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ORIGINAL RESEARCH ARTICLE

Effects of water hardness level on metabolic reserves of post-embryonic life stages of *Culex quinquefasciatus* Say 1826 (Diptera: Culicidae)

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Abstract

Metabolic Reserve (MR) is critical for success of adult mosquitoes, and its availability across life stages of mosquitoes is affected by abiotic factors such as water hardness. We investigated the influence of water hardness levels on MR in *Culex quinquefasciatus, vis-à-vis* its accumulation, mobilisation and utilisation for metamorphosis. First larval instars of the species were reared in five simulated water hardness regimens (0, 30, 90, 150 and 210 mg/L $CaCO_3$) till adulthood following standard protocols. Life stage accumulation of MR components (lipid, protein, glucose and glycogen) and its utilisation for pupation and eclosion were quantified following standard protocols. Analyses revealed significant influence of water hardness on MR at all life stages of *Cx. quinquefasciatus.* MR components increased with development and larval age, and peaked at last larval instar. Water hardness level ≥ 90 mg/L $CaCO_3$ elicited decrease in all reserve components. Reserve components' daily accumulation rates were highest at 90 and and lowest at 210 mg/L $CaCO_3$. Glycogen was the most accumulated MR components at all regimens. Significantly higher reserve component were utilised for pupation and eclosion at regimen ≥ 150 mg/L $CaCO_3$. The information generated from this study would be useful in developing novel vector control technologies through habitat manipulation.

Keywords Metabolic reserve · Body size · Teneral reserve · Accumulation rates · Metamorphosis

Introduction

Culex quinquefasciatus, also known as southern house mosquito, is responsible for the spread of pathogens of important human and livestock diseases such as lymphatic filariasis, and West Nile Equine Virus (Carter Centre 2013). The mosquito continues to constitute a major source of nuisance to man and great public health concern. However, its success as adult and ability to serve as vector depends on energy reserves accumulated during development (Timmermann and Briegel 1999). Due to the ability of this mosquito species to breed in almost any available water habitat, there is

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increased tendency of the species to be exposed to various degree of water conditions/ qualities. These varying water conditions play critical roles in determining energy reserves of the aquatic (larvae and pupae) and terrestrial (adult) life stages.

Teneral metabolic reserves in insects determines most of adult life traits. Further, reserves accumulated during the larval stage are also critical for egg production and thus fecundity, impacting in turn the amount of blood that must be consumed to produce eggs. In holometabolous insects, adult energy reserve is a function of total metabolic reserve accumulated at the final phagoperiod (i.e., fourth larval instar) and the reserves utilised during metamorphosis (pupation and eclosion). Therefore, inefficiency in larval reserves accumulation, affect adult life traits such as host- and blood-meal seeking in females (Briegel 1990a; Naksathit et al. 1999).

Carbohydrate, lipid, protein and glycogen are metabolic reserve components of epidemiological importance in mosquitoes. These components play unique roles in determining the biological fitness of teneral mosquitoes; serving as source of energy for flight and mate location (Briegel 2003), egg development (Aparna et al. 2006) and fecundity (Briegel 1990b). The reserve components of teneral adults have been correlated to onset of blood-meal and host seeking in mosquitoes (Klowden et al. 1988; Zhou et al. 2004).

In mosquitoes, metabolic reserves are determined, principally, by physiological conditions of the larval instars (Telang and Wells 2004) and regulated by prevailing environmental factors (Rejmankova et al. 1991; Washburn 1995). Example of such environmental conditions is the water hardness levels. Water hardness level, has been categorised as 'soft', 'slightly hard', 'moderately hard', 'hard' and 'very hard' water, with values of < 17.0 mg/L CaCO3, 17.0−59 mg/L CaCO3, 60−119 mg/L CaCO3, 120−180 mg/L CaCO3 and > 180 mg/L CaCO3, respectively (Water Quality Association, WQA 2018). Laboratory investigation revealed significant effects of these categories on development (Ukubuiwe et al. 2020) and morphometrics of mosquito (Ukubuiwe et al. 2018a);

In mosquito ecology, water hardness levels determine the quality and productivity of mosquito habitats (Kant et al. 1996; Olayemi et al. 2010). The presence and concentration of calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions are chiefly responsible for the level of water hardness encountered in field studies (Mwangangi et al. 2007). These ions (e.g., Ca^{2+}), apart from being a component of the soil water, constitute the major constituent of insects' cuticles, and affect the availability of ions necessary for growth of aquatic insects, hence, their spatio-temporal distribution (Piyaratne et al. 2005).

According to Tiimub et al. (2012), mosquito larval habitats with high level of hardness does not support mosquito development, while soft waters provide excellent breeding grounds for most mosquitoes (Robert et al. 1998). Field studies have reported various hardness level in mosquito habitats; rain water (20 mg/L), river water (60 mg/L), borehole water (80 mg/L) and well water (230 mg/L) (Oyewole et al. 2009). Presently, quantitative data on the influence of water hardness on metabolic reserve accumulation and utilisation is scanty or probably non-existent for the species. This information is important in understanding the interplay between this factor and biological fitness of the species. It is also important in making informed decision for deployment of control intervention resources and developing novel control protocols of the vector through manipulation of habitats.

