Exxon Valdez Oil Spill
Restoration Project Annual Report

Effects of Oiled Incubation Substrate on Pink Salmon Reproduction
Restoration Project 00476
Annual Report

This annual report has been prepared for peer review as part of the *Exxon Valdez* Oil Spill
Trustee Council restoration program for the purpose of assessing project progress. Peer review
comments have not been addressed in this annual report.

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Study History: This project is in the third year of a multi-year study based at the National Marine Fisheries Service research facility at Little Port Walter (LPW) on Baranof Island in Southeast Alaska. Field activities will continue through early FY 03, and will close out at the end of FY03 .

Abstract: This project examines the effects of oil exposure during embryonic development on the reproductive capacity of pink salmon that survive to spawn. The objective is to determine if exposure to oil during incubation could explain the reduced gamete viability reported for pink salmon in Prince William Sound under Restoration Study 191A. In that study, gametes taken from pink salmon returning to oiled streams had higher mortality rates than gametes taken from salmon in unoiled streams. These field observations suggest a negative effect of oil on vertebrate reproduction that has not previously been described. The importance of those observations is tempered by the fact that the exposure histories of the fish in that study were unknown. However, the plausibility of reduced gamete viability is supported by the results of Restoration Study 191B, which included reduced marine survival and growth of returning adults. The study described in this report (00476) evaluated the viability of gametes taken from fish exposed to known quantities of oil during early development. Trends in average offspring survival suggested an oil effect consistent with results reported in Restoration Study 191B. However, there was insufficient statistical power in the experimental design to demonstrate the effect. Low statistical power resulted from poor marine survival of pink salmon from brood year 1998. Returns of wild fish near the study site were the lowest in 26 years and returns of treated fish were further reduced by oil exposure. Return rates for the various treatment groups were consistent with previous reports of an oil impact on marine survival. In FY01 offspring of exposed fish were marked and released. They will return to the hatchery at the end of FY02 at which time the genetic impacts of embryonic oil exposure will be evaluated.

Key Words: *Exxon Valdez*, pink salmon, *Onchorynchus gorbuscha*, delayed effects, genetic damage, reproductive damage, crude oil.

Project Data: This is a laboratory study involving exposure of pink salmon embryos to known doses of crude oil. Data collected include polynuclear aromatic hydrocarbon and alkane concentrations on oil-coated gravel, and resulting levels in pink salmon tissues and incubator effluents. Chemical observations were made at multiple times during incubation. Biological data include the numbers of eggs exposed, their survival rates to emergence, frequencies of gross abnormalities resulting from exposure, survival of the fry after marking, marine survival after release, size at maturity and offspring survival rates. Data are recorded in databases maintained by the author. All data will be available after publication.

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Executive Summary

This project examines the effects of oil exposure during embryonic development on the reproductive ability of adult pink salmon. The project is motivated by observations of impaired reproductive ability in pink salmon returning to spawn in streams contaminated with oil spilled by the *Exxon Valdez*. In 1993, gametes taken from salmon returning to contaminated and uncontaminated streams were incubated side by side in clean water at a hatchery in Prince William Sound. Gametes taken from fish returning to oiled streams had lower survival rates. This apparent reduction in the reproductive capability of salmon returning to oiled streams was thought to result from their exposure to oil during incubation two years prior to their return. Unfortunately, the exposure levels these fish experienced are unknown. Consequently, the conclusion that oil induced reproductive impairment can only be inferred. In a separate study, Smoker et al. (2000) identified a genetic effect of oil exposure on the reproductive ability of pink salmon whose parents were exposed to oil. However, no corroborating effect was reported for the parental generation. Consequently, this study seeks to test the proposition that embryonic exposure to oil causes delayed impacts on reproduction in exposed populations, and these impacts are relayed genetically to subsequent generations.

Control, low dose and high dose brood-lines were established at the beginning of FY99 with exposure of the parental generation (Table 1). Pink salmon eggs were exposed by incubating them in gravel coated with known amounts of weathered oil. Incubation continued until the middle of FY99 when the surviving fry emerged from the incubators and were released to the wild. Individuals from each brood-line bore a fin mark for future identification. Surviving adults returned to spawn at the end of FY00, at which time their gametes were collected and incubated. Gamete collection and mixing was designed to 1) test the hypothesis that embryonic exposure to oil results in delayed effects on reproductive ability and 2) produce sufficient numbers of offspring for release so that genetic impacts can be evaluated.

