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Research Article

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INTERACTIVE EFFECT OF WATER TEMPERATURE AND NITRATE FERTILIZERS ON GROWTH, SURVIVAL, AND HATCHING SUCCESS OF EGGS, EMBRYOS AND LARVAE OF AFRICAN CATFISH (*CLARIAS GARIEPINUS*, BURCHELL 1822)

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ABSTRACT

The study assessed the interactive effect of water temperature and nitrate fertilizers on growth, survival, and hatching success of eggs, embryos and larvae of African catfish *Clarias gariepinus*. 100 fertilized eggs of *C. gariepinus* were incubated in glass flasks (1000 ml) each filled to 800 ml mark with groundwater containing (0, 5, 10, 15 and 20 mgL^{-1} of NO_3-N) nitrate levels. Flasks were randomly placed in water bath set at (25°C, 28°C, and 31°C) temperature levels in triplicates. 30 larvae were maintained in the same hatching solution and temperature. Data were collected from egg to ten days post-hatching in November 2017. The results showed that the tolerable limit of *C. gariepinus* eggs, embryos and larvae to nitrate fertilizers was 10 mgL^{-1} of NO_3-N at 28°C and 31°C and range between 5 and 15 mgL^{-1} of NO_3-N at 25°C. We concluded that eggs, embryos and larvae of *C. gariepinus* can withstand a maximum of 10 mgL^{-1} of NO_3-N at temperatures ranging from 25°C to 31°C. It is therefore recommended that excess application of nitrate fertilizers in Agricultural fields should be discouraged in order to protect *C. gariepinus* hatchlings from nitrate toxicity.

Keywords: Incubation period, Hatching period, Nitrate toxicity, Growth rate, Hatching rate

Introduction

Eggs, embryos and larvae of shallow-water breeding fishes are at risk of both nitrate toxicity and frequent temperature fluctuations in their environment (Schram et al., 2014). Effluents from agricultural nitrate fertilizers, industrial and human waste have been reported to be the major sources of nitrate (Rouse et al., 1999). Furthermore, some countries in sub-Saharan Africa such as Malawi, Zambia, Zimbabwe and South Africa have almost doubled the application rate of agricultural fertilizers from 2002 to 2014 in order to boost food production (FAOStat 2014). Most of these applied fertilizers are washed or leached into surface and ground waters. Consequently, the concentrations of nitrate in surface waters has increased to the extent that can exceed 25 mgL^{-1} of NO_3-N (107 mgL^{-1} of NO_3^-) (Bogardi et al., 1991). The increasing nitrate levels could be devastating to aquatic life and it has become an issue of global concern, especially at this time when aquatic ecosystems are experiencing increasing and varying water temperatures due to climate change (Rim-Rukeh *et al.,* 2013). Nitrate level above 10 mg/L in drinking water is a potential cause of oxygen deficiency in the blood, a fatal condition known as methemoglobinemia or "blue baby syndrome" in humans less than six months old (Manassaram *et al.,* 2010). Young mammals such as calves, piglets, lamb and horses, birds, especially chicks are also susceptible to nitrate poisoning (Jennings and Sneed 1996). Field studies have further suggested that nitrogen fertilizers have contributed towards the reduction of amphibian populations in agricultural fields (Birge et al., 2000). High levels of nitrate affect amphibian eggs, embryos and larvae in several ways: Nitrate inhibiting egg membrane degradation enzymes, resulting into increased hatching period, anatomical deformities or even death of the egg and embryo stages (Ortiz-Santaliestra et al., 2011; Freda and Dunson. 1985): It also increases the amount of energy required for nitrate detoxification in the eggs and embryos which may result into smaller and reduces survival at larvae stage (Wright and Wright. 1996): It also causes methemoglobinemia resulting into hypoxia and death at all the three stages. Nitrate has also been reported to reduce growth, survival and development in urodele embryos (Ortiz-Santaliestra et al., 2011). On the other hand, recent research findings have reported an increase in both temperature and its varying frequencies in shallow freshwater habitats (Adhikari et al., 2015; Serdeczny et al., 2017). Climate predictions also indicated an increase in the number, frequency and intensity of extreme warm temperature events or heat waves across the world which may last for few days with an increase of about 5°C above the normal temperature. These are likely to maintain higher water temperature in shallow water for longer periods, particularly in the second half of the century (Barlow et al., 2015). High temperature increases metabolic rate resulting into faster; rate of egg and embryonic development, digestive enzymes activities, rate and efficiency at which the yolk sac food reserves are metabolized and utilized (Laurel et al., 2008; da Silva Longo and de Oliveira Nuñer 2010; Okunsebor et al., 2015). However, the effects of nitrate and its interaction with the ever increasing and varying temperature on the early developmental stages of fish are unclear. African catfish (*Clarias gariepinus)* is one of the susceptible fish species because, it naturally breeds in shallow waters, which include edges of lakes, rivers, streams, lagoons, floodplains and estuaries (Konan et al., 2014). The accumulation of nitrate and temperature fluctuation in these areas (Rouse et al., 1999) puts the egg, embryonic and larval stages of *C. gariepinus* at risk of the interactive effects of temperature and nitrate. Therefore, the purpose of this study was to determine the interactive effect of water temperature and nitrate fertilizers on growth, survival and hatching success of eggs, embryos and larvae of African catfish *C. gariepinus.*

