To the graduate council:

I am submitting herewith a thesis written by Russell Joseph Bohl entitled "Negative effects of electroshocking on fish embryos: Implications for threatened and endangered fishes". I have examined the final electronic copy of this thesis and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Wildlife and Fisheries Science.

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NEGATIVE EFFECTS OF ELECTROSHOCKING ON FISH EMBRYOS: IMPLICATIONS FOR THREATENED AND ENDANGERED FISHES

A Thesis
Presented for the
Master of Science Degree
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Russell Joseph Bohl
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Abstract

The purpose of this study was to evaluate the effects of waterborne electric fields on survival of fish embryos. Embryos can be exposed to electric fields during routine electrofishing operations aimed at collecting older fish life history stages to provide data for management of fish populations. Negative effects can occur in fish embryos after electric exposure and this is a particular concern if fish are threatened or endangered species. A primary objective of this study was to develop a model to assist in the prediction of effects of electrofishing on survival of fish embryos. In this investigation, fish embryos were electroshocked over a range of developmental stages to determine the most sensitive developmental stage. The most sensitive developmental stage was then used to assess the effect of current type and electric field intensity on survival and induction of premature hatching in electroshocked embryos. Embryos were most affected by electroshock early in development, particularly near epiboly, and DC was more harmful than 60-Hz PDC. At older developmental stages, embryos were less vulnerable to electroshock-induced mortality, and, in some older developmental stages of spotfin chub *Erimonax monachus*, premature hatching was induced by electroshock. Evidence of premature hatching in other species was inconclusive. Of the species tested, rainbow trout *Oncorhynchus mykiss* were largest in diameter (4.9 mm) and most sensitive to DC electroshock (lethal voltage gradient causing 50% survival (LV-50) = 1.2 V/cm), followed by whitetail shiner *Cyprinella galactura* (1.77 mm; LV-50 = 5 V/cm) and spotfin chub (1.83 mm; LV-50 = 6 V/cm), which were similar in size and sensitivity. A strong relation between embryo diameter and vulnerability to electroshock-induced mortality was found when results from the present investigation were combined with similar results from the literature. Results support use of embryo size as a predictor of vulnerability to electroshock in other species, and indicate that species with large embryos may be particularly vulnerable to lethal effects of electrofishing.

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Chapter 1

Effects of electroshock on cyprinid embryos: Implications for threatened and endangered fishes

Abstract:

The purpose of this study was to evaluate the effects of waterborne electric fields on survival to hatching for embryos of Cyprinidae, which includes numerous species designated as threatened or endangered by the U.S. Fish and Wildlife Service (USFWS). Embryos of three cyprinids were exposed to homogeneous electric fields in the laboratory that were similar in intensity and waveform to electric fields generated during normal backpack and boat electrofishing operations. Zebrafish *Danio rerio* were electroshocked at different developmental stages from fertilization through hatching to evaluate the relationship between survival and developmental stage at the time of electroshock. The most sensitive developmental stage was determined and used to guide subsequent experiments. Embryos of two minnows native to the Southeastern U.S. (spotfin chub *Erimonax monachus* and whitetail shiner *Cyprinella galactura*) were exposed to a range of DC (3-15 V/cm) and 60-Hz PDC (8-15 V/cm) voltage gradients to evaluate the effects of electric field intensity and current type on survival to hatching of embryos. Additionally, the potential for electrofishing to induce premature hatching in late stage cyprinid embryos was investigated by exposing eyed spotfin chub embryos to 8 V/cm DC electroshock. Embryos were most vulnerable to electroshock-induced mortality early in development particularly near epiboly, and DC was more harmful than 60-Hz PDC. At older developmental stages, embryos were less vulnerable to electroshock-induced mortality, although premature hatching was induced in some older developmental stages of electroshocked spotfin chub embryos. Results indicate that cyprinid embryos can be harmed by DC electroshock at electric field intensities commonly generated by electrofishing equipment and electrofishing near spawning grounds of threatened or endangered cyprinids should be avoided when embryos are present.

Introduction:

Electrofishing equipment generates waterborne electric fields used to facilitate capture of fish from many freshwater environments. The electric field is distributed around electrodes and the intensity (voltage gradient, V/cm) decreases with distance from the electrodes (Reynolds 1996). Both efficiency of electrofishing (Miranda and Dolan 2003) and potential for fish injury to occur as a result of exposure (e.g., Henry et al. 2004) increase with increasing intensity of the waterborne electric field. The intensity of voltage gradient vectors around the anodes of electrofishing boats (20 V/cm at 5 cm; Henry et al. 2003) and backpack electrofishing units (13 V/cm at 16 cm; Habera et al. 2008) are similar and are sufficient to induce injuries in exposed fish (e.g., Henry and Grizzle 2003; Henry et al. 2004).

Negative effects of electrofishing on fish are a concern for both target and non-target species and life history stages (Snyder 2003). While it is recognized that fish populations must be sampled for effective management of fisheries and that sampling frequently involves electrofishing, it is also recognized that negative effects on fish must be reduced when possible. In most situations, electrofishing does not place unreasonable stress on fish or lead to population-level effects (McMichael et al. 1998). However, for threatened and endangered fish that reside in small streams, a large proportion of the population could be exposed to electric fields during a single electrofishing event and population level effects are possible. For threatened and endangered fish, loss of even one individual can be unacceptable and considered "take" under the Endangered Species Act (Nielsen 1998).

Fish embryos are not targets of electrofishing but can be unintentionally exposed to electric fields during routine electrofishing and negative effects on embryo survival can occur. Electroshock-induced mortality of fish embryos has been related to developmental stage at time of electroshock and the duration and characteristics of the electric field during exposure (Snyder 2003). Survival of electroshocked embryos can be improved by reducing electric field intensity (Godfrey 1957; Henry and Grizzle 2004) and frequency of pulsed electric currents (Muth and Rupert 1997). Results of previous studies indicate that significant differences in the susceptibility of fish embryos to

electroshock-induced mortality exist among species (Henry and Grizzle 2004); however, comparisons among studies are difficult because of differences in experimental design. Embryos of numerous species can be exposed during electrofishing and effects on some species are particularly important because these species may be threatened or endangered. Cyprinidae is the largest family of fish (Helfman et al. 1997) and this group includes numerous threatened and endangered species (e.g. spotfin chub, Cape Fear shiner, blue shiner, slender chub), but currently there is no information on the effects of electroshock on cyprinid embryo survival.

Effects of electroshock on embryos vary with age at time of exposure. For all species considered, mortality was highest for embryos electroshocked early in development, and the developmental period near epiboly (the period when the blastoderm overgrows the yolk; Warga and Kimmel 1990) has been identified as a particularly sensitive stage in embryonic development (Godfrey 1957; Dwyer et al. 1993; Muth and Rupert 1997; Henry and Grizzle 2004). While survival of embryos improves as development progresses, electroshock-induced premature hatching has been reported for late stage embryos in a centrarchid (bluegill *Lepomois macrochirus*; Henry and Grizzle 2004) and in two salmonids (*Coregonus albula* and *C. lavaretus*; Luczynski and Kolman 1987). Electroshock-induced premature hatching has not been investigated previously for other fish species.

The objectives of this research were to (1) determine if survival of electroshocked embryos varies among three species of cyprinids; (2) identify the electric current types that reduce embryo survival for these species; (3) determine the most vulnerable developmental stages; and (4) to evaluate premature hatching following electroshock. Spotfin chub *Erimonax monachus* (listed as threatened by the U.S. Fish and Wildlife Service-USFWS) and whitetail shiner *Cyprinella galactura* were selected because they are cyprinids native to the southeastern U.S. that spawn readily in captivity and can be used as surrogates for closely related threatened and endangered cyprinids (e.g. blue shiner, *Cyprinella caerulea*, listed as threatened by the USFWS). Zebrafish *Danio rerio* were selected because they are an excellent model organism well-adapted to laboratory

conditions and considerable information is available regarding their embryonic development for comparison with other cyprinids.

Methods:

Experimental animals.—Spotfin chub and whitetail shiner embryos were obtained by spawning adult whitetail shiners and spotfin chubs at Conservation Fisheries, Inc. (CFI), in Knoxville, TN. Following techniques developed by CFI personnel, stacks of rectangular slate (whitetail shiners) or ceramic (spotfin chubs) tiles were placed in the spawning aquaria in an attempt to mimic the crevice habitat where both species spawn in the wild. Adult fish remained in spawning aquaria (510-L for whitetail shiners and 190-L for spotfin chubs) for the duration of the experiments. Spawning aquaria contained gravel substrate and recirculating de-chlorinated tap water with the following water quality characteristics: pH, 7.4-8.0; total alkalinity, 88 mg/L as $CaCO_3$; total hardness, 52 mg/L as $CaCO_3$. Because both species naturally spawn in flowing water, a submersible pump was placed into the spawning aquaria to generate water current. All available whitetail shiner adults (two male and two female fish) were spawned for experiments with whitetail shiner embryos, while 21 of the most fit (based on appearance and relative size) spotfin chub adults (ratio of males to females unknown but at least seven males present) were spawned for experiments with spotfin chub embryos. Tiles were checked for embryos every 24 h and all available embryos were collected. The fractional spawning strategy of these species made the longer spawning time necessary to collect a sufficient number of embryos for the experiments. Embryos of both species adhered to the spawning tiles, and a soft brush was used to gently detach the embryos. After collection, embryos were observed at 10X magnification using a stereomicroscope and all dead, unfertilized, or damaged embryos were discarded.

Zebrafish embryos were obtained by spawning zebrafish broodstock maintained in the Zebrafish Research Facility at the University of Tennessee in Knoxville, TN. Water for holding embryos and conducting experiments (designated as “fish water”) was prepared with purified MilliQ water (Millipore Corp., Bedford, MA) with ions added: 19 mg/L $NaHCO_3$, 1 mg/L sea salt (Instant Ocean Synthetic Seasalt, Mentor, OH), 10 mg/L

$CaSO_4$, 10 mg/L $MgSO_4$, 2 mg/L KCl. Fish water had the following characteristics: pH 7.3–7.9; dissolved oxygen > 6 mg/L; total alkalinity, 30–40 mg/L as $CaCO_3$; and total hardness, 15–20 mg/L as $CaCO_3$. Prior to stocking fish into the 10-L spawning aquaria, a layer of marbles was added to the bottom of each aquaria in order to protect newly spawned embryos from predation by adults. Three male and four female fish were stocked into each aquaria but were kept separate using a glass partition. Fish were allowed to acclimate for 15 h, the partition was removed, and spawning commenced shortly after onset of the photoperiod. Spawning was allowed to occur for no more than 15 min to ensure that all embryos collected were at similar developmental stages. Adult fish were removed from spawning aquaria and newly-spawned zebrafish embryos were collected from the spawning substrate. The marbles and embryos were poured into an aluminum mesh basket, which was then submerged in fish water and gently agitated to rinse the embryos from the marbles. Embryos were rinsed with clean fish water and all dead embryos were discarded.

