Improvement of water quality for mass anopheline rearing: Evaluation of the impact of ammonia-capturing zeolite on larval development and adult phenotypic quality

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Research

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Abstract

Background

Malaria vector control approaches that rely on mosquito releases such as the sterile insect technique (SIT) and suppression or replacement strategies relying on genetically modified mosquitoes (GMM) depend on effective mass production of *Anopheles* mosquitoes. Anophelines typically require relatively clean larval rearing water, and water management techniques that minimise toxic ammonia are key to achieving optimal rearing conditions in small and large rearing facilities. Zeolites are extensively used in closed-system fish aquaculture to improve water quality and reduce water consumption, thanks to their selective adsorption of ammonia and toxic heavy metals. The many advantages of zeolites include low cost, abundance in many parts of the world and environmental friendliness. However, so far, their potential benefit for mosquito rearing has not been evaluated.

Methods

This study evaluated the independent effects of zeolite and daily water changes (to simulate a continuous flow system) on the rearing of *An. coluzzii* under two feed regimes (powder or slurry feed) and larval densities (200 and 400 larvae per tray). The duration of larval development, adult emergence success and phenotypic quality (body size) were recorded to assess the impact of water treatments on mosquito numbers, phenotypic quality and identification of optimal feeding regimes and larval density for the use of zeolite.

Results

Overall, mosquito emergence, duration of development and adult phenotypic quality was significantly better in treatments with daily water changes. In treatments without daily water changes, zeolite significantly improved water quality at the lower larval rearing density, resulting in higher mosquito emergence and shorter development time. At the lower larval rearing density, the adult phenotypic quality did not significantly differ between zeolite treatment without water changes and those with daily changes.

Conclusions

These results suggest that treating rearing water with zeolite can improve mosquito production in smaller facilities. Zeolite could also offer cost-effective and environmental-friendly solutions for water recycling management systems in larger production facilities. Further studies are needed to optimise and assess the costs and benefits of such applications to *Anopheles gambiae* s.l. mosquito rearing programmes.

Background

In sub-Saharan Africa, the primary vectors of malaria are found in the *Anopheles gambiae* s.l species complex, with members, *An. gambiae s.s.*, *An. coluzzii*, and *An. arabiensis* transmitting malaria over vast ranges of sub-Saharan Africa and the surrounding islands [1]. Due to insecticide resistance, there is an increasing demand for complementary or novel approaches to vector control, such as sterile insect technique (SIT), that are effective, sustainable, environmentally-friendly and able to sustain the progress that has been made toward reduction and elimination of malaria transmission [2,3].

The success of SIT or other mass-release based vector control approaches rely on large-scale production of anopheline mosquitoes and are dependent on a reliable supply of constant water of sufficient quality [4–7]. In their natural environment, *An. gambiae* s.s, *An. coluzzii* and *An. arabiensis* typically use larval breeding sites with comparatively cleaner water than *Culex* and aedine species, and their larvae do not survive in water with high organic and bacterial content [8–10]. This requirement is carried over to the insectary, where mosquito larvae succumb at high ammonia levels and do not tolerate heavy bacterial growth [11]. Effective water management in mosquito insectaries that avoids waste and bacteria build-up is key to achieving optimal rearing results for small and large mass-rearing facilities [4,11,12]. This implies that rearing facilities often rely on a water circulating system with continuous quality monitoring and feeding systems that maintain optimal diet availability whilst preventing overfeeding [13]. If only clean water were to be used for this purpose, large amounts of water would be required. For example, the SIT production centres the FAO/IAEA recommends a larval rearing rack (holding up to 200,000 *Anopheles* larvae) using approximately 250l per cohort [4,6,12]. Approximately 100,000l of water is required to produce 10,000,000 sterile males per week [4,12]. In an attempt to reduce water consumption, the FAO/IAEA research laboratory has also tested reusing larval rearing water treated by ultrafiltration (UF) and reverse osmosis (RO) for successive mosquito generations [4,11]. Whilst the results showed success in rearing outcomes; there are other water treatment techniques involving mechanical, chemical and biological filtration that are currently in use for recycling water in fish aquaculture but remain to be evaluated for mosquito rearing [4,11]

Zeolites are microporous crystalline aluminosilicates with chemically neutral basic

honeycomb-like structures [14]. This chemical structure of zeolite forms a network of channels and cavities allowing easy penetration of molecules which are filtered according to size, polarity and shape, thereby serving as an efficient filter which absorbs various substances such as ammonia, heavy metals, pesticides, smells, radioactive cations and many other toxins [15]. Zeolites have an excellent ion exchange capability for cations and prefer those with greater radius and monovalent charge, hence their affinity for cations such as ammonium ion (NH_4^+) [14,16]. Due to their porous nature, the ion exchange occurs not only at the surface but also deep within the zeolite structure, further enhancing its adsorption efficiency [17,18]. There are more than 60 types of naturally occurring zeolites with 150 synthetic types formulated with improved efficiency [14]. Natural zeolites are abundant in many parts of the world where thick deposition and contemporaneous volcanism occurred, such as New Zealand, Japan. Korea, Alaska, western United States, Sakhalin, Kamchatka, Chile and other potential areas in the Tethys region [19].

These properties have caught the aquaculture industry's attention, resulting in an industry-wide application of zeolite in fish and crustacean aquaculture to improve water and feed quality, reduce the negative environmental impacts of aquaculture and improve the quality of seafood [14,15]. In closed-system fish aquaculture research, zeolite has been integrated into biofilters to improve efficiency, for live fish transportation to prevent ammonia accumulation, and as an additive to improve fish growth and health [14,15]. A recent study showed that zeolite's use improved European seabass's survival rate by 12% and growth performance compared to control [20]. Another study showed increased feed consumption and utilisation, improved growth rate, phenotypic quality, and reduced mortality resulting in a 31% increase in economic returns when *Oreochromis niloticus* rearing water was treated with zeolite [21]. In addition, following saturation, zeolite can easily and cheaply be recharged by soaking in 10% NaCl solution and reused [14,17,18].

In this study, the use of zeolite treatment was evaluated by rearing the Mopti strain of *An. coluzzii* in comparison and/or combination with a continuous flow system (simulated by daily water changes). Results showed that under certain conditions, treating rearing water with zeolite could significantly improve production in small-facilities. The possible use of zeolite to compliment or offer a cheaper alternative to water treatment steps such as ultrafiltration, reverse osmosis, or biological filtration which are often part of larger continuous water flow and water recycling systems is discussed.

Methods

Mosquito strain

The Mopti strain of *An. coluzzii*, colonised 17 years ago in 2003 by the Lanzaro Laboratory (UC Davis) from the village of N'Gabacoro droit near Bamako, Mali, West Africa, was used for the experiments. The strain was maintained by the Tripet lab in dedicated insectaries of the Centre of Applied Entomology and Parasitology (CAEP), Keele University, UK. Mosquitoes were maintained at 25 ± 2 °C, relative humidity of 70 ± 5%, with a 12-h light/dark photocycle. Larvae were fed an optimised diet of ground fish food flakes (Tetramin, Tetra, Melle, Germany) at a rearing density of 200 larvae/litre [22]. Pupae were transferred to 5l plastic cages (20.5cm height × 20cm diameter), covered with netting for adult emergence. Cages had sleeved opening for easy management of mosquitoes and accessories. Approximately 600–800 adults were held in a cage, sugar was provided *via* a paper towel soaked in 10% glucose solution, and water *via* a soaked cotton pad in an upturned bowl placed on the cage netting. Female adult mosquitoes were fed with horse blood using an artificial feeding membrane (Hemotek feeding membrane system, Discovery workshops, Blackburn, UK). Styrofoam cups (egg cups) containing filter paper and water were placed in the cages four days post-blood-feeding – to collect eggs. Following the removal of the egg cups, the cages were washed thoroughly and sterilised with bleach. Mouth aspirators were used to transfer adults from one container to another when necessary.