Materials and methods

Source, rearing and maintenance of experimental mosquitoes

Freshly laid egg rafts of *Culex quinquefasciatus* were collected from a mosquito colony in the Entomological Unit of Animal Biology, Federal University of Technology, Minna, Nigeria. The eggs were incubated (at ambient temperature) till hatching by placing them in transparent plastic hatching trays following standard protocols (Ukubuiwe et al. 2016). The newly hatched larvae were reared following standard techniques as described earlier (Ukubuiwe et al. 2012). Briefly, the larvae were reared at the rate of 250 larvae/bowl in 1000 mL of the prepared media in plastic trays (30 cm X 25 cm X 5 cm). The larvae were fed with fish feed (Coppens®) at the rate of 0.32 mg/ 100 larvae every other day, sprinkled on the water surface. On every alternate day, the water from the culture bowls were changed till eclosion. This was to prevent formation of scum and ensure the hardness level is maintained on daily basis. On pupation, the pupae were separated daily and placed in plastic bowl (5 cm height and 20 cm diameter) half filled with the prepared media. These plastic bowls with pupae were labelled and placed in adult-holding cages for emergence.

Simulation of water hardness condition

Five water hardness regimens were produced according to standard classification of water hardness, to mimic varying harness conditions. These are 0, 30 ('soft' water), 90 ('moderately hard' water), 150 ('hard' water), and 210 mg/L $CaCO_3$ ('very hard' water), according to modified methods of Ukubuiwe et al. (2018a). Briefly, the artificial rearing media were prepared by adding the respective weight of Calcium trioxocarbonate, $CaCO_3$ (Chemetrics, Inc., Calverton, VA) to distilled water to make 1000 ml volume. The Control experiment was distilled

Table 1 Accumulation Rates of Metabolic Reserve Components in *Culex quinquefasciatus* Larvae at different Water Hardness Conditions

Hardness Regimen	Metabolic Reserve Component (µg /mosquito/ day)			
(mg/L $CaCO_3$)	Lipid	Glucose	Glycogen	Protein
0	$6.26 \pm 0.29^{b*}{}_{a**}$	$4.79 \pm 0.11^{b}{}_{a}$	$14.92 \pm 0.53^{b}{}_{b}$	$11.26 \pm 0.40^{b}{}_{b}$
30	$5.84 \pm 0.20^{b}{}_{a}$	$4.57 \pm 0.17^{b}{}_{a}$	$14.11 \pm 0.54^{b}{}_{b}$	$10.94 \pm 0.64^{b}{}_{b}$
90	$8.74 \pm 1.29^{d}{}_{a}$	$6.08 \pm 0.97^{d}{}_{a}$	$18.64 \pm 2.71^{c}{}_{b}$	$14.92 \pm 1.90^{c}{}_{b}$
150	$7.86 \pm 0.82^{c}{}_{a}$	$5.67 \pm 0.64^{c}{}_{a}$	$18.55 \pm 2.42^{c}{}_{b}$	$15.56 \pm 1.85^{c}{}_{b}$
210	$2.59 \pm 0.12^{a}{}_{a}$	$1.91 \pm 0.07^{a}{}_{a}$	$6.94 \pm 0.25^{a}{}_{b}$	$5.59 \pm 0.52^{a}{}_{b}$

*Values followed by same superscript alphabet in a column are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). **Values followed by same subscript alphabet in a row are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). All values are expressed as Mean ± SD of Mean

water, with no addition of $CaCO_3$. The hardness levels were monitored every 6 h to regulate fluctuations by using a water hardness meter (Thomas No. 1215Y58).

Quantification of metabolic reserves in the experimental mosquitoes

Four metabolic components namely lipid, carbohydrate (glycogen and glucose) and protein were determined for all post-embryonic life stages (larva, pupa and adult) and larval instars (first, LI to fourth, LIV). At all stage and instar, samples were randomly selected for the analyses following the methods of Van-Handel (1985a, b), Van-Handel and Day (1988) and Kaufmann and Brown (2008) as reported by Ukubuiwe et al. (2018b). To estimate the reserves utilised for pupation, pupae were collected immediately after pupation, while newly eclosed adults were retrieved immediately to estimate reserves utilised for eclosion. Reserves utilised for pupation and eclosion were estimated, as the difference in values of reserve components of late fourth larval instars, and new pupae, and between day old pupae and newly emerged adults, respectively. In estimating reserves for metamorphosis, late fourth larval instars were used as this stage signifies the climax of immature reserve accumulation. Further, newly pupated mosquitoes were also used to ensure that reserves were not utilised for other activities, while newly eclosed adults were used to estimate, closely, reserves of teneral adults.

Data analysis

Data generated were tested for goodness-of-fit before analyses, and processed into means and standard deviation using Statistical Package for Social Scientists (SPSS) version 21. Differences among means of entomologic variables were compared for significance difference using one-way Analysis of variance (ANOVA); coupled with Duncan Multiple Range Test (DMRT). Linear correlation graphs were used to determine regression equations for the rates of accumulation of metabolic components. Mean values of metabolic components were depicted using bar graph. All decisions on statistical comparison of means was taken at $p < 0.05$ level of significance.

