

# Variations of Light and Temperature Regimes and Resulting Effects on Reproductive Parameters in Medaka (*Oryzias latipes*)<sup>1</sup>

C.S. Koger, S.J. Teh, and D.E. Hinton<sup>2</sup>

School of Veterinary Medicine, Anatomy, Physiology and Cell Biology, University of California-Davis, Davis, California 95616

## ABSTRACT

In seasonally breeding fish species, altered fecundity, fertility, and spawning interval are associated with changes in environmental cues such as temperature and photoperiod. To determine quantitative impact of these cues on a suite of reproductive endpoints, groups of medaka (*Oryzias latipes*; two breeding pairs per group) were subjected to varying photoperiod and temperature regimes. Embryo production ceased after photoperiod reduction from 16L:8D to 8L:16D (at 25°C). A severe decline in production was observed after a temperature decrease of 10°C (25°C to 15°C [16L:8D]). Under reduced photoperiod, histologic analysis showed no mature ova and moderate oocyte atresia in all individuals. However, reduced temperature (15°C) produced only mild oocyte atresia and fewer mature ova. Under both reduced photoperiod and reduced temperature regimes, mature spermatozoa were observed. Offspring viability, along with spawning interval, were not affected by photoperiod reduction. Temperature change had no effect on offspring viability but caused an increase in spawning interval. A shortened photoperiod profoundly affected medaka reproduction, whereas decreased temperature reduced, but did not arrest, fertility; reduced photoperiod decreased fecundity. These findings have important implications for culture of medaka as well as use of this teleost model for reproductive toxicology studies.

## INTRODUCTION

Reproduction is sensitive to environmental pollutants via modulation of endocrine function. Recent field and laboratory studies showed that certain compounds mimic estradiol, producing unexpected effects in feral and laboratory fishes [1–3]. In several studies, male fish exposed to sewage effluent or estrogenic pesticides produced the yolk-precursor protein, vitellogenin [4–7]. In addition, fishes living below effluent from paper mills processing pine trees were shown to have modulated endocrine function [8–10]. What is not known is whether these changes are associated with adverse reproductive effects. In order to better determine the significance of the above findings with respect to reproduction, sensitive, well-calibrated fish models are needed.

Medaka (*Oryzias latipes*) are oviparous freshwater teleosts recently proposed as models for detecting adverse effects of chemicals on reproduction [11–13]. These fish breed more freely than other egg-laying aquarium fishes [14] and when maintained under a 16L:8D photoperiod at 25°C, produce 10–25 embryos in a 24-h period, regardless of when the light cycle is initiated [15, 16]. Continuous

light or dark cycles disrupted the normal daily reproductive cycle of medaka. A short photoperiod caused cessation of embryo production despite sufficient reproductive temperature, and a minimum day-length of 11–12 h was required for spawning [17]. Females maintained under conditions with a light phase less than 11–12 h ceased spawning within 20 days. These studies suggest that changes in photoperiod disrupt the normal daily reproductive cycle of medaka.

The goal of the present study was to quantitatively determine the effects that alteration of light and temperature have on egg production and fertilization success. In addition, we characterized the morphology of medaka gonads during periods of sexual activity and inactivity as well as during reproductive recrudescence.

## MATERIALS AND METHODS

Two separate experiments were conducted to quantify effects of altering light or temperature on fecundity, fertility, brood interval, and embryo and fry viability. Preliminary studies demonstrated that conditions of 16L:8D with a water temperature of 25°C promoted a consistent daily production of embryos ( $20 \pm 3$ /group). In the first experiment, effects of photoperiod reduction (from 16L to 8L) while maintaining a temperature of 25°C were determined. In the second experiment, photoperiod was maintained at 16L:8D, but temperature was reduced from 25°C to 15°C. Animals maintained at 16L:8D and 25°C were used as controls for both experiments.

### Environmental System

A table-top tank system (Aquanetics, San Diego, CA) was used for maintaining tightly controlled light and temperature conditions. Water temperatures were maintained at 25 or  $15 \pm 0.3^\circ\text{C}$ . Lighting was provided by two 40-watt Coralite Chromatic bulbs (Candela Corporation, Santa Ana, CA), and photoperiod was maintained with an automatic timer. Light meter (GreenleeTextron Inc., Rockford, IL) measurements within the tank showed zero lumens when lights were off. Fish were housed in aerated 1000-ml beakers and were fed a casein-based purified diet shown to be adequate for normal growth and reproduction in medaka [18] at a rate of 1% body weight three times daily (total = 3% body weight/day). Beakers were siphoned three times daily to control nitrite formation and to remove deposited eggs. Approximately 40% of reconstituted water [19] was replaced daily.

