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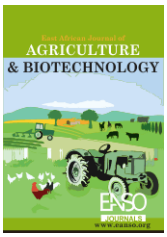

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Original Article

Stress Tolerance of African Catfish (Clarias gariepinus, Burchell, 1822) Larvae Fed on Spirulina platensis or Eisenia fetida in Partial Replacement of Caridina nilotica in Formulated Diets

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ABSTRACT

Enhanced growth and survival indicate a quality diet important in the production of quality fish larvae. Diet's quality heavily depends on the quality of its protein which varies with inclusion levels and sources. This Influences stress responses and compromises wellbeing larvae due to diets suboptimal provision of nutritional requirements. However, there exists a knowledge gap on the performance of different proteins beyond growth and survival. The objective of this study was to determine the effects of partially replacing *Caridina nilotica* with *Spirulina platensis* or *Eisenia fetida* on stress tolerance of *Clarias gariepinus* larvae. This was conducted in 0.0, 0.3, 0.5, 0.7mg/l ammonia concentrations at 28°C and pH 7 within 24-hours using four- and six-weeks old larvae. The larvae were fed on formulated diets and a control at 10% body weight, five times a day. All larvae exposed to 0.7mg/l ammonia concentration died irrespective of the diet fed. A combination of 50%*Caridina nilotica* and 50%*Eisenia fetida* fed larvae posted low ($p<0.001$) total mortality of 78% and 52% in 0.5mg/l, stress indices of 457.3 and 342 and, took the longest time of 12.67 and 18.67-hours for half the number of larvae exposed to die in 0.7mg/l ammonia respectively for four- and six-week-old larvae. However, larvae fed on 50%*Spirulina platensis* and 50%*Caridina nilotica* posted higher ($p<0.001$) total mortality of 97% and 73% in 0.5mg/l, stress indices of 574.3 and 476.3 and, shortest time of 8 and 10.17-hours for half the number of 4- and 6-weeks old (respectively) larvae exposed to 0.7mg/l ammonia to die. Protein source influenced stress tolerance with *Eisenia fetida* diets enhancing better larvae tolerance to ammonia compared to *Spirulina platensis* and *Caridina nilotica* (control) diets. *Caridina nilotica* could be replaced by *Eisenia fetida* up to 50% and 25% by *Spirulina platensis* to enhance *Clarias gariepinus* larvae tolerance to ammonia stress.

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INTRODUCTION

Enhanced growth and high survival rates of larvae in hatcheries are indicators of a balanced diet that meets species' nutritional requirements for the production of quality fish larvae. However, balanced diet does not always guarantee production of quality larvae as they do not reveal the actual condition of fish (Dhert *et al*., 1993). This is probably because fish larvae quality is a product of the environment, management practices, fish social factors, and nutritional status (Oliva-Teles, 2012; Rehman *et al*., 2017). Besides, larval quality remains a restrictive factor in seed production and a hindrance in aquaculture expansion despites its importance in enhancing subsequent production stages (Ramaswamy *et al*, 2013; Munguti *et al*., 2014). This is because, performance of fish in the next stages of growth is dependent on the quality of its larvae as influenced by the nutritional content of starter diets (Mejri *et al*., 2021).

Proteins and lipids are critical nutrients in a diet and they influence fish responses to stress, health, growth, and survival (Tacon, *et al*., 1993; Mesa-Rodrigez *et al*., 2018). However, protein is the most expensive and scarce ingredient in aquafeed. Quality protein is obtained from finite fishmeal with balanced amino acid profile for improved larval health and welfare (Aragão *et al*., 2022). As a result, research on fishmeal alternatives in fish feed formulations has increased considerably due to environmental, social, and economic concerns. However, replacement of fishmeal in a diet by protein alternative may cause negative impacts on larvae behaviour, physiology, metabolism, health, and quality (Rehman *et al*., 2017; Aragão *et al*., 2022). This alters fish larvae stress responses to handling, thermal shock, hypoxia, and chemical shock due to variations in dietary energy content, fatty acid profiles, phospholipids, antioxidants, and composition of bioactive in the diet impacted by protein sources (Rehman *et al*., 2017). Despite use of plant, animal, or novel protein alternatives in aquafeed formulations, the inclusion level of protein in a diet affects stress responses in fish larvae (Aragão *et al*., 2022).

Analysis of stress indictors in fish larvae has been conducted using different methods including enzyme-linked immunosorbent assay (ELISA) with

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reliable results. However, ELISA is time consuming and quite expensive to most hatchery managers (Samaras & Pavlidis, 2020). Exposing fish larvae to air within a specified time has shown no correlation between test results and growth performance. Quantification of yolk sac vitellin reveals the quality of fish larvae as influenced by diet and indicates the energy reserve and structural development (Mhadhbi *et al*., 2010). An evaluation of larval growth, deformities, colour, or appearance are morphological indicators of larval quality. This method is however, time consuming, requires some level of expertise, and does not guarantee determination of larval quality (Dhert *et al*., 1993). Correspondingly, stress effects due to overcrowding and handling of fish larvae are subjective, difficult to standardize and evaluate through correlation and statistical analysis. Hatchery managers have successfully separated quality catfish larvae from the weak ones by exposing batches of hatchling to light where photonegative individuals are considered to be of better quality. Active larvae cross barriers in the trough to the dark areas in search of shelter (Schreck & Tort, 2016). This method is valuable to hatchery managers since it is simple and applicable at early stage of larvae development before exogenous feeding. The method compares batches of larvae from different brooders however, it is not applicable in comparing dietary treatments. Moreover, hatchlings quality has been evaluated based on their resistance to starvation in specific activity index (Aristizabal *et al*., 2009).

