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The effects of pulse exposures to road salt at various stages of early development in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Salmonids spawn in freshwater streams including those in urban areas that are impacted by human activities. In the Vancouver region of British Columbia, Canada, the extensive use of road salt (primarily NaCl) is associated with frequent 24-h "pulses" of salt in streams, some of which may exceed the provincial acute guideline for maximum chloride concentrations (600 mg L^{-1} Cl^-) by up to 11-fold. For some salmonids, road salting coincides with critical developmental stages, as many species spawn between October and January. We explored the concentration-dependent effects of a 24-h salt pulse (600–9600 mg L^{-1} Cl^-) on salmonid development using rainbow trout (*Oncorhynchus mykiss*). Salt pulses were imposed at one of three developmental time points: <1 h post-fertilization, the eyed-stage or 7 days post-hatch. Significant mortality occurred only in the <1 h post-fertilization treatment, at 2400, 4800 and 9600 mg L^{-1} Cl^-, all environmentally relevant salt concentrations. Significant differences in whole-embryo ion concentrations at the end of the salt exposure and at the eyed-stage (17 days post-salt exposure) indicated lasting ionoregulatory effects on embryos. Co-exposure to $CaCO_3$ during the salt pulse, at a level that increased dissolved Ca^{2+} by 2-to 3-fold in the ion poor Vancouver water, greatly reduced mortality and altered whole-embryo ion levels. These findings support the need for site-specific water quality guidelines, as toxicity varies with water's ionic composition. This research also highlights the need for improved road salting practices to reduce salt contamination and its potential adverse effects on developing salmonids.

1. Introduction

Road salt is a common de-icing agent used across North America as a cost-effective solution for preventing ice formation while providing traction on roads and sidewalks during cold seasons (Kelly et al., 2010). In Canada, approximately 5 million tonnes of road salt are used annually, with 20 % and 50 % attributed to use in British Columbia and Ontario, respectively (ECCC, 2022). As the area of impervious surfaces such as roads, sidewalks and parking lots are expanding, the use of road salt is also increasing. In the United States, road salt usage has doubled since 1975, exceeding 20 million tonnes annually (Kelly et al., 2019).

Road salt contamination from runoff is one cause of salinization events that threaten the water quality of freshwater ecosystems (Cañedo-Argüelles, 2020; Evans and Frick, 2001; Tiwari and Rachlin, 2018). The salts readily dissolve and become concentrated in groundwater and surface water, leading to increases in Na^+ and Cl^- concentrations in freshwater streams, rivers, and lakes (Cooper et al., 2014; Dugan et al., 2017; Hintz and Relyea, 2019; Kelly et al., 2019; Kilgour et al., 2025). Although salt contamination is most common during colder months, its penetration into the soil leads to delayed leaching, which can cause elevated salt levels even in the spring and summer (Baltrėnas et al., 2006; Lawson and Jackson, 2021; Williams et al., 2000).

As with many known toxicants, various governments have established water quality guidelines surrounding chloride concentrations to protect aquatic life from potential harm. Canada has acute and chronic chloride guidelines established at 640 and 120 mg L^{-1}, respectively

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(CCME, 2011). In comparison, the United States Environmental Protection Agency's has set acute and chronic chloride guidelines at 830 and 230 mg L^{-1}, respectively (US EPA, 1988). However, chloride guidelines serve more as recommendations, as they are not strictly enforced at the federal or regional level.

Chronic exposure to road salt has been shown to have lethal effects on a variety of fish species including fathead minnow (Corsi et al., 2010) and developing Atlantic salmon (Mahrosh et al., 2018). Similarly, in developing chinook salmon and steelhead trout, survival and development were impaired when salinities surpassed 8 ppt (Morgan et al., 1992). In addition, a chronic increase in salinity has sublethal effects such as a reduction in growth as seen in juvenile rainbow trout (Hintz and Relyea, 2017; Morgan and Iwama, 1991), steelhead trout and chinook salmon (Morgan and Iwama, 1991). However, acute road salt exposures have not shown similar adverse effects on body weight, though a significant decrease in hepato-somatic index was observed in juvenile rainbow trout (Vosylienė et al., 2006). Thus, it is apparent that across species, both chronic and acute salt exposures can have adverse morphological effects.

Fish differ in their salt tolerance depending on the developmental stage. The first few days of embryonic development are particularly sensitive to environmental shifts. Although salmonid eggs have some protection by the chorion and perivitelline fluid (Barrett et al., 2001; Daye and Garside, 1980; Eddy and Talbot, 1983, 1985; Morgan et al., 1992; Potts and Rudy, 1969; Rudy and Potts, 1969; Weisbart, 1968), sufficient ionoregulatory control is not developed until later stages of development (Brauner and Wood, 2002; Degnan and Zadunaisky, 1980; González et al., 1996; Katoh et al., 2000; Rombough, 1999). Salmonids upregulate the expression of gill Na^+/K^+ ATPase in response to higher salinity, enabling them to manage their internal ion levels (Bystriansky et al., 2006; Evans et al., 2005; Hwang et al., 2011). However, salmonid gills are not fully functional until roughly two weeks post-hatch (Gallagher et al., 2021), meaning that embryos and alevins rely on the ionocytes in their skin and lining their yolk sac membrane to ion regulate (Fu et al., 2010; Katoh et al., 2000; Rombough and Moroz, 1990; Shen and Leatherland, 1978a). Consequently, embryos and alevins are ill-equipped to efficiently ion regulate amid salt stress (Mahrosh et al., 2014; Rombough, 1999) and may be most vulnerable to road salt contamination.

Throughout the Vancouver Lower Mainland (VLM), salmonids inhabit soft water streams (hardness < 20 mg L^{-1} as $CaCO_3$; Metro Vancouver, 2024b) that traverse highly urbanized areas where road salt contamination is often observed. Water quality loggers deployed at multiple monitoring locations across the VLM have detected "pulses" in specific conductance during road salting seasons from November to March (DFO, 2024; Kilgour et al., 2025). Road salt pulses are significantly correlated to precipitation events (rain and snowfall) and days when temperature is below 10°C (Kilgour et al., 2025). Road salt pulses overlap with the spawning and rearing period of embryos and alevins of many species of Pacific salmon (Salo, 1991; Sandercock, 1991). The British Columbia Ministry of Environment and Climate Change Strategy (BCMECCS) (2025) has set an acute chloride guideline of 600 mg L^{-1}, yet salt pulses in streams have been observed to exceed this guideline by more than 11 times, with elevated chloride levels persisting for approximately 24 h (DFO, 2024; Kilgour et al., 2025). Although a chronic NaCl exposure at concentrations of 3000 mg L^{-1} NaCl (= 1818 mg L^{-1} Cl^-) has been shown to impair growth in juvenile rainbow trout (Hintz and Relyea, 2017), it remains unclear how more environmentally relevant short pulses at higher concentrations (> 6000 mg L^{-1} Cl^-) may impact the development of Pacific salmon embryos and alevins.

Here we used rainbow trout to investigate the lethal and sublethal effects of a 24-h salt pulse consisting of environmentally relevant salt concentrations at each of three developmental stages: <1 h post-fertilization, the eyed-stage, or 7 days post-hatch. Given that Vancouver's water is extremely soft, we also asked whether an increase in calcium carbonate ($CaCO_3$) could mitigate any adverse effects of a salt exposure as shown in previous studies (Elphick et al., 2011b, 2011a; Erickson et al., 2022a). This study examines the developmental stage at which salmonids are most vulnerable to environmentally realistic chloride pulses while also emphasizing the importance of site-specific water quality guidelines, as water hardness can significantly alter a pollutant's toxicity.

2. Materials and methods

2.1. Experimental design

We performed three independent experiments during early development to examine the effects of a 24-h salt pulse at six different concentrations of rock salt, spanning a geometric concentration range from the BC acute guideline level (600 mg L^{-1} Cl^-) to a 16-fold higher level (Fig. 1). Across all experiments, we used food-grade rock salt to avoid potential contaminates in commercially used road salt. Thus, the ionic composition of experimental rock salt was compared to commercially available road salt. Additionally, to ensure the ionic composition of each experimental solution was near nominal concentrations, analyses of all salt solutions were conducted as described in Section 2.5.

Experiment 1 examined if different developmental stages had different sensitivities to a salt exposure. Experiment 2 repeated the salt exposure at <1 h post-fertilization, as this was observed to be the most sensitive stage in Experiment 1. Experiment 3 was designed to determine whether $CaCO_3$ could protect against the negative effects of road salt. Since each experiment was performed at different times of year and under different conditions, methods for fish husbandry and exposure are described separately below for each experiment.

2.2. Experiment 1

The effects of an acute road salt exposure were investigated at three developmental stages (<1 h post-fertilization, the eyed-stage and 7 days post-hatch). Developmental stages were determined based on accumulated thermal units (ATUs). At each stage, a 24-h salt exposure was imposed at concentrations of 0 = control, 989, 1978, 2967, 3956, 7912 or 15,824 mg L^{-1} rock salt, providing nominal chloride concentrations of 0, 600, 1200, 2400, 4800 and 9600 mg L^{-1} Cl^- in dechlorinated tap water.

