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Hazard/Risk Assessment

STAGE-DEPENDENT DIFFERENCES IN EFFECTS OF CARBARYL ON POPULATION GROWTH RATE IN JAPANESE MEDAKA (*ORYZIAS LATIPES*)

SHOSAKU KASHIWADA,*†‡ HARUKI TATSUTA,† MAIKO KAMESHIRO,†‖ YOSHIO SUGAYA,†
TARA SABO-ATTWOOD,‡ G. THOMAS CHANDLER,‡ P. LEE FERGUSON,§ and KOICHI GOKA†
†Environmental Risk Research Center, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan
‡Department of Environmental Health Sciences, Arnold School of Public Health, §Department of Chemistry and Biochemistry,
University of South Carolina, Columbia, South Carolina 29208, USA
‖The Institute of Environmental Toxicology, Uchimoriya-cho 4321, Johso, Ibaraki 303-0043, Japan

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Abstract—Fish embryo toxicology is important because embryos are considered more susceptible than adult fish to the effects of toxic chemicals. Recently, fish embryo bioassay was proposed to replace the conventional fish acute toxicity chemical test of the Organization for Economic Cooperation and Development guidelines because it offers the advantages of fewer reagents, easy handling, and efficient data production. To accelerate the establishment of a chemical toxicity database for the protection of environmental and human health, we need to determine whether the conventional toxicity test can safely be replaced by such fish embryo toxicity tests. For instance, it is unclear how the presence of the chorion moderates the toxic effects of some chemicals. If such chemical toxicities do differ between embryos and, for example, the larval stage, then different toxic effects should appear in later life. We tested the later-life effects of the neurotoxic insecticide carbaryl at sublethal concentrations (0 [control] and 5 and 10 mg/L) in embryos and posthatch larvae of the freshwater fish medaka, *Oryzias latipes*. Although embryos exposed until hatching showed multiple developmental malformations and reductions in subsequent survival rates over three months, no significant reduction was observed in tolerance to starvation for 7 d and in intrinsic population growth rate (*r*). Exposure of larvae for 96 h resulted in dose-responsive vertebral fracture, significant reduction in tolerance to starvation for 7 d, and reduced three-month survival rate; *r* was reduced significantly and consistently. These results suggested that posthatch larvae were more susceptible than embryos to carbaryl exposure and that the toxic cascades may differ between larvae and embryos. The influences of carbaryl exposure on population growth rate differed significantly with developmental stage.

Keywords—Carbaryl Medaka Population growth rate

INTRODUCTION

Toxicity testing in adult fish is currently performed worldwide in accordance with the Organization for Economic Cooperation and Development (OECD) chemical test guideline 203 [1]; however, earlier life stages, such as the embryo and larva, are known to be more sensitive to chemicals. Because chemical exposure in the early life stages can cause vital and irreversible damage in later lives, we need to understand the toxic effects of chemicals on fish embryos and larvae if we are to successfully regulate hazardous chemicals on the market and manage their ecological risks. Fish embryo toxicity testing is recognized as important because it enables the use of microplates; this means fewer reagents, easier handling, and data production with higher efficiency than with conventional testing. Recently, the German government nationally standardized a 48-h sewage-testing assay in zebrafish (*Danio rerio*) embryos and submitted it for standardization to the International Organization for Standardization [2; http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=37368]. Furthermore, a modified fish embryo test for the regulation of commercial chemicals has been submitted by the German government to the OECD as an alternative to the conventional acute fish toxicity test [3]; the new test is based on the reliable correlation of a single data set between the zebrafish embryo toxicity test and the conventional adult acute toxicity test. Very limited data sets are available on other fish—namely, the medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) [3]. This alternative fish embryo toxicity test is being run under Registration, Evaluation, Authorization, and Restriction of Chemicals in the European Union (http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm) to accelerate the toxicity assessment of chemicals because there are 30,000 existing hazardous chemicals that need toxicity testing [3]. It is important that a toxicity database be established to characterize the effects of chemicals on either embryos or adult fish; however, toxicologists have failed to use simplified individual toxicity data such as the median lethal concentration (LC50) and ecological indicators (i.e., uncertainty factors) to assess the ecological risks of chemicals [4]. Acute and chronic toxicity may reduce the intrinsic growth rate of a population and may therefore increase the probability of population extinction. However, there is no scientific consensus on the extrapolation of individual lethal toxicity data to intrinsic population growth rates. If we are to successfully develop more ecologically relevant risk assessments of hazardous chemicals, then it is important that we use indices for assessing population dynamics such as intrinsic population growth and carrying capacity [4].