Experimental design: Effect of zeolite treatment, water changes, feed regimes and larval density on the development and phenotypic quality of *An. coluzzii*

First instar larvae of *An. coluzzii* were reared at two larval rearing densities (200 and 400 larvae per tray), under four different water treatment regimes, and using two different feed regimes. This resulted in a fully balanced 2 x 4 x 2 design and 16 larval trays per replicate with a total sample size of 19,200 larvae for four replicates. Trays were identified with coloured tapes codes and fully randomised in their positions on the insectary shelves (Figure 1).

Mosquito larvae were reared in mineral water containing natural minerals formed through geological processes and sourced in 5l bottled from a local supplier. Water quality specification for mineral water were: TDS (112.21 ± 2 mg/l), salinity (75.78 ±1 ppm), conductivity (160.40 ± 2 μS) This water contained the following minerals per litre: calcium (11 mg), magnesium (3.5 mg), potassium (2.5 mg), sodium (10 mg), bicarbonate (25 mg), sulphate (11 mg), nitrate (15 mg), chloride (14 mg), dry residue at 180 °C (85 mg) and pH 6.2.

Larvae were reared in four water treatment groups: **Water-change (WC):** first instar larvae were transferred to trays containing 500ml of mineral water on day 1. On day 5, 400ml of water was gently drained from the trays using a low pressured water pump through a filter net to prevent mosquito larvae escaping into the pump after which 900 ml of fresh mineral water was added to the tray. This process of gently draining rearing water and replacing with fresh water was repeated daily from day 5 until all mosquitoes in the tray had pupated (Figure 1). **Water-change-zeolite (WCZ):** first instar larvae were transferred to trays containing 500ml of mineral water on day 1; 1g of finely ground zeolite powder (Natural Clinoptilolite, Minerals-Water, Rainham, United Kingdom) was added to the rearing water on day 4. On day 5, 400ml of water was gently drained from the trays using a low pressured water pump through a filter net to prevent mosquito larvae escaping into the pump after which 900 ml of fresh mineral water was added to the tray. This process of gently draining rearing water and replacing with fresh water was repeated from day 5 until all mosquitoes in the tray had pupated (Figure 1). The draining process did not result in a significant loss of zeolite; water is drained gently, avoiding zeolite particles that have settled at the bottom of the tray. **No-change (NC)** - first instar larvae were initially transferred to trays containing 500ml of mineral water and received an additional 500ml of mineral water on day 5 (Figure 1). **No-change-zeolite (NCZ)** - On day 1, first instar larvae were transferred to trays containing 500ml of mineral water; on day 4, 1g of finely ground zeolite powder was added to the rearing water; and on day 5, 500ml of additional mineral water was added to the rearing trays (Figure 1).

Larvae were fed with two different standardised feeding regimes (slurry and powder feed), except on day 1, where 0.1ml of Liquifry liquid fish food (Interpret Ltd, Surrey, UK) was used to feed first instar larvae. Powder feeding regime consists of daily rations of 'groundfish food', using a spatula to spread on the water surface: 6mg on days 2–3, 30mg on day 4, and 60 mg on day 5 until pupation. Slurry feeding regime consists of the same food quantity dissolved in deionised water (1ml of 60mg/10ml of TetraMin Baby on days 2–3, 1ml of 300mg/10 ml of TetraMin Baby on day 4, and 1ml of 600mg/10 ml of TetraMin Baby on day 5 until pupation) and injected into the larval trays using a pipette. Pupae were picked from larval trays using 3ml plastic pipettes and transferred to styrofoam cups containing mineral water, then placed in adult cages for emergence (Figure 1).

Depending on the mosquitoes' life-cycle stage, the following data were observed and recorded: (i) Larval survival: determined as the percentage of larvae that developed into pupae from the total number of larvae for each water treatment. (ii) Pupal mortality: determined as the number of mosquitoes that died at pupation. (iii) Adult emergence: determined as the percentage of mosquitoes that emerged as adults from the total number of larvae in each water treatment. (iv) Development time: determined as the number of days from placement of first instar larvae in water treatment trays until adult emergence. (v) Wing-length: emerged adults were collected using a mouth aspirator, sexed and stored in 75% ethanol for subsequent wing-length measurement. One wing of each emerged adult was measured from the distal end of the allula to the apical margin (radius veins), excluding the fringe scale using a binocular microscope. A stage micrometre of 1mm ruler length (Graticules Ltd, Kent, UK) was used for calibration at 2.5 magnification on a scale of 1 microscope unit = 0.04mm) [23]. A total of 1280 emerged adults equivalent to 40 males and 40 females per treatment were randomly sampled for wing-length measurements. At the end of the entire experiment, this random selection was made to account for late-emerging adults likely to be bigger.

Physicochemical properties of larval trays

Measurements for ammonia (NH_3) were taken using a Handheld Colorimeter kit (Hanna Instruments, USA), nitrate was measured using API aquarium test kits (Mars Fishcare North America, Inc, Chalfont, USA) on days 4, 6, 8, and 10 (if larvae were still alive in the tray) following experimental set-up (Additional file 1: Table S1).

Statistical analysis

All data collected were analysed using JMP 14 (SAS Institute, Inc., Cary, North Carolina, USA). All data were checked for deviations from normality and heterogeneity of variance, and analyses were conducted using parametric and non-parametric methods as appropriate. The 2 x 4 x 2 design of the experiment allowed for fully balanced multivariate statistical models. In multivariate analyses, replicate effects were tested and only reported when significant. Interactions between independent variables were tested using a stepwise approach, and only those found to be significant were retained in the final models. For analyses of proportion of larvae, pupae and adults, likelihood odds ratios were used for *post-hoc* pairwise group comparisons following logistic regressions. Body size was analysed through general linear models followed by Tukey's HSD *post-hoc* pairwise comparisons. Developmental times (day of emergence) were analysed by Cox Proportional-Hazard models with likelihood odds ratios for *post-hoc* pairwise comparisons. Finally, ammonia and nitrate measurements were analysed through a generalised linear model using standard least squares.

Results

Physicochemical properties of mosquito larval trays

Overall ammonia concentrations in mosquito larval trays were significantly impacted by water treatment (Table 1). *Post-hoc* pairwise comparisons revealed that ammonia concentrations in No-change (NC) and No-change-zeolite (NCZ) treatments were significantly higher compared to those in Daily-change (WC) and Daily-change-zeolite (WCZ) treatments (Tukey HSD test: t-ratio > 9.96, $P < 0.0029$ in all cases). Ammonia concentration was also significantly lower in NCZ compared to NC ($P < 0.0001$); and in WCZ compared to WC ($P = 0.0029$) (Figure 2a; Additional file 1: Table S1). Day of experimentation significantly impacted ammonia concentrations in larval trays resulting in a strong increase from day 4 to day 10 (Table 1; Figure 2a). A significant interaction between water treatment and day of experimentation also impacted ammonia concentration in mosquito larval trays (Table 1). For instance, in NC and NCZ, there was a steady build-up of ammonia, rising above toxicity threshold (0.2mg/l) on day 4 and reaching a peak on day 10; inversely in WC and WCZ ammonia concentrations were relatively low and stable throughout the experiment (Figure 2a; Additional file 1: Table S1). Feed regimes and larval rearing density did not significantly impact ammonia concentrations in larval trays (Table 1).

