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Assessment of carbon dioxide effects on salmon (*salmo salar*) yolk sac fry

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Declaration

I, Ransford Tawiah Frimpong, declare that this thesis is the outcome of my research investigations and discoveries. Other people's sources of information have been recognised, and a reference list has been included. This work has not been previously submitted to any other university for the award of any form of academic degree.

Signature: Ransford Tawiah Frimpong,

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Abstract

Fish are adversely affected by high carbon dioxide concentrations, hence data on their tolerance level are particularly crucial especially for intensive production methods such as flow through and recirculating aquaculture system (RAS) that can accumulate CO_2. The current study assesses how CO_2 affects Atlantic salmon yolk sac fry specifically on their growth in length and weight as well as survival. Yolk sac fry were subjected and grown in different concentrations of carbon dioxide 1.8, 1.9, 5.4, 9.6, 17.7, 29.3 and 60.0 CO_2 mg/l which are also labelled as groups in a replicate for a period of 33 days. A flow through system was used in this study for growing fish. Two methods were to induce the production of carbon dioxide in the fish tanks. The first was the addition of carbon dioxide in tanks to increase the CO_2 concentration to the desired levels and the other was addition of diluted HCl to water in tank which makes water acidic and indirectly increased the CO_2 levels except in the control 1.8mg/l tank that was not given any treatment with a pH range of 6.3 to 7.6 All other major water quality indicators fell within the range that would usually be considered as favorable for fish growth.

In the study it was revealed that pH reduction of water increased the production of carbon dioxide. Fish tanks exposed to 33 days CO_2 at concentration up to 60 mg/l experienced low mortality. The mean ultimate body weight and size in the control treatments 1.8 mg/l with a high pH and 1.9 mg/l with a low pH was significantly higher. For fish length, 1.8 mg/l with high pH was significantly higher to 17.7 mg/l, 29.3 mg/l and 60 mg/l but in terms of weight it was significantly higher to 29.3 mg/l and 60 mg/l. Also, for fish weight, 1.9 mg/l with a low pH was significantly higher to 17.7 mg/l, 29.3 mg/l and 60 mg/l but in terms of length, it was significantly higher to 29.3 mg/l and 60 mg/l. The growth of fish, considering weight gain and size were considerably impeded as carbon dioxide dosage was increased. No effects observed at 9.6 mg/l and below whilst from 17.7 mg/l and above fish had effects during the long period of exposure.

Key words: salmon yolk sac fry; carbon dioxide; pH; fish tank; fish weight; fish length; flow through

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1.0. Introduction

Production of fish in intensive system is being practiced using both flow-through system where there is a continuous flow through of water and recirculation technology to save water (Verdegem, Bosma et al. 2006) thus reducing fish production's environmental impact(Liu, Rosten et al. 2016). Recirculating aquaculture systems (RAS) and flow-through system necessitate a production increase, which raises several water quality concerns that must be considered, including the buildup of carbon dioxide (Mota, Martins et al. 2014). Maintaining good water quality is important and seen as a critical factor for the health, productivity, and welfare in intensive fish farming system.

Water quality in intensive fish farms is affected by the interactions of several physical and chemical components such as temperature, ammonia concentration , metal concentration, conductivity, pH, oxygen and carbon dioxide (Wurts and Durborow 1992). Salmonids are known to have low tolerance to poor water quality such as oxygen, ammonia and carbon dioxide as compared to other group of fish (Wedemeyer and Yasutake 1978). This has called for strict adherence to suggested and recommended levels of the important water quality parameters in intensive fish farming for good fish welfare (Hjeltnes, Bæverfjord et al. 2012) .

The increasing of CO_2 cases in intensive farming has created a need for a comprehensive understanding of the factors, sources and how increased levels of Carbon dioxide affect fish. Poor water quality can increase fish susceptibility to diseases. High levels of CO_2 not only directly damage fish but also indirectly by altering the physico-chemical characteristics of water, such as the pH and chemistry of toxic elements in an aquatic system (Fivelstad, Haavik et al. 1998). For example the toxicity of Carbon dioxide is relatively high at lower pH of water as already found in fresh water studies (Aslam, Navada et al. 2019) and also promotes the solubility of toxic elements like aluminum (Al), copper, lead, and zinc and raises their potential toxicity. (Aslam, Navada et al. 2019)

Carbon dioxide solubility in water depends on several factors including temperature and the chemical composition of the water. When dissolved in water, it reacts with the water

leading to the formation of a mixture of carbonic acid, bicarbonate and carbonate ions as demonstrated in the formulae below.

$CO_2+H_2O[\leftrightarrow]H_2CO[\leftrightarrow]H^+HCO_3+H^+CO_2^{[-1]}$

Hydrogen ions are released as CO_2 dissolves in water, lowering the pH of the water, as the concentration of bicarbonate that can be dissolved in water depends on pH and increase with pH. This means that CO_2 is dissolved as a bicarbonate in water in addition to be present as carbonic acid, and that more CO_2 are present as bicarbonate at high pH compared to low pH. However, from studies, if pH drops the bicarbonate is transformed to carbonic acid and CO_2 again.

The oxidation of carbohydrates, proteins, and lipids by the fish results in the production of carbon dioxide, which is then carried by the circulation and expelled through the gill membrane into water (Ellis, Urbina et al. 2017). From research the major causes of high Carbon dioxide in intensive fish farming includes poor water exchange as a results of limited water supply which leads to Carbon dioxide accumulation, high fish stocking density in tanks and biofilters from the metabolism of microbes in RAS system. It is established that biofilter contributed to 37% of the total production of Carbon dioxide in RAS (Summerfelt, Sharrer et al. 2004) and degassing as a technique to remove CO2 are sometimes insufficient in stripping the quantities of CO_2 produced (Summerfelt, Vinci et al. 2000). Factors such as run off, ground water upwelling, the residence time of CO_2 in water as well as biological and geological process also increase Carbon dioxide concentration.

The recommended safe levels of carbon dioxide requirement for fish farming in Norway issued by the Norwegian food Safety Authority is <15 mg/l as the threshold for fish production in intensive farming system (Hjeltnes, Bæverfjord et al. 2012) It is therefore seen that Carbon dioxide is an important parameter to regulate since it has been reported to have negative effects on aquatic environments and as a result can impact the growth (Smart, Knox et al. 1979)

Carbon dioxide causes wide range impact on fish physiology which is mostly negative (Pörtner, Langenbuch et al. 2004). Again according to research there are few data on the

long-term effects of CO_2 which are reduced growth rate, reproduction, and calcification (Ishimatsu and Kita 1999), short-term effects of CO_2 include, for example, disturbances in the animals' acid-base status, respiration, blood circulation, and nervous activities. Other research affirms substantial differences in the sensitivity of fishes to high levels of CO_2 which can be within and among species of fish (Wittmann and Pörtner 2013). For example there was one study where there was obvious variation in the study of effect of CO_2 on the growth rate of Atlantic salmon, one of the study found no effect (Hannan, Jeffrey et al. 2016) and the other found effect on the growth rate (Fivelstad, Olsen et al. 2003). Similar CO2 levels were employed in both trials, but the fish sizes and housing temperatures were different. So, to these variances, it is challenging to compare the results and forecast how other species would react and tolerate high CO_2 levels.

From research, there have been different predictions and suggestions of CO_2 levels that cause toxic effects on fish. Recent research by (Fivelstad, Kvamme et al. 2015) reported specific growth rates for Atlantic salmon post-smolts cultured for three months period at varying CO_2 levels (up to 34 mg/L) in flow through seawater, and determined that Specific growth rate (SGR) of fish would not be compromised until CO_2 reached 18.6 mg/L. But interestingly the same study also found evidence of nephrocalcinosis at a lower CO_2 concentration of 16 mg/L.

(Ishimatsu, Kikkawa et al. 2004) reported from their research when the following fish exposed to seawater equilibrated with 5% CO_2, adult Japanese amberjack, *Seriola quinqueradiata*, and bastard halibut, *Paralichthys olivaceus*, died after 8 and 48 hours, respectively. Only 20% of the *Starspotted smoothhound, Mustelus manazo*, a cartilaginous fish, did not survive after 72 hours at 7% CO_2. Also, when the larvae and eggs of the marine fish silver seabream *Pagrus major* was tested at the same seawater pH, CO_2-enriched saltwater was far more deadly as compared to the seawater that had been acidified with HCl.

In a 6-month study, by (Good, Davidson et al. 2010) rainbow trout(62.1g) were exposed to CO_2 concentrations of 8 and 24 mg/l in a flow through system. There were no group differences in growth, susceptibility to nephrocalcinosis, or other associated diseases, and survival was great >97%. According to the findings, rainbow trout raised in RAS to market

size can be exposed to Carbon dioxide concentrations as high as 24 mg/L without suffering any appreciable effects on their health or performance.

Also, the exposure of rainbow trout changed their normal swimming style when the carbon dioxide level was to (35 – 60) mg/L. There was total loss of equilibrium at about 150mg/L and also the induction of narcosis after 3 minutes at 14 degree Celsius. (Summerfelt, Sharrer et al. 2004).

Despite this (Aslam, Navada et al. 2019), also proposed dosages as low as 10 mg/L as a precaution. (Fivelstad, Waagbø et al. 2007) and other researchers have shown that CO_2 can build up in intensive production aquaculture systems and reach levels above the ones that are advised.