Average offspring survival tended to decrease with exposure level, but the differences among means were not significant. Observations of mean offspring survival and estimates of variation around those means were consistent with previous reports that identified effects on reproduction. Failure to statistically resolve the differences among brood-lines resulted from low statistical power associated with the small numbers of pink salmon which returned to spawn. This was especially true of those from the high dose brood-line which returned at half the rate of the control brood-line. Release of fish to test the genetic hypothesis began in middle of FY01.

The failure of this study to identify a reproductive impact of embryonic oil exposure should not be construed as evidence of no effect. The low numbers of returning salmon allowed for only one fourth the number of replicates used in previous studies (Bue et al. 1998). Consequently, the study was underpowered. Other lines of evidence are consistent with a reproductive impact, including reports of the effects of benzo-[a]-pyrene on reproduction in fathead minnows (White et al. 1999), altered development of gonads (Marty et al. 1997), genetic impacts on reproduction following oil exposure (Smoker et al. 2000), and reduced offspring survival in gametes collected from pink salmon returning to oil-contaminated streams (Bue et al. 1998). The only counter-example (Brannon et al. 1995) had been previously criticized for lack of statistical power (Rice et al. 2001a).

INTRODUCTION

Embryonic exposure of pink salmon to low doses of crude oil causes a variety of effects in pink salmon including those that are not observed until long after the exposures have ended (Marty et al. 1997, Heintz et al. 1999 ; Heintz et al. 2000). These effects combine to cause reduced marine survival in fish that survive exposure during incubation (Heintz et al. 2000). Therefore, the survivorship during all the life history stages of an exposed population is reduced relative to that of an unexposed population. Reduced survivorship necessarily translates to reduced average fitness of the exposed population because the number of reproducing individuals is reduced. If these reproducing survivors experience reduced reproductive output then the average fitness of exposed populations would be even further reduced. Moreover, if there was a genetic basis to the reduced reproductive output then fitness reductions in exposed populations would be expected to persist even after the oil had dissipated from the environment.

While reduced survivorship in exposed populations has been shown (Heintz et al. 2000), a reproductive impact still requires demonstration. A number of reports suggest embryonic exposure of teleosts to oil may reduce reproductive capability, but all are equivocal. Bue et al. (1998) found gametes taken from pink salmon returning to streams contaminated by crude oil from the *Exxon Valdez* had lower viability than those obtained from fish returning to uncontaminated streams. However, the exposure history of the fish supplying the gametes could not be verified. Genetic damage to germ cells has been cited by Smoker et al. (2000) who reported reduced viability of gametes taken from pink salmon that were never exposed to oil but whose parents had been exposed. However, there are no corroborating data presented for reductions in reproductive output in the parental generation. Such corroboration exists for the genetic effects on reproduction experienced by fathead minnows exposed as embryos to benzo[a]pyrene (White et al. 1999). However, this compound occurs only at very low concentrations in the crude oil spilled from the *Exxon Valdez* (Marty et al. 1997).

Further support for reproductive impacts is suggested by authors that have demonstrated the effects of oil on the DNA of developing embryos of pink salmon. The clastogenic properties of crude oil have been demonstrated (Carls et al. 1999) as well as its mutagenic capability (Roy et al. 1999). In developing embryos, these sorts of impacts may not be lethal, but could lead to impaired tissue function later in life (Rice et al. 2001b). If the function of gonad tissues were impaired then reproductive output might be reduced in individuals that otherwise appear unaffected. Such damage would be phenotypic, in the sense that reduced offspring survival results from damaged gonads. Marty et al. (1997) reported such phenotypic impacts when they observed altered gonad development in pink salmon juveniles exposed to oil during early development. However, if deleterious mutations were to occur in germ cell DNA, then damage acquired in the exposed generation may be inherited by subsequent generations. This genotypic effect would have no impact on the adult fish, but could cause delayed impacts on their offspring. This mechanism was supported by White et al. (1999), who showed reduced reproductive output in fathead minnows whose parents had been exposed to benzo[a]pyrene during embryonic development.

Distinguishing between phenotypic and genotypic impacts on reproductive ability is important, because the effects are likely to persist in affected populations for different amounts of

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time. Phenotypic impacts would persist in the affected populations only as long as toxic effects of the oil persist in the incubating environment. In contrast, genotypic impacts on reproductive ability would have to be removed by natural selection and thereby would require multiple generations for removal. The rate at which selection removes deleterious mutations from populations, removal depends on the severity of the effect, and its degree of dominance (Falconer 1981). Consequently, genotypic effects are apt to persist after the oil has stopped exerting toxic effects .

