Role of Critical Size on Adult life History Traits in Drosophila Melanogaster.

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Research article

Keywords: Drosophila melanogaster, critical size, developmental threshold, silver spoon hypothesis, trade-off

DOI: https://doi.org/10.21203/rs.3.rs-80024/v1

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Abstract

Background

Silver-spoon hypothesis suggests that fitness of individuals is high under good adult conditions provided their development itself has been in good conditions and those who have grown in resource-poor conditions are at a permanent disadvantage. Using two types of Drosophila melanogaster populations grown under two conditions we tested the validity of silver-spoon hypothesis. Three populations were selected for faster pre-adult development as a result of which they had access to food for a shorter duration while the three control populations had access to food for longer duration as growing larvae. In the second set-up the access to food was curtailed immediately on attainment of critical size. We assessed biomolecule levels, copulation latency, copulation duration, life-time realized oviposition and longevity to validate the silver-spoon hypothesis.

Results

Restricted feeding duration as a consequence of selection for faster per-adult development had no fitness consequences in selected populations. However, starvation during post-critical duration resulted in reduced fitness.

Conclusion

Our results show that the silver-spoon model is applicable only under extreme nutrition curtailment and not applicable to biological systems that have genetically evolved to limit food intake.

Background

Early life nourishment is known to affect the adult physiology and various life-history traits in Drosophila melanogaster. For example, nutrient acquisition in the form of dietary sugars (source of C) during the larval stage has contribution in egg provisioning during the early adult life of holometabolous females. In D. melanogaster, early life is divided into pre-adult duration consisting of mobile and voraciously feeding larval phase and non-mobile pupal phase. Further, the larval phase is marked into two stages- (i) pre-critical stage and post-critical (terminal growth period) stage, separated by critical size time point. Larval critical size commit the larva to an irreversible process of metamorphosis, and starvation post attainment of critical size does not affect time course to undergo metamorphosis. The role of critical size as “physiological switch” is well established and is marked by ecdysone pulse. Recently, critical size is reported to also act as “Energy allocation switch” in various species of Drosophila. In another dipteran species, Aedes egypti, it has been previously reported that threshold amount of energy reserves are pre-requisite to the process of metamorphosis in addition to ecdysone level during last larval instar, thus implying significance of energy budgeting in the dipteran species.
‘Developmental threshold model’ states that there is always a minimum size or condition that must be surpassed before the life-history transition occurs in a wide variety of species undergoing metamorphosis\textsuperscript{11,12}. While larger threshold causes a negative relationship between age of transition and growth conditions, the smaller threshold would result in a comparatively positive relationship for higher growth rate. In fast-developing individuals, once this threshold is passed then the excess of energy or resource is translated to overhead threshold and invested in fecundity\textsuperscript{11}.

In \textit{Drosophila melanogaster}, this minimum developmental threshold is represented by critical size/time point\textsuperscript{5,6,13}. Being an inhabitant of rotting fruits and vegetables, it is under direct selection for faster pre-adult development due to over-crowding and food limitation. Selection for faster pre-adult development is known to exhibit reduced development time and subsequently results in the smaller adult body size\textsuperscript{13–18}. In \textit{D. melanogaster}, environmental conditions during larval life are suggested to affect its adult size\textsuperscript{19} and their physiology\textsuperscript{19,20} which in turn affect various life-history traits\textsuperscript{1,3,21–24}. Further, if the larval nutritional environment or developmental diet is rich then they tend to emerge with larger adult body size and attain reproductive maturity at an early age that has a positive implication on fitness\textsuperscript{11,25,26}. In general though not universal\textsuperscript{18} “bigger is better” idea prevails with larvae spending more time in weight (equal to resource/energy) gain under good nourishment conditions, eventually emerging as larger adults with higher fitness. Also ‘silver spoon hypothesis\textsuperscript{25}’ posits that individuals born in good conditions have fitness or performance advantages in later life with many examples\textsuperscript{25} and those born in poor conditions are at a permanent disadvantage\textsuperscript{27}. Varying the quality or quantity of diet during developmental in \textit{D. melanogaster} affects adult body size and its associated life history hence it is known to serve as a tractable model to study dietary manipulation during pre-adult and adult stages\textsuperscript{24,28,29}.