Nitrate concentrations in larval trays were also significantly affected by water treatment (Table 1). Pairwise comparisons showed that nitrate levels were significantly higher in water-changes treatments (WC and WCZ) compared to no-water-changes treatments (NC and NCZ) (Tukey HSD tests: t ratios > -5.62 and P values < 0.0041 in all cases) (Figure 2b; Additional file 1: Table S1). However, there were no significant differences between WC and WCZ (t ratio = -1.79, $P = 0.2819$) nor between NC and NCZ (t ratio = -1.14 , $P = 0.6673$). Day of experimentation significantly affected nitrate concentrations in larval trays resulting in an overall increase from day 4 to day 10 (Table 1). There was a significant interactive effect between water treatment and day of experimentation on nitrate concentrations (Table 1). Nitrate levels increased with time in WC and WCZ from day 4 to day 8, reducing by day 10. In the no-change groups (NCZ and NC), nitrate increased from day 4 to day 6 but already started reducing by day 8 (Figure 2b). Among all water treatment trays, nitrate concentrations were significantly higher in powder feed than slurry feed (Table 1; Figure 2b). Larval rearing density did not have a significant impact on nitrate concentrations (Table 1).

Effect of larval density, water treatment, and feed regimes on larval survival

Nominal logistic regression model showed that larval survival of *An. coluzzii* was significantly impacted by water treatment (Table 2). *Post-hoc* pairwise comparisons (Odds ratio test: $P < 0.0001$) revealed that larval survival was significantly higher in the WC (66%) in comparison to other water treatments (NC –

55%, WCZ - 52%, NCZ – 52%); there were no significant differences between the latter groups (Odds ratio test: $P > 0.3020$ in all comparisons) (Figure 3a; Additional file 2: Table S2).

Feed regime significantly affected larval survival with an overall 10% higher survival in powder feed than slurry feed (Figure 3a; Table 2; Additional file 2: Table S2). Density significantly impacted larval survival which was higher at 200 larvae/tray than 400 larvae/tray (Table 2). The significant interaction between water treatment and feed regime resulted in larval survival affected by water treatments differentially under the two-feed regime (Table 2; Figure 3a; Additional file 2; Table S2). Additionally, a significant interaction between water treatment and density impacted larval survival (Table 2). At 200 rearing density, larval survival was highest in WC (77%) followed by WCZ (67%), then NCZ (67%) and NC (63%) (Figure 3a; Additional file 2; Table S2). Larval survival at 400 rearing density was highest in WC followed by NC, NCZ and WCZ (Figure 3a; Additional file 2; Table S2). Finally, larval survival was affected by a significant interaction between feed and larval density (Table 2).

Pupal mortality, larval density, feed types and water treatments

Overall, the logistic regression model showed that water treatments significantly affected pupal mortality (Table 2). *Post-hoc* pairwise comparisons revealed that pupal mortality was significantly higher for NC (11%) than NCZ (8%), WC (7%) and WCZ (6%) (Odds ratio test: P values < 0.0456 in all cases) (Figure 3b; Additional file 2; Table S2). There was no significant difference in pupal mortality between NCZ and WC ($P = 0.1382$).

Larval rearing density negatively impacted pupal mortality resulting in significantly higher mortality at 200 rearing density (Table 2). For instance, pupal mortality was at least 1% higher in the lower rearing density (200) in all water treatments except for NCZ where percentage pupal mortality was higher at the 400 larval density (Table 2; Figure 3b; Additional file 2; Table S2).

Although feed regimes had no significant effect on pupal mortality, there was a significant interaction between feed and water treatment on pupal mortality (Table 2). This translated in differences associated with feeding regimes occurring only in some water treatment groups (Figure 3b; Table 2; Additional file 2; Table S2).

Adult emergence of *An. coluzzii* across water treatments feed regimes and larval densities

Nominal logistic regression showed that adult emergence was significantly impacted by water treatments (Table 2). *Post-hoc* pairwise comparisons revealed that adult emergence was significantly higher in WC compared to other water treatment groups (Odds ratio test: P values from < 0.0001 in all comparisons) (Figure 3c; Additional file 2: Table S2). Rearing density significantly impacted adult emergence, with higher emergence rate (60%) at 200 rearing density, compared to the 43% adult emergence at 400 larval rearing density (Table 2; Figure 3c). The significant impact of feed regime on adult emergence resulted in 10% more adults emerging from powder feed than slurry feed (Table 2). The significant interaction between water treatment and larval density resulted in varying adult emergence rates among water treatments depending on larval rearing density (Table 2). The differences in adult emergence among water treatment groups were starker at the 400 rearing density with emergence rates as low as 36% in NCZ and 39% in WCZ (Figure 3c; Table 5; Additional file 2; Table S2). The significant interaction between water treatment and feed regime resulted in differences in adult emergence rates among water treatments dependent on feed regime. For slurry feed, emergence was highest in WC, followed by NCZ, WCZ then NC. Likewise, in powder feed, adult emergence was highest in WC followed by WCZ, NC, then NCZ (Figure 3c; Table 2; Additional file 2; Table S2). Finally, the interaction between density and feed also significantly impacted adult emergence resulting in 20% and 16% more adult emergence in slurry and powder feed at 200 rearing density than the 400 rearing density (Figure 3c; Table 2; Additional file 2; Table S2).

Mosquito survival by sex across water treatments and larval densities

Nominal logistic regression revealed that water treatment, rearing densities and feed type had no significant impact on the sex of adult mosquitoes (Table 3). Likelihood ratio tests showed that the sex ratio of surviving mosquitoes did not significantly deviate from the expected 50:50 ratio except at WCZ/400 larval density/powder feed (Chi-square likelihood ratio test: LR= 6.6728, DF = 1, $P = 0.0098$) and NCZ/400 larval density/powder feed (LR= 5.8726, DF= 1, $P = 0.0154$) where females significantly survived more than males.

Effect of water treatments, feed regimes and larval density on adult wing-length

General linear regression model revealed that mosquito adult wing-length was significantly impacted by water treatment (Table 4). *Post-hoc* pairwise comparisons revealed significantly longer wing-length in WC compared to WCZ and NCZ; as well as in NC compared to NCZ (Tukey's HSD tests: t-ratios > 3.62; P values < 0.0020 in all comparisons). No significant difference in wing-length was observed between WCZ and NC, WCZ and NCZ, as well as between WC and NC (t-ratios > -1.36; $P < 0.5479$ in all comparisons) (Figure 4; Additional file 3; Table S3). Larval rearing density significantly impacted emerging adults' wing-length, with longer wing-length in 200 density than 400 (Table 4). The significant interaction between feed and water treatment resulted in variation in adult wing-length in treatment groups depending on feed regimes (Table 4). Adult wing-length significantly differed by sex; females had significantly longer wing-length than males (Table 4; Figure 4; Additional file 3; Table S3).

