Title

Effects of caffeine on mating behavior and sperm precedence in *Tribolium castaneum*

Short running title

Caffeine, mating and sperm precedence

Authors

JI YUHAO[1],

YUKI RYUJI[1],

KENTAROU MATSUMURA[2],

TAKAHISA MIYATAKE[1*]

Address

1 Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan

2 Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki, Kagawa 761-0795, Japan

Correspondence

Takahisa Miyatake

E-mail: miyatake@okayama-u.ac.jp

Funding information

This work was supported by a grant from the Japan Society for the Promotion of

Science KAKENHI 18H02510 to TM.

Abstract

Biogenic amines such as dopamine are physiologically neuroactive substances that affect behavioral and physiological traits in invertebrates, and it has long been known that these substances affect mating behavior in insects. Caffeine is a dopamine activator and thus enhances dopamine receptor activity. However, the effects of caffeine intake on insect mating behavior have been largely unexplored. Therefore, we examined the effect of caffeine on mating behavior in the red flour beetle *Tribolium castaneum*. Caffeine, which activates dopamine, affected the mating behavior of *T. castaneum* males. Males who orally ingested caffeine courted faster than males who did not, resulting in faster mounting of females and less time to a male's external aedeagus protrusion. However, the present results showed no difference in sperm precedence measured as a P2 value between males fed caffeine and males not fed caffeine. We discuss the effects of caffeine on insect mating and the possibility that caffeine consumption may cause males to mate with more females in the laboratory.

KEYWORDS

red flour beetle, mating latency, mounting behavior, number of mating, reproductive success, sperm competition.

1. INTRODUCTION

Males evolve a variety of mating tactics to increase breeding success, and a number of ultimate factors related to fertilization success have been identified (e.g., Blum & Blum, 1979; Thornhill & Alcock, 1983; Choe & Crespi, 2008). Biogenic amines are bioactive substances that influence mating behavior and physiological traits in vertebrates and invertebrates (Evans, 1980; Kravitz, 1988). For example, genetic and pharmacological approaches have shown that increasing dopamine levels in the brain increase the tendency of males to court other males in *Drosophila* species (Liu et al., 2008).

Caffeine is a dopamine activator and thus enhances dopamine receptor activity (Fredholm *et al*. 1999). The activating effect of caffeine on dopamine is well known in vertebrates (Garrett and Griffiths, 1997). In invertebrates, Ho and Sehgal (2005) showed that caffeine affects sleep in *Drosophila melanogaster*. In a study using *Apis mellifera*, when workers were fed caffeine-laden pollen, the bees' cognitive and memory skills increased (Couvillon et al., 2015).

The red flour beetle *Tribolium castaneum* (Herbst) is easy to rear, and its mating behavior is well known (e.g., Fedina & Lewis, 2008; Pai and Bernasconi 2008; Michalczyk et al. 2010; Boukouvala et al. 2019; Pai & Yan, 2020). In addition, adults of *T. castaneum* have been found to shorten their duration of death feigning when fed caffeine (Nishi et al., 2010). In the case of a closely related species, *Tribolium confusum*, caffeine increased walking activity and decreased the duration of death feigning (Nakayama et al., 2012). Thus, although caffeine has been shown to affect insect movement or activity, its effects on mating behavior have not been investigated in *Tribolium* species.

Therefore, in the present study, we aimed to study the effects of caffeine on mating behavior and sperm precedence in *T. castaneum*. At first, we compared mating behaviors of males exposed and unexposed to caffeine. The male mating behavior of this species was measured as courtship time, time until the male successfully mounted the female, time until the male's external aedeagus protruded while mounted, time from the male's external genital protrusion to separation of mating pairs, and number of copulatory trials. We hypothesized that caffeine intake in males would shorten the duration of courtship and increase the number of copulatory trials and the duration of the mating process.