### Animals

Newly hatched golden medaka fry (1200) were raised in a 38-L tank at 25° from hatch in a flow-through system at the Institute of Ecology, University of California-Davis (Davis, CA). At 1 mo, 125 fry were transferred to each of

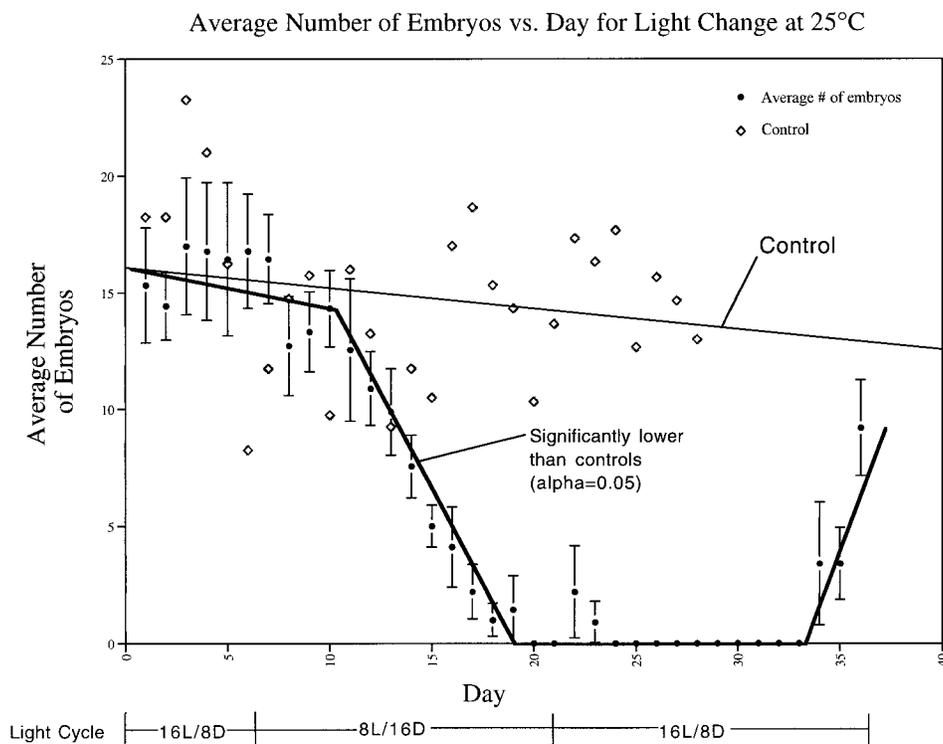
Accepted June 22, 1999.

Received May 17, 1999.

<sup>1</sup>This work was supported by Grant #R823297 from the U.S. Environmental Protection Agency and by National Cancer Institute Grant CA 45131 from the Department of Health and Human Services.

<sup>2</sup>Correspondence: David Hinton, 1 Shields Ave., VM:APC 1321 Haring Hall, University of California, Davis, Davis, CA 95616. FAX: 530 752 9692; e-mail: dehinton@ucdavis.edu

FIG. 1. Effect of photoperiod reduction on embryo production compared to control embryo production under constant temperature conditions. Embryo production ceased in all culture vessels 14 days after the photoperiod was reduced from 16L:8D to 8L:16D at 25°C. After a return to initial light phase duration, embryo production resumed in all culture vessels after 14 days.



four 38-L tanks, for a total of 500 fish. These fish were monitored for 2 mo until egg production was well established in each of the aquaria. The fish were 3 mo old at the beginning of the light experiment and 4 mo old at the beginning of the temperature experiment. Spawning females were selected in the morning when egg clutches were still attached. Males were separated by size, and larger individuals were used. Breeding groups (2 males, 2 females) were transferred to 1000-ml beakers and allowed to acclimate for 4 days before each experiment was initiated. There were a total of 12 breeding groups for the light experiment (3 controls, 9 test beakers) and 13 breeding groups for the temperature experiment (3 controls, 10 test beakers).

#### Embryo Collection and Culture

After the 4-day acclimation period, all resultant egg masses were collected from each culture vessel. To reduce stress to individual fish, siphoning of independent egg masses was done and fish were fed, thereby reducing their tendency to consume eggs. Culture vessels were cleaned by siphoning three times daily, and egg production over 5 days was averaged as an estimate of daily egg production. Embryonated eggs were counted; egg masses were separated using forceps, and after transfer to uncapped 59-ml Qorpak French Square bottles (Fisher Scientific, Pittsburgh, PA) containing 50 ml United States Environmental Protection Agency reconstituted water, were cultured at  $25 \pm 1^\circ\text{C}$  and agitated on an orbital shaker. Unfertilized eggs or dead embryos (opaque) were recorded and discarded. Viable embryos were observed daily for mortality and hatch. To prevent fungal growth, dead embryos were removed from culture vessels upon detection. Approximately 50% of the embryo culture water was changed daily.

#### Photoperiod Change with Constant Temperature

After 5 days at 16L/8D and 25°C, photoperiod was changed to 8L:16D. Embryos were collected and cultured

for the initial 5 days and also from Day 10 to Day 14 as described above to establish baseline embryo production, and were counted throughout the remainder of the experiment. Histologic analysis of the liver and gonads was performed on all individuals of two randomly selected culture vessels from time points after the photoperiod change (Day 6, see Fig. 1) and after all egg production had ceased (Day 20, see Fig. 1). After cessation of egg production, the photoperiod was reversed to the original 16L:8D, and the remaining five culture vessels were maintained under these conditions. Fish were also sampled for histologic analysis after egg production resumed in all vessels (Day 35, see Fig. 1).

