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Modeling the accumulation of CO_2 during high density, re-circulating transport of adult Atlantic salmon, *Salmo salar*, from observations aboard a sea-going commercial live-haul vessel

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ABSTRACT

A major water quality concern for salmon welfare during closed-hold transport at sea is the progressive accumulation of CO_2 generated via fish respiration. Experiments showed that loads in excess of 13,000 adult Atlantic salmon, *Salmo salar*, being transported at an average density of 135 ± 4 kg m^{-3}, experienced a rapid deterioration of water quality with the partial pressure of CO_2 (P_{CO2}) increasing from 0.51 ± 0.04 mm Hg to 2.49 ± 0.38 mm Hg and water pH decreasing from 7.56 ± 0.13 to 7.23 ± 0.14 over a 30-min period of closed-hold transport. Using these data, a mathematical model was generated to establish possible transport times under various fish density and environmental conditions in closed-hold conditions. The model predicted that, if the P_{CO2} level was not to exceed 10 mm Hg (1 mm Hg = approx. 0.133 kPa), a significantly elevated but typically sub-lethal exposure, and if the fish consumed oxygen at a routine rate measured under similar conditions, then closed-hold seawater transport could be extended to between 56 and 150 min for fish densities of 70–170 kg m^{-3}. If oxygen consumption rates were elevated for some reason during the transport at constant temperature, these periods would be reduced proportionately. If they occurred in conjunction with an elevation in temperature which reduces CO_2 solubility, the effects are even greater. Therefore, closed-hold transport is predicted to present significant risks to fish welfare due to the rapid accumulation of CO_2. Given the general inability to accurately measure CO_2 levels onboard current live-haul vessels, the present model provides an important tool to minimize disturbances to fish metabolic status during closed-hold transport.

1. Introduction

Efficient, cost-effective transport in the salmon aquaculture industry requires the live-hauling of large numbers of fish at high densities. If transport conditions are poor, the welfare of thousands of fish is put at significant risk. High fish densities, poor water quality, and excessive handling and disturbances can all cause significant stress during transport. In modern sea-going fish transport vessels, water is supplied using flow-through of ambient water or water re-circulated within the ship. While flow-through conditions are aimed at maintaining a steady-state water quality, they do not prevent the exposure of fish to potentially dangerous ambient water conditions, such as algal blooms or oil spills. Such risks are averted when seawater in the live-haul tanks is re-circulated, but other risks arise. Water re-circulation may result in rapid depletion of oxygen levels because a large mass of fish is being held in a relatively small water volume. While supplemental oxygenation mitigates O_2 depletion, metabolic excretory products such as CO_2 and NH_3 accumulate in the water, resulting in a progressive deterioration of water quality.

Few studies have examined the direct effects of CO_2 buildup during closed-hold transport in salmonids, although numerous have documented the significant stress that closed-hold transport can cause (Barton et al., 1980; Specker and Schreck, 1980; Barton and Peter, 1982; Nikinmaa et al., 1983; Maule et al., 1988; McDonald et al., 1993; Erikson et al., 1997; Iversen et al., 1998; Barton, 2000; Iversen et al., 2005; Nomura et al. 2009). Laboratory studies suggest that elevated water CO_2 concentrations (hypercarbia), which increase the partial pressure of CO_2 (P_{CO2}), will activate the primary stress responses in fish (Perry et al., 1989) and cause significant physiological disturbances including: a reduction in plasma pH (Eddy et al., 1977; Thomas and Le Ruz, 1982); increased ventilation rate and volume (Janssen and Randall, 1975; Thomas et al., 1983; Fivelstad et al., 1999); reduced blood O_2 content and carrying capacity through Bohr and Root effects (Eddy and Morgan, 1969; Eddy et al., 1977; Wedemeyer, 1996);

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narcosis, anesthesia and eventually death at higher levels (Bernier and Randall, 1998).

Surprisingly, non-lethal thresholds for CO_2 exposure have not been described for commercial transport of adult Atlantic salmon, *Salmo salar*. Currently, a CO_2 threshold for Atlantic salmon of 12–30 mg L^{-1} has been suggested for rearing conditions, when the impacts of CO_2 on growth, feed conversion rate, and immune status must be considered (Smart et al., 1979; Wedemeyer, 1996; Fivelstad et al., 1998). Wedemeyer (1996) suggests that salmonids can likely tolerate levels of up to 40 mg L^{-1} under transport conditions due to the short exposure period involved. However, CO_2 thresholds described in mg L^{-1} units cannot be applied universally, as the CO_2 concentration varies in combination with other factors (i.e. water temperature and salinity) to determine the P_{CO2} of a solution. The P_{CO2} of a solution, which is the biologically relevant measure of CO_2 toxicity for fish, can therefore occur within a range of CO_2 concentrations. In this study and others, the CO_2 concentrations given are only representative of the conditions in which they were measured. The P_{CO2} of oceanic surface water is usually very low, in the range of 0.15–0.30 mm Hg and the P_{CO2} of a fish's bodily fluids is only 1–2 mm Hg above that of its environment (Heisler, 1986) despite continuous CO_2 production by tissues. Due to the high ventilation volume required for O_2 uptake and the high capacitance of water for CO_2, the gills can be considered hyperventilated with respect to CO_2 excretion, which maximizes CO_2 removal from the blood. The permeability of fish gills to CO_2 is also high, and hypercarbic conditions are rapidly equilibrated internally, elevating the Pco_2 of the blood and tissues (hypercapnia). The low P_{CO2} of surface waters and fish blood means that fish are highly susceptible to even small elevations in environmental P_{CO2}. The log-linear relationship between P_{CO2} and pH results in large changes in pH with small increases in P_{CO2} at the low P_{CO2} tensions that typically exist in natural waters. Once inside the fish, CO_2 is rapidly equilibrated throughout the body and converted into carbonic acid (catalyzed by carbonic anhydrase), resulting in a hypercapnic acidosis proportional to the degree of hypercarbia experienced.

Therefore, hypercarbia is capable of causing stress and negatively impacting fish welfare. Indeed, studies investigating re-circulating or closed conditions report significant stress during transport as indicated by elevated plasma cortisol concentrations (Barton et al., 1980; Specker and Schreck, 1980; Barton and Peter, 1982; Barton, 2000), plasma glucose and lactate concentrations (Mazeaud et al., 1977; Nikinmaa et al., 1983; Carneiro and Urbinati, 2002), or mortality rates (Specker and Schreck, 1980; Carmichael, 1984; Pavlidis et al., 2003). Despite this knowledge, and the expectation that CO_2 could accumulate to potentially dangerous levels, there is a lack of information regarding the rate of CO_2 accumulation during live-haul transport.