Next, we tested whether oral ingestion of caffeine by male adults of *T. castaneum* altered their sperm precedence when measured as a P2 value. If caffeine intake increases the duration of mating attempt, it may lead to increased sperm precedence measured as a P2 value compared to that in males without caffeine and thus contribute to their fitness. In insects, males generally pass more sperm to females as mating duration increases (e.g., Thornhil,l 1976; Simmons, 2001). Variation in courtship behavior may also affect fitness in males through sperm competition (Blum and Blum, 1979; Birkhead & Møller, 1998; Simmons, 2001). Therefore, we also compared the P2 value and the number of progeny of *T. castaneum* who ingested caffeine and those who did not.

2. MATERIALS AND METHODS

2.1 Insects

The *T. castaneum* beetle culture used in this study has been maintained in the laboratory for more than 40 years according to the rearing method described by Suzuki & Nakakita (1991). This lineage (normal) is the same as what was called the base population in some previous studies (e.g., Miyatake et al. 2004, 2008). The beetles were reared in plastic Petri dishes (53 mm in diameter and 14 mm in height) with a mixture of wholemeal (Graham flour, Nisshin Seifun

Group, Tokyo, Japan) enriched with 5% brewer's yeast (Asahi Beer, Tokyo, Japan) as the rearing medium and kept in an incubator (MIR-154, PHC Holdings Co., Tokyo, Japan) at 25°C and a 60% relative humidity (RH) under a photoperiod of 16:8 (L:D) h (lights on at 0700h, lights off at 2300h). Pupae were sexed based on the shape of the abdominal end (Park, 1934; Sokoloff, 1974). Three weeks after hatching, adults were used for the following experiments.

2.2 Treatments

The oral administration of caffeine was performed according to the method of Nishi et al. (2010). Cotton saturated with a 2% or 5% solution of caffeine with sucrose was provided to *T. castaneum* beetles. As a control, cotton saturated with only sucrose was provided. That is, there were three treatments: 0% (control), 2%, and 5% of caffeine. We placed a few virgin males near the cotton for each treatment. We selected a beetle that had voluntarily stuck its mouth parts into the cotton saturated with caffeine and/or sucrose for more than 10 min, and 2 h later, each male was placed in a well of a 48-well plate (Cell Star, Greiner Bio-One, Kremsmünster, Austria). Each beetle was allowed to acclimate in the wells for 5 min before the experiment. This procedure was repeated for 30 males for each treatment, between 1000 and 1900 h under light within 60 d in a room maintained at 25±2°C.

2.3 Mating behavior

As described in the experiment by Nishi et al. (2010), 2 h after ingesting caffeine or sucrose and then after another 5 min to acclimate in the well for each male, a virgin female was placed in each well, and mating behavior was recorded with a video camera (HDR-PJ590V, Sony, Tokyo, Japan) fixed over the Petri dish on a tripod. Each pair was recorded separately, using a video camera. Recording was performed for 30 min in an insect-rearing room adjusted to 25±2°C. We recorded mating behavior, one pair at a time, between 1100 and 1900 h under light.

We played back the video that we shot and extracted the following data from it: (1) time to initiate courtship, (2) time to mounting, (3) time to protrusion of the male's external aedeagus while mounted, (4) time from the male's external genital protrusion to separation of mating pairs, and (5) the number of times the process from (3) to (4) was repeated in a 30-minute period (=the number of copulation trails).

2.4 Sperm precedence

In the present study, we measured the P2 value, an index of sperm precedence, of males fed caffeine or sucrose solution, respectively. Because the paternity success of *T. castaneum* is often dominated by the last mating male (Fedina & Lewis 2008), we considered that the effects of caffeine on the paternity success can be examined by the P2 value. We used males of a mutant strain that is homozygous for an autosomal, semidominant black body color allele (black males); this phenotype is frequently used as a marker in sperm competition studies in *T. castaneum* (Fedina and Lewis, 2006; Matsumura et al., 2019). One female and one black male were placed in a plastic Petri dishes (30 mm in diameter, 14 mm in depth) lined with filter paper and observed for 24 h to see if they mated. After 24 h, black males were removed from the Petri dishes, and a normal male marked with a white marker that had been orally ingested a 2% caffeine-sucrose solution was allowed to mate with the female (normal) for 24 h. Because the black color is a semi-dominant type, black progeny will emerge when eggs were fertilized with sperm from black males, whereas progeny of normal color will emerge when eggs were fertilized with sperm from normal (caffeine- or sucrose-fed) males. After 24 h, the *T. castaneum* female was removed, placed in a plastic Petri dish containing food, and allowed to lay eggs for 10 d.

