*Asian Fisheries Science* **28** (2015):174-185

ISSN 0116-6514

Asian Fisheries Science
The Journal of the Asian Fisheries Society

# Effects of Chicken Manure Extract on the Population Growth, Mixis Induction and Body Size of the Freshwater Rotifer *Brachionus angularis* Gosse 1851

**ERICK OCHIENG OGELLO[1, 2*] and ATSUSHI HAGIWARA[1]**

[1]Graduate School of Fisheries and Environmental Sciences, Nagasaki University, 1-14 Bunkyo, Nagasaki 852-8521, Japan
[2]Kenya Marine & Fisheries Research Institute (KMFRI), Kegati Aquaculture Research Station, P.O. Box 3259 - 4200 Kisii, Kenya

## Abstract

This study investigated the effects of chicken manure extract (CME) on the population growth, mixis induction and body size of the freshwater rotifer *Brachionus angularis* Gosse 1851. Four concentrations of CME (i.e.0.5, 1.0, 2.0 and 3.0 $ml \cdot L^{-1}$), each in triplicate, were used in glass jars containing 20 mL of sterilised pond water. Thirty clones (24 h old) of *B. angularis* were placed in each jar and the specified concentrations of CME were added except in the control jars. *Chlorella vulgaris* Beijerinck 1890 ($2.5x10^6$ $cells \cdot mL^{-1}$) was supplied daily in all jars. The treatments were incubated at 25 $^oC$ in darkness for 7 days without exchanging water. CME significantly ($P<0.05$) increased the rotifer population density and amictic females from day 4-7 at 2.0 $mL \cdot L^{-1}$ but reduced the mixis induction rate. However, the CME did not significantly ($P>0.05$) affect the unfertilised mictic females. The specific population growth rate was significantly ($P<0.05$) highest at 2.0 $mL \cdot L^{-1}$ of CME. The resting egg production decreased with increasing concentrations of CME. The lorica length increased at 3.0 $mL \cdot L^{-1}$ of CME but the lorica width was unaffected by CME. These results suggest that addition of 2.0 $mL \cdot L^{-1}$ of CME enhances the population growth and regulates lorica size; hence can be applied in the freshwater larviculture initiatives.

## Introduction

The monogonont freshwater rotifer *Brachionus angularis* Gosse 1851 is potentially an excellent live food for small mouthed freshwater fish larvae, thanks to their small size (Ogata et al. 2011) and high reproduction rate (Dumont 2007). These reproductive characteristics are comparable to those of the rotifer, *Brachionus plicatilis* Mueller 1786 (Koiso et al. 2009), which is an important zooplankton widely used as initial food in marine larviculture (Hirayama and Hagiwara 1995).

*Corresponding author. E-mail address: erick.ogello@gmail.com

In semi-intensive fish farming practices, chicken manure has been used to augment pond biological productivity (Lahiri et al. 2014; Elsaidy et al. 2015). The interactions between the manure, soil and the microbial community probably influence the pond's carbon status and carbon sequestration in the system (Lahiri et al. 2014). However, the optimal quantity and mode of application of such manures for maximum pond biological productivity is poorly understood especially in African countries where semi-intensive fish farming is commonly practised (Ogello et al. 2013; Munguti et al. 2014). The effects of the manures are linked to the actions of different sex hormones present in them (Finlay-Moore et al. 2000; Peterson et al. 2000). For example, egg-laying chicken excrete about 50 and 250 $ng \cdot g^{-1}$ dry manure $day^{-1}$ of 17β-estradiol and testosterone respectively (Shemesh and Shore 1994; Hakk et al. 2005), while chicken litter contains between 1 (Jenkins et al. 2006) to 904 $ng \cdot g^{-1}$ (Shore et al. 1993; Bevacqua et al. 2011) of 17β-estradiol and 0.05 to 254 $ng \cdot g^{-1}$ of testosterone (Jenkins et al. 2006). Such chemicals are used by zooplankton to regulate their reproduction, induce predator defense and accomplish selective foraging in their respective habitats (Gilbert 1966).