With the help of the *Sterling Carrier*, a state-of-the-art live-haul vessel operating on the west coast of British Columbia, Canada, we observed fish under closed-hold transport and here provide the first measurements of the rate of CO_2 accumulation under commercial live-haul conditions. These experiments were necessarily limited in duration to ensure the safety of the transported fish, but the collected data provided the basis for a mathematical model of CO_2 accumulation under various salmon transport scenarios. In the model, bulk O_2 consumption rates (bulk $\dot{M}O_2$) of adult Atlantic salmon measured during similar live-haul transport conditions (Tang et al., 2009) were used to estimate CO_2 accumulation rates using the respiratory exchange ratio (RER, the molar ratio of CO_2 excreted and O_2 consumed).

For the purposes of this study, an arbitrary P_{CO2} threshold was set at 10 mm Hg (or a CO_2 concentration of ~17–20 mg L^{-1}, within range of water conditions experienced during live-transport in BC; i.e. water temperatures of 8–14 °C and water salinities of 25–35 ppt; Tang et al., 2009), which is currently suggested as the threshold for rearing conditions (Fivelstad et al., 1998) and represents a significant elevation over environmental levels (~50× normal) but one that is typically non-lethal. By modeling different transport scenarios from the data collected during actual transports, we assessed the risk to fish welfare during high density live-haul transport of adult Atlantic salmon up to a 10 mm Hg threshold of CO_2 exposure though the models we developed can be extrapolated to levels beyond this threshold.

2. Materials and methods

2.1. Transport vessel

A detailed description of the *Sterling Carrier* and its equipment is documented in Farrell (2006). Briefly, the *Sterling Carrier* is a 40 m vessel with two 325 m^3 live-holds that are flooded via a series of 14" hydraulic valves (four forward and three aft valves per hold), producing a directional water flow rate of ~50 $m^3 min^{-1}$ from forward to aft while the vessel is traveling at 9 kn (routine transport speed for the vessel). If the hull valves are closed, water in each hold is re-circulated through separate loops using pumps capable of re-circulating water at 20 $m^3 min^{-1}$. To maintain dissolved O_2 concentrations, pure O_2 gas is injected directly into the re-circulating water through spargers prior to re-entering the holds through a pair of perforated PVC diffusing columns that run lengthwise along the bottom of each hold.

2.2. Closed-hold transport protocol

Six transport experiments were conducted (July 9–13, 2006 and November 13–16, 2006) during routine commercial transports around Vancouver Island, BC. Each experiment started at least 3.5 h into each trip to allow recovery from loading stressors and ended at least 2 h before arrival at the processing plant to allow fish to recover before off-loading. A single experiment was conducted on each trip to ensure true replication. The number of fish per transport was between 13,454 and 14,914 at an average fish density of 135 ± 4 kg m^{-3} (average fish mass of 5.7 ± 0.2 kg, average water temperature of 10.6 ± 1.2 °C, average salinity of 31 ± 1 ppt).

Visual observation of fish was possible from the hatches on deck and from closed-circuit cameras located in the holds. Fish behaviour was monitored by scientists on deck and the captain on the bridge during all experiments to ensure that there were no signs of fish distress.

2.3. Water monitoring and sampling

Hull valves were closed in the starboard hold for 30 min for each re-circulation experiment. During this time, water parameters were recorded and water samples taken every 5 min during re-circulation and then for 15 min following re-establishment of flow-through conditions when hull valves were re-opened, resuming external flushing of the holds.

A water sampling circuit was set up using a submersible pump (5 L min^{-1}) sampling water from the starboard hold at a depth of 2 m. Water pH was measured using a pH probe (Symphony 14002-764, VWR Scientific) attached to a handheld meter (Symphony SP301, VWR) inserted into a 1 L Erlenmeyer flask. A 3-way stop-cock allowed water to be sampled from the circuit into 250 ml BOD (Biochemical Oxygen Demand) bottles. Water samples were drawn such that they overflowed the BOD bottles, a small amount (~10 mL) was poured out so that 2.5 mL of saturated $HgCl_2$ could be added (to prevent microbial respiration) and the lid inserted. All samples were analyzed for total CO_2 content within 7 days. Water leaving the circuit was returned to the hold below the surface.

Dissolved O_2 concentration was maintained above 7.0 mg L^{-1} during the experiments by injecting pure O_2 using onboard oxygenation systems and was monitored by onboard O_2 sensors mounted inside the holds (Oxyguard III, Point Four Systems, Richmond, BC, Canada) and a handheld O_2 meter (Oxyguard Handy, Dynamic Aqua-

Supply, Surrey, BC, Canada). Water pH and temperature were measured during the onboard water sampling and during analysis of total CO_2 using an epoxy combination pH/temperature electrode (Symphony 14002-764, VWR Scientific) and handheld pH meter (Symphony SP301, VWR Scientific).

2.4. Water CO_2 measurements

Total CO_2 (TCO_2—representing the concentration of all species of CO_2 in water) was analyzed at the University of British Columbia from water samples on a gas chromatograph (Carle Model III, Fullerton, California, USA) connected to a chart recorder (Soltec 1241, San Fernando, California, USA) as described in Brauner et al. (2000). A 2 mL water sample, 4 mL of 1 M HCl saturated with N_2 gas and 6 mL of N_2 gas were drawn into a 10 mL syringe and mixed on a rotary mixer for 4 min. As the water was acidified, the CO_2 expelled into the N_2 gas phase was forced through a dehydrating filter directly into the sampling loop of the gas chromatograph.

TCO_2 was calculated from the area under the peak against standard curves measured from fresh bicarbonate standards made daily ($NaHCO_3$, Sigma-Aldrich, Oakville, Ontario, Canada). P_{CO2} was calculated from TCO_2 and pH using the Henderson–Hasselbalch equation (Eq. (1)).

Henderson–Hasselbalch equation:

$$P_{CO2} = \frac{TCO_2}{\alpha 10^{pH-pK^1} + 1} \quad (1)$$

P_{CO2} partial pressure of CO_2 (mmHg)
TCO_2 total CO_2 in solution (mmol L^{-1})
α solubility constant of CO_2 (mmol L^{-1} mm Hg^{-1})
pH pH of solution
pK^1 first dissociation constant of CO_2

2.5. CO_2 accumulation model

A model was created to examine the effects of various recirculating transport conditions on water quality. The input transport water parameters included: temperature, salinity, initial water pH, background TCO_2/P_{CO2}; the transport fish density; and the fish physiological parameters including: RER and $\dot{M}O_2$ (as a specific value or as percentage of the maximum $\dot{M}O_2$).