According to research elevated levels of CO_2 affects and alters physiological process which can reduce population size and the growth rate through failure in reproduction and the activeness of the fish (Ishimatsu, Hayashi et al. 2005). For example, the maximum metabolic rate (MMR) reduced in fresh water pink salmon (Oncorhynchus *gorbuscha)* and Atlantic salmon subjected to elevated CO_2 conditions (Khan, Johansen et al. 2018). These findings may demonstrate that metabolic performance is slowed in physically active freshwater salmonids. This may be explained by the mechanism where the allocation of energy for growth is directed for acid and base regulation.(Hannan, Jeffrey et al. 2016) Thus revealed that prolonged exposure to high levels of CO_2 can have negative effects on the behavior, survival and also lower growth performance (Danley, Kenney et al. 2005), acidosis (Ultsch 1996) and increased ventilation frequency (Smith and Jones 1982). In general, studies show that fish subjected to high carbon dioxide causes gill ventilation. However, recent research has shown that CO_2 itself stimulates gill ventilation (Burleson and Smatresk 2000). It has been hypothesized that the CO_2-driven hyperventilation is caused by lowered blood oxygen content caused by both Bohr and Root effects. Not enough research has been done on all life stages of fish species that are susceptible to Carbon dioxide and this should be a research area of interest in the future.

Upon this background I would like to examine abiotic effects of CO_2 and the effects of varied CO_2 concentrations on salmon yolk sac fry.

1.1. Hypothesis

1. Lowering of the pH level in fish tanks increase the Carbon dioxide concentration.
2. Increased Carbon dioxide concentrations to 15mg/L affect the development and survival of salmon yolk sac fry.

2.0 Materials and method

2.1 Experimental design and set-up

The experiment began on the 10th of January and ended on the 13th of February. Yolk sac fry of Atlantic salmon from the fish lab NMBU were exposed to different water treatments. The day degree of the yolk sac fry from incubation was 550 prior to the start of the experiment. The initial weight and total length of the yolk sac fry was 0.115 ± 0.01g and 20.8 ± 1.24mm respectively. 21fish tanks with different CO_2 concentration were set up. Raw water was added from the fish laboratory to the water tanks treated by adding either diluted HCl to lower the pH which converts HCO_3 to CO_2 or the addition of CO_2 from a CO_2 flask into the water. The yolk sac fries were subjected to the treatment until swim up for 33 days.

Table 1: An overview of groups, treatments, and replicates

Group	Treatment	Number of replications
Control 0mg CO_2 /L	No treatment. High pH 7.6	3 (A B C)
Control 0mg CO_2 /L	Treatment with HCl Low pH 6.5 degassing of water to strip CO_2. Water recycling	3 (A B C)
5mg CO_2 /L	Treatment with HCl pH 7.1	3 (A B C)
10mg CO_2 /L	Treatment with HCl pH 6.8	3 (A B C)
18mg CO_2 /L	Treatment with HCl pH 6.4	3 (A B C)
30mg CO_2 /L	Treatment by adding CO_2 pH6.5	3 (A B C)
60mg CO_2 /L	Treatment by adding CO_2 pH 6.1	3 (A B C)

A B

Figure 1: 2 A) Setup yolk sac exposure B) the fish tanks were covered to prevent exposure to light

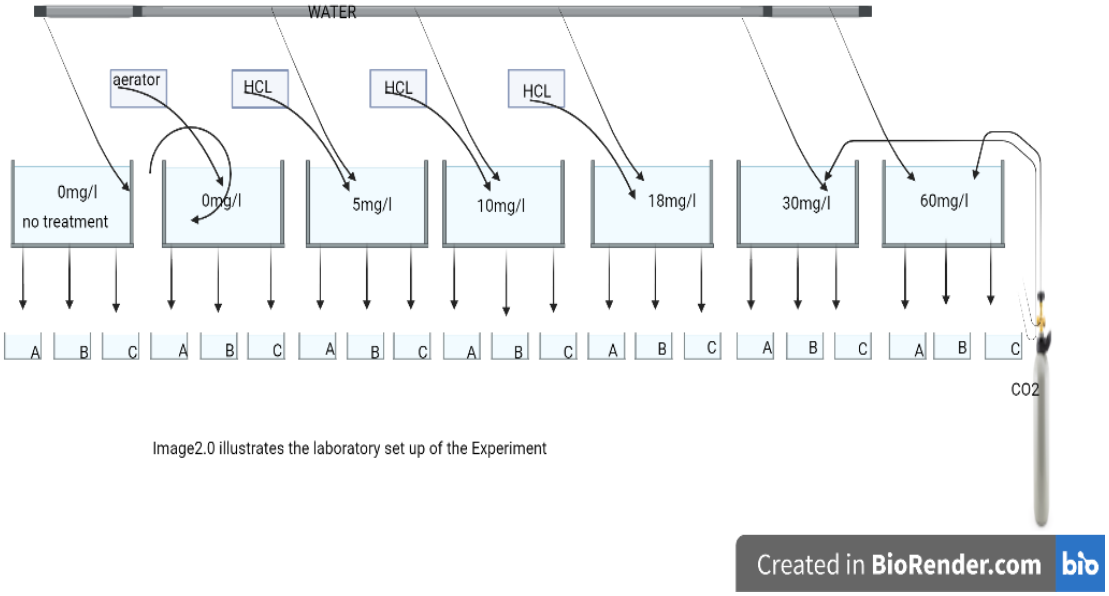

Figure 2: Schematic drawing of experimental set-up

Overview of experimental design. Fig1 (A) and (B) Fish tanks used for the experiment and Fig2 shows the Schematic of the experimental set up

2.2Water treatment

The water source from the main fish laboratory was added into the head tanks and from this head tank the water flowed continually to three replicates fish tanks. Seven main head tanks were used as each head tank represented one group and replicated to have 21 tanks. One outflow on the top of the head tank ensured constant water level in the head tank and thus constant water flow to each fish tank in a flow through system. The experimental set up contained 21 fish tanks (V=1.3L) with 6 carbon dioxide treatments (0, 5, 10, 18, 30, and 60 CO_2 mg/l) and a control with no treatment in replicate and each tank contained 25 yolk sac fry, in all a total of 525 yolk sac fry was used for the experiment. The differences in CO_2 concentration in each fish tank were achieved by addition of acid in the head tank or CO_2 gas as seen in fig2. The different groups are presented in Table1. Tested mobilization of CO_2 due to pH reduction of water. Four groups (0, 5, 10, and 18 CO_2 mg/l) were treated with different concentrations of diluted Hydrochloric acid (HCl) acidification which lowered the pH of the water. This was done to show how pH decrease of water will increase CO_2 by converting bicarbonate to Carbon dioxide. To obtain effects of only pH reduction without increased CO_2, one group control (0mg CO_2/L) was treated with HCl before the CO_2 was stripped off by degassing in the tanks and water was recycled. This tank had low pH and no or very low CO_2 as the CO_2 was stripped off by degassing and only the effects of the acidic water was assessed.

The other three treatments, 5mg CO_2/L, 10mg CO_2/L and 18mg CO_2/L were treated with different HCl concentrations which in effect produced different CO_2 concentrations. In this case both effects of reduced water pH and high CO_2 were assessed on the yolk sac fry. Different concentrations of CO_2 were added to Group 30 mg CO_2/L and 60 mg CO_2/L to assess the effects of the elevated levels of CO_2 concentration on the yolk sac fry.

2.2.1 Stock solution preparation (HCl) for treatment

Stock solution were prepared by adding 3Mol HCl in 10L of water.

- Group 0mg/l CO_2 at low pH: addition of 0.105M HCl (100ml of 3Mol HCl diluted in 10L of water) by peristatic pump to the raw water in the head tank before CO_2 stripping in fish tank.

- Group 5 mg/l CO_2: addition of 0.03M HCl (100ml of 3Mol HCl diluted in 10L of water) by peristatic pump to the raw water in the head tank before into fish tank.
- Group 10 mg/l CO_2: addition of 0.06M HCl (200ml of 3 Mol HCl diluted in 10L of water) by peristatic pump to the raw water in the head tank before into fish tank.
- Group 18 mg/l CO_2: addition of 0.105M HCl (100ml of 3Mol HCl diluted in 10L of water) by peristatic pump to the raw water in the head tank before into fish tank.

2.2.2 Water treatment with Carbon dioxide

Tested addition of Carbon dioxide gas to water. Two groups were treated with CO_2 gas. Different concentrations of CO_2 were added to the group 30mg/l and 60mg/l to assess only the effects of the elevated levels of CO_2 on the yolk sac fry without addition of HCl solution. Carbon dioxide was injected into water by diffusor to attain this concentration in group 60.0mgC02/L and Group 30.0mg/L from a Carbon dioxide flask as seen below in fig3

Figure 3: injection of Carbon dioxide from the flask into water by diffusor.

2.3.Measurement of Water Flow

Main water source was from the fish lab. The water flow from the pipe to the head tanks was 220 ml/min. The ratio of water to HCl addition per minute was 220 ml to 1.7 ml. Water flow from the head tanks to the fish tanks was by means of gravity which was 180 ml/min. The flow of water in the set up was flow through system. A calibrated measuring cylinder and a stopwatch were used to calculate the water flow. Water flow was recorded daily.

