

Krause, P.R., C.W. Osenberg, R.J. Schmitt.  
1992. Effects of produced water on early life  
stages of a sea urchin: gender-specific  
responses and delayed expression. *In* J.P.  
Ray and F.R. Englehardt (eds.), *Produced  
water: technological/environmental issues  
and solutions*, Plenum Press, New York.

## EFFECTS OF PRODUCED WATER ON EARLY LIFE STAGES OF A SEA URCHIN: STAGE-SPECIFIC RESPONSES AND DELAYED EXPRESSION

P. R. Krause<sup>1,2</sup>, C. W. Osenberg<sup>1</sup> and R. J. Schmitt<sup>1</sup>

<sup>1</sup> Coastal Research Center, Marine Science Institute

<sup>2</sup> Department of Biological Sciences  
University of California  
Santa Barbara, California  
USA

### INTRODUCTION

Common objectives of many bioassays are to provide a measure of relative toxicity of various effluents (e.g., Allen, 1971; Kobayashi, 1980; National Research Council, 1983), or to identify components in a given effluent that are most responsible for biological effects (e.g., Higashi et al., 1992). There is an indisputable need for such perspectives for produced waters from different coastal formations (Neff, 1987). However, while such a focus can provide a useful toxicity benchmark, it is not particularly well suited for exploring the nature, extent and generality of biological responses. For example, there is an emerging paradigm that earlier developmental stages of a marine organism are more sensitive to a pollutant than later ones (e.g., National Research Council, 1983; Neff et al., 1976), yet no consistent pattern emerges from the relatively few studies that have examined different life stages of the same species (e.g., Neff et al., 1976; Rossi and Anderson, 1978; see Capuzzo, 1987). Even when multiple life stages of a species have been considered, it is difficult to extend laboratory-based results to natural situations, in part because of uncertainty as to whether the concentrations and exposure durations used are realistic (e.g., Castagna et al., 1981; Neff, 1987).

Another feature of bioassays, and one that has not received a great deal of attention, concerns the endpoint (response variable) chosen to measure an "effect." For a number of logical and logistical reasons, the response variable selected often is a biological "milepost" that coincides with the end of an exposure period. For example, fertilization success often is used as the response variable to estimate effects of toxicant exposure on gametes (e.g., Kobayashi, 1971; Dinnel et al., 1987). However, the extent of deleterious consequences of exposure of gametes will be underestimated if additional effects are manifested at a later developmental stage for successfully fertilized zygotes. Delayed expression of effects, while known to occur in marine organisms (e.g., Castagna et al., 1981; Pagano et al., 1982, 1983), has not been explored systematically, and we therefore lack an appreciation for its potential importance. For similar reasons, we know little about the combined effects that might arise from repeated exposure of an individual in different life stages. To facilitate meaningful biological interpretation of laboratory bioassays, the challenge is to match the laboratory protocol with reasonable

estimates of which life stages are at risk, the concentrations of contaminant they are likely exposed to, and the duration of the exposure under field conditions.

With respect to general classes of lethal and sublethal effects, one of the most important from an ecological perspective is impaired reproductive success of adults. If early life history stages are relatively more sensitive to toxicants than adults, then major effects on the reproductive success of adults could be mediated through effects on early stages of their progeny. For many benthic marine organisms, gametes are released directly into the water column where they undergo syngamy and form zygotes. Thus, important impacts on reproductive success of adults living near a point source outfall might be mediated through early exposure of their gametes and developing progeny. In the present study, we explore whether and how brief exposure to a range of concentrations of produced water affect gametes and early larval stages of the purple sea urchin, *Strongylocentrotus purpuratus*. In particular, we exposed separately and together eggs, sperm, and zygotes to ascertain (a) the relative sensitivities of these life stages to produced water at durations and concentrations realistic to each stage, (b) the nature of the biological responses, and (c) the potential for delayed expression.

We chose the purple sea urchin as our model organism, in part, because there is an extensive body of toxicological work on various life stages of sea urchins (e.g., Cherr et al., 1987; Dinnel et al., 1981, 1982, 1987, 1989; Hagstrom and Lonning, 1973; Hose, 1985; Kobayashi, 1971; McGibbon and Moldan, 1986; Oshida et al., 1981; Pagano et al., 1982, 1983, 1986). Furthermore, the purple sea urchin is representative of many marine organisms in that benthic adults broadcast eggs and sperm into the water column where fertilization and subsequent larval development occur. Purple sea urchin larvae typically develop into the pluteus stage within 48 hours. Up through the late pluteus (~72 hr), larvae are sustained by egg reserves and do not feed (Strathmann, 1987).

## METHODS

Adult purple sea urchins were collected from a pristine reef near Santa Barbara, CA, and maintained in large, continuous flow sea water tanks located outdoors. Animals were supplied *ad libitum* with freshly collected giant kelp (*Macrocystis pyrifera*), a favored food item. For each experiment, gametes were collected from 4 - 6 adults of each sex within 15 minutes following intracoelomic injection of 0.5 - 1.0 ml of 0.5 M KCl (Strathmann, 1987). Eggs were cleaned by passing them twice through a 200  $\mu$ m Nitex mesh and allowing them to settle through 100 ml of filtered sea water. Sperm were collected "dry" (i.e., without sea water, which metabolically activates sperm [Timourian and Watchmaker, 1970]) by pipetting the gametes from the dried surface of a male (Dinnel et al., 1987). Both gamete types were stored on ice until used in a trial, which occurred no later than 3 hours after gamete collection. Separate "control" trials were conducted to determine whether the delay between collection and use (and therefore manner of storage) altered the viability of eggs or sperm; results indicated no difference in viability of eggs or "dry" collected sperm that were stored for three hours compared with freshly released gametes.

Undiluted produced water used for laboratory tests was collected from an oil processing facility in Carpinteria, CA. Samples were collected from an onshore test spigot located on the discharge pipe just after the effluent leaves the final settling tank and just prior to ocean discharge. Produced water was collected without head space in clean amber glass bottles with Teflon lined lids. Samples were maintained on ice and in the dark until experiments were begun within approximately six hours of collection.