Electric fields.—Homogeneous electric fields were generated in the laboratory with an electrofishing pulse box (TEG-10 Proto 1, Coffelt, Flagstaff, Arizona, U.S.A.) modified to be powered by 110 V AC and to produce direct current (DC) or square-pulsed direct current (PDC) electric fields (Figure 1.1a)[1]. The electric fields tested included DC and 60-Hz PDC (pulse width, 3 ms; frequency, 60-Hz) over a range of voltage gradients (0-16 V/cm). Voltage and waveform were confirmed using a voltage gradient probe connected to a digital oscilloscope (THS 720A; Tectronix, Beaverton, Oregon). Embryos were exposed to electric fields in plastic troughs (38.1 cm x 7.7 cm, and 5.1 cm deep) that contained 1 L of water. Electric fields were transferred into exposure troughs by aluminum plate electrodes fixed at 30 cm apart and conformed to the cross-sectional area of the exposure troughs (Figure 1.1b). All electric equipment has been used in previously published electrofishing injury studies on early life history stages of fish (Henry and Grizzle 2003; Henry and Grizzle 2004; Henry and Grizzle 2006), including embryos (Henry and Grizzle 2004). Ambient water conductivity ranged from

[1] All figures and tables are located in the appendix, beginning on page 32

92-108 μS/cm for all experiments conducted with spotfin chub and whitetail shiner embryos and was 40-50 μS/cm for the experiments with zebrafish embryos.

Experimental design.— To avoid natural variation in percent hatch between different spawning events within a species, each experiment was conducted with embryos collected at the same time (i.e. from the same 24-48 h spawning period). An exception was results from experiments conducted with spotfin chub embryos from two separate spawning periods (i.e. embryos collected on different days) that were analyzed together because control survival was similar and results from the separate spawns did not differ significantly (see statistics below). All experiments included three separate, randomly selected troughs as unshocked controls. Un-energized electrodes were placed in each control trough for 20 s at each exposure period, ensuring that differences in survival between treatment and control groups were due to electroshock and not linked to any physical disturbance associated with placing electrodes into the troughs. Treatment troughs were exposed to electric fields at the same time (±15 min); except for the zebrafish experiment, which had time of exposure as the independent variable; and one spotfin chub experiment, which evaluated electroshock-induced premature hatching at two separate time periods. Embryos were monitored daily following stocking and any dead embryos were removed. Survival of embryos was calculated by counting the number of embryos that survived through hatching and dividing by the number initially stocked into the trough. Prior to each exposure period, a random sample of embryos was preserved in 10% neutral buffered formalin for later examination to establish the developmental stage when embryos were electroshocked. All embryos were observed under a stereomicroscope and developmental stage was described following Kimmel et al. (1995) for zebrafish embryos and a staging table appropriate for these experiments was developed for whitetail shiner and spotifn chub embryos (Table 1.1).

The impact of developmental stage on survival of electroshocked cyprinid embryos was investigated using zebrafish embryos. Embryos were collected and stocked into exposure troughs by 0.3 h post-fertilization. No flow-through was provided for zebrafish embryos, and water temperature and ambient water conductivity ranged from 26-28˚C and 40-50 μS/cm, respectively. Each trough containing embryos was randomly

assigned a time of electroshock or designated as an unshocked control. At the beginning of the experiment, each treatment group contained 35 embryos in each trough and each control trough contained 25 embryos. Embryos were exposed once to 20 s of DC electroshock (16 V/cm) from 0.3-24 h post-fertilization. Exposure duration of 20 s was selected to ensure that data from the present study would be comparable to results from Henry and Grizzle (2004).

The impact of voltage gradient on survival to hatching was investigated for late and early stage spotfin chub and whitetail shiner embryos. After being stocked into troughs (20-25 whitetail shiner embryos per trough, 9-15 spotfin chub embryos per trough), embryos were exposed to 20 s of DC electroshock ranging in intensity from 3-15 V/cm. All but one experiment conducted with early stage spotfin chub embryos contained embryos from a single spawn. All experiments with whitetail shiners and all other spotfin chub experiments were conducted with embryos from a single spawn. Exposure troughs were provided with flow-through water and water temperature and conductivity ranged from 24-26˚C and 92-108 μS/cm for these and all subsequent experiments.

Spotfin chub and whitetail shiner embryos were used to investigate effects of DC and 60-Hz PDC electroshock on survival to hatching of cyprinid embryos. Embryos were stocked into exposure troughs and exposed to 20 s of DC or PDC electroshock. For spotfin chub embryos, each trough contained 20 embryos and was exposed to a different voltage gradient of DC or 60-Hz PDC electroshock (8-15 V/cm). For whitetail shiner embryos, each exposure trough was exposed to 10 V/cm DC or 60-Hz PDC electroshock. Three replicate troughs were used for both treatment groups, and each trough contained 25 embryos. The difference in experimental design between species was due to the presence of all blastula stage embryos available for the whitetail shiner experiment, which allowed the selection of a DC voltage gradient (10 V/cm) that had previously been shown to reduce survival of whitetail shiner embryos (see results for the experiment described above). Spotfin chub embryos were from a range of developmental stages, and it was necessary to use a range of voltage gradients to determine differences between DC and PDC.

Susceptibility of spotfin chub embryos to electroshock-induced premature hatching was evaluated. Eight embryos were stocked into each trough and treatment embryos were exposed for 20 s to 8 V/cm DC electroshock at two time periods (1-2 d post-fertilization and 5-6 d post-fertilization; 3 troughs each). Embryos were checked every 24 h and the number hatched was divided by the number initially stocked to obtain percent hatch.

A summary of experimental design for the above experiments is located in Table 1.2.

Statistics.—Statistical analyses were conducted using Statistical Analysis Software (SAS Institute, Cary, North Carolina). For the experiment evaluating differences between the effects of DC and PDC on survival of whitetail shiner embryos, survival data were arcsine transformed (Zar 1984) and homogeneity of variance was assessed by Levene's test prior to performing analysis of variance. Tukey's test was used to find significant differences between groups. For all other experiments, changes in survival as a function of developmental stage or voltage gradient were modeled by logistic regression. The model was

$$\text{logit}(p) = a + bx$$

where logit(p) was the probability of a fish surviving; a was the intercept value; b was a parameter estimate; and x was either time of electroshock (h post-fertilization) or electric field intensity (V/cm). The estimate of logit(p) was used to obtain the predicted probability of embryo survival (p):

$$p = e^{\text{logit}(p)}/(1 + e^{\text{logit}(p)})$$

Model selection was based on the likelihood ratio test, and effects were included in the model if they significantly improved the predictive ability of the model. Voltage gradients that reduced survival to 50% (LV-50 values) were calculated for rainbow trout embryos exposed to DC and PDC using the above equation and finding the value of x that corresponded to 50% survival. For all analyses and model selection, differences were considered significant if $P \leq 0.05$.

Results:

Control survival among all species and experiments ranged from 74-93%. Dead embryos were identifiable by a cloudy appearance which generally became apparent <1 h following electroshock, as opposed to the transparent chorion present in developing embryos. All surviving embryos that were electroshocked within 48 h post-fertilization hatched at the same time as unshocked controls. No deformities of hatched larvae were observed in electroshock treatments or controls. Embryos from late developmental stages moved rapidly inside the chorion during the first 5-10 s during electroshock with DC or 60-Hz PDC, and movements returned to pre-shock levels within 5 min following exposure. Spotfin chub and whitetail shiner embryos used in these experiments ranged in developmental stage from the four cell stage during early cleavage through hatching.

Survival of cyprinid embryos was lowest when electroshock occurred at early developmental stages and increased when embryos were exposed at later developmental stages. Prior to 5 h post-fertilization there was considerable variability in survival of electroshocked zebrafish embryos, and survival ranged from 4-40%. Survival of zebrafish embryos electroshocked after 6 h post-fertilization increased and reached levels similar to control survival by 9-12 h post-fertilization. Survival was >70% for embryos electroshocked after 8 h post-fertilization (Figure 1.2). Hatching of zebrafish embryos occurred between 60 and 72 h post-fertilization. Survival to hatching of electroshocked whitetail shiner and spotfin chub embryos was lower for experiments that contained embryos only from the blastula period of development than when various developmental stages were present (i.e. when there were embryos from late epiboly, tail free, or eyed stages present; Figure 1.3). Early stage (0-24 h post-fertilization) spotfin chub embryos used in this experiment were from two spawns, but because there was no significant difference between the logistic models generated when each spawn was analyzed separately ($df = 1$, $\chi^2 = 1.56$, $P = 0.21$), the data were combined (Figure 1.3). Spotfin chub embryos exposed early in development had an LV-50 value of 6 V/cm, and whitetail shiner embryos exposed early in development (0-24 h post-fertilization) had an LV-50 value of 5 V/cm. There was a significant difference ($P \leq 0.05$) between the models generated to predict survival of spotfin chub and whitetail shiner embryos when

exposed to a similar range of voltage gradients during the first 24 h of development. For electroshocked spotfin chub embryos from a range of developmental stages (1/3 blastula, 1/3 tail free, 1/3 eyed), survival to hatching was related to voltage gradient, and was significantly greater than for early stage embryos exposed during the blastula developmental stage (Figure 1.3). For electroshocked whitetail shiner embryos from two developmental periods (85% blastula, 15% epiboly), survival to hatching was related to voltage gradient, and was significantly greater than for early stage whitetail shiner embryos exposed during the blastula developmental period (Figure 1.3).

Survival of spotfin chub and whitetail shiner embryos was significantly lower when exposed to DC electroshock than 60-Hz PDC electroshock (Figure 1.4). Spotfin chub embryos used in these experiments were from a range of developmental stages (40% blastula, 30% tail free, 30% eyed). Survival was not related to voltage gradient and was significantly higher for spotfin chub embryos exposed to 60-Hz PDC than those exposed to DC over the same range of voltage gradients (8-15 V/cm). Whitetail shiner embryos exposed to DC (10 V/cm) early in development had significantly lower survival than embryos exposed to 60-Hz PDC (10 V/cm) and unshocked control embryos, which were not significantly different ($F=1.66$, $P=0.27$).