If embryo toxicity data are to be used instead of conventional fish toxicity data, then we need to understand the commonalities and differences between embryo and larval toxicity tests because there are likely physiological and toxicological gaps among embryos, larvae, and adult fish. For example, the

* To whom correspondence may be addressed (shosaku@gwm.sc.edu).
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Table 1. Effects of carbaryl exposure on development of medaka embryos

Biomarker	Concn. of carbaryl		
	0 mg/L (control)	5 mg/L	10 mg/L
Tubular heart (%) at embryo development stage 37[a]	0.0 (0.0)	14.8 (6.4)	41.2 (14.2)[b]
Blood clots in ducts of Cuvier (%) at stage 37	0.0 (0.0)	0.0 (0.0)	1.4 (1.4)
Pericardial edema (%) at stage 37	0.0 (0.0)	0.0 (0.0)	11.1 (4.4)
Heart rate per 15 s at stage 37	34.4 (0.6)	32.7 (0.7)	26.6 (0.4)[c]
Hatch rate (%)	94.7 (2.5)	100 (0.0)	91.9 (3.3)
Time to hatch (days)	8.7 (0.2)	8.9 (0.3)	9.3 (0.2)
Spinal deformity (%)	0.0 (0.0)	2.7 (2.7)	5.8 (2.7)
Death rate 24 h posthatch (%)	5.4 (2.5)	9.3 (3.4)	6.8 (3.7)
Body length of posthatch embryos (mm)	4.3 (0.03)	3.7 (0.02)[c]	3.4 (0.02)[c]

[a] Embryo development stage 37 is appropriately 6 d postfertilization, as judged in the control [18]. Standard errors are in parentheses.
[b] Analysis of variance versus control: $p < 0.05$.
[c] Analysis of variance versus control: $p < 0.001$.

liver—a typical target organ—is not developed well enough to protect against xenobiotics in embryos and larvae. Because fish embryos have a chorion to protect themselves, they are less sensitive to chemical exposure than are dechorionated embryos and larvae [5,6]. The chorion plays a protective role against chemical exposure, and the barrier function of the egg chorion increases with the lipophilicity of the chemical [3]. In heavily contaminated aquatic environments, aquatic organisms would be considered damaged in terms of viability and longevity compared with those in a reference area. Several studies have revealed the impacts of pesticides on behavioral and life history traits. For instance, carbaryl and other neurotoxic pesticides decrease swimming ability in rainbow trout [7] and increase the vulnerability of the trout to predation [8]. Carbaryl is a known aquatic environmental contaminant that has been used extensively in agriculture for pest control [9] as a neurotoxic acetylcholinesterase and cholinesterase inhibitor [8,10]. We therefore consider it a suitable model chemical for fish ecotoxicological studies. Although the modes of action of carbaryl are well known, risks to fish populations are still unclear. We examined whether sublethal exposure of Japanese freshwater fish medaka (*O. latipes*) embryos or larvae to carbaryl caused different reductions in later viability and population growth rate.