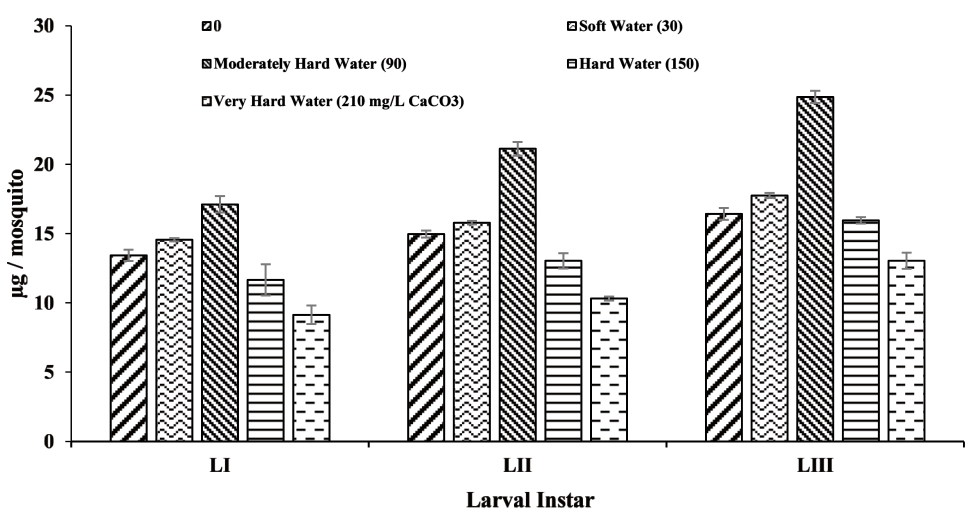

Fig. 1 Variations in Lipid Reserve of Larval Instars of *Cx. quinquefasciatus* at different Water Hardness Conditions. Values are expressed as mean ± SD, n = 30. LI – LIII = Larval instar 1 to 3. Larval instars of *Cx. quinquefasciatus* were exposed to varying water hardness conditions

Table 2 Life Stages' Variations in Lipid Reserve of *Cx. quinquefasciatus* at different Water Hardness Conditions

Hardness Regimen (mg/L $CaCO_3$)	Life Stage Reserve (µg/ mosquito)		
	Fourth Larval Instar	Pupa	Adult
0	$19.22 \pm 0.46^{c*}{}_{b**}$	$17.15 \pm 0.11^{c}{}_{ab}$	$15.54 \pm 1.10^{c}{}_{a}$
30	$20.71 \pm 0.55^{d}{}_{b}$	$18.99 \pm 0.08^{d}{}_{ab}$	$16.20 \pm 0.14^{c}{}_{a}$
90	$25.34 \pm 0.48^{e}{}_{b}$	$22.68 \pm 0.81^{e}{}_{a}$	$21.59 \pm 0.36^{d}{}_{a}$
150	$18.30 \pm 1.18^{b}{}_{c}$	$15.06 \pm 0.08^{b}{}_{b}$	$12.52 \pm 0.40^{b}{}_{a}$
210	$14.47 \pm 0.49^{a}{}_{b}$	$11.75 \pm 0.74^{a}{}_{a}$	$10.03 \pm 0.25^{a}{}_{a}$

Values followed by same superscript alphabet in a column are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). **Values followed by same subscript alphabet within a row are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). Values are expressed as Mean ± SD

Result

Effects of water hardness conditions on larval instars' accumulation rates of metabolic reserve in *Culex quinquefasciatus*

Among the reserve components, the rates of accumulation of glycogen and protein in the larval instars were significantly (df = 4, n = 50, p = 0.002) higher than the rates of lipids and glucose accumulation at all water hardness conditions (Table 1). Accumulation rate of lipid and glucose at the larval stage were highest in 'moderately hard' water condition, and were lowest in 'very hard' water condition. Rates of accumulation of glycogen and protein were highest in 'moderately hard' and 'hard water' conditions; though, there were no significant difference (df = 4, n = 50, p = 0.08) in these values (Table 1). Mosquitoes reared in 'very hard' water condition had the lowest rates of glycogen and protein accumulation (Table 1).

Linear correlation graphs revealed the following equations for larval rates of accumulation of metabolic reserve in *Cx. quinquefasciatus* under different water hardness conditions: glycogen, $y = -1.152x + 18.088$, $r^2 = 0.1459$; protein, $y = -0.672x + 13.67$, $r^2 = 0.0713$; lipid, $y = -0.532x + 7.854$, $r^2 = 0.1266$; and glucose, $y = -0.466x + 6.002$, $r^2 = 0.2047$.

Effects of water hardness conditions on metabolic reserves of life stages of *Culex quinquefasciatus*

Lipid reserve: Growth from LI to LIV recorded significant (df = 4, p = 0.001) increase in lipid reserve. Mosquitoes reared in 'very hard' water condition (210 mg/L $CaCO_3$) had the lowest lipid reserve, while the mosquitoes reared in 'moderately hard' water had the highest lipid reserves. The accumulation of lipid climaxed at LIV, however, at pupal and adult stages, lipid reserves of the species significantly (p = 0.001) reduced (Fig. 1 and Table 2).

Glucose reserve: Life stages' glucose reserves of *Cx. quinquefasciatus* were, negatively, affected by water hardness conditions, especially at 'very hard' water condition (Fig. 2 and Table 3). Glucose reserves increased with age of larval instar; climaxing at LIV. Mosquitoes had the lowest glucose reserve when reared in 'very hard' water condition and the highest at 'moderately hard' water. Pupal and adult life stages witnessed significant reduction in glucose reserves, after moult of larvae to pupae, and pupae to adults, respectively.

Glycogen reserve: The glycogen reserves of the life stages of *Cx. quinquefasciatus* were negatively affected by