Methods and Materials

Source and acclimatization of Brood-stock

Fifteen gravid females and ten mature male broodfish of *C. gariepinus* were obtained from stocks maintained in fish ponds for academic studies at Bunda fish farm of Lilongwe University of Agriculture and Natural Resources (LUANAR) (latitude 14°35 S' and longitude 33°50 E'), Lilongwe District, Malawi. Their individual weights ranged between 401 g and 486 g with an average weight (SD) of 431.5 ± 33.7 g. The timing was between November and March 2016, which is the natural annual breeding season of *C. gariepinus.* Broodfish were acclimatized to tank conditions for seven days. Female and male fish were acclimatized separately in 1000 L plastic circular tanks filled to 800 L with underground water (Table 1) used for breeding fish at LUANAR fish farm hatchery. Acclimatization tanks were aerated and dark covered using a thick black polyethylene. Water temperature was maintained at 28 ± 1°C using heaters and pH at 7.1 ± 0.1 (ssenfuma et al., 2011). The fish were fed a diet containing

Parameters	Unit	Means
Temperature	°C	23.53±0.69
pH	pH units	6.62±0.44
DO	mg/L	5.01±0.67
BOD	mg/L	6.12±0.53
COD	mg/L	7.72±1.37
TDS	mg/L	15.93±7.91
Nitrate	mg/L	1.64±0.40
Phosphates	mg/L	0.62±0.41

Table 1. Physio-chemical characteristics of the Groundwater used for hatching fish at Bunda fish farm, all in a normal rage for fish breeding De Graaf, 1996, WHO (2008) and MBS (2005).

35% crude protein, twice a day (07:00 and 17:00 hours) at a rate of 5% body weight, half at 07:00 and 17:00 hours, respectively (ssenfuma et al., 2011; Aruho et al., 2017). A quarter of the water was replaced to remove faecal material and leftover food on a daily basis.

Preparation of test media

The groundwater used at the fish farm was tested for nitrate concentration before the beginning of the experiment. To obtain the required nitrate concentrations, nitrate fertilizer, i.e., Sodium nitrate, prilled $NaNO_3$ (97%) bought from *Agricultural Trading Company* Limited (ATC) in Malawi was used as the source of nitrate for the experiments. Sodium nitrate, prilled $NaNO_3$ (97%) were also tested to find out whether it had the same concentration as prescribed. A stock solution of 40 mgL^{-1} of NO_3-N (177.072 mgL^{-1} of NO_3-N) was prepared from which five dilutions (control, 5, 10, 15 and 20 mgL^{-1} of NO_3-N) were prepared and put into separate 1000 ml glass flasks filled to 800 ml mark in triplicates. All NO_3^- were converted to NO_3-N by the following formula.

Nitrate-NO_3 (mgL^{-1}): NO_3-N = 4.4268:1 (Weschler 1968)

Broodfish management hormonal admission and fertilization

A total of three females and two male broodfish were carefully selected, acclimatized and injected with Aquaspawn® (manufactured by Spawnrite Ltd, Touwsrivier, Southern Cape, South Africa) according to methodology by Haniffa and Sridhar (2002) and ssenfuma et al., (2011).

Fertilization

Twelve hours after injection, eggs and sperm were obtained (according to methodology by Viveiros et al., (2002) and Dunham and Masser (2012)) and then mixed with a clean and soft feather. Saline (0.9%) was added and stirred for a few seconds. Eggs from three females were fertilized by sperm from two males. The entire process, from disassembly to fertilization, took about three minutes.

Experimental design

This experiment design was a 5 × 3 factorial. After fertilization, 100 fertilized eggs of *C. gariepinus* were incubated in glass flasks (1000 ml) each filled to 800 ml mark with groundwater containing one of the five concentrations of inorganic nitrate (control, 5, 10, 15 and 20 mgL^{-1} of NO_3-N) levels and the flask were randomly placed in a water bath set at one of the three temperature levels (25, 28, and 31°C) levels in triplicates. After hatching, 30 hatched larvae were maintained in the same hatching solution and temperature for the larval stage experiments. Air stones were placed in each flask and in each water bath to ensure oxygen supply and temperature homogeneity respectively. Mercury thermometers were also placed in each water bath to monitor temperature. The test solutions were renewed at the end of the hatching period for the egg and embryonic stages and after every two days for the larval stage until the yolk sac was fully absorbed.