The model predicted changes in water P_{CO2} due to fish respiration over time according to the following equation:

$$P_{CO2} = \frac{TCO_{2ac} + TCO_{2bg}}{\alpha 10^{pH-pK^1} + 1} \quad (2)$$

TCO_{2ac} moles of accumulated total CO_2 from respiration (mmol L^{-1})
TCO_{2bg} moles of background total CO_2 (mmol L^{-1})

The amount of CO_2 created through respiration is calculated from the equation:

$$TCO_{2ac} = \frac{(\dot{M}O_2 \times RER \times mass) \times t}{V_r} \quad (3)$$

$\dot{M}O_2$ O_2 consumption rate (input as mg O_2 $min^{-1} kg^{-1}$, converted to mmol O_2 $min^{-1} kg^{-1}$)
RER respiratory exchange ratio (moles CO_2 excreted per moles O_2 consumed). RER can vary between 0.7 and 1.3, increasing with activity level and in relation to the tissue level O_2 consumption and CO_2 production ratio, or respiratory quotient (RQ). An RER of 1.0 was used in the present modeling, but this can be varied.
mass total fish mass per hold (kg)
t time (min)
V_r water volume of hold (l)

The solubility (α) and first dissociation constant of CO_2 (pK^1) are derived from tables presented in Boutilier et al. (1984) and Mehrbach et al. (1973) (Table 1). The accumulation of TCO_2 in the system drives both an increase in P_{CO2} and decrease in pH. However, in order to calculate the P_{CO2} at a given TCO_2 concentration using the Henderson–Hasselbalch equation, we need to know the water pH. Due to the variability in starting water pH values and the variability inherent in the TCO_2 measurement method, we found that our ability to predict water pH from TCO_2 was relatively poor outside of the range of the collected samples. Instead, we generated a water pH/P_{CO2} curve from the collected water samples (Fig. 1) which had a much better fit, and which allowed us to accurately predict changes in water pH as water P_{CO2} increased. However, since the model provides a TCO_2 concentration (Eq. (3)), in order to calculate the water P_{CO2} (Eq. (2)) from the modeled TCO_2, we need to first predict the water pH, from some P_{CO2} value. In order to overcome this complication, we generated TCO_2 values in 1-min increments and calculated P_{CO2} using the water pH from the previous increment (with the first P_{CO2} calculated from the starting water pH, which is given by the user). As this method will inherently overestimate water pH and thus underestimate the increase in water P_{CO2}, we minimized this error by using small time increments, as the change in water pH between each incremental increase in TCO_2 would be very small and thus more accurately reflect the actual pH conditions from which P_{CO2} was calculated. The amount of error in the water P_{CO2} accumulates with each progressive calculation, reaching nearly 10% after 1000 calculations (1000 min). Shortening the time increments reduced the error introduced in water P_{CO2} between each calculation, but increased the accumulated error over a set period of time since more calculations were required. By using 1-min increments in this study, water P_{CO2} was modeled for extended periods, while limiting total calculation error to less than 2% for the longest time period relevant to closed-hold transport.

For the modeling experiments, we used $\dot{M}O_2$ values that spanned the range previously measured as bulk $\dot{M}O_2$ for adult Atlantic salmon during transport (Tang et al., 2009). By using a full range of $\dot{M}O_2$ values, we considered fish under different 'stress' levels. Highly stressed fish are consuming large amounts of O_2, producing proportional amounts of CO_2, increasing the rate of CO_2 accumulation in the hold and decreasing the amount of time required to reach a specific P_{CO2} threshold. Stressful conditions preceding or during closed-hold transport that elevate bulk $\dot{M}O_2$ can therefore have serious consequences for the health of transported fish. The routine $\dot{M}O_2$ (2.5 mg O_2 $min^{-1} kg^{-1}$) modeled during transport was based on the bulk $\dot{M}O_2$ measurements made by Farrell (2006) and the average bulk $\dot{M}O_2$ measured from 45 transports of 10 h or longer (Tang et al., 2009). The maximum bulk $\dot{M}O_2$ was estimated from the maximum bulk $\dot{M}O_2$ measured by Farrell (2006) (>8 mg O_2 $min^{-1} kg^{-1}$) during the first few minutes of transport and the knowledge that $\dot{M}O_2$ values greater than 8 mg O_2 $min^{-1} kg^{-1}$ have been measured from individuals of numerous salmonid species. For example: 12 mg O_2 $min^{-1} kg^{-1}$ in adult sockeye salmon, *Oncorhynchus nerka* (Farrell et al., 2003); 13 mg O_2 $min^{-1} kg^{-1}$ in adult pink salmon, *O. gorbuscha* (Farrell et al.,

Table 1
Constants used in CO_2 accumulation model.

Constant used in model	Value	Source
CO_2 solubility (α)	Temperature 0–20 °C	Boutilier et al. (1984)
CO_2 dissociation (pK)	Salinity 25–35 ppt	Mehrbach et al. (1973)
Hold volume (V_W) =	325 m^3	
Molar weight O_2	32.00 g mol^{-1}	
Molar weight CO_2	44.01 g mol^{-1}	

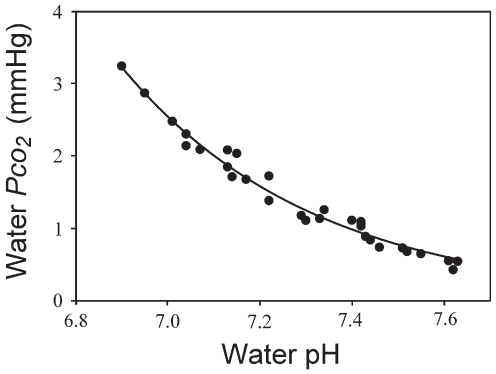

Fig. 1. Relationship between water P_{CO2} and water pH in closed-hold water samples. Water P_{CO2} was calculated from total CO_2 and water pH measured from water samples taken during 30 min closed-hold experiments aboard the *Sterling Carrier*. Solid circles represent individual data points.

2003); and 10 mg O_2 $min^{-1}kg^{-1}$ in adult coho salmon, *O. kisutch* (Lee et al., 2003). Adult Atlantic salmon are likely capable of a maximal $\dot{M}O_2$ of at least 8 mg O_2 $min^{-1}kg^{-1}$, justifying its use in the modeling experiments. These $\dot{M}O_2$ values were applied to a range of loading densities (70–170 kg m^{-3}) that were being routinely used during live-haul aboard the *Sterling Carrier*.

3. Results

3.1. Closed-hold transport of adult Atlantic salmon

P_{CO2} of water re-circulating in the live-haul holds of the *Sterling Carrier* increased at a near linear rate and water pH decreased non-linearly during the 30-min re-circulation periods (Fig. 2A–B). During this period, P_{CO2} increased from 0.51 ± 0.04 mm Hg ([CO_2] of 1.2 mg L^{-1} under the tested conditions) to 2.49 ± 0.38 mm Hg ([CO_2] of 5.3 ± 0.2 mg L^{-1}, under the tested conditions) and water pH decreased by 0.3 units from 7.56 ± 0.13 to 7.23 ± 0.14. Incorporating water pH data from 3 additional experiments (from which we were unable to analyze the preserved water samples for TCO_2) showed that mean water pH dropped from 7.76 ± 0.11 to 7.34 ± 0.09 over a 30-min period of re-circulation (Fig. 2C). During the later stages of re-circulation, fish were observed to increase activity and flush to a brighter blue color, suggesting that they were responding to the changing water quality conditions (i.e. P_{CO2} ~ 2.5 mm Hg and pH ~ 7.3).

