International Journal of Fisheries and Aquatic Studies 2016; 4(4): 219-226

ISSN: 2347-5129
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.352
IJFAS 2016; 4(4): 219-226

www.fisheriesjournal.com
Received: 29-05-2016
Accepted: 30-06-2016

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Reproductive rate of *Brachionus calyciflorus* under the influence of salinity, temperature, feed type and feed concentration

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Abstract
The impact of salinity, temperature, feed type and feed concentration on reproductive rate ‘r’ of *Brachionus calyciflorus* were examined in the present study. Three salinity levels (0.5%, 5.0% and 10.0%) and three temperature regimes (25 ± 1 ºC, 29 ± 1 ºC and 35 ± 1 ºC) were evaluated. Four microalgal diets, *Chlorella ellipsoidea*, *Ankistrodesmus convolutes*, *Scenedesmus protuberans* and *Chlorococcum infusorium* at a concentration of 1x 10^6, 2x 10^6, 3x 10^6, 4x 10^6, 6x 10^6, 8x 10^6, 10x 10^6 and 12x 10^6 were tested in the experiment. The findings showed that all these parameters have significant influence on reproductive potential of *Brachionus calyciflorus*. The optimum salinity and temperature required for r_{max} for *B. calyciflorus* were of 0.5% and 29 ± 1 ºC, respectively. The intrinsic rate of reproduction of *B. calyciflorus* for the best salinity x feed concentration with *C. ellipsoidea* (8 x 10^6 cell mL^{-1}), *A. convolutus* (6 x 10^6 cells mL^{-1}), *C. infusorium* (8 x 10^6 cells mL^{-1}) and *S. protuberans* (4 x 10^6cells mL^{-1}) at the optimal salinity and temperature were 1.91, 1.83, 1.77 and 1.62. Based on the study, the maximum reproductive potential of *B. calyciflorus* can be achieved by feeding with *C. ellipsoidea* at a salinity and temperature of 0.5% and 29 ± 1 ºC, respectively for the larviculture of fishes.

Keywords: Reproductive rate *Brachionus calyciflorus Chlorella ellipsoidea Ankistrodesmus convolutes Scenedesmus protuberans Chlorococcum infusorium*

1. Introduction
Zooplanktons constitute the primary food sources to fish and crustacean larvae [1]. Among them, Rotifers are considered as an ideal food for fish larvae due to its small size, shape, slow mobility, ability to grow in high density and their ability to produce resting eggs in adverse conditions [2, 3]. In addition, they are nutritionally rich with all essential nutrients and can be enriched to meet the nutritional requirement of the fish larvae [4]. Several rotifer species such as *Brachionus plicatilis*, *B. rotundiformis*, *B. calyciflorus*, *B. angularis*, *B. rubens* and *B. patulus* are used as live food for various marine, estuarine and freshwater larval fishes or even in rearing brackishwater crustaceans [4, 5]

The prime factor that determines the effectiveness for mass culture of rotifers is its intrinsic rate of reproduction or reproductive rate ‘*r*’. It forms the totality of all life table parameters because it combines several factors like survival, fecundity and reproduction of rotifers [6]. In natural waters, rotifers experience wide fluctuations in both abiotic (e.g. temperature, salinity) and biotic factors (e.g., food availability, predation) that results in changes in their survival rate and reproduction [7]. Major factors that influence the reproductive rate of rotifers are temperature [8, 9], salinity [10], food quality and its quantity [11-14].

In general, rotifers are mainly restricted to freshwaters. Therefore, salinity plays a vital role in the diversity, survival and reproductive potential of an individual rotifer [15, 16]. Several factors have been suggested for the poor survival of freshwater rotifers in saline waters [11]. Other important factors such as temperature, feed type, feed concentration and particle size of the feed also determines the reproductive rate of rotifers, thereby affecting species composition and richness of rotifers in natural water bodies [17-19].

B. calyciflorus is a widely distributed rotifer found in many freshwater bodies around the world including India [20]. Moreover, small size, slow swimming velocity and ability to form dense population make this rotifer a suitable live feed for rearing fish larvae. In addition, the use of freshwater rotifers like *B. calyciflorus* is likely to enable intensive production of freshwater ornamental fish species having small larvae [21]. The key factor responsible for developing mass production of *B. calyciflorus* under controlled condition is to determine its reproductive rate. Hence, the aim of our study was to evaluate the reproductive rate '*r*' of *B. calyciflorus* against different temperature, salinity, feed and feed concentrations on the reproductive potential.