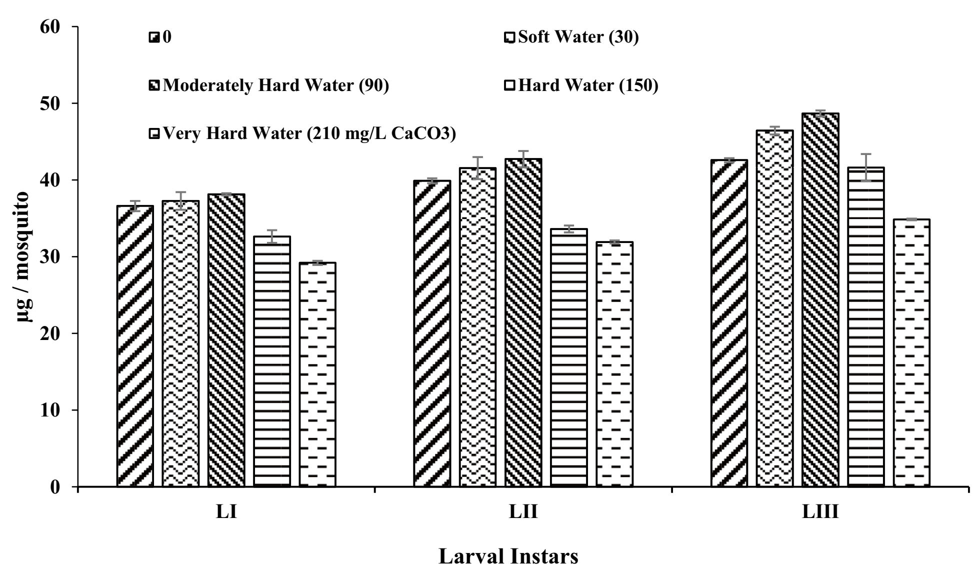

Fig. 2 Variations in Glucose Reserve of Larval Instars of *Cx. quinquefasciatus* at different Water Hardness Conditions. Values are expressed as mean ± SD, n = 30. LI – LIII = Larval instar 1 to 3. Larval instars of *Cx. quinquefasciatus* were exposed to varying water hardness conditions

Table 3 Life Stages' Variations in Glucose Reserve of *Cx. quinquefasciatus* at different Water Hardness Conditions

Hardness Regimen (mg/L $CaCO_3$)	Life Stage (µg/ mosquito)		
	Fourth Larval Instar	Pupa	Adult
0	$14.72 \pm 0.07^{c*}_{a**}$	$14.10 \pm 0.61^{c}_{a}$	$12.45 \pm 0.43^{c}_{a}$
30	$16.21 \pm 0.32^{d}_{b}$	$15.21 \pm 0.44^{d}_{ab}$	$14.57 \pm 0.41^{d}_{a}$
90	$19.74 \pm 0.25^{e}_{a}$	$17.78 \pm 0.60^{e}_{a}$	$17.33 \pm 0.32^{e}_{a}$
150	$13.19 \pm 0.44^{b}_{c}$	$9.00 \pm 0.52^{b}_{b}$	$7.94 \pm 0.49^{b}_{a}$
210	$10.71 \pm 0.15^{a}_{b}$	$6.30 \pm 0.45^{a}_{a}$	$5.98 \pm 0.62^{a}_{a}$

Values followed by same superscript alphabet in a column are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). **Values followed by same subscript alphabet within a row are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). Values are expressed as Mean ± SD

Table 4 Life Stages' Variations in Glycogen Reserve of *Cx. quinquefasciatus* at different Water Hardness Conditions

Hardness Regimen (mg/L $CaCO_3$)	Life Stage (µg/ mosquito)		
	Fourth Larval Instar	Pupa	Adult
0	$45.82 \pm 0.54^{c*}_{b**}$	$44.11 \pm 0.32^{c}_{ab}$	$43.06 \pm 0.08^{c}_{a}$
30	$50.08 \pm 0.82^{d}_{a}$	$48.10 \pm 0.61^{d}_{a}$	$47.46 \pm 0.48^{d}_{a}$
90	$54.07 \pm 0.84^{e}_{a}$	$51.45 \pm 0.61^{e}_{a}$	$49.97 \pm 0.14^{e}_{a}$
150	$43.03 \pm 0.15^{b}_{c}$	$38.99 \pm 0.32^{b}_{b}$	$35.09 \pm 0.10^{b}_{a}$
210	$38.80 \pm 0.62^{a}_{c}$	$32.29 \pm 0.23^{a}_{b}$	$27.66 \pm 0.42^{a}_{a}$

Values followed by same superscript alphabet in a column are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). **Values followed by same subscript alphabet within a row are not significantly different at $p < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA). Values are expressed as Mean ± SD

increasing water hardness condition (Fig. 3 and Table 4). Glycogen levels increased significantly ($p < 0.05$) as the larvae progressed from LI to LIV (Fig. 3); this was, however, dependent on water hardness level.

Glycogen reserve level of the species also peaked at fourth larval instar, LIV, and at 'moderately hard' water. The pupal life stage had significantly ($p = 0.01$) lower glycogen level than LIV. Teneral adult had significantly lower glycogen reserves than LIV and pupae. Glycogen reserves of LIV, pupae and adult stages at 30 and 90 mg/L $CaCO_3$ were not significantly ($p = 0.08$) different; while at hardness conditions > 90 mg/L $CaCO_3$, significant life stages' variation in glycogen reserves values were observed (Table 4).

Protein reserve: Analyses revealed significant ($df = 4$, $n = 50$, $p = 0.002$) effects of water hardness conditions on protein reserve of the life stages of *Cx. quinquefasciatus* (Fig. 4 and Table 5). Protein reserves of LI to LIII instars of the species differed significantly between among and within treatments.

The protein reserve of fourth larval instar was also significantly ($p = 0.02$) higher than those of the succeeding life stages (pupae and adult). The pupal protein reserve varied significantly ($p = 0.001$) among the treatments. Teneral adult protein reserve followed similar trend as the preceeding life stages.

Analyses also revealed no significant ($p = 0.07$) difference among the protein reserves of LIV and pupae at ≤ 30 mg/L $CaCO_3$ ('soft water' range). At 'moderately hard' water (90 mg/L $CaCO_3$), protein reserves of the life stages (LIV, pupae and adult) did not differ significantly. However, at hardness conditions > 90 mg/L $CaCO_3$, there were significant ($p = 0.072$) difference in life stages' protein reserves of the species.