Ambient water P_{CO2} and pH conditions could be quickly restored after each experiment. After just 15 min of flushing the live-holds with fresh seawater, P_{CO2} had recovered to 0.78 ± 0.10 mm Hg (Fig. 2A) and pH had recovered to 7.82 ± 0.09 (Fig. 2C), neither of which were significantly different from ambient water conditions ($p > 0.05$).

3.2. CO_2 accumulation model

As expected, the time for the re-circulated water to reach the 10 mm Hg P_{CO2} threshold was directly proportional to fish density. The 10 mm Hg P_{CO2} threshold (a [CO_2] of 19.6 mg L^{-1} under the tested conditions) was reached after 150 min at low fish density (70 kg m^{-3}) at a routine bulk $\dot{M}O_2$ (2.5 mg O_2 $min^{-1}kg^{-1}$). Increasing the density to 170 kg m^{-3} correspondingly reduced the time to reach the threshold to 56 min (Fig. 3A).

Elevating bulk $\dot{M}O_2$ in the model similarly reduced the time required to reach the 10 mm Hg P_{CO2} threshold. At the maximal rate of $\dot{M}O_2$ used here, the 10 mm Hg P_{CO2} threshold was exceeded at 48 min with a fish density of 70 kg m^{-3} and after only 19 min with a fish density of 170 kg m^{-3} (Fig. 3B).

Water pH decreases in conjunction with increases in P_{CO2} and, under the modeled conditions, reached pH 6.60 ± 0.01 at 10 mm Hg P_{CO2}. Changes in fish density or bulk $\dot{M}O_2$ affected the rate of water pH decrease (Fig. 3C–D) but had no significant effect on the final water pH since it was constrained by the 10 mm Hg P_{CO2} threshold.

The time to reach the 10 mm Hg P_{CO2} threshold was examined with respect to different ambient water temperature and salinity conditions. An increase in temperature elevates $\dot{M}O_2$ (thereby simultaneously elevating $\dot{M}CO_2$) and also reduces CO_2 solubility. Consequently, higher water temperatures resulted in a large decrease in the time required to reach the P_{CO2} threshold. Changes in salinity have a smaller effect on the rate of P_{CO2} increase within the range of the model (25–35 ppt) as it

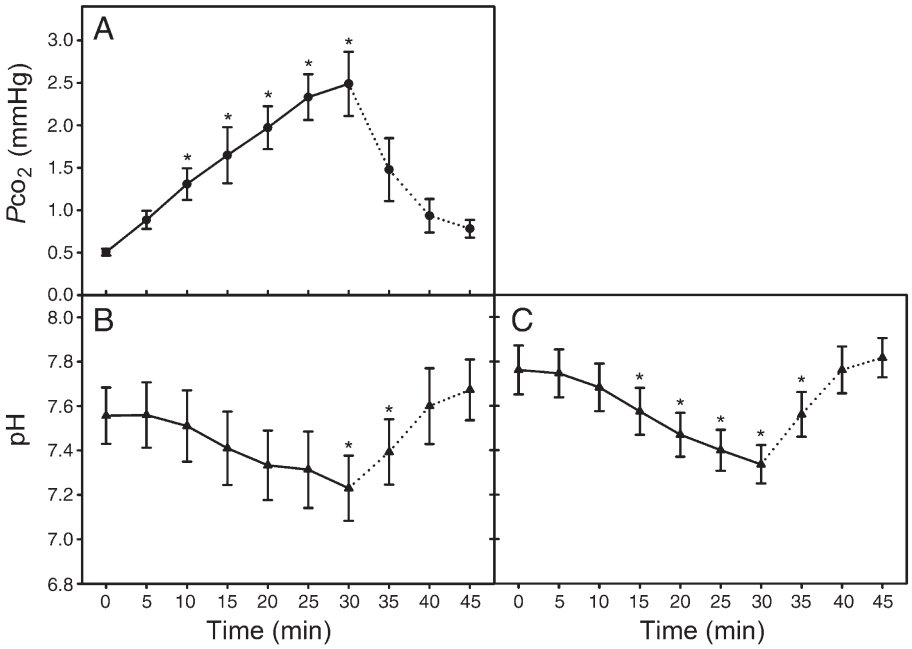

Fig. 2. Changes in water P_{CO2} and water pH during 30 min re-circulating transport. Measured changes in A) water P_{CO2} calculated from total CO_2 in water samples ($n = 3$) and B) water pH ($n = 3$) during 30 min re-circulating transport of 6.0 ± 0.1 kg Atlantic salmon at 135 ± 4 kg m^{-3} (8.7 ± 0.2 °C). C) Water pH measured for all 30 min re-circulating transports of 5.7 ± 0.2 kg Atlantic salmon at 127 ± 5 kg m^{-3} (11.0 ± 0.2 °C, $n = 6$, we were unable to measure total CO_2 for 3 trips). Broken line represents first 15 min of recovery following resumption of open circulation. *indicates values significantly different from starting conditions (paired *t*-test, $p < 0.05$).