We performed these procedures using 35 caffeine-consuming males and 35 sucrose-consuming males, respectively (i.e., n = 70). After approximately 50 d, the number and color of adults that emerged (= offspring) in each Petri dish were recorded. Paternity was calculated by the proportion of progeny of normal color in all offspring (Matsumura et al. 2019; Matsumura and Miyatake 2019). Because 16 pairs (9 pairs in the caffeine treatment, 7 pairs in the sucrose treatment) did not produce fertilized eggs, we removed those data from the statistical analysis. Finally, therefore, we could analyzed 54 pairs.

2.5 Statistics

For comparisons of behavioral traits, the Dunn's post-hoc multiple comparison after the Kruskal-Wallis test was used at the P = 0.05 level of significance. JMP version 12.2 (SAS Institute Inc., 2015) statistical software was used for these analyses.

Effects of caffeine and sucrose (i.e., the negative control) on the paternity success and the number of progeny of normal color were tested by a generalized linear model (GLM) with a binomial distribution and logistics function. To test the effects of treatment on the paternity success and the number of progeny of normal color, we used a GLM with a Poisson distribution and log link function. These analyses were conducted in R version 3.4.3 (R Core Team, 2017).

3 RESULTS

Males who consumed caffeine orally had a significantly shorter time to courtship

(Figure 1A, time to courtship: *d.f.* = 2, χ^2 = 13.66, $P < 0.01$) and time to mounting (Figure 1B, time to mount: *d.f.* = 2, χ^2 = 14.77, $P < 0.01$) and a significantly shorter time to the male's external aedeagus protrusion while mounted (Figure 1C, time to mating, *d.f.* = 2, χ^2 = 18.47, $P < 0.01$) than males who did not consume caffeine. There was no difference in these behaviors between the 2% and 5% caffeine intake treatments. The time from male's external genital protrusion to separation of mating pairs was unaffected by oral caffeine intake (Figure 1D, duration from male's external genital protrusion to separation of mating pairs: *d.f.* = 2, χ^2 = 2.62, $P = 0.27$). Males who orally consumed caffeine (2%) mated significantly more times in the process from (C) to (D) than males who did not consume caffeine (Figure 1E, number of matings: *d.f.* = 2, χ^2 = 9.92, $P < 0.01$). There was no difference in the length of the process from (C) to (D) between the 2% and 5% caffeine intake treatments.

Whether caffeine was given to males did not affect the paternity or the number of progeny of normal color (Figure 2). The results of GLMs for paternity success of males and the number of progeny of normal color oviposited by females mated with males exposed and unexposed to caffeine are as follows. There were no significant differences in paternity success (Treatment: *d.f.* = 1, χ^2 = 0.70, $p = 0.42$, Error: *d.f.* = 52), and in the number of progenies of normal color (Treatment: *d.f.* = 1, χ^2 = 0.38, $p = 0.54$, Error: *d.f.* = 52).

4 DISCUSSION

Caffeine, which activates dopamine, affected the mating behavior of *T. castaneum* males (Figure 1). Males who ingested caffeine orally courted faster than males who did not, resulting in faster mounting of females and earlier time to male's external aedeagus protrusion while mounted. In previous studies, it was reported that individuals who ingested caffeine orally had a shorter death-feigning duration in *T. castaneum* (Nishi et al., 2010) and the closely related species *T. confusum* (Nakayama et al., 2012). In addition, the caffeinated adult of *T. confusum* displayed a greater locomotor activity in 30 minutes than the males and females who did not ingest caffeine (Nakayama et al., 2012). Therefore, caffeine may activate the movement of *Tribolium* beetles. The present results suggest that males who ingest caffeine were able to find females earlier, resulting in earlier courtship and mounting as a result of being more mobile on the Petri dish.

