Exxon Valdez Oil Spill
Restoration Project Annual Report

Injury to Pink Salmon Eggs and Preemergent Fry Incubated in Oiled Gravel (Laboratory Study).

Restoration Project 94191B
Annual Report

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Study History: Restoration Project 94191B was initiated as part of the continuation of NRDA Study Fish Shellfish 2 (Inujury to Salmon Eggs and Preemergent Fry in PWS). The objectives of that study included estimating the egg mortality and overwinter survival of pink salmon eggs in oiled streams after the spill. In FY 93 FS 2 was continued as Restoration Study 93003A, and it was combined with the initiation of this study (93003B), because the objectives were complementary. An objective of Study 93003A was to continue monitoring oiled and unoiled streams to document differences in embryo survival, while 93003B represented the beginning of a long-term investigation into the potential causes for these differences in embryo survival. In the Spring of 1994 the initial results of 93003B were submitted to the Trustees in a report titled: **Interim Report for Restoration Science Study Number 93003: Injury to Pink Salmon Eggs and Preemergent Fry Incubated in Oiled Gravel (Laboratory Study).** This report covered the initial results of the laboratory study. In FY 94 the project was funded under Restoration Science Project 94191B, and it continued to be identified with the field analysis funded as 94191A. This report covers the laboratory results obtained in FY 94. In FY 95 the project continues ~~to identified~~ as 95191B, a complement to the field work covered under 95191A.

Abstract: Analysis of the marine growth of pink salmon exposed to oil during incubation demonstrates delayed effects on growth 4 to 6 months after the exposures have ceased. In addition, incubating in oiled gravel reduces embryo survival, disrupts developmental stability and alters emergence timing. Oil's delayed effect on growth has rarely been documented, and in the case of pink salmon, suggests a mechanism for observations of reduced fitness in Prince William Sound. Unfortunately, all fish from the 1992 brood succumbed to disease before sexual maturity, so analysis of survival and growth to maturity remains incomplete. The 1993 brood will mature in September 1995, and the analysis will continue at that time.

Key Words: *Oncorhynchus gorbuscha*, growth, incubation, *Exxon Valdez*, oil, survival, development.

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Current Status

1992 Brood Year:

Oil exposures were completed in spring 1992, rearing continued until June 1994 when all of the fish succumbed to bacterial kidney disease. Previously unreported results for this brood include impaired growth and low survival of high dose fish.

1993 Brood Year:

Oil exposures were begun in September 1993, and emergence was complete by May 15, 1994. Results of incubation support findings with the 1992 brood. More than 6,000 fish are currently being cultured in netpens, and more than 14,000 were coded wire tagged and released. Spawning of mature fish is planned for September 1995.

I. Introduction

Comparisons of embryo survival between oiled and unoiled streams in Prince William Sound (PWS) since the *Exxon Valdez* oil spill led Sharr et al. (1991) to hypothesize that pink salmon embryos and larvae incubating in oiled substrates may have experienced genetic damage resulting in "functional sterility." Sharr's work revealed persistent differences in embryo survival between oiled and unoiled streams. In 1989 and 1990, survival differences were limited to strata below the high oil mark, but work done in 1991 through 1993 identified survival differences in all stream strata, including those above the high oil mark. Sharr et al. (1992) hypothesized that incubation in oiled gravel reduced fertility in the intertidally spawning fraction of the 1989 brood, and their progeny (1991 brood) inherited this trait.

A laboratory study, designed to test the plausibility of the "functional sterility" hypothesis began in July 1992 at the National Marine Fisheries Service (NMFS) research hatchery at Little Port Walter (LPW) in southeastern Alaska. Pink salmon eggs from the 1992 brood year were exposed to either clean or oiled incubation substrates. A similar experiment, initiated the following year, was intended to replicate the 1992 brood year experiment and increase the ability to resolve differences between doses in emergent fry size and survival to emergence. If genetic damage in laboratory oil exposures is verified, the cause of poor embryo survival in oiled streams of PWS in 1991 and 1992 could be ascribed to oil with more confidence.

II. Project Objectives

This project is a laboratory study intended to simulate the intertidal incubation environment of Prince William Sound pink salmon in 1989. Resultant fry are reared to adulthood, and subsequent matings used to verify the findings of Sharr et al. (1991). The specific objectives are:

1. Evaluate the reproductive success of two brood years of pink salmon incubated in a substrate contaminated with different amounts of oil by rearing them to maturity and observing their fertilization rates and offspring survival rates.

2. Determine the amount of hydrocarbon uptake in two broods of pink salmon incubating with different levels of oil in their incubation substrate.

3. Determine the effects of different levels of oiled incubation substrate on all the major life history stages of the exposed fish including:
   a. survival to eyeing, emergence from gravel, and maturity
   b. size at emergence and maturity
   c. growth rate from juvenile stage to maturity, and fecundity
   d. degree of histopathological damage and mixed function oxidase activity in emergent fry.

IIIa. Methods Common to Both Brood Years

The experiment requires culturing two brood years of pink salmon from fertilized eggs to maturity. Pink salmon eggs are collected from an intertidally spawning stock. Aliquots of fertilized pink salmon eggs are taken from a randomized pool and placed in incubators containing either oiled or non-oiled gravel. Incubation continues until all surviving salmon emerge from the substrate, and these "emergent fry" are cultured until maturity. Gametes are collected from the mature fish, and differences in survival between the progeny of oiled and non-oiled parents may be ascribed to the oil.

This report documents the progress of both the 1992 and 1993 brood years. Procedures common to both years are described below, and details unique to each brood year are discussed later (Table 1).

Incubation

Fertilized eggs were subject to standard hatchery practices. After fertilization, eggs were placed on the gravel substrate. After the eye had developed (eyeing) eggs were removed from the incubators, shocked, and counted. Surviving eggs were replaced in the incubators and permitted to continue developing. When hatching began, chorions were removed from the incubators, and alevins were permitted to burrow into the substrate (some exceptions are noted below). Emergence was volitional; all emerging fish were counted and inspected for gross deformities.