The general laboratory protocol involved cross-designed experiments in which produced water concentration (1, 0.01 and 0.0001%) was crossed with a second factor designating the life stage(s) that was exposed (e.g., eggs only, sperm only, zygote only, and/or a combination of these treatments). A control treatment was included and consisted of unexposed life stages kept in filtered sea water but otherwise handled identically to the other treatments. Produced water concentrations were made by serial dilution of the raw effluent with filtered sea water in

volumetric labware (Allen, 1971). All sea water was filtered through a 0.45  $\mu$ m autoclaved filter. In all experiments, there were three replicates of each treatment, and each replicate typically involved approximately 500 eggs. The incubation units were 15 ml plastic Falcon multi-well culture dishes into which the sperm and eggs were introduced. All experiments were conducted in a cold (15° C) room on a continuous shaker table.

Ten minute gamete exposures were accomplished by adding sperm (or eggs) to a glass test tube containing a given concentration of produced water. Unexposed gametes were added to filtered sea water for 10 minutes. Following the 10 minute exposures, gametes were incubated together in 10 ml of filtered sea water. To assess fertilization success, replicates were fixed with 10% formalin 25 minutes after gametes had been added to the incubation units. Fertilization success was estimated by microscopic identification of the fertilization membrane.

In experiments that examined zygote performance, we manipulated zygote exposure by varying the concentration of produced water in which sperm and eggs were incubated (i.e., exposed eggs and sperm were introduced into incubation units that contained a gradient of produced water concentrations). Developing zygotes remained in these units for up to 96 hours. Thus, our "zygote exposures" included brief exposures of gametes during the period between introduction into the incubation chamber and fertilization. After a specified time, replicates were fixed with 10% formalin, and 100 individuals from each replicate were inspected and their developmental stage noted. Modification to these general protocol are noted below.

To facilitate comparisons among experiments and to avoid polyspermy, we standardized fertilization rates by adjusting the ratio of sperm to eggs (Cherr et al., 1987; Dinnel et al., 1987). Prior to each experiment, batches of eggs were flooded with varying amounts of a stock sperm solution to establish the relationship between fertilization success and the ratio of sperm to eggs. Each experiment was then conducted at the sperm:egg ratio that produced 90% fertilization in filtered sea water.

To prevent microbial contamination, 0.05% Penicillin G was added to each replicate in all experiments. An initial experiment was conducted to establish whether the added antibiotic altered normal fertilization or development, and whether there was a synergistic response of penicillin and produced water. The experiment involved two factors: the presence/absence of penicillin (0 or 0.05%) was crossed with the presence/absence of produced water (0 or 10%). Eggs and sperm were separately exposed to produced water (or filtered sea water) for 10 minutes and then incubated together in filtered sea water. Two endpoints were measured: (a) the proportion of successfully fertilized eggs (as measured by the presence of the fertilization membrane) after 25 minutes of adding sperm to the eggs, and (b) the proportion of embryos that had reached the pluteus stage after 48 hours.

The first main experiment explored the effects of produced water on fertilization success. Produced water concentrations used were 1, 0.01, and 0.0001%. Three exposure regimes were crossed with the produced water treatment: sperm exposed but eggs unexposed, eggs exposed but sperm unexposed, and both sperm and egg exposed. A 0% gamete exposure (filtered sea water only) served as the control, and all incubations were done in filtered sea water.

The second main experiment addressed (a) whether effects from the exposure of gametes might be delayed to a later developmental stage, (b) the relative sensitivities of eggs, sperm and zygotes, and (c) the magnitude of effects arising from combined exposure of different life stages. Unlike the first experiment, the incubation period (following the 10 minute exposure of gametes) was increased from 25 minutes to 48 hours. In addition, there were five exposure treatments: 1) sperm (i.e., eggs and zygotes unexposed); 2) eggs; 3) zygotes; 4) sperm and eggs; 5) sperm, eggs and zygotes. After 48 hours, each replicate was fixed in 10% formalin and the proportion of embryos that had reached the pluteus stage was determined microscopically.

In this experiment, the response parameter (proportion of larvae in the pluteus stage) was based on the relative developmental states of only the eggs and larvae that were still intact after 48 hours. Furthermore, the endpoint of this experiment was time dependent (i.e., assessed after only 48 hours). Thus, it was not possible to separate the relative contributions of mortality from

delayed development. Consequently, a third experiment was conducted to estimate directly the separate effects of produced water on embryo survivorship and developmental rates. To do this, initial cohorts of eggs were counted and the fates of all initial eggs followed through time. Treatments consisted of exposure to 1% produced water of eggs only, sperm only, and of zygotes. A control treatment consisted of exposure to filtered sea water only. The number of eggs in each replicate was known at the beginning of the experiment, and eighteen "copies" of each treatment were initiated; at specified intervals, three replicates per treatment were fixed for later analysis. The intervals were 25 min, 12 hr, 24 hr, 48 hr, 72 hr and 96 hr. Mortality (1 minus survivorship) was estimated as the number of "dead" eggs or embryos divided by the number of eggs initially present. "Dead" eggs and embryos were defined as those missing or obviously dead (i.e., damaged or broken). The numbers of individuals in the pluteus stage were counted at each sampling time and developmental success expressed as the proportion of the initial cohort that had entered the pluteus stage.

In general, results of each experiment were analyzed by analysis of variance. Comparisons with the control treatment were made using Dunnett's two-tailed *t* test, and *a posteriori* comparisons among treatment main effects were made using the Ryan-Elinot-Gabriel-Welsch multiple *F* test (REGWF: SAS, 1988). All data were arcsine-square root transformed prior to analysis.

## RESULTS

### Methodological Tests of Penicillin Effects

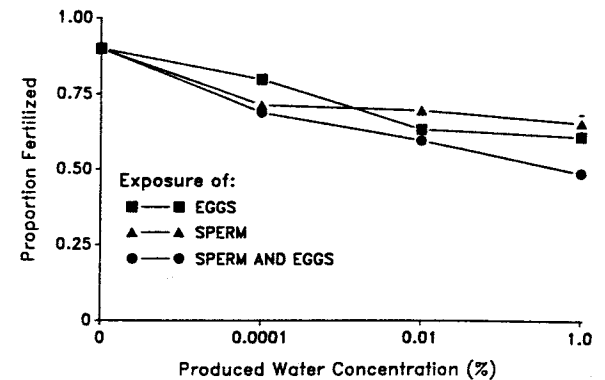
Before considering effects of produced water, we first present evidence that the addition of penicillin to control bacterial contamination does not obscure interpretation of laboratory experiments. Penicillin G had no detectable effect on the proportion of eggs that were successfully fertilized (Table 1;  $F_{1,8} = 1.30$ ;  $P > 0.25$ ). More importantly, there was no interaction between the antibiotic and the effect of produced water on fertilization (Table 1;  $F_{1,8} = 0.43$ ;  $P > 0.50$ ). Similarly, the proportion of embryos that reached the pluteus developmental stage in 48 hrs was not affected by the antibiotic (Table 1; in sea water,  $F_{1,4} = 1.47$ ;  $P > 0.25$ ). There were, however, marked effects of 10% produced water on fertilization success ( $F_{1,8} = 98.4$ ;  $P < 0.0001$ ) and development (Table 1).