Development of salinity and chemical stress shocks (such as ammonia test) have provided simple criteria of evaluating larval resistance to inherent stressors in culture conditions for both fish and shrimp (Racotta *et al*., 2003, Liu *et al*., 2020) and helps to indicate fish larvae quality necessary for aquaculture. These stress shocks are carried out at minimal time and financial costs (Dhert *et al*., 1993). Ammonia stress test has been reported to be sensitive and easily reproducible for the establishment of larval quality (Racotta *et al*, 2003). Ammonia levels in aquaculture systems depends on the rate of metabolic activities and organic decomposition (Boyd, 2017), leading to compromised larvae welfare depending on ammonia concentration, temperature, pH, dissolved oxygen, carbon (IV) oxide, and salinity in the culture system (Eddy *et al*., 1999). Effects of ammonia in fish larvae normally manifests through gill lamellae lesions or increased endocrine secretion of cortisol and catecholamine hormones (Fletcher, 1997). These hormones prepare fish larvae to develop stress coping strategies depending on toxicity levels or initiates recovery processes and/or death from stress (Wendelaar, 1997). However coping strategies consume more energy in fish larvae leading to reduced growth, compromised immune system and interrupted body hydromineral balance (Boerrigter, 2015). Consequently, larvae respond to ammonia by actively excreting it, cellular adaptation to ammonia or by detoxifying its quantities in the body through glutamine synthesis (Schram *et al*., 2010). The effectiveness of these coping strategies depends on fish age, immune capacity, interactions of intestinal microbes involved in digestion process, physiological, and nutritional status (Randall & Tsui, 2002; Oliva-Teles, 2012; Rehman *et al*., 2017). Ordinarily, quality diet should provide extra energy and essential fatty acids necessary for fish larvae adjustment and adaptation to rearing conditions. However, main energy and fatty acids provider in a diet is the expensive fish oil therefore, different oil alternative have been explored. Plant and animal oils have been used with variable results and have been found to alter larvae stress responses due to provision of inadequate polyunsaturated fatty acid ratios or unavailability of the nutrient to larvae due to its digestibility (Mejri *et al*., 2021). Crude lipid is a probable indicator of quantities of fatty acid and dietary energy available in a diet for enhanced stress tolerance though it varies with protein source (Montero *et al*., 2003)

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Generally, information on crustacean and general animals stress exists but it is yet to be extended to fish (Samocha *et al*., 1998; Abreu *et al*., 2012; Baßmann *et al*., 2017). Fish larvae stress information remains minimal because of limited data and lack of consensus from researchers on the assessment techniques to use in evaluating larval quality (Arizabal *et al*., 2009; Baßmann *et al*., 2017; Rehman *et al*., 2017; Liu *et al*., 2020). This is despite increased fish larvae vulnerability to stress (Rehman *et al*., 2017). Thus, there is a need to evaluate seed robustness so as to verify the effectiveness of hatchery management practices applied and appraise diet nutritional content as they influence stress tolerance in fish larvae (Dhert *et al*., 1993; Fletcher, 1997; Liang *et al*., 2013).

Clarias gariepinus, is a globally cultured freshwater species and an ecotoxicological model organism (Nguyen & Jassen 2002; Kreutz *et al*., 2008). The species' biology is well understood and documented. It has faster growth and higher tolerance to different environmental conditions (Irina, 2014; Audu *et al*., 2017; Baßmann et *al*, 2017). *Clarias gariepinus* larvae has been reported to have a toxic tolerance range between 0.05-0.5mg/l of unionized ammonia exposure (Ngugi *et al*., 2007; Komugisha & Rajts, 2021). This large range of tolerance is probably because of the species undefined larval stage given variable phase indicators (Chepkirui-Boit *et al*., 2013). However, *C. gariepinus* larvae is a stress vulnerable stage and therefore, requires optimal diet and water quality (Schreck & Tort, 2016; Rehmann *et al*., 2017).

Mass production of *C. gariepinus* larvae has been successful due to its ease of manipulation in captivity and hardiness. The *C. gariepinus* larvae depends on live feed although this feed has been associated with mass mortalities and low energy in larvae of many fish species owing to nutritional deficiency (Takeuchi, 2014). This has led to a general agreement among researcher on the development of dry diets that sustains growth and survival to replace live feed. The performance of these diets has been reported with considerable success (Kong *et al*., 2020). Currently, the use of microalgae, insects, and worm ingredients in fish larval diets have intensified. However, information of these formulated diets performance beyond growth and survival rates is limited (Aragão *et al*., 2022). Yet, information from such evaluations could contribute to the development of aquafeeds that guarantee fish larvae health and quality. This is because nutritional content in a diet has proportionate effects on fish larvae growth, immunity, and general robustness (Kreutz *et al*., 2008; Kumlu *et al*., 2021). This study aimed at determining the effects of partially replacing *C. nilotica* with *S. platensis* or *E. fetida* on stress tolerance of *C. gariepinus* larvae. This information will improve our understanding on the influence of diet on *C. gariepinus* larvae robustness in culture environment.

MATERIALS AND METHODS

Production of the Experimental Larvae

Clarias gariepinus were produced and reared at the University of Nairobi (UoN), Aquaculture laboratory as described in Nyangate *et al.*, (2022). The hatchlings were reared in glass aquaria of 50 l filled to 40 l in controlled hatchery conditions at a density of 25 larvae/l. Aquarium tanks were randomly assigned one of the isonitrogenous and isocaloric formulated diets (*Table 1*) in triplicate (T_1 *(25%S. platensis +75%C. nilotica),* T_2 *(50%S. platensis +50%C. nilotica),* T_3 *(75%S. platensis + 25%C. nilotica),* T_4 *(25%E. fetida +75%C. nilotica),* T_5 *(50%E. fetida +50%C. nilotica),* T_6 *(75%E. fetida +25%C. nilotica),* T_7 *(100%C. nilotica).* Larvae were fed at 10% wet body weight five times a day in standing water for eight weeks. The feeding ration was adjusted weekly based on wet body weight. Water quality and 12-hours light and 12-hours dark photoperiod maintained as detailed in Nyangate *et al*., (2022). A total of 30 four- and six-weeks old larvae per replica were randomly sampled with replacement and

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individually weighed to the nearest 0.001g using an ASB=220=C2 electric weighing balance (Shambhavi impex, Mumbai, India) and their total length measured to the nearest 0.1 cm using a ruler on a wet table.