Incubators were designed to simulate upwelling water flows found in stream environments. The incubators were constructed from 30 cm sections of 16 cm polyvinylchloride pipe. The pipe was stood on end and the bottom sealed. Water was admitted through an inlet tapped into the side near the bottom and exited from an outlet near the top. A perforated (3 mm perforations) aluminum plate, fixed inside the pipe immediately above the water inlet and below the incubation substrate, prevented the water from channeling through the substrate. To optimize alevin survival, pipes were filled with 10 kg of shot gravel. Gravel sizes differed between years, diameters were < 5.0 cm in 1992 and < 2.5 cm in 1993.

To simulate the intertidal environment, fresh and salt water were cycled through the incubators. Incubators were supplied with fresh water for eight hours followed by four hours of salt water. Fresh water was directly drawn from Sashin Creek, and salt water was taken from a 10 m depth in the estuary at Little Port Walter. All water was filtered (mesh size = 10 microns) to remove macroscopic debris. Both fresh and saltwater temperatures were monitored daily, flow rates were checked every other day, salinity and DO were monitored weekly. Flow rates to incubators were maintained at approximately 150 ml/min from fertilization to eyeing, and at 200 ml/min thereafter. Salinities typically reached 28 ppt 20 minutes after the saltwater cycle began.

After emerging from the incubators, the fish were reared in small freshwater raceways at the NMFS Osprey Bay facility located approximately 5 km north of LPW. Fry from each incubator were kept separate and cultured using standard techniques. When the fish weights averaged 6.0 g they were tagged with passively induced transponders (PIT tags). Two weeks after tagging, the fish were moved to saltwater netpens for continued culture. Fish were reared in fresh water to avoid exposure to *Vibriosis*. All fish retained for netpen culture were vaccinated against *Vibriosis* two weeks prior to moving them to salt water.

Development of Dose Response Curves

Dosing levels were established by analyzing hydrocarbon concentrations in incubator effluent, substrate, and fish tissue with gas chromatograph and mass spectroscopy (GC/MS). Hydrocarbon concentrations in substrate, effluent and tissue were estimated for each major developmental stage; development of the eye (eyeing), hatching, and emergence from the substrate. Pooled samples of effluent, gravel and fish tissue were collected from each of the replicate incubators in an exposure group and analyzed. In addition, effluent samples were collected at each major developmental stage for spectrophotofluoremetry, to provide estimates of variability between incubators within an exposure group.

IIIb. Methods and Results Unique to Each Brood Year

1992 Brood Year

EXPERIMENTAL DESIGN (Experiment 92-1)

In this, the main experiment, developing pink salmon eggs were exposed to oiled incubation gravel from fertilization to emergence. Exposures consisted of control and four doses ranging from 0.1 to 5.7 g oil/kg gravel, with two to four replicate incubators per dose. A total of 16 incubators was used. Results of incubation and some hydrocarbon analysis were reported in the 1993 interim report (Heintz et al. 1993). Alevins were permitted to burrow into the substrate after hatching, in some incubators significant numbers of alevins continued development on the surface of the gravel. Fish were sampled for histopathological damage and immunoflorescent activity at two weeks prior

to, during the peak of, and two weeks after emergence. The remaining fish were cultured in raceways, one per incubator, until they were large enough to tag with PIT tags (approximately 6 g). By the end of emergence, the size distribution of fish in raceways was highly variable. After tagging (August 1993), the fish were moved to one of two netpens: one at Osprey Bay, the other at Little Port Walter. Fish were sampled to determine size and survival in October 1993 and April 1994. The discussion below reports results collected since the fish were PIT tagged.

ACCESSORY EXPERIMENT (Experiment 92-2)

In this experiment, developing pink salmon embryos were exposed to oiled incubation substrate after they have developed eyes. Exposures consisted of a control and one dose of 6.1 g oil/kg gravel with four replicate incubators per dose. Results from the incubation phase of this experiment were reported in the 1993 interim report. As with Experiment 92-1, these fish were sampled for histopathological and immunoflorescent analysis. Remaining fish were moved to freshwater raceways, as in experiment 92-1, and similar size variation existed in the raceways by the end of the emergence period. Subsequent culture was identical to Experiment 92-1. Discussion below reports results since the fish were PIT tagged.

CONTRACT STUDIES

Two contract studies using animals from both experiments described above are near completion. The first examines the histopathological and immunohistochemical effects of oiled incubation substrate on embryos near and immediately after emergence. The contractor (UC Davis) reports that pink salmon exposed to oiled incubation substrate from fertilization to emergence delayed embryo development and increased cytochrome P450IA activity. The second contract study examines immunohistochemical effects of short term exposure to acute doses of oiled incubation substrate, and links the lab results to direct field observations made in PWS.

RESULTS TO DATE

Results from incubating and culturing the 1992 brood suggest that incubation in an oiled substrate had deleterious effects on survival and altered metabolism. In a previous report (Heintz et al. 1993) we concluded that incubation in an oiled substrate had negative impacts on embryo survival, developmental stability, emergence timing and size (Table 3). After continued culture, higher dose fish demonstrated reduced vigor exemplified by reduced growth and survival. An epizootic infection by *Renibacterium salmoninarum* (Bacterial kidney disease) killed all populations of 1992 brood pink salmon by June 1994, preventing analysis of their gamete viability. A health plan was developed to protect the 1993 brood from a similar problem.