**Table 1.** The effect of 0.05% Penicillin G on fertilization success and embryo development in the presence and absence of 10% produced water; data are the mean ( $\pm 1$  SE) proportion of eggs fertilized and of embryos that were in the pluteus developmental stage at 48 hours

Produced Water	Fertilization Success		Pluteus Stage	
	Penicillin		Penicillin	
	Absent	Present	Absent	Present
Absent	0.900 (0.040)	0.840 (0.056)	0.720 (0.034)	0.773 (0.026)
Present	0.434 (0.020)	0.409 (0.020)	0	0

### Fertilization Assay for Effects of Produced Water

Ten minute exposure of eggs and/or sperm to produced water resulted in statistically significant depressions in the fraction of successful fertilizations (Fig. 1;  $F_{2,18} = 83.23$ ;  $P < 0.0001$ ). As expected, the magnitude of the depression increased with increasing concentrations of produced water (Fig. 1), although a substantial fraction (>50%) of eggs still were fertilized at the highest concentration used (1%). However, even at the lowest produced water concentration (0.0001%)



**Figure 1.** Fertilization success of gametes exposed for 10 minutes to a range in concentrations of produced water. Data are the mean ( $\pm 1$  SE,  $n=3$ ) proportion of eggs fertilized after 25 minute incubations of eggs and sperm in filtered sea water.

fertilization success was reduced by as much as ~10 - 20% (Fig. 1); each treatment response in the lowest concentrations differed significantly from the control (Dunnett's test,  $P < 0.05$ ).

There was also a significant difference attributable to which gamete type(s) was exposed (Fig. 1;  $F_{2,18} = 42.42$ ;  $P < 0.0001$ ). *A posteriori* comparison of main effects (using REGWF) shows that exposure of sperm gave similar results as egg exposure, but that exposure of both sperm and eggs yielded a significantly lower fertilization rate. That is, based on fertilization success averaged across produced water concentrations, the sensitivity of sperm was about equal to that of eggs. Further, the greatest impairment occurred when both gamete types were exposed, but the combined effect appeared to be less than the sum of individual effects on each gamete type (Fig. 1). There was, however, an interaction between the gamete type exposed and concentration of produced water ( $F_{4,18} = 11.27$ ;  $P < 0.0001$ ), suggesting a cross-over in relative sensitivity of eggs and sperm to increasing produced water concentrations. It appears that sperm may be relatively more sensitive at low toxicant levels, whereas eggs may be more sensitive at higher concentrations (Fig. 1).

### Embryonic Development Assay for Effects of Produced Water

Produced water reduced the fraction of larvae that were in the pluteus developmental stage after 48 hours (Fig. 2). For all gamete and zygote exposure combinations, the occurrence of pluteus larvae decreased with increasing concentration of produced water ( $F_{2,30} = 919.37$ ;  $P < 0.0001$ ). The reductions in pluteus larvae were statistically significant even at the exceedingly low concentration of 0.0001% (Dunnett's test,  $P < 0.05$ ).

Within a concentration of produced water, the smallest reduction in pluteus development occurred when the zygote only was exposed, and the greatest reduction occurred when sperm, egg and the zygote stage all were exposed (Fig. 2; REGWF comparison shows all main effects are significantly different from one another). These data indicate that the brief 10 minute exposure of gametes reduced larval development to a greater degree than did the subsequent 48 hour exposure of zygotes. Thus, gametes were exceedingly more sensitive than zygotes to produced water. Furthermore, most of the effect arising from exposure of gametes was due to sperm, and very little from exposure of eggs (Fig. 2). Based on the pluteus development assay, the rank of sensitivity of each life stage to produced water was:

sperm >> egg >> zygote.

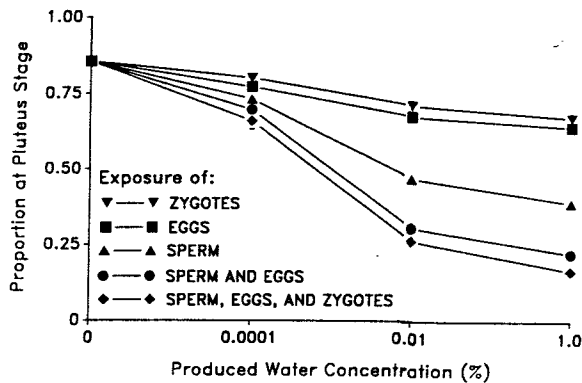


Figure 2. The mean ( $\pm 1$  SE,  $n=3$ ) proportion of embryos that are in the pluteus stage of development after 48 hours as a function of life stage exposed and produced water concentration. Gametes were exposed to produced water for 10 minutes prior to fertilization and zygotes were exposed for the entire 48 hour incubation period.

### Cohort Analysis for Effects of Produced Water

The effect noted above could be due to a combination of increased mortality of developing embryos and/or a reduction in the development rate of survivors. To distinguish between direct mortality and reduced developmental rates, cohorts of eggs of known number were followed through time and the fraction of the initial number of eggs that eventually reached the pluteus larval stage was estimated. This was done using 1% produced water, which was the effluent concentration that yielded the greatest effects in both our fertilization (Fig. 1) and embryo development (Fig. 2) assays.

The results indicate that 1% produced water had no discernible effect on mortality (Fig. 3). Regardless of the life stage exposed, > 85% of the initial cohort survived to 96 hours, and there was no significant difference among the control or exposure treatments ( $F_{3,8} = 1.23$ ;  $P > 0.35$ ). It is important to note that zygotes were exposed to produced water for the entire 96 hr period, yet still did not show an increased death rate.