2.2.1. Fertilization protocol

Rainbow trout gametes were sourced from Trout Lodge Hatchery in Bonney Lake, Washington, USA, in August 2023. The gametes were delivered to the University of British Columbia in Vancouver, BC in a Styrofoam container with ice packs to maintain a cold temperature during transit. The gametes arrived on the same day as when they were strip spawned. Eggs from four females were pooled in a dry glass container. Milt from four males was also pooled and then added to the eggs using a Pasteur pipette. The eggs and milt were gently mixed and left to fertilize in the dark for 10 min at 11°C. Next, the fertilized eggs were separated into weigh boats, with each weigh boat representing one replicate. 36–40 embryos were added to each replicate, with four replicates per exposure group. 300 mL of water or the respective salt solution for those undergoing salt exposure was poured into each weigh boat and left for 5 min initiating the egg hardening process. After 5 min, the embryos were transferred to labelled mesh baskets (50 cm^2) in Heath trays (8-tray Vertical Incubator, MariSource, Burlington, Washington, USA). When embryos were not in a 24-h salt exposure, they were reared in a flow-through system in dechlorinated Vancouver city tap water (Na, 0.009 mM; Cl, < 0.5 mg L^{-1}; Ca, 0.03 mM; Mg, 0.007 mM; K, 0.005 mM; alkalinity, 3.2 mg as $CaCO_3$ L^{-1}; hardness 4.8 mg as $CaCO_3$ L^{-1}; SO_4, 0.5–1.4 mg L^{-1}; dissolved organic carbon, 1.7 mg L^{-1}; pH 6.5–7.2; Metro Vancouver, 2024a, 2024b). Chillers (EcoPlus Commercial Grade Water Chiller, 1 ½ HP, Sunlight Supply Inc., Vancouver, Washington, USA) were used to maintain a water temperature of 14 ± 1°C.

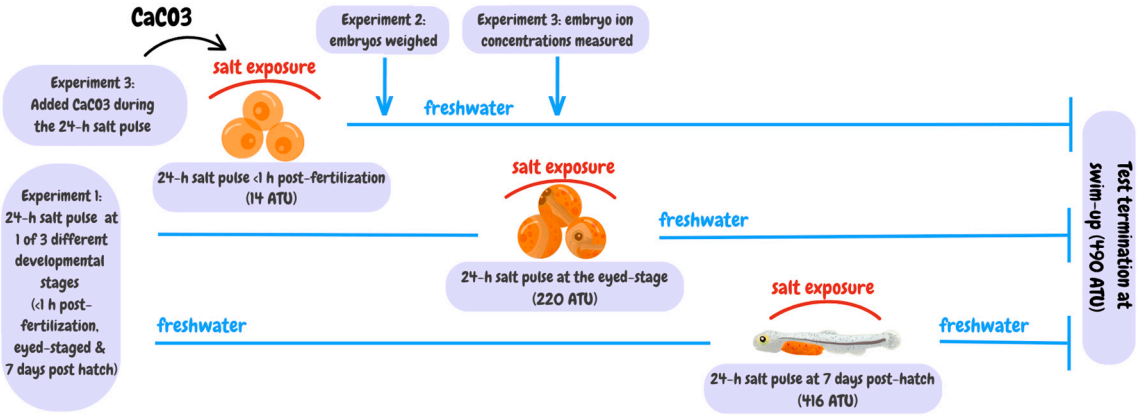

Fig. 1. Experimental design for Experiment 1, 2 and 3. Experiment 1 consisted of a 24-h salt pulse at one of three developmental stages. Experiment 2 replicated the 24-h salt pulse at <1 h post-fertilization as performed in Experiment 1 with the addition of embryo weight measurements. Experiment 3 explored if the addition of $CaCO_3$ could have protective effects during a salt exposure at <1 h post-fertilization, and whole-embryo ion concentrations were measured.

Temperature, conductivity and dissolved oxygen were recorded daily, while ammonia, nitrite and nitrate were measured weekly.

2.2.2. Experimental protocol

All salt solutions were aerated overnight to ensure the salt was fully dissolved. Conductivity, salinity, dissolved oxygen and pH were measured across experimental concentrations. Rainbow trout were exposed to one of the six test solutions starting either at <1 h post-fertilization (14 ATUs), the eyed-stage (220 ATUs), or 7 days post-hatch (416 ATUs) for 24 h. While in their respective egg baskets, embryos and/or alevins were transferred, without air or light exposure, into each respective salt solution. To account for any mechanical disruption, control groups were handled in the same way as salt-exposed groups but in the absence of salt-exposure. Individuals were statically exposed to test solutions in dim-lighting and with aeration for 24 h. Following 24 h, water samples of each experimental solution were taken and stored at 4°C for later analysis. Egg baskets were moved back to heath trays to rear in the flow-through system with dechlorinated tap water until the yolk sac was absorbed (~490 ATUs). All baskets were checked daily, and embryos which appeared dead (identified by an opaque white colour) were immediately removed to prevent any fungal growth.

2.3. Experiment 2

Unlike Experiment 1 which had begun in the summer, Experiment 2 was initiated later into the calendar year, so air and water temperatures naturally began to decline allowing us to maintain this experiment at 11 ± 1°C. The primary purpose of this experiment was to replicate the salt exposure that experienced the greatest mortality in Experiment 1, a 24-h salt pulse at <1 h post-fertilization. Rainbow trout embryos underwent the same fertilization and experimental procedures as Experiment 1, except that rainbow trout gametes were obtained from Trout Lodge Hatchery on September 2023.

2.3.1. Embryo weight 24-h post-fertilization

In Experiment 2, embryos were weighed at the end of the 24-h salt exposure. Embryos (egg + embryo) were sampled from each salt concentration (n = 8; 0, 600, 1200, 2400, 4800 and 9600 mg L^{-1} Cl^-). Embryos were rinsed in ultrapure water, blotted dry and individually weighed to 0.001 mg.

2.4. Experiment 3

Experiment 3 investigated if calcium could mitigate the negative effects of a salt exposure at <1 h post-fertilization as previously observed in Experiments 1 and 2. A 24-h salt exposure was conducted at nominal concentrations of 0 (control), 1200, 1800 and 2400 mg L^{-1} Cl^-. One group had no added $CaCO_3$, while the other received 200 mg L^{-1} of $CaCO_3$ (nominal) added to each of the four test solutions.

2.4.1. Fertilization protocol

Rainbow trout gametes were obtained from Trout Lodge Hatchery on August 15, 2024, but due to a shipping delay, they arrived the morning after the parents were strip spawned. However, the gametes were properly insulated in a Styrofoam box with several ice packs and were still cold when received. In dim lighting and at 11°C, eggs from 10 females were pooled and mixed gently in a dry, glass container. For each of the five replicates, 5 g of eggs were weighed (~100 eggs). 300 µL of pooled milt from 10 males was pipetted to each replicate. Eggs were gently mixed and then left to fertilize in the dark for 10 min at 11°C. After 10 min, eggs were exposed to 300 mL of dechlorinated tap water or if undergoing an exposure to salt, or salt + $CaCO_3$, their respective test solution. After a further 5 min, embryos were transferred to their egg baskets in their respective heath tray. All embryos were reared in static conditions in an environmental chamber holding a temperature of 11 ± 1°C. Each individual heath tray was constantly aerated. Water changes occurred daily; temperature, conductivity and dissolved oxygen were recorded daily. Ammonia, nitrite and nitrate were measured weekly.

2.4.2. Experimental protocol

Salt solutions were prepared at the following nominal concentrations: 0 (control), 1200, 1800 and 2400 mg L^{-1} Cl^- with either no added $CaCO_3$ or $CaCO_3$ at 200 mg L^{-1} $CaCO_3$. All salts were dissolved in dechlorinated tap water and aerated overnight. Embryos were exposed to one of the eight test solutions right after fertilization (10 ATUs) for 24 h. Exposures took place statically in the respective heath tray, so embryos were not physically moved to and from a separate vessel. At the end of the 24-h salt exposure, water samples from each experimental solution were collected and stored for analysis. Over the course of an hour, dechlorinated tap water was added to the heath trays to gradually reduce salinity until all salt was eliminated. The embryos were then reared in dechlorinated tap water until the eyed-stage (~220 ATUs). The experiment remained static with daily water changes, and dark lighting and aeration were maintained consistently. Conductivity, salinity, dissolved oxygen and pH were measured across experimental concentrations. All baskets were checked daily and dead embryos were promptly removed.