Water quality pH was measured and recorded daily. Water was drawn from the head tanks by using a syringe into a beaker and immersing the pH probe in the water and read the pH from the beaker. (: WTW Profoline pH /cond3320 with pH probe)

Carbon dioxide was measured and recorded daily. Water was drawn from the head tanks by using a syringe into the oxyguard calibration beaker and dissolved CO_2 was measured. Oxyguard CO_2 analyzer (Oxyguard international A/S Denmark) was used for the measurement. The instrument was calibrated before use.

Temperature was measured daily with a thermometer by lowering the thermometer in the head tanks and using a logger as well to measure and record temperature. Temperature logger Onset HOBO pendant was used to record temperature.

The conductivity of water was measured using a conductivity meter by lowering the conductivity probe in head tanks and measured.

Oxygen probe was immersed in fish tanks to measure oxygen levels every 3 days. Oxyguard oxygen analyzer (Oxyguard international A/S Denmark) was used for measurement.

To obtain information about ion composition in the water, water samples were collected in 50ml Sarstedt tube before analysis in the laboratory in NMBU. Major cations were determined using ICP-MS (agilent 8900) in acidified water samples (5%HNO_3), while anions were determined using Ion chromatography

2.4 Sampling of fish and measurement of body weight and size

Fish were sampled on day 10, 20 and 33. The fish were randomly taken using aquarium fish net (fine mesh suitable for fry) from the tanks and placed in the MS-222 solution in a beaker for sedation. On day 10 and 20 of sampling, five fish were taken from each of the 21 tanks. In all a total of one hundred and five fish were sampled for each day of sampling. On day 33 fifteen fish were sampled in each tank and a total of 306 fish were sampled as nine mortality was recorded for the entire experiment.

Figure 4: measurement of weight of yolk sac fry

Measurement of fish weight (fig 4) The fish were sampled and euthanized in MS-222 solution. The MS-222 solution was prepared at the day of sampling by adding 0.2g to 1L of water. The fish were immersed and euthanized in the MS-222 solution. Forceps was gently used to take fish from the MS-222 solution and placed on tissue paper for blotting before weighed on electronic scale balance. (Precisca HA Nerliens Switzerland)

Figure 5: Measurement of fish length on a millimeter sheet

Fish length was measured by placing the fish on a millimeter sheet (fig 5), picture was taken to record the size of the fish on day 10,20 and 33.

2.5. Data Analysis.

The data obtained from the studies were analyzed using IBM Corp. Released 2022. IBM SPSS Statistics for Windows, version 29.0. Armonk, NY: IBM Corp. All water quality parameters, growth indicators and mortality were averaged to obtain a grand mean and standard deviation. One way analysis of variance ANOVA was used to determine significant differences across groups. Also, for growth performance (fish weight and size) Tukey post hoc test and multiple comparison were performed for data collected at each sampling day to identify differences between treatment groups. Means and standard errors for growth performance were based on replication of fish within treatments (n=3). For all analysis significance was accepted at $P>0.05$

3.0. Results

This chapter presents observations and findings from the experimental work. The source of water used for the experiment was from the NMBU fish laboratory. Prior to the start of the experiment, water was subjected to treatments.

3.1 Water Quality and water flow

3.1.1 Water flow

Differences in the average of water flow were observed (table4). The variation was in an average of 50±2.1 ml/min to 54±7.7 ml/min. No significant difference between group ($p>0.05$)

3.1.2 Water ions conductivity

The composition of water ions included cations which are calcium, magnesium, potassium, and sodium. The anions included sulphate (SO_4), nitrate (NO_3) and chloride. The major ions were constant and no significant difference except chloride that was high in group 1.9 CO_2 mg/l with low pH. The high level of chloride was mainly due to the addition of HCl. Conductivity variation was in average of 315.0 ± 0.67 to 328 ± 0.77(µS/cm). There was significant difference ($p>0.001$). The group 1.9mg/l where water was recycled has a high conductivity level, and this was due to evaporation and high chloride levels from the addition of HCl.

Table 2: Average concentration of ions for treatment groups

Group	Sodium Na mg/l	Magnesium Mg mg/l	Potassium K mg/l	Calcium Ca mg/l	Chloride Cl mg/l	Sulphate SO_4 mg/l	Nitrate NO_3 mg/l	Conductivity (µS/cm)
1.8 mg CO_2/l	31.5	3.26	2.92	21.0	33.7	45.7	5.21	315.0
1.9 mg CO_2/l	30.3	3.38	2.98	22.6	61.7	47.1	5.19	328.0
5.4 mg CO_2/l	30.4	3.17	2.83	20.4	36.8	45.4	5.17	315.0
9.6 mg CO_2/l	31.0	3.23	2.90	22.1	40.0	45.7	5.19	315.0
17.7 mg CO_2/l	31.2	3.25	2.85	22.0	44.3	45.4	5.16	315.0
30mgCO_2/l	31.6	3.26	2.90	21.5	33.7	45.7	5.21	315.0
60 mg CO_2/l	31.0	3.23	2.83	20.9	34.0	45.9	5.24	315.0

3.1.3 Temperature and Oxygen

Temperature was in average of 9.1 ± 0.1°C to 9.2 ± 0.1°C but was not significant different between groups (P>0.998). The oxygen variation was in average of 97 ± 0.21% to 101 ± 0.44%. The oxygen was close to saturation and some variation was mainly due to temperature. There was no significant difference.

3.1.4 pH

The pH levels varied, and this was influenced by the addition of HCl and CO_2 in the various group as already described in chapter 2. In the group 1.9 mg/l, 5.4 mg/l, 9.6 mg/l and 17.7 mg/l, addition of HCl increased the H^+ ion concentration and reduced the pH in an average from 7.6 ±0.06 to 6.5 ± 0.07, to 7.2±0.06, to 6.9 ±0.05, to 6.4±0.05 respectively. The H^+ concentration and low pH depended on the different concentration of HCl added in these groups. In the groups 30mg/l and 60mg/l, CO_2 gas was added in their tanks which led to the production of carbonic acid and hence a low pH in the tanks. In the group 30mg/l the pH changed from an average of 7.6 ±0.06 to 6.5±0.1 and in group 60mg/l it was changed from 7.6 ±0.06 to 6.1±0.1. There was a significant difference between the groups. (P≥0.001) Table 3 and fig 6

3.1.5 Carbon dioxide

Carbon dioxide levels were different between groups because of different treatment in the various groups. Addition of HCl and CO_2 caused these changes. When HCl was added the pH dropped due to the increase in H^+ ions which made water acidic and as a result increased the CO_2 levels. The increase in CO_2 is mainly due to the dropping of solubility of bicarbonate where the bicarbonate is converted to CO_2 through carbonic acid in the acidic water medium. In the group 1.9mg/l addition of HCl dropped the pH and CO_2 was increased. CO_2 was stripped off by recycling of water many times and very low CO_2 1.9mg/l was recorded. Groups 5.4 mg/l, 9.6mg/l, and 17.7 mg/l have an average CO_2 level changed from 1.8±0.38 to 5.4±0.68, to 9.6±1.10, to 17.7±0.10 mg/l respectively. In the group 29.3mg/l and 60mg/l, CO_2 was added from carbon dioxide gas flask which increased the CO_2 concentration. Also increased concentration CO_2 dropped water pH and the solubility of bicarbonate dropped, and this induced the conversion of bicarbonate to CO_2

and hence also contributed to the increase level of CO_2. The average CO_2 level of group 30mg/l and 60mg/l was increased from 1.8±0.38 to 29.3±2.5, to 60±4.7 mg/l respectively after addition of carbon dioxide

Table 3: Water quality measurement and water flow

Group	pH	Carbon dioxide(mg/l)	TEMPERATURE (°C)	OXYGYEN (%)	Water flow (ml/min)
1.8mgCO_2/l	7.6±0.06	1.8±0.38	9.1±0.10	98±0.61	52.3±3.21
1.9mgCO_2/l	6.5±0.07	1.9±0.19	9.1±0.10	101±0.44	47.3±3.32
5.4mgCO_2/l	7.2±0.06	5.4±0.68	9.2±0.10	97±0.14	51.6±2.12
9.6mgCO_2/l	6.9±0.05	9.6±1.10	9.1±0.10	97±0.40	52.0±3.70
17.7mgCO_2/l	6.4±0.05	17.7±0.10	9.1±0.10	97±0.21	49.33±3.51
29.3mgCO_2/l	6.5±0.1	29.3±2.5	9.1±0.10	98±0.14	52.0±3.20
60.0mgCO_2/l	6.1±0.1	60±4.7	9.1±0.10	98±0.42	52.1±4.16

* Significant ($p \geq 0.05$) differences between the different groups.

Table 3 shows the average ± standard deviation of the water quality in the seven treatment groups averaged over the 33 days period.