The inverse relationship between time to maturity (metamorphosis) and size is expected in species that live in ephemeral habitat\textsuperscript{12}. A physiological change from resource-dependent (pre-critical) to resource independent (post-critical duration) rate of development occurs at critical size or threshold time point\textsuperscript{7}. Further, the energetically costly process of reproduction is favoured over survival under rich (nutritional) environment than in dietary restriction condition where longevity (somatic maintenance) is favoured\textsuperscript{30–32}. However, dietary restriction (DR) is also known to result in short term adaptive response where in females that were deprived of yeast diet as larvae had reduced fecundity but mortality rate was unaltered\textsuperscript{24} suggesting that longevity-fecundity trade-off may not be universal.

In the present study, we tested the applicability of ‘silver spoon hypothesis\textsuperscript{27,33} using six outbred populations of \textit{D. melanogaster}. Three of the six populations were under conscious selection for faster pre-adult development and extended reproductive longevity while the other three were their ancestral control populations. The selected populations were internally driven to stop feeding in about 17-hour post attainment of critical size thus curtail food intake, while the control populations fed for 42-hours post attainment of critical size. Critical size time is an important switch point that commits the organism to irreversible metamorphosis process\textsuperscript{5,7}. Further, we also tested the hypothesis under food curtailment post
attainment of critical size in both the control and selected populations. The realized life-time oviposition of the populations selected for faster pre-adult development thus under food restriction was not significantly different from those of the control populations, refuting the silver spoon hypothesis.

**Results**

**Biochemical assays during larval life**

There was no significant effect of selection ($F_{1, 2} = 14.756, p = 0.062$, Fig. 1c) on glycogen levels. However, there was a highly significant effect of larval growth time point ($F_{4, 8} = 64.419, p = 0.000$, Fig. 1c) on glycogen content (Fig.1c). Further, selection × larval growth time point interaction was not significant. Interestingly, selection ($F_{1, 2} = 20.394, p = 0.046$), larval growth time ($F_{4, 8} = 46.663, p < 0.001$) and selection × larval growth time point interaction ($F_{4, 8} = 31.564, p < 0.001$) had significant effect on protein content (Fig.1d). Similarly, selection ($F_{1, 2} = 189.34, p < 0.005$), larval growth time ($F_{4, 8} = 104.435, p < 0.001$) and selection × larval growth time point interaction ($F_{4, 8} = 86.645, p < 0.001$) had significant effect on lipid content (Fig.1e). The energy equivalents of the biomolecules were summed and the effects of selection, larval growth time and their interaction were ascertained. There was a significant effect of selection ($F_{1, 2} = 200.037 p < 0.005$; Fig 1f), larval growth time ($F_{4, 8} = 128.801, p < 0.001$; Fig. 1f) and selection × larval growth time point interaction ($F_{4, 8} = 79.65, p < 0.001$).

**Realized life-time oviposition**

There was no significant effect of selection ($F_{1, 2} = 11.83, p = 0.075$, Fig 2b) on realized life-time oviposition. We observed significant effect of fly type on realized oviposition with flies that emerged from larvae fed up to critical time point showing significantly lower oviposition ($F_{1, 2} = 54.24, p = 0.017$, Fig. 2b). However, there was no selection × fly type interaction effect ($F_{1, 2} = 7.98, p = 0.106$).

**Longevity and Survival Probability**

There is no significant effect of selection and diet curtailing post attainment of critical size on average longevity of both male and female flies. Overall CS females had significantly higher median longevity than NS flies. However, there was no significant effect of selection on median longevity. The maximum lifespan of critical-sized males was significantly altered (Additional Table S1).

Average, median and maximum longevity are descriptive statistics that can at best provide overall comparison but not compare the progression of the biological process of aging\(^{34}\). We compared the survival probabilities of the different type of flies to ascertain the progress of the biological process. There was a significant effect of selection and fly type on survival probabilities of both male and female flies (see Table 1, Fig. 2c and 2d).
Table 1
Chi square analysis using Kaplan Meier analysis with comparison of age dependent survival of males and females under selection for faster pre-adult development and extended longevity, and larval diet curtailing at critical size.

| Population & fly type | FEMALE | MALE |
|-----------------------|--------|------|
|                       | $S_{NS} & C_{NS}$ | $S_{CS} & S_{NS}$ | $C_{CS} & C_{NS}$ | $S_{NS} & C_{NS}$ | $S_{CS} & S_{NS}$ | $C_{CS} & C_{NS}$ |
| $\chi^2$              | 47.78  | 47.67 | 31.38 | 21.87 | 20.41 | 20.10 |
| $p$                   | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

Copulation latency

There was no significant effect of selection on both the male ($F_{1,2} = 1.69, p = 0.322$, Fig. 3b) and female ($F_{1,2} = 5.33, p = 0.147$, Fig. 3c) copulation latency. In addition, there was no significant effect of fly type on copulation latency of male ($F_{1,2} = 10.69, p = 0.082$, Fig. 3b) as well as female ($F_{1,2} = 0.286, p = 0.646$, Fig. 3c) flies.