There are scientific evidences that sex hormones influence the population growth, mixis induction and body size of zooplankton species (Preston et al. 2000; Yang and Snell 2010). Gallardo et al. (1997) reported a 2.3 fold increase in mictic female production of *B. plicatilis* exposed to 50 $mg \cdot L^{-1}$ of 17β-estradiol. Up to 7.8 fold enhancement of resting egg production was reported for *Brachionus manjavacas* Fontaneto, Giordani, Melone & Serra 2007 exposed to 5 $mg \cdot L^{-1}$, and complete inhibition at 14 $mg \cdot L^{-1}$ of progesterone (Snell and DesRosiers 2008). Huang et al. (2012) reported that 1 $\mu g \cdot L^{-1}$ of 17β-estradiol produced optimal demographic parameters for the freshwater rotifer, *Brachionus calyciflorus* Pallas 1766. A significant increase in amictic population growth rate of *B. calyciflorus* was observed under the influence of combined progesterone and estradiol hormones (Yang and Snell 2010). Apparently, such studies are limited for the rotifer *B. angularis*, which are potential live food for small mouthed freshwater larval fishes (Ogata et al. 2011). Chicken manure extract (CME) is cheap and blends varieties of hormones (Hakk et al. 2005; Hagiwara et al. 2014 unpubl data) hence eliminates the need to purchase commercial growth promoting compounds. The aim of this study was to determine the effects of CME on the population growth, mixis induction and body size of the freshwater rotifer, *B. angularis.*

## Materials and Methods

### *Rotifers*

The stock culture of the rotifer *B. angularis* was available in the laboratory of aquaculture biology, Nagasaki University, Japan. From this stock, about 200 rotifers were isolated and acclimatised for 4 days in 100 mL culture medium at 25±1°C using $2.5 \times 10^6$ algal cells $\cdot mL^{-1}$ of *Chlorella vulgaris* Beijerinck 1890. The culture medium (pond water) was GF/C filtered (Whatman) prior to autoclave sterilisation at 121 °C for 15 min.

From this population, egg bearing rotifers were shaken in a screw-capped bottle, and the detached amictic eggs were collected and hatched in a petri dish (50 mm diameter) with similar *C. vulgaris* suspensions as stated previously. Twenty four hours old hatched clones were used in this study.

### *Preparation of chicken manure extract (CME)*

One kg of fermented chicken manure (Shitama Inc., Fukuoka, Japan) was mixed with 10 g of fossil coral powder (Coral international Co. Ltd., Okinawa, Japan). This mixture was boiled in 5 L of sterilised pond water for 40-50 min and then kept overnight at room temperature. The supernatant liquid was filtered off the sludge using nylon net of pore size 100 μm. The sludge was re-filtered and the liquid was mixed with the previous supernatant. The CME was preserved at 4 °C for subsequent use during the experiment. Table 1 shows the quantities of sex hormones in the CME (Hagiwara et al. 2014 unpubl data).

**Table 1**: Sex hormones ($\mu g \cdot kg^{-1}$) in chicken manure extract (CME); ND = not determined.

| Sex hormones | CME | Limit of detection |
|---|---|---|
| 17 α-estradiol | 0.16±0.04 | 0.05 |
| 17 β-estradiol | 0.53±0.35 | 0.05 |
| Estron | 2.20±1.65 | 0.05 |
| Estriol | ND | 0.05 |
| Progesterone | ND | 0.1 |
| Testosterone | ND | 0.5 |
| Methyltestosterone | ND | 0.5 |

### *Experimental design*

Bioassay experiment was conducted at nominal concentrations of CME (i.e. 0.0: control, 0.5, 1.0, 2.0 and 3 $mL \cdot L^{-1}$). These concentrations were obtained after a series of prior range finding tests in the laboratory. Each CME concentration and the control were triplicated. Thirty single egg bearing amictic females were placed in different 100 mL glass jars containing 20 mL of culture medium. The CME concentrations were introduced into the glass jars once at the start of experiment without exchanging water. All the rotifers were incubated at 25±1 $^{o}$C under total darkness with daily feeding on *C. vulgaris* at $2.5x10^6$ $cells \cdot mL^{-1}$ for 7 days. The density of *C. vulgaris* was monitored twice daily and adjusted accordingly to maintain a constant density of $2.5x10^6$ algal $cells \cdot mL^{-1}$ in the culture medium.