The purposes of this study were to determine if embryonic exposure to crude oil causes a delayed effect on the reproductive ability of pink salmon and if such impacts have a genotypic or phenotypic basis. Consequently, this is a multiple year study, involving three generations (Table 1). The first generation (P1) was exposed to oil beginning in 1998. They were released to the marine environment in the spring of 1999 and they returned to spawn in the fall of 2000. Evaluation of their fertility and offspring survival represents an evaluation of their reproductive output. Their offspring are the second generation (F1). The F1 incubated in clean water beginning in fall 2000; they were released in the spring of 2001 and will return in the fall of 2002 to spawn. Their reproductive output will be evaluated by measuring the survival of their offspring, which is the third generation (F2). Reductions in reproductive output in the P1 generation could result from either genotypic or phenotypic impacts of oil. Reductions in reproductive output of the F1 generation can only result from genotypic impacts, because they will never be exposed to oil.

This project is at its midpoint. The exposure and early marine survival of the of the P1 have already been described (Heintz 2000). This report evaluates the reproductive output of the P1 generation by providing an analysis of the marine survival of the P1, their size at maturity, fertility and estimates of their offspring survival. In addition, fish from the donor stock, Lovers Cove Creek, were also spawned so that offspring survivals could be related to previous experiments. Future reports will describe the incubation of the F1, and their reproductive output (Table 1).

METHODS

Marking, release and recovery of exposed pink salmon

Adult pink salmon representing control, low and high levels of embryonic exposure to oil returned to the Sashin Creek weir in the fall of 2000. The fish had been exposed to oil-contaminated water in a nearby hatchery when they incubated between September 1998 and April 1999. The low dose represent exposure to an initial aqueous total PAH concentration of 5.0 ppB and the high dose represents a 13.3 ppB concentration. After incubation, the surviving fish were marked by excising combinations of their adipose and pelvic fins and released. Marking was intended to facilitate identification of their exposure histories when they returned as adults. Only fish that appeared healthy at the time of marking were released, and fish with obvious deformities were counted and discarded. See Heintz (2000) for more detailed description of the incubating, marking and release procedures.

Each fish trapped by the weir was inspected, and those bearing any deformity to either their adipose or pelvic fins were retained in a holding-pen located adjacent to the weir and

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supplied with water drawn from Sashin Creek. The run began on August 26, and the middle two quartiles arrived between September 4 and 15. On September 14, all the fish in the holding-pen were inventoried and only those with unambiguous fin clips were retained for spawning. Experimental designs were subsequently developed based on the total number of fish available for spawning from each of the treatment groups.

Prior to spawning, fish were transferred from the holding-pen to a holding-tank locatd next to the spawning area at the hatchery. On a given day, male and female fish representing each of the three treatment groups were netted from the holding- pen and placed into a 1200 L tank supplied with oxygen and transferred by boat to the hatchery. After the ~5 minute trip to the hatchery, fish were netted with dipnets and separated by sex into 4000 L holding-tanks near the spawning area. The holding-tanks were supplied with fresh water flowing at a rate of approximately 40 L per minute. Fish were held in the holding-tanks overnight and spawned the next day.

Wild fish from Lovers Cove were also held in the holding-tanks prior to spawning. Treated fish returning to the weir were derived from the wild stock spawning the stream at Lovers Cove. Therefore, we also sampled fish from Lovers Cove as a control. These fish were collected from the stream at high tide with a beach seine. Fish were captured on the day before spawning and transferred to the hatchery in a 1200 L tank supplied with oxygen. These fish were held overnight in the same holding-tanks as the treated fish.

Spawnings took place on September 15, 18 and 20. On a given day, females from a randomly selected group were removed from the holding tank, sacrificed and bled. Their lengths (middle of eye to fork of tail) and weights were recorded. Their gametes were removed, weighed and stored in a refrigerator. Gamete weights for females reflected the mass of eggs and ovarian fluid recovered, and for males reflected the summed weight of testes and expressed milt. Males were processed with a similar procedure after all the females from a given dose had been processed. Milt taken from males was stored on oxygen in a refrigerator.

Mixed gametes were incubated using standard hatchery procedures and incubated in clean water. By November 8, the eggs had completed development of their eyes and their survival rates could be assessed following standard husbandry practices (ADFG 1983).