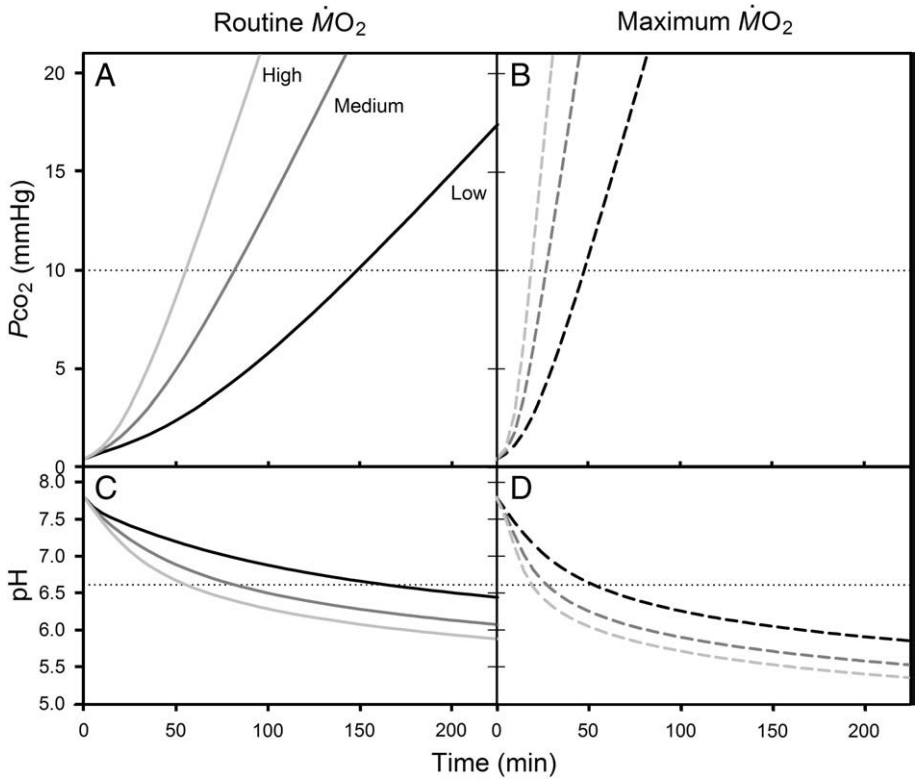

Fig. 3. Modeled changes in water quality during various closed-hold transport scenarios. Simulated P_{CO2} accumulation at routine $\dot{M}O_2$ (2.5 mg O_2 $min^{-1}kg^{-1}$, solid lines) and maximum $\dot{M}O_2$ (8.0 mg O_2 $min^{-1}kg^{-1}$, dashed lines) across a range of loading densities; low density (70 kg m^{-3}), medium density (120 kg m^{-3}) and high density (170 kg m^{-3}). (A) Under low stress conditions, low loading density allows a re-circulation time of up to 150 min while at a high density this time is reduced to 56 min. (B) Under high stress conditions, low loading density levels allow re-circulation time of 48 min while high loading densities further reduce the time to reach the 10 mm Hg CO_2 threshold to 19 min. The associated water pH changes at routine $\dot{M}O_2$ (C) and maximum $\dot{M}O_2$ (D) at low, medium and high loading densities show that water pH decreases with increasing water P_{CO2}. The arbitrary threshold P_{CO2} of 10 mm Hg is reached at a water pH of 6.6 (dotted lines) under all scenarios. All trips were modeled using the same water conditions: temperature = 10 °C, salinity = 30 ppt, starting water pH = 7.8.

affects only CO_2 solubility and not $\dot{M}O_2$ or $\dot{M}CO_2$. Thus, temperature had substantial effects on the rate of CO_2 accumulation and P_{CO2} increase over the range of the model (0–20 °C) (Table 2).

4. Discussion

During transport with re-circulated water, fish welfare is at risk because water quality deteriorates with the continuous accumulation of CO_2. This study represents the first time that the potential impact of hypercarbia on welfare has been assessed and modeled for closed-hold conditions aboard a sea-going, live-haul vessel carrying adult Atlantic salmon at commercial densities. We found that water P_{CO2} increased in a nearly linear fashion, with a concomitant drop in water pH during a 30-min period of re-circulation. Even so, re-opening the hull valves (to flush the holds with ambient seawater) rapidly reduced P_{CO2} levels and completely restored water quality within 15 min. During closed-hold experiments, the water pH was continuously monitored, and any experiment was terminated if water pH fell below pH 7.0, indicating a P_{CO2} of ~6.5 mm Hg based on local water conditions. After a 30-min period of re-circulation, P_{CO2} was still well below the recommended CO_2 threshold of 10 mm Hg. Other salmonids have been exposed to P_{CO2} levels ranging from 15 mm Hg (Eddy et al., 1977) to 78 mm Hg (Bernier and Randall, 1998) without mortality and full recovery was possible following return to normocarbic conditions. Water pH was also substantially higher than the sub-lethal pH limits of 4.0–4.5, as assessed by Butler et al. (1992) for brown trout (*Salmo trutta*) in freshwater, and still within the recommended pH range of 6.5–8.5 (Wedemeyer, 1996). Thus, the P_{CO2} and pH levels fish experienced during the present experiments were not expected to present a serious threat to fish welfare.

The 10 mm Hg water P_{CO2} threshold was based on the findings of Fivelstad et al. (1998), who assessed safe levels of CO_2 exposure during the rearing of post-smolt Atlantic salmon. Their results showed that exposure to P_{CO2} levels up to 12 mm Hg did not produce any significant decreases in growth factors or increases in mortality rate. However, their observed mortality rate of 1.1% at 12 mm Hg (i.e. one fish died after 43 days of exposure in one of the two replicate groups) would be unacceptable during typical transports, where mortalities are <1%. The mortality rate on the *Sterling Carrier* was only 0.21% for 1.44 million fish transported in 116 trips during the period of October 2005–March 2006.

Table 2
Effect of water temperature and salinity on time required to reach P_{CO2} threshold.

Activity level	Temperature (°C)	$\dot{M}O_2$	Salinity	Time required to reach 10 mm Hg P_{CO2} threshold (min)	Final pH at 10 mm Hg P_{CO2}
Routine	5	1.8	30	153	6.60
	10	2.5	30	83	6.61
	15	3.5	30	42	6.64
	10	2.5	25	97	6.62
	10	2.5	35	71	6.60
Maximum	5	5.7	30	49	6.61
	10	8.0	30	27	6.62
	15	11.3	30	15	6.66
	10	8	25	32	6.64
	10	8	35	23	6.61

The time to reach the P_{CO2} threshold decreases as water temperature increases because of the associated increase in $\dot{M}O_2$ (and associated increase in $\dot{M}CO_2$) and decrease in CO_2 solubility. Increasing salinity also reduces the time to reach the P_{CO2} threshold by reducing CO_2 solubility, but has no effect on $\dot{M}O_2$ in this range. Transport conditions were modeled at medium density (70 kg m^{-3}), using a temperature coefficient of 2.0 for $\dot{M}O_2$ (Tang et al., 2009) and a starting water pH of 7.8.

Monitoring P_{CO2} during closed-hold live-haul presents its own challenges. Accurate methods of measuring P_{CO2} are complicated, time-consuming and expensive and are currently impractical for a commercial live-haul setting. The widely used colorimetric titration methods are time-consuming, can be inaccurate and are poorly suited for monitoring progressively increasing P_{CO2} levels. Since water P_{CO2} and water pH are closely related in a closed-system, changes in water P_{CO2} are almost immediately reflected by changes in water pH. Water pH measurements can be taken quickly, accurately and could be used to continuously estimate P_{CO2} levels in hauling water during a closed-hold event, provided there was an accurate calibration between water pH and P_{CO2} for the ambient salinity and temperature conditions. Under the water conditions examined here, it is evident that once water pH dropped below pH 6.6, the 10 mm Hg threshold was exceeded (Fig. 3C–D). The concomitant decrease in water pH with CO_2 addition also affects the rate of P_{CO2} increase. In typical seawater, with fish providing a steady rate of CO_2 addition, P_{CO2} will increase in a not quite linear fashion over time as TCO_2 increases and water pH decreases. This property of the system is important when considering P_{CO2} thresholds, as increasing the threshold level does not provide a proportional increase in safe transport time (i.e. doubling the P_{CO2} threshold to 20 mm Hg would not provide closed transport times that were exactly twice as long as those reported here).