2. Materials and methods

2.1 Rotifer

The Veli Lake (latitudes 8°25' – 8°35'N & longitudes 76° 50' 76° 58'E), is a freshwater lake situated approximately 8 Km North-West of Thiruvananthapuram, Kerala, India having a length of 2 km long, 0.3 km broad and with a mean depth of 2-3m. During south west monsoon, the lake opens to the sea through a narrow outlet and seawater exchange takes place during this time. *B. calyciflorus* (Length 220-340 µm; width 120-180 µm) collected and identified [20] from the aforesaid lake were employed for the study by using a starter culture of an amitic female. The cultures were fed with *Chlorella ellipsoidea* and maintained at room temperature (29±1 °C) under continuous fluorescent illumination (1000 lux).

2.2 Design of experiment

Salinity, temperature and microalgal diets at different concentrations of the algae were the variables tested. Based on a series of preliminary salinity tolerance tests, three salinity levels (0.5%, 5.0% and 7.0%) were selected and the required salinities were prepared by diluting sterile sea water with distilled water. Three temperatures (25 ± 1 °C, 29 ± 1 °C and 35 ± 1 °C) were selected based on the temperature levels of the lake from which the rotifer was collected. The experimental animals were maintained by thermostatically controlled water baths under diffused and continuous fluorescent illumination (1000 lux).

Four algal diets, *Chlorella ellipsoidea*, *Ankistrodesmus convolutus*, *Chlorococcum infusorium* and *Scenedesmus protuberans* were employed for the study. The stock cultures of *C. ellipsoidea* and *A. convolutus* were provided from the Vizhinjam Research Centre of CMFRI, Kerala (INDIA) while, *S. protuberans* and *C. infusorium* were isolated from Veli Lake by serial dilution method [22]. All the algal cultures were maintained in the laboratory by using Walne's medium under constant illumination. The required cell densities were prepared by centrifuging the algal cultures at 3000 *g* and then introduced the cells into the experimental vials [15 ml] of appropriate salinities. The experimental feed concentrations employed were 1 x 10^6, 2 x 10^6, 3 x 10^6, 4 x 10^6, 6 x 10^6, 8 x 10^6, 10 x 10^6 and 12 x 10^6 cells mL^{-1} for all the algal diets and the algal cell counts were taken with a hemocytometer.

The rotifers were acclimatized to the experimental salinities, temperature and feed concentrations for one month prior to the experiment. Five ml of the medium of each feed type with appropriate feed concentrations and salinities were taken in vials. Five numbers of previously acclimatized amitic females (with a single egg) of *B. angularis* (one animal per one ml) was carefully transferred to the appropriate medium with a micropipette. A total of 216 vials were set up for each feed as follows: 1 feed type x 8 feed concentrations x 3 salinities x 3 temperatures x 3 replicates. After three days (72 hours incubation), the experimental vials were fixed using 4% formaldehyde solution. The final populations of rotifers were estimated by counting the animals under a stereomicroscope with an overall magnification of 40X. Intrinsic rate of reproduction (*r*) was estimated [6].

$$r = \frac{\ln(Nt) - \ln(No)}{t}$$

Where, r = instantaneous growth rate; ln (Nt) = natural logarithm of population density after time t; ln (No) = natural logarithm of initial population; t = duration of experiment (3 days).

2.3 Statistical analysis

Each set of experiment was carried out in triplicate and the mean values were taken. Two-way ANOVA was used to compare the reproductive potential in relation to salinity and feed concentration against three temperatures. All analysis were done using SPSS 14.0.

3. Results

The intrinsic rate of reproduction in relation to salinity, temperature and feed concentration using four feed types *viz*., *C. ellipsoidea*, *A. convolutus*, *C. infusorium* and *S. protuberans* are presented below. The optimum '*r*' value was found at room temperature (29 ± 1 °C) and at salinity (0.5%) for all the feed concentrations.

3.1 *C. ellipsoidea*

The reproductive rate, r ± SD of *B. calyciflorus* fed with *C. ellipsoidea* against three different levels of salinity and temperature are shown in Fig 1A-C. From the results, we observed no significant influence in salinity or feed concentration in determining the *r* at low temperature (25 ± 1 °C) (Fig 1A). Meanwhile, at room temperature (29 ± 1 °C), the rate of reproduction was prominently affected by salinity (F= 58.0; $p<0.001$) and feed concentration (F= 26.3; $p<0.001$) both independently and in combination (F= 6.0; $p<0.001$) (Fig 1B). Similar results were also observed at higher temperature (35 ± 1 °C), where salinity (F= 191.7; $p<0.001$), feed concentration (F= 56.2; $p<0.001$) and in combination of these factors (F= 19.7; $p>0.001$) exert marked change in r_{max} of *B. calyciflorus* (Fig 1C).