From the first day, the population density of rotifers was daily defined by counting all live rotifers in 1 mL from each replicate jar using a graduated counting plate with lugol fixation, under stereo microscope at x 25 magnification. In the same sample, the number of amictic females, fertilised and unfertilised mictic females were counted based on the type of egg they carried (Hagiwara et al. 1988). At the end of experiment (day 7), the total number of resting eggs were counted in each replicate jar and the population density of all live rotifers and amictic females were reported as individuals·$mL^{-1}$ ± standard deviation of the three replicates in each treatment. The rates of fertilised and unfertilised mictic females were reported as mixis percentages using these formulae:

$$\text{Fertilised mictic females (\%)} = \left[ \frac{fertilised\ \ mictic\ \ females}{amictic\ \ females + fertilised\ \ mictic\ \ females} \right] x\,100\ \%$$

$$\text{Unfertilised mictic females (\%)} = \left[ \frac{unfertilised\ \ mictic\ \ females}{amictic\ \ females + unfertilised\ \ mictic\ \ felales} \right] x\,100\ \%$$

The specific population growth rate of rotifers was also calculated as $r = \left[ \frac{\ln N_t - \ln N_o}{t} \right]$ where $N_t$= Number of individuals in culture after t days, $N_o$ = initial number of individuals, and t = time in days (7 days).

***Body size measurements***

Upon onset (before application of CME) as well as termination of the experiment, the lorica length and width of 10 rotifers randomly sampled from each replicate treatments were measured using microscope (Axioskop, Zeiss, Germany) with an ocular micrometer at x40 magnifications. The rotifers were fixed with 10 % formalin before taking the measurements.

***Data analysis***

The data were analyzed using R statistical software (version 3.2.1 of the R Foundation for Statistical Computing Platform © 2015) after test for normality and homogeneity of variance using the Bartlett test. $Log_{10}$ transformation was performed on the proportions of ovigerous fertilised and unfertilised mictic females, and the number of resting eggs before performing ANOVA tests to identify significant differences among the treatment means. Multiple comparisons were conducted using Tukey HSD test to determine where the differences were situated. *P* value of $\leq 0.05$ was accepted as being significantly different in all the tests.

## Results

The total population density was significantly affected by the days of culture ($F$ = 404.02, $P$ = 0.00), CME ($F$ = 99.72, $P$ = 0.00) and the interaction between culture days and CME ($F$ = 10.94, $P$ = 0.00). The density of amictic females were also affected by days of culture ($F$ = 429.17, $P$ = 0.00), CME ($F$ = 78.51, $P$ = 0.00) and the interaction between the culture days and CME ($F$ = 7.81, $P$ = 0.00). Both the population density and that of amictic females were significantly higher ($P$<0.000) at 2.0 $mL \cdot L^{-1}$ of CME compared to other CME concentrations and the control from day 4-7 (Fig. 1 and 2). However, each day from day 4-7, there was no significant difference in the population density ($P$>0.05) among the control, 0.5 and 1.0 $mL \cdot L^{-1}$of CME (Fig. 1 and 2). From day 1-3, the total population density and that of amictic females at all CME concentrations were similar ($P$>0.05), but reduced significantly from day 4-7 at 3.0 $mL \cdot L^{-1}$ of CME (Fig. 1 and 2).

The highest total population density (248.7±16.4 $individuals \cdot mL^{-1}$) (Fig. 1) and amictic females (183.3±8.5 $individuals \cdot mL^{-1}$) (Fig. 2) were obtained on day 5 at 2.0 $mL \cdot L^{-1}$ of CME. The fertilisation rate of mictic females significantly increased with days of culture ($F$ = 26.65, $P$ = 0.00), where up to 32.9±2.3 % of mictic females were fertilised on day 7 in the control experiment (Fig. 3). On the contrary, application of CME significantly ($P$<0.000) reduced the fertilisation rate of mictic females from day 5-7 (Fig. 3) but did not affect the proportion of unfertilised mictic females ($F$ = 0.66, $P$ = 0.61) (Fig 4).