By contrast with no affect on mortality, produced water greatly altered developmental rates of sea urchin embryos (Fig. 4). For example, the fraction of initial eggs that had reached the pluteus stage by 48 hours differed markedly among treatments ( $F_{3,8} = 17.65$ ;  $P < 0.001$ ); Once again, zygote exposure yielded the smallest effect, while sperm exposure gave the greatest effect. These patterns at 48 hours (Fig. 4) were both qualitatively and quantitatively similar to those observed in the embryonic development bioassay reported above (Fig. 2). However, the effluent did not affect the number of eggs that ultimately reached the pluteus stage by 96 hours (Fig. 4;  $F_{3,8} = 1.02$ ;  $P > 0.40$ ). More than 70% of the initial cohort of eggs in each treatment had developed into pluteus larvae by 96 hours, and all treatments appeared to be asymptoting near 85%. Thus produced water slowed the developmental rates of sea urchin embryos but did not contribute directly to their mortality. Based on the slowing of developmental rates relative to exposure duration, this experiment indicates that the rank sensitivity of life stages was:

sperm > eggs >> zygotes.

The cohort analysis directly contradicted the finding in our fertilization assay regarding the fraction of eggs that ultimately underwent development: e.g., we observed only 61% of eggs fertilized after exposure of eggs to 1% produced water (Fig. 1), but we subsequently found that

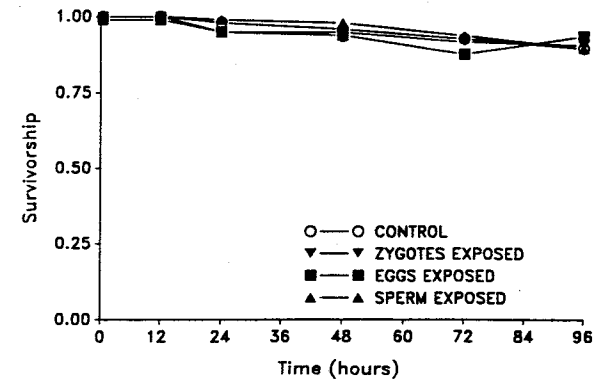


Figure 3. Survivorship of sea urchin eggs and embryos. Data are the mean proportion ( $\pm 1$  SE,  $n=3$ ) of cohorts of sea urchins (beginning at the egg stage) alive at each time interval. Gamete treatments involved exposure of eggs or sperm for 10 minutes prior to fertilization (no subsequent exposure of zygotes), and the zygote treatment involved exposure for the entire 96 hour period.

83% of all eggs developed to the pluteus stage (Fig. 4). It is possible that, in the cohort analysis, sperm continued to fertilize eggs long after the 35 minute exposure and incubation period used in the fertilization bioassay. A direct test of this hypothesis indicated that only ~10% of sperm remain viable 35 minutes after activation, and none were capable of fertilizing eggs after 45 minutes (Fig. 5). Exposure of gametes to 1% produced water lowered the fraction of viable sperm of a given age, and did not prolong the length of time that sperm remained capable of fertilization (Fig. 5). The estimate of age-specific sperm viability was independent of the endpoint (i.e., 25 or 120 mins) used to measure successful fertilization (Fig. 5). This experiment also indicated that the effect of produced water on viability of sperm was virtually instantaneous,

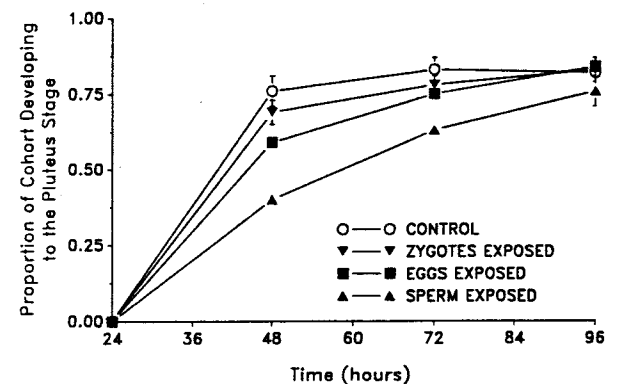
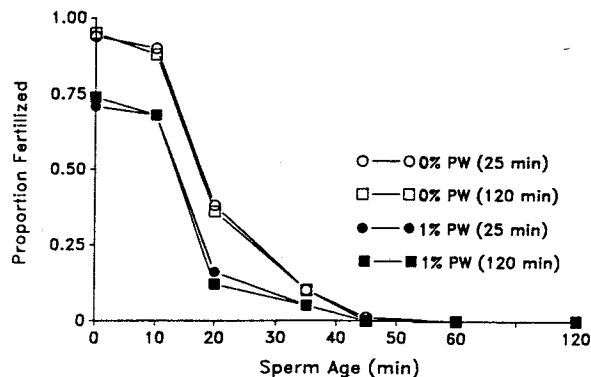


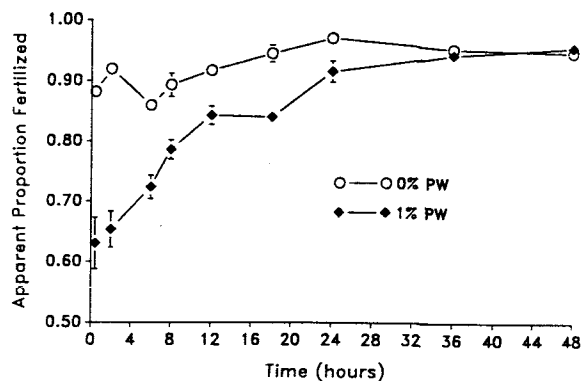
Figure 4. Mean proportion ( $\pm 1$  SE,  $n=3$ ) of initial sea urchin eggs that had entered the pluteus larval stage at different times. Gamete treatments involved exposure of eggs or sperm for 10 minutes prior to fertilization (no subsequent exposure of zygotes), and the zygote treatment involved exposure for the entire 96 hour period.



**Figure 5.** Viability of purple sea urchin sperm as a function of time since activation. Data are the mean ( $\pm 1$  SE,  $n=3$ ) proportion of eggs fertilized by sperm in the absence (circles) and presence (squares) of 1% produced attributable water. Only sperm were exposed to produced water; eggs and zygotes were kept in filtered sea water. Fertilization was assayed after 25 (open symbols) and 120 (closed symbols) minute incubations.

and suggests that at least part of the "zygote" sensitivity we have observed might be due to brief exposure of sperm that occurs when we expose zygotes.