Table 1: Chemical composition (%) formulated diets (dry matter basis) for *C. gariepinus* larvae feeding

Ingredients	Formulated diets						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Dry Matter	88.6	91.8	94.6	94.3	94.2	95	93.1
Crude Protein	54.8	54.6	54.2	54.9	55	54.8	54.8
Ether Extract	8.82	8	7.57	8.97	8.98	10.4	8.79
Calcium	2.71	2.37	2.03	2.47	1.89	1.31	3.03
Phosphorus	1.29	1.17	1.04	1.24	1.09	0.92	1.42
Crude fiber	1.67	1.77	1.83	1.49	1.44	1.35	1.63
Ash content	13.8	14.6	15	14.2	14.3	12.9	14.2
NFE	21	21	21.4	20.4	20.3	20.5	20.6
DE (kCal/kg)	3804	3802	3801	3804	3800	3802	3802

*NFE*Nitrogen-free extract, DE*=calculated Digestible energy*

Source: (Nyangate *et al*., 2022)

Ammonia Stress Test

Preliminary Trials

In aquaculture, ammonia is a stressor because of its ecological and environmental relevance besides its ability to influence the digestive physiology of fish (IP & Chew, 2010; Turner *et al*., 2019). Ammonia stress concentrations were prepared by dissolving ammonium chloride (batch number 213330, 99.9% purity), 0.0 (control/culture water), 0.1, 0.5, 1, 1.5 and 2g/l that were converted to 0.0 (control), 0.03, 0.2, 0.3, 0.5 and 0.7mg/l respectively using a conversion factor of 0.0502 as described by Francis-Floyd *et al*., (2009), in plastic buckets filled to five litres with tap water. The pH of the prepared ammonium chloride solutions was increased from 6.2 to 7 by an addition of few drops of sodium hydroxide (1N NaOH) to avoid any changes as the experiment progressed. The test solutions were then left in dissolving plastic buckets for a minimum of 2 hours to ensure uniformity and stabilization. Subsequently, the solutions were respectively transferred into 250 ml disposable plastic cups filled to 200 ml in triplicate and suspended in a 100-l water bath maintained at 28°C. The solutions in test cups remained suspended in the water bath until their temperatures matched that in the water bath before introducing test larvae.

Weeks four and six old *C. gariepinus* larvae were subjected to variable ammonia concentrations above to select appropriate ammonia levels for use in subsequent stress test. The larvae were starved for 18 h prior to ammonia exposure to ensure uniform physiological state at the start of the trial. Thereafter, 18 larvae per treatment (six per replica) were randomly sampled using a scoop net, concentrated in a strainer without exposing them to air so as to minimize stress from sampling. Afterward, three larvae per treatment were randomly placed in each of the unionized ammonia concentration for 12 hours. There was no aeration to avoid ammonia oxidation, no feeding to reduce nitrogenous waste burden on the larvae and no handling during the trials to minimizing stress due to manipulation. Death of *C. gariepinus* larvae in each test solution were observed every 30 minutes

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and recorded. Death was defined as no response to touch by plastic ruler.

After 12 hours of exposure, no deaths were recorded in 0.0g/l, a total of 8 larvae had died in 0.5mg/l ammonia solution compared to 100% (18 larvae) mortality in 0.7mg/l. larvae exposed to 0.7mg/l started dyeing after four hours compared to all other ammonia concentrations. The number of deaths and the time when larvae started dying in every ammonia concentration informed the choice of 0.0, 0.5 and 0.7mg/l for ammonia stress test while 0.3mg/l was an intermediate.

Ammonia Stress Test

Stock solutions of 0.0, 0.3, 0.5 and 0.7mg/l unionized ammonia concentration were prepared according to the preliminary trial stress test but scaled up to 20 l in plastic buckets. Thereafter, 350 ml ammonia solutions of each concentration were respectively transferred into 500 ml disposable plastic cups in triplicate. The disposable cups with their content were heated in four different water baths of 100 l glass aquaria at 28 ^{0}C until their temperatures matched that of the water bath.

Twenty *C. gariepinus* larvae per replicate, a total of 60 larvae per treatment were randomly sampled and transferred into each of the experimental cups according to preliminary trial. Also, larvae deaths were observed and recorded as described in the preliminary trial but within 24-hour experimental period. Timing that is practical, easy to replicate by the farmers and economical compared to conventional life-cycle chronic toxicity. Time taken for 50% of larvae to die in each of the replicates were recorded and the total dead per treatment was summed up to calculate mean time for 50% of the larvae to die per treatment. At the end of the 24-hour period, number of dead larvae per triplicate were used to calculate mean total mortality per treatment. The stress index compressed start of larvae mortality, total mortality, and progressive mortalities. This stress index was computed per treatment as described in Dhert *et al*. (1993).

Data Presentation and Analysis

Statistical analysis was done using the GenStat edition 13.3 software programme. One-way ANOVA was performed to test the difference in stress tolerance of *C. gariepinus* larvae fed on formulated diets containing different ingredients. Significant differences between treatments were determined by the Tukey HSD and considered significant at $p < 0.05$. Prior to data analysis, all data sets were subjected to normality and homogeneity of variance tests using Kolmogorov Smirnov's (Zar, 1999), and Levene's tests (Levene, 1960) respectively. All data posted $p>0.05$ and were considered not significant and therefore fitted a normal distribution and had equality of variance. A two-sample T-test was used to compare stress index of four- and six-weeks-old *C. gariepinus* larvae.