Growth rates

Growth rates (% increase in wet body weight per day) from tagging to October did not differ between doses. This is likely due to the large variability in growth rates

of the high dose fish which were few in number. Growth between tagging and April 1994 were significantly different between doses ($F = 3.27$ $P = 0.023$), note that no high dose fish survived the winter, and they are not included in this analysis. Control fish averaged 0.8% compared to 0.7% for fish from the 1.5 ppt dose (Figure 1). Growth rate differences were detected for the Experiment 92-2 fish for the period between tagging and the following October, but not the following April. The results for the tagging to April period may result from the small number of fish from experiment 92-2 surviving the winter.

Survival

Survival was poor for the high dose fish during all culture phases. Of 161 high dose fish placed into raceways, only 11 survived to October 1993 leading to significant differences in survival rates existed between doses between tagging and October 1993 (Figure 2a) ($F = 8.23$ $P= 0.003$). Between October 1993 and April 1994 all the high dose fish died and no significant differences existed between the remaining doses (Figure 2b) ($F = 1.23$ $P =0.361$). We do not believe that differences in survival between the high dose and remaining fish, during the latter period, were related to related to differences in susceptibility to *Renibacterium*.

1993 Brood year

EXPERIMENTAL DESIGN (Experiment 93-1)

This experiment followed the basic procedures of experiment 92-1, but with several modifications. The number of doses was increased; they consisted of a control and 6 doses of Prudhoe Bay Crude ranging from 0.1 to 5.3 g oil/kg gravel. In addition, a seventh dose, consisting of the highest dose (5.7 g/kg) gravel from the 1992 experiments was also used. There were eight replicate incubators for each dose, except the 5.3 g/kg dose which had 15. Immediately prior to hatching, incubator lids were removed, and the tops were exposed to light on an ambient cycle, stimulating all the newly hatched alevins to burrow into the substrate. All fry were incubated until emergence. After emergence, a group of 14,000 fish representing the control, 0.1, 0.5 and 1.125 g/kg doses was coded wire tagged and released. Additionally, aliquots of 200 fish were collected from each of the incubators during the peak of emergence and ponded into small raceways, as with the 1992 brood, assuring much greater size consistency among the cultured fish. These fish were cultured until they were large enough to PIT tag in August 1994. Subsamples of 42 fish were tagged from each raceway, and the remaining fish were given a fin clip (Table 2). All fish were vaccinated against *Vibriosis*. PIT tagged fish were handled the same as the 1992 brood year, fin marked fish were pooled into a separate, net pen. Both PIT tagged and fin clipped fish are being fed food treated with erythromycin for prophylaxis against *Renibacterium*.

Table 2:

Control	Left Pelvic
0.1 g oil/kg gravel	Adipose + Upper Caudal
0.225 g oil/kg gravel	Adipose + Left Pelvic
0.5 g oil/kg gravel	Upper Caudal
1.125 g oil/kg gravel	Right Caudal
2.5 g oil/kg gravel	Right Pelvic
5.625 g oil/kg gravel	Adipose
5.7* g oil/kg gravel	Adipose + Lower Caudal

ACCESSORY EXPERIMENT (Experiment 93-2)

The objective of this experiment was to determine if embryo survival was reduced among eggs exposed to the water contaminated by percolating through oiled gravel. Exposures consisted of a control and two doses (0.5 and 1.25 g oil/kg gravel), with four replicate incubators per treatment. Eggs were loaded into the incubators, and cultured identically to those from experiment 93-1, except the developing eggs were placed on perforated plates resting on top of the column of oiled gravel. Embryos from this experiment were cultured to emergence, counted and sacrificed.

RESULTS TO DATE

Results to date support observations made with the 1992 brood: incubation in oiled substrates decreases embryo survival, disrupts emergence timing, and increases the proportion of deformities among emerging fry (Table 3). These effects still hold for oiled gravel used in the 1992 brood study. In addition, survival is also reduced in embryos exposed only to water after it has percolated through oiled gravel.

Experiment 93-1

Survival

Survival was greatly affected by dose. Significant differences existed among the different doses for survival between fertilization and eyeing ($F = 20.03$ $P <= 0.001$), as well as fertilization to emergence. ($F=26.64$ $P<0.001$). Controls demonstrated the highest survival and the high dose the lowest. Surprisingly, embryos developing in the gravel reused from experiment 92-1 showed relatively poor survival (Figure 3).

Temperature units required for emergence

There were significant differences between the doses in the average number of degree days required for emergence ($F = 18.01$ $P < 0.001$). The lowest dose fish

required the fewest, and the highest dose required the most while control incubators were intermediate (Figure 4).

Size at emergence

No differences among the doses were detected (1.09 P = .378). However, there are slight differences in the change in wet weight with time (F = 2.42 P = 0.031).

Frequency of deformities

There were significantly more deformities among emerging fry exposed to higher doses (F = 5.74 P < 0.001). The deformities that drove this relationship included edema and scoliosis. Figure 5 shows 95% confidence intervals for this analysis.

Growth

Dose related differences in growth between tagging and October 1994 were detected. Control fish grew at approximately 3.2% increase in wet weight per day while 1.125 and 5.63 ppt dose fish grew at 2.9% and 2.7%, respectively.

Experiment 93-2

Survival to emergence

Survival was significantly reduced in the 1.25 ppt (nominal) dose (F=9.74 P = 0.006). The 95% confidence interval and names of the doses tested are shown in Figure 6.

Planned Work

Culture of the 1993 brood will continue until maturity. Cultured fish are being given prophylactic treatments of erythromycin to prevent an epizootic of bacterial kidney disease. In addition, conservative estimates suggest over 700 coded wire tag fish will return to Little Port Walter at the same time. Maturation of the 1993 brood will occur in September 1995. At maturity, gametes will be collected, mixed and incubated at Little Port Walter. These progeny will permit evaluation of the relationship between gamete viability and dose.