MATERIALS AND METHODS

Collection of fertilized eggs and hatched larvae of medaka

The small laboratory fish species medaka (*O. latipes*) has a small body (3–4 cm in adults), a hardy nature (wide temperature and salinity tolerances), and a short generation time (two to three months); therefore, it is frequently used to investigate waste water toxicology [11], endocrine disruptors [12], liver carcinogenesis [13], germ cell mutagenesis [14], gene mutagenesis [15], and developmental and functional genomics [16]. The medaka orange-red strain that we used was obtained from broodstock at the National Institute for Environmental Studies (Ibaraki, Japan). Details of cultivation conditions are given in our previous publications [17,18]. Briefly, breeding groups of medaka were fed *Artemia salina* nauplii twice daily and maintained under a 16:8-h light:dark cycle at 26 ± 0.5°C in a culture room. After female medaka had spawned eggs, the external egg clusters were removed. Fertilized eggs were selected and rinsed and then placed in embryo-rearing medium (ERM) [19]. Eggs in development stage 10 (early blastula stage) [20] were isolated and subsequently used for exposure. Medaka larvae were obtained by the incubation of embryos at 26 ± 0.1°C in an incubator (Multi Thermo Incubator MTI-202, Tokyo Rikakikai, Tokyo, Japan) under a 16:8-h light:dark photoperiod until hatching and subsequently used for exposure. Fish and eggs were treated humanely and with regard for the alleviation of suffering.

Exposure of embryos and larvae

Carbaryl (methyl carbamic acid-1-naphthyl ester, technical-grade purity >99%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The U.S. Environmental Protection Agency database (AQUIRE, http://cfpub.epa.gov/ecotox) reports the lethal concentration of carbaryl to be approximately 20 mg/L in temperate fish. The sublethal concentrations that we used were defined as 5 and 10 mg/L. Carbaryl was dissolved in dimethylsulfoxide (Wako) to 0, 5, and 10 mg/ml as stock solutions, and then each stock solution was diluted with ERM at a ratio of 1:1,000 to make 0, 5, and 10 mg/L, respectively, of carbaryl–ERM solution. The concentration of dimethylsulfoxide in each carbaryl solution was 0.1% (v/v). Five milliliters of each carbaryl solution (0, 5, and 10 mg/L carbaryl) were prepared in five glass scintillation vials (20-ml volume), to each of which 15 egg embryos were added and exposed until hatching or 14 d. For the larval exposure experiment, five 100-ml solutions of carbaryl at each concentration (0, 5, and 10 mg/L) were prepared in five glass dishes (volume 200 ml, diameter 100 mm, depth 50 mm), to each of which 15 larvae were added and exposed for 96 h. Embryos and larvae were incubated at 26 ± 0.1°C in an incubator (Multi Thermo Incubator MTI-202) under a 16:8-h light:dark photoperiod. Test solutions were renewed daily. Embryos and larvae were exposed simultaneously.

Estimation of exposure effects and postexposure incubation

Embryos were observed daily through the glass vials under a dissecting microscope (Olympus SZ-ET, Tokyo, Japan). To assess embryo toxicity, a number of biomarkers—percent of occurrences of tubular heart, blood clots in the ducts of Cuvier, and pericardial edema in embryos; heart rate, hatch ratio, and time to hatch in embryos; and percent occurrence of spinal deformity, death rate, and body length in posthatch larvae—were measured and summarized (Table 1). Posthatch larvae were anesthetized with ice-cold water, and their body lengths were immediately measured under a dissecting microscope equipped with a micrometer. After measurement, all larvae (five groups of 15 larvae per embryo treatment) were immediately moved to fresh ERM (26°C), which was aerated for 5

min. After all larvae had been confirmed to be alive, each group was moved to 200 ml of fresh ERM in the respective glass dishes and fed with a rotifer, *Brachionus urceolaris*, once a day for 3 d and then with *A. salina* nauplii. At 7 d posthatch, the larvae were moved to 500-ml acrylic resin aquariums (90 × 150 × 90 mm, height × width × depth) in a medaka culture system (Small Fish Culture System Type Meito-Hikosaka), Meitosuien, Nagoya, Japan) and fed with *A. salina* nauplii three times a day for three months until the medaka reached sexual maturity. The numbers of surviving medaka were recorded at 30, 60, and 90 d posthatch. Tap water, which was dechlorinated with an activated carbon filter and temperature controlled to 26 ± 1°C, was supplied (sodium ions 19.3 mg/L, chloride ions 32.7 mg/L, hardness 76 mg/L, total organic carbon 0.9 mg/L, pH 7.8) to the medaka culture system.