RESULTS

Total Mortalities of *C. gariepinus* Larvae Exposed to Different Ammonia Concentrations

Total mortality increased with ammonia concentration and the control solution (0.0 mg/) posted a mortality range of 10% to 16% for both four- and six-weeks-old *C. gariepinus* larvae. Larvae exposed to the highest ammonia concentration of 0.7mg/l posted 100% mortality within 24-hour period for all treatments and for both four- and six-week-old larvae (*Figure 1a* and *1b*). The larvae fed on a combination of combination of 50%*E. fetida* and 50%*C. nilotica* (T_5) posted significantly lower ($p<0.001$) mortalities of 78% and 52% (for four and six-weeks old larvae respectively) as compared to larvae fed on all other formulated diets exposed to 0.5mg/l ammonia solution. However, the larvae fed on 50%*S. platensis* and 50%*C. nilotica* (T_2) recorded the highest mortalities of 97% and 73% respectively compared to all other fed diets in 0.5mg/l ammonia. Though total mortalities posted by diet T_2 fed larvae were not different from those larvae fed on a combination of 75%*S. platensis* and 25%*C. nilotica*

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(T_3) in similar ammonia concentration (*Figure 1a* and *1b*). *Clarias gariepinus* larvae exposed to 0.3mg/l and 0.5mg/l ammonia posted higher total mortalities for four-week-old compared to the six-weeks-old larvae. Larvae fed on diets T_1, T_2 and T_3 containing *S. platensis* recorded higher mortality for both the four- and six-weeks-old *C gariepinus* larvae compared to those fed on *E. fetida* containing diets and the control. The increase of *S. platensis* levels in a diet decreased mortality from 77% to 65% for six-week-old larvae (*Figure 1b*) compared to those of four-week-old where diet T_1 had the lowest mortality of 83% in 0.5mg/l ammonia solution (*Figure 1a*). Six weeks old larvae fed on all diets posted lower mortalities compared to those of four weeks old larvae (*Figures 1a* and *1b*).

Figure 1a: Total mortality (mean ± SE) for four-week-old C. gariepinus larvae exposed to different ammonia concentrations

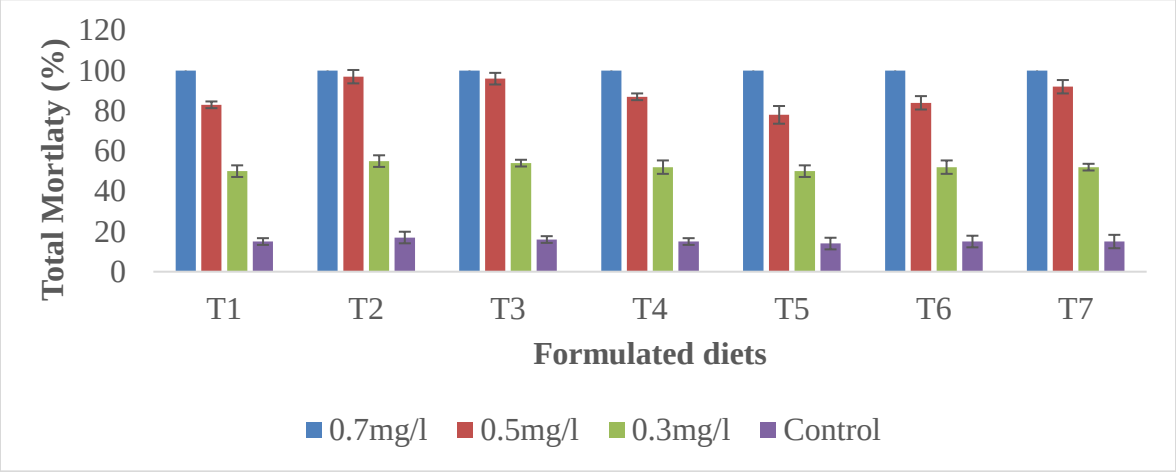
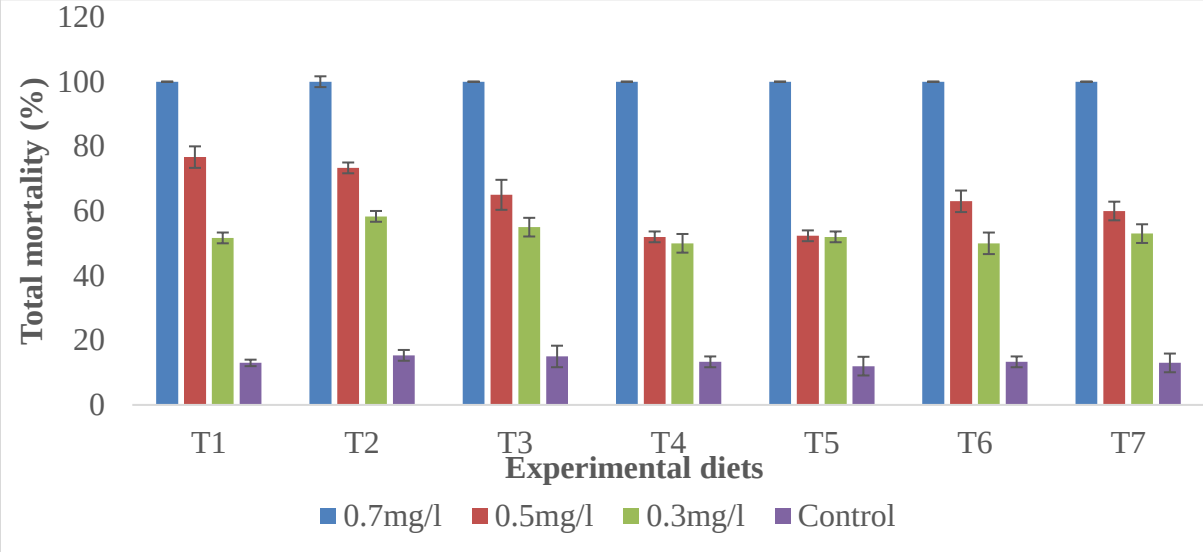