Impact of larval density, water treatment and feed regimes on development time

The duration of development from first instar larvae until adult emergence (development time) was significantly impacted by water treatment (Table 5). *Post-hoc* pairwise comparisons revealed that development time was significantly longer in WCZ compared to WC and NC (Risk ratio tests: *P* ³ 0.0007 in both cases) but not compared to NCZ (P = 0.0671) (Figure 5; Additional file 4; Table S4). Larval rearing density significantly impacted development time which was 1-day longer in the 400 compared to 200 rearing density (Table 5; Figure 5; Additional file 4; Table S4). Development time was also significantly impacted by feed regimes with mosquitoes taking more prolonged time (half-day) to complete development in slurry feed than powder feed (Table 5; Figure 5; Additional file 4; Table S4). Significant interactions between water treatment and density resulted in the shortest development time at the 200 rearing density in WC; development time was more succinct in NCZ than NC. At 400 rearing density, the shortest development time occurred in the NC water treatment (Table 5; Figure 5; Additional file 4; Table S4). Additionally, the significant interaction between water treatment and feed regimes resulted in longer development time in NCZ than NC for mosquitoes fed with slurry feed and longer in NC than NCZ for powder feed (Table 5; Figure 5; Additional file 4; Table S4).

Discussion

As expected, mosquitoes reared in the trays where water was continuously refreshed provided a better larval environment for optimal mosquito growth and development. Consistently lower ammonia concentrations and higher nitrate concentrations in these trays indicated efficient conversion of toxic ammonia to nitrate [24]. Mosquito survival and adult body size were maximised in groups where water was continuously refreshed due to the absence or minimal presence of toxic compounds such as ammonia [25]. Nitrogenous wastes are known to be poisonous to aquatic organisms above certain concentrations. Ammonia, a by-product of protein metabolism by aquatic animals, is toxic to fish and other freshwater animals above 0.2mg/l, in closed aquatic systems [24,26,27]. In larval trays without water replacement, ammonia concentrations increased steadily, exceeding toxicity threshold on the fourth day, and reaching a peak on the tenth day. Zeolite added to the NCZ water treatment significantly decreased ammonia concentrations than NC trays where zeolite was not applied. Similarly, nitrate concentrations were higher from day 4 in NCZ than NC, indicating greater ammonia conversion to the less toxic nitrate [28]. The cause of overall higher mortality in *Anopheles* larval trays without-water-change (NC and NCZ) in comparison to those with water-change (WC and WCZ) could range from hypoxia, ammonia toxicity, inability to transport oxygen, pathogenicity, nutrient enrichment, and competition for food resource [27,29–31]. In addition, the bacterial build-up that typically accompanies waste accumulation could compound these effects by increasing ammonia production and/or potential direct bacterial toxicity [26,32–34].

Although not observed for overall mosquito survival, the impact of ammonia-absorbing zeolite in improving water quality in larval trays without-water-change was evident at the 200 larval rearing density. Adult emergence was significantly higher in NCZ than NC at the 200 larval density, thus validating zeolite's ability to improve water quality in an aquaculture system based on small larval rearing trays [14,15]. However, at higher larval density (400), the effect of zeolite was not evident for mosquito adult emergence, possibly due to two factors. Firstly, zeolite saturation as ammonia concentration produced in the 400-larval-density-trays was higher than at 200. The overcrowded trays (400 larval density) resulted in the production of relatively more elevated amounts of toxic ammonia due to the increased metabolism and waste production. Reports from the use of fish and crustacean aquaculture revealed that the greater the concentration of initial ammonia, the less the ammonia removal efficiency, providing a possible explanation for the reduced effect of ammonia adsorption by zeolite in these trays since the same amount of zeolite was used at both rearing densities [15,20,35].

A second but not exclusive explanation for the lack of zeolite's impact at higher density may be that ammonia reduction benefits were obscured by intra-specific competition for food and space [36]. Here, starvation resulting from intra-instar competition may have accounted for the reduced survival in trays with 400 larvae [37,38]. Larval overcrowding is relatively common in insectaries due to lack of space and/or standardised rearing protocols, leading to suboptimal emergence rates and phenotypic quality [36,39]. Our results suggest that zeolite might allow for rearing at higher larval densities but require higher doses of zeolites. Further studies are needed to optimise the timing and dosage of zeolite water treatment and maximise its beneficial impact at different larval densities.

Zeolite water treatment also favourably impacted on the duration of mosquito development time. Development time was not significantly longer in NCZ compared to the more effective continuous change WC group. This allowance for synchronous hatching and pupation using zeolite ideal for smaller insectaries and mass-rearing facilities [40]. Any additive that can shorten pre-imaginal development time is welcome as it will reduce labour costs and enhance accelerated production of adults [36]. This is particularly desirable in the mass rearing of adult mosquitoes for vector control/research programmes where efficient rearing systems which balance larval density, nutrition and water quality are needed [36,41].

A crucial factor to consider for applying zeolite to improve water quality for mosquito production without water replacement is that zeolites can significantly influence the abundance and development of nitrifying microorganisms [26,33,34]. Additionally, un-ionised ammonia can inhibit the action of nitrifying bacteria, resulting in increased ammonia levels in aquatic habitats, thereby intensifying the harmful effects on aquatic animals and beneficial bacteria [27]. In this study, the use of zeolite prevented these ammonia spikes hence reducing any adverse carry-over effects. There is a need to understand the complex interactions between zeolite use and bacterial communities' dynamics in these mosquito larval trays.

In this study, there was surprisingly little difference in the effect of feed regime on ammonia content in mosquito larval trays. However, powder feed was better than slurry feed for mosquito development and phenotypic quality for all water treatment types. This is likely due to the greater ammonia conversion in the powder feed trays indicated by higher nitrate concentrations [28]. This is not significant for continuous flow systems that routinely use slurry feed but might be for smaller insectaries employing powder feed without daily water changes [6,12].

Overall, the higher developmental success in NCZ compared to NC (at the 200 larval rearing density) and similar phenotypic quality in NCZ compared to WC showed zeolite could be beneficial for mosquito mass-rearing. Zeolite can be particularly useful to prevent ammonia accumulation in medium or small scale rearing facilities constrained by space or water, allowing the rearing of anopheline mosquitoes at higher densities. This may be relevant to the often overcrowded insectaries of smaller research institutions and infrastructures in malaria-endemic countries with low GDIs (Gross Domestic Income) in arid regions [4,42,43].

Currently, there is a dearth of literature on water management systems and water recycling and conservation in larger mosquito-rearing infrastructures [11] In contrast to that; zeolite applications are common in closed-system fish aquaculture, which uses larger amounts of water and are more advanced regarding water treatment and reuse (Table 6). In future, larger mosquito production facilities might benefit from similar zeolite applications, particularly those that can decrease their reliance on freshwater and generally improve sustainability [11]. Zeolite as a biofilter media is cheaper and more effective than activated carbon and sandbed filters and reduces both the cost of operation and backwash maintenance required [15,20,35,44,45] (Table 6). Compared with sand or plastic biofilter media, zeolite provides a hundredfold more total surface area (TSA) for microbial attachment, thus, doubling their nitrifying efficiency, and producing purer water at higher throughput rates and lower cost [15,44]. For example, zeolite with pore size 0.05 – 0.1 mm retains up to $\leq$ 3 mm compared to sand (particle size 0.5 -2mm) retaining 20 - 40 mm [15,45,46]. Zeolites are also simpler to use than activated carbon, requiring less skill, and no pre-conditioning [20,35]. In water systems aiming for a high proportion of water recycling, zeolite, combined with biological filters, prevents the accumulation of nitrates and may thus eliminate the need for denitrification chambers [47,48]. Where the more expensive RO or UF are employed, pre-filtration with zeolite commonly prevents organic build-up and membrane fouling, thereby decreasing maintenance costs [48,49] (Table 6). Following saturation, zeolitic materials are recharged by soaking in a 10% NaCl solution, thus renewing their capacity and subsequently reused [17,18,50]. Alternatively, the ammonia-saturated zeolite media can be used as organic fertiliser, serving as an environmentally useful by-product [15,44]. These examples suggest that zeolite has many potential applications to improve water quality and decrease production costs in mosquito production facilities, particularly those large infrastructures proposing to recirculate a substantial proportion of rearing water.