Copulation duration

There was non-significant effect of selection on the male ($F_{1,2} = 6.72, p = 0.122$, Fig. 3d) and female ($F_{1,2} = 1.266, p = 0.377$, Fig. 3e) copulation duration. Further, there was no significant effect of fly type on copulation duration of male ($F_{1,2} = 4.56, p = 0.166$, Fig. 3d) and female ($F_{1,2} = 4.92, p = 0.157$, Fig. 3e) flies.

Discussion

Recently, it has been reported that glycogen metabolism is required during third larval instar for normal body size growth and even the developmental delay would not rescue the arrest of body size due to reduced glycogen levels in the larval stage\(^35\). Further, it has been evidenced that defects in glycogen metabolism are known to affect larval physiology and hence the adult fitness\(^35\). While the fat body, muscles and CNS (Central Nervous System) in larva act as the site for glycogen storage\(^36\), glycogen synthesis occurs during late larval life in fat body\(^37\) lending support to our interpretation. Furthermore, fat bodies act as a peripheral system for ecdysone metabolism\(^38\). Despite smaller larval size than controls\(^39\), the selected populations had comparable levels of glycogen (Fig. 1c) throughout the third instar suggesting that glycogen is the primary source of energy that is possibly driving the physiological processes leading to early expression and release of ecdysone in selected populations\(^39\) that might, in turn, facilitate faster development.
When we compared the trend of lipid content during larval life, it is comparable at first (48 h post-hatching of synchronized eggs), 2nd (early L3) and 3rd larval time point (Critical size time point-developmental threshold/metamorphosis commitment time point) suggesting the utilization of lipids for metabolic processes during these periods. The lower level of lipid post-critical size attainment at 4th and 5th larval time point is perhaps due to decrease in the post-critical growth duration of the selected populations\textsuperscript{13}. It is known that lipids are majorly stored in fat body to be utilized further during the pupal duration and early adult life\textsuperscript{23}. The energy equivalent (in terms of calories) of lipids are highest (Supplementary Fig. 1c) among the three macromolecules. Selected populations with comparable and energy levels till critical time point undergo early metamorphosis without compromising on pupal duration\textsuperscript{13}.

The protein levels during larval life are comparable at 1st, 2nd and 3rd time point representing the time points of higher wet weight gain. The low level at 4th and 5th larval time point in selected populations might be due to the utilization of proteins in the growth and development of imaginal discs\textsuperscript{39}. It is likely that as protein content during late larval life in selected populations catabolized for preparing the organism for metamorphosis while the control flies continue to accumulate proteins during nearly 40 hours long post-critical duration\textsuperscript{13}. For example, proteins like LSP (Larval serum protein) increases drastically in this late Larval (l3) stage, which is the most abundant protein during the post-critical duration and contributes to the major wet weight of larva\textsuperscript{40}.

The major macromolecules and energy results indicate that our selected populations have evolved mechanisms to maintain the requisite levels of molecules and energy till attainment of critical size- a time point at which the organisms commit to an irreversible process of metamorphosis. This is in agreement with Hironaka et al. who proposed that the investment value in larval tissue is more during the pre-critical period than terminal growth period (a.k.a. post-critical duration)\textsuperscript{8}. Further, supporting the view that critical size in \textit{Drosophila melanogaster} is acting as an optimal switch for energy allocation during larval life\textsuperscript{8, 9}. Furthermore, following the model for developmental threshold- with the age of maturity (metamorphosis- in the present context) and size at transition, it is likely that selection for faster pre-adult development evolved smaller developmental threshold (Critical size- in this case) through higher growth rate during second larval instar\textsuperscript{13} thus accumulating sufficient energy reserves to sustain the development without compromising their metamorphosis duration\textsuperscript{11, 13}.