Figure 1b: Total mortality (mean ± SE) for six-week-old C. gariepinus larvae exposed to different ammonia concentrations



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Time Taken for Half of *C. gariepinus* Larvae Exposed to Different Ammonia Concentration to Die

Time taken for half the numbers of *C. gariepinus* larvae fed on *S. platensis* or *E. fetida* diets to die was influenced by ammonia concentrations (*Table 2*). Four- and six-weeks-old larvae fed on a combination of 50%*E. fetida* and 50%*C. nilotica* (T_5) recorded significantly longer (<0.001) time of 12.67 and 18.67 hours respectively, for 50% of larvae exposed to die in 0.7mg/l and 24 hour in 0.3mg/l (p=0.012) ammonia compared to all other diets. Larvae fed on a combination of 75%*E. fetida* and 25%*C. nilotica* (T_6) were more sensitive to ammonia and took a shorter time of 10.83 and 16.67 hours respectively, for half the numbers exposed to 0.7mg/l to die compared to those fed on diet T_5. However, four- and six-weeks-old larvae fed on a combination of 50%*S. platensis* and 50%*C. nilotica* (T_2) were significantly (p<0.001) more sensitive to ammonia exposure and took the shortest time of 8 and 10.17 hours respectively, for 50% of the larvae exposed to 0.7mg/l ammonia solution to die compared to all other diets fed larvae. However, a combination of 50%*S. spirulina* and 50%*C. nilotica* (T_2) fed larvae sensitivity in 0.7mg/l ammonia was not different from that fed on a combination of 75%*S. spirulina* and 25%*C. nilotica* (T_3). Further, a blend of 25%*E. fetida* and 75%*C. nilotica* (T_4) and 100%*C. nilotica* (T_7) fed larvae took a similar time for 50% of the larvae exposed to die in the three ammonia solutions (0.3, 0.5 and 0.7mg/l) in both the four- and six-weeks-old larvae. Larvae fed on *S. platensis* containing diets had half the number dying faster for both four- and six-weeks-old larvae compared to *E. fetida* fed larvae.

Stress index for C. *gariepinus* Larvae Exposed to Ammonia Concentrations

The stress index increased with ammonia concentration for both four- and six-weeks-old *C. gariepinus* larvae as shown in *Table 3*. A combination of 50%*E. fetida* and 50%*C. nilotica* (T_5) posted significantly lower (p<0.001) stress index in the ranges of 81.67 – 457.3 and 83.33 – 342 for weeks four-and- six old larvae respectively compared to all other dietary treatments. However, a significantly higher (p<0.001) stress index was posted by combination of 50%*S. platensis* and 50%*C. nilotica* in ranges of 89 – 586 and 83-500 for four- and six-weeks-old larvae respectively in *Table 3*. Nonetheless, larvae fed on a combination of 75%*S. platensis* and 25%*C. nilotica* (T_3) posted a significantly lower(p<0.001) stress index at 0.5mg/l and 0.7mg/l ammonia for both four- and six-weeks-old larvae compared to those fed on 50%*S. platensis* and 50%*C. nilotica* (T_2) in similar concentrations.

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Table 2: Time taken (mean hours, SEM, n=60) for half of the numbers of *C. gariepinus* larvae to die in different ammonia solutions

Larvae age (weeks)	Ammonia solutions	Formulated diets							LSD	SEM	p
		T_1	T_2	T_3	T_4	T_5	T_6	T_7			
4	0.7 mg/lg	9^{bc}	8^{a}	8.17ab	9.33^{c}	12.67e	10.83d	9.83^{c}	32.43	10.69	<.001
	0.5mg/lg	11.67^{ab}	10.83^{a}	10.83^{a}	13.33^{c}	15.67^{d}	13.83c	12.83^{bc}	45.86	15.12	<.001
	0.3mg/l	23.67^{ab}	23.28^{a}	23^{a}	23.83^{ab}	24^{b}	23.83^{ab}	23.83^{ab}	25.35	8.36	0.012
	control	-	--	-	-	-	-	-	-	-	-
6	0.7mg/l	11.67^{b}	10.17^{a}	10.67^{a}	15.33^{c}	18.67^{e}	16.67^{d}	14.83^{c}	36.25	11.95	<.001
	0.5mg/l	17.33^{b}	15^{a}	15.67^{a}	19.83^{c}	23.83^{e}	21^{d}	19.17^{c}	32.43	10.69	<.001
	0.3mg/l	21.33^{bc}	20^{a}	20.33^{ab}	21.83^{c}	24^{d}	23.5^{d}	22.17^{c}	42.9	14.14	<.001
	Control	-	-	-	-	-	-	-	-	-	-

T_1- T_7 are formulated diets described in materials and methods, SEM =standard error means and LSD=least significance difference, numbers with similar superscript are not significantly different

Table 3: Stress index for (mean, SEM, n=60) weeks four and six old *C. gariepinus* larvae fed on the different formulated diets

Time (Weeks)	Ammonia fractions	Formulated diets							LSD	SEM	p
		T_1	T_2	T_3	T_4	T_5	T_6	T_7			
4	control	89.33^{a}	89^{a}	88^{a}	90^{a}	81.67^{a}	84^{a}	85.33^{a}	5.994	1.976	0.072
	0.3mg/l	194.3^{d}	265.7^{e}	265.3^{e}	156^{c}	115.3^{a}	147.3^{b}	153.3^{c}	5.2	1.741	<.001
	0.5mg/l	475^{d}	531.3^{f}	497.3^{e}	443^{c}	370.7^{a}	394^{b}	451^{c}	8.88	2.93	<.001
	0.7mg/l	554.3^{d}	586f	574.3^{e}	521.7^{c}	457.3^{a}	482.7^{b}	516.7^{c}	5.212	1.718	<.001
6	Control	82^{a}	83^{a}	84^{a}	81.67^{a}	83.33^{a}	82^{a}	82.33^{a}	2.729	0.9	0.529
	0.3mg/l	170^{d}	219.7^{f}	212.3^{e}	142^{bc}	111.3^{a}	127.3^{b}	151.3^{bc}	4.633	1.52	<.001
	0.5mg/l	259.3^{d}	334^{f}	318e	239.7^{c}	185^{a}	211^{b}	236^{c}	6.474	2.134	<.001
	0.7mg/l	429.7^{e}	500^{g}	476.3^{f}	406.3^{c}	342^{a}	374.7^{b}	418.7^{d}	6.018	1.984	<.001