Mating designs

Two different mating designs were employed to evaluate the survival of offspring from the treated fish. The first design is based on the approach taken by Bue et al. (1998) and the second the approach employed by Brannon et al. (1995). These are hereafter referred to as the pooled and pairwise approaches, respectively. Both approaches attempt to measure the mean survival of the offspring derived from treated and untreated fish. The mean estimated by the first approach represents the average drawn from all the possible crosses that could be made in the population. The estimate made by the second approach is calculated from a number of randomly selected matings representing a given treatment..

The first approach used pools of gametes as the experimental units. On a given day, gametes from at least 5 females and 10 males were pooled together to create all the possible crosses. The pools were generated by mixing the eggs from each female representing a given dose on each of the spawning days. The mixed eggs were divided into a number of aliquots equal

to the number of males. A 2 ml aliquot of milt from a male was used to fertilize the first two aliquots, milt from the second male fertilized the second and third aliquots and so on. The fertilized eggs were pooled into a common container and allowed to water harden for 2 h. Once hardened, 7 aliquots of approximately 120 eggs were withdrawn and incubated in vertical incubators. The procedure was repeated on each of the three spawning dates, using all of the males and females listed in Table 2. The remaining eggs were used to create production lots for future release. Two samples of approximately 150 eggs were drawn from the production lots 18 hours after fertilization to evaluate fertilization rates in the pools. Estimates of fertilization rate follow the procedures described in ADFG (1983).

The second approach used randomly selected pairs of individuals from each of the treatment groups. On each of the three spawning dates, approximately 400 eggs were taken from a given female and crossed with sperm taken from a randomly selected male. The fertilized gametes were divided into two aliquots and incubated in separate vertical incubators. This procedure was repeated for each of the females spawned on each of the three spawning dates. Each female was crossed with 3 different males, and a minimum of 5 females were used for each treatment on a given day. Males were crossed with at least 2 females, and by the end of the three spawning dates all individuals had been mated. Table 3 provides details on the number of crosses made for each treatment and date.

Statistical analysis

Marine survival was evaluated by Chi-square analysis with two degrees of freedom and an $\alpha = 0.05$. The proportion of individuals surviving to maturity from a given dose was calculated as the total number returning bearing unambiguous fin marks, divided by the total number released. Heintz (2000) provides the number of releases. The expected number of returning fish was estimated by first dividing the total number of fish returning with unambiguous fin marks by the total number released. This proportion was then multiplied by the number released to obtain an expected number returning for a given dose.

Size at maturity, and gonadosomatic indices (GSI) were compared among the treatment groups by one-way ANOVA with dose as a fixed factor. Size was estimated by the length at spawning. GSIs were calculated for each fish as the percentage of the total weight accounted for by the combined weight of the gonads and gametes. Lengths and GSI were compared only for fish of the same sex.

Fertilization and offspring survival were evaluated by ANOVA. Offspring survival was estimated by dividing the number of eggs alive at eyeing by the total number of eggs placed into the incubator. The statistical model used to evaluate the pairwise estimates of offspring survival used spawning date as a random variable nested within dose and randomly selected crosses nested within spawning date. The doses were fixed factors and included control, low and high dose groups. The model used to evaluate the offspring survival based on the pooled approach used spawning date as a random variable nested within treatment. Treatment groups included control, low and high dose groups and Lover's Cove. Fertilization rate was estimated by the number of eggs found to have reached cleavage divided by the number evaluated. The model used to evaluate fertilization was identical to that used for the pooled approach to evaluating offspring survival.

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RESULTS

Marine survival and size at maturity of the exposed generation

   Marine survival depended on exposure history (P < 0.001) and those fish incubating in an initial aqeuous PAH concentration of 13 ppB survived at about half the rate of unexposed fish. A total of 89 control fish out of 66,391 survived to maturity. Low dose fish had similar survival, with 93 fish surviving out of 64,087 released. In contrast, only 35 high dose fish survived to maturity out of the 55,362 originally released. Therefore, exposures to 13 ppB concentration of PAHs in water during embryonic develpment led to decreas

   Despite differences in the survival rate no difference in their size at maturity or gonadosomatic index was observed (P > 0.095). Females averaged 465 ± 1 mm long and an average 16.2 ± 0.3% of their body weight was taken up by gonads. Males averaged 446 ± 3 mm long with testes and milt averaging 6.3 ± 0.1 % of their entire body weight.