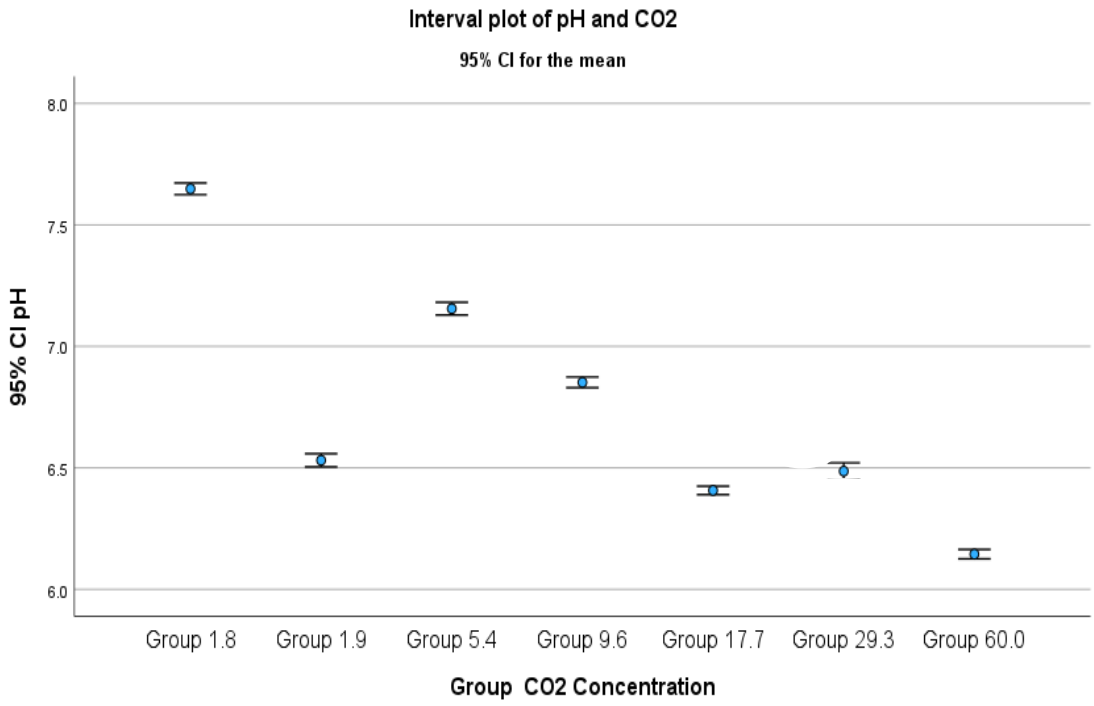

Figure 6: Shows relationship between different group's treatment and their pH levels over the 33 days period using an interval plot.

There was significant difference between all groups compared to the pH of control 1.8 mg/l by comparing the average mean. From the plot it can be seen as pH decreases, carbon dioxide increases and the vise-versa $P>0.001$.

3.2 Yolk sac fry response to treatment.

The yolk sac responded to the treatment differently. Generally, the results showed significant differences in average fish weight and size of the fish between sampling days for the experimental period. (Fig7)

A

B

C

D

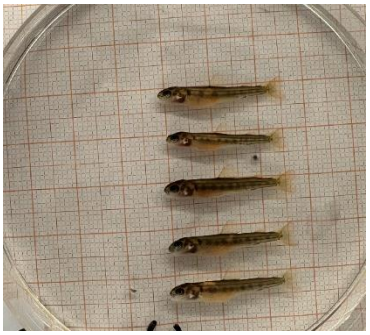

Figure 7: Fish growth and development over time A) fish growth at the start of the experiment B) fish growth and development at day 10 C) fish growth and development at day 20 (D fish growth and development at day 33..

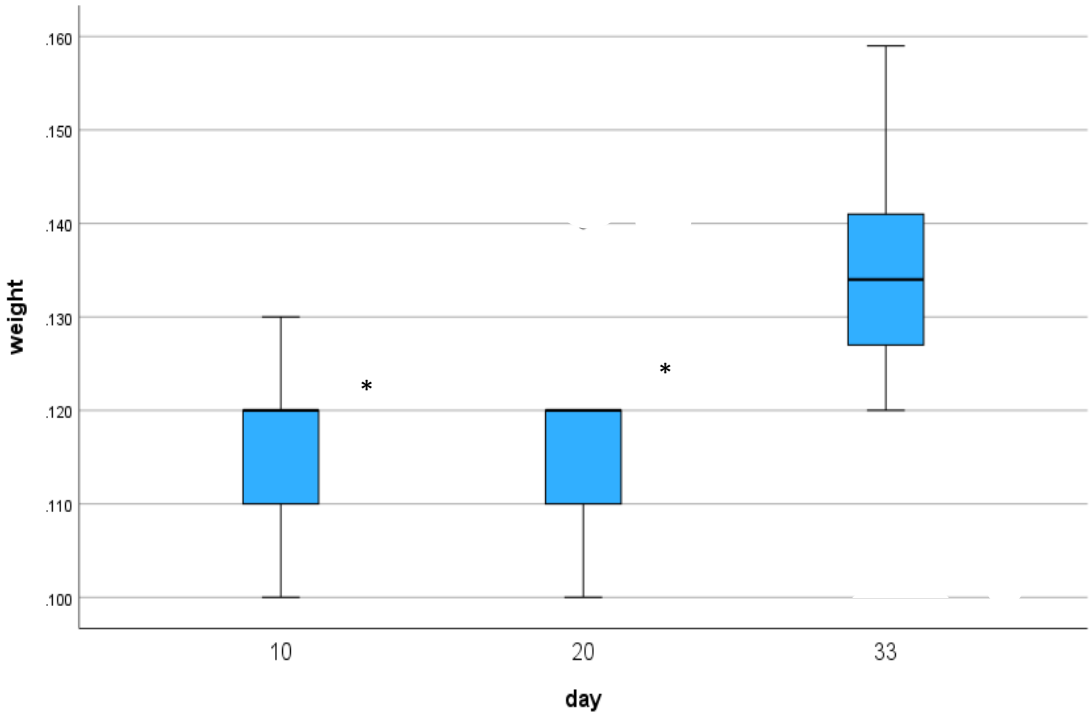

Figure 8: fish weight in the control group 1.8 mg/l with high pH showing weight differences of yolk sac fry between sampling days. From the box plot

** Shows day 10 and 20 are statistically significant different (as seen in the median vertical Black thick line in the blue box) from day 33. P>0.001*

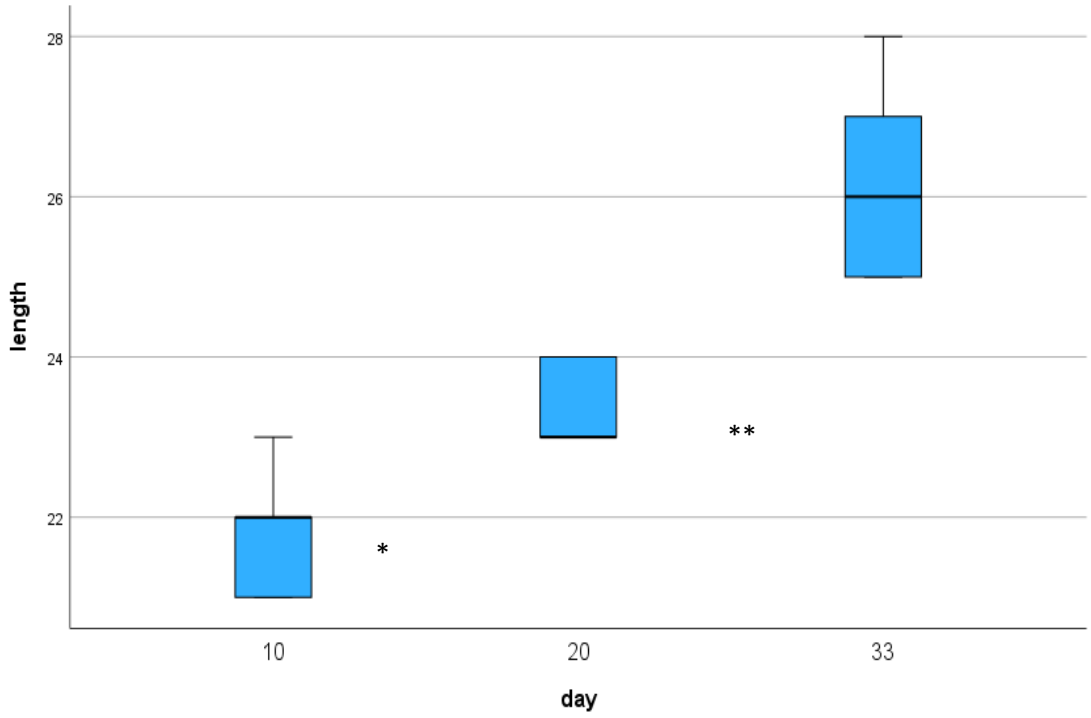

Figure 9: Fish length in control group 1.8 mg/l showing length of yolk sac fry differences between sampling days.

** Shows day 10 is significantly different from day 20 and 30. ** shows day 20 is significant different from day 30 P>0.001*

3.2.1Control group

In the control group 1.8mg/l with high pH, the salmon yolk sac fry was observed to have normal development whereby they increased in weight and size as expected. Mortality was very low, which was 1 % and mortality less than 5% is generally considered positive in an experiment (OECD 2013). Comparatively, group 1.9 mg/l with low pH also had no negative effect on the yolk sac fry and normal fish development and low mortality was recorded. It was thus observed pH 6.5 has no effect on fish growth and development.

3.2.2 Effects of carbon dioxide exposure

The effects of carbon dioxide on the fish weight and size were observed and were different between groups**.**

3.2.3 Effects of carbon dioxide on weight

At days 10 and 20, there was no differences in effect of carbon dioxide on the weight of fry between all groups as seen in table 5. However, there were observed effects on the fish weight in the group where high carbon dioxide was high which was group 17.7 mg/l, 29.3 mg/l and 60 mg/l. The difference in effect was significant as compared to the control groups and this was observed in day 33 only. Group 5.4 mg/l, 9.6 mg/l which has relatively low carbon dioxide had no effects on fish weight compared to the control group. It was discovered that carbon dioxide had a tendency to have a dose-effect over time.