Spotfin chub embryos hatched early when exposed to DC electroshock at approximately 5-6 d post-fertilization. These embryos were electroshocked with 8 V/cm DC at 5-6 d post-fertilization, and hatched within 30 min of the exposure, 48 h earlier than both control embryos and those electroshocked at 1-2 d post-fertilization, which hatched at 7-8 d post-fertilization. All embryos used in this experiment survived until hatching.

Discussion:

Zebrafish embryos were a useful model for investigating the effect of developmental stage on survival to hatching for electroshocked cyprinid embryos. Survival to hatching was related to developmental stage, and was lowest when embryos were electroshocked early in development. Survival improved as development progressed, which is consistent with results of investigations on embryos of other species

(Godfrey 1957; Dwyer et al. 1993; Muth and Rupert 1997; Henry and Grizzle 2004). The developmental period most susceptible to electroshock included early epiboly in zebrafish, which is consistent with other studies (Muth and Rupert 1997; Henry and Grizzle 2004). However, previous investigations have reported high survival (similar to control survival) of embryos electroshocked at some developmental stages prior to the stage of peak sensitivity (Dwyer et al. 1993; Cho et al. 2002). In zebrafish, there was considerable variability in survival when exposure occurred before epiboly, and only 4-40% of embryos survived when electroshock occurred during these earlier stages. Survival of zebrafish embryos did not approach control levels until epiboly was nearly completed. The differences between our results and those of Dwyer et al. (1993) and Cho et al. (2002) could be related to species differences or differences between the electric fields to which embryos were exposed, as both of those studies tested effects of PDC electroshock on salmonid embryos, whereas the present study tested DC electroshock on embryos of a cyprinid.

Experiments to determine the embryonic stage most susceptible to electroshock-induced mortality were focused on the first 12 h post-fertilization (fertilization through epiboly) to attempt to better define the stage of highest sensitivity No other study has electroshocked embryos at as many early developmental stages as the present study. However, the variance in sensitivity during the first 12 h of development prevented precise identification of the most sensitive developmental stage. Zebrafish embryos develop rapidly (hatching occurred between 60 and 72 h post-fertilization; 28°C), and the variability observed early in development could be due in part to differences in developmental stage among individual embryos that were not detected in the staging process. Despite the variability of embryo sensitivity, it was clear that zebrafish embryos were most sensitive to DC electroshock early in development and that early epiboly was a particularly sensitive developmental stage.

The effect of developmental stage on survival of electroshocked embryos was difficult to determine precisely for spotfin chub and whitetail shiner because both species spawn fractionally and obtaining embryos all at the same stage of development was not possible. In experiments with these species, the mixture of developmental stages of

embryos was determined by examining a sample of the embryos preserved at the time electroshock occurred. While this approach allowed the stages of exposed embryos to be determined, it did not enable determination of which of the stages represented in the sample had higher mortality. However, survival of spotfin chub and whitetail shiner embryos exposed to a range of DC voltage gradients was related to age at time of exposure, indicated by differences in survival between experiments containing embryos from different developmental stages. For experiments where embryos of spotfin chubs and whitetail shiners were exposed to DC voltage gradients, survival was higher when there were embryos from later developmental stages (late epiboly, tail free or eyed) present in the exposure troughs. Because of the fractional spawning strategy of the native cyprinids tested in this study, there are likely to be a range of developmental stages present in the nests of these species, increasing the likelihood that both sensitive and insensitive stages will be exposed to electric fields during electrofishing.

Voltage gradient of DC was inversely related to survival of whitetail shiner and spotfin chub embryos. The highest voltage gradients tested in these studies were within the range commonly produced by electrofishing equipment (Henry et al. 2003; Habera et al. 2008), and 0% of spotfin chub and whitetail shiner embryos survived 10 V/cm DC electroshock when exposed early in development (cleavage or blastula period). Direct current voltage gradients have previously been shown to reduce survival of exposed fish embryos (Dwyer and Erdahl 1995; Henry and Grizzle 2004). Henry and Grizzle (2004) exposed embryos of three warmwater fishes to DC voltage gradients similar to those tested in the present study and found that survival decreased with electric field intensity, and that level of response differed among species.

Survival of whitetail shiner and spotfin chub embryos was not reduced when exposed to 60-Hz PDC electroshock over the range of voltage gradients tested in the present study. Whitetail shiner embryos were exposed early in development (the blastula period) to 10 V/cm 60-Hz PDC, and spotfin chub embryos were from a range of developmental stages (30% eyed, 30% tail free, 40% blastula) when exposed to 8-15 V/cm 60-Hz PDC. Other studies have also indicated that PDC has less impact on survival of embryos than DC (Dwyer and Erdahl 1995; Henry and Grizzle 2004).

Although embryos in this study were not affected by PDC voltage gradients as high as 15 V/cm, Muth and Rupert (1997) found that survival of razorback sucker (*Xyrauchen texanus*) embryos was reduced following exposure to 1.2 V/cm PDC (650 µS/cm) of various pulse frequencies (including 60-Hz), indicating that PDC can reduce survival of embryos in some species.

Whitetail shiner and spotfin chub embryos responded similarly to DC electroshock, with LV-50 values of 5 and 6 V/cm, respectively. There was a small but significant difference between the logistic models generated for spotfin chub embryos and whitetail shiner embryos exposed to similar DC voltage gradients early in development (between 0 and 24 h post-fertilization), with whitetail shiner embryos being more sensitive than spotfin chub embryos. Although differences in sensitivity among embryos of different species have been reported when exposed to DC electroshock (Henry and Grizzle 2004), considerable similarities exist between whitetail shiners and spotfin chubs (embryo development times, embryo sizes, spawning strategies), and it is unlikely that the difference in survival reported in the present study indicates a difference in sensitivity between species. The difference is more likely due to slight differences in developmental stage when embryos were electroshocked. Differences in developmental stage between species could not be estimated precisely due to the small number of spotfin chub embryos (9) that were preserved for staging. However, it was evident that two different developmental periods were present in the preserved sample of spotfin chub embryos (5/9 embryos were from the cleavage period; 4/9 blastula), while all 36 preserved whitetail shiner embryos were from the blastula period, indicating that there were some differences in developmental stage between the experiments being compared.

Whitetail shiners and spotfin chubs deposit their eggs into natural crevices in bedrock and boulder for protection from predators (Etnier and Starnes 1993), and developing embryos might receive protection from the full intensity of electrofishing fields because of this spawning adaptation. Godfrey (1957) found that buried (8 cm of gravel) Atlantic salmon embryos exposed during pre-cleavage had significantly higher survival than embryos left uncovered when exposed to similar DC electric fields, but voltage gradient was not reported in that study. Dwyer et al. (1993) reported that

significant mortality occurred when cutthroat trout eggs in artificial redds (buried under 15 cm of gravel) were exposed to 340 V of 250-Hz PDC, and that electric field intensity was similar between artificial redds and exposure chambers (1 V/cm). Whitetail shiners and spotfin chubs can deposit their eggs more than 7 cm into crevices between ceramic tiles during hatchery spawning, but most naturally spawning fish deposit eggs into crevices ranging from 1-3 cm deep (P.L. Rakes, CFI; personal communication). There is insufficient evidence that crevice spawning will provide any protection from electrofishing for cyprinid embryos, and perhaps embryos in crevices are more vulnerable if the electric field is concentrated in the aqueous medium.

Sublethal effects of electrofishing can occur in posthatching life history stages of fish, but have rarely been reported for fish embryos. Embryos in the present study that survived through hatching did not have gross external deformities, and this observation is consistent with other studies that have reared electroshocked embryos through hatching (Muth and Rupert 1997; Henry and Grizzle 2004). In the present study, premature hatching was induced in spotfin chub embryos exposed to electroshock on day 5 post-fertilization, and this result is consistent with previous observations of premature hatching (Luczynski and Kolman 1987; Henry and Grizzle 2004). The observations of Henry and Grizzle (2004), in electroshock experiments with bluegill embryos, were consistent with release of enzymes from hatching glands within 30 min of electroshock. Premature hatching did not directly impact survival in their laboratory experiment, but they suggested that increased predation risk could result from premature hatching. The effect on survival after premature hatching should be investigated before it is concluded that later stages of embryonic development are less vulnerable to consequences of electroshock than early stages.

Results from the present study indicate that electrofishing in the spawning habitat of threatened and endangered cyprinids can reduce embryo survival if sensitive stages are present and DC electric fields are used. Direct current has been suggested as a means of minimizing the negative effects of electrofishing (Reynolds 1996); however, based on the results of the present study and others (Dwyer et al. 1993; Henry and Grizzle 2004), use of DC should be avoided when fish embryos may be present. In light of the results from

this research, it is recommended that electrofishing not be conducted when embryos of threatened or endangered cyprinids are known to be present in a sampling location. The spawning strategies of some cyprinids (e.g., nesting and aggregating to spawn) could lead to exposure of a larger proportion of the population and an increase in the likelihood of population-level effects from electrofishing.

Chapter 2

Vulnerability of fish embryos to electroshock-induced mortality is a function of embryo size

Abstract:

Principles guiding the use of electrofishing to reduce fish injury are limited and presently based on information obtained from numerous separate studies that are difficult to compare and apply to actual electrofishing conditions. The purpose of this study was to develop a model to predict vulnerability of fish embryos to electroshock-induced mortality based on results from the present study and previously published results that used consistent methodology. Fish embryos can be unintentionally exposed to electric fields during routine electrofishing operations aimed at collecting older life history stages, and survival can be reduced under some conditions. In this investigation, sauger *Sander canadensis* and rainbow trout *Oncorhynchus mykiss* were exposed to homogeneous electric fields in the laboratory that were similar in intensity and waveform to those produced by electrofishing equipment. Rainbow trout were exposed to 2 V/cm and 3 V/cm DC electroshock over a range of developmental stages from pre-cleavage through hatching to determine the most sensitive developmental stage. Embryos from this stage were then used to test the effect of DC and 60-Hz PDC voltage gradients on survival of rainbow trout embryos. Sauger embryos were similarly exposed to a range of DC and 60-Hz PDC voltage gradients during early epiboly. Rainbow trout eggs were most sensitive to electroshock early in development and were particularly susceptible to electroshock-induced mortality during early epiboly. Based on results for these species and previously published results in other species (total of eight species), embryo size (diameter) was positively related ($P = 0.0005$, $R^2 = 0.87$) to vulnerability to electroshock-induced mortality. This relation suggests that the interaction of electric fields with fish embryos may be a function of physical characteristics of the embryo, which is consistent with observations of electrical injury in cells that indicate larger cells are most vulnerable. A strong relation between fish embryo size and vulnerability to electroshock-induced mortality indicates that, with knowledge of embryo size, fisheries biologists may

be able to predict the vulnerability of species of concern and modify electrofishing procedures to reduce negative effects.