The specific population growth rate was significantly affected by CME (one-way ANOVA, $F$ = 27.85, $P$ = 0.00). The specific population growth rate was higher (0.71±0.01 $day^{-1}$) at 2.0 $mL \cdot L^{-1}$ than the control, 0.5 and 1.0 $mL \cdot L^{-1}$ of CME (one-way ANOVA, $P$<0.02) but reduced significantly to 0.51±0.03 $day^{-1}$ at 3.0 $mL \cdot L^{-1}$ of CME (one-way ANOVA, $P$=0.000) (Fig 5). The CME significantly affected the mean number of resting eggs (one way ANOVA, $F$ = 37.14, $P$ = 0.00), where the number of resting eggs reduced with the increasing CME concentrations. At 2.0 and 3.0 $mL \cdot L^{-1}$of CME, the resting eggs were x1.7 and x3.1 lower than in the control respectively (Fig. 5).

The CME significantly increased the lorica length (one-way ANOVA; $F$=7.042, $P$=0.000) but not the width (one-way ANOVA; $F$=1.558, $P$=0.201). At the end of day 7, the lorica length of the rotifers exposed to 3.0 $mL \cdot L^{-1}$ of CME was higher ($P$<0.003) compared to the rest of the treatments, which were similar to each other (Fig. 6). At this concentration, the lorica length and width were 97.21±1.76 µm and 84.26±1.34 µm respectively (Fig. 6).

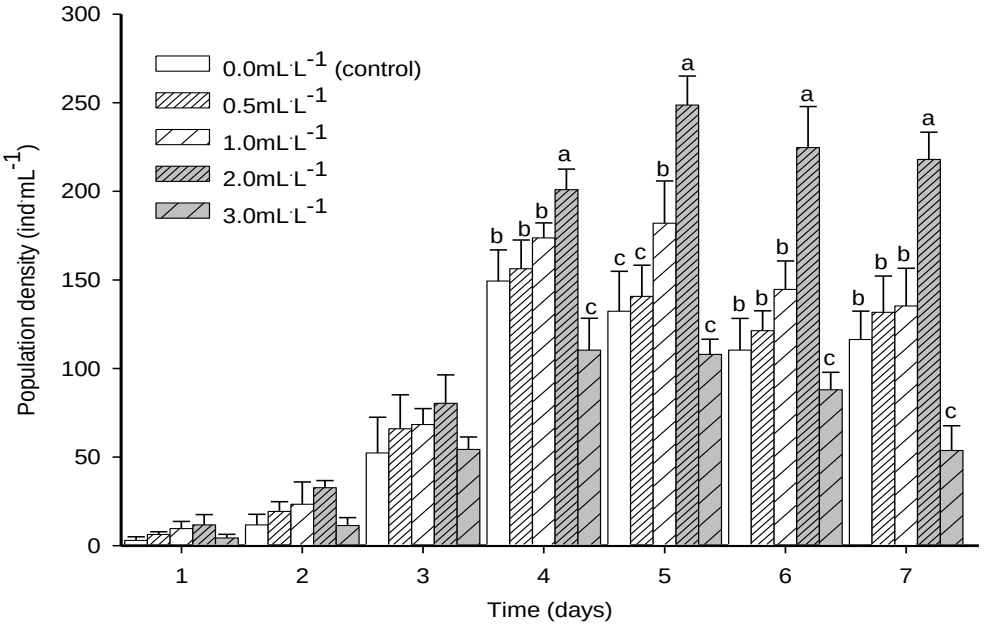


**Fig. 1**. Effects of different concentrations of CME and days of culture on the population density of the rotifer *Brachionus angularis*. Data shown are means ± standard deviation of three replicates; two-way ANOVA, Tukey HSD test. Different letters on top of the bars each day denote significant differences at $P<0.05$; a>b>c