Salinity plays an inverse relationship with reproduction of *B. calyciflorus*. The density of rotifers reduced as the salinity increased from 0.5% to 7.0% irrespective of temperature. The concentrations of *C. ellipsoidea* fed to *B. calyciflorus* also influence a major role in determining the *r* value. The intrinsic rate of reproduction increased with increase in the density of algae from 1 x 10^6 to 8 x 10^6 cells mL^{-1} and decreased with further elevation in the levels of algae. Meanwhile, the optimum level of *C. ellipsoidea* required for r_{max} were found to be 8 x 10^6 cells mL^{-1} at room temperature with a salinity of 0.5% (323 ± 102 individuals).

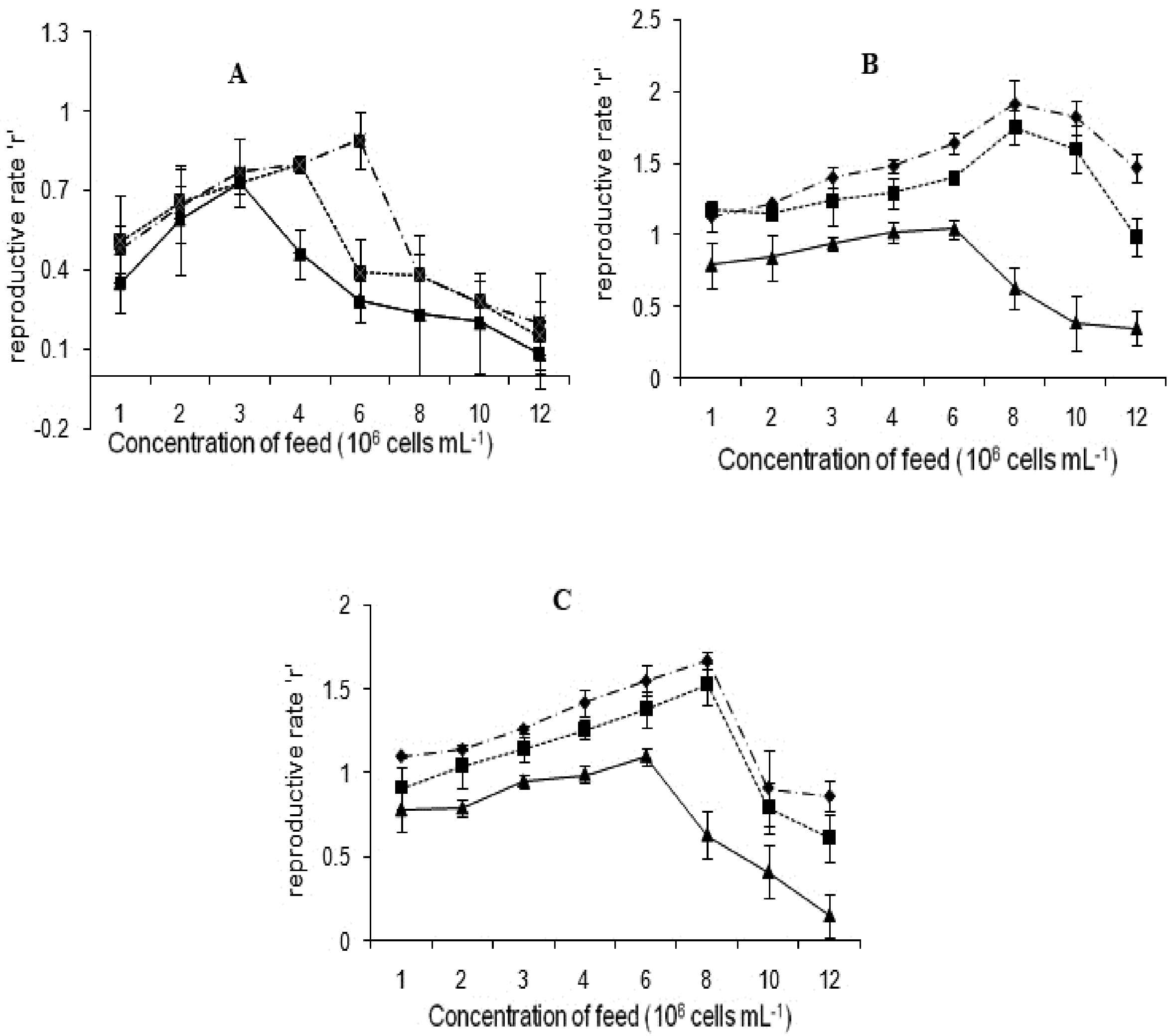

Fig. 1. Reproductive potential (r ± SD) of *Brachionus calyciflorus* fed with *Chlorella ellipsoidea* at three temperatures **(A)** 25 ± 1°C **(B)** 29 ± 1°C and **(C)** 35 ± 1°C and at three salinities (–◆– 0.5‰ ---■--- 5.0‰ —▲— 7.0‰)