2.4.3. Whole-embryo ions

Whole-embryo (egg + embryo) ion concentrations were measured in Experiment 3. Sampling took place at the end of the 24-h salt exposure at <1 h post-fertilization and at test termination (eyed-stage; 226 ATUs

=18 days post-salt exposure). Six embryos from each of the five replicates were sampled across all eight exposure groups. Embryos were rinsed with ultrapure water produced by a Milli-Q system (EMD Millipore, Massachusetts, United States), blotted dry, and two embryos from each replicate were pooled and placed in pre-weighed polystyrene tubes to obtain wet weight. Given the relatively low survival at the eyed-stage in some of the salt exposed groups without $CaCO_3$, sample sizes ranged from n = 8–15. Embryos were then placed in a drying oven at 60°C until a constant dry weight was observed. Embryos were digested in 1 mL of 2 M trace-metal grade HNO_3 (CAS 7697-37-2) and placed back in a drying oven at 60°C for five days. Following digestion, samples were diluted in ultrapure water and 1 % $LaCl_3$; the samples were then vortexed and analyzed using flame atomic absorption spectroscopy (FAAS; Varian, California, USA) to measure concentrations of sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}). All samples were run according to the manufacturer's suggested settings within the Spectra AA software (Agilent Technologies, Santa Clara, California, USA). All samples were run in duplicate.

2.5. Water analysis

In these experiments, we used food grade rock salt to avoid the possibility that commercially available road salt might contain unknown additives that would confound interpretation. We therefore compared the composition of both salts, and we validated the ionic composition of all experimental salt solutions as detailed below.

2.5.1. Salt comparison

The ionic compositions of rock salt (Sifto® Gros sel 9000 Coarse Salt; Sifto Canada, Ontario) used in all experiments and commercially available road salt (Sifto® Safe Step® Ice Salt®, Compass Minerals Canada Corp, Mississauga, Ontario. Product# 067568-673404) were compared. 5 g of each salt type were weighed, dried in the drying oven at 60°C for 24 h and reweighed to obtain moisture content. The 5 g was then dissolved and diluted with ultrapure water and 1 % $LaCl_3$ into the optimal concentrations of the standard curves for the various ions. Salt samples were analyzed with FAAS for sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}; Table 1). Certified reference materials for the aqueous matrix were run alongside all water analyses to evaluate the accuracy, maintaining ± 10 % of the certified value. Water samples were run in duplicate and according to the manufacturer's suggested settings within the Spectra AA software. Chloride (Cl^-) concentrations were measured in the undiluted, intact samples using a chloridometer (Chlorocheck model 3400; ELITechGroup Inc., Utah, USA).

2.5.2. Experimental water samples

To validate that experimental salt solutions were near nominal concentrations, water samples were collected from each salt exposure and diluted with ultrapure water and 1 % $LaCl_3$ to bring them into the optimal ranges of the standard curves for the various ions. The samples were then acidified to 1 % vol/vol using trace-metal grade HNO_3 and ions were analyzed following the same protocol as detailed in Section 2.5.1, except that, in control samples containing relatively low chloride concentrations, chloride (Cl^-) was measured using the colorimetric assay of Zall et al. (1956). The concentrations of total CO_2 (representing $HCO_3^- + CO_3^{2-}$) resulting from $CaCO_3$ addition in Experiment 3 were measured on non-acidified, undiluted water samples using a model 965 total CO_2 analyzer (Corning Instruments, Corning, New York, USA), calibrated with $NaHCO_3$ standards.

2.6. Statistical analyses

Graphing and statistical analyses were conducted using R version 4.3.1. Data are presented as mean ± SEM. For Experiments 1 and 2, One-way Analysis of Variance (ANOVA) was used to evaluate survival across salt exposures at each developmental stage (<1 h post-fertilization, the eyed-stage and 7 days post-hatch), followed by a Tukey's post-hoc test. In Experiment 3, a two-way ANOVA was used to assess survival in salt-exposed groups with and without added $CaCO_3$, followed by a Tukey's post-hoc test. A one-way ANOVA, followed by Tukey's post-hoc test, was used to evaluate embryo weight. For each ion (Na^+, Ca^{2+}, & K^+) sampled 24 h post-fertilization and at the eyed-stage, analyses were conducted using a two-way ANOVA and a Tukey's post-hoc test. If assumptions of normality and variance were violated, data were log-transformed prior to analysis. Significant differences were defined by $p \leq 0.05$.

3. Results

3.1. Water analysis

3.1.1. Road salt comparison

The ionic composition of rock salt (Sifto® Gros Sel 9000 Coarse Sal) used in all experiments, was analyzed to compare it with commercially available road salt. Sifto® Safe Step® Ice Salt® (Product# 067568–673,404). In both types of salt, 1 g contained 0.389 g Na^+ and 0.600 g Cl^-, but the content of minor ions differed. Each gram of rock salt contained of 0.0020 g K^+ and 0.00040 g Mg^{2+}, which is higher compared to that measured in road salt (0.00004 g K^+ and 0.00004 g Mg^{2+}). By contrast, the levels of Ca^{2+} were higher in road salt (0.0003 g Ca^{2+}) than in rock salt (0.0001 g Ca^{2+}). Additionally, 1 g of road salt had a greater water content (0.0008 mL) compared to the experimentally used rock salt (0.00012 mL; Table 1). Overall, the rock salt used in the present study, which is a food grade, has slightly higher levels of trace ions, but our choice avoided the possibility of unknown additives that could confound interpretation.

3.1.2. Experimental water samples

Measured chloride concentrations in all experimental salt solutions deviated ≤ 15 % from their nominal concentrations (Table 2). Concentrations of K^+ and Mg^{2+} increased geometrically and in approximate proportion to the concentrations of Na^+ and Cl^-, paralleling the geometrically increasing exposures to rock salt in all experiments. This reflected the trace levels of these two cations in the rock salt. Ca^{2+} concentrations in the water were elevated when 200 mg L^{-1} $CaCO_3$ (= 80 mg L^{-1} Ca^{2+}) was added to four salt treatments; however, the total Ca^{2+} concentration measured in the solution was only ~20 mg L^{-1} Ca^{2+} (Table 2). In parallel, the total CO_2 representing dissolved HCO_3- + CO_3^{2-} in the water was only about 40 mg L^{-1}. Thus, the elevations resulted in dissolved Ca^{2+} and $HCO_3^- + CO_3^{2-}$ concentrations that were 2- to 3-fold above levels in the controls. The $CaCO_3$ addition also

Table 1
Rock salt used for Experiments 1, 2 & 3 (Sifto® Canada Gros sel 9000 Coarse Salt) was compared to commercially available road salt (Sifto® Safe Step® Ice Salt^T®). The composition of sodium (Na^+), chloride (Cl^-), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}) of 1 g of salt was measured and compared for each salt type. Additionally, salt was dried to determine the water content.

Salt type	Na^+ (g)	Cl^- (g)	K^+ (g)	Mg^{2+} (g)	Ca^{2+} (g)	Water (mL)
Sifto® Rock Salt (used in salt exposures)	0.3886	0.6000	0.0020	0.00040	0.00010	0.00012
Sifto® Road Salt (commercially available)	0.3886	0.6000	0.00004	0.00004	0.00030	0.00080

Table 2
Water analysis of the 24-h salt exposures conducted in Experiments 1, 2 & 3 to measure concentrations of sodium (Na^+), chloride (Cl^-), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}). In Experiment 3, total CO_2 ($HCO_3^- + CO_3^{2-}$) was measured in solutions with added $CaCO_3$.

Treatment (mg L^{-1} rock salt)	Nominal chloride (mg L^{-1})	Specific conductance (μS/cm)	pH	Na^+ (mg L^{-1})	Cl^- (mg L^{-1})	K^+ (mg L^{-1})	Mg^{2+} (mg L^{-1})	Ca^{2+} (mg L^{-1})	Total CO2 (mg L^{-1})
Experiments 1 & 2									
0	**0**	56.0–62.5	7.1–7.4	1.88–5.52	5.1–8.6	0.39–0.83	0.20–0.38	7.38–8.07	NA
989	**600**	2000.0–2014.0	7.2–7.4	350.1–419.88	567.20–638.10	1.06–1.86	0.61–0.68	8.07–8.86	NA
1978	**1200**	3800.0–3910.0	7.2–7.4	695.52–914.16	1134.40–1169.85	2.77–3.34	0.97–1.16	7.34–9.82	NA
3956	**2400**	7315.0–7500.0	7.2–7.4	1440.72–1600.32	2162.45–2481.50	6.32–7.28	1.81–2.09	8.66–10.06	NA
7912	**4800**	1394.0–14,250.0	7.2–7.4	2785.44–3222.72	4254.00–4750.30	9.67–16.62	2.51–4.05	7.58–10.47	NA
15,824	**9600**	26,700.0–2980.0	7.2–7.4	5135.04–6647.04	8153.50–9677.85	24.26–34.77	5.28–7.49	10.06–12.00	NA
Experiment 3									
0	**0**	90.0	7.4	4.48	7.2	0.16	0.05	9.56	NA
1978	**1200**	4310.0	7.4	898.08	1205.30	3.85	1.31	9.62	NA
2967	**1800**	5820.0	7.4	1146.12	1737.05	5.97	1.82	9.92	NA
3956	**2400**	7440.0	7.4	1560.00	2304.25	7.50	2.22	9.10	NA
0 + 200 mg L^{-1} $CaCO_3$	**0**	122.0	7.4	2.56	7.3	0.16	0.08	15.08	42.00
1978 + 200 mg L^{-1} $CaCO_3$	**1200**	4350.0	7.8	850.68	1276.20	3.84	1.34	22.44	76.80
2967 + 200 mg L^{-1} $CaCO_3$	**1800**	6000.0	7.9	1209.66	1807.95	6.20	2.30	24.18	76.80
3956 + 200 mg L^{-1} $CaCO_3$	**2400**	7450.0	8.0	1377.12	2268.80	7.38	2.47	25.87	90.00

resulted in about a 0.5 unit rise in water pH. Throughout all experimental solutions, ammonia remained below 0.2 mg L^{-1} and nitrate and nitrite remained below detection limits (ammonia: 0.1 mg L^{-1}, nitrite: 0.1 mg L^{-1} and nitrate: 1 mg L^{-1}). Additionally, dissolved oxygen remained above 90 % saturation for the duration of each experiment.