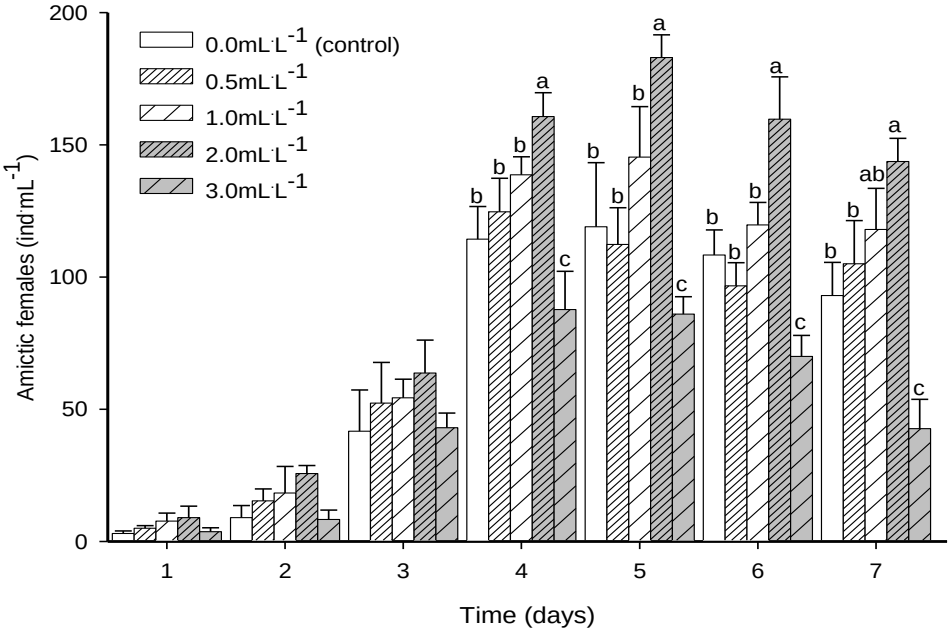


**Fig. 2**. Effects of different concentrations of CME and days of culture on the population density of amictic females of the rotifer *Brachionus angularis*. Data shown are means ± standard deviation of three replicates; two-way ANOVA, Tukey HSD test. Different letters on top of the bars each day denote significant differences at $P<0.05$; a>b>c

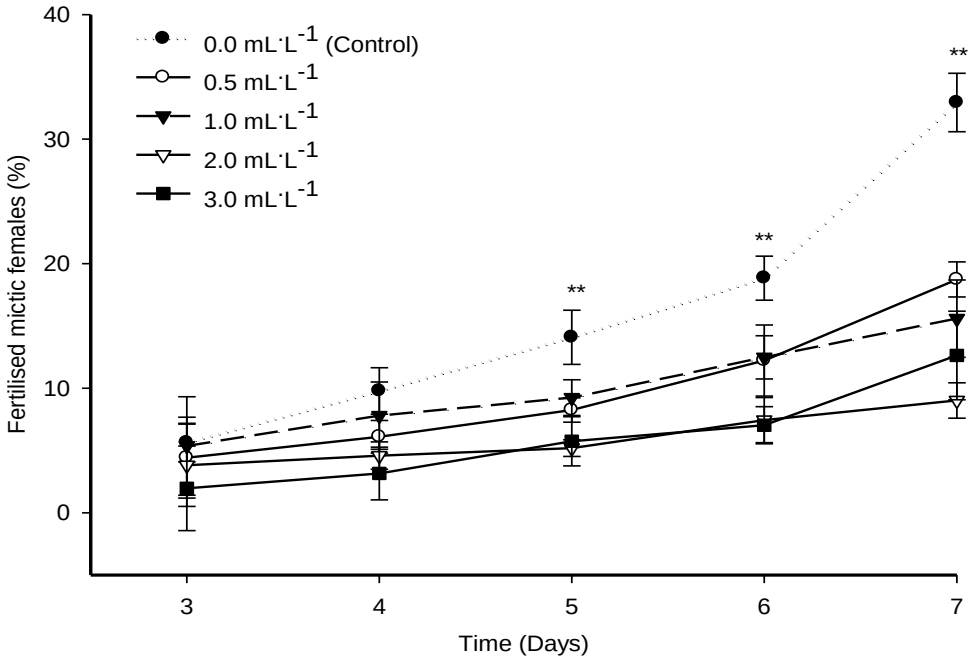


**Fig. 3**. Effects of different concentrations of CME and days of culture on the fertilised mictic female production rate of the rotifer *Brachionus angularis*.Data shown are means ± standard deviation of three replicates; two-way ANOVA, Tukey HSD test. **denote significant differences at P<0.003

