pm 0.08^{**,a}$	$4.01 \pm 0.05^{**,b}$	$4.13 \pm 0.04^{**,b}$	DNH	$3.63 \pm 0.12^{*,a}$

* Superscripted letters indicate values which represent the mean length ± S.E.M., expressed in millimeters, for all larvae.
** Indicates value significantly different from control ($P<0.05$). For a given diazinon concentration, groups that are not followed by the same superscripted letter are statistically different ($P<0.05$). Grossly deformed larvae, i.e. those having spinal curvature, were not used for the calculation of total length.

tion, the question of where and by which steps this reaction occurs is particularly important. Cantrell et al. (1996) demonstrated that TCDD caused DNA degradation and loss of vascular integrity. These same workers later localized P4501A to sites of DNA damage and reported that piperonyl butoxide, a P450 inhibitor, blocked both DNA degradation and embryotoxicity (Cantrell et al., 1998). Cytochrome P4501A was first localized to the endothelium of heart in scup (*Stenotomous chrysops*) (Stegeman et al., 1989) and later in *Poeciliopsis monacha* and *P. lucida* (Smolowitz et al., 1992). Cantrell et al. (1996) also reported apoptosis within the endothelium. We reported apoptosis within retina of medaka embryos at sites of AChE activity following diazinon exposure (Hamm et al., 1998). Perhaps endothelial P450(s) are responsible for local bioactivation of diazinon and this reaction might lead to apoptosis and a loss of integrity in endothelial cells resulting in edema.

Early embryonic exposure had the greatest effect on hatch success. For example, when embryos were exposed to 26 ppm diazinon from days 1 to 5, only 16% hatched whereas 100% hatched when exposed to the same concentration from days 5 to 9. Our results support earlier findings by Takimoto et al. (1984) who demonstrated that pulse exposure of medaka embryos to fenitrothion between days 0 and 3 was associated with a decrease in hatch which was quantitatively greater than that occurring in exposures conducted from days 3 to 6 or days 5 to 8. The question which now arises concerns the mechanism whereby diazinon exposure early in development can lead to decreased hatch. Here, it will be necessary to have detailed data on the time of appearance of cytochrome P450 and/or to determine whether the parent compound itself is interacting to inhibit hatching success.

Fig. 5. Fixed larvae: control (on top) with larva exposed to 26 ppm from days 3 to 9. Note the curvature in the tail of exposed larva (arrowhead). Other structures noted are the eye (E) and yolk (Y). Bar = 0.24 mm.

Table 6
Incidence of spinal deformities among medaka embryos exposed to diazinon[a]

Diazinon concentration (mg/l)	Time of embryo exposure				
	Days 1–5	Days 3–7	Days 5–9	Days 1–9	Days 3–9
0	0	0	0	0	0
1	0	0	0	0	0
5	0	0	0	0	0
7	1	0	0	0	0
9	0	1	0	0	0
13	1	0	0	0	0
17	0	0	0	0	0
22	1	0	1	1	0
26	0	4	5	0	7

[a] Values represent the number of larvae out of the 25 embryos exposed per group with curvature of the spine.

To better understand alterations related to decreased hatching success, embryos exposed to 26 ppm diazinon were examined histologically. Hatching glands from 7-day-old embryos exposed to diazinon granules containing the hatching enzymes. Examination of 5 to 6-day-old control embryos revealed the appearance of granules within the hatching gland. In contrast, no granules were seen to develop within exposed embryos. Physiological processes including neural control of hatching remain unclear. However, exposure of teleost embryos to neurotransmitter agonists and antagonists has suggested such a role. Schoots et al. (1983) reported that dopaminergic agonists increase the time of hatch and antagonists cause a decrease, whereas DiMichele and Taylor (1981) reported that epinephrine decreased average time to hatch. In addition, DiMichele and Taylor (1981) demonstrated that atropine, a muscarinic receptor antagonist (Katzung, 1992), inhibited hatch in Fundulus heteroclitus. More work is needed to understand the normal biology of the hatching process and how diazinon interferes with the development of the hatching gland.

While hatch success was decreased by diazinon exposure, effects on the time to hatch were obscured by delayed hatching of control embryos inserted into vials on day 5. Further information on the possible role of this factor in embryotoxicity tests is warranted. However, low diazinon concentrations, 1–7 ppm, clearly caused premature hatch. The pattern of effect seen with diazinon, early hatch at low concentrations and delayed hatch at higher concentrations, may be classified as hormesis. Hormesis is a condition in which low level exposure to a toxicant increases growth or activity and with increasing concentrations the organism experiences impaired function (for a review see Stebbing, 1982). Based upon observations with a dissecting microscope, as is characteristic with OP poisoning, diazinon exposure appeared to increase body movements of the embryos (Katzung, 1992). It is possible that the increased movement leads to the embryos breaking through the chorion sooner.