Introduction:

Electric fields can negatively affect organisms and the extent of injury produced upon exposure is related to intensity and type of the electric field. Freshwater fish are deliberately exposed to electric fields during electrofishing, which is conducted to sample fish from the environment, and negative effects on fish survival can occur. Despite significant efforts to understand the effects of electric fields on fish and recommendations on how to reduce injury (reviewed in Snyder 2003), fish injury can still occur during electrofishing. Reducing the electric field intensity, reducing frequency of pulsed direct current (PDC), or use of DC have been suggested to minimize fish injury (Reynolds 1996); however, less understanding on effects in different fish species and life history stages presents limitations to these recommendations. The recommendation to use DC to minimize injury (Reynolds 1996) in older life stages does not apply for fish embryos as evidence indicates they are more vulnerable to electroshock-induced injury from DC than PDC (Dwyer and Erdahl 1995; Henry and Grizzle 2004). Testing the vulnerability of all potentially exposed fish species and life history stages is not feasible, but predictive models based on results from key species and life stages should be developed to improve guidance of electrofishing operations.

Fish embryos can be unintentionally exposed to electric fields during electrofishing operations, but no unifying principle exists to predict embryo sensitivity to electric fields. Some studies have concluded that electroshock has little impact on embryos (Roach 1999; Cho et al. 2002), but others have indicated that embryo survival can be reduced significantly following electroshock (Dwyer and Erdahl 1995; Muth and Rupert 1997; Henry and Grizzle 2004). Fish embryos can be more vulnerable to electroshock early in development, and epiboly (when the blastoderm begins to overgrow the yolk; Warga and Kimmel 1990) has been identified as a particularly sensitive developmental stage (Muth and Rupert 1997; Roach 1999; Henry and Grizzle 2004; Bohl et al. 2008). Both DC and pulsed PDC electroshock can decrease survival of fish

embryos, but DC reduces survival of exposed embryos more than PDC (Dwyer and Erdahl 1995; Henry and Grizzle 2004). To minimize impacts of electrofishing on embryos, reducing electric field intensity (Godfrey 1957; Henry and Grizzle 2004) and frequency of pulsed electric currents (Muth and Rupert 1997) are recommended.

Differences in sensitivity to electroshock among species are not well understood for fish embryos, and most electrofishing injury studies are difficult to compare because of differences in experimental design. Henry and Grizzle (2004) electroshocked embryos of three warmwater species and noted that differences in sensitivity were consistent with differences in embryo size: catfish (*Ictalurus punctatus*) embryos were larger and more sensitive to electroshock than largemouth bass (*Micropterus salmoides*) embryos, which were larger and more sensitive to electroshock than bluegill (*Lepomis macrochirus*) embryos. Results from Chapter 1 of this thesis indicated that of the three cyprinid species tested, the smaller zebrafish (*Danio rerio*) embryos were less sensitive to DC electroshock than spotfin chub (*Erimonax monachus*) and whitetail shiner (*Cyprinella galactura*) embryos, which were similar in size and sensitivity to electroshock. Developing a model to predict sensitivity of embryos to electroshock based on embryo characteristics (such as size) would enable fisheries managers to better understand the potential for electrofishing to reduce embryo survival in species that have not been previously tested in an electrofishing injury study.

The objectives of this research were to identify differences in sensitivity to electroshock between embryos of two fish species, and to develop predictive models of fish embryo vulnerability to electroshock based on the present results and comparable results from other studies. Experiments were conducted with embryos of rainbow trout *Oncorhynchus mykiss* and sauger *Sander canadensis*, and these data were combined with published results for embryos of the following species to develop the models: largemouth bass, bluegill, and channel catfish (DC only; Henry and Grizzle 2004); spotfin chub, whitetail shiner, and zebrafish (DC only; Chapter 1); cutthroat trout *Oncorhynchus clarki* (PDC only; Dwyer and Erdahl 1995); and razorback sucker *Xyrauchen texanus* (PDC only; Muth and Rupert 1997).

Methods:

Experimental animals.—Rainbow trout embryos from three strains (Arlee, Eagle Lake, and Fish Lake) were used in these experiments, and were obtained from the Erwin National Fish Hatchery in Erwin, TN. Adult broodstock were strip spawned by hatchery personnel, and all embryos used in a single experiment were fertilized within 1 h. Following fertilization, embryos were allowed to water harden for 1.5 h before being transported to the Joe Johnson Agricultural Research and Teaching Facility (JARTU) at the University of Tennessee in Knoxville, TN. Prior to stocking into exposure troughs, all dead embryos were discarded. Remaining embryos were stocked into exposure troughs by 4 h post-fertilization, where they remained for the duration of the experiment. All experiments with rainbow trout embryos were terminated at the eyed stage. Temperature ranged from 12-13.5˚C for all experiments with rainbow trout embryos.

Sauger embryos were obtained from the Eagle Bend Hatchery in Clinton, TN. Adult broodstock were injected with human chorionic gonadotropin (HCG) and strip spawned by hatchery personnel, and all embryos were fertilized within 0.5 h. Diatomaceous earth was added to the water to inhibit embryos from sticking to each other in an attempt to inhibit spread of the water mold *Saprolegnia* (Oomycetes) among embryos. Embryos were transported to JARTU and were placed into exposure troughs by 5 h post-fertilization, where they remained for the duration of the experiment (until embryos reached the eyed stage). Water temperature ranged from 16-17˚C.

Embryos of both species were provided with flow-through, dechlorinated tap water with the following water quality characteristics: pH, 7-8; total alkalinity, 250 mg/L as CaCO3; hardness, 120 mg/L as CaCO3. Except during daily observation periods and exposure periods, troughs containing embryos were covered in order to limit direct exposure to light.

Electric Fields.—Homogeneous electric fields were generated in the laboratory with an electrofishing pulse box (TEG-10 Proto 1, Coffelt, Flagstaff, Arizona, U.S.A.) modified to be powered by 110 V AC and to produce direct current (DC) or square-pulsed direct current (PDC) electric fields. The electric fields tested included DC ranging in intensity from 0-9 V/cm and PDC (pulse width, 3 ms; frequency, 60-Hz) ranging in

intensity from 0-14 V/cm. Voltage and waveform were confirmed using a voltage gradient probe connected to a digital oscilloscope (THS 720A; Tectronix, Beaverton, Oregon). Embryos were exposed to electric fields in plastic troughs (38.1 cm x 7.7 cm, and 5.1 cm deep) that contained 1 L of water. Electric fields were transferred into exposure troughs by aluminum plate electrodes fixed at 30 cm apart that conformed to the cross-sectional area of the exposure troughs. All electric equipment has been used in previously published electrofishing injury studies on early life history stages of fish (Henry and Grizzle 2003; Henry and Grizzle 2004; Henry and Grizzle 2006), including fish embryos (Henry and Grizzle 2004). Ambient water conductivity ranged from 170-230 μS/cm for all experiments conducted with rainbow trout embryos and 230-240 μS/cm for the experiment with sauger embryos.

Experimental design.—All experiments contained 3 randomly selected troughs to serve as unshocked controls. Control and treatment embryos were handled identically, and at each exposure period, un-energized electrodes were placed in each control trough for 20 s to simulate electroshocking and ensure that differences between controls and treatments were due to electroshock and not to any physical disturbance caused by placing electrodes into the troughs. Except for the rainbow trout experiments which had developmental stage (i.e. time of electroshock; h post-fertilization) as the independent variable, all treatment troughs were exposed at the same time (±15 min). Immediately prior to each electroshock period, ten embryos were removed from the exposure trough that was to be electroshocked and preserved in 10% neutral buffered formalin to establish the developmental stage at the time of electroshock. Rainbow trout embryos were observed under a stereomicroscope and were staged according to Ballard (1973) and Kunz (2004). For all experiments, survival was calculated by dividing the number of embryos that survived until the eyed stage by the number of embryos initially stocked into the exposure troughs.

To identify the developmental period when rainbow trout embryos are most sensitive to electroshock, two experiments were conducted. The only differences between the experiments were voltage gradient and the strain of rainbow trout tested (3 V/cm, Arlee strain; and 2 V/cm, Eagle Lake strain). Embryos were stocked into the

exposure troughs (60-97 embryos per trough) as described above, and each trough was electroshocked (20 s of DC) at a different developmental stage (time post-fertilization).

One experiment was conducted to evaluate the effects of voltage gradient and current type on survival of rainbow trout embryos (Fish Lake strain) electroshocked at 150 h post-fertilization (25-50% epiboly). Embryos were stocked into the exposure troughs (61-105 embryos per trough) and exposed to a range of DC or 60-Hz PDC voltage gradients (DC, 0.5-3 V/cm; PDC, 2-8 V/cm).

The effects of current type and electric field intensity on survival of sauger embryos were evaluated in a single experiment. Sauger embryos were stocked into exposure troughs (270-760 embryos per trough), and a random sample (n =10) of embryos were removed from an unused exposure trough every 3-4 h in order to determine developmental stage. Electroshock was applied at 22-24 h post-fertilization (25-50% epiboly), and embryos were exposed for 20 s to a range of DC and 60-Hz PDC voltage gradients (1-14 V/cm).

Models were developed to test the effect of embryo size on sensitivity to electroshock for DC and 60-Hz PDC. Effect of embryo diameter on sensitivity to DC electroshock was modeled using data from the present study (Fish Lake strain rainbow trout and sauger), data reported in Chapter 1 (zebrafish, whitetail shiner, and spotfin chub), and data from Henry and Grizzle (2004; channel catfish, largemouth bass, and bluegill). Experimental design was similar among these studies (identical electric equipment and exposure duration). A separate model was developed for 60-Hz PDC using data from the present study (Fish Lake strain rainbow trout and sauger), data from Dwyer and Erdahl (1995; cutthroat trout), and data from Muth and Rupert (1997; razorback sucker). Exposure duration and pulse width were not identical between data included in the 60-Hz PDC model, and differences were as follows: the present study used 20 s and 3 ms, Dwyer and Erdahl (1995) used 10 s and approximately 8 ms, and Muth and Rupert (1997) used 10 s and 4 ms. Because water conductivity varied by study (40-235µS/cm), power density (mW/cm^3) was used as the measure of electric field intensity. For the DC model, power density that resulted in survival ≤ 30% of control survival was the dependent variable, and embryo diameter (mm) was the independent

variable. For the 60-Hz PDC model, power density that resulted in survival 25-50% of control survival was the dependent variable, and embryo diameter (mm) was the independent variable. At the most sensitive developmental stage for each species, the voltage gradient that caused survival to be reduced to the designated level (see above) of control survival was used to calculate the power density as follows:

$$D = (E^2 c)/1{,}000$$

where D was power density (mW/cm^3), E was voltage gradient (V/cm), and c was ambient water conductivity (μS/cm). Data used in developing the models is described in Table 2.1.