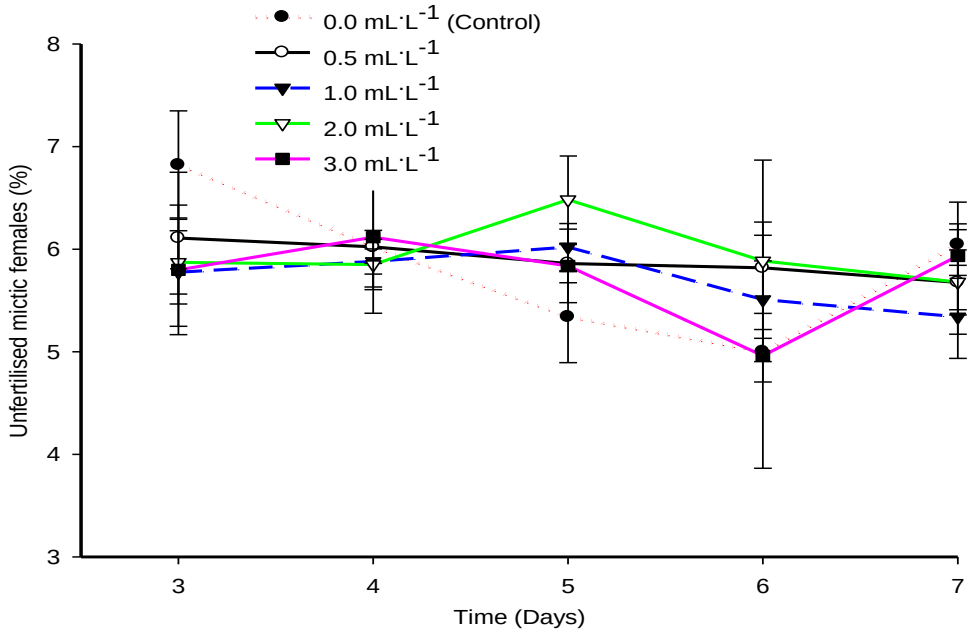


**Fig. 4**. Effects of different concentrations of CME and days of culture on the unfertilised mictic female production rate of the rotifer *Brachionus angularis*. Data shown are means ± standard deviation of three replicates; two-way ANOVA