Fertility of the exposed generation and survival of their offspring to eyeing

   Average fertilization rates decreased with exposure level, but were not significantly different (P = 0.261). Fertilization rates were lowest for gametes drawn from high dose fish averaging 77.7 ± 7.3% compared with 85.3 ± 1.7 % and 91.8 ± 1.5% for control and Lovers Cove groups, respectively (Table 3). There was significant variation among the spawning dates (P = 0.028) indicating that fertilization rates were more dependent on spawning time than dose. This was best illustrated by the high dose gametes which had fertilization rates ranging between 90.2 ± 4.4% and 69.4 ± 5.7 % of the first and second days, respectively. In contrast, the remaining groups had relatively consistent fertilization rates ranging between 80.4 ± 5.0% and 94.8 ± 2.8%. Gametes taken from the Lovers Cove fish had the most consistent fertilization rates (Table 3).

   Offspring survival rates to the eyed stage as estimated by the pooled approach were not influenced by dose (P = 0.711). Mean survival rates were 68.5 ± 20.1%, 71.2 ± 3.1% and 75.4 ± 7.6% for the high, low and control doses, respectively. The Lover's Cove offspring had the highest average survival at 83 ± 6.6% (Figure 1). Survival on different spawning dates varied significantly (P< 0.001), indicating that offspring survival was more influenced by the date they were spawned than by exposure history (Table 5). Variation among spawning dates was most extreme for the high dose fish which experienced a mean survival of 88.6 ± 1.2% on the first date, and 48.5 ± 2.1% on the second date. However, survivals did not change consistently with date, survival on the second date was half the value observed on the first and third dates for the low dose fish (Table 5). The least variable survivals were observed among the Lover's Cove offspring, ranging between 78.9 ± 1.1% and 88.9 ± 0.5% on the first and third dates, respectively (Figure 2).

   The pair-wise approach revealed that differences among spawning dates (P < 0.001) were driven by significant variation among crosses (P < 0.001) on a given date. As with the pooled approach, the mean survival rates were lowest for the exposed fish but differences between treatment groups were not significant (P = 0.985) (Figure 3). While accounting for a significant amount of the total error, the amount of variation associated with spawning dates was consistent among treatment groups (P = 0.717).

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DISCUSSION

　　　　Release and recovery data for the exposed P1 generation in this study confirm conclusions drawn by Heintz et al. (2000) that incubating in oil-contaminated water leads to delayed effects which ultimately reduce marine survival. Consistent with that study, the size of the returning fish was unaffected by oil exposure, but survival was significantly reduced. Heintz et al. (2000) theorized that reduced growth rate in the marine environment put a larger proportion of the population at risk to predation, concluding only the largest and fastest growing of the exposed fish survived to maturity. The same argument can be drawn for these fish. However, observations of elevated mortality in the treated fish populations prior to release Heintz (2000) suggest the effects of reduced growth may be compounded by other injuries that result in delayed mortality.

　　　　Its unlikely that the observed differences in survival result from differential mortality assoicated with the different fin clips. The control and low dose had similar survivals, but controls had a more extreme mutilation than the low dose. Control and high dose fish had both their adipose and pelvic fins amputated in contrast to low dose fish which were missing only their adipose fins. If the degree of mutilation influenced survival (Pacific Salmon Commission 1995), then control and high dose fish from this study should have had similar survivals, and both of these should have been lower than the low dose. Wertheimer et al. (1999) showed that different fin marking crews can influence the survival of fish released with similar marks, but all fish in this study were marked by the same crew.

　　　　Despite the identification of an influence of marine survival on the returning adults, no impact of oil on the survival of their offspring (F1) could be statistically resolved. However, mean offspring survival declined with exposure level in both approaches. This outcome results from either the true absence of an effect, or an insufficiently powered experiment. If the treatments truly result in differences in mean offspring survival, then the power to statistically resolve the differences will depend the variation around the means, and the number of replicates examined. Normally, the number of replicates used in an experiment is determined by assuming some level of variation around the mean response. *A priori* expectations for variation around the means can exceeded if experimental protocols provide an additional source of variation, which would reduce experimental power for a given number of replicates. The following three sections examine these issues in more detail by first examining the *a priori* expectations for variation and replication, then the potential for additional error arising from experimental protocols and finally the potential for there to truly be no difference among the means.

Evaluation of observed variation and numbers of replicates

　　　　Comparison of the pooled approach employed here with results reported by Bue et al. (1998) indicate this study had lower statistical power as a result of a limited ability to provide replicates. Estimates of the variation around the mean are comparable between the two studies. Standard errors for the survival estimates for each combination of spawning date and dose ranged between 0.005 and 0.029 while those reported by Bue et al. (1998) range between 0.005 and 0.036. In addition, the difference in the mean survival rate of control and high dose offspring was 11.6%, which is comparable to the 9% observed by Bue et al. (1998). Consequently, the only

meaningful difference in the two studies is in the number of replicates. We employed only two replicates for our high dose in contrast to Bue et al. (1998) who employed eight. Differences in the details of the spawning designs between our paired approach and that of Brannon et al. (1995) prevent a similar comparison of power.