Marty et al. (1990) reported that altered swim bladder inflation was the most sensitive measure of exposure. In contrast to the observations of Marty et al. (1990), growth as evidenced by total length of larvae, proved to be the most sensitive indicator of effect. In addition, this endpoint showed a dose response relationship. Decreased growth can have a profound effect on larval survival because the longer fish spend in a vulnerable developmental stage the greater their chance of predation (Houde, 1987). As fish grow, their predation by invertebrate predators decreases (Paradis et al., 1996) and field studies have demonstrated larval predation to be a major determinant of recruitment (Hewitt et al., 1985; Houde, 1987; Purcell, 1990). If diazinon exposure slows growth of larvae, this could influence recruitment and thereby affect the population. In addition to the change in length, exposure pro-

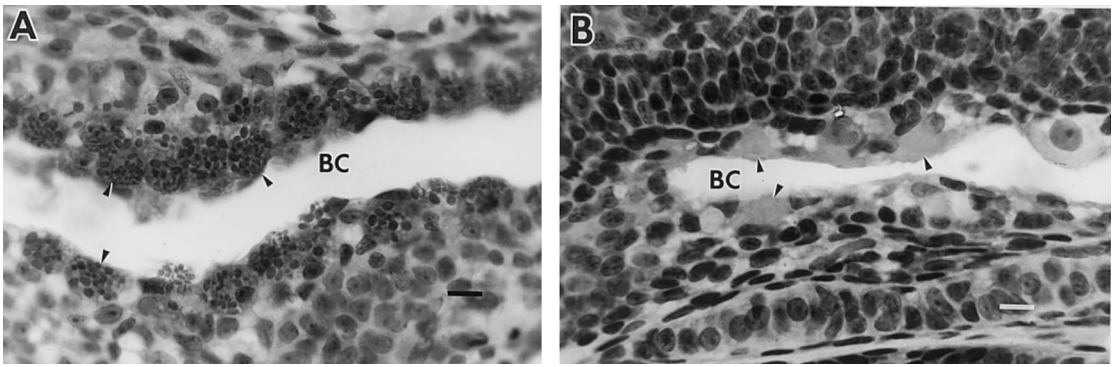


Fig. 6. (A,B) Section through a control embryo on day 7. Note numerous hatching gland cells (denoted by arrow heads) lining the buccal cavity (BC). Fig. 6B is a section through an embryo exposed to 26 ppm from days 1 to 7. Note the absence of granules within the hatching gland cells (denoted by arrow heads) lining the buccal cavity (BC). H&E. Bars = 8 mm.

duced larvae with spinal curvatures. A similar outcome was reported in medaka embryos exposed to fenitrothion (Hiraoka, 1989) and medaka fry were shown to develop a lump in the notochord following parathion exposure (Tomita and Matsuda, 1961).

Previous results from this lab demonstrated that as medaka embryos develop, equimolar concentrations of diazinon caused a greater degree of AChE inhibition and mortality (unpublished observations this laboratory) and based upon these findings, the range of diazinon concentrations used in the current study was selected. This range allowed us to use the advantages of medaka as a model teleost embryo to define gross morphological targets of embryonic diazinon exposure and to determine sensitive tissues and embryonic windows of opportunity for toxicity. Future work should examine the effect of alterations of environmental factors on toxicity. Cameron and Hunter (1984) demonstrated conditions that influence the permeability of the chorion and these conditions influence uptake of organophosphorus compounds by fish embryos (Takimoto et al., 1984) suggesting effects could occur in some feral fish species at much lower levels.

In conclusion, diazinon exposure resulted in a suite of developmental alterations with developmental sensitivity dependent on the endpoint. At similar diazinon concentrations, early stage embryos proved most sensitive to decreases in hatching success. Histological examination of embryos exposed to diazinon concentrations that prevented hatch revealed the absence of granules within hatching gland cells. Late stage embryos had an increased incidence of edema and the formation and progression of edema appears strongly tied to developmental changes in the circulatory system. Growth, as determined by total length of larvae, although a nonspecific response, was a sensitive indicator of exposure. In contrast, curvatures of the spine could be indicative of organophosphate exposure. Data presented herein on developmental sensitivity and toxicological endpoints may guide the development of experimental protocols employing species of concern.

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