Statistics.—Statistical analyses were conducted using Statistical Analysis Software (SAS Institute, Cary, North Carolina). Simple linear regression was used to relate embryo diameter to power density (Log_{10} transformed). For results reported in this study, changes in survival as a function of developmental stage or voltage gradient were modeled by logistic regression. The model was

$$\text{logit}(p) = a + bx$$

for all experiments except the developmental stage experiment that used 2 V/cm DC electroshock to evaluate effect of developmental stage on survival to hatching of rainbow trout embryos, which was modeled by a quadratic equation

$$\text{logit}(p) = a + b\text{x} + cx^2$$

For both models, logit(p) was the probability of a fish surviving; a was the intercept value; b was a parameter estimate; c was a parameter estimate for the quadratic term, and x was either time of electroshock (h post-fertilization) or electric field intensity (V/cm). The estimate of logit(p) was used to obtain the predicted probability of embryo survival (p):

$$p = e^{\text{logit}(p)}/(1 + e^{\text{logit}(p)})$$

Voltage gradients that reduced survival to 50% of control survival (LV-50 values) were calculated for rainbow trout embryos exposed to DC and PDC using the above equation and finding the x value that corresponded to 50% of the control survival. Model selection was based on the likelihood ratio test, and effects were added to the model if they significantly improved the predictive ability of the model. Higher order terms were

added to the model if their addition produced an increase in the index of rank correlation (c) over the model that had only the first order term. For all analyses and model selection, differences were considered significant if $P \leq 0.05$.

Results:

Control survival among all rainbow trout experiments ranged from 65-84%, and control survival for the sauger experiment was 30% (SE=3.6%). Dead rainbow trout embryos were identifiable by a cloudy appearance, which generally became apparent in the chorion <1 h following electroshock, as opposed to the transparent chorion present in developing embryos. The chorion of dead and developing sauger embryos appeared similarly opaque throughout the experiment, and dead embryos could only be identified when visibly infected with the water mold *Saprolegnia* (Oomycetes). All surviving embryos of both species hatched at the same time as unshocked controls, and no deformities of hatched larvae were observed among electroshock treatments or controls. There was no difference in calculated survival when counted at the eyed stage or at hatching. Rainbow trout embryos from late developmental stages could be observed moving rapidly inside the chorion during the first 5-10 s during exposure to DC or 60-Hz PDC. Rainbow trout embryos electroshocked in these experiments ranged in developmental stage from pre-cleavage (prior to blastomere divisions) to the eyed stage of development, and sauger embryos were electroshocked during the first half of epiboly (24 h post-fertilization).

Survival of rainbow trout embryos was related to the developmental stage when electroshock occurred (Figure 2.1). When electroshock intensity was 2 V/cm DC, the quadratic model (c = 0.605) indicated a better fit to the data compared to the first order model (c = 0.575). Prior to 150 h post-fertilization there was considerable variability in survival (16-72%). Survival was 14% at 168 h post-fertilization and increased to 68% by 218 h post-fertilization. For all electroshock periods after 200 h post-fertilization (218-337 h post-fertilization), survival was within 10% of control survival (76%; Figure 2.1a). When electroshocked with 3 V/cm DC, a first order logistic model adequately described data (c = 0.906), and adding a quadratic term did not improve the fit of the model (c =

0.904). Survival ranged from 0-4% during the first 150 h post-fertilization, then increased to 52% by 243 h post-fertilization. Survival of embryos electroshocked after 250 h post-fertilization (266-341 h post-fertilization) ranged from 54-69% and was within 11% of control survival (65%; Figure 2.1b).

For rainbow trout embryos exposed to DC or 60-Hz PDC at 158 h post-fertilization (25-50% epiboly), voltage gradient was significantly related to survival (Figure 2.2). The LV-50 value for DC was 1.3 V/cm, and the LV-50 value for PDC was 2.6 V/cm. The logistic models generated for DC and PDC differed significantly, and control survival was 84%.

Survival of sauger embryos exposed to DC or 60-Hz PDC at 24 h post-fertilization decreased as voltage gradient increased (Figure 2.3). From 0.5-3 V/cm for DC and PDC, survival was variable and ranged from 100-160% of control survival (control survival = 30%, SE=3.6%). Survival was lower for embryos exposed to DC (1-9 V/cm) than those exposed to PDC (2-14 V/cm).

Power density that reduced embryo survival was related to embryo size for DC and 60-Hz PDC electroshock (Figure 2.4). As embryo size increased, the power density required to reduce survival of fish embryos decreased for both current types. Power density ranged from 0.7-25.6 mW/cm^3, and embryo size ranged from 1.1-5 mm.

Discussion:

Results of the present study are consistent with previous research on the susceptibility of fish embryos to electroshock. Embryos of rainbow trout were most sensitive early in embryonic development and especially during early epiboly, and were more susceptible to DC than 60-Hz PDC. Some studies have found that survival is high (similar to control survival) when embryos are electroshocked earlier than the most sensitive developmental stage (Dwyer et al. 1993; Cho et al. 2002). For early developmental stages (before epiboly) of rainbow trout in the present study, survival was low (<5%) and did not approach control survival until epiboly was nearly complete when exposed to 3 V/cm DC electroshock; when embryos were exposed to 2 V/cm DC, survival was variable and ranged from 14-72% prior to the onset of epiboly, indicating

that some developmental periods prior to peak sensitivity are sensitive to electroshock. Differences between studies are likely due to differences in the electric fields tested, as both Dwyer et al. (1993) and Cho et al. (2002) exposed embryos to 10 s of PDC, and survival was not reduced to lower than 40% at any developmental stage tested. The present study tested 20 s of DC, and survival was <15% when exposed during early epiboly (2 V/cm). Sauger embryos were not tested for stage sensitivity; however, they were tested during early epiboly (22-24 h post-fertilization) and also were found to be more susceptible to DC than PDC electric fields. The consistency of the present results with previous comparable investigations suggests that there are some common characteristics of developing embryos that result in higher vulnerability to electric fields during early developmental stages.

Control survival for sauger embryos was low (30%; SE=3.6%); however, the low variance among control troughs indicated that non-electroshock related mortality was consistent among embryos in different troughs. Immediately following fertilization, diatomaceous earth was added to the sauger embryo water to inhibit embryos from adhering to each other, giving all embryos an opaque appearance. This made it difficult to identify dead embryos until they were overgrown by the water mold *Saprolegnia* (Oomycetes), and the high embryo density present in the exposure troughs facilitated its spread between embryos and likely caused the low control survival.

The DC power density required to reduce survival to ≤ 30% of control survival was modeled with data from the present study and other similar data from other studies. Selection of this survival range was made to enable the use of all available comparable data, and it is recognized that comparing LV-50 values for each species would result in greater model precision. However, due to the difficulty in obtaining embryos and conducting experiments there was insufficient data to determine LV-50 values in all species. In bluegill embryos 0% survival was observed at 25 mW/cm^3 DC electroshock (the value used in the model), while survival did not differ from controls when exposed to 6.4 mW/cm^3 (Henry and Grizzle 2004). Thus the power density required to induce partial survival between 0% and 30% is between 6.4 and 25 mW/cm^3, and this likely explains the lack of fit of bluegill in the model. Based on the model, partial survival (i.e., between

0% and 30% survival) of bluegill embryos would be expected at 12.7 mW/cm^3 for embryos of bluegill and other species with similarly sized embryos (1.1 mm in diameter).

Sensitivity to DC electroshock was modeled as a function of embryo size for eight species (three separate studies, see methods). The studies from which data were taken were similar in experimental design (identical equipment, exposure duration, handling techniques) and electric field characteristics (DC electric fields produced and measured using identical equipment), and all data included in the model were from embryos electroshocked early in development (i.e. during peak sensitivity). Differences in water conductivity were accounted for by converting voltage gradient (V/cm) to power density (mW/cm^3) and survival was calculated as percent of control survival to account for differences in control survival among experiments. The largest and most sensitive embryos were rainbow trout (4.97 mm in diameter; 0.7 mW/cm^3 to reduce survival to $\leq$ 30%). Zebrafish embryos were smaller (1.36 mm) and required a higher power density (11.7 mW/cm^3) to reduce survival to $\leq$ 30%. Bluegill were the smallest (1.1 mm) embryos tested, and required the highest power density (25 mW/cm^3) to reduce survival to $\leq$ 30%. To test how the present model reflects the vulnerability of fish embryos from other studies, results for cutthroat trout exposed to DC during the most sensitive stage of development (Dwyer and Erdahl 1995) are included (but not used to compute the model, Figure 2.4a). The results for cutthroat trout fit the model well despite the different electroshocking equipment and experimental design (10 s as opposed to 20 s exposure duration). Unfortunately, comparable data for other species could not be obtained from the literature.

Developing a similar model for PDC was difficult because of differences in electric field characteristics (frequency, pulse width, exposure duration) tested between separate studies. The 60-Hz PDC power density required to reduce embryo survival to 25-50% of control survival was modeled as a function of embryo size for four species (three separate studies). Pulse width and exposure duration varied among the studies as follows: the present study used 20 s and 3 ms, Dwyer and Erdahl (1995) used 10 s and approximately 8 ms, and Muth and Rupert (1997) used 10 s and 4 ms. Differences in exposure duration may be of little importance, as Henry and Grizzle (2004) found that

survival was similar when embryos were electroshocked at the developmental stage of peak sensitivity, regardless of exposure duration (5, 10, or 20 s). However, effect of pulse width on embryo survival has not been adequately tested (Snyder 2003) and it is possible that the effect of embryo size for the 60-Hz PDC model is being confounded by the effect of different pulse widths between the studies. Although these differences could affect the predictive ability of the 60-Hz PDC model, it is clear that there is a relationship between embryo size and sensitivity that is similar to the relationship demonstrated for DC. Other studies that tested effects of PDC on fish embryos could not be included because they tested frequencies other than 60-Hz (Dwyer et al. 1993) or did not use electric field intensities that reduced survival to the levels included in the model (Cho et al. 2002; Roach 1999).