Survival of fertilized eggs, Incubation period, hatching period and hatching rate

A separate sample of fertilized eggs were incubated alongside with the experimental flasks at all temperatures. A sample of 40 to 60 eggs (average) was take from these eggs and observed under stereo-loupe microscope connected to a video screen. Fertilized eggs were those which cleaved or differentiated 30 to 80 minutes after fertilization. Another sample of 30 eggs, 10 at a time were collected from each replicate after every 4 hours. Eggs were replaced after counting was completed. Dead and unfertilized eggs (showed no signs of development after 4 hours and turned cloudy 8 hours post-fertilization) were counted twice, using a stereo-loupe microscope connected to a video screen. Survival was determined using (Eqn 1) (Okunsebor *et al.*, 2015).

$$\text{Survival rate of the fertilized eggs} = \frac{\text{Number of live eggs just before hatching}}{\text{Number of fertilized eggs 14 h post-fertilization}} \times 100 \quad \text{(Equation 1)}$$

The incubation period began from the time of egg fertilization to the first hatch while the hatching period is defined as the period from egg fertilization to the time when 50% of *C. gariepinus* embryos had hatched. At the end of hatching period all unhatched embryos, hatched larvae and dead larvae were counted, and the hatching rate

(HR) was determined by the equation (2) (Radonic et al., 2007).

$$HR=\frac{\text{hatched larvae}}{\text{Hatched larvae + dead larvae + non hatched eggs}}\times 100 \quad \text{(Equation 2)}$$

Yolk absorption rate

The yolk absorption rate was determined by the rate of reduction in the yolk volume. Immediately after fertilization, 20 eggs were randomly selected, and their yolk sac length (L) and yolk sac height (H) were measured using a stereo-loupe microscope connected to a video screen with a graduated slide. The Yolk sac volume was calculated using the formula (Eqn 3, Borode and Oyintoke 2005).

$$V=\pi/6\cdot LH^2 \quad \text{(Equation 3)}$$

Where;

L is yolk sac length (mm)

H is yolk sac height (mm)

This was repeated in each replicate after the first larvae had hatched after 50% of the larvae have hatched and every day for the larval stages. Linear regressions were also made to predict the yolk absorption rate at 50%, 80% and 100% (Kamler et al., 1994) but this was proved by the actual measurements. The average rate of yolk absorption was determined by measuring the yolk volume of three larvae on a daily basis using the formula below by Borode and Oyintoke (2005):

$$\text{Yolk absorption rate}=(nI - nF)/t \quad \text{(Equation 4)}$$

Where:

- nI=Average initial yolk size,
- nF=Average final yolk size,
- t=rearing period

Yolk absorption period and survival rate

The yolk-sac period begins from the end of the hatching period until when 50% of the larvae fully absorbed their yolk-sac. This was determined by visual observation, then confirmed using a stereo-loupe microscope connected to a video screen and the time taken was recorded.

Mortality was determined by counting and recording on a daily basis the number of dead larvae for ten days of larvae life. Percentage survival was determined by the formula below.

$$(\%)\ \text{Survival}=\frac{\text{Number of live larvae at the end of yolk absorption}}{\text{Number of live larvae after hatching}}\times 100 \quad \text{(Equation 5)}$$

Growth rate

The rate of growth (GR) was determined daily by measuring the length of nine larvae from each treatment (thus three from each replicate) using an ocular micrometer mounted on a light microscope for ten days. The average rate of growth in length was calculated using the following formula (Borode and Oyintoke 2005):

$$GR=(Lf - Li)/t \quad \text{(Equation 6)}$$

Where:

Lf=Average final length of the larvae,

Li=Average initial length of the larvae

t=rearing period.

Feeding

After 50% of the larvae had absorbed two-thirds of their yolk-sac, larvae were fed on naturally occurring zooplankton. These were collected from a well-fertilized fishpond from Bunda fish farm using a plankton net (64 micro net). The fry was fed every 3 times a day adlibitum for each treatment (Oyelese 2006; Adewumi 2014).

Morphological development of newly hatched African catfish larvae

Morphological development was also observed. Immediately after hatching, the number of deformed larvae were counted, both dead and live deformed larvae were counted again and recorded daily. A stereo-loupe microscope connected to a video screen with a graduated slide was used. (Blaxter and Hempel 1963; Matsumoto et al., 2001).

Water quality parameters

Water temperature was measured and monitored using a portable digital thermometer (PTM-806, Lutron Electronics, New Delhi, India). pH of the water was determined using a digital pH meter (pH-208, Lutron Electronics, New Delhi, India). Dissolved Oxygen (DO) was measured using the Winkler's titration method and maintained using a RESUN low noise air pump (LP-100, RESUN aquarium, Guangdong, China). Nitrate was measured using the Spectrophotometric (UV/Vis Spectrophotometer, Lambda-650, PerkinElmer Life and Analytical Sciences, New York, USA) screening method.