An alternative explanation to prolonged sperm viability is that the presence of a vitelline membrane underestimated successful syngamy when gametes had been exposed to produced water. This was supported by re-examining data from the cohort analysis and expressing fertilization success as the fraction of eggs that either had a fertilization membrane or had undergone cleavage. The initial depression of fertilization in the produced water treatment diminished through time such that by 36 hours there was no difference between treatments in



**Figure 6.** Apparent fertilization success as a function of time. Data are the mean ( $\pm 1$  SE,  $n = 3$ ) proportion of egg cohorts known to be fertilized based on the presence of either a vitelline membrane or cleavage at each interval in the absence (open circles) and presence (solid diamonds) of 1% produced water. Sperm, eggs, and zygotes were all exposed to produced water in this example.

the fraction of initial eggs known to have been successfully fertilized (Fig. 6). The results indicate that early indicators of fertilization (e.g., presence of the vitelline membrane) grossly underestimated the fraction of eggs that were actually fertilized and subsequently began embryonic development (Fig. 6).

## DISCUSSION

### Interpretation of Bioassay Studies

Bioassays typically use a response variable that coincides with the end of the planned exposure period. For example, tests of effects on gametes usually involve estimating changes in fertilization ability immediately after gamete exposure (e.g., Adams and Slaughter-Williams, 1988; Allen, 1971; Dinnel et al., 1981; Hagstrom and Lanning, 1973; Kobayashi, 1971; Pagano et al., 1982, 1983), and fertilization success has become a standard toxicity measure (e.g., Cherr et al., 1987; Dinnel et al., 1982, 1987; Dinnel and Stober, 1987; McGibbon and Moldan, 1986; Pagano et al., 1985, 1986). Post-fertilization assays often measure the fraction of young individuals that reach some developmental "milepost" in a specified time period (e.g., Adams and Slaughter-Williams, 1988; Byrne and Calder, 1977; Dinnel et al., 1989; Hose, 1985; Oshida et al., 1981; Pagano et al., 1985, 1986). However, our application of these event and time-dependent bioassays to produced water revealed several limitations of this general approach.

Our fertilization assay did not capture accurately the effect of produced water on syngamy. The apparent effect on gametes was to reduce successful fertilization (Fig. 1). However, our embryo cohort analyses revealed that the fraction of eggs eventually fertilized was unaffected by produced water (Fig. 4). Failure of the fertilization test to properly convey the lack of an overall affect on syngamy could have been due either to the particular design of the bioassay (i.e., sperm fertilized eggs long after the 35 minute exposure/incubation period used in our fertilization trials), or from the use of an inaccurate measure of syngamy. The first hypothesis can be rejected (Fig. 5). Other workers have also found that, once activated, sea urchin sperm remains viable for a very short period, usually  $< 25$  minutes (e.g., Tyler, 1953; Timourian and Watchmaker, 1970; Pennington, 1985). By contrast, our measure of fertilization appeared to be imprecise. As is typical in these types of assays (e.g., Dinnel et al., 1987; McGibbon and Moldan, 1986; Oshida et al., 1981), we used the presence of a fertilization membrane to score successful fertilization. However, following exposure of gametes to produced water, the fraction of initial eggs that eventually underwent embryonic development was grossly underestimated by the fertilization membrane criterion (Fig. 6). It appears that produced water inhibited the vitelline membrane from lifting off the surface of some eggs, yet did not preclude syngamy. Alternative techniques to estimate fertilization success, such as nuclear staining, may be more reliable (though less practical) measures of syngamy.

With respect to differential sensitivity of gametes, our fertilization assay also incorrectly suggested that effects arising from pre-fertilization exposure on eggs and sperm were roughly equivalent (Fig. 1). However, our embryo development tests demonstrated that effects arising from sperm exposure were far greater than those from egg exposure (Figs. 2 & 4). The inconsistency resulted from an effect from sperm exposure that was manifested much later in development, and which was not evident at the time of fertilization. These results cogently illustrate that a delay in expression of an effect cannot be discovered when the assay endpoint, such as fertilization success, coincides with the end of the exposure period. The scope and magnitude of biological responses from a toxicant can be underestimated by failing to determine whether exposure at one life stage of an individual has an effect that is expressed in a later stage, yet this aspect is largely unexplored (but see Adams and Slaughter-Williams, 1988). In the case of fertilization, this limitation was perhaps presaged by the suggestion of certain authors that fertilization assays may be less sensitive indicators of effects from gamete exposure than exploration of abnormalities later in development (e.g., Allen, 1971; Hose, 1985).

However, the interpretation of bioassays that examine delayed effects can still be problematic. For example, we found that the fraction of sea urchin embryos reaching the pluteus larval stage in 48 hours was greatly impaired by produced water (Fig. 2). A logical interpretation of these results is that produced water exposure killed sperm, eggs, or embryos in some developmental phase prior to the pluteus stage. Our cohort analyses revealed that this interpretation was incorrect. The only effect of produced water was to impede developmental rates of embryos (Fig. 4): it did not directly increase mortality of developing zygotes (Fig. 3) or reduce fertilization of eggs (Fig. 6).

These results underscore a crucial but little appreciated difficulty with standard bioassay approaches; without additional evidence, the existence and contribution of various biological effects (e.g., delayed expression, reduced developmental rates, increased mortality) cannot be inferred safely from bioassays with fixed-time endpoints. While such techniques are useful in ranking the relative toxicity of various effluents, their utility in providing an understanding of the nature of biological responses is more limited, and alternative approaches should be sought. Endpoints and exposure regimes should be selected that enhance biological insight and therefore either help pinpoint mechanisms of toxicity or permit extrapolation to field situations where biological effects arise from exposure of particular life stages (e.g., Somerville et al., 1987; Higashi et al., 1992; Raimondi and Schmitt, 1992).

### Biological Effects of Produced Water

Predicting or understanding the potential for long-term environmental impacts arising from the discharge of produced water into coastal waters is impeded because biological effects of whole produced waters have received little attention (Neff, 1987). To the extent that laboratory bioassays have been applied to produced waters, most produced waters do not appear to be acutely toxic (for review see Neff, 1987). Results of our study support this notion for produced water from oil fields in the Santa Barbara Channel; brief (10 minute) exposure of sea urchin gametes and prolonged (96 hr) exposure of zygotes did not result in increased mortality, even at the moderately high concentration of 1% produced water (Fig. 3). We did, however, detect sublethal responses to produced water at substantially lower levels; statistically significant responses of young purple sea urchins were detected at produced water concentrations as low as 1 part per million, with the magnitude of the response(s) increasing with increased concentration of the effluent.