Fewer replicates were employed here because of the small return of salmon. Only 13 high dose females returned to the weir in 2000. Of those, only 11 could be spawned and these were subsequently used to generate two pools. The poor return rates were consistent with the low numbers of pink salmon returning to southeastern Alaska in 2000. The 2000 harvest of pink salmon in southeastern Alaska was the lowest in the last 12 years (Rigby et al. 1991, Doug Eggers Alaska Department of Fish and Game, personal communication), similarly the escapement to Sashin Creek was the lowest since 1974 (Bradshaw and Heintz 2000). Our expectation was that survival would be within two standard errors of the mean survival observed for the even year pink salmon run at Sashin Creek. This mean, based on 17 brood years, was expected to produce at least 125 spawners from the high dose group, after assuming oil exposure caused a 50% reduction in marine survival relative to the controls. While we did observe a 50% reduction in marine survival for the fish exposed to the high dose, survival of controls was approximately one fourth of the expected low value for survival.

Influence of experimental protocols on offspring survivals

The offspring survivals reported here are consistent with those of Bue et al. (1998) and Brannon et al. (1995) indicating that experimental procedures do not account for our inability to detect effects. The estimated fertilization rates can be used to estimate the total number of eggs that were actually fertilized in the pooled approach. Consequently, survival after fertilization can be estimated for each of the groups on each of the days. Doing so, produces mean post-fertilization survival rates of 88.9%, 93.9%, 86.7% and 89.0% for the control, low dose, high dose and Lover's Cove groups, respectively. Bue et al. (1998) reported values of survival for their pooled groups ranging 78% and 94%. Brannon et al. (1995) observed mean values of 83.9% to 98.1% using their pairwise approach. No estimates of fertilization rate were made for our pairwise approach, so estimates of post-fertilization mortality include eggs that were dead at fertilization, unfertilized eggs and those that died after fertilization. However these values are similar to those for the pooled groups.

The effect of spawning date on offspring survival is best explained by variation in ovulation time among individuals selected on a given date. Ovulation time is closely linked to fertility (Leitriz and Lewis 1980), thus increased asynchrony in ovulation time in a sample leads to greater variation in and reduced fertility. Collecting Lovers Cove fish from redds meant that we selected females that had behaviorally demonstrated their readiness to spawn, thus ensuring they recently ovulated. This probably explains their tendency towards higher and less variable fertility and offspring survival. In contrast, the treated fish were accumulated over a two week period and randomly assigned to spawning dates, with no assurance that fish allocated to a given date had any synchrony in ovulation. Typically, ovulation can be detected in females by palpating abdomens and checking to see if they extrude eggs. If the process is repeated every few days, then the time of ovulation can be identified. This was not possible with these fish, because it took the entire period to obtain sufficient numbers for the experiments. The increased variation in

mean offspring survival that resulted from increased asynchrony in ovulation further supports the notion that the experiments described here were underpowered, because Bue et al. (1998) selected fish from redds.

The lack of a consistent pattern between spawning date and offspring survival for the fish collected from the weir (Table 3) further supports the idea that the effect of spawning date resulted from increased asynchrony in ovulation. If the effect was simply one of holding the fish too long, then offspring survival rates should have declined with time for all the groups. Similarly, if the effect of spawning date was the result of procedural differences among days, then one day would be noteworthy by low survival across the board. Similarity in the survival rate of control and Lover's Cove offspring for the first two days also demonstrates that differences in the maturation environments were negligible.

Potential for no effect of oil on reproduction

The absence of an effect is not consistent with our observations of delayed effects on growth (Heintz et al. 2000) and marine survival. Nor is the absence of an effect consistent with conclusions drawn by Bue et al. (1998) who found a reproductive impairment in adult salmon returning to oil-contaminated streams. Evidence of delayed effects suggest that, at a minimum, phenotypic impacts on reproduction should exist. In fact, Marty et al. (1997) reported impaired development of gonads in fry surviving embryonic exposure to oil. Futhermore, Smoker et al. (2000) provides evidence of a genetic effect of embryonic oil exposure on reproduction, which is supported by controlled studies demonstrating genetic impacts of benzo-[a]-pyrene on reproduction in an exposed generation of fathead minnows (White et al. 1999). A possible mechanism to account for these effects has been described by Roy et al. (1999) who demonstrated that incubating under conditions similar to those used here led to genetic mutation, and Carls et al. (1999) who demonstrated the clastogenic properties of Alaska North Slope crude oil. In contrast, only Brannon et al. (1995) provide evidence of no effect, but that study has been criticized for its lack of statistical power (Rice et al. 2001a).