Data analysis

Data analysis was done using R and R-studio statistical analysis software (version R 3.0.2 and R-studio 0.9.8). ANOVA was used to test for two-way interactions between means. When interactions were found to be significant after (*P<0.05)*, the Tukey's test was used to compare interaction means in a one-way ANOVA. Orthogonal polynomial contrasts were used to partition significant nitrate rates into linear, quadratic, cubic and quartic components.

Results

The results showed that there were significant interaction ($P<0.05$) between the temperature and nitrate concentration and their orthogonal polynomial component on hatching rate, incubation period, hatching period growth rate and larval survival on *C. gariepinus* eggs, larvae and embryos (Table 2).

Interactive effect of temperature and nitrate on hatching rate of *C. gariepinus* eggs

The significant linear trend of nitrate in temperature and nitrate interactions showed an inverse relationship between the hatching rate of *C. gariepinus* eggs and increasing levels of nitrate at all temperature treatments (Figure 1). The results further showed that, at all incubation temperatures, there were no significant differences ($p>0.05$) in the hatching rates of *C. gariepinus* eggs between the control and nitrate concentration up to 10 mgL^{-1} of NO_3-N. Significant differences ($p<0.05$) were noted between 10 and 20 mgL^{-1} of NO_3-N at 28°C and 31°C, but no significant differences ($p>0.05$) were noted in the incubation periods between the control and all other nitrate concentrations (control-20 mgL^{-1} of NO_3-N) at 25°C. These results suggest that, at 28°C and 31°C, *C. gariepinus* eggs are resilient (resistant/not affected) to nitrate concentration levels up to 10 mgL^{-1} of NO_3-N beyond which they were susceptible (vulnerable/affected negatively). However, at 25°C, the embryos were resilient up to 20 mgL^{-1} of NO_3-N. This implies that the effect of nitrate on *C. gariepinus* eggs and embryos increases with increasing temperature.

Interactive effect of temperature and nitrate on incubation period of *C. gariepinus* eggs

The significant cubic non-cross-over trend of nitrate in temperature and nitrate interactions showed that the incubation period of *C. gariepinus* eggs and embryos increased with increasing nitrate concentrations at all incubation temperatures (Figure 2). Significant differences

Parameters	T	N	TN
Survival of Fertilized eggs (%)	L***	Q**	ns
Incubation period (h)	Q***	Q*	C*
Hatching period (h)	Q*	Q**	Q***
Hatching rate (%)	Q***	Q*	L*
Yolk absorption period (h-1)	L***	L***	ns
Yolk absorption rate (Day-1)	Q***	L***	ns
Growth Rate (mm day-1)	Q***	Q***	Q***
Larval Survival (%)	Q***	Q***	Q**
Larval deformities	L**	QR*	ns

***p-value<0.001, **p-value<0.01, *p-value<0.05, T: Temperature, N: Nitrate, L: Linear, Q: Quadratic, C: Cubic, QR: Quartic, ns: Not Significantly

Table 2. Two-way ANOVA for temperature and nitrate, and their orthogonal polynomial contrasts on growth, survival, and hatching success of eggs, embryos and larvae of African catfish *C. gariepinus*.

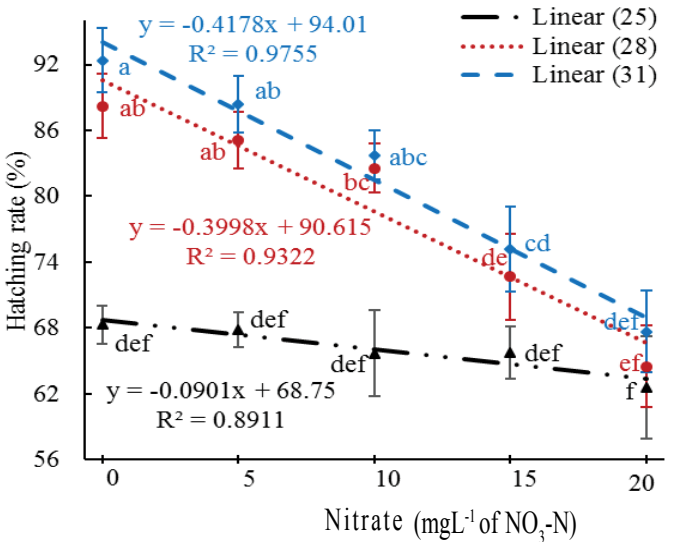

Figure 1. Linear effects of nitrate in Temperature × nitrate interaction on hatching rate of *C. gariepinus* eggs incubated at 25°C, 28°C and 31°C. Means (mean ± SE, n=45) with the same letters don't differ significantly

($P<0.05$) were noted between the control and 20 mgL^{-1} of NO_3-N at all incubation temperatures. However, no significant differences ($P>0.05$) were noted in the incubation period when *C. gariepinus* eggs and embryos were incubated in water containing 5 to 15 mgL^{-1} of NO_3-N at all incubation temperatures. This direct relationship between the incubation period of *C. gariepinus* eggs and increasing levels of nitrate at all temperature treatments suggest that the presence of nitrate in incubation water delays the rate of development and growth of *C. gariepinus* eggs and embryos.