3.2.4 Effects of carbon dioxide on fish length

The effects of carbon dioxide after day10 and 20 were not significantly different between all groups. However, at day 33 it was observed that in the group 29.3 mg/l and 60 mg/l length of fish was significantly different from both controls 1.8 mg/l with high pH and 1.9mg/l with low pH.

Table 4: Overview length and weight of yolk sac fry after10, 20 and 33 day of exposure

Group	Length day 10 (mm)	Length day 20 (mm)	Length day 30 (mm)	Weight day 10 (g)	Weight day 20 (g)	Weight day 30 (g)
	(N=3x5)	(N=3x5)	(N=3x15)	(N=3x5)	(N=3x5)	(N=3x15)
1.8 mg/l *	21.7±0.7	23.5±0.5	26.7±0.8	0.116±0.01	0.117±0.01	0.138±0.01
1.9 mg/l **	21.7±0.4	23.4±0.6	26.5±0.8	0.117±0.01	0.117±0.01	0.141±0.01
5.4 mg/l	21.6±0.8	23.6±0.4	26.4±0.8	0.118±0.01	0.121±0.01	0.136±0.01
9.6 mg/l	21.7±0.5	23.1±0.7	26.3±0.8	0.116±0.01	0.118±0.02	0.135±0.01
17.7 mg/l	21.6±0.6	23.4±0.8	26.2±0.8[c]	0.123±0.01	0.121±0.01	0.131±0.01[c]
29.3 mg/l	21.8±0.9	23.6±0.7	26.0±0.7 [b]	0.121±0.01	0.118±0.01	0.125±0.01[b]
60.0 mg/l	22.4±0.7	23.2±0.9	24.7±0.7 [a]	0.116±0.01	0.115±0.01	0.116±0.01 [a]

* control high pH, ** control low pH

Mean weight and height at day 33. Mean difference is significant at 0.05 level. a= significant different to all groups P<0.001, b = significant different to * And **, p<0.001 c = significant different to *and** , p>0.02

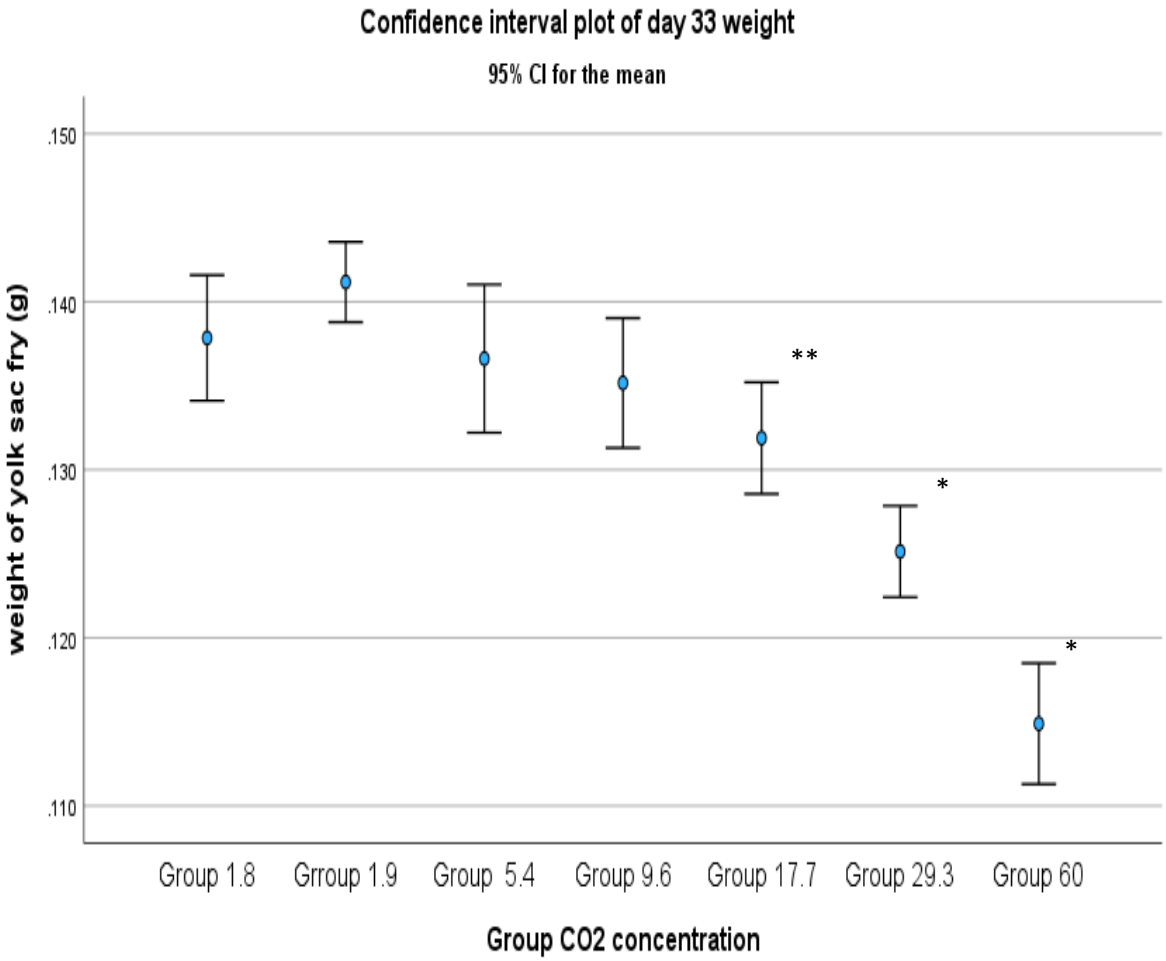

*Figure 10: Shows the response of fish weight to carbon dioxide dosage at day 33. *Mean weight is Statistically significant different compared to control groups 1.8 mg/l and 1.9 mg/l, **statistically different from the control 1.9 mg/l P>0.05*

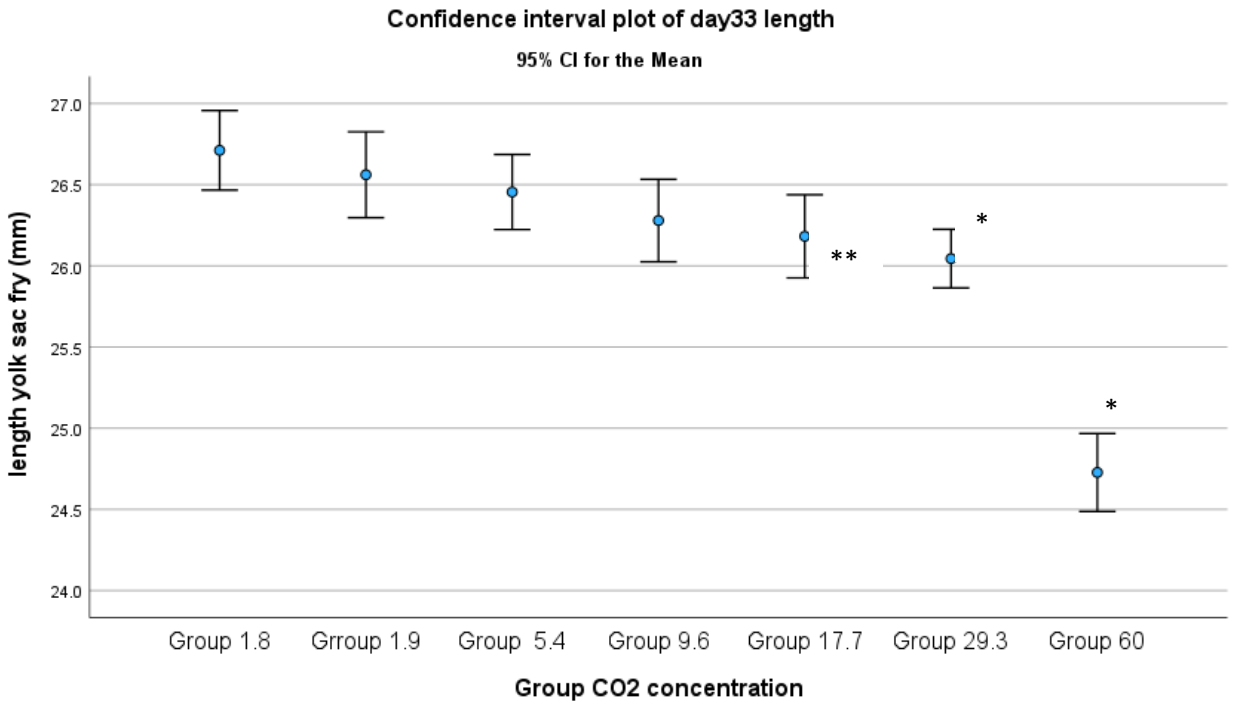

*Figure 11: Shows the response of fish length to carbon dioxide dosage at day 33. *Mean length is Statistically significant different compared to control groups 1.8 mg/l and 1.9 mg/l , **statistically different from the control 1.8 mg/l P>0.05,*

4.0 Discussion

The main finding of this study was that salmon yolk sac fry raised to the swim up stage in freshwater showed appreciable differences in growth and development in the long term 33 days and no significant differences in the short term 10 t0 20 days when exposed to the different concentrations of carbon dioxide while major water quality and water flow was monitored.