Conclusions

Under the no-water-change condition, zeolite reduced ammonia build-up and resulted in improved larval, pupal, and adult mosquito survival as well as development time. However, this effect was not observed under all water treatment conditions; hence, further optimisation would be required for broader applications in mosquito-rearing. Zeolite can be integrated into various water management scenarios for small to medium and large scale rearing facilities to improve water quality and reduce costs. The results of this first application of zeolite water treatment to anopheline rearing are auspicious; further studies are needed to optimise zeolite dosage in relation to larval density and feed type, especially when targeting mass rearing. Similarly, a better understanding of the complex interactive effects between the use of zeolite, ammonia fluctuations and the population dynamics of beneficial and detrimental bacteria is needed to fully understand the potential benefits of zeolite and other additives for anopheline mosquito mass production.

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and raw materials

All datasets generated and/or analysed during this study are included in this published article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Experiments were planned by FT and NOA, conducted by NOA, analysed by FT and NOA. The manuscript was written by NOA and FT. Both authors edited, read and approved the final manuscript.

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Tables

Table 1: General linear model of ammonia and nitrate concentrations across water treatments

Parameter	Source	df	F-ratio	*P*-value
Ammonia(mg/l)	Feed	1	0.605	0.4374 ^{ns}
	Larval density	1	0.4077	0.5238^{ns}
	Water treatment	3	88.361	<0.0001***
	Day	1	171.397	<0.0001***
	Water treatment*Day	3	56.165	<0.0001***
	Day*Day	1	82.230	<0.0001***
Nitrate (mg/l)	Feed	1	40.497	<0.0001***
	Larval density	1	3.167	0.0764 ^{ns}
	Water treatment	3	12.992	<0.0001***
	Day	1	128.072	<0.0001***
	Water treatment*Day	3	6.202	0.0005**
	Day*Day	1	61.472	<0.0001***

P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant)
Abbreviation: df, degrees of freedom

Table 2: Nominal logistic regressions of the effects of water treatments, larval density and feed regimes mosquito survival.

Parameter	Source	DF	Likelihood ratio	P-value
Larval survival	Larval density	1	610.267	<0.0001***
	Feed	1	195.915	<0.0001***
	Water treatment	3	221.067	<0.0001***
	Water treatment*Feed	3	39.440	<0.0001***
	Water treatment*Larval density	3	31.839	<0.0001***
	Feed*Larval density	1	7.118	0.0076*
Pupal mortality	Larval density	1	5.007	0.0252*
	Feed	1	2.049	0.1523^{ns}
	Water treatment	3	76.424	<0.0001***
	Water treatment*Feed	3	42.522	<0.0001***
	Water treatment*Larval density	3	8.433	0.0379*
	Feed*Larval density	1	7.885	0.0050*
Adult emergence	Larval density	1	544.058	<0.0001***
	Feed	1	187.584	<0.0001***
	Water treatment	3	258.443	<0.0001***
	Feed*Larval density	1	4.801	0.0285*
	Water treatment*Feed	3	44.096	<0.0001***
	Water treatment*Larval density	3	46.674	<0.0001***

P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant)

Table 3: Nominal logistic regression of mosquito survival by sex

Source	DF	Likelihood ratio	P-value
Water treatment	3	0.470	0.9255^{ns}
Feed	1	3.001	0.0832^{ns}
Larval density	1	0.141	0.7075^{ns}

P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant)

Table 4: General linear model of the effect of water treatments, feed regimes and larval density on wing-length

Parameter	Source	df	F-ratio	*P*-value
Wing length	Larval density	1	17.106	<0.0001***
	Feed	1	1.973	0.1603^{ns}
	Water treatment	3	9.852	<0.0001***
	Sex	1	232.853	<0.0001***
	Water treatment*Feed	3	2.998	0.0298*

P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant)
Abbreviation: df, degrees of freedom

Table 5: Cox Proportional-Hazard analyses of the effect of water treatments, feed regimes and larval density on development time

Parameter	Source	df	Chi-Square	*P*-value
Day of emergence	Larval density	1	614.460	<0.0001***
	Feed	1	142.292	<0.0001***
	Water treatment	3	18.179	0.0004**
	Water treatment*Feed	3	8.365	0.0390*
	Water treatment*Larval density	3	21.040	<0.0001***
	Feed*Larval density	1	14.973	0.0001**

P- value: *** < 0.0001 (most significant), ** < 0.005, * < 0.05, ^{ns} > 0.05 (not significant)

Table 6: Comparison of water management steps in representative examples of infrastructures for mosquito production and closed-system fish aquaculture highlighting possible uses of zeolite for mass-rearing.

cility type	Mosquito rearing			Closed system fish aquaculture
	Small to medium-scale	Medium to large-scale		Large-scale
ter nagement	No or little water replacement	Continuous water-replacement	Water recycling and recirculation	
urcing	Tap water [51,52] Mineral water [30,52,53]	Tap water [51].	Proportion of rearing water from previous cohort* [4,11].	Proportion of water from fish rearing* [47,54].
dimentation	Tap or well water allowed to settle or age [55]	NA	NA	NA
rification	None (mineral water) [53] or DI water from RO unit or DS water [36,56] or DC water or UV sterilised water [57].	DI water from RO unit or distilled water [12] or DC water [6].	**Mechanical filtration**	
			Removal of coarser wastes using cloth [58]	Removal of coarser wastes using plastic, fibre sieves [47,59]
			Biological aerated filtration BAF (Ammonia removal)	
			Currently no literature available on the use of biological filtration in mosquito insectaries. As in fish aquaculture, biofilters can be used for recycling mosquito rearing water [11].	Various biological filters convert ammonia to nitrites and nitrates, including sand, ceramic, activated carbon, plastic, and sponge media [20,35,44]. **Zeolite offers a higher surface area than sand and is cheaper than activated carbon, not requiring conditioning, and yielding purer water at higher throughput with less maintenance required** [15,20,35,44–46]
			Nitrate removal	

cility type	Mosquito rearing			Closed system fish aquaculture
	Small to medium-scale	Medium to large-scale		Large-scale
ter nagement	No or little water replacement	Continuous water-replacement	Water recycling and recirculation	
			Denitrification, the conversion of BAF-produced nitrates to atmospheric nitrogen has not an issue in mosquito production but will be required when a higher proportion of water is recycled.	Nitrates from BAF are converted to nitrogen using denitrification chamber allowing more extensive water reuse [47,48,60] **Using zeolite before or in BAF removes ammonia by adsorption hence reduces the production of nitrates. If kept below 100mg/l, a denitrification chamber may not be necessary** [47,48]
			Removal of toxic gases	
			Currently not used in mosquito rearing	Removal of accumulated gases (CO_2 and nitrogen from biofilters and denitrification) and water re-oxygenation [47].
			Removal of pathogens	