While the hypercapnia accompanying hypercarbia is associated with the activation of primary stress responses in fish, it is not hypercapnia *per se* but the concurrent reduction in blood O_2 content due to pH-related Bohr and Root shifts of hemoglobin saturation and binding properties that are primarily responsible for increases in plasma catecholamine levels in trout (Perry et al., 1989). It has been suggested that the stress associated with elevated levels of CO_2 can be alleviated if blood O_2 content can be maintained above hypoxic levels, possibly by combining hypercarbia with hyperoxia (Dejours, 1975). However, when O_2 is below saturation levels, hypercarbia will exacerbate the effect of environmental hypoxia due to pH effects on blood O_2 transport, making the monitoring of O_2 and CO_2 levels equally important during closed-hold transport.

Prolonged exposures to high CO_2 levels can also directly affect O_2 consumption rates. Elevating P_{CO2} was found to depress $\dot{M}O_2$ at higher tensions (Basu, 1959), even though comparable reductions in water pH have been shown to elevate $\dot{M}O_2$ by as much as 40% (Butler et al., 1992). The anesthetic properties of CO_2 on fish were first described by Fish (1943), although relatively high tensions (>30 mm Hg) are required for anesthetic action (Bernier and Randall, 1998). It is thought that CO_2 narcosis may occur during severe hypercapnic acidosis by reducing brain pH (Yoshikawa et al., 1991; Yoshikawa et al., 1994). With P_{CO2} tensions below 10 mm Hg, the anesthetic effect is minimal, and the gradual accumulation of CO_2 during transport is much less likely to induce narcosis than abrupt changes.

The initial introduction of fish to hypercarbic conditions can trigger the stress response, resulting in a transient elevation of $\dot{M}O_2$ (Thomas et al., 1983). Kikkawa et al. (2006) found that up to 85% of juvenile Japanese sillago (*Sillago japonica*) were able to survive step-wise increases in P_{CO2} to over 50 mm Hg over a period of 18 h, but an immediate introduction to the final P_{CO2} tensions resulted in 100% mortality within 15 min. Moreover, the juvenile Japanese sillago also experienced significant mortality when they were abruptly returned to normocapnic water from P_{CO2} tensions of above 50 mm Hg.

We did not expose fish to 10 mm Hg P_{CO2} because of the concern for fish welfare. Indeed, we observed behavioural changes at a level well below this threshold. This meant that we had to model the closed-hold conditions that would result in a threshold of 10 mm Hg P_{CO2} being reached. The results of our model show that threshold conditions can be reached after about 2.5 h under the most favorable conditions (lowest fish density and MO_2) and in as little as 19 min (highest fish density and $\dot{M}O_2$; Table 3).

Table 3
Estimated times to reach 10 mm Hg P_{CO2} threshold under various transport conditions compared to values predicted by Sanni and Forsberg (1996).

Density ($kg\ m^{-3}$)	Bulk $\dot{M}O_2$ ($mg\ O_2\ min^{-1} kg^{-1}$)	Time required to reach 10 mm Hg P_{CO2} threshold (min)	
		Current model	Sanni and Forsberg (1996)
70	2.5	150	153
70	8.0	48	48
170	2.5	56	63
170	8.0	19	20

(Modeling conditions; temperature = 10 °C, salinity = 30 ppt, volume = 325 m^3).

Moran et al. (2008) showed that simulated transport of juvenile yellowtail kingfish at high density resulted in elevations in P_{CO2} of up to 38 mm Hg partway through a 5-h transport. Despite these adverse conditions, fish experienced low mortality (0.5%), no significant changes in secondary stress indicators (glucose, lactate) and were able to recover from initial haematological disturbances during both constant and variable hypercapnic exposure. Barton and Peter (1982) found that while water CO_2 increased (from 6 mg L^{-1} to 48 mg L^{-1}) during the closed transport of juvenile rainbow trout, plasma cortisol peaked at 0.5 h and had recovered to pre-transport levels after 8 h.

Other studies have used modeling to predict CO_2 production in freshwater systems (Colt and Orwicz, 1991) and flow-through aquatic culture systems (Sanni and Forsberg, 1996). Colt and Orwicz (1991) determined that the capacity for O_2 consumption would be reduced with the addition of CO_2 in a freshwater closed production system, when $\dot{M}O_2$ is the limiting factor. Sanni and Forsberg (1996) developed a predictive model for changes in water CO_2 and water pH in high-intensity flow-through seawater production systems, using a similar approach to modeling changes in water pH and TCO_2 in response to the CO_2 added by fish respiration. The Sanni and Forsberg model also used input water pH, TCO_2 and $\dot{M}O_2$ to directly calculate output water pH and a comparison to our approach yielded comparable results for estimating output water pH and times to reach a 10 mm Hg P_{CO2} threshold under various conditions (Table 3). The main difference in our approaches was Sanni and Forsberg calculated output water pH directly from the inputs (water pH, TCO_2, $\dot{M}O_2$) by re-arranging the equations describing simple aqueous carbonate solutions and relating carbonate alkalinity to carbonate equilibrium constants, whereas we used water quality data to generate a pH/Pco_2 equation combined with the Henderson–Hasselbalch equation to derive output water pH from the inputs in a series of interative, step-wise calculations.

The effects of water temperature and/or salinity on the pH/P_{CO2} relationship were small. However, as water temperature increases, CO_2 solubility decreases which alone would decrease the time to reach the 10 mm Hg P_{CO2} threshold. Furthermore, water temperature also increases $\dot{M}O_2$ (and therefore $\dot{M}CO_2$) which exacerbates the problem. Many studies have shown that $\dot{M}O_2$ is highly temperature dependent in salmonids, typically with a temperature coefficient (Q_{10}) of around 2.0 (Clarke and Johnston, 1999; Lee et al., 2003; MacNutt et al., 2006). Tang et al. (2009) also showed that the Q_{10} of bulk $\dot{M}O_2$ of adult Atlantic salmon during transport was 1.9–2.0. We would then expect a 10 °C increase in water temperature to potentially double the rate of $\dot{M}O_2$, doubling the rate of CO_2 (and ammonia) production, which in conjunction with the reduction in CO_2 solubility, would substantially reduce the time required to reach the P_{CO2} threshold as illustrated in Table 2. In comparison, the effects of salinity are less marked but still important (Table 2).