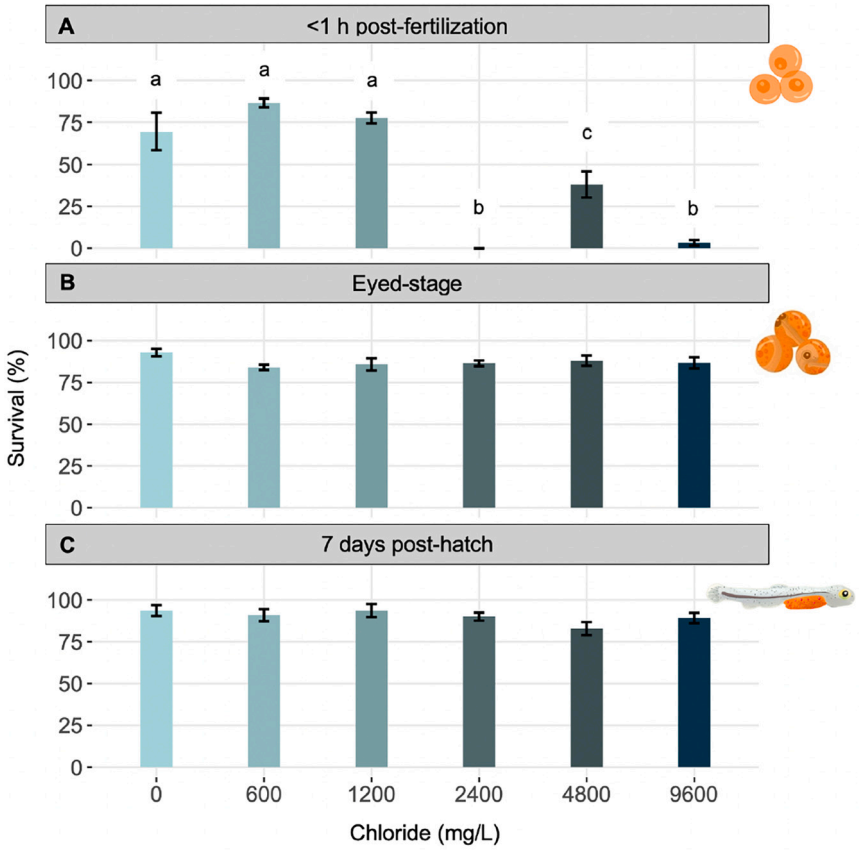

Fig. 2. Mean survival (%) ± SEM of developing rainbow trout when exposed to different salt concentrations, at different developmental stages, in Experiment 1. Percent survival at swim-up from individuals exposed to a 24-h salt pulse at <1 h post-fertilization (panel A), at the eyed-stage (panel B), or 7 days post-hatch (panel C) (N = 4, with ~40 embryos per replicate for each panel). Lowercase letters indicate significant differences ($p \leq 0.05$) in survival among the groups exposed to different concentrations of salt.

3.2. Experiment 1 - survival

Experiment 1 explored differences in salt sensitivity across developmental time points. The survival at swim-up of rainbow trout embryos exposed to a 24-h salt exposure starting at <1 h post-fertilization (10 ATUs) was significantly reduced at 2400 mg L^{-1} (0 %, $p < 0.0001$), 4800 mg L^{-1} (38.2 %, $p = 0.011$) and 9600 mg L^{-1} Cl^- (3.3 %, $p < 0.0001$) compared to the control (i.e., no salt exposure; 70.0 %; Fig. 2A). The two lowest salt exposures (600 and 1200 mg L^{-1} Cl^-) showed survival rates of 86.5 % and 77.6 %, respectively, which were not significantly different from the control ($p > 0.30$; Fig. 2A). The average survival of groups exposed to 2400 mg L^{-1} Cl^- did not significantly differ from the survival of groups exposed to 9600 mg L^{-1} Cl^- ($p = 0.99$) but did significantly differ from the survival of groups exposed to 4800 mg L^{-1} Cl^- ($p = 0.002$; Fig. 2A). By contrast, salt exposures (600 to 9600 mg L^{-1} Cl^-) imposed at the eyed-stage (220 ATUs) did not significantly impact survival compared to the control group ($p = 0.193$; Fig. 2B). Similarly, salt exposures at 7 days post-hatch (416 ATUs) did not significantly impact survival when compared to the control or across salt exposures ($p = 0.719$; Fig. 2C).

3.3. Experiment 2

Experiment 2 replicated Experiment 1's <1 h post-fertilization salt exposure, but at a slightly lower temperature.

3.3.1. Survival

As was observed in Experiment 1, survival at swim-up for fish exposed to salt at <1 h post-fertilization was significantly lower in the highest three salt exposures 2400, 4800 and 9600 mg L^{-1} Cl^-, with average survival of 12.0 %, 5.2 % and 1.5 %, respectively (Fig. 3) compared to the control, 600 mg L^{-1} and 1200 mg L^{-1} Cl^- exposures which had 66.7 %, 74.1 % and 62.4 % survival, respectively ($p < 0.001$). The latter three did not differ significantly from one another ($p > 0.5$; Fig. 3). The decline in survival was not apparent until ~7 days post-salt exposure (Fig. 4).

The survival of the control groups in both Experiments 1 and 2 (from the <1 h post-fertilization exposures only), did not significantly differ from each other ($p = 0.99$). In Experiment 2, survival in the treatment groups exposed to 4800 mg L^{-1} Cl^- at <1 h post-fertilization was significantly lower than in the same salt exposure in Experiment 1 ($p = 0.002$), highlighting the apparent anomaly at this concentration in Experiment 1. In the remaining groups (controls, 600, 1200, 2400 and 9600 mg L^{-1} Cl^-), survival did not significantly differ between Experiment 1 and Experiment 2 ($p > 0.5$; Fig. 3).

3.3.2. Embryo wet weight

In Experiment 2, at the end of the 24-h salt exposure starting at <1 h post-fertilization, the wet weight of embryos was measured across all concentrations. As the salt concentration increased, there was a progressive decline in embryo wet weight. The average wet weight of embryos of the control was 55.4 mg, while embryos exposed to 600, 1200, 2400, 4800 and 9600 mg L^{-1} Cl^- had average wet weights of 51.8, 49.1, 47.1, 44.7 and 44.5 mg, respectively. A significant decline in wet weight relative to the control was first observed at 1200 mg L^{-1} Cl^- ($p = 0.05$; Fig. 5).