For the larval exposure experiments, larvae were observed daily under the dissecting microscope to detect any morphological changes, and mortalities were recorded daily during the 96 h of exposure. After the exposure period, the larvae were rinsed with fresh ERM and moved to the medaka culture system. The number of surviving medaka was recorded at 30, 60, and 90 d posthatch.

Impact of carbaryl on growth of medaka populations

To evaluate the impact of the different carbaryl exposures on medaka populations, we first estimated *r*, a summary index that represents the ability of each population to proliferate. The index *r* was estimated by fitting the life table data for each exposure treatment to the Euler–Lotka equation [21]. The equation is

$$\sum_t l_t m_t e^{-rt} = 1$$

where t is age in days, l_t is survivorship until age t, and m_t is per-capita fecundity. Since females began to spawn eggs at age around 75 d posthatch, we used the number of eggs from 91 to 105 d posthatch for estimating *r*. The number of newborn females is usually taken as m_t, but in our experiment we did not know the exact number of female newborns. Therefore, we extrapolated the sex ratio data from the exposure experiment (i.e., estimated number of newborn females = number of hatched individuals × sex ratio) and the fecundity of eggs produced by each replicate in order to estimate m_t. The estimated sex ratio was 1:1.283 male to female.

Five mating pairs were randomly selected from each treatment at 90 d posthatch and incubated for two weeks. We then measured the number of eggs spawned and fertilized and obtained the fertilization ratio, and we calculated the arithmetic mean of fecundity over the pairs in order to estimate m_t.

Data were analyzed using Dunnett's test to evaluate the impact of carbaryl at each concentration compared with the control at each developmental stage. Subsequently, two-way analysis of variance (ANOVA) was used to examine the impacts of carbaryl on all biomarkers and *r*. All calculations were performed by using a code developed for the statistical program R (http://www.R-project.org).

Effects of carbaryl on embryo osteogenesis and larval vertebrae

To obtain information on the effects of carbaryl exposure on osteogenesis (as demonstrated by changes in body length) and on the vertebrae of medaka, additional carbaryl exposures of the same series of medaka embryos and larvae were performed. Larvae hatched from embryos exposed to carbaryl and directly exposed larvae were immersed in 0.02% calcein (3,3′-*bis*[*N,N*′-bis(carboxymethyl)aminomethyl]fluorescein, Dojindo Molecular Technologies, Kumamoto, Japan) solution for 10 min and then rinsed in fresh ERM for 30 min. The stained larvae were anesthetized with ice-cold water, and we then observed the effects of exposure on osteogenesis and vertebral structure under a fluorescence microscope (Leica MZ FL III, Leica Microsystems, Tokyo, Japan) equipped with a green fluorescent protein filter (wavelength range: excitation 480 nm, emission 510 nm). Fluorescence images were captured for 100 ms with a digital camera (Leica DC 350FX, Leica Microsystems) attached to the microscope. To identify the body shape and skeleton from the fluorescence, regular light images were also captured for 5 ms, and both images were overlaid. The captured fluorescence was pseudocolored with yellow-green by using the Leica FW 4000 software (Ver 1.0.3, Leica Microsystems).

Effects of carbaryl on larval tolerance to starvation

To obtain information on the effects of carbaryl exposure on larvae viability under altered environmental conditions, additional carbaryl exposures of the same series of medaka embryos and larvae were performed. Larvae hatched from embryos exposed to carbaryl and directly exposed larvae were subjected to a starvation tolerance test. Larvae posthatched from exposed embryos were moved to five 200-ml solutions of ERM, each in a glass dish (diameter 100 mm, depth 50 mm) and reared for 7 d without feeding. Larvae exposed to carbaryl had already been subjected to 96 h (4 d) of starvation during the exposure period, so they were reared without food for only an additional 3 d under fresh and clean conditions to complete the 7-d starvation test. After the starvation, the survival rates were measured.