#### Temperature Change with Constant Photoperiod

After 5 days at 16L:8D and 25°C, water temperature was reduced to 15°C. Embryos were collected and cultured for the initial 5 days and also from Day 16 to Day 20 as described above, and were counted but not cultured throughout the remainder of the experiment. After the temperature change (Day 6, see Fig. 6) histologic analysis of the liver and gonads was performed on all individuals of randomly selected culture vessels. Samples were taken on Days 14, 15, 16, 21, 23, and 25. After 14 days (Day 21, see Fig. 6) at 15°C, the temperature was increased to 20°C to determine whether fish would produce embryos comparable to those of control fish. After egg production resumed, the temperature was then increased to 25°C (Day 25, see Fig. 6). Fish from the remaining four beakers were sampled after 4 days (Day 29, see Fig. 6).

#### Fry Viability

After hatch, fry were observed for 1 wk to detect developmental abnormalities and to monitor viability.

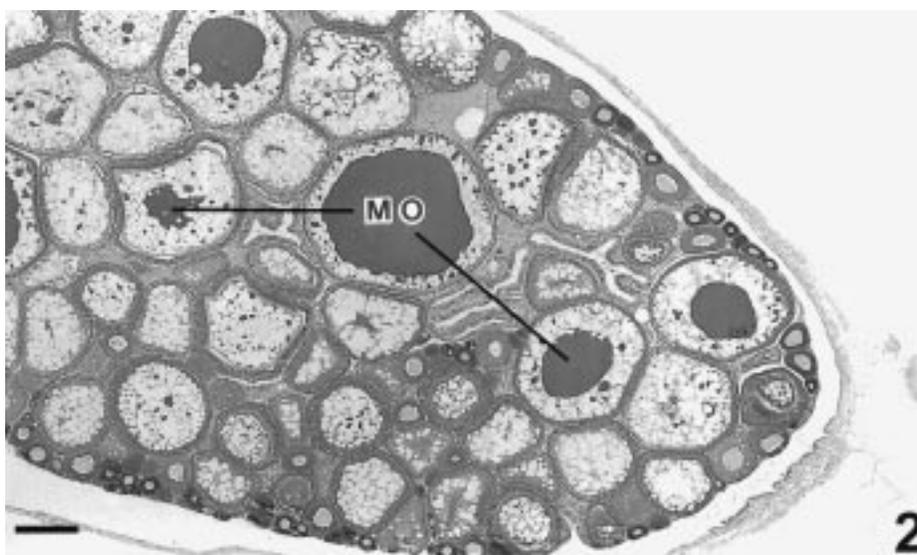


FIG. 2. Normal adult ovary with numerous mature (MO) and immature oocytes. Bar = 270  $\mu\text{m}$ .

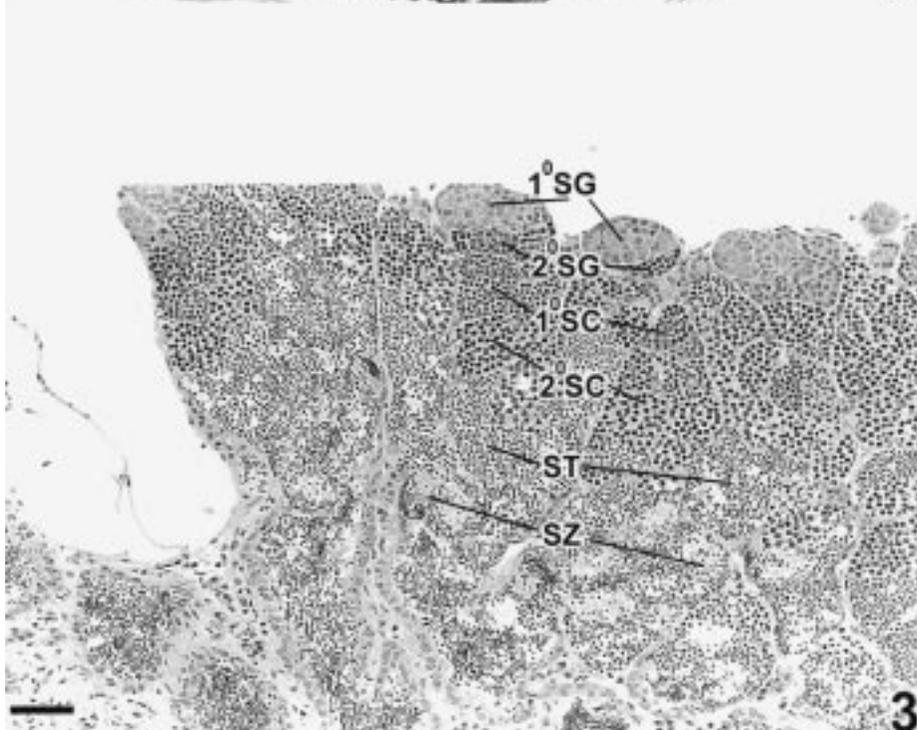


FIG. 3. Normal adult testis, representative of all male testes examined, showing the progression of immature germ cells to mature sperm. 1°SG, primary spermatogonia; 2°SG, secondary spermatogonia; 1°SC, primary spermatocytes; 2°SC, secondary spermatocytes; ST, spermatids; SZ, spermatozoa. Bar = 54  $\mu\text{m}$ .