3.2 *A. convolutes*

The reproductive potential, r ± SD of *B. calyciflorus* fed with various densities of *A. convolutus* in three different salinity and temperature levels are shown in Fig 2 A-C. The '*r*' value of the rotifer exposed to low temperature (25 ± 1 °C) showed significant influence to salinity (F= 10.32; p<0.001) and feed concentration (F= 31.41; p<0.001) (Fig 2A). Meanwhile, the reproductive rate was prominently decreased in *B. calyciflorus* exposed to various levels of salinity (F= 52.65; p< 0.001), density of algae (F= 17.98; p<0.001) and in combination of both (F= 6.06; p<0.001) at 29 ± 1 °C (Fig 2B). Similarly, at high temperature (36 ± 1 °C), the r value changed significantly with salinity (F= 49.25; p<0.001) and concentration of *A. convolutus* (F= 83.86; p<0.001) by both independently and in combination (F= 17.43; p<0.001) (Fig 2C).

Salinity influenced the reproduction of *B. calyciflorus* greatly. The optimum salinity required for r_{max} was found to be 0.5% and further increase in this variable resulted in reduced number of animals. The experiment showed that density of *A. convolutus* prominently determines the reproductive success of *B. calyciflorus*. The maximum number of rotifers was obtained after feeding the animals with 4X10^6 cells mL^{-1} of algae at all tested temperature regimes.