While not statistically significant, the trends in both experiments are consistent with expectations for oil impacts. Rice et al. (2001b) posited a damage mechanism whereby superoxide ions produced by PAH metabolism causes random genetic damage in exposed embryos and these mutations are passed to daughter cells. This suggests that impacts on reproduction should result in reduced average viability of offspring and increased variation around that average. This is the result reported by Bue et al. (1998). In this report mean offspring survival tended to decline with dose and errors around those means tended to increase (Figures 1 and 2). In contrast, the results reported by Brannon et al. (1995) fail to reveal any trend in error.

A possible explanation for the absence of differences in reproductive ability between the treatment groups is that only unaffected salmon survived to reproduce. Only 0.05% of the high dose fish survived to maturity, and these apparently grew at the same rate as the control fish and produced gonads of similar mass. The model for damage proposed by Rice et al. (2001b) indicates impacts should be randomly distributed suggesting some individuals could be unaffected. The poor returns for pink salmon in 2000 indicate poor marine conditions, which could compound any oil impacts relative to years when marine conditions are more conducive to pink salmon production. Thus, the increased severity of marine conditions may have further

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exacerbated any oil effects thereby filtering out all of the affected individuals.

Such a dependency between expression of delayed effects and marine conditions illustrates the sorts of problems that must be overcome in evaluating impacts of oil on the fitness of wild populations. The absence of any control over the survival of experimental animals during the marine phase requires the rearing and marking of tens of thousands of pink salmon fry and releasing them to the environment. The numbers required for release are based on best guesses of future survival providing adequate numbers of spawners. These procedures have great appeal, because they provide a essential element of realism to an otherwise controlled experiment. However, real world variation provides no guarantee that our best predictions of the future will be accurate.

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Table 1. Time line of biological endpoints and reports for study 476. Shaded boxes describe this report.

	1998	1999		2000		2001		2002		2003
	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
Biological endpoints	Expose P1 generation	Release P1 generation	P1 generation at sea.	P1 generation at sea	P1 generation matures, incubate F1	Mark and release F1 generation	F1 generation at sea	F1 generation at sea	F1 generation matures, incubate F2	Closeout
Report and contents	Heintz 2000 Exposure levels, survival during incubation and release numbers	Annual report 00476 Marine survival, size at maturity, fertility and reproductive ability of P1				Annual report 01476 Incubation, marking and release of F1		Final Report 02476 Marine survival, size at maturity and reproductive ability of F1.		

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Table 2. Numbers of dams and sires used to generate pools using the pooled approach to estimate average offfspring survival.

Spawn Date	Treatment	Number of Dams	Number of Sires	Number of Crosses in Pool
September 15	Control	8	12	96
	Low	8	12	96
	High	6	9	54
	Lovers Cove	5	10	50
September 18	Control	10	15	150
	Low	10	15	150
	High	5	9	45
	Lovers Cove	5	10	50
September 20	Control	18	13	54
	Low	14	21	42
	Lovers Cove	5	10	50

Table 3. Numbers of individuals used for generating crosses using the pairwise approach to evaluate mean offspring survival.

Spawning Date	Treatment	Number of Dams	Number of Sires	Number of Crosses in Pool
September 15	Control	8	12	24
	Low	8	12	24
	High	6	9	18
September 18	Control	10	15	30
	Low	10	15	30
	High	5	9	13
September 20	Control	18	13	54
	Low	14	21	42
	High	0	0	0

Table 5. Average fertilization and offspring survival rates (%) for offspring generated under the pooled and pairwise approaches on each of the spawning dates.

Pooled Approach

	September 15		September 18		September 20	
	Fertilized	Survival	Fertilized	Survival	Fertilized	Survival
Lover's Cove	92.8	78.9	89.2	81.0	93.3	89.1
Control	83.8	82.8	83.2	83.1	88.8	60.2
Low Dose	84.6	79.4	66.7	59.0	87.9	78.2
High Dose	89.2	88.6	66.1	48.4	-	-

Pairwise Approach

Exposure Group	Survival	Survival	Survival
Control	82.0	76.0	51.3
Low Dose	82.0	48.1	74.1
High Dose	90.6	44.0	-

Table 5. ANOVA table for pooled approach to comparing mean offspring survival of fish exposed to different amounts of oil. Abbreviations: CO control, LD low dose, HD high dose and LC Lover's Cove. Asterisks depict mean squared errors that differ from the expected mean square error when $\alpha = 0.05$.