In many re-circulating systems, the accumulation of metabolically derived ammonia is also a concern as un-ionized ammonia (NH_3) is highly toxic to fish. Elevated levels of ammonia may also affect $\dot{M}O_2$ in fish due to the activation of the stress response, increased activity levels or increased metabolic costs associated with ion regulation. Smart (1978) found that rainbow trout exposed to lethal concentrations of

ammonia (273 mg L^{-1} TAN in freshwater at 15 °C, pH 6.9) had a greater than 3-fold increase in $\dot{M}O_2$. During re-circulated water transport, the effects of ammonia on $\dot{M}O_2$ will probably be negated by the narcotic effects of CO_2, which is excreted at 5–10 times the rate of ammonia during respiration (Randall and Wright, 1989), and the toxic effects of CO_2 will be manifested much earlier than those of ammonia. In addition, the concomitant decrease in water pH with increasing CO_2 will serve to reduce ammonia toxicity by reducing the proportion of ammonia existing in the un-ionized form (Thurston et al., 1981). Finally, the starvation of fish prior to transport minimizes ammonia production and excretion during transport. Our preliminary re-circulation study conducted during a commercial transport on Dec. 4, 2004 (density = 115 kg m^{-3}, average fish mass = 5.9 kg), found that total ammonia concentration did not exceed 0.36 mg NH_3–N L^{-1} during a 90-min re-circulation experiment, which was well below the acute lethal concentration of 22 mg NH_3–N L^{-1} and recommended safe concentration for intensive rearing purposes of 3.4 mg NH_3–N L^{-1} for rainbow trout (in sea water at 15 °C, pH 8.0; from Smart, 1981).

We conclude that closed-hold transport for limited periods of time can be accomplished at the typical densities and masses involved in commercial live-haul of adult Atlantic salmon without seriously compromising fish welfare. The ability to measure CO_2 levels is crucial to this process, as factors such as density and stress levels can significantly affect the rate of CO_2 accumulation. By measuring water pH as a surrogate for P_{CO2}, the water quality, and the risk to fish welfare can be accurately and continuously monitored during closed-hold transport conditions as water quality conditions during such transports will quickly deteriorate beyond typical conditions for salmonids. However, due to the log-linear relationship of pH and P_{CO2}, as pH drops towards the pK of the CO_2/HCO_3^- reaction (~5.9 in sea water at 10 °C, 30 ppt, from Mehrbach et al. 1973) much larger increases in P_{CO2} are required to produce relatively small decreases in water pH, reducing the effectiveness of using water pH as a monitoring indicator in high Pco_2/low pH conditions. Up to a P_{CO2} of 10 mm Hg, changes in water pH would still be large enough to accurately monitor incremental changes in P_{CO2}. Therefore there is a need to establish accurate relationships between pH and CO_2 across the range of water conditions routinely encountered during transport and to calculate pH thresholds for specific P_{CO2} levels. This approach will enable live-haul operators to avoid exposing fish to CO_2 levels above 10 mm Hg during closed-hold transport in order to protect fish welfare.