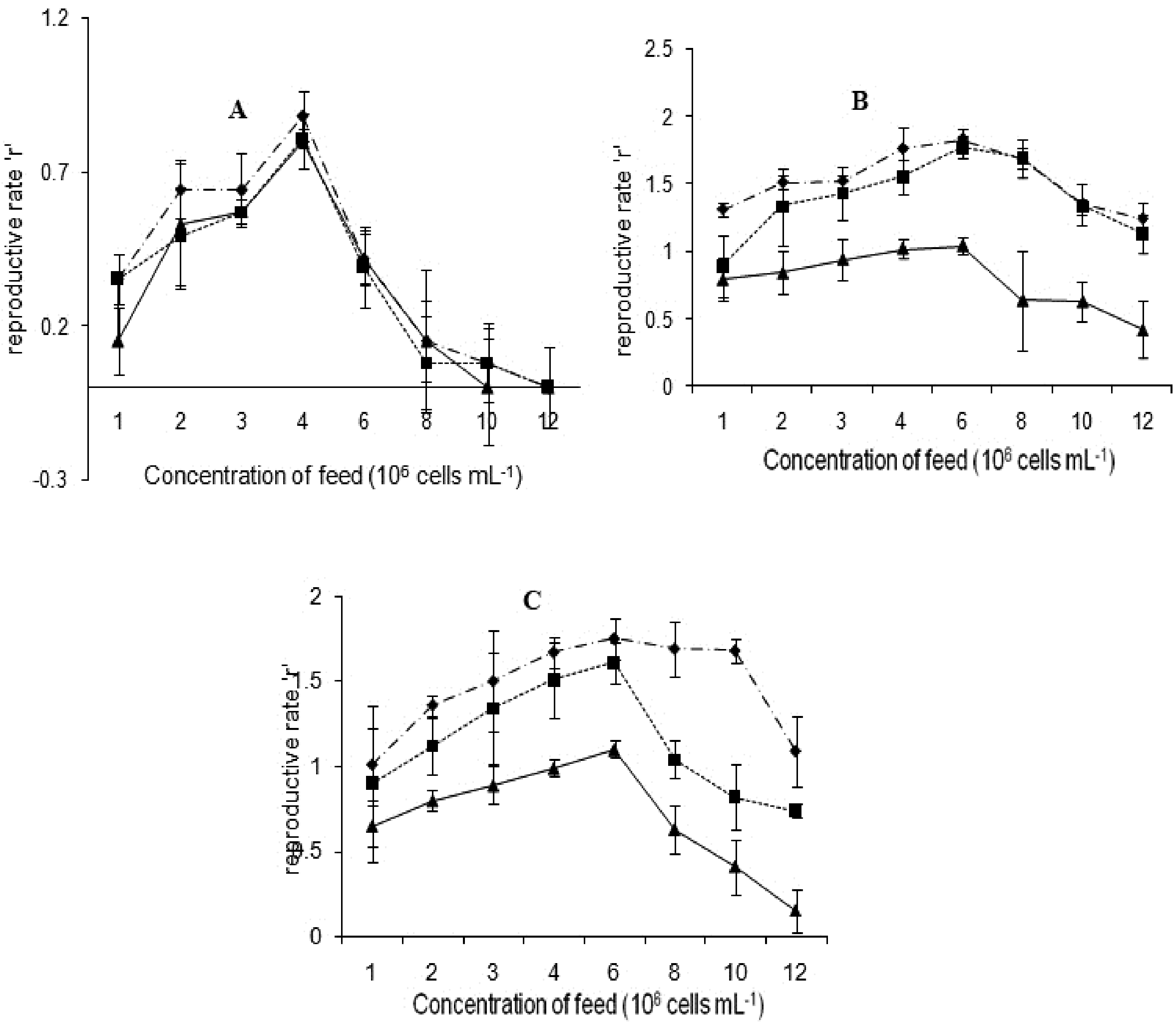

Fig. 2. Reproductive potential (r ± SD) of *Brachionus calyciflorus* fed with *Ankistrodesmus convolutes* at three temperatures **(A)** 25 ± 1°C **(B)** 29 ± 1°C and **(C)** 35 ± 1°C and at three salinities (–◆– 0.5‰ ---■--- 5.0‰ —▲— 7.0‰)

3.3 *C. infusorium*

The intrinsic rate of reproduction r ± SD of *B. calyciflorus* at different salinities and feed concentrations of *C. infusorium* exposed to different temperatures are shown in Fig 3A-C. The reproductive behavior of *B. calyciflorus* depends greatly on temperature of the ambient medium. At lower temperature, the '*r*' value of rotifer was significantly affected by concentration of the algae (F= 17.95; p<0.001) (Fig 3A) while at room temperature it was influenced by salinity (F= 55.34; p< 0.001), density of algal diet (F= 28.56; p<0.001) and in combination of both (F= 8.17; p<0.001) (Fig 3B).

Irrespective of temperature and feed concentration, the reproductive rate of *B. calyciflorus* decreased with increase in salinity. The density of rotifers reduced from 217 ± 94 to 37 ± 13 individuals as the salinity elevated from 0.5% to 7.0%. The concentration of *C. infusorium* fed to *B. calyciflorus* also determines the *r*. The r_{max} were observed at a density of 8 x 10^6 cells mL^{-1} and further increase in concentration of feed resulted in reduced reproductive rate of rotifer.