T_1- T_7 formulated diets described in materials and methods, SEM = standard error mean, LSD = least significance difference, numbers with similar superscript are not significantly different, control=0.0mg/l ammonia concentration

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A contrasting trend was observed for larvae fed on a combination of 75%*E. fetida* and 25%*C. nilotica* (T_6) which recorded higher stress index in all ammonia solutions compared for those larvae fed on *E. fetida* and 50%*C. nilotica* (T_5). It was interesting to observe no differences in stress indices for diets T_4 (25%*E. fetida* and 75%*C. nilotica*) and T_7 (100% *C. nilotica*) fed larvae in all ammonia solutions except 0.7mg/l for six-weeks-old larvae. *Clarias gariepinus* larvae fed on *S. platensis* containing diets had higher stress indices in all ammonia solutions compared to *E. fetida* containing diets and the control diet (100%*C. nilotica*). In general. Week six old larvae posted lower stress index in all test solutions compared week four old larvae.

A Comparison of weeks four- and six- old *C. gariepinus* Larvae Stress Indices in Different Ammonia Concentrations

The age of *C. gariepinus* larvae revealed a substantial ($p<0.05$) effect on stress indices for all diets evaluated (*Table 4*). Larvae fed on all diets posted meaningfully higher stress index for four-weeks-old compared to six-weeks-old larvae. Furthermore, a combination of *50%E. fetida* and 50%*C. nilotica* posted notably lower ($p=0.004$) stress indices of 414 and 265.5 for four- and six-week-olds larvae respectively compared to all other diets tested. Nevertheless, larvae fed on a blend of 50% *S. platensis* and 50%*C. nilotica* (T_2) recorded the highest stress indices of 545.3 and 417 in four- and six-week-olds respectively. This was followed by stress indices of 535.3 and 413.8 recorded for larvae fed on a combination of 75%*S. platensis* and 25%*C. nilotica* (T_3). Both four- and six-week-old, larvae fed on *S. platensis* had particularly higher stress indices compared to those fed on *E. fetida.*

Table 4: A comparison of stress indices (Equality test, mean ± SEM, df=58, SED, t-value) between four- and six-weeks old *C. gariepinus* larvae fed on the formulated diets

Diets	Equality of variance	week 4	week 6	SED	t value	p-value
	p value	mean	mean			
T_1	0.8	478.8±33.76	344.5±38.10	50.92	2.64	0.025
T_2	0.15	545.3±18.30	417±37.14	41.41	3.1	0.011
T_3	0.12	535.8±17.27	413.8±37.06	40.89	2.98	0.014
T_4	0.13	482.3±17.61	323±37.28	41.23	3.86	0.003
T_5	0.22	414±19.39	263.5±35.16	40.15	3.75	0.004
T_6	0.14	443.3±17.74	292.8±36.6	40.68	3.7	0.004
T_7	0.2	490±12.02	327.3±40.85	42.58	3.82	0.003

T_1- T_7 formulated diets described in materials and methods, SED= Standard error of difference

DISCUSSION

Formulated diets influenced *C. gariepinus* larvae nutritional status and physiological wellbeing as demonstrated by stress indices reported over a 24-hour period (*Table 3*). A combination of 50%*E. fetida* + 50%*C. nilotica* (T_5) produced stress tolerant larvae that posted the lowest ($p<0.001$) stress indices within ranges of 115.3 - 475.3 and 113.3-342 for week four and six old larvae respectively compared to all other diet fed larvae. The observation could be accredited to high energy reserves due to the synergetic advantages of combining animal proteins, increaseFd growth reported in Nyangate *et al*, (2022) and higher lipid content of 8.98% (*Table 1*). According to Abaho *et al*., (2016), higher lipids in a combination of 50%*E. fetida* + 50%*C. nilotica* (T_5) could have provided more polyunsaturated fatty acids (PUFA) in *C. gariepinus* larvae for enhanced

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growth, immunity, survival, and stress tolerance (Tacon *et al*., 1983, Conceicao et al., 2012). Nyangate *et al*., (2022), reported significantly higher growth with a weight gain of 0.43±0.05g for larvae fed on 50%*E. fetida* + 50%*C. nilotica* and higher methionine (1.12g/100g/diet) and lysine (2.88g/100g diet) compared to all other formulated diets. This improved growth suggested improved development of gills, skin, kidney, and liver for enhanced ammonia excretion during the ammonia exposure in larvae fed on diet T_5. According to Conceição *et al*., (2012), methionine and lysine levels in diet T_5 could have provided necessary energy and enhanced innate immune system to reduce ammonia sensitivity during the 24-hour ammonia exposure. These findings were comparable to enhanced survival and stress tolerance reported for longfin yellow tail (*S. rivoliana*) by Mesa-Rodriguez *et al.,* (2018). Possibly because both studies used diets with lipid levels of 9% that could have increased energy reserves for effective physiological processes to manage ammonia effects by maintaining normal larval metabolic processes and health state.