cility type	Mosquito rearing			Closed system fish aquaculture
	Small to medium-scale		Medium to large-scale	Large-scale
ter nagement	No or little water replacement	Continuous water-replacement	Water recycling and recirculation	
			RO and UF have been applied successfully for recycling mosquito rearing water, but are comparably more expensive than UV treatment [4]. **Using pre-treated feed water (mechanical, biological filtration-including zeolite) for RO and UF prevents membrane fouling and reduce maintenance costs** [49,61].	RO and UF removes pathogens, but UV preferred due to high throughput and low cost [47,49,61] **Using pre-treated feed water (mechanical, biological filtration including zeolite) for RO and UF prevent membrane fouling and reduce maintenance costs** [49,61]
rameter ustment	Re-mineralisation (where deionised water is used) [30,53,57].		pH adjustments can be made using NaOH or Re-mineralization is required [30,53,57].	pH adjustment to balance the acidity created by the nitrification process using lime water or Sodium hydroxide [47].

cility type	Mosquito rearing			Closed system fish aquaculture
	Small to medium-scale	Medium to large-scale		Large-scale
ter nagement	No or little water replacement	Continuous water-replacement	Water recycling and recirculation	
tribution rearing	Manual distribution in larval trays (water replaced intermittently or topped up at a later point during rearing) [53]. The added water also reduces the nitrate content generated due to nitrification [48]. **Zeolite can be added to prevent ammonia spikes and to increase rearing larval density and quality.**	Regular partial replacement of water in stacked larval trays systems [6,12] **Zeolite could be applied to larval trays as an emergency measure in case of continuous flow system failure**		Regular partial replacement of water in fish tanks and enclosures (Bregnballe, 2015)
st rearing	Discarded, or reused for agricultural purposes		Collected and recycled to increase overall production sustainability, particularly where freshwater is scarce. Organic matter derived from the filtration process can be used as agricultural fertiliser [15]	

Notes: DI (deionised); DS (distilled); DC (dechlorinated); RO (Reverse osmosis); UF (Ultrafiltration); UV (Ultraviolet rays); BAF (Biological aerated filters); Small to medium-scale facility (mosquito insectaries in research institutions, egg-to-adult rearing units); Large-scale facility (Sterile insect release and genetically modified mosquito programmes).

*****The proportion of recycled water varies and is combined with freshwater sourced and purified as described in the 2nd column describing water management in 'continuous water replacement' mosquito rearing system

Figures

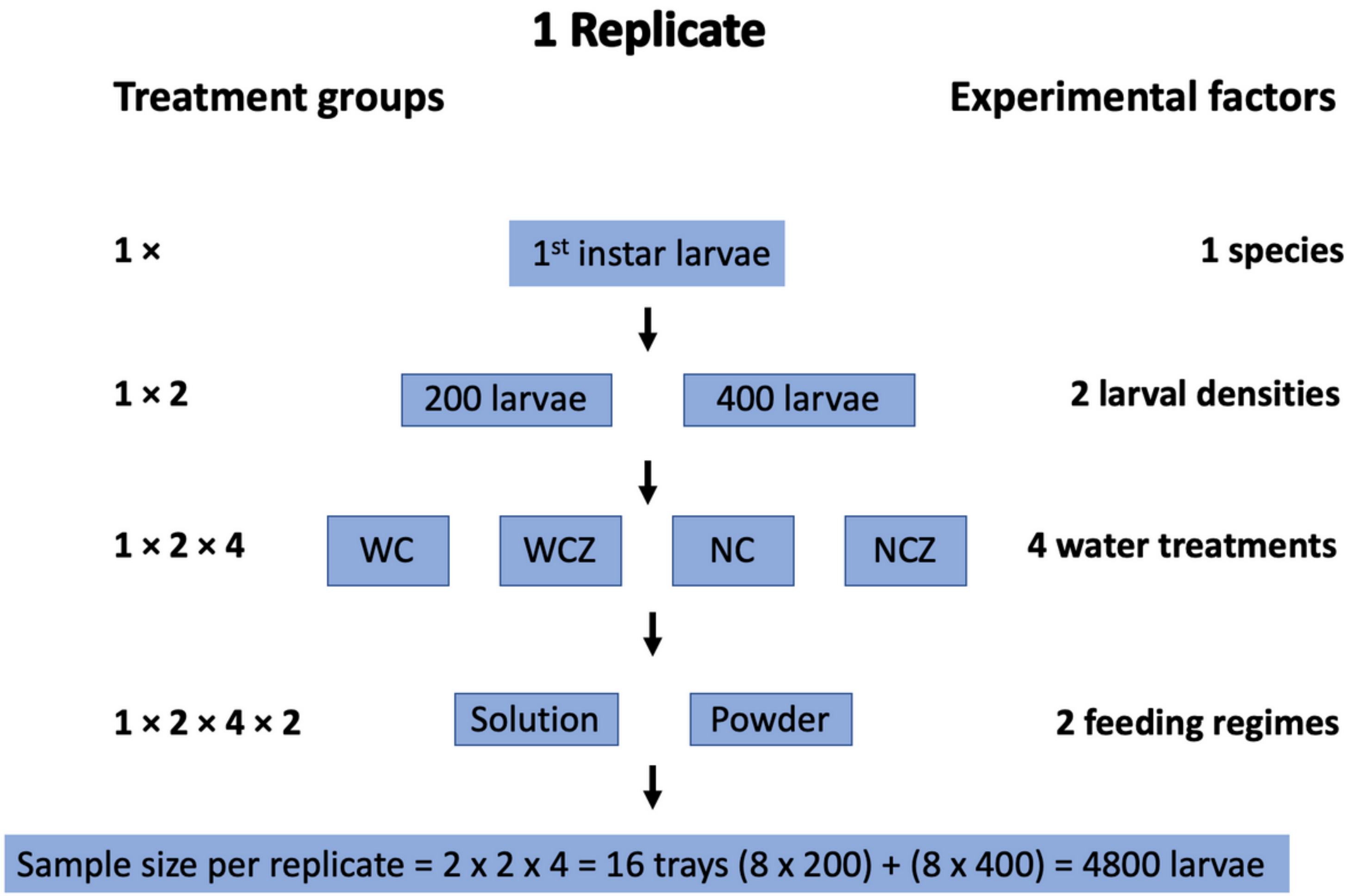

Figure 1

Experimental design showing experimental factors combined in one replicate resulting in 2 larval densities, four experimental water treatments (WC, WCZ, NC, NCZ) and 2 feeding regimes (powder and slurry).