Interactive effect of temperature and nitrate on hatching period of *C. gariepinus* eggs

The significant quadratic non-cross-over trend of nitrate in temperature and nitrate interactions showed that the

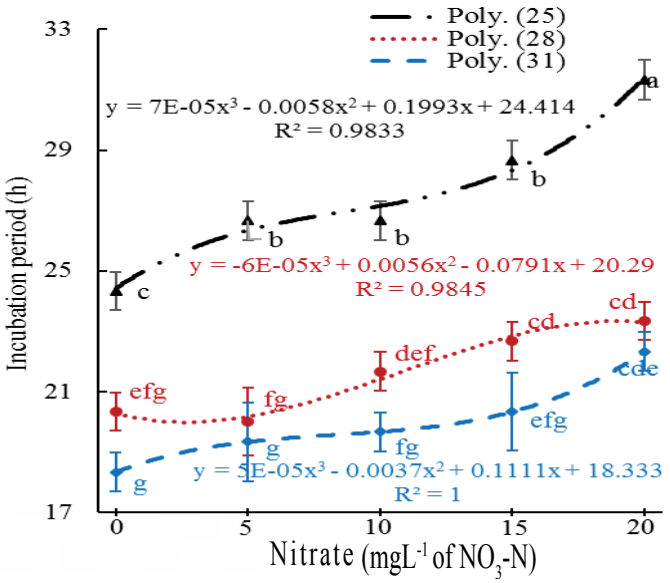

Figure 2. Cubic effects of nitrate in Temperature × nitrate interaction on incubation period of *C. gariepinus* eggs incubated at 25°C, 28°C and 31°C. Means (mean ± SE, n=45) with the same letters don't differ significantly

hatching period of *C. gariepinus* eggs and embryos increased with increasing nitrate concentrations at all incubation temperatures (Figure 3). Significant differences ($P<0.05$) were noted between the control and all nitrate polluted water (5 to 20 mgL^{-1} of NO_3-N) at all incubation temperatures. However, no significant differences ($P>0.05$) were noted in hatching period when *C. gariepinus* eggs and embryos were incubated in water containing 10 to 20 mgL^{-1} of NO_3-N at all incubation temperatures. Significant differences ($P<0.05$) were noted in hatching period when *C. gariepinus* eggs and embryos were incubated in the control and water containing 0 to 10 mgL^{-1} of NO_3-N at 28 and 31°C. These results suggest that the presence of nitrate fertilizers in hatching water delayed the development, growth and hatching process of *C. gariepinus* eggs and embryos.

Interactive effect of Temperature and nitrate on the growth rate of *C. gariepinus* larvae

The significant quadratic non-cross-over trend of nitrate in temperature and nitrate interactions indicated that there were no significant differences ($P>0.05$) in the growth rate of *C. gariepinus* larvae between the control and nitrate concentration up to 10 mgL^{-1} of NO_3-N at all incubation temperatures (Figure 4). Significant differences ($P<0.05$) were noted when *C. gariepinus* larvae were raised in water containing 10 and 20 mgL^{-1} of NO_3-N at all incubation temperatures. Furthermore, significant differences ($P<0.05$) were also noted when *C. gariepinus* larvae were raised in the control and water containing 15 mgL^{-1} of NO_3-N at 28 and 31°C while at 25°C, significant differences ($P<0.05$) were noted when *C. gariepinus* larvae were raised in the control and water contain 20 mgL^{-1} of NO_3-N (Figure 4). The results suggest that C. gariepinus larvae could be successfully grown in water containing a maximum of 10 mgL^{-1} of NO_3-N at all incubation temperatures (25°C to 31°C) beyond which they are susceptible to nitrate toxicity. However, at 25°*C. gariepinus* larvae could even be raised successfully at levels as high as 15 mgL^{-1} of NO_3-N.

Interactive effect of temperature and nitrate on survival of *C. gariepinus* larvae

The significant quadratic non-cross-over trend of nitrate in temperature and nitrate interactions indicated that there were no significant differences ($P>0.05$) in the survival of *C. gariepinus* larvae between the control and nitrate concentration up to 10 mgL^{-1} of NO_3-N at all incubation temperatures (Figure 5). Significant differences ($P<0.05$) were noted when *C. gariepinus* larvae were raised in the control and water containing 15 mgL^{-1} of NO_3-N at all nursing temperature. Furthermore, Significant differences ($P<0.05$) were also noted when *C. gariepinus* larvae were raised in water containing 10 and 20 mgL^{-1} of NO_3-N at all incubation temperatures (25°C to 31°C).