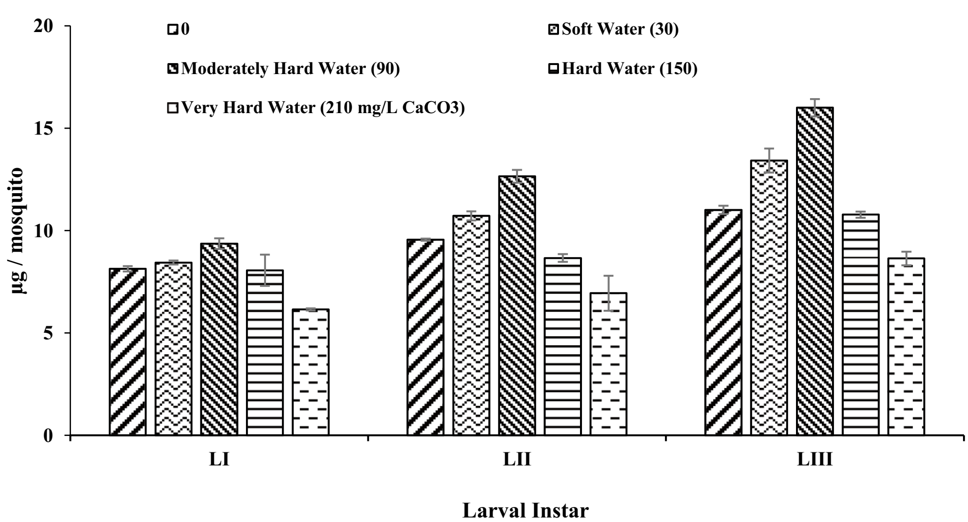

Fig. 3 Variations in Glycogen Reserve of Larval Instars of *Cx. quinquefasciatus* at different Water Hardness Conditions. Values are expressed as mean ± SD, $n = 30$. LI – LIII = Larval instar 1 to 3. Larval instars of *Cx. quinquefasciatus* were exposed to varying water hardness conditions

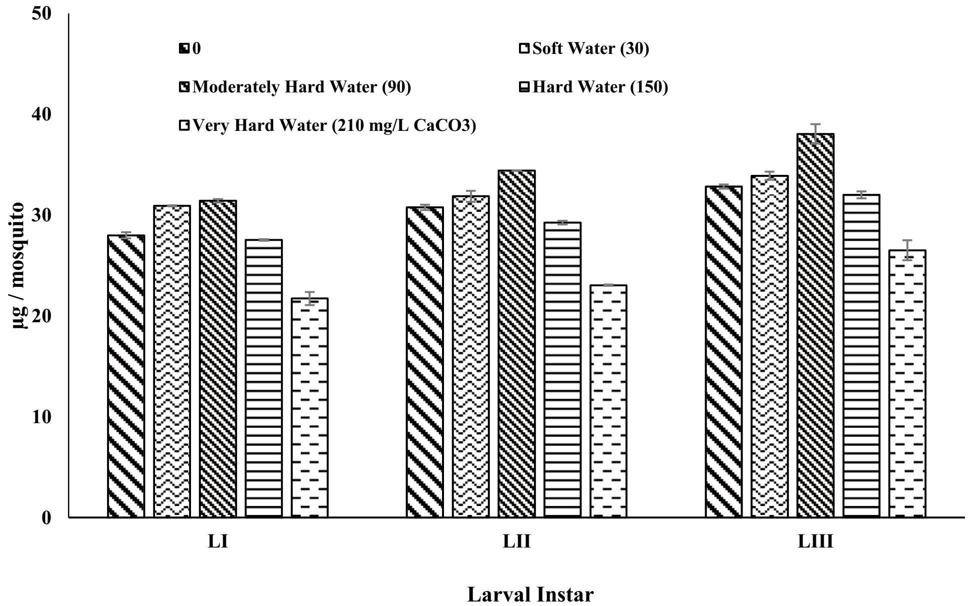

Fig. 4 Variations in Protein Reserve of Larval Instars of *Cx. quinquefasciatus* at different Water Hardness Conditions. Values are expressed as mean ± SD, n = 30. LI – LIII = Larval instar 1 to 3. Larval instars of *Cx. quinquefasciatus* were exposed to varying water hardness conditions

Effects of water hardness on reserves utilised for pupation and eclosion in *Culex quinquefasciatus* mosquito

Pupation: The reserve components and total reserves utilised for pupation in *Cx. quinquefasciatus* were significantly (df = 4, n = 50, p = 0.002) affected by water hardness conditions (Fig. 5 and Table 6). Mosquitoes reared in water hardness levels ≤ 90 mg/L $CaCO_3$ utilised significantly (df = 4, n = 50, p = 0.008) lower aggregate reserves for pupation; while the mosquito cohorts reared at hardness level ≥ 150 mg/L $CaCO_3$ utilised significantly (p = 0.004) higher reserves (Table 6).

Table 5 Life Stages' Variations in Protein Reserves of *Cx. quinquefasciatus* at different Water Hardness Conditions

Hardness Regimen (mg/L $CaCO_3$)	Life Stage (µg/ mosquito)		
	Fourth Larval Instar	Pupa	Adult
0	$34.58 \pm 0.42^{b*}{}_{b^{**}}$	$33.34 \pm 0.58^{c}{}_{b}$	$30.46 \pm 0.47^{c}{}_{a}$
30	$38.80 \pm 0.59^{c}{}_{b}$	$37.35 \pm 0.11^{d}{}_{b}$	$33.32 \pm 0.45^{d}{}_{a}$
90	$43.37 \pm 0.56^{d}{}_{a}$	$42.47 \pm 0.55^{e}{}_{a}$	$41.18 \pm 0.88^{e}{}_{a}$
150	$36.14 \pm 0.66^{b}{}_{c}$	$30.45 \pm 4.04^{b}{}_{b}$	$26.08 \pm 0.24^{b}{}_{a}$
210	$31.29 \pm 2.69^{a}{}_{c}$	$24.69 \pm 0.66^{a}{}_{b}$	$20.24 \pm 0.07^{a}{}_{a}$