We used caffeinated males in this experiment but did not measure how much caffeine each male ingested. Therefore, we need to investigate the effects of caffeine on mating behavior by injecting a fixed amount of caffeine into adults of *T. castaneum*.

As a result of artificial selection on locomotion measured as walking distance in *T. castaneum* (Matsumura et al., 2019), males with a longer walking distance were more successful in mating with females in a given period of time than males with a shorter walking distance. That result should relate to the results of the present experiment. On the other hand, *T. castaneum* males who were artificially selected for short walking distance ejaculated more competitive sperm into females than did males of strains with a higher walking distance (Matsumura et al., 2019). This result showed an association between mating success, which is a prefertilization source of sexual selection, and fertilization success, which is a postfertilization source of sexual selection (Matsumura et al., 2019), but no difference in sperm precedence was detected between males fed caffeine and males not fed caffeine. An outcome of the previous study (Matsumura et al. 2019), which showed that males with lower moving ability increased mating duration, would have something to do with the result in which males ejaculated more into the females (Matsumura et al. 2019). The fact that caffeine intake had no effect on duration from a male's external genital protrusion to separation of mating pairs may be consistent with the lack of a caffeine effect on the number of sperm released into the female.

In this experiment on sperm precedence, only the sperm precedence of males mated only once was considered. However, we found that males who consumed caffeine orally were more likely to mate with females in a given time period than males who did not consume caffeine (Figure 1). This indicates that males fed caffeine are able to mate with many more females than males not fed caffeine, strongly suggesting that caffeine intake increases the fitness (reproductive success) of males. In the future, it will be necessary to test whether males fed caffeine have higher fitness than males not fed caffeine by allowing mating with multiple females, as predicted in this study.

The present study revealed the effect of caffeine on the mating behavior of male *T. castaneum*. However, the effect of caffeine on mating traits including the remating receptivity of females is unknown. Therefore, it would be interesting to assess how feeding females caffeine affects their mating traits in the future.

In summary, *T. castaneum* males who ingested caffeine orally (1) courted females sooner, (2) mounted and protruded more quickly his external genital aedeagus, and (3) repeated more often per time period than males who did not ingest caffeine. However, there was no evidence that caffeinated males had an advantage in terms of sperm precedence measured as a P2 value. These findings indicate that oral consumption of caffeine altered the mating behavior of *T.*

castaneum males but did not affect sperm precedence.

CONFLICT OF INTEREST

The authors declare no conflicts of interest for this study.

REFERENCES

Birkhead, T.A., & Møller, A.P. (1998). Sperm Competition and Sexual Selection. Academic Press, Oxford.

Boukouvala, M.C., Romato, D., Kavallieratos, N.G., Athanassiou, C.G., Stefanini, C., Canale, A., & Benelli, G. (2019). Asymmetric courtship boosts male mating success in the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Journal of Stored Products Research*, 81, 1-6.

Blum, M.S., & Blum, N.A (1979). Sexual Selection and Reproductive Competition in Insects. Academic Press, Oxford.

Choe, J.C., & Crespi, B.J. (1997). The Evolution of Mating Systems in Insects and Arachnids. Cambridge University Press, Cambridge.

Couvillon, M.J., Toufailia, H.A., Butterfield, T.M., Schrell, F., Ratnieks, F.L.W., & Schürch, R. (2015). Caffeinated forage tricks honeybees into increasing foraging and recruitment behaviors. *Current Biology, 25* (2), 2815–2818.

Evans, P.D., (1980). Biogenic amines in the insect nervous system. *Advances in Insect Physiology, 15*, 317–473.