It has been argued that such sublethal or chronic effects may be a better gauge of potential environmental significance than the acute lethality of an effluent (e.g., Capuzzo, 1987; Neff, 1987). Extrapolation from laboratory demonstration of sublethal effects to assessment of ecological consequences under natural conditions is not, however, straightforward. For example, exposure concentrations and durations used in a laboratory study do not necessarily bear any relationship to exposure regimes that arise under field conditions. Two lines of evidence suggest that our laboratory exposures spanned conditions likely to occur in the field. First, our results show that the effect of produced water on gametes, particularly sperm, was virtually instantaneous (Fig. 5). Therefore, adult urchins that spawn in the vicinity of the produced water outfall are likely to produce offspring with delayed developmental rates even though those developing larvae may soon be transported away from the outfall. Second, we have examined effects under field conditions by exposing urchin gametes and larvae to water collected at different distances from the produced water diffuser and compared these results with those obtained from known concentrations of produced water (Krause, unpublished data). These results suggest that although produced water is rapidly diluted (the diffuser is designed for an initial seawater:effluent dilution of 125:1), detectable developmental effects can persist out to 100 - 500 m, where produced water concentrations drop to approximately 1 ppm. Thus, the produced water concentrations we used in our laboratory experiments nicely matched with the concentrations that can occur in the field at sites within 500 m of the outfall.

Another potential difficulty in extrapolating laboratory findings of developmental delays to field consequences is that delayed development can lead to an indirect increase in mortality: e.g., by increasing the length of time that early life stages remain vulnerable to natural sources of mortality, such as size-selective predators (e.g., Werner et al., 1983). Thus, slight sublethal effects, such as delayed development, could indirectly contribute to significant reductions in the number of larvae available to recruit into benthic populations. However, processes linking larval and benthic dynamics are poorly understood and therefore the ecological significance of larval mortality is largely unknown (e.g., Capuzzo, 1987; Nisbet et al., 1993; Raimondi and Schmitt, 1992). Much work remains before impacts on benthic populations can be predicted based on sublethal responses of larval stages.

Because so little is known regarding sublethal effects of produced water, it is not yet possible to assess whether a reduction in developmental rate is a common response of early life stages. Lowered growth or developmental rates have been observed for individuals exposed to certain constituents of produced water. For example, petroleum hydrocarbons can retard individual growth of adult copepod crustaceans (Hay et al., 1988), larval decapod crustaceans (Caldwell, 1977; Cucci and Epifano, 1979; Katz, 1973; Laughlin et al., 1978; Laughlin and Neff, 1979; Wells, 1972; Wells and Sprague, 1976) and larval bivalve mollusks (Byrne and Calder, 1977). In each of these cases, the life stage exposed was capable of feeding, and hydrocarbons are known to reduce feeding rates of planktotrophic larvae (e.g., Johns and Pechenik, 1980; Wells and Sprague, 1976). Hence, reduced growth or developmental rates could have resulted primarily from an indirect effect on food intake. This process was not involved in the reduced developmental rates of purple sea urchins; larvae of this species do not feed until the late pluteus stage (Strathmann, 1987), which is not attained until ~72 hours after fertilization in normally developing embryos. The response of young purple sea urchins, especially that arising from exposure of gametes, must have involved direct alteration of normal cellular activity. Delayed development of embryos mediated through exposure of gametes to PCBs has been observed for the sea urchin *Arbacia punctulata* (Adams and Slaughter-Williams, 1988). Like produced water, the actual mechanism producing this effect of PCB exposure is unknown, but like certain petroleum hydrocarbons (Capuzzo, 1987), PCBs are highly lipophilic. Lipid reserves are of primary importance in embryonic development (e.g., Holland, 1978), and it has been suggested that larvae may shunt energy reserves away from differentiation to be used to detoxify such lipophilic compounds as petroleum hydrocarbons (e.g., Sharp et al., 1979).

Produced water, of course, contains numerous potential toxicants in addition to petroleum hydrocarbons (e.g., Neff, 1987), and the particular constituents of the Carpinteria effluent responsible for the observed effect on purple sea urchins remains to be determined. Higashi et al. (1992) explored "toxicity" of various fractions of produced water from Carpinteria, and found that the majority of biological effects arose from the water-only soluble fraction that contained divalent cations. Interestingly, Pagano et al. (1982) found results for other sea urchin species exposed to cadmium that were qualitatively similar to ours. Although cadmium is not common in the Carpinteria produced water, analogous elements, such as barium and strontium, are present (Higashi et al., 1992). Furthermore, barium and strontium are known to impair development (Conrad and Davis, 1980), and recent work suggests that these ions might mediate their effects through modification of microtubule function (Tamm, 1989; Tamm and Tamm, 1990). Because we observed that sperm exposure yielded the greatest effect on development, and because this effect appeared to arise very early in development, we suggest that the toxicological mechanisms most likely involve a feature specific to sperm that involves microtubule function. One likely candidate is the sperm centriole, which is responsible for the transport of the sperm pronucleus to the egg pronucleus (Bestor and Schatten, 1981). If the function of the centriole were impaired, this could lead to an early retardation of development by delaying nuclear fusion. Other studies using produced water from this same source have found responses that might involve microtubule-mediated effects: e.g., swimming and chemoreception of abalone larvae (Raimondi and Schmitt, 1992), swimming of kelp spores (Reed, 1992), migration of kelp nuclei (Pillai et al.,

1990), and growth (possibly feeding) of mussels (Osenberg et al., 1992). If these diverse biological effects of produced water were mediated through a similar toxicological mechanism, it would provide a strong unifying theme to the study of this complex effluent.

## ACKNOWLEDGMENTS

We thank G. Cherr, A. Ebeling, T. Fan, R. Higashi, S. Holbrook, A. Martinez, D. Morse, M. Pillai, and D. Steichen for discussions and criticisms, M. Krause for assistance in the laboratory, B. Williamson for technical support, and J. Wallace for facilitating our collections of produced water. This research was funded by the UC Coastal Toxicology Program, and by the Minerals Management Service, U.S. Department of Interior under MMS Agreement No. 14-35-0001-3071. The views and conclusions in this paper are those of the authors and should not be interpreted as necessarily representing the official policies, either express or implied, of the U.S. Government.