*Values followed by same superscript alphabet in a column are not significantly different at p < 0.05 according to Duncan multiple range test following analysis of variance (ANOVA). **Values followed by same subscript alphabet within a row are not significantly different at p < 0.05 according to Duncan multiple range test following analysis of variance (ANOVA). Values are expressed as Mean ± SD

The reserve components (lipid, protein, glucose and glycogen) utilised for pupation varied significantly (df = 4, n = 50, p = 0.001) among the larvae from the different water hardness conditions. Glucose appeared to be the most utilised for pupation by the species, this was evidenced from the significantly (df = 4, n = 50, p = 0.01) higher values for the components at all hardness conditions. However, mosquitoes reared at 'hard' and 'very hard' water, utilised significantly (p = 0.002) higher amounts of protein for pupation. Lipid, on the other hand, were most utilised by mosquitoes reared at hardness level ≥ 90 mg/ L $CaCO_3$ and least by those reared at ≥ 150 mg/ L $CaCO_3$ (Fig. 5).

Eclosion: Water hardness conditions also significantly affected total reserves utilised for eclosion (df = 4, n = 50, p = 0.001) in *Cx. quinquefasciatus* (Table 6). Unlike during pupation, mosquitoes reared in 'moderately hard' water utilised the lowest amount of reserves for eclosion, while those reared in 'very hard' water utilised the highest. There were, however, no significant difference (df = 4, n = 50, p = 0.085) in the reserve expenditure (for eclosion) among pupae at hardness level ≤ 30 mg/L $CaCO_3$ and ≥ 150 mg/L $CaCO_3$ (Table 6).

Water hardness condition also affected the reserve components expended during eclosion (df = 4, n = 50, p = 0.008). Mosquitoes reared in 'moderately hard' water utilised the lowest reserves components for eclosion (Fig. 6). At all water hardness conditions tested, protein reserve was the most utilised for eclosion; though, there were no definite trend or pattern for utilisation of this reserve. This was followed by glycogen, especially at hardness level ≥ 90 mg/L $CaCO_3$. Glucose, on the other hand was the least utilised by all mosquito cohorts at eclosion (Fig. 6).

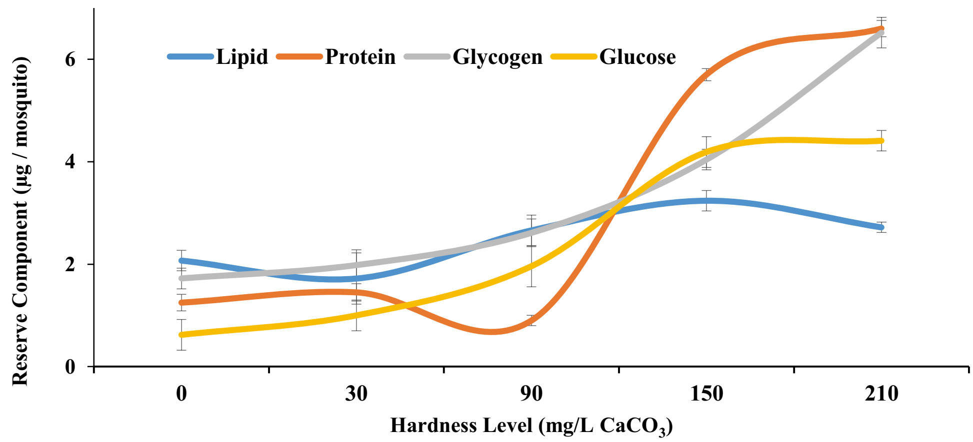

Fig. 5 Effect of Water Hardness on Reserves utilised for Pupation in *Cx. quinquefasciatus.* Values are expressed as mean ± SD. Larval instars of *Cx. quinquefasciatus* were exposed to varying water hardness conditions

Discussion

Effect of water Hardness on larval accumulation rates of metabolic reserve in *Culex quinquefasciatus*

In the present study, rates of accumulation of metabolic reserves in larvae of the species were affected by water hardness levels. Mosquito cohorts reared in 'moderately hard' and 'hard' water conditions had the highest rates of accumulation of glycogen and protein. Larvae reared in 'moderately hard' water condition had the highest rates of lipid and glucose accumulation. On the other spectrum, larvae reared in 'very hard' water condition had the lowest rates of metabolic components' accumulation. These results suggest that 'moderately hard' water condition is the optimum for accumulation of reserves, while very hard' water condition affects it negatively.

Rates of reserves accumulation are affected by factors such as feeding rates (Day and Van-Handel 1986) and environmental stress level (Mpho et al. 2002). Since the rates of reserve accumulation were highest in 'moderately hard' water condition, it follows, therefore, that this water condition favoured feeding in the species, and presented the lowest (if any) stress conditions than other water hardness conditions.