V. Summary

A laboratory study designed to test the hypothesis that pink salmon embryos exposed to oil during early developmental stages experience "functional sterility" resulting from genetic damage began in July 1992 at the National Marine Fisheries Service (NMFS) research hatchery at Little Port Walter (LPW) in southeastern Alaska. The 1992 and 1993 brood years of pink salmon eggs were exposed to either clean or oiled incubation substrates. Fish from the 1992 brood succumbed to bacterial kidney disease prior to maturation. Several steps have been taken to prevent the recurrence of the disease in the 1993 brood including: coded wire tagging and releasing a group of 14,000 fish in May 1993, and culturing another group of 7,000 fish to maturity in netpens. Cultured fish are being given prophylactic treatments of erythromycin. Fish from the 1993 brood will mature in September 1995, at which time, their gametes will be collected, mixed and the

viability of the resulting embryos will be observed.

Comparisons of results for each of the experiments must be made with caution (Table 3). All of the newly hatched alevins in experiment 93-1 burrowed into the substrate, while similar behavior was seen in only a subset of incubators from experiment 92-1. Direct comparisons between the two brood years must be limited to incubators where alevins behaved similarly, greatly reducing the number of replicates used to calculate variances in experiment 92-1. Consequently, experiment 93-1 has much greater power to resolve differences between doses, and should be given more weight in the comparisons. Additionally, any differences among doses observed consistently across brood years are extremely reliable. An additional difference between brood years is that all emergent fry from the 1992 brood were retained for continued culture, while fry retained from the 1993 brood represented a small subset collected at the peak of emergence. This difference resulted in much less size variability during the culture of the 1993 brood. Consequently, analyses of growth and marine survival for the 1993 brood should be considered much more conservative.

Incubation in oiled gravel reduces embryo survival, disrupts developmental stability, and alters emergence timing (Table 3). Several observations of the effects of oiled gravel on pink salmon embryo development are consistent across brood years. Incubation in oiled gravel decreased pink salmon embryo survival to eyeing, increased the frequency of malformed emergent fry, but did not appear to affect the weight of fry emerging from gravel. Initial analysis of results for the 1992 brood year suggested that dose may also affect survival to emergence and emergence timing (Heintz et al. 1993), the increased resolution provided by experiment 93-1 confirms these conclusions.

Initial analyses of pink salmon marine growth rates suggest continued effects from the oiled incubating environment (Table 3). Growth between August and October was inhibited in pink salmon from experiments 92-2 and 93-1. Dose related differences in growth for the same period in experiment 92-1 appear if the 11 fish remaining from the high dose are removed from the analysis. Note that the analysis for growth between August and April for experiment 92-1 does not include any high dose fish, because none survived the winter.

Literature Cited

Sharr, S., B. Bue, and S. Moffitt. 1991. Injury to salmon eggs and preemergent fry in Prince William Sound. State/Federal Natural Resources Damage Assessment Draft Preliminary Status Report. Cordova, Alaska, USA.

Sharr, S., B.G. Bue, S.D. Moffitt, and A. Craig. 1992. Injury to salmon eggs and preemergent fry in Prince William Sound. State/Federal Natural Resources Damage Assessment Final Report. Cordova, Alaska, USA.

Table 1: Comparison of procedures for experiment performed on brood years 1992 and 1993 pink salmon.

Procedure	Experiment			
	92-1	92-2	93-1	93-2
Duration of exposure:	Fertilization to emergence.	Eyeing to emergence.	Fertilization to emergence.	Fertilization to emergence.
Method of exposure:	Direct contact with gravel.	Direct contact with gravel.	Direct contact with gravel.	Contact with effluent only.
Nominal doses (g oi/kg gravel):	control, 0.1, 0.5 1.5 and 5.7.	control and 6.1	control, 0.1, 0.225, 0.5,1.125, 2.5, 5.63, and 5.7 (reused from experiment 92-1)	control, 0.5 and 1.125.
Number of replicate incubators:	control: 3; 0.1: 3; 0.5: 2; 1.5 : 4; 5.7 : 4;	4 per dose	8 per dose except 15 for 5.63 dose.	4 per dose
Number of replicate incubators where alevins burrowed into gravel:	control: 2; 0.1: 3; 0.5: 1; 1.5 : 3; 5.7 : 0;	control: 2; oiled: 4;	8 per dose except 15 for 5.63 dose.	4 per dose
Endpoints:	Embryo and marine survival, marine growth, gamete viability, histopathology, immunofloresence, flow cytometry.	Embryo and marine survival, marine growth, gamete viability, histopathology, immunofloresence, flow cytometry.	Embryo and marine survival, marine growth, gamete viability, flow cytometry.	Embryo survival,
Disposition of emergent fry:	Sample for histopathological and immunoflorescence analysis, culture rest to maturity.	Culture to maturity.	Coded wire tag and release, culture a subset, collected at peak emergence, to maturity.	Sacrifice.

Table 3: Comparison of results for experiments performed on brood years 1992 and 1993 pink salmon. Cells display F values and associated probabilities from one way ANOVA's where dose was the independent variable. All analyses were performed on raw data. Emergence related analyses include only incubators where alevins burrowed into the gravel.

Measurement	Experiment			
	92-1	92-2	93-1	93-2
Survival to eyeing	F = 16.51 P < 0.001	N/A	F = 20.09 P < 0.001	F = 2.42 P = 0.144
Survival to emergence.	F = 0.48 P =0.642	F = 3.50 P = 0.110	F = 26.64 P < 0.001	F = 10.70 P = 0.004
Proportion of emerging fry with visible lesions:	F = 27.33 P < 0.001	F = 1.70 P = 0.240	F = 5.74 P < 0.001	Not measured
Average number of degree days required for emergence:	F = 0.57 P = 0..655	F = 0.01 P = 0.992	F = 18.01 P < 0.001	Not measured.
Average wet weight during peak emergence:	F = 0.84 P = 0.503	F = 1.01 P = 0.325	F = 1.09 P = 0.378	Not measured.
Marine growth (Tagging - October)	F = 0.74 P = 0.563	F = 21.69 P < 0.001	F = 22.37 P < 0.001	N/A
Marine growth (Tagging - April)	F = 3.27 P = 0.023	F = 0.40 P = 0.503	N/A	N/A
Marine Survival (Tagging - October)	F = 8.23 P = 0.003	F = 0.54 P = 0.495	F = 1.08 P = 0.385	N/A

Figure 1: 95% confidence intervals for percent increase in body weight per day between tagging and April 1994, of 1992 brood pink salmon exposed to oiled incubation substrate.