Owing to short post-critical duration- a period during which most of the growth and weight increase occurs\textsuperscript{13} the selected populations have significantly reduced protein and lipid content during the mid and late-L3 stages. Although the selected populations are under the physiological trigger to complete development due to higher ecdysone levels throughout L3\textsuperscript{38} they can accumulate adequate proteins and lipids to maintain their phenotypic integrity albeit emerge as small adults\textsuperscript{13, 38}. Despite small size due to curtailed post-critical duration their copulation latency (i.e., time to attain sexual maturity) and copulation duration were unaffected supporting the claim that sexual maturity and copulation duration are species-specific traits\textsuperscript{18}. However, copulation latency and duration were affected when \textit{Drosophila melanogaster}
was starved during early adult life\textsuperscript{41}. The differences in the two findings might be due to diet restriction at different life stages ascertained in the two studies. Two studies in the past have reported decoupling of body size and copulation duration\textsuperscript{18,42}.

A developmental dietary history is known to influence adult physiology\textsuperscript{1}. In general, the trade-off between longevity and lifetime oviposition are well documented in \textit{Drosophila melanogaster}\textsuperscript{28,44–45} and other holometabolous insects like \textit{Speyeria mormonia}\textsuperscript{46}. Thus following various studies on dietary restriction, CS flies had lower oviposition (Fig. 2b). However, exception to larval dietary manipulation has also been reported. For example in holometabolous Lepidopteran butterfly, \textit{Speyeria mormonia}, there was no independent effect of semi-starvation on realized egg laying\textsuperscript{46} suggesting an indirect effect of larval dietary restriction on oviposition. In our study, conscious selection for faster pre-adult development that resulted in small-sized adults (Fig. 2e and 2f) did not affect the life-time realized oviposition (Fig. 2b) although the selected flies had significantly smaller ovaries\textsuperscript{18}. Despite being small, they had realized oviposition comparable to their ancestral controls, suggesting that they might have evolved mechanisms to maximize their fitness under restrictive growth conditions perhaps through better resource acquisition and utilization during the adult phase. Our results are in agreement with Min et al. where they reported the contribution of larval resources in early life oviposition in addition to adult diet\textsuperscript{47}. However, a recent study reported reduced reproductive fitness as a consequence of small adult size due to dietary manipulation during larval growth period\textsuperscript{48}.

Females have higher longevity under larval diet curtailing post attainment of critical size than females fed \textit{ad libitum} food during larval life. This is in sharp contrast to the study on \textit{Speyeria mormonia} where larval food restriction resulted in smaller adults and larval feeding duration affected lifespan but not realized fecundity\textsuperscript{46}. A recent study reported the non-universal nature of the trade-off between longevity and oviposition\textsuperscript{29}. However conscious curtailing of diet duration post attainment of critical size in larval life resulted in an extension of adult longevity accompanied by a reduction in lifetime oviposition (Fig. 2b-2d). Our populations that were under direct selection for faster pre-adult development and indirect selection for late reproduction have longevity extension as against populations selected for faster pre-adult development and early reproduction\textsuperscript{17}. The indirect selection pressure for late reproduction might be responsible for life span extension\textsuperscript{44} while conscious selection for faster pre-adult development for more than 134 generations has led to the evolution of smaller critical size\textsuperscript{13}. Further, early attainment of high ecdysteroids titer and large Prothoracic gland at comparable time points during larval development in selected populations might be the reason for the evolution of small adult body size and reduced developmental duration\textsuperscript{38}.

Taken together, fitness (measured as longevity and life-time realized oviposition) of our selected populations are comparable to their ancestral control thus refuting the widely accepted ‘bigger is better’ hypothesis\textsuperscript{49,50} as well as the silver spoon effect\textsuperscript{27,33}. 


Conclusion

Overall, our study provides insight into the role of (gradually evolved) critical size on adult life-history in *Drosophila melanogaster* populations. The populations that are under conscious selection for faster pre-adult development and thus under curtailed food intake have not compromised on their Darwinian fitness thus refuting the silver spoon hypothesis\(^{27}\).