On the other hand, *C. gariepinus* larvae fed on a blend of 50%*S. platensis* and 50%*C. nilotica* (T_2) had notably higher ($p<0.001$) stress indices in the ranges of 89-586 and 83-500 for four- and six-weeks old larvae respectively (*Table 3*). This could be attributed to low energy reserves to mobilize ammonia stress coping strategies in larvae fed on diets T_2. This could be supported by significantly low growth gain of 0.20±0.05g and methionine and lysine concentration of 0.73g/100g and 2.49g/100g respectively compared to all other formulated diets reported by Nyangate *et al*., (2022). Probably because diet T_2 fed larvae could have used up their energies and diet nutrients in other physiological processes other than growth and immune system for enhanced ammonia tolerance. Thus, diet T_2 fed larvae were possibly weak and vulnerable to ammonia stress perhaps because of poor nutrient utilization (Dhert *et al*., 1993; Sushma *et al*., 2021). This is because stress tolerance in fish species has been reported to be proportional to its nutritional status (Kreutz *et al*., 2008). Similarly, low growth for diet T_2 fed larvae did suggest low energy reserves that could not efficiently stimulate oxygen uptake to minimize stress effects through ammonia oxidation processes.

According to reports by Mesa-Rodriguez *et al*., (2018), larvae fed on a mixture of 75%*E. fetida* +25%*C. nilotica* (T_6) were expected to be less sensitive to ammonia stress owing to diets high lipid content of 10.4% dry matter shown in *Table 1*. However, diet T_6 fed larvae were more sensitive to ammonia stress and posted higher stress indices in the ranges of 147.3 – 482.7 and 127.3 – 374.3 for four- and six-week old larvae respectively, compared to 50%*E. fetida* +50%*C. nilotica*. Presenting larvae fed on a combination of 75%*E. fetida* +25%*C. nilotica* as less robust compared to those fed on a combination of 50%*E. fetida* +50%*C. nilotica* (T_5) . This could be attributed to poor growth and increased chitin content (non-digestible protein) in diet T_6 due high inclusion levels of *E. fetida* that could have decreased its digestibility (Musyoka *et al*., 2019). Thus, reduced supply of nutrients for development and enhanced stress tolerance in *C. gariepinus* larvae (Kumlu *et al*., 2021). Furthermore, high stress indices were recorded possibly because lipid (10.4/% dry matter) content could have increased PUFA beyond optimal levels for *C. gariepinus* larvae and could have caused physiological malfunctioning and suppression of stress management abilities of the larvae (Rufchaei *et al*., 2019). Higher dietary lipids have been reported to decrease growth, survival, and stress tolerance in larval trout (Dhert *et al*., 1993). On the contrary, larvae fed on a diet containing higher inclusion of *S. platensis* (75%*S. platensis* + 25%*C. nilotica*) posted lower stress indices of 265.3 – 574.3 and 212.3 – 476.3 respectively for weeks four and six old larvae compared to those fed on 50%*S. platensis* +50%*C. nilotica* (T_2). This observation was not expected since their growth was not different as reported by Nyangate *et al*., (2022). The current study did not have evidence to explain this observation as this diet had the lowest lipid content of 7.57% (*Table 1*) compared to all other formulated diets. Consequently, the diet could not

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provide optimal PUFA for enhanced ammonia stress tolerance. Moreover, the study did not analyse the bioactive compounds in *S. platensis* because of financial constrictions. Thus, the study only hypothesized low stress index for diet T_3 fed larvae to be due to increased antioxidant (carotenoids, beta-carotene and phycocyanin) quantities in the diet which could have reduced ammonia effects in the larvae (Awed *et al*., 2020; Sushma *et al*., 2021).

Stress indices posted by week six old larvae for all formulated diets were statistically lower ($p<0.05$) compared to those of week four as shown in *Table 4*. This observation could be attributed to improved gill functionality over time. At earlier days of *C. gariepinus* larvae, ammonia is effectively excreted through the skin because gills remain elementary with reduced surface area and low density of ionocytes (Zimmer *et al*., 2017; Alexandrova *et al*., 2021). Therefore, six weeks old *C. gariepinus* larvae could have had efficient branchial systems to enhance oxygen uptake for ammonia oxidation hence increased tolerance to ammonia stress compared to ammonia clearance in week four old larvae (Alexandrova *et al*., 2021). This study findings deviated from higher stress tolerance reported for five-day old tilapia larvae compared to 25 days old tilapia (Luz *et al*., 2012). The increased stress tolerance at fifth day in tilapia could be due to optimal nutritional content supplied by the yolk sack compared to the later life stages. However, the current study findings were comparable to those reported for earlier life stages of *C. gariepinus* exposed to different toxicants within seven days difference (Nguyen & Janssen, 2002). Therefore, the study proposes stocking of older larvae that could have passed the most sensitive stage that could be threatened by inherent environmental toxicants in culture waters.

Spirulina platensis containing diets had higher total mortalities (*Figure 1a* and *1b*), stress indices (*Table 3*) and shorter time for half the number of *C. gariepinus* larvae exposed to ammonia concentrations to die (*Table 2*) compared to *E. fetida* diets. These results could be attributed to low lipids (*Table 1*) and energy reserve, PUFA alteration before (sun drying) and during diet formulation, compromised digestive physiology, limited availability of bioactive and nutrients due to variable digestibility and assimilation of plant and animal protein in the larvae gastrointestinal tract (Verreth *et al*., 1992; Munguti *et al*., 2021). This observation does not conform to earlier reports that *S. platensis* alleviates toxic effects in *C. gariepinus* through antioxidant activities (Sayed & Authman, 2018). According to Awed *et al*., (2020), antioxidants enhance specific biochemical and physiological activities that neutralize effects of the toxicant. Therefore, *S. platensis* fatty acid supply could have been compromised for effective stimulation of larval immunity against ammonia stress effects (Diraman *et al*., 2009). Further, *S. platensis* diets could have failed to stimulate intestinal flora to aid in digestion of indigestible feed components and production of enzymes that could neutralize ammonia effects in *C. gariepinus* larvae (Hasanein *et al*., 2018). On the other hand, *E. fetida* containing diets improved tolerance to ammonia stress could be attributed to higher lipid levels and corresponding fatty acids in *E. fetida* diets which enhances fish resistance (Tacon *et al*., 1983; Abaho *et al*., 2016; Ebm *et al*., 2021). Observations that could be supported by Kumlu *et al*., (2018), findings of 51-53% PUFA in *E. fetida* compared to 28-42% of fatty acids reported for *S. platensis* by Diraman *et al*., (2009). This could have been a possible explanation for the observed differential total mortalities and stress indices between these protein alternatives fed to *C. gariepinus* larvae. Enhanced ammonia tolerance by larvae fed on *E. fetida* containing diets was comparable to those reported for Caspian roach (*Rutilus caspicus*) larvae by Rufchaei *et al*., (2019) and mirror carp where *E. fetida* replaced herring meal (Rawling *et al*., 2014). This was probably due to higher levels of Arachidonic acids (ARA) and bioactive molecules like peroxidase and lysozymes positively influencing larvae's stress tolerance and growth performance in both studies (Tacon *et al*., 1983; Rufchaei *et al*., 2019).