### Histologic Evaluation

After fish were killed with a lethal concentration of MS-222 (100 mg/L), abdomens of all fish were surgically opened to ensure optimum fixation in 10% buffered formalin. After 24 h, fish were then transferred to Bouin's fixative for another 24 h to decalcify bones, allowing sectioning of entire fish. Tissues were then dehydrated with alcohol. All samples were then embedded in either paraffin or glycol methacrylate (GMA). Serial sections (4–4.5  $\mu\text{m}$ ) through the viscera were stained with hematoxylin and eosin and viewed under a light microscope. The percentage of ovarian atresia was scored as 1) none, 2) mild (5–10%), 3) moderate (11–25%), and 4) severe (> 25%).

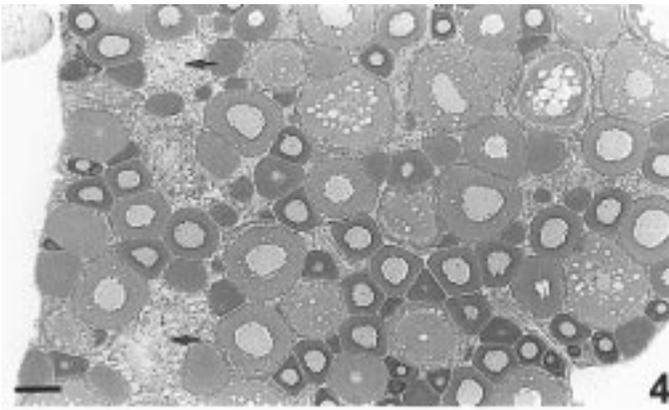
## RESULTS

### Photoperiod Change Experiment

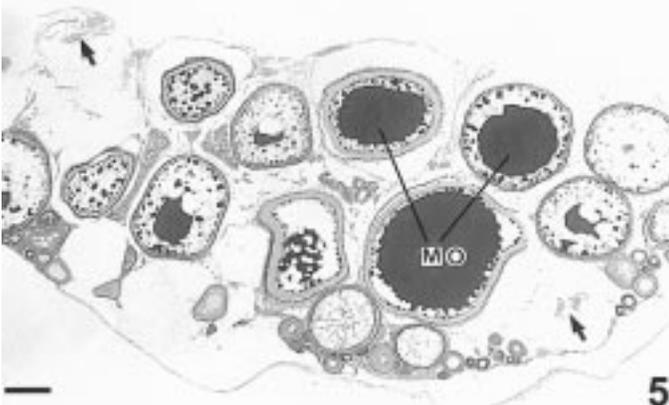
When a 16L:8D photoperiod was used, consistent production of embryos was observed before photoperiod re-

duction. After photoperiod was decreased, embryo production gradually decreased for 14 days until production ceased in all nine culture vessels (Day 20, Fig. 1). Along with a decrease in total number of embryos, there was an increase in the number of unfertilized eggs collected. After the photoperiod was reset to 16 h (Day 21, Fig. 1), all culture vessels produced embryos after a period of 14 days (Day 35, Fig. 1), and production of embryos was at average levels approaching that of control vessels. There was no statistical difference ( $\alpha = 0.05$ , Tukey-Kramer, JMP software, SAS Institute, Cary, NC) between the viability of embryos or hatched fry collected during a 16-h photoperiod compared to those collected during an 8-h photoperiod. No developmental abnormalities were observed in collected embryos or hatched fry.

Histologic evaluation of the gonads of control fish (6 per gender) showed abundant mature and immature germ cells in both ovaries (Fig. 2) and testes (Fig. 3). All female fish (10 fish) maintained at a lower photoperiod displayed atret-



4



5

FIG. 4. Ovary under reduced photoperiod (8L:16D, 25°C) displaying mild oocyte atresia (arrows) and no mature oocytes. Bar = 270 μm.

FIG. 5. Ovary returned to 16L:8D from 8L:16D displaying severe oocyte atresia (arrows) despite the presence of mature oocytes (MO). Bar = 270 μm.