Physical-chemical parameters

Mean pH ranged from 6.94 in nitrate treatments to 8.16 in the control. The average nitrate concentration in borehole water used at Bunda farm for fish breeding was 1.6 mgL^{-1} of NO_3-N. Temperatures were controlled on average at 25 ± 0.2°C, 28 ± 0.4°C and 31 ± 0.5°C from the least to the highest, respectively. Other factors that were measured included pH, DO, BOD, COD, Nitrite, Phosphates, TDS, ammonia and salinity (Table 1). pH, correlated to nitrate levels; however, it didn't differ significantly ($p>0.05$) from each other in all treatments. Nitrate levels were attained by adding $NaNO_3$.

Discussion

The results of the present experiment may have been affected or pronounced by the ionic imbalance between K^+ and Na^+ (in the internal and external environment of the experimental organisms) if the concentration of Na^+ increased sustainably. However, this was explained by Romano and Zeng (2009) who carried out a similar

experiment on highly susceptible shrimp, and they added KCl in a mole ratio of 46:1, Na: K in order to balance K^+ and $Na^{+.}$ They discovered that both ions had the same effect. Therefore the findings of this experiment were as a result of the two factors that varied, temperature (25-31°C) and nitrate (0-20 mgL^{-1} of NO_3-N).

Interactive effect of temperature and nitrate on hatching rate of *C. gariepinus* eggs

The results reveal that at 28 and 31°C, *C. gariepinus* eggs and hatching embryos were resilient to nitrate levels up to 5 mgL^{-1} of NO_3-N beyond which they are susceptible. However, at 25°C the embryos were resilient to 20 mgL^{-1} of NO_3-N (Figure 1). The explanation for this may be linked to the passive diffusion of nitrate (NO_3^-) via the egg and embryos envelop to the developing tissues and the toxic products its metabolism. The accumulation of these substances which include nitrate (NO_3^-), nitrite (NO_2^-) and nitric oxide (NO) in the tissues may have resulted into death of the developing eggs and embryos in the current study. Increased levels of NO may cause Carcinogenesis by diffusing into the tissues to form nitrosalting species, which have the potential to damage DNA (Lundberg et al., 2009). Nitrite (NO_2^-) and nitric oxide (NO) can also transform Haemoglobin (Hb) into MetHb, this reduces Hb-Oxygen binding and transporting capacity (Kamstra and van der Heul 1998). At this stage, the embryos and its circulation system were in their early stages of developmentmaking them unable to withstand nitrate levels higher than 5 mgL^{-1} of NO_3-N at 28°C and 31°C. These results seem to be contradicting with those reported by (Ohio Environmental Protection Agency 2002) which reported that nitrate level below 90 mgL^{-1} of NO_{3-} (20 mgL^{-1} of NO_3-N) may have no effect on warm water fishes. However, in the current work, this seemed to be true when eggs were incubated at 25°C, but at higher temperature of 28°C and 31°C nitrate poses a significant effect at 10 mgL^{-1} of NO_3^-. The reason for this could be as suggested by von Westernhagen (1988) who reported that high levels of nitrate reduces physiological activities which retards growth and weakens the embryo making them unable to break the chorion hence reducing hatching rate. Temperature may have accerelated the rate of diffusion via the egg and embryo envelop and matabolism of nitrate into its toxic products (Haylor and Mollah 1995), resulting into death at higher temperaturethan at lower temperature.

Interactive effect of temperature and nitrate on incubation and hatching period of *C. gariepinus* eggs and embryos

The result of the present work showed that the presence of nitrate in incubation water reduced the development and growth rate of *C. gariepinus* eggs and embryos (Figures 2 and 3). The explanation for this may be attributed to what was suggested by von Westernhagen (1988) who reported that diffusion and metabolism of nitrate in the eggs and developing embryos may have resulted into high concentrations of nitrate (NO_3^-), nitrite (NO_2^-) and nitric oxide (NO) in their tissues. In an effort to eliminate these substances, eggs and developing embryos respond by increasing the energy requirements for cellular metabolism defense, homeostatic mechanism and growth restoration potential. This may have made them bioactive by increasing their maintenance energy (Lundberg et al., 2009; Hickey 2013; Sowers 2009). High maintained energy results into reduced growth and weakens the embryos making them unable to break the chorion. These results are in agreement with those reported by Backer and Waight (1993) who reported that exposure of common toads (*Bufo bufo)* to nitrate levels ranging between 9 and 22.6 mgL^{-1} of NO_3-N (2 to 5.1 mgL^{-1} of NO_3-N), resulted into reduced growth. They further reported that nitrate has the same effect at both low and high nitrate levels. The similarity of their