The major water quality concentrations were normal and were not significantly different between groups except pH, chloride, and conductivity Table2 and 3. It was obvious in all groups that as pH of water was reduced, the Carbon dioxide level also increased as seen in (table2 and fig6) and the vice -versa. From fig 9 and 10, the total average weight and length of the two controls were not significantly different which shows that the growth of yolk sac fry was not affected by pH down to 6.5. This can also be related to studies (Ishimatsu, Kikkawa et al. 2004) reported from their research when the following fish exposed to seawater equilibrated with 5% CO_2, adult Japanese amberjack, *Seriola quinqueradiata*, and bastard halibut, *Paralichthys olivaceus*, died after 8 and 48 hours, respectively. Only 20% of the *Starspotted smoothhound, Mustelus manazo*, a cartilaginous fish, did not survive after 72 hours at 7% CO_2. Also, when the larvae and eggs of the marine fish silver seabream *Pagrus major* was tested at the same seawater pH, CO_2-enriched saltwater was far more deadly as compared to the seawater that had been acidified with HCl. It is helpful to differentiate between the effects of CO2 and the impacts of water acidification. Also findings from (Norrgren and Degerman 1993) showed when Atlantic salmon and brown trout were exposed in low PH alumium rich water, Atlantic salmon were more sensitive to the acidic water than brown trout both at hatching and yolksac fry. Prolonged exposure caused 100% mortality.

Carbon dioxide influenced fish growth as both the weight and length were affected. Salmon yolk sac was sensitive to elevated levels of carbon dioxide29.3 mg/l and 60 mg/l. However, the yolk sac fry had limited effects to 17.7 mg CO_2/l during 33 days of exposure as compared to 29.3 and 60 mg/l. (Table4). Comparing my findings to other studies, according to (Foss, Røsnes et al. 2003), juvenile spotted wolffish growth was slower at 39 mg/l than at 21mg/l and below. Growth was significantly slower for European seabass at 46 mg/1

(Lemarie, Dosdat et al. 2004). Again, from research, (Fivelstad, Haavik et al. 1998) found out that there were no effect on growth when Atlantic salmon post smolts were subjected to carbon dioxide concentrations between 8 and 12 mg/l, while adverse effects were seen at CO_2 concentrations of 21 mg/l and higher .

The increase in weight and length from the initial stage to the swim up in both control groups with total mortality was less than1% and indicates the experimental condition were adequate to achieve closer to optimum normal for growth. The total mortality of this study was very low and was the same as the levels recorded in study of comparative experimental conditions using juvenile Atlantic cod.(Foss, Kristensen et al. 2006)

Again, comparisons between the current study and earlier research are frequently challenging due to variations in, among other things, life stage, water quality parameters such ions, alkalinity, and pH conditions where the experiments were conducted. Finally, number of yolk sac fry subjected to treatment replication was low on day 10 and 20 and this frequently lack a high level of statistical power, some findings of this study should be interpreted with some caution. As a result, significant differences between some groups might have been hidden. It would be beneficial to investigate the effects of 10mg/l and15mg/l carbon dioxide on a larger number of fish over a longer exposure time and to know if yolk sac fry will be sensitive to such long period.

5.0 Conclusion

My study seeks to know if decreased water pH increases carbon dioxide concentration hypothesis 1. It was obvious from the results which showed carbon dioxide increased as pH was reduced. Salmon yolk sac fry exhibited variable behavior between treatment groups up to a swim up stage. After 33 days of exposure, there was evidence of negative effects of elevated carbon dioxide (>17.7 mg/l) and physiological adaptations to the increased CO_2 conditions, this affected the total mean weight and length. Thus it is important to check carbon dioxide concentration if pH drops occur in fish tanks or during recycling of water.

This work thus demonstrates that efforts to lower CO_2 concentrations to below 15 mg/l may be necessary and as it can be seen in group 17.7 mg CO_2/l which was closer to the hypothesis two, showed a significant difference of growth when compared to control group 1.8 mg/l with high pH in day 33.

Elevated levels of carbon dioxide from moderate 17.7 mg/l to very high level 29.3 mg/l and 60 mg/l affected growth of yolk sac fry. Other findings also contradict where same fish species and same stage of development exposed to same dosages of carbon dioxide varies though sometime the variation is attributed to different water environment.

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Appendix A: Descriptives of growth of control 1.8 mg co2/l for day 10, 20 and 33

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
weight	10	15	.11600	.010556	.002726	.11015	.12185	.100	.130
	20	15	.11733	.008837	.002282	.11244	.12223	.100	.140
	30	45	.13593	.012410	.001850	.13220	.13966	.101	.159
	Total	75	.12823	.014762	.001705	.12483	.13162	.100	.159
length	10	15	21.73	.594	.153	21.40	22.06	21	23
	20	15	23.40	.507	.131	23.12	23.68	23	24
	30	44	26.07	1.065	.161	25.74	26.39	25	28
	Total	74	24.65	2.017	.234	24.18	25.12	21	28

Appendix B: Descriptives of growth in treatments groups of sampling days

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
DAY10 WEIGHT	Group 1.8	15	.1160	.01056	.00273	.1102	.1218	.10	.13
	Grroup 1.9	15	.1173	.00799	.00206	.1129	.1218	.10	.13
	Group 5.4	15	.1187	.00640	.00165	.1151	.1222	.11	.13
	Group 9.6	15	.1167	.00816	.00211	.1121	.1212	.10	.13
	Group 17.7	15	.1233	.01345	.00347	.1159	.1308	.11	.16
	Group 29.3	15	.1213	.00990	.00256	.1158	.1268	.10	.13
	Group 60	15	.1160	.01298	.00335	.1088	.1232	.10	.14
	Total	105	.1185	.01026	.00100	.1165	.1205	.10	.16
DAY20 WEIGHT	Group 1.8	15	.1173	.00884	.00228	.1124	.1222	.10	.14
	Grroup 1.9	15	.1173	.00961	.00248	.1120	.1227	.10	.14
	Group 5.4	15	.1213	.00516	.00133	.1185	.1242	.11	.13
	Group 9.6	15	.1180	.01821	.00470	.1079	.1281	.10	.17
	Group 17.7	15	.1207	.01163	.00300	.1142	.1271	.11	.15
	Group 29.3	15	.1187	.01246	.00322	.1118	.1256	.10	.14
	Group 60	15	.1153	.00834	.00215	.1107	.1200	.10	.13
	Total	105	.1184	.01110	.00108	.1162	.1205	.10	.17
DAY33 weight	Group 1.8	45	.13784	.012441	.001855	.13411	.14158	.101	.165
	Grroup 1.9	41	.14117	.007573	.001183	.13878	.14356	.117	.154
	Group 5.4	44	.13661	.014497	.002185	.13221	.14102	.109	.179
	Group 9.6	42	.13517	.012390	.001912	.13131	.13903	.111	.156
	Group 17.7	44	.13189	.010938	.001649	.12856	.13521	.096	.157
	Group 29.3	45	.12513	.009042	.001348	.12242	.12785	.104	.144

	Group 60	44	.11489	.011837	.001784	.11129	.11848	.084	.145
	Total	305	.13170	.014110	.000808	.13011	.13329	.084	.179
DAY 10 Length	Group 1.8	15	21.67	.724	.187	21.27	22.07	21	23
	Grroup 1.9	15	21.67	.488	.126	21.40	21.94	21	22
	Group 5.4	15	21.60	.828	.214	21.14	22.06	20	23
	Group 9.6	15	21.73	.594	.153	21.40	22.06	21	23
	Group 17.7	15	21.60	.632	.163	21.25	21.95	21	23
	Group 29.3	15	21.87	.915	.236	21.36	22.37	20	23
	Group 60	15	22.40	.737	.190	21.99	22.81	21	24
	Total	105	21.79	.743	.072	21.65	21.93	20	24
Day20 length	Group 1.8	15	23.53	.516	.133	23.25	23.82	23	24
	Grroup 1.9	15	23.47	.640	.165	23.11	23.82	22	24
	Group 5.4	15	23.67	.488	.126	23.40	23.94	23	24
	Group 9.6	15	23.13	.743	.192	22.72	23.54	22	24
	Group 17.7	15	23.40	.828	.214	22.94	23.86	21	24
	Group 29.3	15	23.67	.724	.187	23.27	24.07	23	25
	Group 60	15	23.20	.941	.243	22.68	23.72	22	25
	Total	105	23.44	.720	.070	23.30	23.58	21	25
DAY33 length	Group 1.8	45	26.71	.815	.122	26.47	26.96	25	28
	Grroup 1.9	41	26.56	.838	.131	26.30	26.83	24	28
	Group 5.4	44	26.45	.761	.115	26.22	26.69	25	28
	Group 9.6	43	26.28	.826	.126	26.02	26.53	24	28
	Group 17.7	44	26.18	.843	.127	25.93	26.44	24	28
	Group 29.3	45	26.04	.601	.090	25.86	26.23	25	27
	Group 60	44	24.73	.788	.119	24.49	24.97	24	27
	Total	306	26.13	.991	.057	26.02	26.25	24	28

Appendix C: ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DAY10 WEIGHT	Between Groups	.001	6	.000	1.165	.331
	Within Groups	.010	98	.000		
	Total	.011	104			
DAY20 WEIGHT	Between Groups	.000	6	.000	.505	.803
	Within Groups	.012	98	.000		
	Total	.013	104			
DAY33 weight	Between Groups	.021	6	.004	27.018	<.001
	Within Groups	.039	298	.000		
	Total	.061	304			
DAY 10 Length	Between Groups	7.257	6	1.210	2.364	.036
	Within Groups	50.133	98	.512		
	Total	57.390	104			
Day20 length	Between Groups	3.981	6	.663	1.304	.263
	Within Groups	49.867	98	.509		
	Total	53.848	104			
DAY33 length	Between Groups	115.420	6	19.237	31.245	<.001
	Within Groups	184.086	299	.616		
	Total	299.507	305			

Appendix D: Multiple comparisons for treaments groups of sampling days.