It is unknown what physiological response or physical damage occurs in electroshocked embryos to cause mortality. Dwyer et al. (1993) found that rainbow trout embryos were most sensitive to electroshock and physical shock (dropping embryos onto a soft plastic bumper from 15 cm) at the same developmental period (8 d at 10.4°C). Hayes (1949) suggested that mortality of salmonid embryos caused by physical shock during early stages was due to the rupture of the vitelline membrane of the yolk sac, which becomes covered by a protective layer of cells at the completion of epiboly, and (Godfrey 1957) postulated that this was also the case for electroshocked embryos. Results of the present study agree that survival is improved for embryos electroshocked after epiboly is complete, and this could be due to a protective layer of cells encompassing the yolk sac, as suggested by Godfrey (1957). It is evident from other disciplines that electric shock can cause damage to cell membranes (e.g. Gaylor et al. 1988). Electroporation is the use of electric fields to create temporary pores in biological membranes, and can be used to transport foreign substances into cells (Chen et al. 2006). When the electric field exceeds a certain intensity during electroporation of cells, the pores fail to reseal, and breakdown of the cell membrane can occur (Chen et al. 2006). Electroporation can be used to transport materials into zebrafish embryos (Cerda et al. 2006), and breakdown of the vitelline membrane may help explain the cause of electroshock-induced mortality of fish embryos. According to Gaylor et al. (1988)

transmembrane potential increases with cell radius, and this suggests that larger cells are more vulnerable to membrane rupture than smaller cells. Similar mechanisms may explain the observation that sensitivity of embryos to electroshock increases with embryo size (Henry and Grizzle 2004).

Fish embryos can be negatively impacted by electroshock, and sensitivity to electroshock-induced mortality can be related to embryo size. Based on the models developed in the present study, fisheries managers may be able to use embryo size to determine the sensitivity of fish embryos that may be present in a sampling location for species that have not been tested directly. The model presented in this investigation should be verified in future studies that use a similar experimental design and particular emphasis should be on testing species of intermediate size (2-4 mm; e.g. fantail darter *Etheostoma flabellare*, northern pike *Esox lucius*, slender madtom *Noturus exilis*) to establish that the vulnerability of these species is consistent with this model.

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Appendices

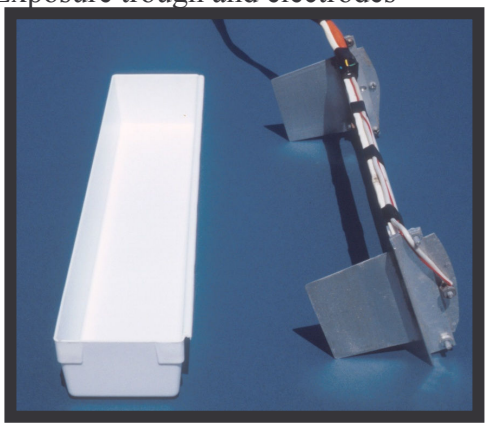
Appendix 1:

Figures and Tables, Chapter 1

a) Electrofishing pulse box



b) Exposure trough and electrodes



c) Exposure troughs set up with recirculating water at CFI



Figure 1.1. Equipment used to expose fish embryos to electroshock.

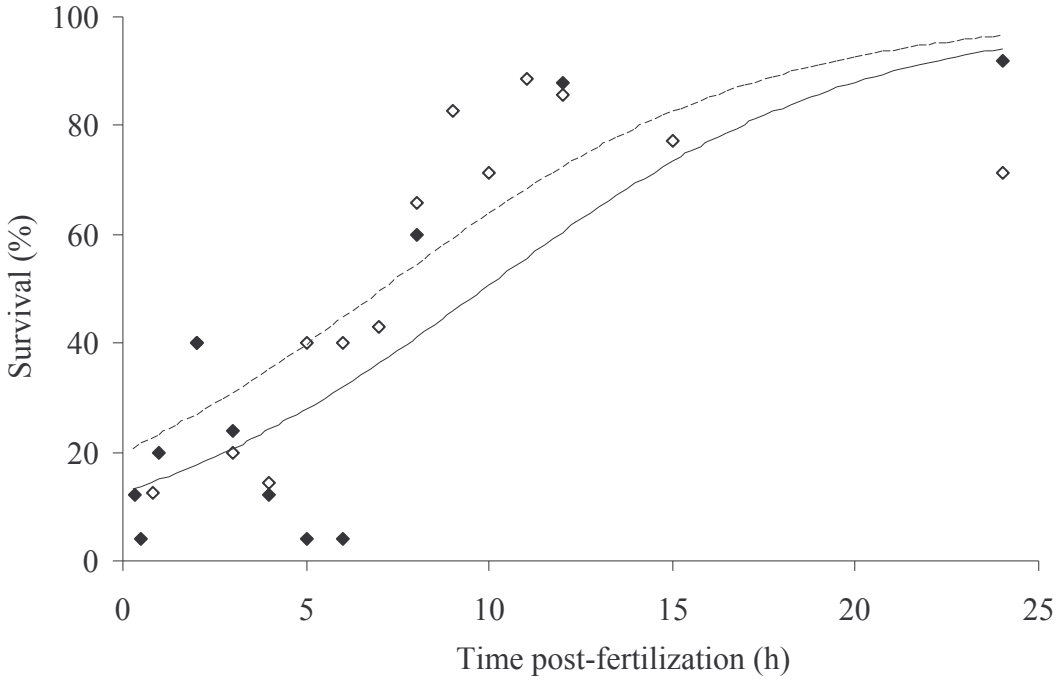


Figure 1.2. Survival to hatching of zebrafish embryos exposed to 20 s of 16 V/cm DC electroshock relative to time post-fertilization (h). Open data points and dashed line represent observed survival and logistic model, respectively, for the first experiment (80% control survival); filled data points and solid line represent observed survival and logistic model for the second experiment (93% control survival). Ambient conductivity of water was 40-50 μS/cm for both experiments.

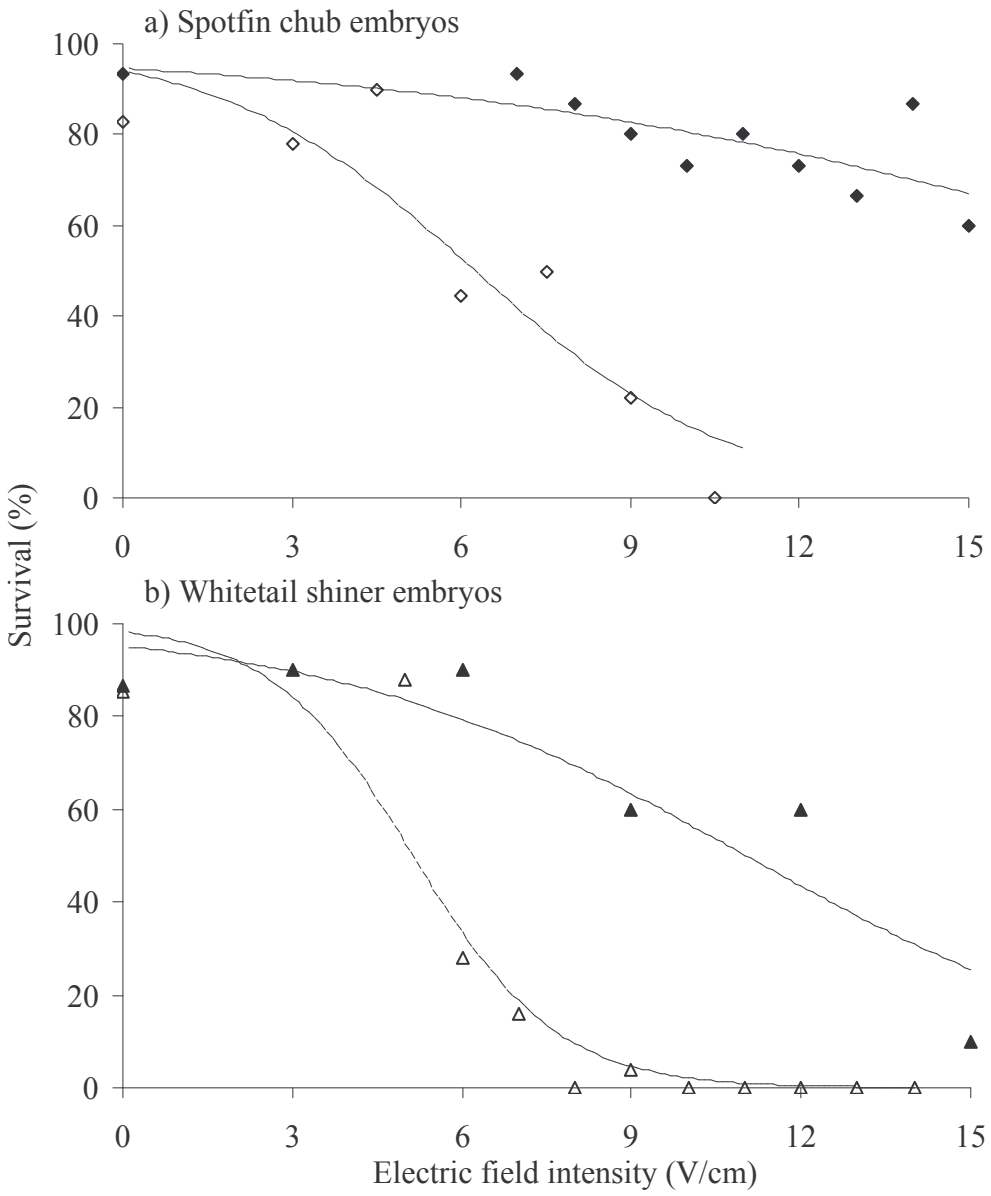


Figure 1.3. Survival of a) spotfin chub embryos and b) whitetail shiner embryos exposed to DC electroshock in water with ambient conductivity of 92-108 µS/cm. Points at 0 V/cm represent survival of unshocked control embryos. a) The open data points and dashed line represent observed survival and the logistic model, respectively, for electroshocked early stage (56% cleavage, 44% blastula) spotfin chub embryos. Filled data points and the solid line represent observed survival and logistic model, respectively, for survival of electroshocked later stage (33% blastula, 33% tail free, 33%% eyed) spotfin chub embryos. b) The open data points and dashed line represent observed survival and the logistic model, respectively, for electroshocked early stage (100% blastula) whitetail shiner embryos. Filled data points and the solid line represent observed survival and logistic model, respectively, for survival of electroshocked later stage (85% blastula, 15% early epiboly) whitetail shiner embryos.