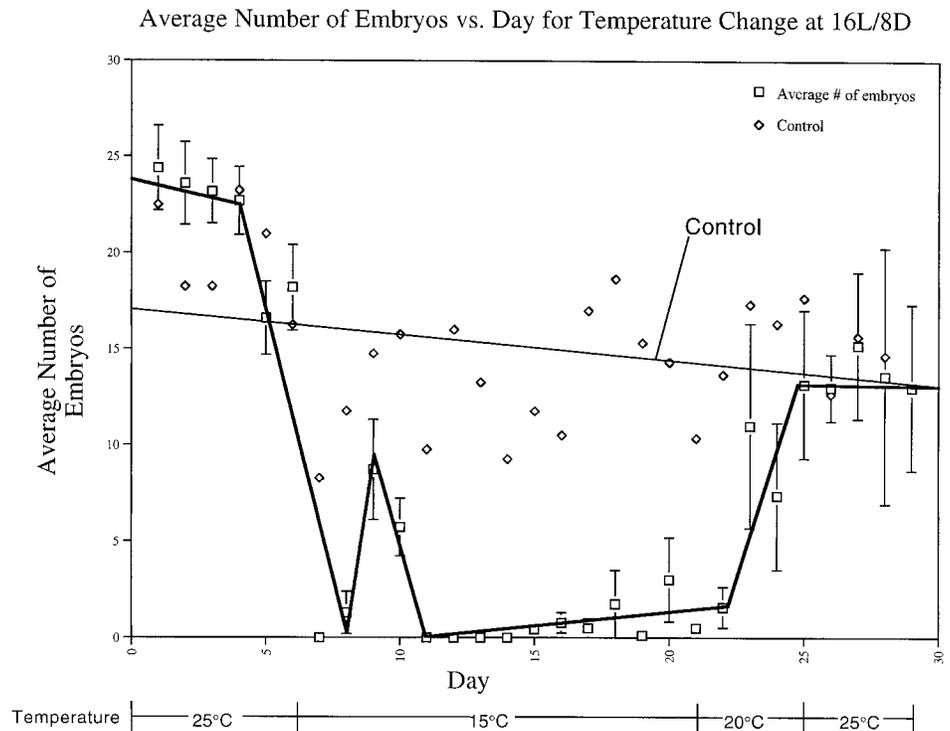
ic oocytes, and absence of mature ova was observed (Fig. 4). Oocyte atresia was characterized by the presence of large numbers of macrophages within the hypertrophic follicles as shown in Figures 4, 7, and 8. Atretic oocytes were still present in higher numbers than in control fish after a return to 16L (8 fish). Along with increased atresia and interoocyte edema, there were fewer immature oocytes (Fig. 5). All male fish (10 fish) maintained at a low photoperiod had mature spermatozoa present in the testicular ducts (Fig. 3).

*Temperature Change Experiment*

During the initial 5 days of collection, embryo production was consistent (Day 6, Fig. 6). The day after the temperature reduction, all embryo production ceased. Sporadic production was then observed for the next 3 days followed by a 4-day cessation of embryo production. Subsequently, embryo production was followed for 1 wk and shown to occur randomly and at levels significantly lower than that of controls. No change in the number of unfertilized eggs occurred after temperature reduction. After the temperature was increased to 20°C (Day 21, Fig. 6) embryo production reached average control levels after 3 days, despite severe oocyte atresia observed in histologically evaluated fish (Fig. 7, 4 fish). On Day 25 (Fig. 6) the temperature was returned to the initial 25°C, and embryo production remained consistent with that of controls. There was no statistical difference (alpha = 0.5) between the viability of embryos or hatched fry collected from vessels maintained at 25°C and those collected from vessels at 15°C. No developmental abnormalities were observed in embryos or fry collected at reduced temperature.

Histologic evaluation of gonads of fish (8 fish) maintained at a lower temperature revealed an increase in the number of atretic oocytes in ovaries, although mature and abundant immature ova were present (Fig. 8). Again, ma-

FIG. 6. Effect of temperature reduction on embryo production compared to control embryo production. Embryo production ceased after a temperature reduction of 10°C (from 25°C to 15°C with a 16L:8D cycle). Embryo production resumed at significantly lower numbers than observed in control culture vessels. After a temperature increase from 15 to 25°C, embryo production resumed in all culture vessels.



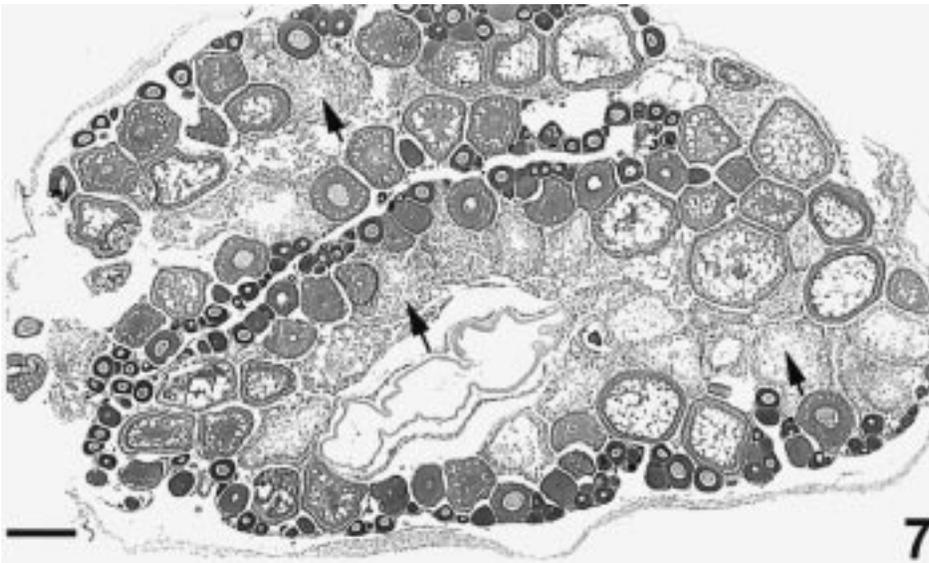


FIG. 7. Ovary raised from 15°C to 20°C. Note the severe oocyte atresia (arrows) and the presence of several mature oocytes. Bar = 270  $\mu$ m.