RESULTS

Toxicities on medaka embryos and larvae and survival

With exposure of embryos to carbaryl at 10 mg/L, there was a significant ($p < 0.05$) increase in the rate of formation of tubular heart, a significant ($p < 0.001$) reduction in heart rate, and a significant ($p < 0.001$) shortening of body length compared with those in the controls; however, there was no effect on hatch rate (Table 1). Tubular heart formation and heart rate reduction are typical phenomena that occur in medaka embryos in response to chemical exposure [22–24]. Fluorescence microscopy with calcein staining was used to examine the shortening of body length in posthatch larvae due to disruption of osteogenesis as a result of carbaryl exposure during the embryo stage (Fig. 1). Fractured vertebrae were observed in medaka larvae exposed to carbaryl solutions for 96 h (Fig. 2A and B), although there were no lethal effects (Table 2). Vertebral fracture was caused by dislocation due to muscle cramping (Fig. 2C), and the rate of fracture increased with the exposure dose (Table 2). Shortened body length and fractured vertebrae disappeared in the adult stage (data not shown).

Carbaryl exposure at the embryo stage had no influence on tolerance to starvation after hatching; however, tolerance was significantly ($p < 0.001$) reduced when fish were exposed at the larval stage (Table 3). Although the exposure period at the embryo stage was much longer than that at the larval stage, the embryos were less susceptible than the larvae to carbaryl exposure in terms of starvation.

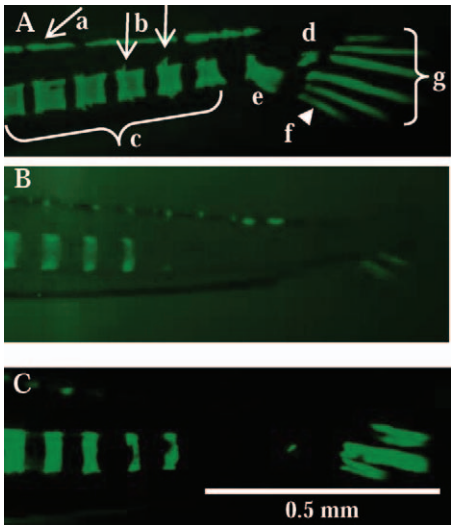

Fig. 1. Morphological effects of carbaryl on medaka osteogenesis. Posthatch larvae were stained with 0.02% calcein solution immediately after hatching. (**A**) Larva hatched from control embryo, showing a, pigmentation (indicated by diagonal arrow) on tail; b, neural spines (indicated by two downward arrows); c, vertebral centrum; d, uroneural; e, urostyle; f, parhypural (indicated by a arrowhead); and g, hypural. (**B**) Larva hatched from embryo exposed to 5 mg/L carbaryl. (**C**) Larva hatched from embryo exposed to 10 mg/L carbaryl. Osteogenesis was disrupted in tails of larvae hatched from embryos exposed to carbaryl.

Ninety-day survival of medaka was reduced by carbaryl exposure. The rates were 77.5, 60.8 ($p < 0.05$, in comparison with control), and 55.9% ($p < 0.005$) for exposure to 0, 5, and 10 mg/L carbaryl, respectively, at the embryo stage and 90.7, 73.3 ($p < 0.05$), and 74.7% ($p < 0.05$) for the same exposures at the larval stage. Carbaryl exposure had no effect on sex ratio, which was 1:1.283 male to female.

Impacts of carbaryl on population growth rate

The mean r was significantly different among treatments in the case of exposed embryos (one-way ANOVA, $F = 38.18$, $p < 0.001$). Dunnett's tests showed that mean r was significantly lower than in controls when embryos were exposed to 5 mg/L carbaryl (mean absolute difference = 0.014, $p < 0.05$), whereas it was significantly higher than in controls when embryos were exposed to 10 mg/L carbaryl (mean absolute difference = 0.076, $p < 0.001$). Least-squares regression analysis revealed that there was a significant positive relationship between dose rate and r ($F = 38.184$, $p < 0.001$; Fig. 3). Thus, the impact of carbaryl on r on medaka embryos varied with the concentration.