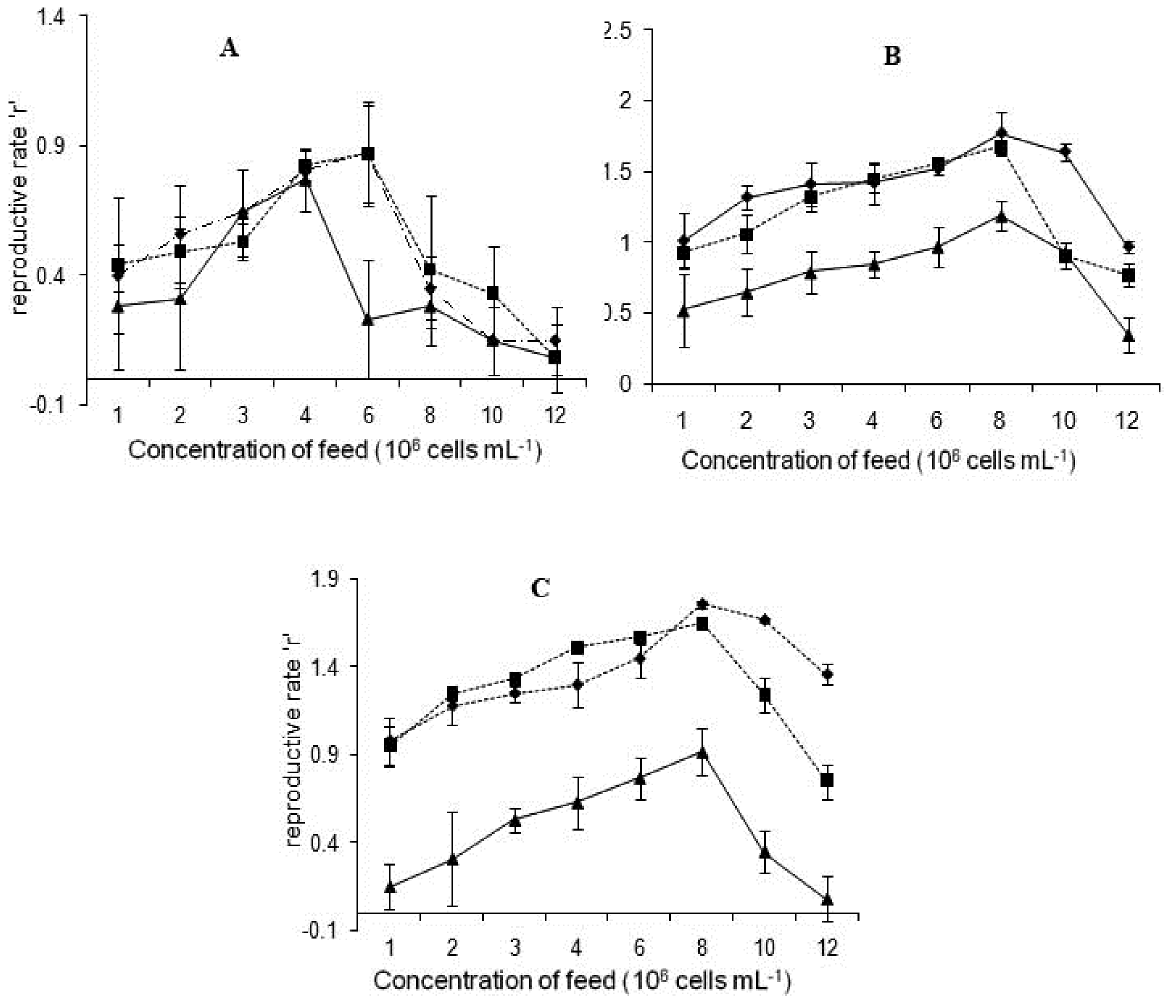

Fig. 3. Reproductive potential (r ± SD) of *Brachionus calyciflorus* fed with *Chlorococcum infusorium* at three temperatures **(A)** 25 ± 1°C **(B)** 29 ± 1°C and **(C)** 35 ± 1°C and at three salinities (0.5‰ 5.0‰ 7.0‰)

3.4 *S. protuberans*

The specific growth rate '*r*' ± SD of *B. calyciflorus* in different regimes of salinity, temperature and feed concentrations of *S. protuberans* are shown in Fig 4A-C. At low temperature, the feed concentration of algae showed significant variation in intrinsic rate of reproduction ($F= 49.05$; $p<0.001$) (Fig 4A), while at room temperature, the reproduction was prominently influenced by salinity ($F= 32.42$; $p<0.001$), density of feed ($F= 53.57$; $p<0.001$) and in combination of both factors ($F= 16.24$; $p<0.001$) (Fig 4B). Similar results were observed at high temperature where r_{max} was significantly changed by salinity ($F= 68.37$; $p<0.001$), feed concentration ($F= 18.32$; $p<0.001$) and in combination ($F= 5.32$; $p<0.05$) of both these variables (Fig 4C).

The concentrations of *S. protuberans* fed to *B. calyciflorus* also influence a major role in determining the reproductive rate. The intrinsic rate of reproduction increased with increase in the density of algae from 1 x 10^6 to 4 x 10^6 cells mL^{-1} and decreased with further elevation in the algal levels. The maximum number of individuals were obtained after feeding with 4X10^6 cells mL^{-1} at 25 ± 1 ºC (14 ± 3), 29 ± 1 ºC (130 ± 32) and 36 ± 1 ºC (100 ± 18).