3.4. Experiment 3

Experiment 3 investigated the impact of added $CaCO_3$ in embryos

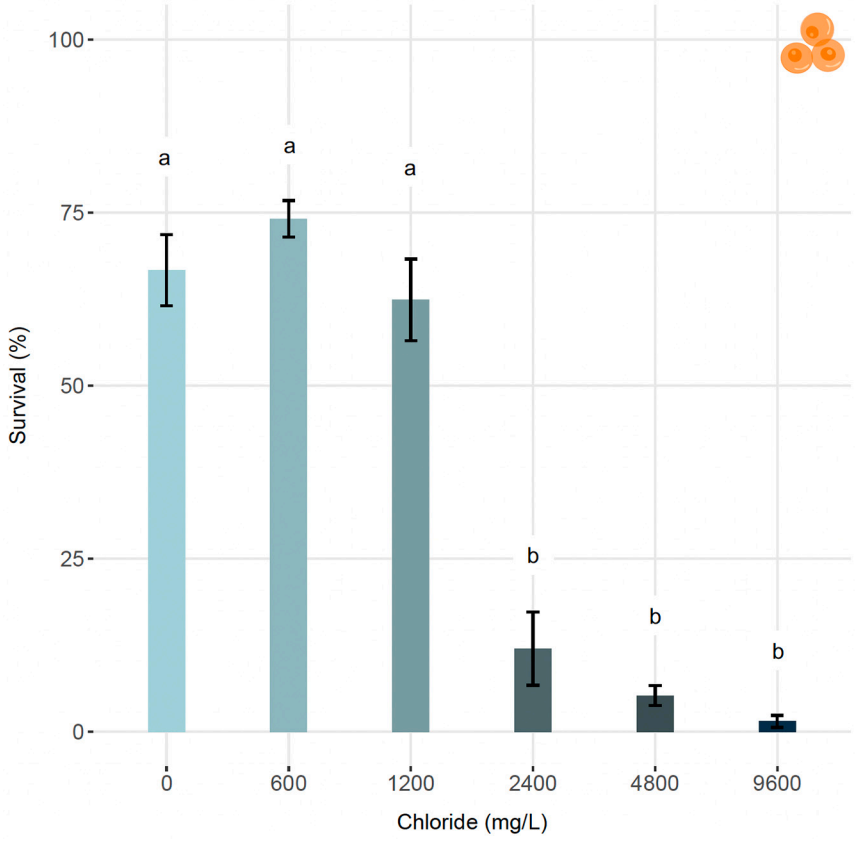

Fig. 3. Mean survival (%) ± SEM of rainbow trout following a salt exposure <1 h post-fertilization in Experiment 2 (same protocol as Experiment 1). Embryos were exposed to a salt pulse for 24-h, beginning <1 h post-fertilization (N = 4, with ~40 embryos per replicate). Lowercase letters that differ indicate statistically significant differences ($p \leq 0.05$) in survival among the different concentrations of salt exposure.

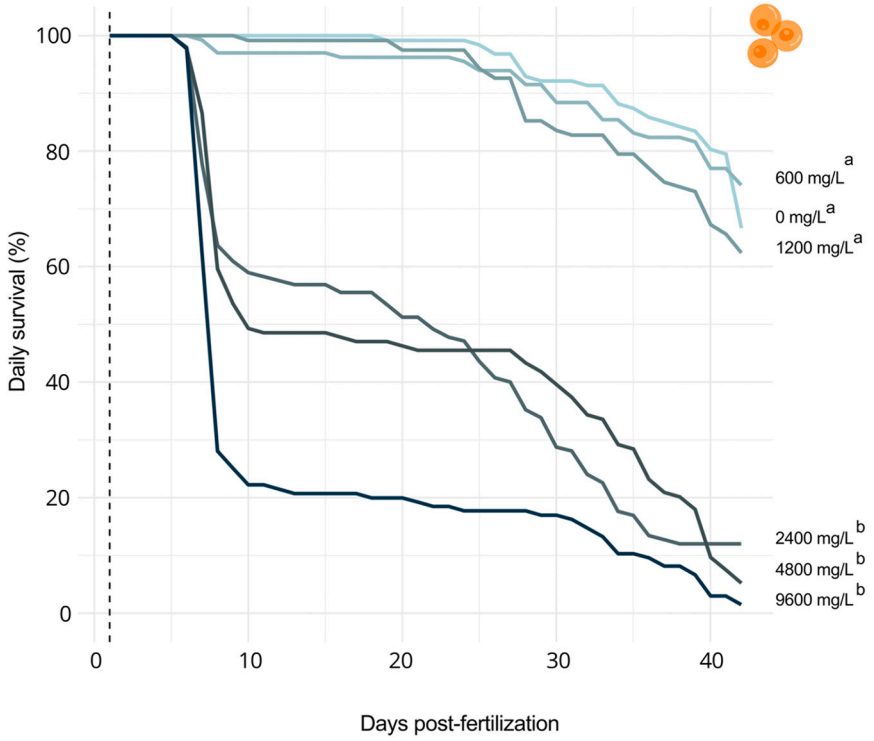

Fig. 4. Daily survival (%) of rainbow trout following a 24-h salt exposure <1 h post-fertilization in Experiment 2 (same protocol as Experiment 1). The dashed lined indicates the end of the 24-h salt exposure that began <1 h post-fertilization (N = 4, with ~40 embryos per replicate). Lowercase letters indicate statistically significant differences ($p \leq 0.05$) in survival among the different concentrations of salt exposure.

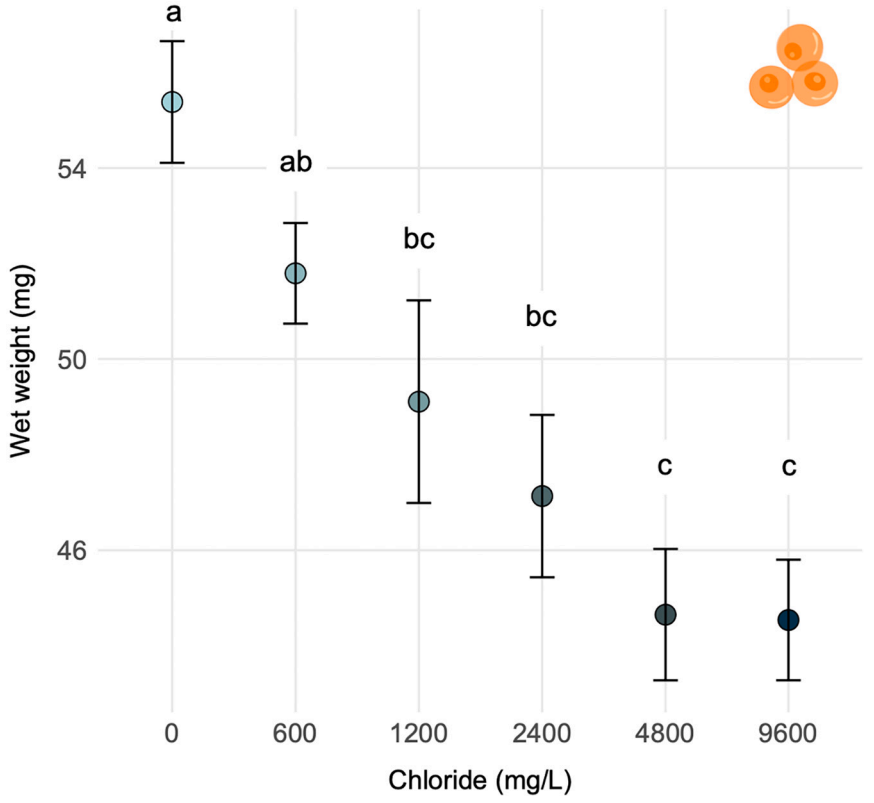

Fig. 5. Experiment 2, mean embryo wet weight ± SEM at the end of the 24-h salt exposure starting at <1 h post-fertilization (n = 8). Lowercase letters indicate significant differences ($p \leq 0.05$) among groups.

exposed to rock salt at <1 h post-fertilization.

3.4.1. Survival

There was a significant effect of $CaCO_3$ on survival at the eyed-stage (Fig. 6), as detected using a two-way ANOVA. There was a significant effect of $CaCO_3$ ($p < 0.0001$), chloride exposure ($p < 0.0001$), and a significant interaction between these factors ($p < 0.0001$). The control groups without added $CaCO_3$ had an average survival of 98.5 % which did not significantly differ from the 98.8 % survival of controls with added $CaCO_3$ ($p = 1.0$). All three salt exposure groups with added $CaCO_3$ exhibited significantly higher survival compared to each corresponding salt exposure without $CaCO_3$ ($p < 0.03$). Embryos exposed to 1200, 1800 and 2400 mg L^{-1} Cl^- without added $CaCO_3$ had an average survival of 32.2 %, 14.1 % and 1.9 %, respectively, all differing significantly compared to their control ($p < 0.001$). Average survival for 1200, 1800 and 2400 mg L^{-1} Cl^- with added $CaCO_3$ was 90.6 %, 62.3 % and 18.8 %, respectively (Fig. 6).

3.4.2. Whole-embryo ions 24-h post-fertilization

In Experiment 3, we examined the ion concentrations in embryos immediately following their 24-h salt exposure at <1 h post-fertilization. A two-way ANOVA for embryo Ca^{2+} concentrations found no significant effects of the presence or absence of $CaCO_3$ ($p = 0.348$), chloride exposure ($p = 0.648$), or interaction ($p = 0.372$). Ca^{2+} concentrations in salt exposed-embryo did not significantly differ compared to their respective controls with or without the presence of $CaCO_3$ and were all about 15 μmol g^{-1} ($p > 0.05$; Fig. 7A; Table 1S A).

Embryo K^+ concentrations were significantly affected by the presence or absence of $CaCO_3$ ($p < 0.0001$; Two-way ANOVA) and there was an insignificant effect of chloride concentration ($p = 0.475$), but a significant interaction ($p = 0.023$). Embryo K^+ concentrations (~40 μmol g^{-1}) did not differ in control solutions with or without $CaCO_3$ (all $p > 0.05$; Fig. 7B). However, embryo K^+ concentrations were significantly elevated to about 43 μmol g^{-1} in the 1800 and 2400 mg L^{-1} Cl^- exposures in the absence of $CaCO_3$, significantly higher compared to the same salt exposures with $CaCO_3$ present (~38 μmol g^{-1}, $p < 0.003$; Fig. 7B; Table 1S A).