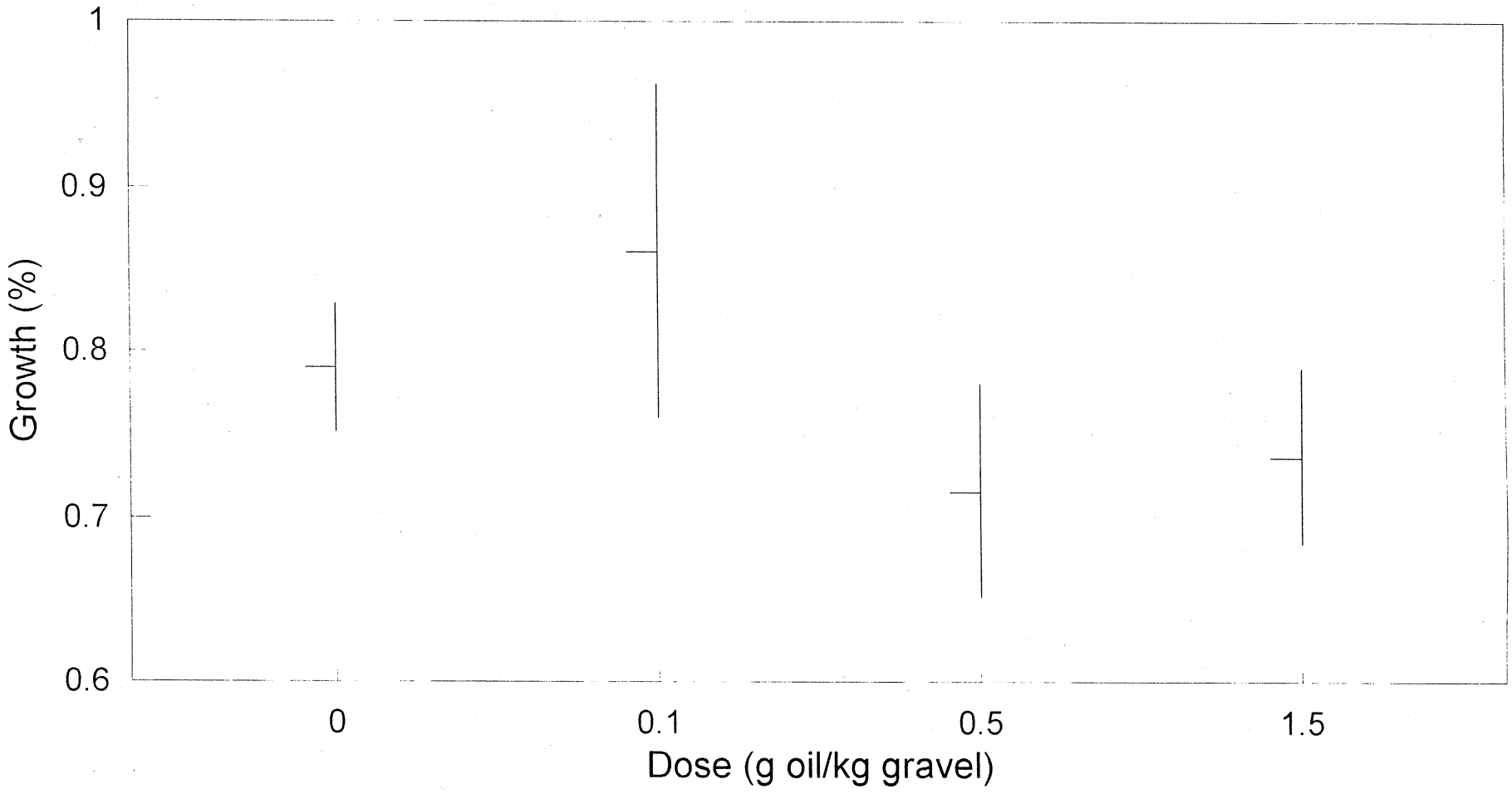


Figure 2a: 95% confidence intervals for mean survival from tagging to October 1993 of pink salmon exposed to oiled substrate.