Methods

Fly husbandry

Two kinds of laboratory *Drosophila melanogaster* populations were used in this study. The Control (C) populations were on 21 days egg to egg discrete generation cycle, while the Selected (S) populations were derived from the controls by direct selection for faster pre-adult development and indirect selection for extended longevity. Detailed protocols adopted in rearing and maintenance of C and S populations are explained previously\(^{13}\). Briefly, each of the three C populations were cultured in 40 vials with 6 ml standard banana-jaggery media (SM) at a density of 40-50 eggs per vial and incubated at SLC for full 12 days\(^{13}\). At the end of 12 days, all emerging flies from the 40 vials were transferred to pre-labeled plexiglass population cages and provided with fresh food every alternate day till day 18. On day 18, fresh food plate was supplemented with live yeast-acetic acid paste. Eggs for initiating the next generation were collected on day 21 from the previous egg collection day. Each of the three S populations was derived from the three C populations by collecting 160 vials of 60-80 eggs per 6 mL banana-jaggery food vial. The vials were incubated at SLC. The early emerging 15-20 flies (as ascertained by empty pupal cases) from each vial were transferred to pre-labeled population cages. Two sister cages were maintained per S population to avoid adult crowding. The S population cages too were provided with fresh food plates every alternate day till 50% mortality was noticed in any of the cages, at which point all cages were provided fresh food plates supplemented with live yeast-acetic acid paste for three days following which eggs for starting next generation were collected. The eggs obtained from the two sister cages of a given population were mixed and redistributed into 160 vials.

Originally, the Control populations (also called as JB populations) were derived from IV populations\(^{51}\) and are described in detail in Prasad et al.\(^{17}\). The selected and control populations had been through 134 and 242 generations respectively at the time of being used in this study. To remove non-genetic parental effects which might appear due to the differences in maintenance regime, both the S and C populations were run through common rearing conditions for 1 generation at a moderate density of 50 eggs per 6 mL banana-jaggery media vial and 40 vials per population before being used in this study. The egg collection from the S and C populations were staggered by their developmental time difference to synchronize the emergence of adults. All adults emerging from each of the 40 vials of a given population were transferred to pre-labeled population cage with fresh SM plate. These populations are referred to as standardized flies\(^{13, 17}\). Embryos for all the experiments were obtained from these standardized flies (SF).
Generation of Critical size adults and Normal-sized adults

Synchronized eggs were collected from SF, evenly spread on agar-agar plates and incubated at SLC. Freshly hatched larvae (~22 h post-egg-laying) were harvested using a fine camel hair brush and transferred to Petri-plates (5.5 mm diameter, Tarson) with 2000 µL of LSM (Liquid Standard Media) at a density of 30 larvae per plate\textsuperscript{13}. Twenty such plates per population were incubated at SLC. The larvae from all the 20 plates were re-harvested from LSM plates after 64 and 72 h (post-egg-lay) for S and C populations respectively, washed with RO (Reverse osmosis) water, rolled on tissue towel and randomly transferred (25 larvae per vial) to vials containing 6 mL non-nutritive agar-agar or SM and incubated at SLC\textsuperscript{13}. At every 6 hour interval, the emerging flies from these vials were collected, sorted according to their gender and held as virgins in pre-labeled holding vials with 6mL SM till use in further assays. The adults that emerged from vials containing non-nutritive agar are referred to as critical-sized (CS) adults, while those that emerged from vials containing SM are referred to as normal-sized (NS) adults.

Macromolecule quantification during larval life

Glycogen and lipid content were estimated using Van Handel's method\textsuperscript{52} and the protein content was estimated using Smith's method\textsuperscript{53} with minor modifications.

All three macromolecules were quantified for pre-critical stages (L2 to L3 transition stage- 48 h for both C and S populations; Early L3 stage-56 h and 64 h for S and C populations respectively), critical size stage (64 h and 72 h for S and C) and post-critical stages (Late L3 stages - 72 h and 104 h; before pupation L3-80 h and 112 h for S and C populations respectively) of larval life. All the sampling time intervals mentioned are from the time of transfer of newly hatched larvae to LSM plates. Assays are presumed to have been performed using synchronized larvae from standardized ies in both control and selected populations. The detailed protocols used in the estimation of the macromolecules are as follows:

1. Glycogen estimation

Five randomly chosen larvae were homogenized in 400 µL of 2% Na\textsubscript{2}SO\textsubscript{4}. 80 µL of homogenate was aliquoted into 5 mL Eppendorf tube, to which 184 µL of Na\textsubscript{2}SO\textsubscript{4} and 3736 µL of the (fresh) mixture of chloroform and methanol (1:1) was added. The tubes with the mix were centrifuged (Eppendorf, 5430R) at 14000 r.p.m. for 10 minutes at 4 °C. The supernatant was discarded and the pellet was air-dried for 10 minutes. The pellet was resuspended in 2000 µL Anthrone reagent and heated at 90 °C in water-bath for 10 min. Aliquots were kept on ice for 5 min following which absorbance was measured at 625 nm on ELISA plate reader (ECIL micro scan, MS5605A). The assay was repeated in triplicate per population and means of the triplicate measures were used in statistical analysis.