Tukey HSD

Dependent Variable	(I) Group CO2 concentration	(J) Group CO2 concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
DAY10 WEIGHT	Group 1.8	Grroup 1.9	-.00133	.00373	1.000	-.0126	.0099
		Group 5.4	-.00267	.00373	.991	-.0139	.0086
		Group 9.6	-.00067	.00373	1.000	-.0119	.0106
		Group 17.7	-.00733	.00373	.443	-.0186	.0039
		Group 29.3	-.00533	.00373	.784	-.0166	.0059
		Group 60	.00000	.00373	1.000	-.0112	.0112
	Grroup 1.9	Group 1.8	.00133	.00373	1.000	-.0099	.0126
		Group 5.4	-.00133	.00373	1.000	-.0126	.0099
		Group 9.6	.00067	.00373	1.000	-.0106	.0119
		Group 17.7	-.00600	.00373	.677	-.0172	.0052
		Group 29.3	-.00400	.00373	.935	-.0152	.0072
		Group 60	.00133	.00373	1.000	-.0099	.0126
	Group 5.4	Group 1.8	.00267	.00373	.991	-.0086	.0139
		Grroup 1.9	.00133	.00373	1.000	-.0099	.0126
		Group 9.6	.00200	.00373	.998	-.0092	.0132
		Group 17.7	-.00467	.00373	.872	-.0159	.0066
		Group 29.3	-.00267	.00373	.991	-.0139	.0086
		Group 60	.00267	.00373	.991	-.0086	.0139
	Group 9.6	Group 1.8	.00067	.00373	1.000	-.0106	.0119

		Grroup 1.9	-.00067	.00373	1.000	-.0119	.0106
		Group 5.4	-.00200	.00373	.998	-.0132	.0092
		Group 17.7	-.00667	.00373	.560	-.0179	.0046
		Group 29.3	-.00467	.00373	.872	-.0159	.0066
		Group 60	.00067	.00373	1.000	-.0106	.0119
	Group 17.7	Group 1.8	.00733	.00373	.443	-.0039	.0186
		Grroup 1.9	.00600	.00373	.677	-.0052	.0172
		Group 5.4	.00467	.00373	.872	-.0066	.0159
		Group 9.6	.00667	.00373	.560	-.0046	.0179
		Group 29.3	.00200	.00373	.998	-.0092	.0132
		Group 60	.00733	.00373	.443	-.0039	.0186
	Group 29.3	Group 1.8	.00533	.00373	.784	-.0059	.0166
		Grroup 1.9	.00400	.00373	.935	-.0072	.0152
		Group 5.4	.00267	.00373	.991	-.0086	.0139
		Group 9.6	.00467	.00373	.872	-.0066	.0159
		Group 17.7	-.00200	.00373	.998	-.0132	.0092
		Group 60	.00533	.00373	.784	-.0059	.0166
	Group 60	Group 1.8	.00000	.00373	1.000	-.0112	.0112
		Grroup 1.9	-.00133	.00373	1.000	-.0126	.0099
		Group 5.4	-.00267	.00373	.991	-.0139	.0086
		Group 9.6	-.00067	.00373	1.000	-.0119	.0106
		Group 17.7	-.00733	.00373	.443	-.0186	.0039
		Group 29.3	-.00533	.00373	.784	-.0166	.0059
DAY20 WEIGHT	Group 1.8	Grroup 1.9	.00000	.00411	1.000	-.0124	.0124
		Group 5.4	-.00400	.00411	.959	-.0164	.0084

		Group 9.6	-.00067	.00411	1.000	-.0131	.0117
		Group 17.7	-.00333	.00411	.983	-.0157	.0091
		Group 29.3	-.00133	.00411	1.000	-.0137	.0111
		Group 60	.00200	.00411	.999	-.0104	.0144
	Grroup 1.9	Group 1.8	.00000	.00411	1.000	-.0124	.0124
		Group 5.4	-.00400	.00411	.959	-.0164	.0084
		Group 9.6	-.00067	.00411	1.000	-.0131	.0117
		Group 17.7	-.00333	.00411	.983	-.0157	.0091
		Group 29.3	-.00133	.00411	1.000	-.0137	.0111
		Group 60	.00200	.00411	.999	-.0104	.0144
	Group 5.4	Group 1.8	.00400	.00411	.959	-.0084	.0164
		Grroup 1.9	.00400	.00411	.959	-.0084	.0164
		Group 9.6	.00333	.00411	.983	-.0091	.0157
		Group 17.7	.00067	.00411	1.000	-.0117	.0131
		Group 29.3	.00267	.00411	.995	-.0097	.0151
		Group 60	.00600	.00411	.768	-.0064	.0184
	Group 9.6	Group 1.8	.00067	.00411	1.000	-.0117	.0131
		Grroup 1.9	.00067	.00411	1.000	-.0117	.0131
		Group 5.4	-.00333	.00411	.983	-.0157	.0091
		Group 17.7	-.00267	.00411	.995	-.0151	.0097
		Group 29.3	-.00067	.00411	1.000	-.0131	.0117
		Group 60	.00267	.00411	.995	-.0097	.0151
	Group 17.7	Group 1.8	.00333	.00411	.983	-.0091	.0157
		Grroup 1.9	.00333	.00411	.983	-.0091	.0157
		Group 5.4	-.00067	.00411	1.000	-.0131	.0117

		Group 9.6	.00267	.00411	.995	-.0097	.0151
		Group 29.3	.00200	.00411	.999	-.0104	.0144
		Group 60	.00533	.00411	.852	-.0071	.0177
	Group 29.3	Group 1.8	.00133	.00411	1.000	-.0111	.0137
		Grroup 1.9	.00133	.00411	1.000	-.0111	.0137
		Group 5.4	-.00267	.00411	.995	-.0151	.0097
		Group 9.6	.00067	.00411	1.000	-.0117	.0131
		Group 17.7	-.00200	.00411	.999	-.0144	.0104
		Group 60	.00333	.00411	.983	-.0091	.0157
	Group 60	Group 1.8	-.00200	.00411	.999	-.0144	.0104
		Grroup 1.9	-.00200	.00411	.999	-.0144	.0104
		Group 5.4	-.00600	.00411	.768	-.0184	.0064
		Group 9.6	-.00267	.00411	.995	-.0151	.0097
		Group 17.7	-.00533	.00411	.852	-.0177	.0071
		Group 29.3	-.00333	.00411	.983	-.0157	.0091
DAY33 weight	Group 1.8	Grroup 1.9	-.003326	.002471	.829	-.01066	.00401
		Group 5.4	.001390	.002426	.998	-.00581	.00859
		Group 9.6	.002678	.002455	.931	-.00461	.00997
		Group 17.7	.005958	.002426	.180	-.00124	.01316
		Group 29.3	.012711*	.002413	<.001	.00555	.01987
		Group 60	.022958*	.002426	<.001	.01576	.03016
	Grroup 1.9	Group 1.8	.003326	.002471	.829	-.00401	.01066
		Group 5.4	.004716	.002484	.483	-.00266	.01209
		Group 9.6	.006004	.002512	.207	-.00145	.01346
		Group 17.7	.009284*	.002484	.004	.00191	.01666

	Group 29.3	.016037*	.002471	<.001	.00870	.02337
	Group 60	.026284*	.002484	<.001	.01891	.03366
Group 5.4	Group 1.8	-.001390	.002426	.998	-.00859	.00581
	Grroup 1.9	-.004716	.002484	.483	-.01209	.00266
	Group 9.6	.001288	.002469	.999	-.00604	.00862
	Group 17.7	.004568	.002440	.500	-.00267	.01181
	Group 29.3	.011321*	.002426	<.001	.00412	.01852
	Group 60	.021568*	.002440	<.001	.01433	.02881
Group 9.6	Group 1.8	-.002678	.002455	.931	-.00997	.00461
	Grroup 1.9	-.006004	.002512	.207	-.01346	.00145
	Group 5.4	-.001288	.002469	.999	-.00862	.00604
	Group 17.7	.003280	.002469	.838	-.00405	.01061
	Group 29.3	.010033*	.002455	.001	.00274	.01732
	Group 60	.020280*	.002469	<.001	.01295	.02761
Group 17.7	Group 1.8	-.005958	.002426	.180	-.01316	.00124
	Grroup 1.9	-.009284*	.002484	.004	-.01666	-.00191
	Group 5.4	-.004568	.002440	.500	-.01181	.00267
	Group 9.6	-.003280	.002469	.838	-.01061	.00405
	Group 29.3	.006753	.002426	.082	-.00045	.01396
	Group 60	.017000*	.002440	<.001	.00976	.02424
Group 29.3	Group 1.8	-.012711*	.002413	<.001	-.01987	-.00555
	Grroup 1.9	-.016037*	.002471	<.001	-.02337	-.00870
	Group 5.4	-.011321*	.002426	<.001	-.01852	-.00412
	Group 9.6	-.010033*	.002455	.001	-.01732	-.00274
	Group 17.7	-.006753	.002426	.082	-.01396	.00045