Table 6 Effects of Water Hardness on Total Reserves (µg / mosquito) utilised for Pupation and Eclosion in *Culex quinquefasciatus*

Hardness Regimen (mg/L $CaCO_3$)	Pupation	Eclosion	Total
0	5.66 ± 1.41[a*]	7.17 ± 3.23[b]	12.83 ± 4.64[a]
30	6.15 ± 3.19[a]	8.10 ± 1.77[b]	14.25 ± 4.96[a]
90	8.13 ± 0.92[a]	4.31 ± 1.70[a]	12.44 ± 2.62[a]
150	17.17 ± 5.77[b]	9.87 ± 5.60[c]	27.04 ± 11.37[b]
210	20.25 ± 4.50[b]	11.12 ± 2.37[c]	31.37 ± 6.87[b]

[*]Values followed by same superscript alphabet in a column are not significantly different at $P < 0.05$ according to Duncan multiple range test following analysis of variance (ANOVA); Values are expressed as Mean ± SD

In an earlier study, 'moderately hard' water produced big mosquitoes (Ukubuiwe et al. 2018a), supported the fastest growth rates and high survivorship in *Cx. quinquefasciatus* (Ukubuiwe et al. 2020). These observations and the high rates of accumulation of metabolic reserves (observed in the present study) suggest that *Cx. quinquefasciatus* mosquitoes from 'moderately hard' water conditions may be better vector of disease in the wild; given these good indices of fitness in this water condition. These adults would also be expected to be more fecund. Further studies in this line isrecommended..

In the present study, irrespective of water hardness condition, glycogen and protein reserves had the highest accumulation rates. This phenomenon may underscore the developmental and physiological roles of these two components in the species and insects in general. Protein, for example, serve as major building blocks for most anatomic structures and physiologic processes, while glycogen is a great store of energy for the species (Tauber and Kyriacou 2001; Gillott 2005).

Effect of water hardness on metabolic reserve accumulation and distribution across life stages of *Culex quinquefasciatus*

Mineral elements, such as calcium present in $CaCO_3$, are essential for normal physiology and growth of organisms. There are, however, minimum and maximum tolerance limits; outside which, serious abnormalities will occur. Excess of these chemicals are sources of toxicity, eliciting environmental stress conditions which alter feeding rate (Merritt et al. 1992; Poteat and Buchwalter 2014). This impaired feed intake negatively affects the amount of metabolic reserve (Day and Van-Handel 1986), its life stages' mobilisation in insects (Briegel 1990b), and, hence, energy availability for normal activities.

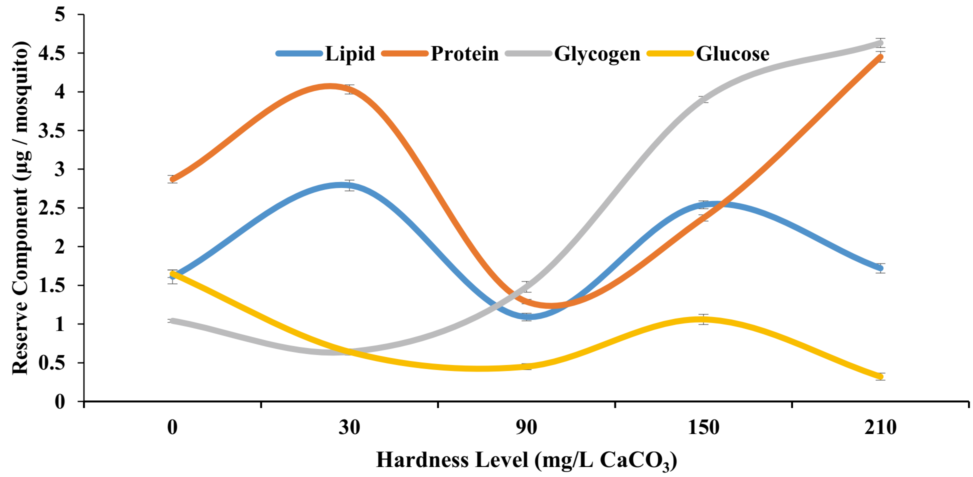

Fig. 6 Effect of Water Hardness on Reserves utilised for Eclosion in *Cx. quinquefasciatus*. Values are expressed as mean ± SD. Larval instars of *Cx. quinquefasciatus* were exposed to varying water hardness conditions

The present study revealed significant negative effects of increasing water hardness condition on the metabolic reserves components (lipid, glycogen, sugar, and protein) across the life stages of *Cx. quinquefasciatus*. First, at all hardness conditions, metabolic reserve components gradually increased as the larval instars progressed from LI to LIV instar, and reduced, significantly, during pupation and eclosion to pupal and adult stages, respectively. Secondly, metabolic reserves at each life stage, exhibited an inverse relationship with water hardness level. We opined that increasing level of toxicity and/ or hardness-induced stress conditions elicited by increasing water hardness conditions could have elicited these inverse associations (Wesner et al. 2014). The possibility of stress induction and/or toxic condition was evident in the differential rates of accumulation of all metabolic component by the active feeding stage (larvae) observed in this study. Thirdly, mosquito larvae reared in ‘very hard’ water had the least of all metabolic components, while those from ‘moderately hard’ water had the highest of reserves, closely followed by those from ‘soft’ water. Interestingly, ‘moderately hard’ water had been reported to support development of the species (Ukubuiwe et al. 2020). These reports suggests that ‘moderately hard’ water is the optimum water hardness condition that supports growth and metabolic reserve accumulation in *Cx. quinquefasciatus*; while ‘very hard’ water discourages it. However, field evaluations are advocated to consolidate these possibilities.

In addition, in mosquitoes, accumulation of metabolic reserve during the phagoperiods (larval stages) determines its availability at subsequent life stages. It, therefore, follows that subsequent life stages (e.g., pupae and adult mosquitoes) from ‘moderately hard’ water conditions may have higher metabolic reserves than the cohorts from other water hardness conditions.

The present observations may explain the differential productivity of domestic well water of different hardness levels in the study area (unreported) and reported elsewhere in Dakar, Senegal (Robert et al. 1998). Water hardness status can provide insight on the productivity of mosquito habitats. Water ‘hardening’ agents can also be applied to increase hardness level, thereby reducing productivity of less hard habitats, this is, though, subject to field experimentation. This information could be incorporated in mosquito vector survellance and control programs.