## REFERENCES

- Adams, J.J., and Slaughter-Williams, S., 1988, The effects of PCB's (Aroclors 1254 and 1016) on fertilization and morphology in *Arbacia punctulata*, Water, Air, and Soil Pollut. 38:299-310.
- Allen, H., 1971, Effects of petroleum fractions on the early development of a sea urchin, Mar. Poll. Bull. 2:138-140.
- Bestor, T.M., and Schatten, G., 1981, Anti-tubulin immunofluorescence microscopy of microtubules present during the pronuclear movements of sea urchin fertilization, Dev. Biol. 88:80-91.
- Byrne, C.J., and Calder, J.A., 1977, Effects of the water-soluble fractions of crude, refined, and waste oils on the embryonic and larval stages of the Quahog clam *Mercenaria* sp, Mar. Biol. 40:225-231.
- Caldwell, R.S., Calderone, E.M., and Mallon, M.H., 1977, Effects of seawater-soluble fraction of Cook Inlet crude oil and its major aromatic components on larval stages of the Dungeness crab, *Cancer magister* (Dana), in: "Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms," D.A. Wolfe, ed., Pergamon Press, New York.
- Capuzzo, J.M., 1987, Biological effects of petroleum hydrocarbons: assessments from experimental results, in: "Long-Term Environmental Effects of Offshore Oil and Gas Development," D.F. Boesch and N.N. Rabalais, eds., Elsevier Applied Science, NY.
- Castagna, A., Sinatra, F., Scalia, M., and Capodicasa, V., 1981, Observations of the effect of zinc on the gametes and various development phases of *Arbacia lixula*, Mar. Biol. 64:285-289.
- Cherr, G.N., Shenker, J.M., Lundmark, C., and Turner, K.O., 1987, Toxic effects of selected bleached kraft mill effluent constituents on the sea urchin sperm cell, Env. Toxicol. Chem. 6:561-569.
- Conrad, G.W., and Davis, S.E., 1980, Polar lobe formation and cytokinesis in fertilized eggs of *Ilyanassa obsoleta*, Devel. Biol. 74:152-172.
- Cucci, T.L. and Epifano, C.E., 1979, Long term effects of water-soluble fractions of Kuwait oil on the larval and juvenile development of the mud crab *Eurypanopeus depressus*, Mar. Biol. 55:215-220.
- Dinnel, P.A., Stober, Q.J., and DiJulio, D.H., 1981, Sea urchin sperm bioassay for sewage and chlorinated seawater and its relation to fish bioassays, Mar. Env. Res. 5:29-39.
- Dinnel, P.A., Stober, Q.J., Crumley, S.C., and Nakatani, R.E., 1982, Development of a sperm cell toxicity test for marine waters, in: "Aquatic toxicology and hazard assessment: Fifth Conference, ASTM STP 766," J.G. Pearson, R.B. Foster, and W.E. Bishop, eds., Amer. Soc. Testing and Materials, Philadelphia, PA.
- Dinnel, P.A., Link, J.M., and Stober, Q.J., 1987, Improved methodology for sea urchin sperm cell bioassay in marine waters, Arch. Environ. Contam. Toxicol. 16:23-32.
- Dinnel, P.A., and Stober, Q.J., 1987, Application of the sea urchin sperm bioassay to sewage treatment efficiency and toxicity in marine waters, Mar. Env. Res. 21:121-133.
- Dinnel, P.A., Link, J.M., Stober, Q.J., Letourneau, M.W., and Roberts, W.E., 1989, Comparative sensitivity of sea urchin sperm bioassay to metals and pesticides, Arch. Environ. Contam. Toxicol. 18:748-755.
- Hagstrom, B.E., and Lonning, S., 1973, The sea urchin egg as a testing object in toxicology, Acta. Pharm. et Toxicol. 32(Suppl. 1):1-49.
- Hay, S.J., Evans, G.T., and Gamble, J.C., 1988, Birth, growth and death rates for enclosed populations of calanoid copepods, J Plank. Res. 10(3):431-454.
- Higashi, R., Cherr, G., Bergens, C., Fan, T., and Crosby, D., 1992, Toxicant isolation from a produced water source in the Santa Barbara Channel, in: "Produced Water: Technological/ Environmental Issues and Solutions," J.P. Ray and F.R. Engelhardt, eds., Plenum Publishing Corp., New York, New York, USA.
- Holland, D.L., 1978, Lipid reserves and energy metabolism in the larvae of benthic marine invertebrates, in: "Biochemical and Biophysical Perspectives in Marine Biology, Volume 4," D.C. Malins and J.R. Sargent eds., Academic Press, New York.
- Hose, J.E., 1985, Potential uses of sea urchin embryos for identifying toxic chemicals: Description of a bioassay incorporating cytologic, cytogenetic and embryonic endpoints, J. Appl. Toxicol. 5:245-254.
- Johns, D.M., and Pechenik, J.A., 1980, Influence of the water accommodated fraction of No. 2 fuel oil on energetics of *Cancer irroratus* larvae, Mar. Biol. 55:247-254.
- Katz, L.M., 1973, The effects of water-soluble fractions of crude oil on larvae of the decapod crustacean *Neopanope texana* (Say), Environ. Pollut. 5:199-204.
- Kobayashi, N., 1971, Fertilized sea urchin eggs as an indicative material for marine pollution bioassay, preliminary experiment, Publs. Seto Mar. Biol. Lab. 18:379-406.
- Kobayashi, N., 1980, Comparative sensitivity of various developmental stages of sea urchins to some chemicals, Mar. Biol. 58:163-171.
- Laughlin, R.B. Jr., and Neff, J.M., 1979, Interactive effects of salinity, temperature and polycyclic aromatic hydrocarbons on the survival and development rate of larvae of the mud crab *Rhithropanopeus harrisi*, Mar. Biol. 53:281-291.
- Laughlin, R.B., Jr., Young, L.G.L., and Neff, J.M., 1978, A long-term study of the effect of water-soluble fractions of No.2 fuel oil on the survival, development rate, and growth of the mud crab *Rhithropanopeus harrisi*, Mar. Biol. 47:87-89.
- McGibbon, S., and Moldan, A.G.S., 1986, Routine toxicity testing of toxicants using a sea urchin gamete bioassay, Mar. Pollut. Bull. 17(2):68-72.
- National Research Council, 1983, "Drilling Discharges in the Marine Environment," National Academy Press, Washington, D.C.
- Neff, J.M., Anderson, J.W., Cox, B.A., Laughlin, R.B., Jr., Rossi, S.S., and Tatum, H.E., 1976, Effects of petroleum on survival, respiration and growth of marine animals, in: "Sources, Effects and Sinks of Hydrocarbons in the Aquatic Environment," American Institute of Biological Sciences, Washington, D.C.
- Neff, J.M., 1987, Biological effects of drilling fluids, drill cuttings and produced waters, in: "Long-Term Environmental Effects of Offshore Oil and Gas Development," D.F. Boesch and N.N. Rabalais, eds., Elsevier Applied Science, New York.
- Nisbet, R. M., Murdoch, W.W., and Stewart-Oaten, A., 1993, Consequences for Adult Fish Stocks of Human Induced Mortality on Immatures. Ecol. Appl. in review.
- Oshida, P.S., Goochey, T.K., and Mearns, A.J., 1981, Effects of municipal wastewater on fertilization, survival, and development of the sea urchin, *Strongylocentrotus purpuratus*, in: "Biological Monitoring of Marine Pollutants," F.J. Vernberg, A. Calabrese, F.P. Thumberg, and W.B. Vernberg eds., Academic Press, New York.
- Osenberg, C.W., Schmitt, R.J., Holbrook, S.J., and Canestro, D. 1992, Spatial scale of ecological effects associated with an open coast discharge of produced water, in: "Produced Water: Technological/ Environmental Issues and Solutions," J.P. Ray and F.R. Engelhardt, eds., Plenum Publishing Corp., New York, New York, USA.
- Pagano, G., Esposito, A., and Giordano, G.G., 1982, Fertilization and larval development in sea urchins following exposure of gametes and embryos to cadmium, Arch. Env. Contam. Toxicol. 11:47-55.
- Pagano, G., Esposito, A., Bove, P., DeAngelis, M., Rota, A., and Giordano, G.G., 1983, The effects of hexavalent and trivalent chromium on fertilization and development in sea urchins, Env. Res. 30:442-452.
- Pagano, G., Cipollaro, M., Corsale, G., Esposito, A., Ragucci, E., Giordano, G.G., and Trieff, N.M., 1985, Comparative toxicities of chlorinated biphenyls on sea urchin egg fertilization and embryogenesis, Mar. Env. Res. 17:240-244.
- Pagano, G., Cipollaro, M., Corsale, G., Esposito, A., Ragucci, E., Giordano, G.G., and Trieff, N.M., 1986, The sea urchin: Bioassay for the assessment of damage from environmental contaminants, in: "Community toxicity testing, ASTM STP-920," J. Cairns ed., Amer. Soc. Testing and Materials, Philadelphia, PA.
- Pennington, J.T., 1985, The ecology of fertilization of echinoid eggs: The consequences of sperm dilution, adult aggregation, and synchronous spawning, Biol. Bull. 169(10):417-430.
- Pillai, M.C., Baldwin, J.D., and Cherr, G.N., 1990, Role of cytoskeleton in germtube elongation and nuclear translocation in a marine algal gametophyte, J. Cell Biol. 111:(No.5, Pt. 2) 415a.
- Raimondi, P.T., and Schmitt, R.J., 1992, Effects of produced water on settlement of larvae: field tests using red abalone, in: "Produced Water: Technological/ Environmental Issues and Solutions," J.P. Ray and F.R. Engelhardt, eds., Plenum Publishing Corp., New York, New York, USA.
- Reed, D.C., and Lewis, R.J., 1992, Water column effects of produced water discharges on the production and dispersive potential of giant kelp (*Macrocystis pyrifera*). In preparation.
- Rossi, S.S., and Anderson, J.W., 1978, Petroleum hydrocarbon resistance in the marine worm *Neanthes arenaceodentata* (Polychaeta: Annelida) induced by chronic exposure to No. 2 fuel oil, Bull. Environ. Contam. Toxicol. 20:515-521.