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References

Barton, B.A., 2000. Salmonid fishes differ in their cortisol and glucose responses to handling and transport stress. N. Am. J. Aquac. 62, 12–18.
Barton, B.A., Peter, R.E., 1982. Plasma-cortisol stress response in fingerling rainbow trout, *Salmo gairdneri* Richardson, to various transport conditions, anesthesia, and cold shock. J. Fish Biol. 20, 39–51.
Barton, B.A., Peter, R.E., Paulencu, C.R., 1980. Plasma-cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subjected to handling, confinement, transport, and stocking. Can. J. Fish. Aquat. Sci. 37, 805–811.
Basu, S.P., 1959. Active respiration of fish in relation to ambient concentrations of oxygen and carbon dioxide. J. Fish. Res. Board Can. 16, 175–212.
Bernier, N.J., Randall, D.J., 1998. Carbon dioxide anaesthesia in rainbow trout: effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. J. Fish Biol. 52, 621–637.
Boutilier, R.G., Heming, T.A., Iwama, G.K., 1984. Appendix: physicochemical parameters for use in fish respiratory physiology. Academic Press, Orlando, Florida. 403–430 pp.
Brauner, C.J., Thorarensen, H., Gallaugher, P., Farrell, A.P., Randall, D.J., 2000. CO_2 transport and excretion in rainbow trout (*Oncorhynchus mykiss*) during graded sustained exercise. Respir. Physiol. 119, 69–82.
Butler, P.J., Day, N., Namba, K., 1992. Interactive effects of seasonal temperature and low pH on resting oxygen uptake and swimming performance of adult brown trout *Salmo trutta*. J. Exp. Biol. 165, 195–212.
Carmichael, G.J., 1984. Long-distance truck transport of intensively reared largemouth bass. Prog. Fish-Cult. 46, 111–115.
Carneiro, P.C.F., Urbinati, E.C., 2002. Transport stress in matrinxa, *Brycon cephalus* (Teleostei: Characidae), at different densities. Aquac. Int. 10, 221–229.
Clarke, A., Johnston, N.M., 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. J. Anim. Ecol. 68, 893–905.
Colt, J., Orwicz, K., 1991. Modeling production capacity of aquatic culture systems under fresh-water conditions. Aquac. Eng. 10, 1–29.
Dejours, P., 1975. Principles of comparative respiratory physiology. North-Holland Publishing Company, Amsterdam.
Eddy, F.B., Lomholt, J.P., Weber, R.E., Johansen, K., 1977. Blood respiratory properties of rainbow trout (*Salmo gairdneri*) kept in water of high CO_2 tension. J. Exp. Biol. 67, 37–47.
Eddy, F.B., Morgan, R.I.G., 1969. Some effects of carbon dioxide on blood of rainbow trout *Salmo gardneri* Richardson. J. Fish Biol. 1, 361–372.
Erikson, U., Sigholt, T., Seland, A., 1997. Handling stress and water quality during live transportation and slaughter of Atlantic salmon (*Salmo salar*). Aquaculture 149, 243–252.
Farrell, A.P., 2006. Bulk oxygen uptake measured with over 60,000 kg of adult salmon during live-haul transportation at sea. Aquaculture 254, 646–652.
Farrell, A.P., Lee, C.G., Tierney, K., Hodaly, A., Clutterham, S., Healey, M., Hinch, S., Lotto, A., 2003. Field-based measurements of oxygen uptake and swimming performance with adult Pacific salmon using a mobile respirometer swim tunnel. J. Fish Biol. 62, 64–84.
Fish, F.F., 1943. The anesthesia of fish by high carbon dioxide concentrations. Trans. Am. Fish. Soc. 25–29.
Fivelstad, S., Haavik, H., Lovik, G., Olsen, A.B., 1998. Sublethal effects and safe levels of carbon dioxide in seawater for Atlantic salmon postsmolts (*Salmo salar* L.): ion regulation and growth. Aquaculture 160, 305–316.
Fivelstad, S., Olsen, A.B., Kloften, H., Ski, H., Stefansson, S., 1999. Effects of carbon dioxide on Atlantic salmon (*Salmo salar* L.) smolts at constant pH in bicarbonate rich freshwater. Aquaculture 178, 171–187.
Heisler, N., 1986. Acid–base regulation in animals. Elsevier Science Publishers B.V., Amsterdam.
Iversen, M., Finstad, B., McKinley, R.S., Eliassen, R.A., Carlsen, K.T., Evjen, T., 2005. Stress responses in Atlantic salmon (*Salmo salar* L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. Aquaculture 243, 373–382.
Iversen, M., Finstad, B., Nilssen, K.J., 1998. Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts. Aquaculture 168, 387–394.
Janssen, R.G., Randall, D.J., 1975. Effects of changes in pH and pCO_2 in blood and water on breathing in rainbow trout, *Salmo gairdneri*. Respir. Physiol. 25, 235–245.
Kikkawa, T., Sato, T., Kita, J., Ishimatsu, A., 2006. Acute toxicity of temporally varying seawater CO2 conditions on juveniles of Japanese sillago (*Sillago japonica*). Mar. Pollut. Bull. 52, 621–625.
Lee, C.G., Farrell, A.P., Lotto, A., MacNutt, M.J., Hinch, S.G., Healey, M.C., 2003. The effect of temperature on swimming performance and oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*) salmon stocks. J. Exp. Biol. 206, 3239–3251.
MacNutt, M.J., Hinch, S.G., Lee, C.G., Phibbs, J.R., Lotto, A.G., Healey, M.C., Farrell, A.P., 2006. Temperature effects on swimming performance, energetics, and aerobic capacities of mature adult pink salmon (*Oncorhynchus gorbuscha*) compared with those of sockeye salmon (*Oncorhynchus nerka*). Can. J. Zool. (Revue Canadienne De Zoologie) 84, 88–97.
Maule, A.G., Schreck, C.B., Bradford, C.S., Barton, B.A., 1988. Physiological-effects of collecting and transporting emigrating juvenile chinook salmon past dams on the Columbia river. Trans. Am. Fish. Soc. 117, 245–261.
Mazeaud, M.M., Mazeaud, F., Donaldson, E.M., 1977. Primary and secondary effects of stress in fish—some new data with a general review. Trans. Am. Fish. Soc. 106, 201–212.
McDonald, D.G., Goldstein, M.D., Mitton, C., 1993. Responses of hatchery-reared brook trout, lake trout, and splake to transport stress. Trans. Am. Fish. Soc. 122, 1127–1138.
Mehrbach, C., Culberso, C.H., Hawley, J.E., Pytkowic, R.M., 1973. Measurement of apparent dissociation-constants of carbonic-acid in seawater at atmospheric-pressure. Limnol. Oceanogr. 18, 897–907.
Moran, D., Wells, R.M.G., Pether, S.J., 2008. Low stress response exhibited by juvenile yellowtail kingfish (*Seriola lalandi* Valenciennes) exposed to hypercapnic conditions associated with transportation. Aquac. Res. 39, 1399–1407.
Nomura, M., Sloman, K.A., von Keyserlingk, M.A.G., Farrell, A.P., 2009. Physiology and behaviour of Atlantic salmon (*Salmo salar*) smolts during commercial land and sea transport. Physiol. Behav. 96, 233–243.
Nikinmaa, M., Soivio, A., Nakari, T., Lindgren, S., 1983. Hauling stress in brown trout (*Salmo trutta*)—physiological-responses to transport in fresh-water or salt-water, and recovery in natural brackish water. Aquaculture 34, 93–99.
Pavlidis, M., Angellotti, L., Papandroulakis, N., Divanach, P., 2003. Evaluation of transportation procedures on water quality and fry performance in red porgy (*Pagrus pagrus*) fry. Aquaculture 218, 187–202.
Perry, S.F., Kinkead, R., Gallaugher, P., Randall, D.J., 1989. Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). Respir. Physiol. 77, 351–363.
Randall, D.J., Wright, P.A., 1989. The interaction between carbon-dioxide and ammonia excretion and water pH in fish. Can. J. Zool.-(Revue Canadienne De Zoologie) 67, 2936–2942.

Sanni, S., Forsberg, O.I., 1996. Modeling pH and carbon dioxide in single-pass seawater aquaculture systems. Aquac. Eng. 15, 91–110.
Smart, G.R., 1978. Investigations of toxic mechanisms of ammonia to fish gas exchange in rainbow trout (*Salmo gairdneri*) exposed to acutely lethal concentrations. J. Fish Biol. 12, 93–104.
Smart, G.R., Knox, D., Harrison, J.G., Ralph, J.A., Richards, R.H., Cowey, C.B., 1979. Nephrocalcinosis in rainbow trout *Salmo gairdneri* Richardson—the effect of exposure to elevated CO_2 concentrations. J. Fish Dis. 2, 279–289.
Specker, J.L., Schreck, C.B., 1980. Stress responses to transportation and fitness for marine survival in coho salmon (*Oncorhynchus kisutch*) smolts. Can. J. Fish. Aquat. Sci. 37, 765–769.
Tang, S., Brauner, C.J., Farrell, A.P., 2009. Using bulk oxygen uptake to assess the welfare of adult Atlantic salmon, *Salmo salar*, during commercial live-haul transport. Aquaculture 286, 318–323.
Thomas, S., Fievet, B., Barthelemy, L., Peyraud, C., 1983. Comparison of the effects of exogenous and endogenous hypercapnia on ventilation and oxygen-uptake in the rainbow trout (*Salmo gairdneri* R.). J. Comp. Physiol. 151, 185–190.
Thomas, S., Le Ruz, H., 1982. A continuous study of rapid changes in blood acid–base status of trout during variations of water pCO_2. J. Comp. Physiol., B. Biochem. Syst. Environ. Physiol. 148, 123–130.
Thurston, R.V., Russo, R.C., Vinogradov, G.A., 1981. Ammonia toxicity to fishes—effect of pH on the toxicity of the un-ionized ammonia species. Environ. Sci. Technol. 15, 837–840.
Wedemeyer, G.A., 1996. Physiology of Fish in Intensive Culture Systems. Chapman & Hall, New York. 60–109 pp.
Yoshikawa, H., Kawai, F., Kanamori, M., 1994. The relationship between the EEG and brain pH in carp, *Cyprinus-carpio*, Subjected To Environmental Hypercapnia At An Anesthetic Level. Comp. Biochem. Physiol., A Comp. Physiol. 107, 307–312.
Yoshikawa, H., Yokoyama, Y., Ueno, S., Mitsuda, H., 1991. Electroencephalographic spectral-analysis in carp, *Cyprinus-carpio*, Anesthetized With High-Concentrations Of Carbon-Dioxide. Comp. Biochem. Physiol., A Comp. Physiol. 98, 437–444.