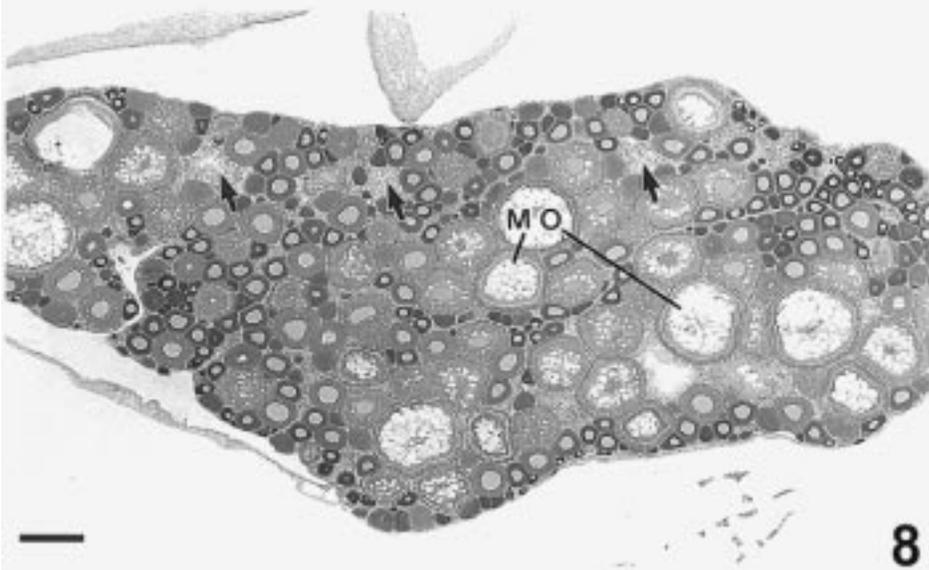


FIG. 8. Ovary under reduced temperature conditions (15°C, 16L:8D) displaying mild to moderate oocyte atresia (arrows) while still producing mature oocytes (MO). Bar = 270  $\mu$ m.

ture spermatozoa were present in each testis evaluated (Fig. 3, 20 fish) at 15°C.

#### *Histologic Evaluation of the Kidney*

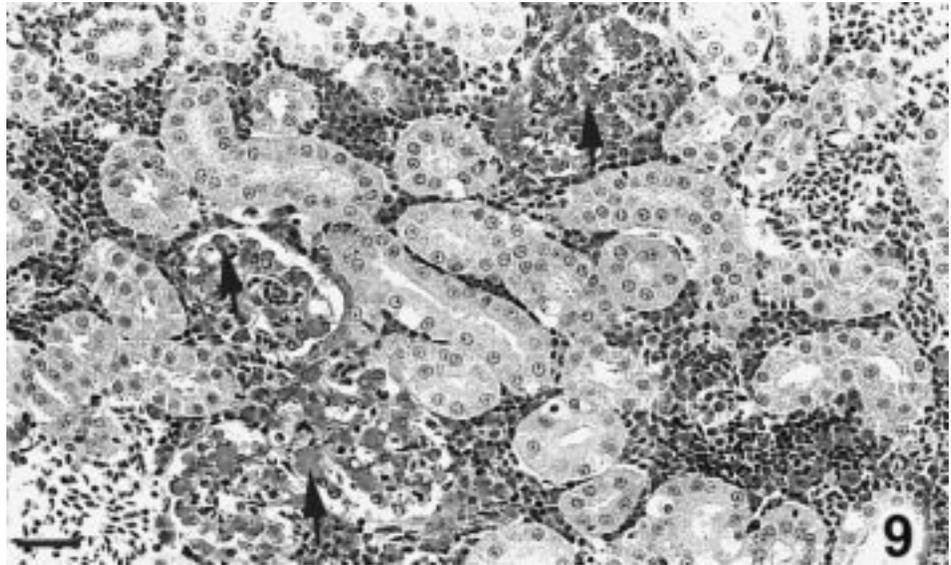
Eosinophilic proteinaceous droplets were found in the glomeruli and proximal tubular epithelium of kidneys of all fish maintained under reduced photoperiod or temperature conditions (Fig. 9). Fish transiently held under altered environmental conditions that were returned to the original photoperiod or temperature also displayed the same eosinophilic droplets in the renal sites as described above.

#### **DISCUSSION**

While numerous studies have determined effects of photoperiod and/or temperature on certain aspects of medaka reproduction [15, 16, 20–24], the current study demon-

strates the effects these environmental cues have on 1) gonadal morphology during periods of sexual activity and inactivity, 2) fecundity, and 3) fertility. The assessment of a suite of parameters under defined environmental conditions makes this study unique. Results demonstrated that male gonads maintained a functional microscopic morphology, indicated by mature sperm production and large numbers of various stages of maturing germ cells, and that this appearance was maintained regardless of environmental conditions. However, female gonads displayed obvious morphologic changes in response to variation of either of the two environmental cues, and this variation was associated with reduced numbers of embryos produced. An increase in the number of unfertilized eggs following a photoperiod reduction suggests that females were either producing unfertilizable eggs or that males were not fertilizing oviposited eggs because of a behavioral change, since all males

FIG. 9. Kidney of a female under reduced photoperiod conditions (8L:16D) displaying eosinophilic (proteinaceous) droplets in the glomerular spaces (arrows). Bar = 54  $\mu$ m.



were producing mature sperm. Further studies are required to determine which of the above circumstances occurs.