Fedina, T.Y., & Lewis, S.M. (2006). Proximal traits and mechanisms for biasing paternity in the red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Behavioral Ecology and Sociobiology 60*, 844-853.

Fedina, T.Y., & Lewis, S.M. (2008). An integrative view of sexual selection in *Tribolium* flour beetles. *Biological Reviews*, *83* (2), 151-171.

Fredholm, B.B., Bättig, K., Holmé n, J., Nehlig, A., & Zvartau, E.Z. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacological Reviews, 51*, 83–133.

Garrett, B.E., & Griffiths, R.R. (1997). The role of dopamine in the behavioral effects of caffeine in animals and humans. *Pharmacology Biochemistry and Behavior*, *57* (3), 533–541.

Ho, K.S., & Sehgal, A., (2005). *Drosophila melanogaster*: an insect model for fundamental studies of sleep. *Methods in Enzymology, 393*, 772–793.

Kravitz, E.A. (1988). Hormonal control of behavior: amines and the biasing of behavioral output in lobsters. *Science 241* (4874), 1775–1781.

Liu, T., Dartevelle, L., Yuan, C., Wei, H., Wang, Y., Ferveur, J. & Guo, A. (2008). Increased dopamine level enhances male-male courtship in *Drosophila*. *The Journal of Neuroscience*, *28* (21), 5539 -5546.

Matsumura, K., Hosken, D.J, Archer, C.R., & Miyatake, T. (2019). Artificial selection on walking distance suggests a mobility-sperm competitiveness trade-off. *Behavioral Ecology, 30* (6), 1522-1529.

Michalczyk, L., Millard, A.L., Martin, O.Y., Lumley, A.J., Emerson, B.C., & Gage, M.J.G. (2010). Experimental evolution exposes female and male responses to sexual selection and conflict in *Tribolium castaneum*. *Evolution*, 65, 713-724.

Miyatake, T., Katayama, K., Takeda, Y., Nakashima, A., Sugita, A., & Mizumoto, M. (2004). Is death-feigning adaptive? Heritable variation in fitness difference of death-feigning behaviour. *Proceedings of the Royal Society London Series B, 271* (1554), 2293-2296.

Miyatake, T., Tabuchi. K., Sasaki, K., Okada, K., Katayama, K., & Moriya, S. (2008). Pleiotropic antipredator strategies, fleeing and feigning death, correlated with dopamine levels in *Tribolium castaneum*. *Animal Behaviour, 75* (1), 113-121.

Nakayama, S., Sasaki, K., Matsumura, K., Lewis, Z., & Miyatake, T. (2012). Dopaminergic system as the mechanism underlying personality in a beetle. *Journal of Insect Physiology*, *58*, 750-755.

Nishi, Y., Sasaki, K., & Miyatake, T. (2010). Biogenic amines, caffeine and tonic immobility in *Tribolium castaneum*. *Journal of Insect Physiology 56* (5), 622–628.

Pai, A., Bernasconi, G. (2008). Polyandry and female control: the red flour beetle *Tribolium castaneum* as a case study. *Journal of Experimental Zoology*, 310B, 148-159.

Pai, A., Yan, G. (2020). Long-term study of female multiple mating indicates direct benefits in *Tribolium castaneum*. *Entomologia Experimentalis et Applicata*, 168, 398-406.

Park, T. (1934). Observations on the general biology of the flour beetle, *Tribolium confusum*. *Quarterly Review of Biology*, *9*, 36-54.

R Core Team (2017). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org/

SAS Institute Inc. (2015). JMP 12.2.0. SAS Institute Inc., Cary

Simmons, L.W. (2001). Sperm Competition and Its Evolutionary Consequences in the Insects. Princeton University Press.

Sokoloff, A. (1974). The biology of *Tribolium*: with special emphasis on genetic aspects. Volume 2. Oxford University Press, London.