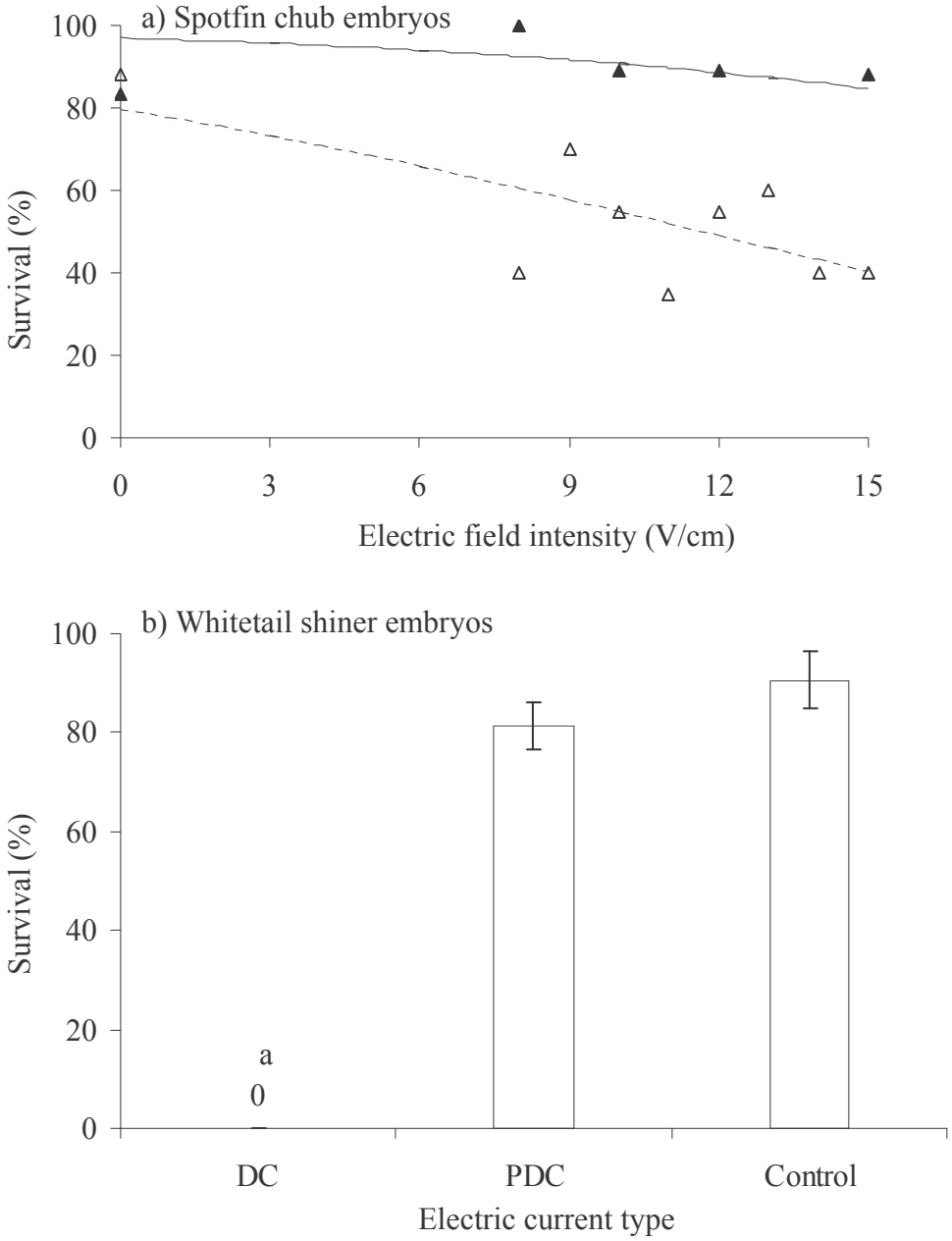


Figure 1.4. Survival to hatching of a) spotfin chub embryos and b) whitetail shiner embryos following DC and PDC electroshock in water with ambient conductivity of 92-108 µS/cm. a) Spotfin chub embryos from a single spawn and a range of developmental stages (30% eyed, 30% tail free, 40% blastula) were exposed to voltage gradients from 8-15 V/cm. The filled data points and solid line represent the observed survival and corresponding logistic model, respectively, for embryos exposed to PDC electroshock. The open data points and dashed line represent the observed survival and corresponding logistic model, respectively, for embryos exposed to DC electroshock. b) Whitetail shiner embryos were from the blastula developmental stage and were exposed to 10 V/cm DC or 10 V/cm 60-Hz PDC. Means with a letter were significantly different from unshocked controls.

Table 1.1. Developmental stage descriptions of preserved (10 % neutral buffered formalin) spotfin chub and whitetail shiner embryos collected during electroshock experiments. Embryos of both species were incubated at 24-26ºC.

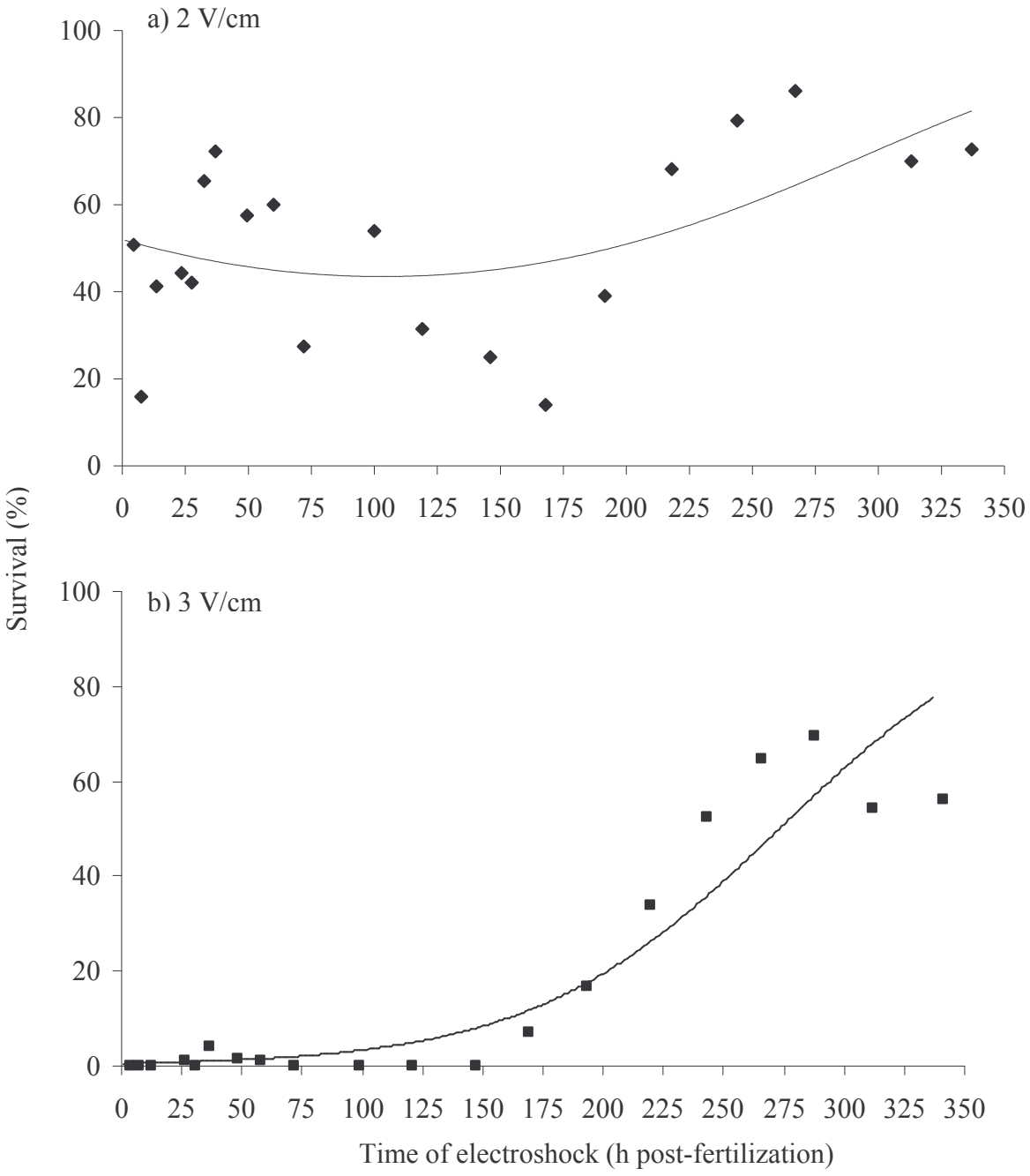
Stage title	Approximate time post-fertilization (h)	Description of developmental period
Cleavage	0-24	Early cleavage of the blastoderm
Blastula	0-24	Blastoderm appears as a ball of cells on top of the yolk sac and cleavage is nearly complete through the onset of epiboly (<30%)
Epiboly	0-24	30% epiboly through 90% epiboly
Tail-free	24-48	The time when the embryo is formed (can tell head from tail, tail usually free from yolk sac) through the eyed stage
Eyed	48-168	Embryo develops eye pigmentation through hatching

Table 1.2. Summary of experimental design. The number of fish embryos stocked into each trough is *N*, and the dependent and independent variables are *Dep. variable* and *Ind. variable*, respectively.

Species	Dep. variable	Ind. variable	N	Number preserved	Current type	Voltage gradient (V/cm)	Developmental period
Zebrafish	Survival	Time of exposure	25-35	10/period	DC	16	Pre-cleavage through eyed
Zebrafish	Survival	Time of exposure	25-35	10/period	DC	16	Pre-cleavage through eyed stage
Whitetail shiner	Survival	Voltage gradient	25	36	DC	3-15	Blastula (100%)
Whitetail shiner	Survival	Voltage gradient	20	7	DC	5-14	Blastula (85%), Epiboly (15%)
Whitetail shiner	Survival	Voltage gradient	25	22	DC and PDC	10	Blastula (100%)
Spotfin chub	Survival	Voltage gradient	9-10	9	DC	3-9	Cleavage (56%), Blastula (44%)
Spotfin chub	Survival	Voltage gradient	15	9	DC	7-15	Blastula (33%), Tail free (33%), Eyed (33%)
Spotfin chub	Survival	Voltage gradient	9-20	12	DC and PDC	8-15	Blastula (40%), Tail free (30%), Eyed (30%)
Spotfin chub	Early hatching	Time of exposure	8	9	DC	8	Tail free; eyed

Appendix 2:

Figures and Tables, Chapter 2



Fgure 2.1. Survival of rainbow trout embryos exposed to a) 2 V/cm DC electroshock and b) 3 V/cm DC electroshock for 20 s over a range of developmental stages. Control survival for the 2 V/cm group was 76% (SE=2%) and was 65% (SE=0.7%) for the 3 V/cm group.