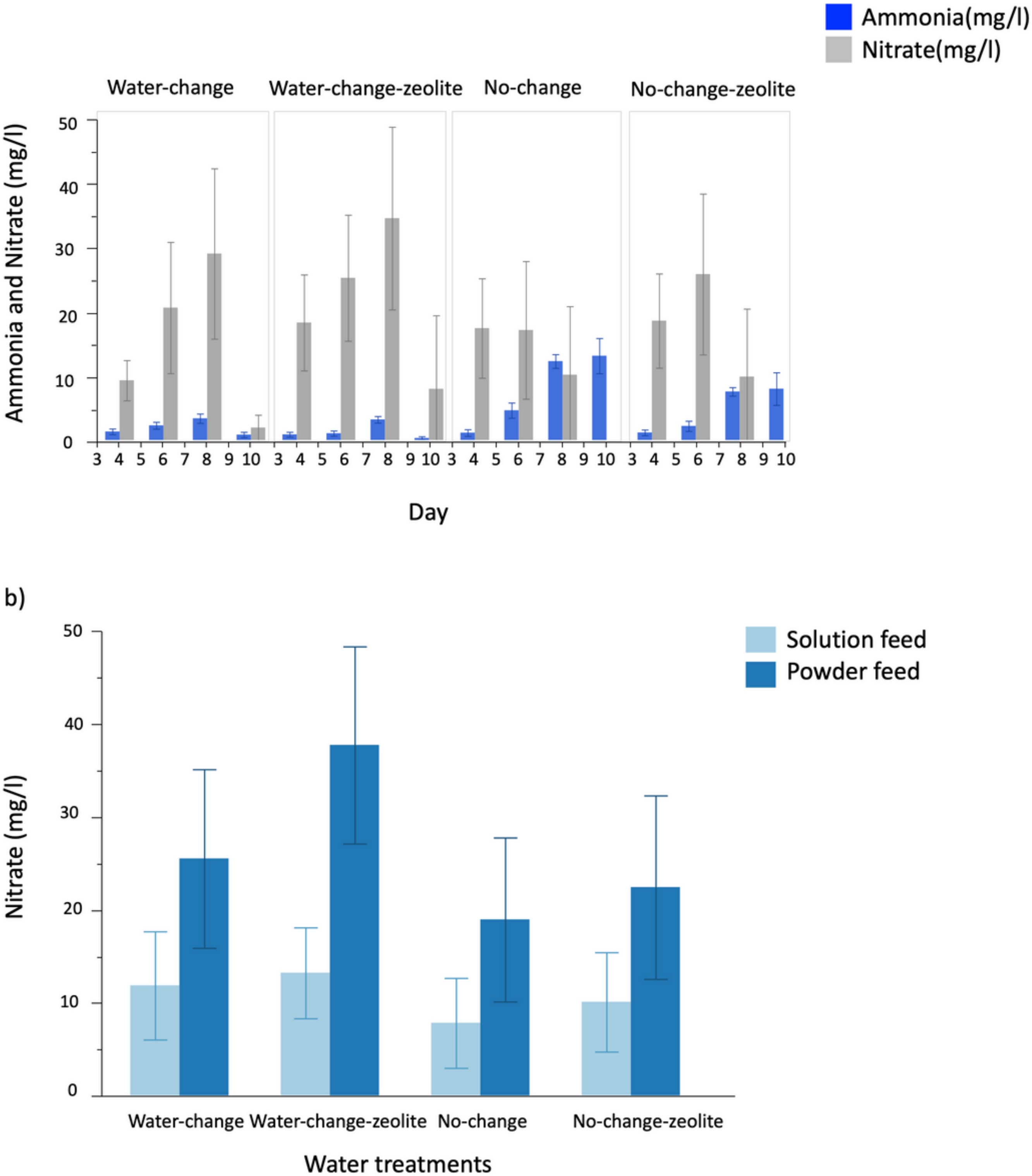

Figure 2

a,b: Ammonia and nitrate concentration across water treatments. a) Ammonia (blue bars) and nitrate (grey bars) concentrations across water treatments b) Nitrate concentration by feed regimes (slurry feed- light blue bars, powder feed- dark blue bars) across water treatments. Whiskers represent 95% confidence intervals (CI).

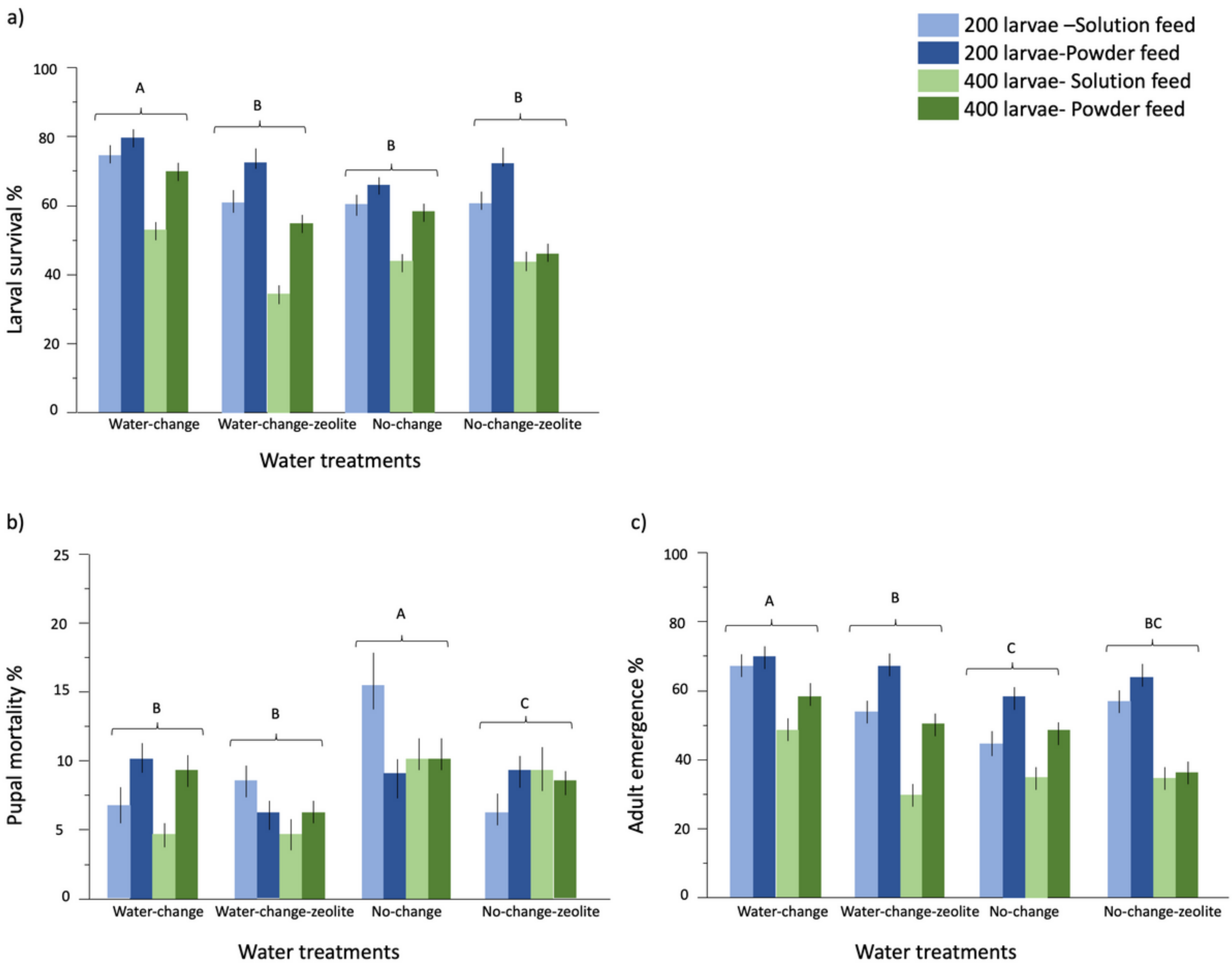

Figure 3

a-c: Developmental success of An. coluzzii across water treatments, feed regimes and larval densities. Whiskers represent 95% confidence intervals (CI).