By 14 days, under shortened photoperiod, embryo production ceased in all culture vessels. Subsequent ovarian histologic examination, after cessation of production, revealed no mature oocytes. Further, mild oocyte atresia, in conjunction with a reduction in number of immature oocytes, demonstrated that fecundity (capacity for reproduction, number of oocytes in the ovary) was reduced by decreased photoperiod. Our findings confirmed the Yoshioka [21] study, which demonstrated that oocyte regression (defined as atresia) was associated with a reduction of photoperiod. Upon restoring light phase to original photoperiod conditions, we observed ovaries with severe oocyte atresia, interoocyte edema, and few mature oocytes. These findings suggest that the ovary may need additional time to restore reproductive activity, and that an interval of overlap exists in which elements of regression and renewed folliculogenesis appear in the same organ. Upon return to original photoperiod, gonads of newly restored, reproductively active females resembled those of control fish with one exception, presence of residual atretic oocytes.

In response to a decrease in temperature, ovaries of spawning medaka showed a decrease in the number of mature oocytes and increased oocyte atresia compared to controls. Gerking [25] demonstrated that fish have an optimal temperature associated with optimal reproductive output.

Since oocyte maturation is dependent on the enzymatic reactions involved in steroidogenesis and synthesis of yolk proteins, and an alteration in temperature impacts these reactions, the change in embryo production was expected [26]. Although embryo production did not cease, spawning interval increased from one to approximately 3 days. The abrupt cessation of embryo production following temperature reduction was most likely due to the rapid 10°C temperature reduction. These findings indicate that reduced temperature affected fertility (number of offspring produced), but failed to affect fecundity since no reduction in immature germ cell number was observed. When temperature was increased, embryo production was comparable to that seen in control fish. However, moderate oocyte atresia was observed, as above, suggesting that the ovary contained some residual, atretic oocytes despite re-entering gametogenesis. The viability of offspring was not significant-

ly different under altered photoperiod or temperature in this study, indicating that once fertilization occurred, embryos developed normally.

Eosinophilic (proteinaceous) droplets found in the glomeruli of female fish after light or temperature was reduced suggests that vitellogenin produced during this period of depressed sexual activity could not be incorporated into oocytes, remained in plasma, and crossed the blood-urine barrier in the glomeruli. These findings are similar to those of Wester and Canton [27], who found proteinaceous droplets in the kidneys of juvenile medaka exposed to estrogenic compounds. Studies using guppies (*Poecilia reticulata*) yielded similar results, as well as histochemical confirmation that the droplets observed were indeed yolk-precursor proteins [28]. Normally, proteins at or above the molecular mass of albumin (66 kDa) are not found in the glomerular filtrate. Vitellogenin is much larger than albumin (180 kDa) and should not be found in epithelial cells of the proximal tubule. However, some yolk-precursor subunits are small enough to cross the blood-urine barrier and could be re-sorbed in the proximal tubule. There were two possible sources of the vitellogenin found in medaka kidneys in the present study: 1) recently synthesized vitellogenin that was unable to be incorporated into oocytes because of decreased folliculogenesis, and 2) vitellogenin that had previously been transported into developing oocytes and was cleaved to yolk but was subsequently released during oocyte atresia. To our knowledge, the second source has not been investigated. If all vitellogenin from atretic oocytes is phagocytosed by granulosa cells and broken down in lysosomes, it may not enter the intercellular space and thereafter appear in the circulation.

The advantages of using medaka to measure reproductive output are numerous. First, aspects of this study showed that reproductive capacity can be calibrated before exposure, thereby establishing a baseline for a given set of breeding groups (Figs. 1 and 6). During and after exposure, reproductive output can be compared to both that of control fish and pre-exposure output from exposed groups. Second, the small size of medaka enables the use of reasonable numbers of replicates, permitting quantification of a variable endpoint. Third, daily production of embryos permits tracking of reproductive progress. Fourth, medaka have a relatively short maturation cycle, enabling multigeneration-

al studies in reasonable time periods. For example, a three-generation study can be completed in under 1 yr.

Associating toxicant exposure with effects on reproduction is often difficult because of reproductive variability, and few studies have shown such effects in a dose-dependent manner. The current approach may be used to determine the adverse effects of toxicants on reproduction in this model during specific windows of exposure. Sexually active fish could be exposed to assess real-time effects of a toxicant on both adults and on resultant offspring. Conversely, effects on recrudescence could be ascertained by cessation of reproductive activity via a photoperiod and/or temperature reduction and exposure during this period of sexual inactivity. This approach mimics seasonal flux with environmental factors. Furthermore, data from this experiment show that alteration of environmental cues alone can lead to certain changes that could be erroneously attributed to toxicant exposure. The current experimental design permits exposure, in a controlled setting, of breeding groups of medaka to water samples from the field, thereby retaining environmental relevance.

## ACKNOWLEDGMENTS

We would like to thank Ching Teh, Christine Wang, Sun Wong, Brian Enriquez, Can Nhan, Felix Chu, and Jeaneal Davis for their help with this project.