2. Protein estimation
Precipitation assay was done before quantification of protein followed by BCA method of protein quantification. Five randomly chosen larvae were homogenized in 400 µL of 2% Na₂SO₄. Then 80 µL of homogenate was aliquoted and 500 µL of 0.15% Deoxycholate was added to the aliquot. After the incubation period of 10 min on ice, 1000 µl 3M Trichloroacetic acid (TCA) was added. The aliquots were centrifuged at 8500 r.p.m. (Eppendorf, 5430R) for 15 min at 4 °C. Protein was precipitated at the base of each aliquot. Pellets were washed with HCl and air-dried. BCA reagent was added to each of the pellets, resuspended and heated in water-bath at 60 °C for 10 min. Absorbance was recorded at 562 nm using ELISA plate reader (ECIL micro scan, MS5605A). The assay was repeated in triplicate per population and means of the triplicates were used in statistical analysis.

3. **Lipid estimation**

Five randomly chosen larvae were homogenized in 200 µL of PBS (1X). 1 mL of freshly prepared methanol and chloroform mixture (1:1) was added to the homogenate and vortexed. This was followed by centrifugation at 4 °C at 4000 r.p.m. (Eppendorf, 5430R). Two phases of the solution were separated. From the lower layer of solution, 200 µL of the solution was taken in an aliquot and was evaporated completely at 90 °C, using water-bath. 50 µL of H₂SO₄ was added post evaporation and incubated at 60 °C for 10 min, cooled on ice for 5-7 min., following which 1000 µL of Vanillin reagent was added and incubated at room temperature for 30 min. Absorbance was taken at 525 nm using ELISA plate reader (ECIL micro scan, MS5605A). The assay was repeated in triplicates per population and means of triplicate were used in statistical analysis.

All the biomolecules converted to their energy equivalents and compared.

**Adult life-history traits**

1. **Copulation latency and copulation duration**

Copulation latency (the time lag between the time of emergence and initiation of copulation) and copulation duration (the time difference between initiation and termination of copulation) were ascertained by pairing freshly emerged flies Zero-day flies with 3-day old mature flies of the opposite gender.

2. **Life-time oviposition and longevity**

One day old virgins from holding vials were used in this assay. The flies were anaesthetized using CO₂ and a female and male pair were transferred to fresh vials with 3 mL SM. A total of 20 pairs per treatment were set up. Any fly that did not wake up within 1 h of transfer was replaced with a new fly of the same gender. Further, any fly that died within the first 24 h of set up was also replaced by a fresh fly. The pair of flies were transferred to fresh 3 mL SM vials every 24 hours. The eggs laid in the preceding 24 h were counted under stereo zoom microscope (Carl Zeiss Binocular stereozoom microscope, Stemi 305) and
recorded. Census records were also maintained till the death of all flies. The average life-time oviposition and life-span were estimated from this primary data (Fig. 2a).

Data analysis

In all cases except survival probability function, univariate analysis of variance, under general linear model (GLM) using SPSS v. 22 was carried out and population means were used as units of analysis with selection, larval growth stage and fly type as fixed variables and replication as a random variable\textsuperscript{55-57}. Hence, only fixed-factor effects and interactions could be tested for significance\textsuperscript{13,17}. The significance of adult survival probability curves was analyzed using Kaplan-Meier log-rank test\textsuperscript{58}.

Abbreviations

GLM- General Linear Model, SLC- Standard Laboratory Conditions, LSM- Liquid Standard Media, RO-Reverse Osmosis, CS- Critical size, NS- Normal Size, S –Selected, C-Control, LSP- Larval Serum Protein, CNS- Central Nervous System

Declarations

Acknowledgement

We thank Council for Scientific and Industrial Research, Government of India and University of Delhi for financial support.

Authors’ contribution

KS and MS conceived the study, designed the experiments, analysed the data and interpreted the results; KS performed the experiments and wrote the first draft of the manuscript, and MS finalised the manuscript. Authors read and approved the final manuscript.

Funding

KS was supported by Council for Scientific and Industrial Research [CSIR-Grant number: 37(1495/11/EMR-II)] for doctoral work. There was no role of funding bodies in the design of experiments, data analysis and interpretation of results, and further writing of the manuscript.

Availability of data and materials

Following is the link for review:
Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interest
Authors declare no competing interests.

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