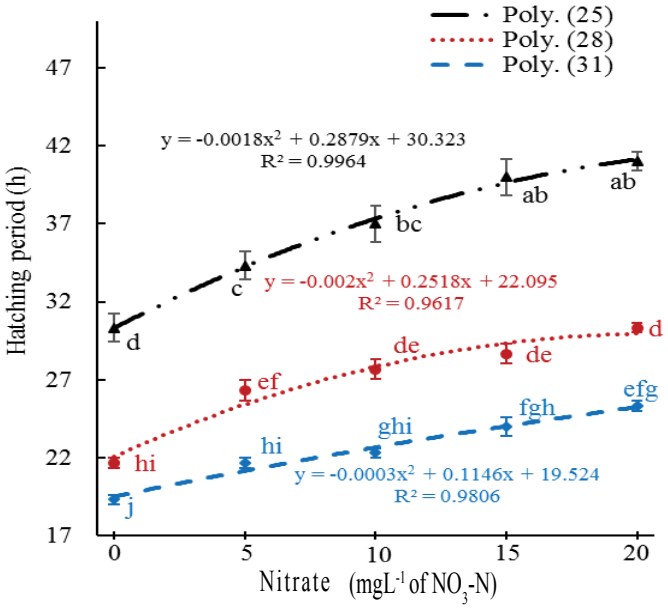

Figure 3. Quadratic effects of nitrate in Temperature × nitrate interaction on hatching period of *C. gariepinus* eggs incubated at 25°C, 28°C and 31°C. Means (mean ± SE, n=45) with the same letters don't differ significantly

study with the present work is that nitrate retarded growth rate of *C. gariepinus* embryos at nitrate levels as low as 5 mgL^{-1} of NO_3-N (22.14 mgL^{-1} of NO_3^-) but it differs in that in the current work, growth retardation increased with increasing levels of nitrate. The present results are also in line with those reported by Hecner (1995) who reported that the severity of nitrate on toads increased with increasing nitrate concentration.

Interactive effect of temperature and nitrate on growth rate of *C. gariepinus* larvae

The observed inverse trend between the growth rate of *C. gariepinus* larvae and increasing levels of nitrate at all temperature treatments (Figure 4) is in line with the recommendations of Westin (1974). Westin recommended that a maximum concentration 5.7 mgL^{-1} of NO_3-N for the optimal growth and health of trout. These findings are also in line with those of Kamstra et al., (1998), who reared eel at 22-24°C and reported retardation in growth at high levels of nitrate (250 mgL^{-1} of NO_3-N) than at lower levels of nitrate (50 mgL^{-1} of NO_3-N). Kamstra further explained that growth retardation was as a result of the formation and reduction of Methemoglobin (MetHb); in most aquatic animals, nitrate transforms Hemoglobin (Hb) into MetHb; this reduces Hb-Oxygen binding and transporting capacity. The reversal of this process by MetHb reductase in fish is an energy-consuming process (Camargo et al., 2005; Van Bussel et al., 2012) which gives an explanation for the observed retarded growth at higher nitrate levels in the present study. Further explanation was given by van Bussel et al., (2012), who reported that the presence of nitrate in water decreases feed conversion efficiency (FCE). This suggests that nitrate is a toxic substance to fish, and it requires a substantial amount of energy to be detoxified. The current study, further shows that the effect of nitrate beyond 10 mgL^{-1} of NO_3-N increases sharply at high temperature 28°C and 31°C than at low temperature 25°C. Related results were published by Schram et al., (2014) who cultured juvenile African catfish (154.3 ± 7.5 g) at 25.4-25.6°C and recommended a maximum of 140 mgL^{-1} of NO_3-N for proper growth. However, the differences between the results of these two experiments could have arisen due to differences in the age of fish cultured and temperature. In the same experiment, Schram cultured juvenile African catfish at increasing levels of nitrate. They found out that the concentration of nitrate in juvenile African catfish blood plasma increased nearly linearly with

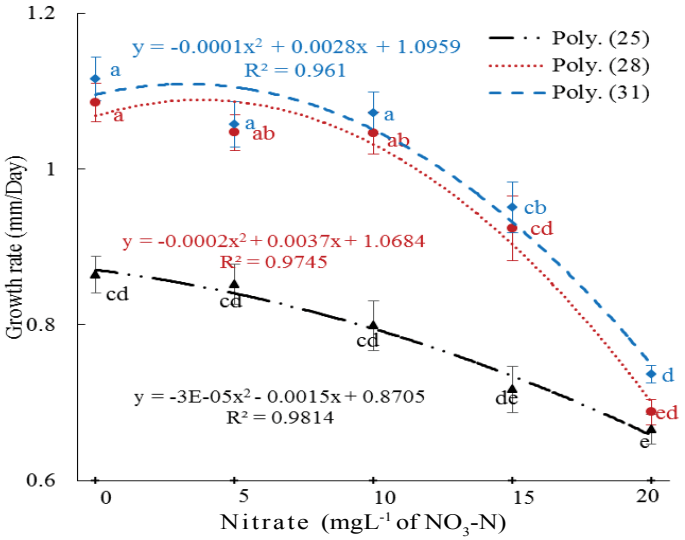

Figure 4. Quadratic interactive effect of nitrate in Temperature × nitrate interaction on Growth rate of *C. gariepinus* larvae reared at 25°C, 28°C and 31°C. Means (mean ± SE, n=45) with the same letters don't differ significantly