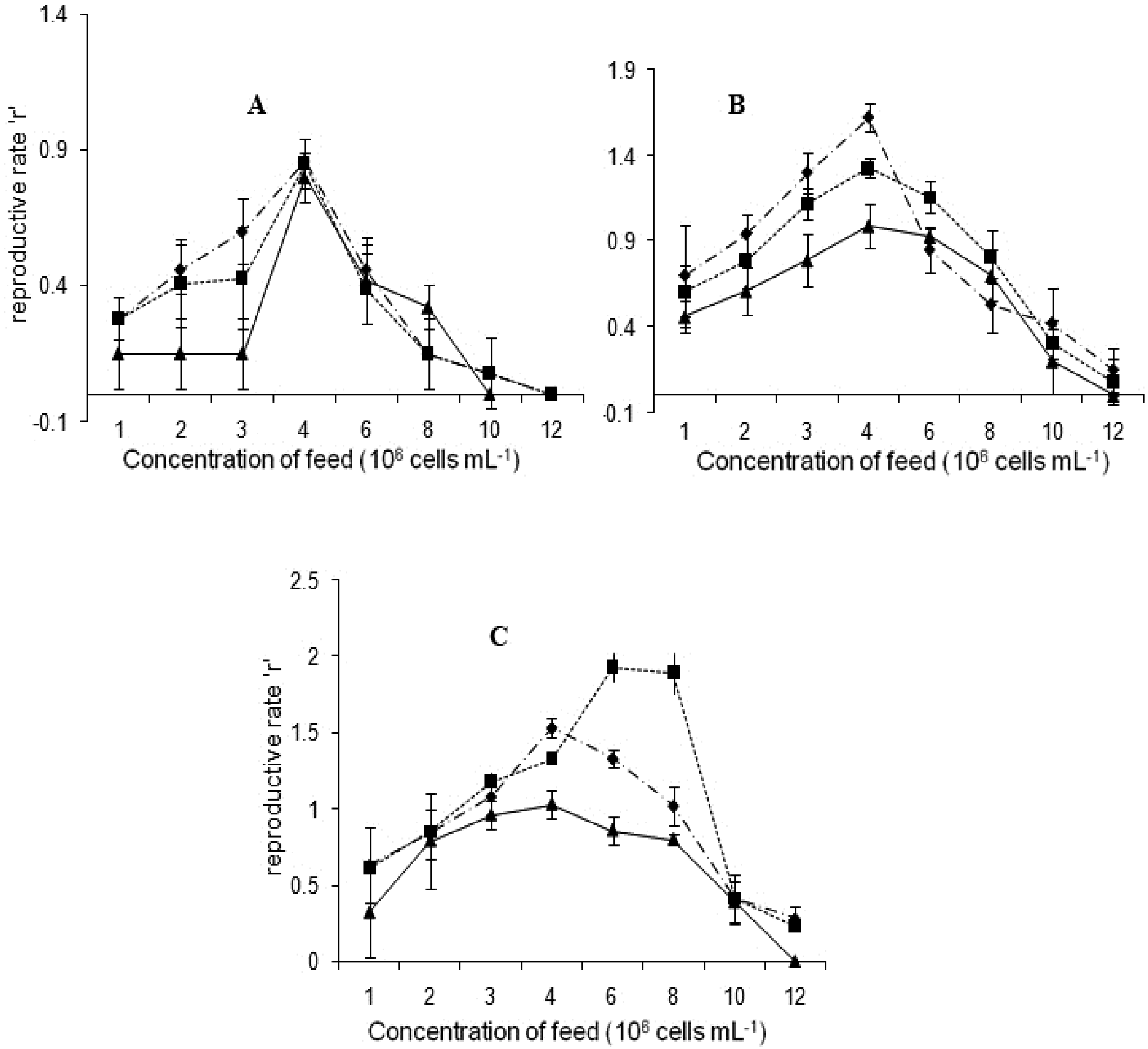

Fig. 4. Reproductive potential (r ± SD) of *Brachionus calyciflorus* fed with *Scenedesmus protuberans* at three temperatures **(A)** 25 ± 1°C **(B)** 29 ± 1°C and **(C)** 35 ± 1°C and at three salinities (–◆– 0.5‰ ---■--- 5.0‰ —▲— 7.0‰)

4. Discussion

Intrinsic reproductive rate (*r*) of rotifers indicates the most suitable environmental conditions for reproduction as well as their changes in the reproductive performances under stress conditions [12, 23]. Furthermore, the rate of population increase (*r* day^{-1}) provides information about population dynamics in several life table and demographic studies [11, 24]. Due to their small size and parthenogenic mode of reproduction, rotifers show high intrinsic rate of reproduction than other zooplanktons [6]. In addition, certain biotic and abiotic factors like temperature, salinity, feed type and feed concentration also exert prominent variations on this parameter [12, 13, 15]. Moreover, *r* value also helps to determine the levels of abiotic and biotic requirements for successful mass culture of rotifers [12, 13, 15]. In the present study, the reproductive rate of *B. calyciflorus* was strongly affected by temperature, salinity, type of feed and its concentration.

The reproductive rate of *B. calyciflorus* varies with temperature and the highest *r* value was observed at room temperature (29 ± 1 ºC). Several reports suggest that an increase in intrinsic rate of population coincides with increase in temperature for *B. calyciflorus*. In their study, the r_{max} was observed at a temperature of 28 ºC to 30 ºC [25, 26]. Meanwhile, a lower temperature of 20 ºC was also reported for this rotifer [27]. A possible reason for this variation could be due to the strain difference used in the study. The survival or reproductive performance of rotifers varies between the strains of the same species in response to temperature as reported in Anging strain of *B. calyciflorus* [28]. Additionally, the direct effect of temperature on *r* of rotifers is difficult to determine as this parameter is mostly influenced by other features such as population density, food supply, variation in developmental stages and metabolic activities of rotifer [11].