In contrast, a significant and constant reduction in mean r was found among treatments in the case of exposure of medaka larvae ($F = 34.383$, $p < 0.001$; Fig. 3). Dunnett's test indicated that the difference in mean growth rate between controls and fish exposed as larvae to 5 mg/L was not significant (mean absolute difference = 0.051, $p = 0.35$), whereas a significant difference was detected between the controls and fish exposed as larvae to 10 mg/L carbaryl (mean absolute difference = 0.300, $p < 0.001$). Two-way ANOVA demonstrated that both of the main factors—dose rate and developmental stage—had significant effects on r (Table 4). The interaction of these two factors had a highly significant effect, showing that the response of r was greatly dependent on medaka developmental stage and carbaryl dose rate.

Table 2. Rates of survival and vertebral fracture in medaka larvae exposed to carbaryl for 96 h[a]

Concn. of carbaryl (mg/L)	Total *n*	Survival (%)	Vertebral fracture (%)
0 (control)	75	100 (0.0)	0.0 (0.0)
5	75	100 (0.0)	8.4 (2.5)[b]
10	75	100 (0.0)	28.9 (6.4)[b]

[a] Standard errors are in parentheses.
[b] Analysis of variance versus control: $p < 0.001$.

DISCUSSION

We examined whether exposure of embryonic and larval medaka to carbaryl at sublethal dose rates led to different reductions in later viability and reproduction. We demonstrated that carbaryl has growth stage–dependent influences on medaka embryos and larvae. Medaka larvae were more susceptible to the effects of carbaryl than were larvae exposed as embryos in terms of the exposure-starvation test and population growth rate. Embryos exposed to carbaryl at sublethal concentrations exhibited increased rates of tubular heart, blood clots, and pericardial edema and a reduction in heart rate, increased rate of spinal deformity, and shortened body length. These abnormalities have been reported in similar embryo stages, including in other fish species [24–26]. Weis et al. reported that spinal deformity caused by carbaryl was more common when fish were exposed at the blastula stage than in the early eight- to 16-cell stages [25,26]. Gonzáles-Doncel et al. also reported that the organogenesis stage of the medaka embryo was more sensitive than the early stages to permethrin exposure [22]. Sensitivity to carbaryl appeared to depend on the developmental stage of the medaka embryos. However, the biological damage observed after exposure in the embryo stage was not as severe as that after exposure in the larval stage in terms of subsequent starvation testing and especially intrinsic population growth rate. Interestingly, larvae were more susceptible in terms of these two parameters even though the period of larval exposure was only 96 h. In freshwater adult/immature fish, carbaryl has been reported to cause energy crises and disruption of cholesterol metabolism [27,28] and to decrease glycogen and protein concentrations in the muscles and liver [29].

The results in our present study indicate that the yolk plays a role in combating exposure damage. The posthatch medaka larva consumes the yolk more rapidly than does the prehatch embryo, almost exhausting it almost within 72 h posthatch at 26°C (data not shown). If medaka larvae were exposed to carbaryl and damaged, they would become emaciated by exhausting the yolk to mitigate the damage from chemical stress; subsequently, they would fall into a state of energy deficit because at this time they are not exotrophic (i.e., they are still obtaining nutrition from the yolk alone) and do not have an abundance of nutrients that can help them combat chemical damage. The larvae therefore had less starvation tolerance than the embryos (Table 3), and subsequence effects may eventually have substantially reduced their r (Fig. 3). Our statistical analyses indicated that the response of r differed significantly with growth stage (Table 4). These results suggested that exposure of embryos to carbaryl did not necessarily reduce their population numbers. In contrast, we can expect that the suscep-