Embryo Na^+ concentrations were significantly affected by the presence or absence of $CaCO_3$ ($p = 0.036$) and a significant effect of chloride exposure ($p < 0.0001$) and a significant interaction between these factors ($p = 0.006$) as assessed using two-way ANOVA. Regardless of $CaCO_3$ exposure, embryos exposed to rock salt had higher Na^+ concentrations (~20 μmol g^{-1}) compared to their respective controls (~14 μmol g^{-1}) (all $p < 0.001$; Fig. 7C), as detected in post-hoc tests. In general, there were few differences in embryo Na^+ concentration across the three salt exposures either with or without $CaCO_3$, although embryos exposed to the highest salt concentration (2400 mg L^{-1} Cl^-) had significantly higher Na^+ concentrations than embryos exposed to 1200 mg L^{-1} and 1800 mg L^{-1} Cl^- ($p = 0.041$ and 0.002, respectively; Fig. 7C; Table 1S A).

3.4.3. Whole-embryo ions at the eyed-stage

In Experiment 3, we examined ion concentrations in embryos at the eyed-stage that had been previously exposed to elevated salinities for 24-h starting at <1 h post-fertilization and then subsequently allowed to develop to the eyed-stage in dechlorinated tap water with no added salt. A two-way ANOVA for Ca^{2+} concentrations in embryos found an insignificant effect of the presence or absence of $CaCO_3$ ($p = 0.089$), a significant effect of chloride concentration ($p = 0.018$), and a significant interaction ($p < 0.0001$). The control value without $CaCO_3$ (19.80 μmol g^{-1}) differed significantly from the control value with $CaCO_3$ (16.52 μmol g^{-1}; $p = 0.0001$), but no other significant differences associated with the presence of $CaCO_3$ at the same salt level were seen in any of the experimental treatments ($p > 0.5$; Fig. 8A). In the absence of $CaCO_3$, the concentration of Ca^{2+} in the eyed-stage embryos progressively decreased, reaching 18.13 μmol g^{-1}; $p = 0.004$) in those previously

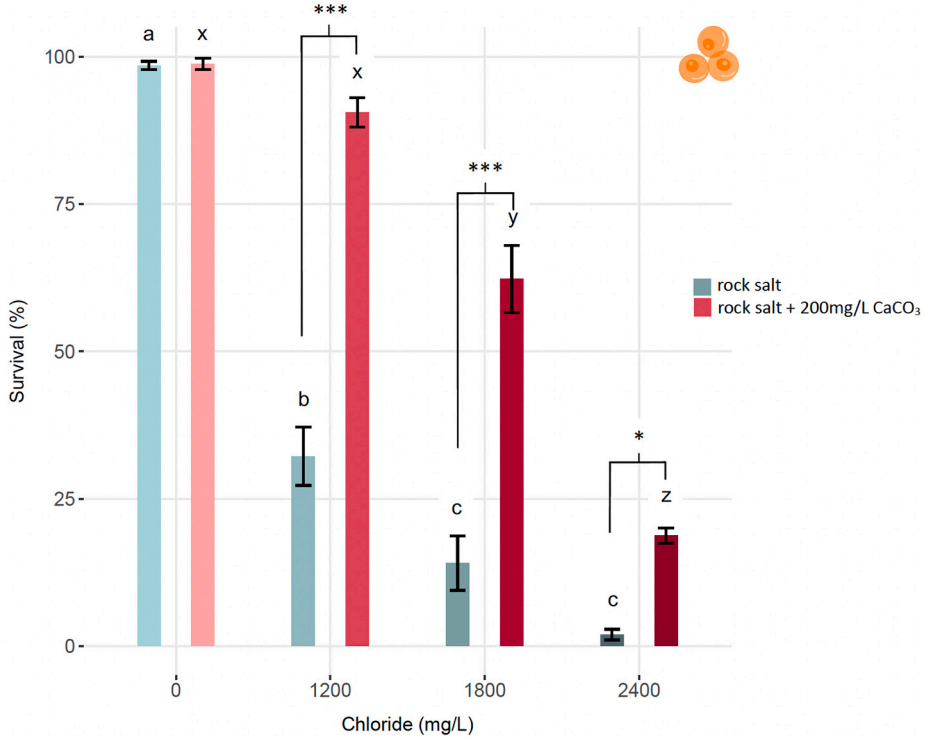

Fig. 6. Percent survival in Experiment 3 at the eyed-stage of embryos previously exposed to a 24-h salt pulse <1 h post-fertilization in the presence (red gradient) or absence (blue gradient) of 200 mg L^{-1} $CaCO_3$ (N = 5, with ~100 embryos per replicate). Lowercase letters that differ indicate statistically significant differences ($p \leq 0.05$) in mean survival ± SEM among the different concentrations of salt exposure (a, b, c and x, y, z, respectively). Asterisk (*) indicates significant differences in mean survival (%) across test solutions with and without 200 mg L^- $CaCO_3$. Significance $p < 0.0001$ and $p < 0.01$ indicated by '***', '*', respectively.

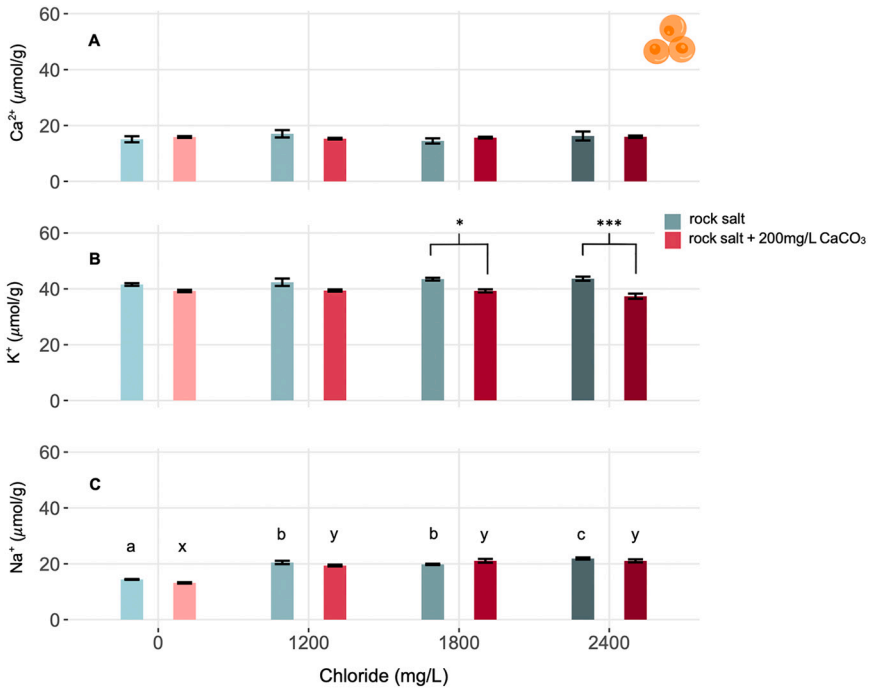

Fig. 7. Whole-embryo calcium (Ca^{2+}), potassium (K^+), and sodium (Na^+) ion concentrations relative to egg wet weight at the end of the 24-h salt exposure starting at <1 h post-fertilization in Experiment 3. Mean ion concentrations ± SEM in salt exposures without $CaCO_3$ are shown as blue bars and in corresponding salt exposures with 200 mg L^{-1} $CaCO_3$ are shown as red bars (n = 15). Lowercase letters that differ indicate statistically significant differences ($p \leq 0.05$) in embryo ion concentrations among the different concentrations of salt exposure (a, b, c and x, y, z, respectively). Asterisk (*) indicates significant differences in mean ion (µmol g^{-1}) between test solutions at the same chloride concentration with and without 200 mg L^{-1} $CaCO_3$. Significance $p < 0.0001$ and $p < 0.01$ indicated by '***', '*', respectively.