Salinity is an important abiotic factor that strongly determines the abundance and diversity of rotifers. In general, rotifers are very sensitive to salinity [7] and any slight variation in this factor results in changes in populations [15, 16]. Investigations on the impacts of salinity changes on r_{max} of rotifers are mainly reported from few euryhaline species such as *B. rotundiformis* and *B. plicatilis* while little information is available on *B. calyciflorus*. The results of the present experiment showed that the intrinsic rate of reproduction of *B. calyciflorus* exhibit an inverse relationship with salinity with the maximum at 0.5% and reduced with further elevation in salinity level till 7%. *B. calyciflorus* are known to tolerate less salinity with LC_{50} value of 3.75 ± 0.04% [16]. Several physiological mechanisms are involved in the lower salinity tolerance of freshwater rotifers. This includes failure of osmoregulation, reduced swimming rate, less hatching success, mass mortality of neonates and biochemical changes [10] and a possible reason for the low salinity tolerance by *B. calyciflorus* could be due to any of the above factors.

Effects of feed and its concentrations were tested in this experiment to determine the most effective algae as food for maximum production of *B. calyciflorus*. Generally, green algae are widely used for the culture of rotifers [11-13] and the reproductive rate depends on the type of algae employed for its production [29]. In the present study, *B. calyciflorus* attained the highest rate of population increase per day with *C. ellipsoidea* followed by *A. convolutus*, *C. infusorium* and *S. protuberans*. Among the algal diets used for mass production of *B. calyciflorus*, the most commonly used microalgae have been *C. vulgaris* and *S. obliquus* and *C. vulgaris* were found to offer better results than other algae [16, 30]. The results of our experiment showed that a higher *r* value was produced by feeding *C. ellipsoida* (*r* =1.91) than *C. vulgaris* (*r* = 0.27 - 0.52) [2] and *S. obliquus* (*r* =1.6) [31]. The principle factors responsible for the preference of microalgae as food for rotifers include size, shape, digestibility, motility and nutrient composition of the algal diet employed [11]. The preference to *C. ellipsoidea* by *B. calyciflorus* in this study could be attributed to its small size (2 - 5 µm) compared to *C. infusorium* (2 - 7 µm), *A. convolutus* (10 - 20 µm) and *S. protuberans* (30 - 37µm).

B. calyciflorus attained r_{max} at by feeding *C. ellipsoidea* (8 x 10^6 cell mL^{-1}), *A. convolutus* (6 x 10^6 cells mL^{-1}), *C. infusorium* (8 x 10^6 cells mL^{-1}) and *S. protuberans* (4 x 10^6cells mL^{-1}). Earlier workers showed that *C. vulgaris* and *Scenedesmus obliquus* at a concentration of 4.5 x 10^6cells mL^{-1} and 8 x 10^6 cells mL^{-1} had a prominent influence on *r* value [30, 32] while a higher concentration of *C. ellipsoidea* was necessary to reach the r_{max} in our study. A possible reason for this variation could be due to the smaller particle size of the feed and the ingestion ability of *B. calyciflorus* used in the experiment. Several reports suggested that the density of rotifers increase with increase in the concentration of microalgae, reach at high densities at a certain level and then remain stable or decrease [29, 30]. A similar pattern of feeding behavior and population growth was exhibited by *B. calyciflorus* against four different algae used in our study. This low density of rotifers at high levels of microalgae could be due to the toxic effect of algae on rotifers [33], interfere with the feeding apparatus of rotifer [34], inhibit the ciliary activity, thereby leading to reduced swimming speeds [35] or reduced assimilation rate due to rapid movement of food through gut, eventually leading to malnutrition [36].

In conclusion, *B. calyciflorus* attained optimum maximum *r* at salinity (0.5%) and temperature (29 ± 1 ºC) with *C. ellipsoidea* as feed at a level of 4 x 10^6 cells ml^{-L}. Determination of optimum abiotic and biotic variables for maximum *r* is required for mass production and the application of *B. calyciflorus* for larviculture of freshwater ornamental fishes with small larvae. Moreover, *B. calyciflorus* can also be used as a suitable live feed for fish larvae due of its smaller size and tolerance to salinity.

5. Acknowledgements
The authors are thankful to the Director, CMFRI, for providing facilities to carry out this work. The first author is grateful to ICAR for providing Senior Research Fellowship.

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