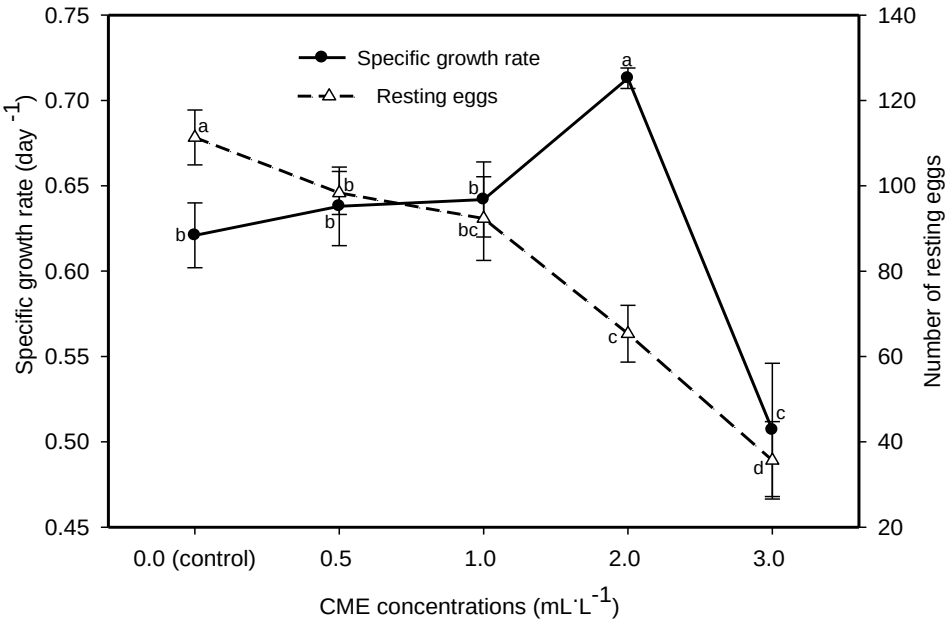


**Fig. 5**. Effects of different concentrations of CME on specific population growth rate and resting egg production of the rotifer *Brachionus angularis.* Data shown are means ± standard deviation of three replicates; one-way ANOVA, different letters in each curve denote significant differences at P<0.05; Tukey HSD test; a>b>c>d

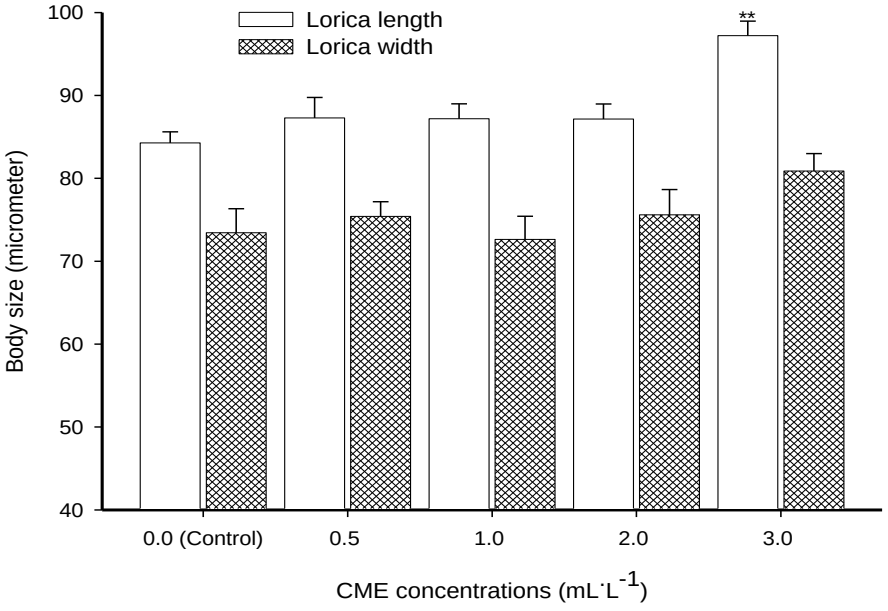


**Fig. 6**. Effect of different concentrations of CME on the body size of the rotifer *Brachionus angularis.* The values represent mean lorica length and width (µm) ± standard deviation of 10 individuals; one- way ANOVA, Tukey HSD test. The symbol ** denote significant difference at P<0.003.

## Discussion

Application of organic manures enhances the biological productivity in the fishponds (Elsaidy et al. 2015). The current study has demonstrated that CME influences the population density, mictic induction, and specific population growth rate and body size of the rotifer, *B. angularis*. Treatment of rotifers with 2.0 $mL \cdot L^{-1}$ of CME prolonged the rotifer population density and amictic female population, suggesting that only specific amount of CME is effective for the increased parthenogenetic reproduction of this rotifer species. This observation is consistent with the persistent nature of growth promoting compounds contained in the CME (Shemesh and Shore 1994). According to Pentikainen et al. (2006), the estradiol compounds act as growth hormone for the female reproductive tissues, hence resulting in high viability of oocytes. The biomolecules in the CME can be recovered and applied in the production of the rotifer *B. angularis*, where harvesting can be done on day 5. It is probable to suggest that CME acts like a capsule of hormones (Hakk et al. 2005) that synergistically augments each other to produce stunning growth and reproduction effects on rotifers. Moreover, literature indicates that chicken manure is an excellent substrate for the heterotrophic production of probiotic bacteria (e.g. *Bacillus* sp.) that can be utilised by rotifers to improve survival and reproduction (Gatesoupe 1991; Moriarty 1997; Rapatsa and Moyo 2013; Elsaidy et al. 2015).