Effects of water hardness on reserve utilised for metamorphosis in *Culex quinquefasciatus*

According to Grodzicki and Walentynowicz (2011), physiological processes in insect are strongly influenced by changes in the prevailing environmental condition; these fluctuations affect metabolic rates and physiological needs of insects (Bartholomew and Casey 1978; Saunders 2002). In the present study, water hardness conditions affected the total metabolic reserves required for metamorphosis (pupation and eclosion) in *Cx. quinquefasciatus.* Among the reserve components, protein and glycogen – which had the highest rates of accumulation – were the most expended, while glucose the least. These observations could represent the significant functions of these components as highlighted above.

For pupation: Hardness level ≤ 90 mg/L $CaCO_3$ significantly lowered reserves utilised for pupation. Unlike at hardness level ≥ 150 mg/L $CaCO_3$, where the species utilised more for the process. The reason for this was unclear. Although, in our earlier study, hardness level ≥ 150 mg/L $CaCO_3$ presented stress-like conditions for the species, eliciting greater mortality and prolonged duration of development (Ukubuiwe et al. 2020). We, therefore, opined that this observation (increased utilisation of reserves for pupation) at ≥ 150 mg/L $CaCO_3$ may represent intensified physiological demands for tissue repairs processes due to the hardness induced stress condition, and/ or developmental needs of the mosquito at this hardness level.

Among the reserve components, glucose appeared to be the most utilised for pupation by the species. This could represent increased utilisation due to the non-feeding habits of pupae. Glucose, available in form of trehalose, is a readily available source of energy during non-feeding periods. At hardness conditions ≥ 150 mg/ L $CaCO_3$, protein was the most utilised for pupation by the species, this may be correlated to increased need for tissue repairs due to damage caused by toxic conditions (Wesner et al. 2014) and prolonged duration of development as observed and reported earlier (Ukubuiwe et al. 2020).

Lipid, a foremost requirement for vitellogenesis, was most utilised by mosquitoes reared at hardness level ≥ 90 mg/ L $CaCO_3$ and least by those reared at ≥ 150 mg/ L $CaCO_3$. The reason for this was not understood; but may represent physiological needs at these hardness conditions. However, we postulate that those individuals that utilised lower metabolic components may have had 'normal' physiological developmental process, devoid of 'stress'; hence, demanded lower reserves, unlike those in water conditions with ≥ 150 mg/ L $CaCO_3$. These observations are epidemiological important for disease transmission in mosquitoes; as increased expenditure for pupation will ultimately determine the amount available for adult life stage (Bartholomew and Casey 1978).

For eclosion: In the present study, mosquito cohorts reared in 'moderately hard' water utilised the lowest reserve for eclosion, while those reared in 'very hard' water utilised the highest. According to Marron et al. (2003), physiological processes which reduces energy availability in tenerals limit the ability of species to carry out adult life traits. This observation is epidemiologically significant, as the former group would have higher reserves as tenerals and efficient as vectors and adults, unlike the latter group (Tauber et al. 1986). These results could also explain the low adult daily survivorship and short post-emergent longevity observed in the latter group in an earlier study (Ukubuiwe et al. 2020).

Though, there were no significant difference in reserves utilised by the pupae reared at hardness level ≤ 30 mg/L $CaCO_3$ and ≥ 150 mg/L $CaCO_3$ for eclosion, the former groups of mosquitoes utilised significantly lower total reserves than the latter for the process. Further, the tenerals from the hardness condition ≤ 30 mg/L $CaCO_3$ had significantly higher reserves than the latter group. These observations portend that mosquitoes from water hardness conditions ≤ 30 mg/L $CaCO_3$ have more metabolic reserves as adults than those from conditions ≥ 150 mg/L $CaCO_3$.

The present study demonstrated that the reserve components expended during the process of eclosion is also dependent on water hardness condition; this was evident in mosquitoes reared in 'soft' water and hardness level conditions below it. These mosquitoes utilised more lipid and protein, while mosquitoes reared in 'moderately hard' water utilised higher protein and glycogen reserves. For the mosquitoes reared in 'hard' and 'very hard' water conditions, lipid, protein and glycogen were the most expended. The reasons for these observations need further investigation, but could mark various physiological need of these mosquitoes occasioned by the hardness conditions.

Conclusion

This study revealed that larvae of *Cx. quinquefasciatus* reared in 'moderately hard' water condition had the highest rates of accumulation of metabolic reserves, while those reared in 'very hard' water condition had lower reserve accumulation rates. The mosquito larvae in the former water hardness conditions had the highest of all metabolic component measured. At hardness level ≤ 90 mg/L $CaCO_3$, the species utilised lower aggregate reserves for pupation, while at hardness level ≥ 150 mg/L $CaCO_3$, the species utilised significantly higher reserves. Field evaluations of these results are recommended, and the genetic bases of these interactions should be investigated for the development of novel genetic control protocols.

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Authors' contribution Azubuike Christian Ukubuiwe (ACU), Israel Kayode Olayemi (IKO), Francis Ofurum Arimoro (FOA), and Innocent Chukwuemeka James Omalu (ICJO) conceived and designed the experiments. ACU, Chinenye Catherine Ukubuiwe (CCU), Bulus Musa Baba (BMB) and Bright Ugbede Sule (BUS) performed the experiments. ACU, IKO, FOA, ICJO and CCU analyzed the data. ACU, CCU and BUS wrote the first draft of the manuscript. IKO, FOA, and ICJO corrected the draft copy. All AUTHORS agreed to the final state of the manuscript.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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