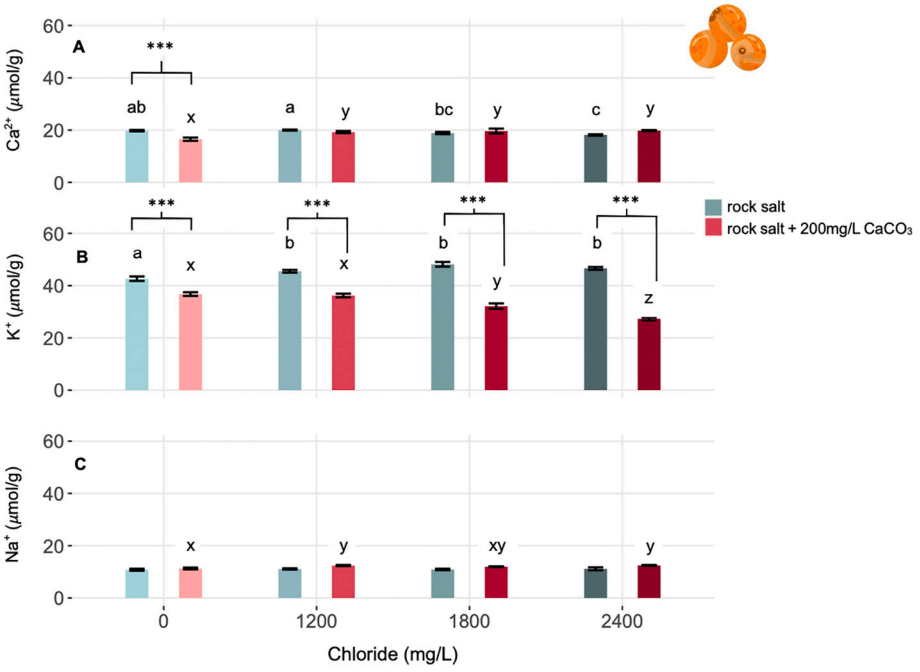

Fig. 8. Whole-embryo calcium (Ca^{2+}), potassium (K^+) and sodium (Na^+) ion concentrations relative to egg wet weight measured at the eyed-stage in embryo previously exposed to a 24-h salt pulse starting at <1 h post-fertilization, and then allowed to develop to the eyed-stage in Vancouver tap water without added Cl^- for 17 days in Experiment 3. Mean ion concentrations ± SEM of individuals in exposures without $CaCO_3$ are shown as blue bars and in corresponding salt exposures with 200 mg L^{-1} $CaCO_3$ are shown as red bars (n = 8–15). Lowercase letters that indicate statistically significant differences in embryo ion concentrations among the different concentrations of salt exposure ($p \leq 0.05$; a, b, c and x, y, z, respectively). Asterisk (*) indicates significant differences in mean ion (µmol g^{-1}) between test solutions at the same chloride concentration with and without 200 mg L^{-1} $CaCO_3$. Significance $p < 0.0001$ indicated by '***'.

exposed to 2400 mg L^{-1} Cl^-. In the presence of $CaCO_3$ during the exposures, this did not occur, with all chloride treatments having Ca^{2+} concentration of ~19.5 μmol g^{-1}, significantly higher relative to their control (16.52 μmol g^{-1}; all $p < 0.02$; Fig. 8A; Table 1S B).

K^+ concentrations in embryos showed a significant effect of the presence or absence of $CaCO_3$ ($p < 0.0001$), a significant effect of chloride concentration ($p < 0.0001$), and a significant interaction ($p < 0.0001$; two-way ANOVA). Embryos without $CaCO_3$, exhibited an increase in K^+ concentrations with elevated chloride concentrations in the earlier salt exposure, rising from 42.63 μmol g^{-1} in the controls to 46.61 μmol g^{-1} in those previously exposed to 2400 mg L^{-1} Cl^- ($p = 0.008$; Fig. 8B). With $CaCO_3$, embryos exposed to elevated chloride concentrations, exhibited a progressive decline in K^+ concentrations reaching 27.33 μmol g^{-1} in those previously exposed to 2400 mg L^{-1}, significantly lower compared to the $CaCO_3$ control, (36.78 μmol g^{-1} $p < 0.0001$; Fig. 8B; Table 1S B).

A two-way ANOVA for Na^+ concentrations detected a significant effect of the presence or absence of $CaCO_3$ ($p < 0.0001$), a significant effect of chloride concentration ($p = 0.007$) and an insignificant interaction ($p = 0.413$). Na^+ concentrations in embryos did not differ across salt exposures in treatments without $CaCO_3$ ($p > 0.9$) and were all ~11 μmol g^{-1}. However, embryos that had been exposed to 1200 mg L^{-1} and 2400 mg L^{-1} Cl^- with $CaCO_3$ (both ~12 μmol g^{-1}) exhibited significantly higher Na^+ concentrations compared to the $CaCO_3$ control (11.30 μmol g^{-1}; $p < 0.003$, Fig. 8C; Table 1S B).

4. Discussion

4.1. Survival

A key finding of this study is that a 24-h salt exposure at <1 h post-fertilization resulted in significant mortality at concentrations of 2400, 4800 and 9600 mg L^{-1} Cl^- (Fig. 2A), whereas none of the salt exposures negatively affected survival when applied at the eyed-stage or 7 days post-hatch (Fig. 2B, C). These data are consistent with the suggestion that as development progresses, embryos become increasingly resilient to a range of environmental stressors (Giroux and Schlenk, 2021; Park et al., 2023; Shen and Leatherland, 1978b, 1978a; Shephard and McWilliams, 1989; Zotin, 1958). At both <1 h post-fertilization and at the eyed-stage, embryos possess the chorion, perivitelline membrane, and perivitelline fluid (PVF), providing some protection against changes to external salinity. However, at <1 h post-fertilization, ionocytes are not yet differentiated, unlike at the eyed-stage (Ballard, 1973; González et al., 1996; Kaneko et al., 2002; Rombough, 1999). Ionocytes that line the yolk sac play a crucial role in regulating Na^+ and Cl^- transport (Degnan and Zadunaisky, 1980; Kaneko et al., 2002; Katoh et al., 2000). For example, eyed-chum embryos can reduce the osmolality of their blood with the activation of ionocytes in the yolk sac membrane amid a saltwater exposure (Kaneko et al., 2002). This may explain why mortality was observed following a salt pulse <1 h post-fertilization, but not at the eyed-stage (Fig. 2A, B).

Another vulnerable period is suggested to occur at hatch, with some studies indicating that alevins have a lower salt tolerance compared to even the eyed-stage (Brown and Lynam, 1981; Kitancharoen et al., 1997; Rombough, 1983). However, at both the eyed-stage and 7 days post-hatch, survival was not significantly affected by a salt pulse (Fig. 2B, C). While alevins showed no mortality following a 24-h salt pulse at even the highest salt concentration tested (9600 mg L^{-1} Cl^- at 14°C), Croke and McDonald (2002) reported low mortality in juvenile rainbow trout after a 24-h salt exposure at approximately 260 mM NaCl (~9200 mg L^{-1} Cl^-) at 16°C. Qualitatively, alevins in this study that were exposed to a 24-h salt pulse appeared normal at swim-up, but no sublethal measurements were taken.

Our finding that trout embryos are particularly sensitive to a 24-h salt pulse starting <1 h post-fertilization, as consistently observed in Experiments 1–3 (Figs. 2A, 3, & 6), aligns with earlier studies showing that immediately following fertilization, salmonid embryos are vulnerable to both elevated salinity (Girard and Gatti-Favereau, 1993; Mackinlay, 2004; Rudy and Potts, 1969; Yamamoto and Kobayashi, 1996; Zotin, 1958) and handling disturbance (Musialak et al., 2024). Additionally, gamete quality has been shown to affect toxicant sensitivity (Baker et al., 2015; Brix et al., 2010; Schreck et al., 2001), potentially explaining the higher mortality from salt pulses at 1200 and 1800 mg L^{-1} Cl^- in Experiment 3, in contrast to the salt pulses of the same concentrations in Experiments 1 and 2. In Experiment 3, milt motility appeared good upon arrival (see criteria in Environment Canada, 1998), but the shipment delay of the gametes likely led to a decline in egg quality, resulting in a greater salt sensitivity and consequently higher mortality (Fig. 6).

Immediately after fertilization, embryos undergo cellular division (Danner, 2008), so exposure to elevated NaCl at this time may impact blastomere formation, as observed in chum salmon embryos (Yamamoto and Kobayashi, 1996). Although blastomere adhesion was found to recover once embryos were returned to a hypotonic solution, the embryos were not reared beyond 48 h of development in that study, so it is unknown if the embryos would have experienced any delayed mortality. In the present study, significant mortality did not become evident until approximately seven days post-salt exposure in rainbow trout subjected to a salt pulse at <1 h post-fertilization (Fig. 4), which highlights the importance of monitoring organisms following acute exposures for longer periods than those often used in standardized regulatory testing.

4.2. Osmotic and ion regulation in embryo exposed to road salt

At the end of the 24-h salt exposure at <1 h post-fertilization, embryos decreased in weight when exposed to increasing salt concentrations (≥1200 mg L^{-1} Cl^-; Fig. 5). Mackinlay (2004) observed a similar trend with coho salmon, in which a decrease in fertilized egg weight was associated with softened eggs. Although a decline in embryos weight indicates that salt may have adverse effects on egg swelling and egg hardening, it is unclear whether these changes directly or indirectly contribute to the mortality observed in this study. Egg swelling is impacted by ionic solutions, but not solutions of similar osmolarities (e.g. urea, glucose or mannitol) (Bogucki, 1930; Girard and Gatti-Favereau, 1993; Potts and Rudy, 1969). Additionally, while a prolonged decrease in the perivitelline space (PVS) can adversely affect development and survival due to spatial restrictions on the embryo (Li et al., 1989), the composition of the perivitelline fluid (PVF) is seen to match external conditions within hours (Kaneko et al., 2002; Winnicki and Cykowska, 1973). Thus, when embryos in our study were transferred back to freshwater after a 24-h salt exposure, egg swelling would likely have resumed as the chorion is permeable to water. As a result, water would be drawn into the PVS until turgor pressure matching the osmotic pressure gradient is attained (Li et al., 1989; Potts and Rudy, 1969). However, regained turgidity and the re-establishment of the PVS do not ensure a recovery in egg hardness, as egg hardness requires mediated cortical granule release (Yamagami et al., 1992; Yamamoto, 1962), a process found to be blocked by NaCl (Blaxter, 1969; Zotin, 1958). As such, it is unlikely that a decrease in egg size is the primary cause of high mortality following a 24-h salt exposure <1 h post-fertilization, but an ion-specific disruption to the egg hardening process may be a contributing factor. Further research should explore if egg hardening can recover following acute salt exposures.