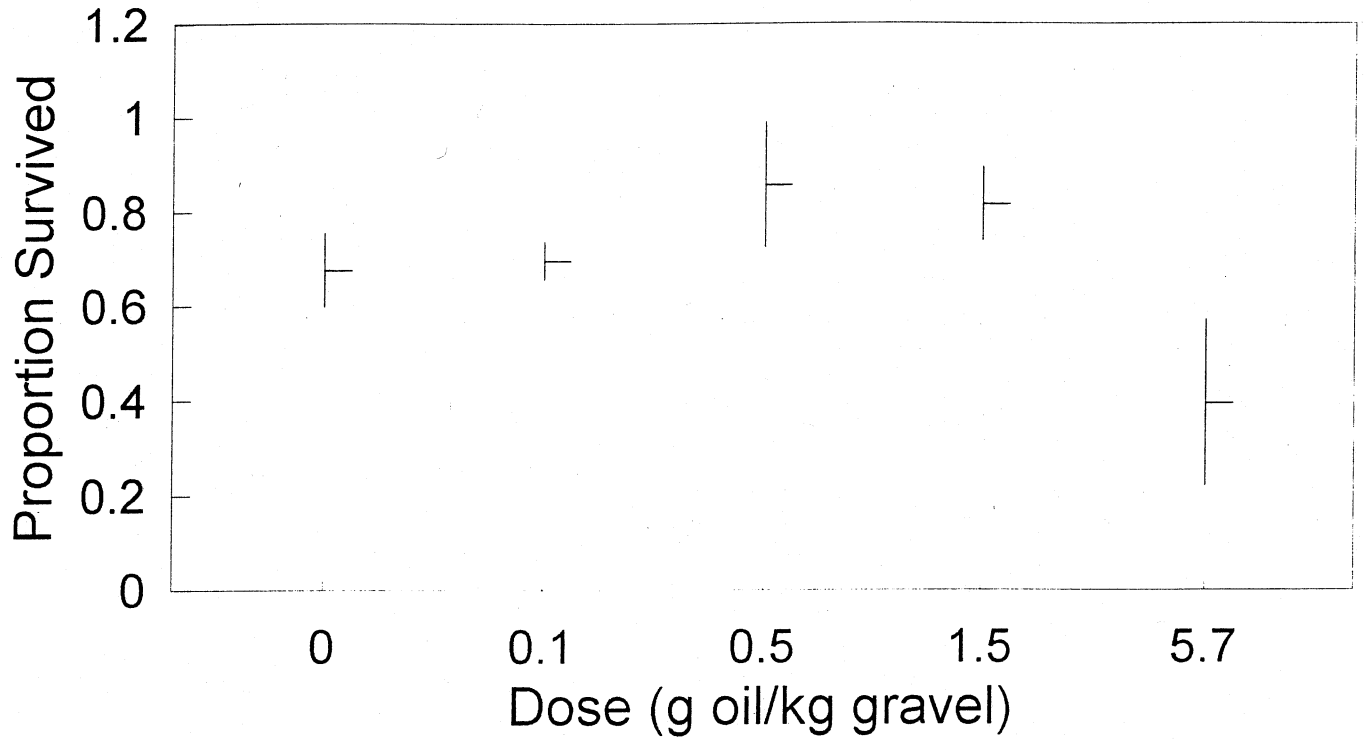


Figure 2b: 95% confidence intervals for mean survival from October 1993 to April 1994 