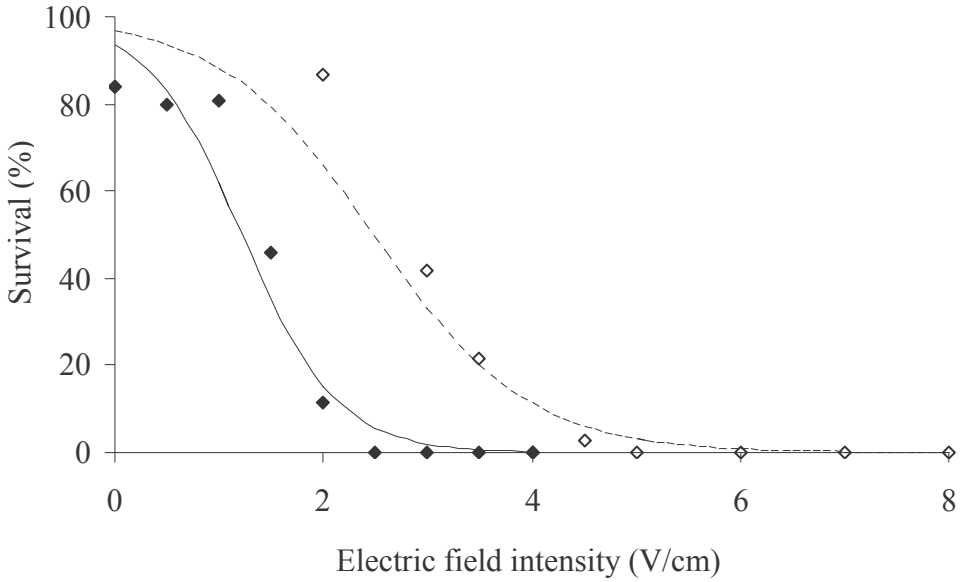


Figure 2.2. Survival of rainbow trout embryos after exposure to DC and 60-Hz PDC electroshock at 25-50% epiboly (158 h post-fertilization). Filled data points and solid line represent observed survival and logistic model, respectively, for embryos exposed to DC. Open data points and dashed line represent observed survival and logistic model, respectively, for embryos exposed to PDC. Control (0 V/cm) survival was 84%.

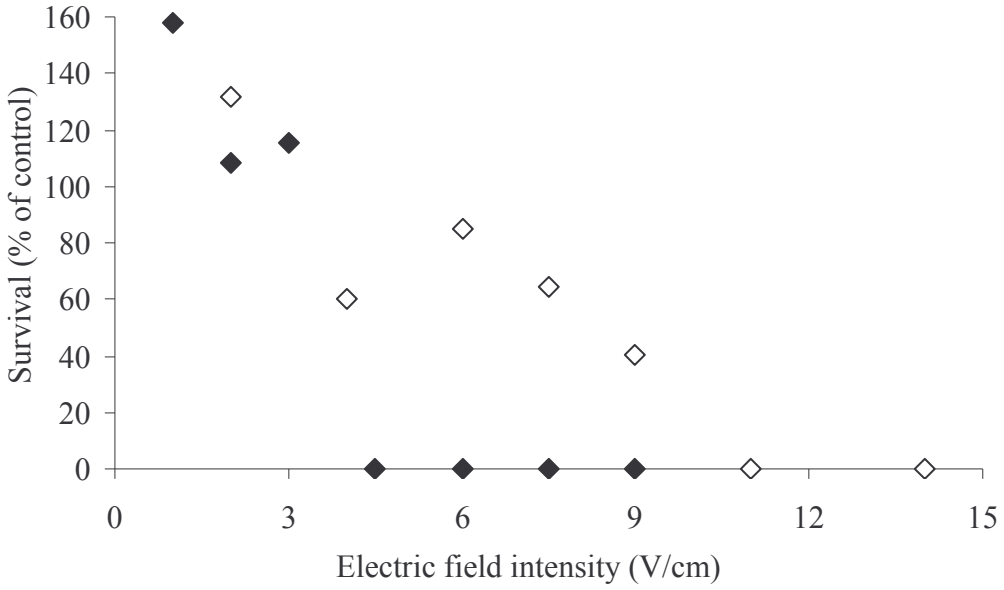


Figure 2.3. Survival (as % of control) of sauger embryos exposed to a range of DC (filled points) and 60-Hz PDC (open points) voltage gradients at 24 h post-fertilization. Control survival was 30% (SE=3.6%).

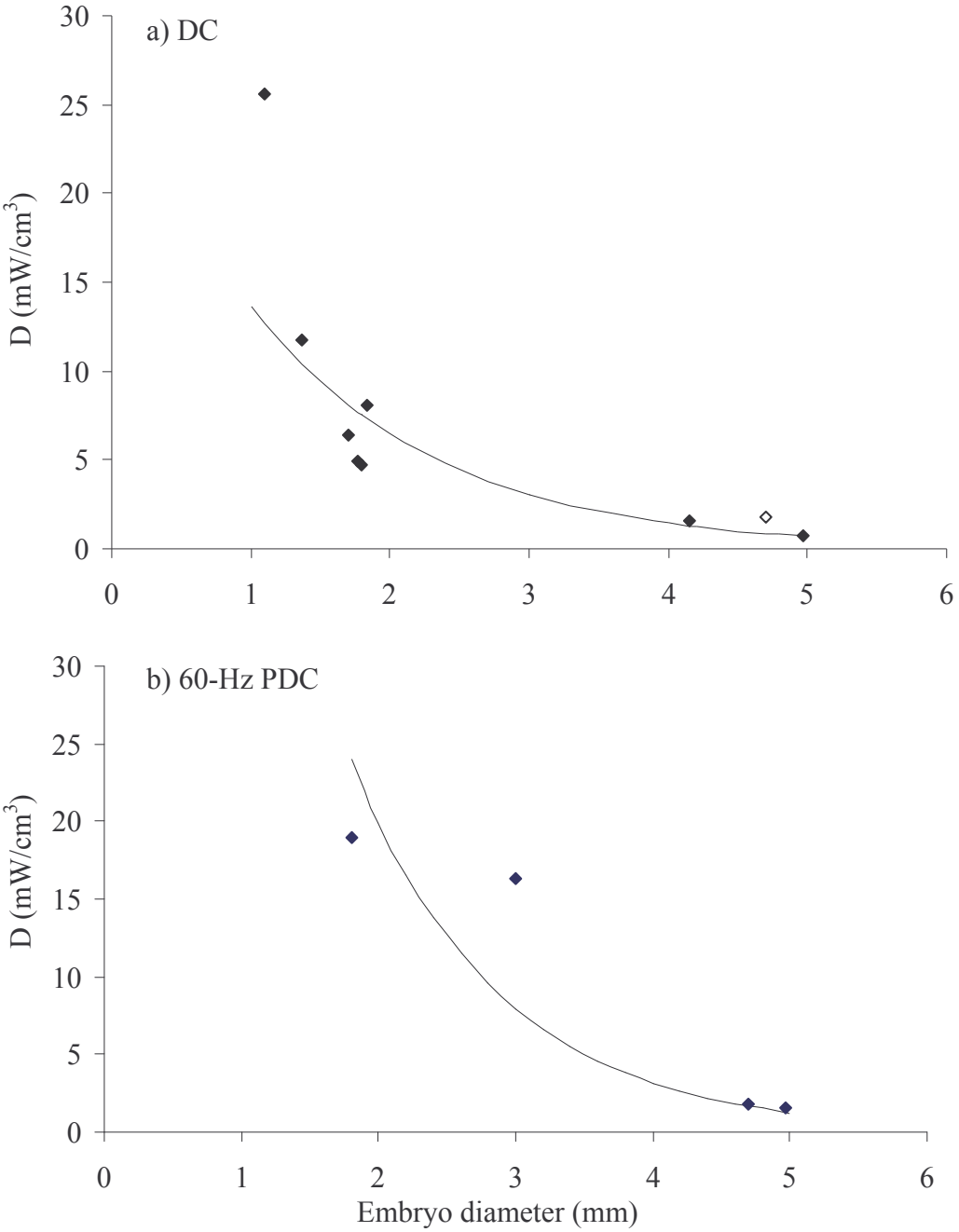


Figure 2.4. a) Direct current power density (mW/cm^3) required to reduce embryo survival to 30% or less for embryos of different diameters (mm). Filled points represent observed power densities for species used to generate the model, and the open point represents DC power density that reduced cutthroat trout survival to 30 % or less using data from Dwyer et al. (1993). b) Pulsed DC power density (mW/cm^3) required to reduce embryo survival to 25-50% for embryos of different diameters (mm). Filled points represent observed power densities for species used to generate the model.

Table 2.1. Summary of the data used to develop models predicting embryo sensitivity based on embryo size for DC and 60-Hz PDC. Survival was calculated as percent of control survival, and when two survival values are listed, the species was used in development of both models and the order is DC/PDC. Superscripts refer to the source from which data was taken, and results for species without a superscript are described in the present study. Where superscripts are placed next to embryo diameters, embryo size was not directly measured in the study from which power density was calculated, and diameters (mean or range) were obtained from sources listed in the footnotes.

Species	Diameter (mm)	Conductivity (μS/cm)	Power D (mW/cm^3) DC	PDC	Survival (%)
Sauger	1.8	235	4.8	19	0/41
Rainbow trout	5	175	0.7	1.9	13/50
Zebrafish[a]	1.4	45	11.8[a]	-	4
Spotfin chub[a]	1.8	100	8.1[a]	-	30
Whitetail shiner[a]	1.8	100	4.9[a]	-	19
Bluegill[b]	1.1[e]	100	25.6[b]	-	0
Largemouth bass[b]	1.7[e]	100	6.4[b]	-	2
Channel catfish[b]	4.15 (3.2-5.1)[f]	100	1.6[b]	-	3
Razorback sucker[c]	3[g]	650	-	16.3[c]	49
Cutthroat trout[d]	4.7[h]	388	-	1.8[d]	35

[a] Chapter 1
[b] Henry and Grizzle (2004)
[c] Muth and Ruppert (1997)
[d] Dwyer and Erdahl (1995)
[e] Merriner (1971)
[f] Reagan and Conley (1977)
[g] Mueller et al. (2006)
[h] Pauley et al. (1989)

Vita

Russell J. Bohl was born in Norway, ME on January 6, 1981. He was raised in Brockton, MA, and attended high school at New Testament Christian School, where he graduated in 1999. From there he went to Gordon College where he earned a Bachelor of Science in biology in 2003, then spent one year teaching middle school math and science in Tegucigalpa, Honduras. After returning to the United States, he completed a one year AmeriCorps national service program in Southern NJ, during which he participated in environmental education and stream monitoring. From there he went to the University of Tennessee, Knoxville and received his Master of Science degree in Wildlife and Fisheries Science with a minor in statistics in 2008.

Russ is currently pursuing his doctorate in Conservation Biology at the University of Minnesota, St. Paul, MN.