At the end of the 24-h salt exposure at <1 h post-fertilization, Na^+ concentration in whole-embryos increased in the salt-exposed groups, regardless of the presence or absence of added $CaCO_3$ (Fig. 7C). Normally, salmonid embryos excrete Na^+ and K^+ after fertilization (Girard and Gatti-Favereau, 1993); however, the rise in internal Na^+ concentrations associated with salt exposure could disrupt embryo's ionoregulatory capabilities. In this regard, monovalent cations have been shown to inhibit the osmoregulatory function of embryo (Eddy and Talbot, 1983; Girard and Gatti-Favereau, 1993), while this negative

effect has not been observed with divalent cations (Eddy and Talbot, 1983; Rudy and Potts, 1969; Yamamoto and Kobayashi, 1996). Alternatively, since the egg chorion is relatively permeable to Na^+, but the vitelline membrane appears to have some ion regulatory control of Na^+ (Barrett et al., 2001), the increase in whole-embryo Na^+ concentrations (Fig. 7C) may be due to Na^+ accumulating in the PVF rather than in the tissue of the embryo, as seen in trout embryos exposed to 11 % saltwater three days post-fertilization (Shen and Leatherland, 1978b). Interestingly, in embryos sampled at the eyed-stage, Na^+ concentrations in whole-embryos were elevated, but only in those that had been co-exposed to $CaCO_3$ (Fig. 8C), indicating a persistent response.

4.3. Protective effects of $CaCO_3$ and persistent sublethal effects of an earlier salt exposure

The addition of $CaCO_3$ led to a significant increase in survival when individuals experienced a 24-h salt pulse <1 h post-fertilization (Fig. 6). An increase in external Ca^{2+} has been shown to mitigate the toxicity of other environmental salts (Brown and Lynam, 1981; Elphick et al., 2011b, 2011a; Erickson et al., 2022a). Erickson et al. (2022b, 2022a) found that NaCl exhibited specific ion-related toxic effects in solutions with relatively low concentrations of Ca^{2+}, similar to the Vancouver water used in the present study. In fish, external Ca^{2+} has been demonstrated to reduce toxicity by decreasing gill permeability to major ions (Carrier and Evans, 1976; Cuthbert and Maetz, 1972; Hunn, 1985; Po and Wood, 2021; Potts, 1984), though it is unclear if similar effects of Ca^{2+} apply to salmonid embryo. Girard and Gatti-Favereau (1993) observed that the addition of Ca^{2+} or a Ca^{2+} ionophore recovered Na^+ efflux and chorion elevation. Since concentrations of Na^+ and Ca^{2+} did not differ in embryos sampled immediately after their salt exposure with or without $CaCO_3$ (Fig. 7A, C), the protective effect is challenging to decipher. However, the advantages of Ca^{2+} may be occurring at the embryonic level, where such effects cannot be detected through whole-embryo (embryo, PVF and chorion) measurements.

The K^+ concentration of whole-embryos differed significantly in embryos exposed to 2400 and 4800 mg L^{-1} Cl^- with and without $CaCO_3$ (Fig. 7B). Interestingly, this difference in K^+ concentrations was exacerbated at the eyed-stage, approximately 17 days after the salt pulse (Fig. 8B). In the absence of $CaCO_3$ during the salt exposures, K^+ concentrations in embryos increased (Figs. 7B, 8B). Shen and Leatherland (1978b) found that K^+ concentrations increased at both the level of the egg and embryonic tissue during a chronic seawater exposure, though it should be noted that seawater K^+ concentrations (~380 mg L^{-1}) are more than an order of magnitude higher than the water K^+ concentrations measured in the present study (Tables 1 & 2). Conversely, when $CaCO_3$, was present during the salt exposures (Figs. 7B, 8B), embryo's K^+ concentrations decreased as the previous salt concentration increased. This suggests that either the Ca^{2+} ion interfered with K^+ uptake from the water during the salt exposure, or that the 0.5 unit rise in pH (Table 2) and/or elevated HCO_3^-/CO_3^{2-} concentrations had this effect. Thus, elevated K^+ concentrations in embryos exposed to a salt pulse without $CaCO_3$ reflects disrupted development, suggesting that some embryos may not successfully develop come hatch. To conclude, the mechanisms behind the disturbances in embryo's K^+ concentration that persisted 17 days after the salt exposure, and the interactive influence of co-exposure to $CaCO_3$ during the salt pulses are "legacy effects" that cannot be explained at present but deserve further investigation.

It is well known that across vertebrates, an increase in internal Ca^{2+} occurs following fertilization of the egg, which is essential for the onset of embryonic development (Kashir et al., 2013; Paudel et al., 2018; Stricker, 1999; Wakai et al., 2011; Yamamoto, 1962). Research has shown that salmonid embryo uptake Ca^{2+} after fertilization (Danner, 2008; Khlebovich et al., 1977; Martemyanov, 2014). While the chorion seems to be permeable to Na^+, it has a mediated transport mechanism for Ca^{2+} (Barrett et al., 2001). This may explain why there was no significant difference in Ca^{2+} concentrations in embryos exposed to a 24-h salt pulse <1 h post-fertilization, regardless of added $CaCO_3$, as carrier-mediated transport could be saturated (Fig. 7A). Despite numerous studies investigating the role of Ca^{2+} in embryonic development, there is no clear consensus on the function of external Ca^{2+}, complicating the interpretation of how Ca^{2+} is mitigating mortality caused by a 24-h salt pulse at <1 h post-fertilization. Some researchers propose that Ca^{2+} is vital for egg activation (Zotin, 1958), water hardening (Kusa, 1949; Luchi et al., 1991; Warren et al., 1947) and blastula development (Alderdice, 1988). Conversely, others suggest that development can persist in Ca^{2+} free environments with the substitution of other divalent cations (Girard and Gatti-Favereau, 1993; Iwamatsu et al., 1985; Yamamoto and Kobayashi, 1996). The impact of external Ca^{2+} on early salmonid development and its reduction of salt toxicity is intricate and emphasizes the need for further research. Nevertheless, the increased survival observed with the addition of $CaCO_3$ during a 24-h salt pulse at <1 h post-fertilization, underscores the significance of developing site-specific water quality guidelines, as toxicity varies considerably with water hardness and ionic composition (Elphick et al., 2011b, 2011a; Erickson et al., 2022b, 2022a).

4.4. Environmental relevance

This study reveals that road salt contamination may harm developing salmonids, as the ecologically relevant salt concentrations that we tested resulted in significant mortality when embryos were exposed immediately following fertilization (Fig. 2A). Mortality did not occur at levels up to 2× BC's acute guideline (1200 mg L^{-1} Cl^-), but a substantial decrease in survival was observed once chloride concentrations reached 4× the acute guideline (2400 mg L^{-1} Cl^-). Recent water quality data for the Vancouver Lower Mainland (VLM) area indicate that approximately 6 % of salt pulses reach or surpass 2400 mg L^{-1} Cl^- through the late fall and winter (Kilgour et al., 2025). Although the majority of salt pulses recorded in that field data set occurred at colder temperatures, our study was conducted at water temperatures between 10 and 15°C, representative of the temperatures at which rainbow trout spawn from March to late June (Scott and Crossman, 1973; Roberge et al., 2002). Based on field data, salt pulses exceeding 2400 mg L^{-1} Cl^- rarely occur in the VLM at water temperatures above 9°C, while the majority occur at ~6°C (Kilgour et al., 2025). The occurrence of these road salt pulses coincides with the spawning period of other salmonid species such as coho and chum salmon in local streams. In the VLM, Pacific salmon spawn in very soft, ion-poor water and this study demonstrates that this has implications for salt toxicity. Additionally, the physiological effects of a salt exposure may have synergistic effects when combined with low temperatures, as low temperatures could exacerbate ionoregulatory stress (Cossins et al., 1995). Therefore, results in this study may be conservative in estimating the threats of road salt contamination on developing Pacific salmon.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110334.

CRediT authorship contribution statement

Carley E. Winter: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Clare L. Kilgour:** Writing – review & editing, Conceptualization. **Colin J. Brauner:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Patricia M. Schulte:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Chris M. Wood:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All raw data for this publication are publicly available at https://borealisdata.ca/dataset.xhtml?persistentId=doi:10.5683/SP3/2M97XM.

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