of pink salmon exposed to oiled substrate.

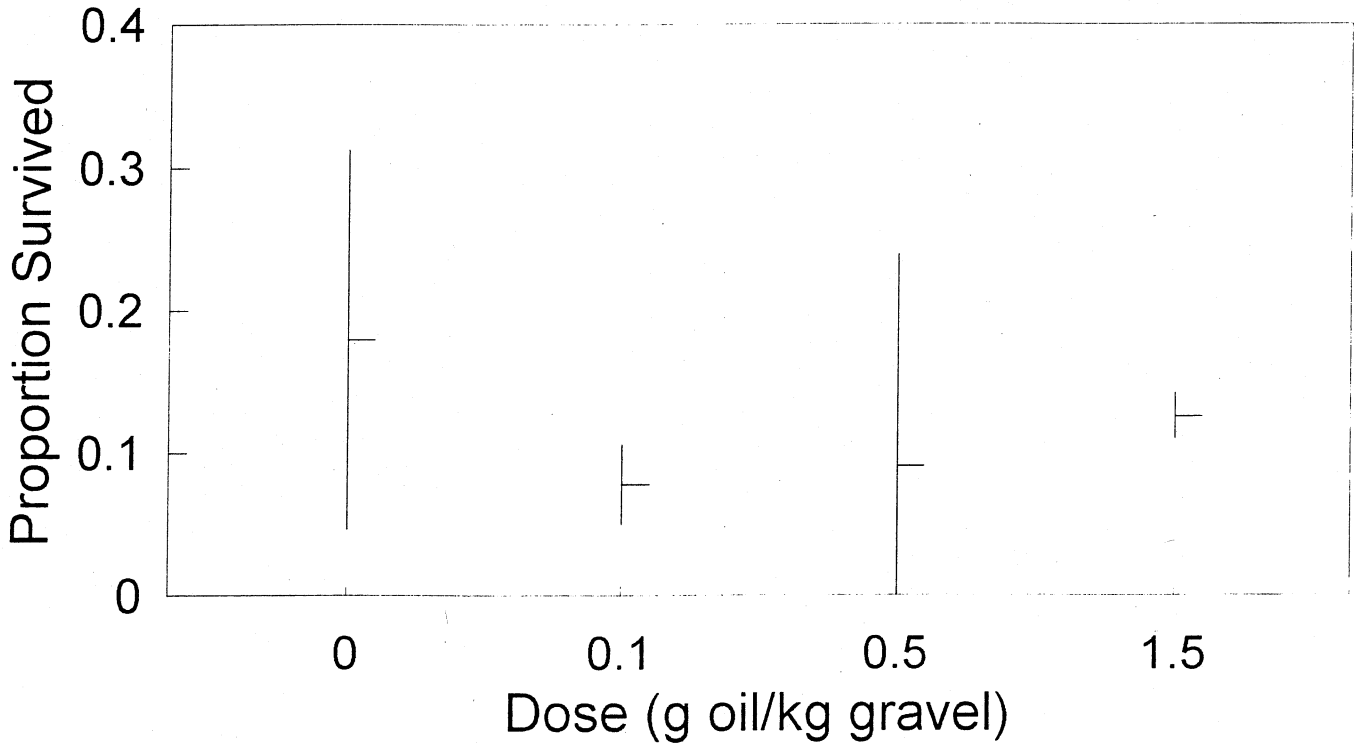
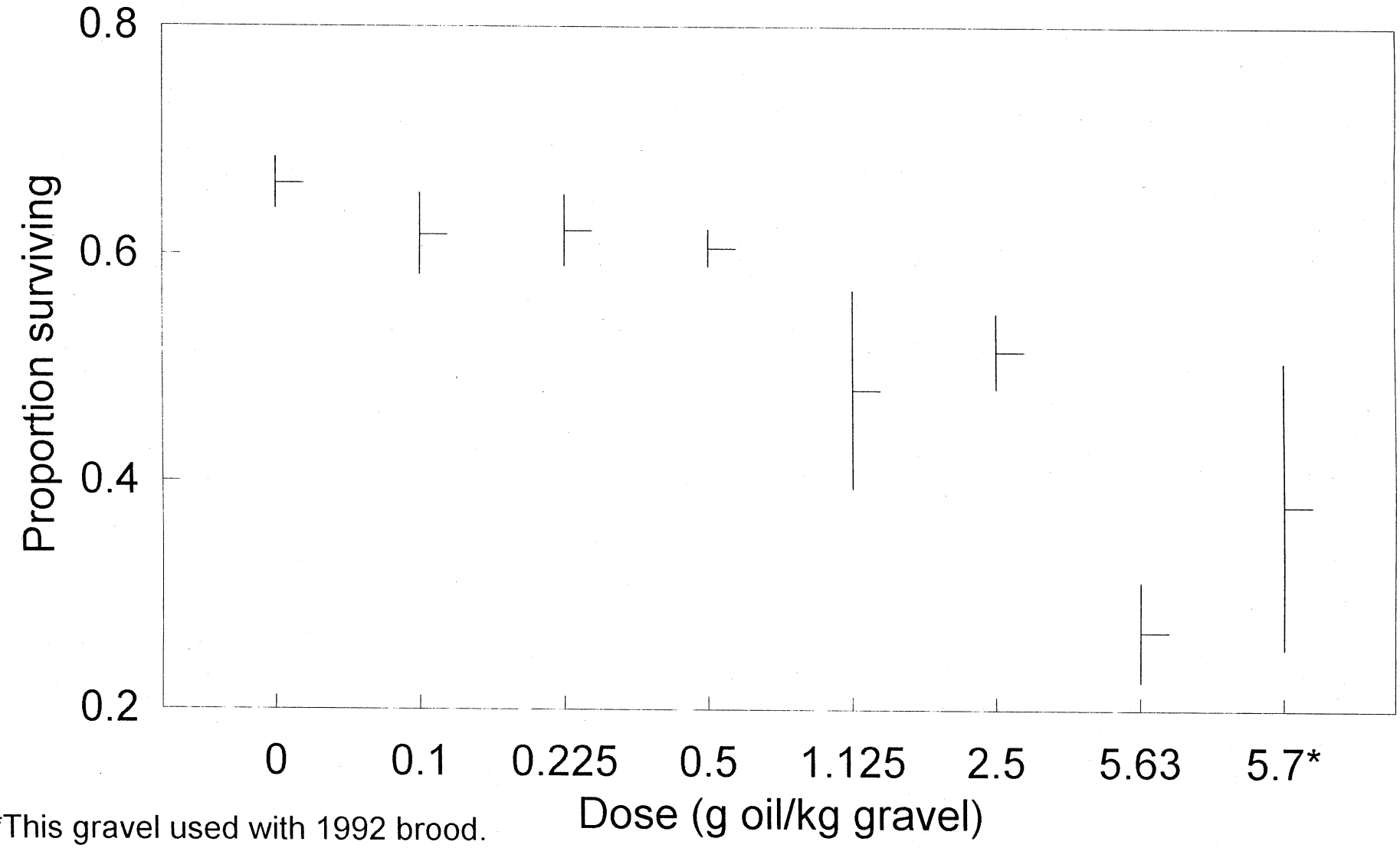