Suzuki, T. & Nakakita, H. (1991). *Tribolium castaneum* (HERBEST), *T. confusum* J. du V., *T. freemani* HINTON. In Rearing methods of insects (ed. K. Yushima, S. Kamano & Y. Tamaki), pp. 251–254. Tokyo: Nihon Shokubutsu-Boueki Kyokai. [In Japanese].

Thornhill, R. (1976). Sexual selection and paternal investment in insects. The *American Naturalist 110*, 153-163.

Thornhill, R. & Alcock, J. (1983). Evolution of Insect Mating Systems. Evolution of Insect Mating Systems, Harvard University Press, Cambridge.

Legends of Figures

Figure 1. Comparison of mating behavior of males without caffeine (0%) and after ingesting a 2% or 5% solution of caffeine. (A) time to initiate courtship (sec.), (B) time to mount (sec.), (C) time to male's external genital protrusion (sec.) , (D) duration from male's external genital protrusion to separation of mating pairs (sec.), and (E) the number of times the process from (3) to (4) was repeated per 30 min. All the experiments were performed with 30 pairs of males and females. The numerals in brackets below the table show the number of each behavior observed. Different letters in each graph show significant differences ($p < 0.05$). The box-beard diagram shows the median and quartiles.

Figure 2. Effects of caffeine and sucrose (i.e., negative control) on paternity success (A) and the number of progeny of normal color (B), respectively. Error bars show the standard error.

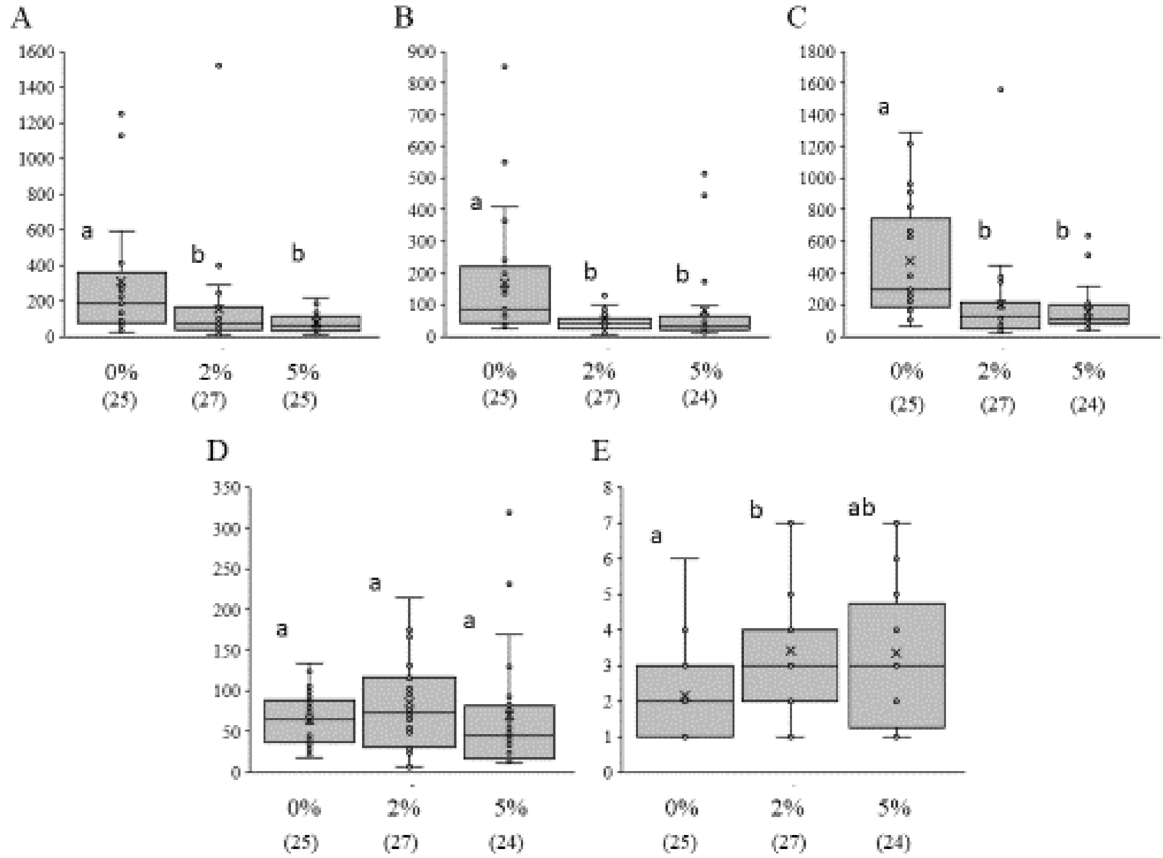
A
1600
1400
1200
1000
800
600
400
200
0
a
b
b
0%
(25)
2%
(27)
5%
(25)
B
900
800
700
600
500
400
300
200
100
0
a
b
b
0%
(25)
2%
(27)
5%
(24)
C
1800
1600
1400
1200
1000
800
600
400
200
0
a
b
b
0%
(25)
2%
(27)
5%
(24)
D
350
300
250
200
150
100
50
0
a
a
a
0%
(25)
2%
(27)
5%
(24)
E
8
7
6
5
4
3
2
1
0
a
b
ab
0%
(25)
2%
(27)
5%
(24)