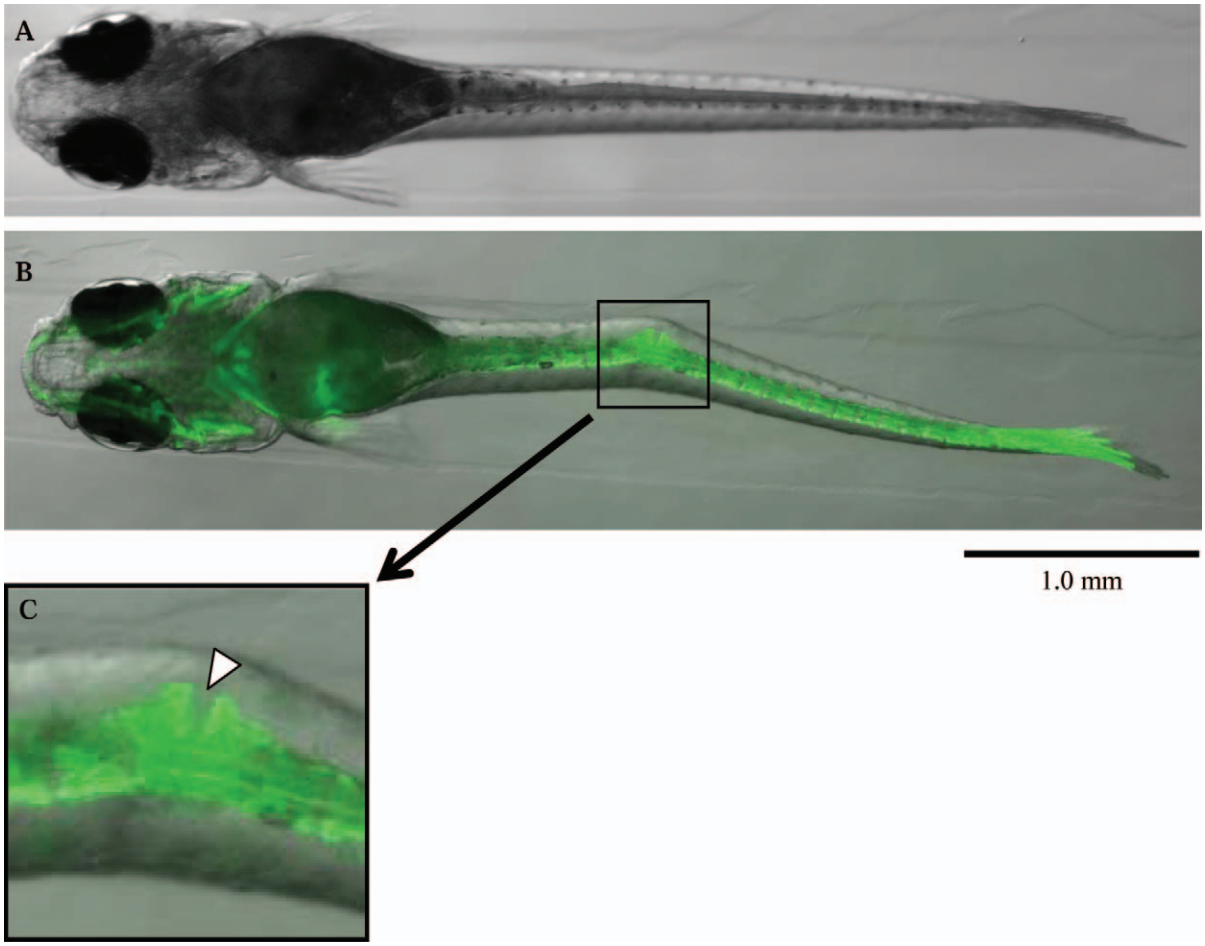

Fig. 2. Vertebral fracture in medaka larva exposed to carbaryl. Posthatch medaka larvae were exposed to 10 mg/L carbaryl for 96 h. (**A**) Control, (**B**) carbaryl-exposed, and (**C**) a partial enlarged image from **B** (white arrowhead indicates vertebral fracture). Exposed larvae were stained with 0.02% calcein solution and observed under a fluorescence dissecting microscope (**B** and **C**). Images **B** and **C** are overlapping images of regular light and fluorescence images.

tibility of medaka larvae would have a longitudinal negative influence on future population growth.