- SAS Institute Inc, 1988, "SAS/STAT User's Guide, Release 6.03 Edition," SAS Institute Inc, Cary, North Carolina.
- Sharp, J.R., Fucik, K.W., and Neff, J.M., 1979, Physiological bases of differential sensitivity of fish embryonic stages to oil pollution, *in*: "Biological Monitoring of Marine Pollutants," F.J. Vernberg, A. Calabrese, F.P. Thurnberg, and W.B. Vernberg, eds., Academic Press, New York.
- Somerville, H.J., Bennett, D., Davenport, J.N., Holt, M.S., Lynes, A., Mahieu, A., McCourt, B., Parker, J.G., Stephenson, R.R., Watkinson, R.J., and Wilkinson, T.G., 1987, Environmental effect of produced water from North Sea oil operations, *Mar. Poll. Bull.* 18(10):549-558.
- Strathmann, M.F., 1987, Phylum Echinodermata, Class Echinoidea, *in*: "Reproduction and Development of the Marine Invertebrates of the Northern Pacific Coast: Data and Methods for the Study of Eggs, Embryos, and Larvae," M.F. Strathman ed., University of Washington Press, Seattle, Washington.
- Tamm, S.L., 1989, Control of reactivation and microtubule sliding by calcium, strontium and barium in detergent-extracted macrocilia of *Beroe*, *Cell Motil. Cytoskel.* 12:104-112.
- Tamm, S.L., and Tamm, S., 1990, Ca/Ba/Sr-induced conformational changes of ciliary axonemes, *Cell Motil. Cytoskel.* 17:187-196.
- Timourian, H., and Watchmaker, G., 1970, Determination of spermatozoan motility, *Dev. Biol.* 21:62-72.
- Tyler, A., 1953, Prolongation of life span of sea urchin spermatozoa and improvement of the fertilization reaction by treatment of the spermatozoa and eggs with metal chelating agents, *Biol. Bull.* 104:224-239.
- Wells, P.G., 1972, Influence of Venezuelan crude oil on lobster larvae, *Mar. Pollut. Bull.* 3:105-106.
- Wells, P.G., and Sprague, J.B., 1976, Effects of crude oil on American lobster (*Homarus americanus*) larvae in the laboratory, *J. Fish. Res. Board Can.* 33:1604-1614.
- Werner, E.E., Gilliam, J.F., Hall, D.J., and Mittelbach, G.G., 1983, An experimental test of the effects of predation risk on habitat use in fish, *Ecology* 64(6):1540-1548.