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Clarias gariepinus larvae fed on all formulated diets posted total mortalities within the of range of 10% to 16% for both four- and six-weeks-old larvae exposed to the control solution (0.0mg/l), though it was not expected (*Figure 1a* and *1b*). This was attributed to incidental mortalities due to natural factors or experimental circumstances. These factors could have compromised oxygen and energy distribution to vital organs for effective functioning and survival of the larvae in the control solution (Schreck & Tort, 2016). This study's findings were comparable to 1-21% reported for *C. gariepinus* larvae in a control solution during ammonia toxicity test (Nguyen & Jassen, 2002). This serves as a warning to farmers and hatchery managers that mortalities may still occur in the absence of toxic substances in the culture water. Therefore, larvae demand extra care in the hatcheries to avoid economic losses.

Four- and six-weeks-old larvae fed on all formulated diets posted 100% mortality in 0.7mg/l ammonia solution. An indication of ammonia effects being proportional to its concentration and all diet's inability to enhance C. *gariepinus* larvae tolerance in 0.7mg/l ammonia. This confirms 0.7mg/l ammonia to be above the optimal tolerance limit of 0.5mg/l unionized ammonia reported for *C. gariepinus* larvae by Ngugi *et al.,* (2007). Though, this value was still higher than 0.05mg/l reported for hatcheries by Komugisha and Rafts (2021). According to Wang *et al*., (2021), short period exposure to ammonia could have caused severe effects on amino-acid metabolism therefore, disrupting physiological functioning of the larvae leading to death. This is probably because of neuron depolarization and activation of glutamate receptors leading to acid-base disturbance and subsequent central nervous system cell deaths (Eddy *et al*., 1999; Randal and Tsui 2002). Furthermore, the death of larvae could be attributed to excessive use of brain energy due to ineffective physiological process to convert toxic ammonia to non-toxic substances like glutamine through glutamine synthetase or destruction of ammonia excretion system (Irina, 2014; Chithambaran *et al*., 2015; Turner *et al*., 2019).

All formulated diets fed larvae registered low total mortalities in low ammonia concentrations (*Figure 1a* and *1b*). This could be attributed to the ability of *C. gariepinus* larvae to adapt and tolerate low ammonia levels in culture water (Sanderson *et al*., 2010). In culture, there exists some level of ammonia due to continuous decomposition of excess feed and faeces. This could have stimulated an increase of immunity genes and antioxidant enzymes responsible for enhanced stress tolerance in low ammonia exposure (Qi *et al*., 2017). This study results were comparable to those reported for *C. gariepinus* larvae in low ammonia concentrations by Terjesen *et al*., (2001) and Kreutz *et al*., (2008). This similarity could be attributed to the capacity of *C. gariepinus* larvae to down-regulate stress effects through ornithine urea cycle enzymes (Sayed & Authman, 2018).

CONCLUSIONS AND RECOMMENDATIONS

It is possible to partially replace *C. nilotica by E. fetida* up to 50% or 25% of *S. platensis* in a diet for enhanced *C. gariepinus* larvae stress tolerance. *Clarias gariepinus* larvae fed on a combination of 50%*E. fetida* and 50%*C. nilotica* were more tolerant to ammonia stress as indicated by significantly low mortalities, stress indices and longest time for half the number of larvae exposed to ammonia to die for both weeks four and six old larvae. This was conceivably because of energy synergy from combined animal protein and high lipid content that improved nutritional status, survival, and stress resistance of the larvae. However, 50%*S. platensis* and 50%*C. nilotica* fed larvae were the most sensitive to ammonia stress probably because of low growth which could have resulted low energy reserves to boost stress coping strategies. Enhanced ammonia tolerance by week six-old *C. gariepinus* larvae for all formulated diets was possibly because of improved gill development and branchial system for increased oxygen uptake to counter ammonia toxicity. Furthermore, *C. gariepinus* larvae sensitivity to ammonia stress was proportional to ammonia concentration. Larvae enhanced sensitivity at

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0.7mg/l ammonia concentration was due to intense stress that compromised ammonia excretion and detoxifying mechanisms in the larvae.

Protein alternatives in the current study influenced stress tolerance in *C. gariepinus* larvae in ammonia. *Eisenia fetida* containing diets fed larvae were less sensitive to ammonia stress compared to *S. platensis* containing diets. Possibly because of differences in protein structure and larval abilities in utilizing algae and worm derived proteins. The study recommends worm containing diets for *C. gariepinus* larvae and proposes stocking of older larvae to prevent economic losses while ensuring aquaculture sustainability and environmental health. The study also, recommends an evaluation of *E. fetida* or *S. platensis* enriched with PUFA effects on stress tolerance on *C. gariepinus* larvae.

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