Figure 1

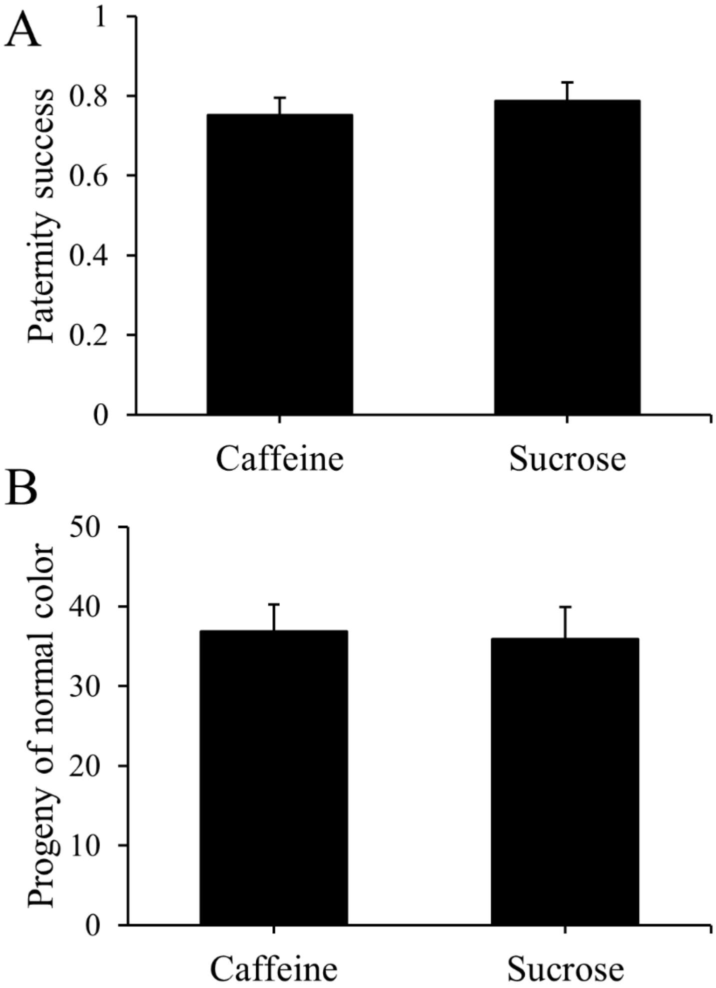
A
1
0.8
0.6
0.4
0.2
0
Paternity success
Caffeine
Sucrose
B
50
40
30
20
10
0
Progeny of normal color
Caffeine
Sucrose

Figure 2