Figure 3: Survival from fertilization to emergence of 1993 brood pink salmon exposed to different doses of crude oil.



*This gravel used with 1992 brood.

Figure 4: Average number of degree days required for emergence by 1993 brood pink salmon exposed to different doses of oil.

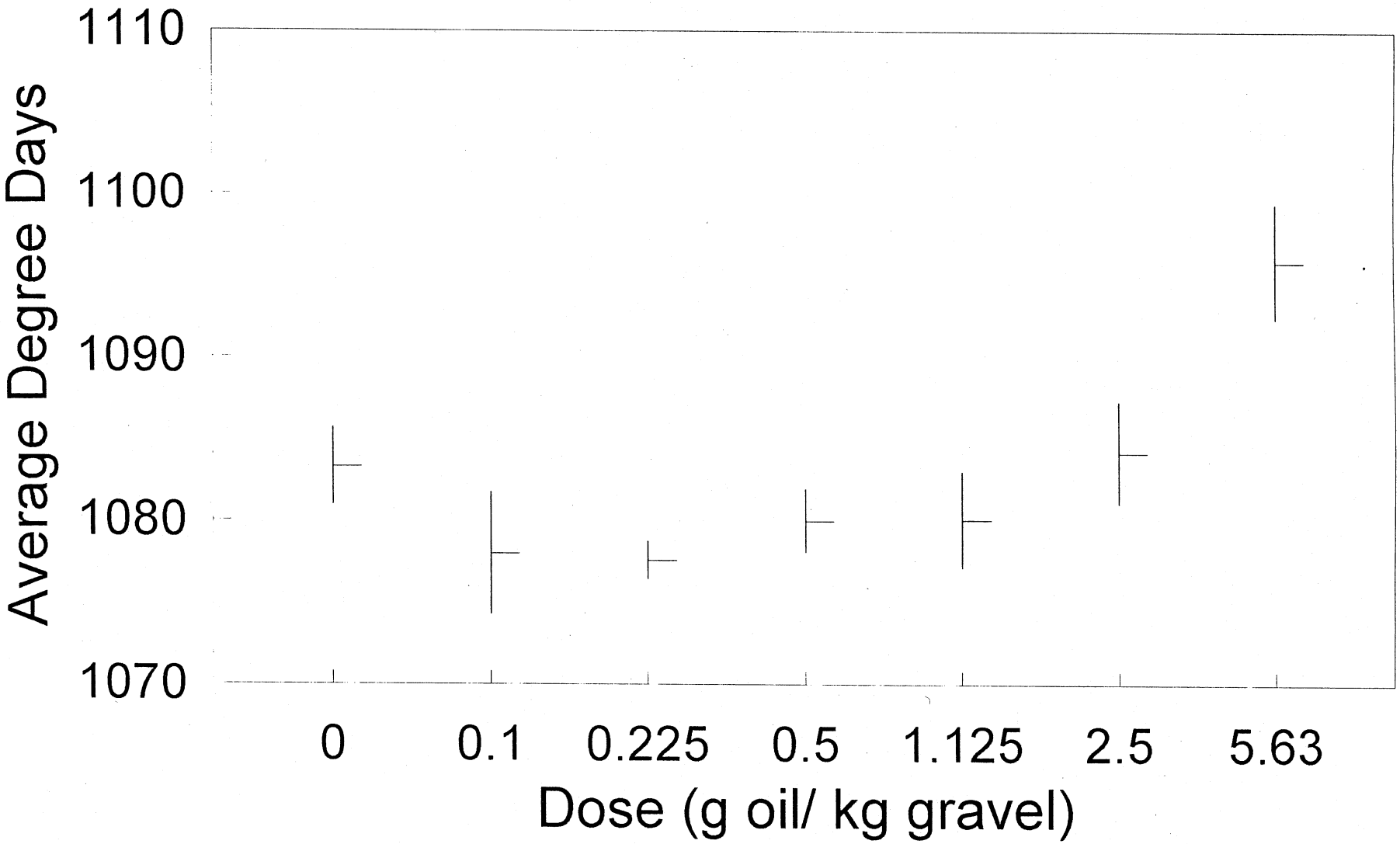
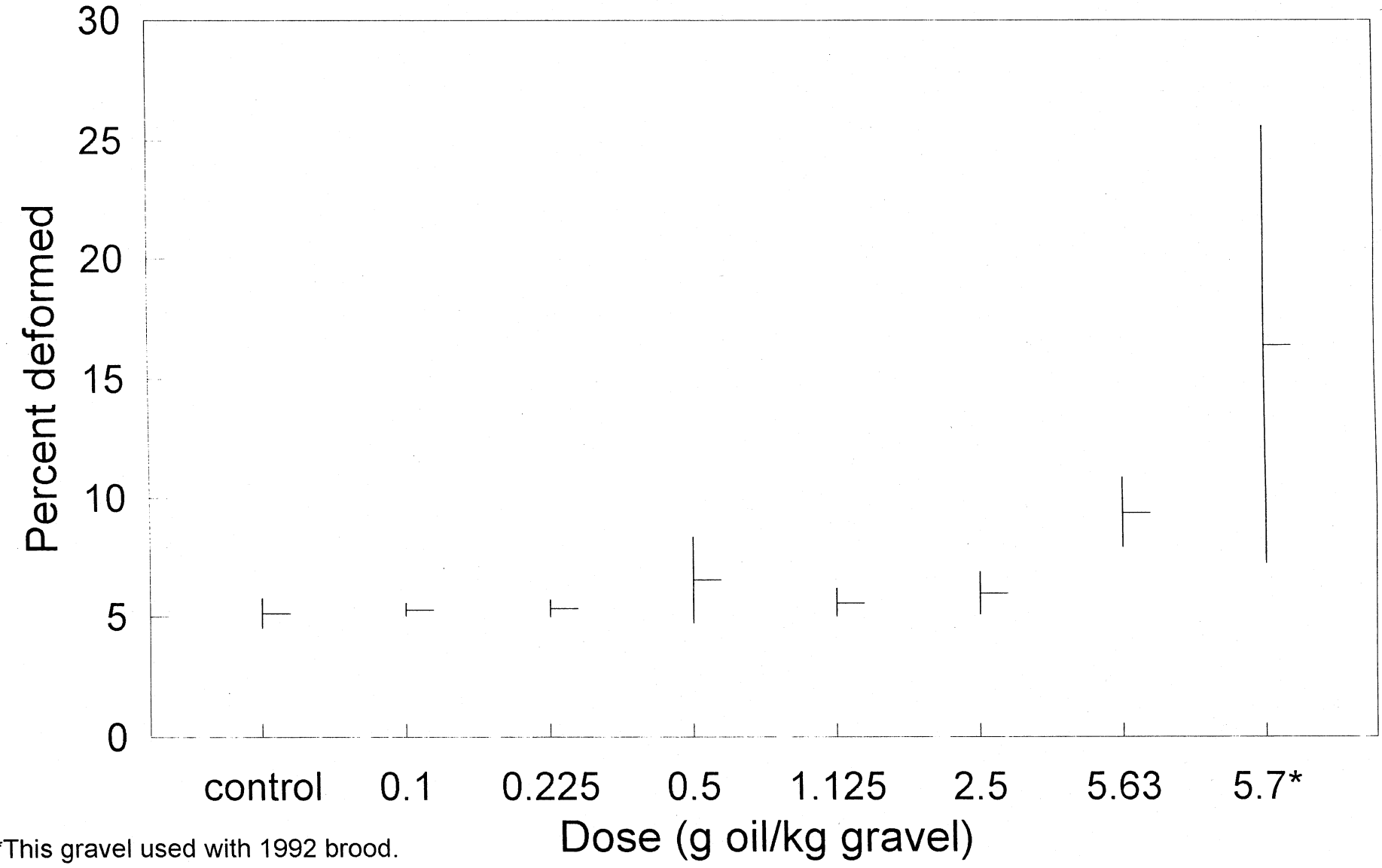


Figure 5: 95% confidence intervals for the average percent of emerging 1993 brood pink salmon displaying deformities.



*This gravel used with 1992 brood.

Figure 6: Survival from fertilization to emergence of pink salmon incubated in water exposed to oiled gravel. Lines depict 95% confidence interval.