Source	Sum of Squares		Degrees of Freedom		Mean Squared Error	
Dose	0.208		3		0.069	
Spawn Day (Dose)	1.030		7		0.147*	
Spawn Date (CO)		0.241		2		0.121
Spawn Date (LD)		0.182		2		0.091
Spawn Date (HD)		0.566		1		0.566
Spawn Date (LC)		0.041		2		0.020
Error	0.149		66		0.002	
Total Error	1.388		76			

Table 5. ANOVA table for pairwise approach to comparing mean offspring survival of fish exposed to different amounts of oil. Abbreviations: CO control, LD low dose, HD high dose and LC Lover's Cove. Asterisks depict mean squared errors that differ from the expected mean square error when $\alpha = 0.05$.

Source	Sum of Squares	Degrees of Freedom		Mean Squared Error	
Dose	0.074	2		0.037	
Spawn Day (Dose)	10.80	5		2.16**	
Spawn Day (CO)	3.65		2		1.82
Spawn Day (LD)	3.62		1		1.81
Spawn Day (HD)	3.52		2		3.52
Cross (Spawn Day-Dose)	26.30	226		0.12**	
Cross (Spawn Day-CO)	14.05		102		0.14
Cross (Spawn Day-LD)	9.83		93		0.12
Cross (Spawn Day-HD)	2.42		31		0.08*
Error	0.870	233		0.004	
Total Error	38.032	466		0.833	

Figure 1. Mean survival (± 1 s.e.) of offspring generated under the pooled approach for the Lover's Cove, Control, Low dose and High Dose groups.
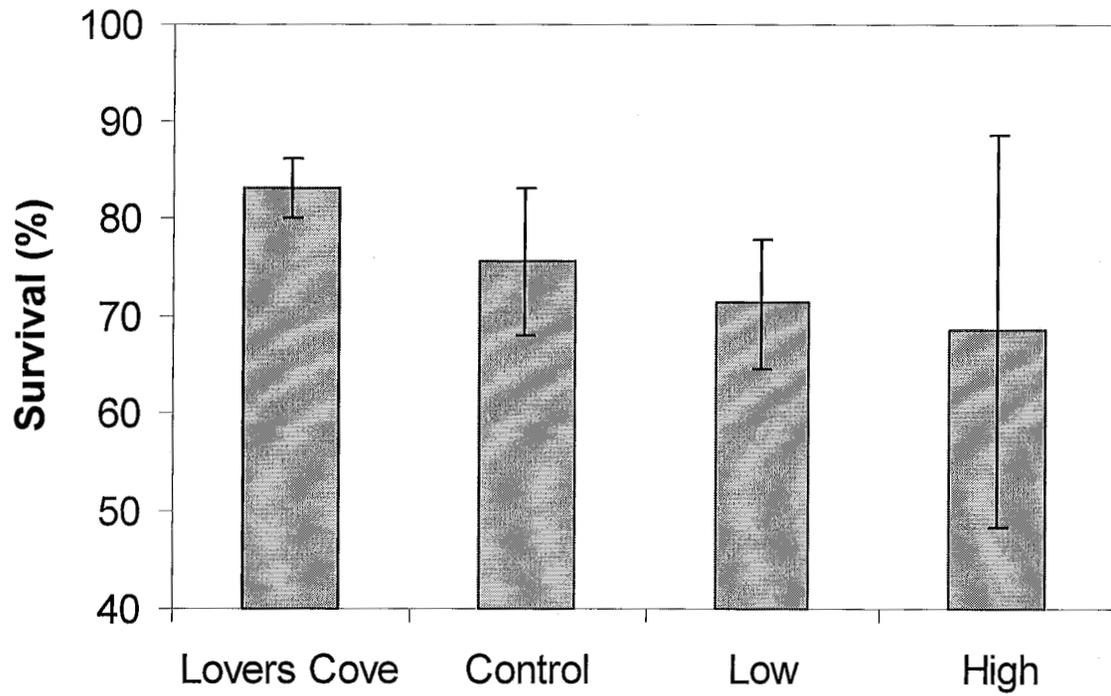
Figure 2. Mean survival (± 1 s.e.) of offspring generated under the pairwise approach for the Control, Low dose and High Dose groups.