The European Union REACH legislation proposes the bioassay of embryos of zebrafish, medaka, and fathead minnow as an alternative to fish toxicity testing [3]. Use of zebrafish embryos for acute toxicity testing has advantages in terms of cost-, space-, and time-effectiveness because zebrafish can produce greater numbers of embryos than medaka and complete their hatching within 2 or 3 d (shorter than the 7–10 d required for medaka). The new zebrafish embryo assay will become an alternative to the conventional fish acute toxicity test. Conventional LC50 data have been applied to assessments of the ecological risks of hazardous chemicals; hence, LC50 data on embryos will be used as well. It is therefore important that we demonstrated here that the toxic effects from carbaryl exposure

Table 3. Growth-dependent effects of carbaryl exposure on tolerance of larvae to starvation (7 d)

Tolerance	Carbaryl concn. (mg/L)	Exposure at	
		Embryo stage[a]	Larval stage[b]
Tolerance to starvation (% survival rate after 7 d)	0 (control)	95.3 (2.9)	98.7 (1.3)
	5	93.9 (1.6)	85.3 (5.3)[c]
	10	96.5 (2.1)	81.3 (3.9)[d]

[a] Embryos were exposed to carbaryl from immediately after fertilization until hatch. Hatched larvae were immediately moved to fresh and clean water conditions and kept for 7 d without feeding.
[b] Immediately after hatching, larvae were exposed to carbaryl for 96 h without food and then moved to fresh and clean water, where they were kept for a further 3 d without feeding (total 7 d starvation). Standard errors are in parentheses.
[c] Analysis of variance versus control: $p < 0.05$.
[d] Analysis of variance versus control: $p < 0.001$.

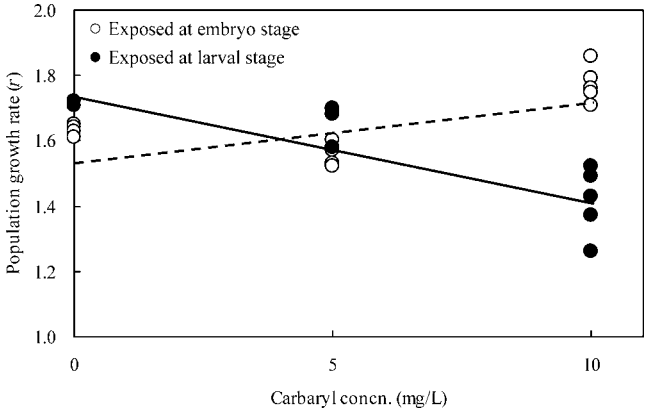

Fig. 3. Population-level effects of carbaryl exposure on medaka exposed as embryos and larvae as measured by the intrinsic rate of natural increase.

Table 4. Two-way analysis of variance table: comparison of the effects of developmental stage and carbaryl dose level on intrinsic growth rate, *r*

Factor	*df*[a]	MS[b]	*F*[c]	*p*[d]
Dose	2	0.0359	6.7878	0.0046
Stage	1	0.0286	10.8185	0.0031
Dose × stage	2	0.3397	64.2278	<0.0001

[a] Degrees of freedom.
[b] Mean squares.
[c] *F* statistic.
[d] Probability of significance.

of medaka at the embryo stage were lower than from larval exposure in terms of population growth rate. Carbaryl exposure of embryos and larvae led to different ecological scenarios. The early establishment of a toxicity database to characterize the effects of chemicals on adult fish or embryos is therefore important; it is also important that we pay attention to population dynamics in the ecological risk assessment of hazardous chemicals.

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