## REFERENCES

1. Jobling S, Sumpter JP. Detergent components in sewage effluent are weakly oestrogenic to fish: an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquat Toxicol* 1993; 27:361–372.
2. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 1996; 15:194–202.
3. Ashfield LA, Pottinger TG, Sumpter JP. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environ Toxicol Chem* 1998; 17:679–686.
4. Denison MS, Chambers JE, Yarbrough JD. Persistent vitellogenin-like protein and binding of DDT in the serum of insecticide resistant mosquitofish (*Gambusia affinis*). *Comp Biochem Physiol* 1981; 69C:109–112.
5. Donohoe RM, Curtis LR. Estrogenic activity of chlordecone, o,p'-DDT and o,p'-DDE in juvenile rainbow trout: induction of vitellogenesis and interaction with hepatic estrogen binding sites. *Aquat Toxicol* 1996; 36:31–52.
6. Toy NH, Washburn BS. Effects of waste water on vitellogenin levels of Sacramento suckers. In: UC Davis Undergraduate Research Conference; 1996; Davis, CA.
7. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* 1994; 135:175–181.
8. Van der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol Appl Pharmacol* 1992; 115:224–233.
9. Gagnon M, Bussieres D, Dodson JJ, Hodson PV. White sucker (*Catostomus commersoni*) growth and sexual maturation in pulp mill-contaminated and reference rivers. *Environ Toxicol Chem* 1995; 14:317–327.
10. LeBlanc J, Couillard CM, Brethes J-CF. Modifications of the reproductive period in mummichog (*Fundulus heteroclitus*) living downstream from a bleached kraft pulp mill in the Miramichi Estuary, New Brunswick, Canada. *Can J Fish Aquat Sci* 1997; 54:2564–2573.
11. Papoulias DM, Tillitt DE, Jones S, Noltie D. Use of medaka (*Oryzias latipes*) as a model to identify reproductive endpoints indicative of exposure to endocrine disrupting chemicals. In: Program of the 16th annual meeting of the Society of Environmental Toxicology and Chemistry; November 5–9, 1995; Vancouver, BC, Canada. Page 197.
12. Arcand-Hoy LD, Benson WH. Fish reproduction: an ecologically relevant indicator of endocrine disruption. *Environ Toxicol Chem* 1998; 17:49–57.
13. Metcalfe CD, Gray MA, Kiparissis Y. The Japanese medaka (*Oryzias latipes*): an in vivo model for assessing the impacts of aquatic contaminants on the reproductive success of fish. In: Rao SS (ed.), *Impact Assessment of Hazardous Aquatic Contaminants: Concepts and Approaches*. Boca Raton, FL: Lewis Publishers; 1999: 29–52.
14. Myers GS. Easiest of all to spawn and raise-the medaka. *Aquarium J* 1952; 9:13.
15. Robinson EJ, Rugh R. The reproductive processes of the fish *Oryzias latipes*. *Biol Bull* 1943; 84:115–125.
16. Egami N. Effect of artificial photoperiodicity on time of oviposition in the fish *Oryzias latipes*. *Annot Zool Jpn* 1954; 27:57–62.
17. Yoshioka H. On the effects of environmental factors upon the reproduction of fishes: I. Effects of short and long day-lengths on *Oryzias latipes* during spawning season. *Bull Fac Fish Hokkaido Univ* 1963; 14:137–151.
18. DeKoven DL. Evaluation of conventional and purified rations for the Japanese medaka, *Oryzias latipes*: refining a fish model for toxicological research. *Lab Anim Sci* 1992; 42:180–189.
19. Horning WB, Weber CI. Short-term methods for estimating chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014 1985: 58–75.
20. Egami N. Effects of exposure to low temperature on the time of oviposition and the growth of the oocytes in the fish, *Oryzias latipes*. *J Fac Sci Univ Tokyo Sect IV Zool* 1959; 8:539–548.
21. Yoshioka H. On the effects of environmental factors upon the reproduction of fishes: I. The effects of day-length on the reproduction of the Japanese killifish, *Oryzias latipes*. *Bull Fac Fish Hokkaido Univ* 1962; 13:123–136.
22. Yoshioka H. On the effects of environmental factors upon the reproduction of fishes: III. The occurrence and regulation of refractory period in the photoperiodic response of medaka, *Oryzias latipes*. *J Hokkaido Univ Univ Education* 1966; 17:23–33.
23. Chan KK-S. A photosensitive daily rhythm in the female medaka, *Oryzias latipes*. *Can J Zool* 1976; 54:852–856.
24. Weber DN, Spieler RE. Effects of the light-dark cycle and scheduled feeding on behavioral and reproductive rhythms of the cyprinodont fish, Medaka, *Oryzias latipes*. *Experientia* 1987; 43:621–624.
25. Gerking SD. Fish reproduction and stress. In: Ali M (ed.), *Environmental Physiology of Fishes*. New York: Plenum Press; 1980:18–22.
26. Kime DE. The control of gonadal androgen biosynthesis in fish. In: Richter CJ, Goos HJT (eds.), *Proceedings of the International Symposium on the Reproductive Physiology of Fish*. The Netherlands: Pudoc, Wageningen; 1982:133.
27. Wester PW, Canton JH. Histopathological study of *Oryzias latipes* (medaka) after long-term  $\beta$ -hexachlorocyclohexane exposure. *Aquat Toxicol* 1986; 9:21–45.
28. Wester PW, Canton JH. Histopathological study of *Poecilia reticulata* (guppy) after long-term  $\beta$ -hexachlorocyclohexane exposure. *Aquat Toxicol* 1985; 6:271–296.