However, the reduced fertilization rate of mictic females (Fig. 3) suggests that the CME could have suppressed mixis stimulus. Other studies have identified estrogen agonist chemicals such as nonylphenol in the freshwater *B. calyciflorus*, which have no effect on amictic reproduction but reduces mictic induction (Preston et al. 2000). They further observed stoppage of fertilisation rate at 50 $\mu g \cdot L^{-1}$ of nonylphenol. Similar reduction of fertilisation rate of females has been reported in *B. plicatilis* under the influence of 30-50 $\mu g \cdot 1L^{-1}$ of nonylphenol (Marcial 2004). Even though these hormones were not tested in their pure form, it is suspected that this could probably explain the reduced mixis under the influence of CME in the current study. However, further investigations should unravel this speculation. According to Gilbert (1963), it is the environmental stimuli, and not internal rhythmic cycle that causes mictic female production in the rotifer *B. calyciflorus*. Mictic reproduction is an inherently more complicated process, which depends on the fertility of both male and female and on their successful mating behavior (Sugumar and Munuswamy 2006). Thus, rotifer mictic reproduction is likely to be a factor of signals rather than amictic reproduction (Preston et al. 2000). The reduced production of resting eggs (Fig. 5) could have been a direct consequence of suppressed fertilisation of mictic females. Rotifer resting egg production is believed to be the most sensitive endpoint for a number of chemicals (Preston et al. 2000) probably due to the complexity of rotifer mictic reproduction, which integrates toxicant effects over the full life cycle (Preston and Snell 2001). The higher specific population growth rate of $0.71 \pm 0.01$ $day^{-1}$ obtained at 2.0 $mL \cdot L^{-1}$ of CME (Fig. 5) was probably due to higher parthenogenetic reproduction at that condition. This value is comparable to the findings of Yang and Snell (2010) for freshwater *B. calyciflorus* exposed to 1,000 $\mu g \cdot L^{-1}$ of combined progesterone and estradiol hormones.

The increase in lorica length at 3.0 $mL \cdot L^{-1}$ of CME (Fig. 6 and 7) coincided with reduced population density, hence the reduced competition for food enabled the rotifers to achieve their full growth potentials, and not directly influenced by CME. Nonetheless, we could not explain why the lorica width was not affected. More studies should focus on the elongation preference of lorica length at the expense of the width under the influence of CME. Gallardo et al. (1997) observed reduced body size of the rotifer *B. plicatilis* with different hormonal treatments and attributed the results to high competition for food caused by increased population densities.

Even though the observed reproductive effects of CME in this study seem consistent with the influence of sex hormones e.g.17β-estradiol, testosterone, oestrogens and androgens that are significantly represented in chicken manure (Shemesh and Shore 1994; Hakk et al. 2005), we can only speculate that such effects were related to endocrine disruption mechanisms because no molecular essay was done to authenticate these observations. According to Preston et al. (2000), in the absence of a molecular assay for interactions with a rotifer endocrine receptor, the observed effects should be considered as consistent with those of endocrine disruption mechanisms but not as proof of such mechanisms.

## Conclusion

The results of this study show that 2.0 $mL \cdot L^{-1}$ of CME is optimal for enhanced rotifer population density, amictic female population and specific growth rate. The optimal CME concentration is most effective on day 5 day of culture and does not increase the rotifer size, hence applicable in aquaculture.

## Acknowledgements

This study was supported by the Japan Society for the Promotion of Science (JSPS) Asia-Africa and JSPS Kakenhi grant No. 24380108. Mr. Erick Ochieng Ogello is grateful to the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for providing the PhD scholarship. We thank the anonymous reviewers for improving the quality of this article.

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*Received: 17/09/2015; Accepted: 19/11/2015 (MS15-75)*