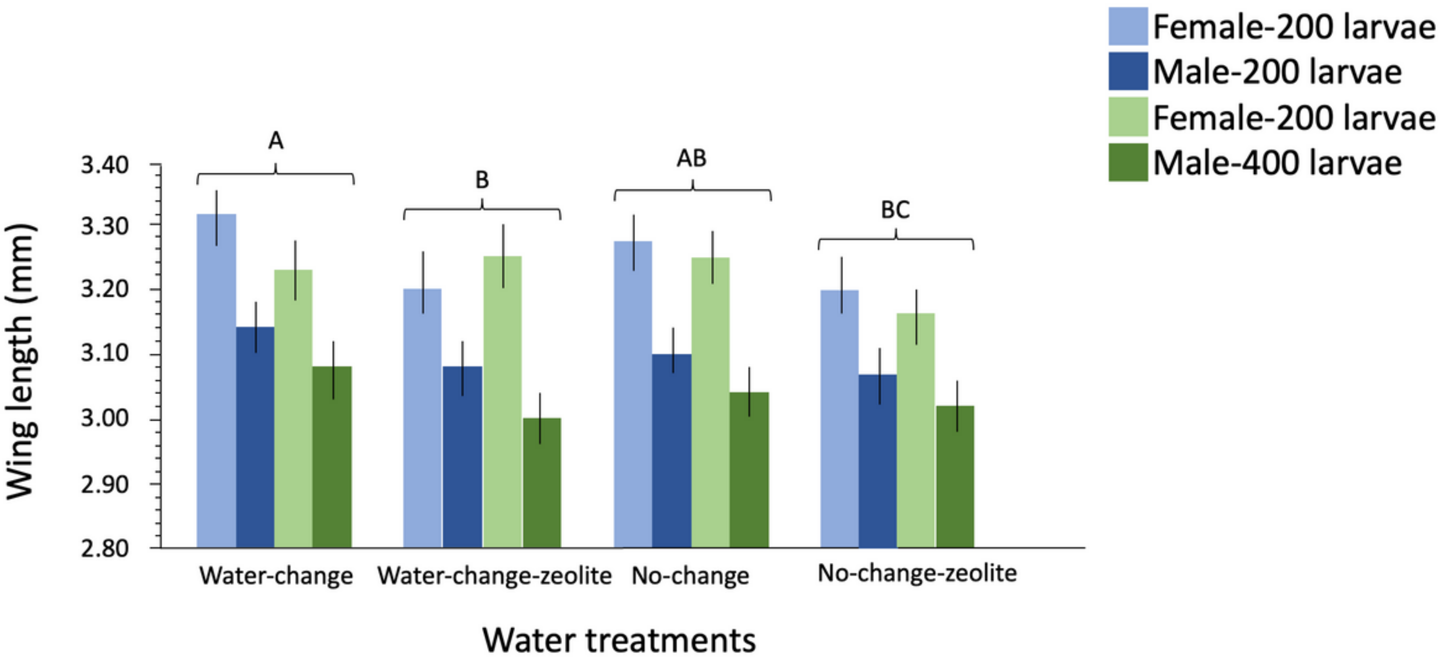

Figure 4

Mean wing-length of emerged mosquitoes across water treatments for two larval rearing densities. Whiskers represent 95% confidence intervals (CI). Significant differences among water treatments are represented by different letters.

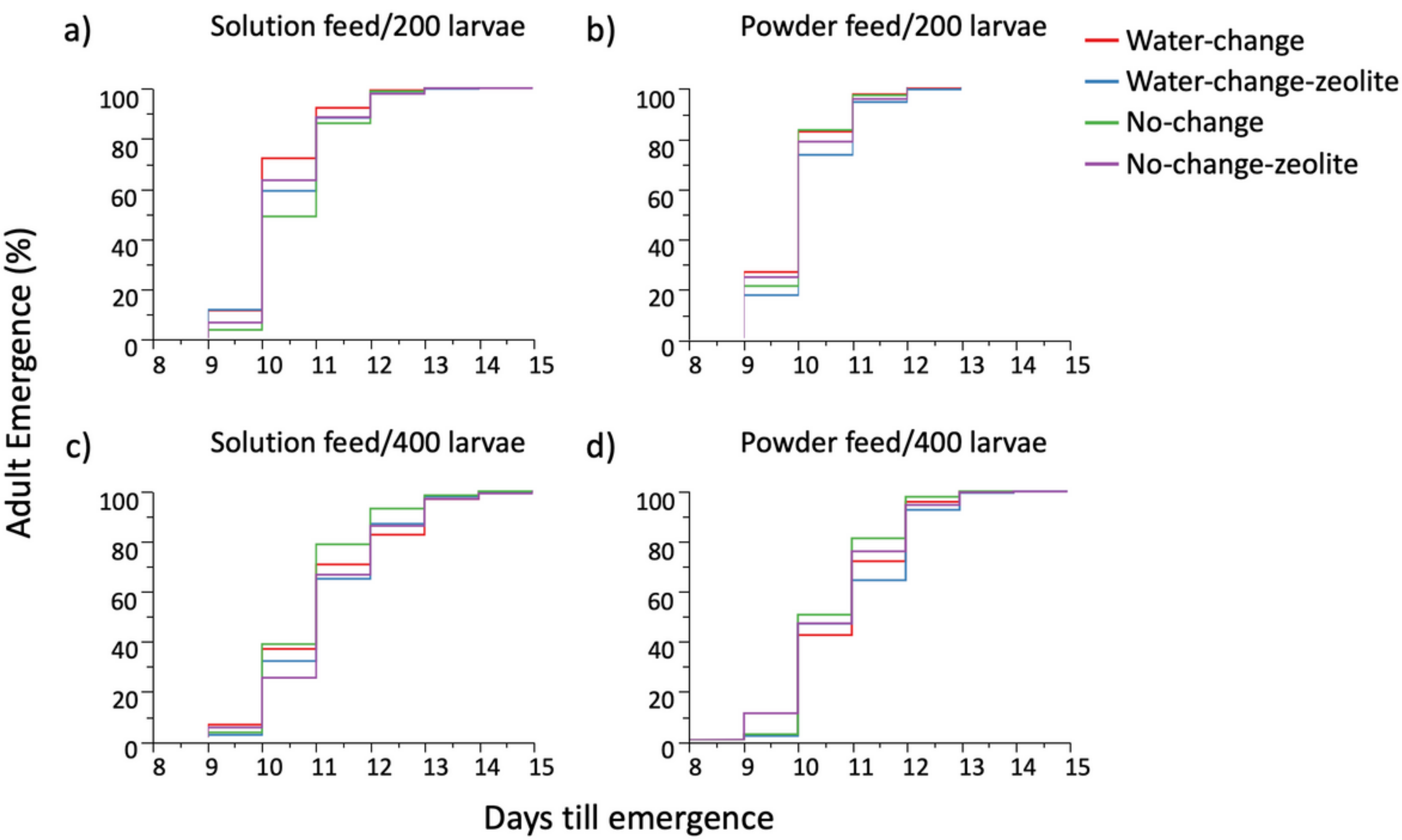

Figure 5

Survival curves of mosquito larvae in water treatment types by larval densities and feed regimes, a) 200 larvae/slurry feed; b) 200 larvae/powder feed; c) 400 larvae/slurry feed; d) 400 larvae/powder feed.

Supplementary Files

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