		Group 60	.010247*	.002426	<.001	.00304	.01745
	Group 60	Group 1.8	-.022958*	.002426	<.001	-.03016	-.01576
		Grroup 1.9	-.026284*	.002484	<.001	-.03366	-.01891
		Group 5.4	-.021568*	.002440	<.001	-.02881	-.01433
		Group 9.6	-.020280*	.002469	<.001	-.02761	-.01295
		Group 17.7	-.017000*	.002440	<.001	-.02424	-.00976
		Group 29.3	-.010247*	.002426	<.001	-.01745	-.00304
DAY 10 Length	Group 1.8	Grroup 1.9	.000	.261	1.000	-.79	.79
		Group 5.4	.067	.261	1.000	-.72	.85
		Group 9.6	-.067	.261	1.000	-.85	.72
		Group 17.7	.067	.261	1.000	-.72	.85
		Group 29.3	-.200	.261	.988	-.99	.59
		Group 60	-.733	.261	.084	-1.52	.05
	Grroup 1.9	Group 1.8	.000	.261	1.000	-.79	.79
		Group 5.4	.067	.261	1.000	-.72	.85
		Group 9.6	-.067	.261	1.000	-.85	.72
		Group 17.7	.067	.261	1.000	-.72	.85
		Group 29.3	-.200	.261	.988	-.99	.59
		Group 60	-.733	.261	.084	-1.52	.05
	Group 5.4	Group 1.8	-.067	.261	1.000	-.85	.72
		Grroup 1.9	-.067	.261	1.000	-.85	.72
		Group 9.6	-.133	.261	.999	-.92	.65
		Group 17.7	.000	.261	1.000	-.79	.79
		Group 29.3	-.267	.261	.948	-1.05	.52
		Group 60	-.800*	.261	.043	-1.59	-.01

	Group 9.6	Group 1.8	.067	.261	1.000	-.72	.85
		Grroup 1.9	.067	.261	1.000	-.72	.85
		Group 5.4	.133	.261	.999	-.65	.92
		Group 17.7	.133	.261	.999	-.65	.92
		Group 29.3	-.133	.261	.999	-.92	.65
		Group 60	-.667	.261	.152	-1.45	.12
	Group 17.7	Group 1.8	-.067	.261	1.000	-.85	.72
		Grroup 1.9	-.067	.261	1.000	-.85	.72
		Group 5.4	.000	.261	1.000	-.79	.79
		Group 9.6	-.133	.261	.999	-.92	.65
		Group 29.3	-.267	.261	.948	-1.05	.52
		Group 60	-.800*	.261	.043	-1.59	-.01
	Group 29.3	Group 1.8	.200	.261	.988	-.59	.99
		Grroup 1.9	.200	.261	.988	-.59	.99
		Group 5.4	.267	.261	.948	-.52	1.05
		Group 9.6	.133	.261	.999	-.65	.92
		Group 17.7	.267	.261	.948	-.52	1.05
		Group 60	-.533	.261	.395	-1.32	.25
	Group 60	Group 1.8	.733	.261	.084	-.05	1.52
		Grroup 1.9	.733	.261	.084	-.05	1.52
		Group 5.4	.800*	.261	.043	.01	1.59
		Group 9.6	.667	.261	.152	-.12	1.45
		Group 17.7	.800*	.261	.043	.01	1.59
		Group 29.3	.533	.261	.395	-.25	1.32
Day20 length	Group 1.8	Grroup 1.9	.067	.260	1.000	-.72	.85

		Group 5.4	-.133	.260	.999	-.92	.65
		Group 9.6	.400	.260	.723	-.38	1.18
		Group 17.7	.133	.260	.999	-.65	.92
		Group 29.3	-.133	.260	.999	-.92	.65
		Group 60	.333	.260	.860	-.45	1.12
	Grroup 1.9	Group 1.8	-.067	.260	1.000	-.85	.72
		Group 5.4	-.200	.260	.987	-.98	.58
		Group 9.6	.333	.260	.860	-.45	1.12
		Group 17.7	.067	.260	1.000	-.72	.85
		Group 29.3	-.200	.260	.987	-.98	.58
		Group 60	.267	.260	.947	-.52	1.05
	Group 5.4	Group 1.8	.133	.260	.999	-.65	.92
		Grroup 1.9	.200	.260	.987	-.58	.98
		Group 9.6	.533	.260	.392	-.25	1.32
		Group 17.7	.267	.260	.947	-.52	1.05
		Group 29.3	.000	.260	1.000	-.78	.78
		Group 60	.467	.260	.557	-.32	1.25
	Group 9.6	Group 1.8	-.400	.260	.723	-1.18	.38
		Grroup 1.9	-.333	.260	.860	-1.12	.45
		Group 5.4	-.533	.260	.392	-1.32	.25
		Group 17.7	-.267	.260	.947	-1.05	.52
		Group 29.3	-.533	.260	.392	-1.32	.25
		Group 60	-.067	.260	1.000	-.85	.72
	Group 17.7	Group 1.8	-.133	.260	.999	-.92	.65
		Grroup 1.9	-.067	.260	1.000	-.85	.72

		Group 5.4	-.267	.260	.947	-1.05	.52
		Group 9.6	.267	.260	.947	-.52	1.05
		Group 29.3	-.267	.260	.947	-1.05	.52
		Group 60	.200	.260	.987	-.58	.98
	Group 29.3	Group 1.8	.133	.260	.999	-.65	.92
		Grroup 1.9	.200	.260	.987	-.58	.98
		Group 5.4	.000	.260	1.000	-.78	.78
		Group 9.6	.533	.260	.392	-.25	1.32
		Group 17.7	.267	.260	.947	-.52	1.05
		Group 60	.467	.260	.557	-.32	1.25
	Group 60	Group 1.8	-.333	.260	.860	-1.12	.45
		Grroup 1.9	-.267	.260	.947	-1.05	.52
		Group 5.4	-.467	.260	.557	-1.25	.32
		Group 9.6	.067	.260	1.000	-.72	.85
		Group 17.7	-.200	.260	.987	-.98	.58
		Group 29.3	-.467	.260	.557	-1.25	.32
DAY33 length	Group 1.8	Grroup 1.9	.175	.165	.939	-.31	.66
		Group 5.4	.325	.162	.411	-.15	.80
		Group 9.6	.432	.163	.113	-.05	.91
		Group 17.7	.529*	.162	.020	.05	1.01
		Group 29.3	.667*	.161	<.001	.19	1.14
		Group 60	1.984*	.162	<.001	1.50	2.46
	Grroup 1.9	Group 1.8	-.175	.165	.939	-.66	.31
		Group 5.4	.150	.165	.971	-.34	.64
		Group 9.6	.258	.166	.716	-.24	.75

		Group 17.7	.355	.165	.330	-.14	.85
		Group 29.3	.492*	.165	.047	.00	.98
		Group 60	1.809*	.165	<.001	1.32	2.30
	Group 5.4	Group 1.8	-.325	.162	.411	-.80	.15
		Grroup 1.9	-.150	.165	.971	-.64	.34
		Group 9.6	.107	.163	.995	-.38	.59
		Group 17.7	.205	.163	.870	-.28	.69
		Group 29.3	.342	.162	.346	-.14	.82
		Group 60	1.659*	.163	<.001	1.18	2.14
	Group 9.6	Group 1.8	-.432	.163	.113	-.91	.05
		Grroup 1.9	-.258	.166	.716	-.75	.24
		Group 5.4	-.107	.163	.995	-.59	.38
		Group 17.7	.097	.163	.997	-.39	.58
		Group 29.3	.235	.163	.778	-.25	.72
		Group 60	1.552*	.163	<.001	1.07	2.04
	Group 17.7	Group 1.8	-.529*	.162	.020	-1.01	-.05
		Grroup 1.9	-.355	.165	.330	-.85	.14
		Group 5.4	-.205	.163	.870	-.69	.28
		Group 9.6	-.097	.163	.997	-.58	.39
		Group 29.3	.137	.162	.979	-.34	.62
		Group 60	1.455*	.163	<.001	.97	1.94
	Group 29.3	Group 1.8	-.667*	.161	<.001	-1.14	-.19
		Grroup 1.9	-.492*	.165	.047	-.98	.00
		Group 5.4	-.342	.162	.346	-.82	.14
		Group 9.6	-.235	.163	.778	-.72	.25

		Group 17.7	-.137	.162	.979	-.62	.34
		Group 60	1.317*	.162	<.001	.84	1.80
	Group 60	Group 1.8	-1.984*	.162	<.001	-2.46	-1.50
		Grroup 1.9	-1.809*	.165	<.001	-2.30	-1.32
		Group 5.4	-1.659*	.163	<.001	-2.14	-1.18
		Group 9.6	-1.552*	.163	<.001	-2.04	-1.07
		Group 17.7	-1.455*	.163	<.001	-1.94	-.97
		Group 29.3	-1.317*	.162	<.001	-1.80	-.84

*. The mean difference is significant at the 0.05 level.

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