increasing levels of nitrate in their environment, though the ratio of nitrate concentration in the culture environment to the blood plasma was considerably low, i.e., 0.15 to 0.25. Scott (1993), Schram et al., (2014) and Davidson et al., (2014), further explained that the difference between the ratios was due to the fact that nitrate uptake into the fish's body through the gills, and surface body cells is a passive process and that nitrate permeability into the bronchial of the freshwater fish gills is low. In the present study, the effect of nitrate on the fish larvae increased with increasing temperature; this could have been due to the fact that temperature increases the rate of diffusion into the animal cell (Scott 1993). This increased the energy required for nitrate detoxification resulting in retarded growth.

Interactive effect of temperature and nitrate on larvae survival of *C. gariepinus*

The results revealed that larvae survival of *C. gariepinus* was not affected by nitrate up to 10 mgL^{-1} of NO_3-N at temperature treatments 28°C and 31°C, beyond which larval survival reduced. However, at 25°C larvae survival was not affected by nitrate up to 15 mgL^{-1} of NO_3-N (Figure 5). This could have been due to the fact that temperature increases the rate of nitrate diffusion and metabolism resulting into the accumulation of nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) and other toxic oxides into the cells of the developing *C. gariepinus* larvae (Lundberg et al., 2009; Scott 1993). This may have increased nitrate

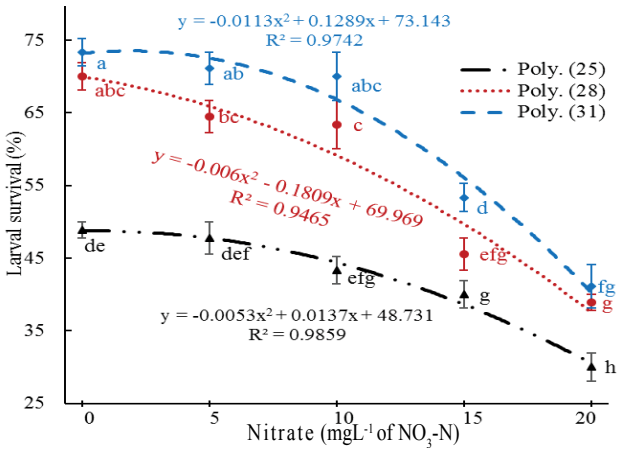

Figure 5. The quadratic effects of nitrate in Temperature × nitrate interaction on survival of *C. gariepinus* larvae reared at 25°C, 28°C and 31°C. Means (mean ± SE, n=45) with the same letters don't differ significantly.

poisoning, resulting into death of the larvae. These results contradicts with those reported by both Davidson et al., (2011a, 2011b) and Pedersen et al., (2012). Davidson, cultured rainbow trout at low level nitrate of 13 mgL^{-1} of NO_3-N and high level nitrate 99 ± 7 mgL^{-1} of NO_3-N at 12.9-14.0°C while Pederson, cultured rainbow trout at low-level nitrate of 50 mgL^{-1} of NO_3-N and high-level nitrate 200 mgL^{-1} of NO_3-N at 15.5°C and both reported no difference in survival. The contradiction in the results could have arisen from the differences in susceptibility of fish to nitrate at different ages. Embryos, larvae, and juveniles have been reported to be the most susceptible life stages to toxicants than adult fishes (Sprague, 1985). However, results related to the present study were published by Davidson et al., (2014) who cultured rainbow trout at low-level nitrate of 30 mgL^{-1} of NO_3-N and high-level nitrate 91 mgL^{-1} of NO_3-N and found that there was a cumulative reduction of survival at 91 mg L^{-1} NO_3-N. However, the cause of this was not clear to him.

Conclusions and Recommendations

The study concluded that *C. gariepinus* eggs, embryos and larvae can with stand a maximum of 10 mgL^{-1} of NO_3-N at incubation temperatures ranging from 25°C to 31°C. This puts the fish at risk of nitrate toxicity in their natural environment, especially at this time when temperatures are dramatically fluctuating. It is therefore recommended that the application of nitrate fertilizers in Agricultural fields should be discouraged in order to protect the eggs, embryos and larvae of *C. gariepinus* from nitrate toxicity.

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