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The impact of cold storage on mating behavior of emerging adults and progeny fitness, in a parasitoid wasp, *Lysiphlebus fabarum*, under constant and fluctuating temperatures

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Abstract

Cold storage is a valuable method for reducing the cost of producing large amounts of biological agents. Fluctuating thermal regimes (prolonged exposures at low temperatures combined with periodic short higher temperature) are reported to improve cold survival and fecundity in some parasitoid species. In this study, the pupal stage of the parasitoid wasp *Lysiphlebus fabarum* (Marshall) (Braconidae: Aphidiinae), within mummies of *Aphis fabae* were stored for a period of two weeks at 6±1°C (50–60% RH, L14:D10 photoperiod), and the mating behavior of emerging adults and fitness of their progeny were compared under fluctuating thermal regime (2 h daily at 21°C) versus constant low temperature. Results revealed that the males emerging from fluctuating temperature regime, in contrast to constant temperature treatment, were not significantly different from the control males in odour recognition, walking speed, and number of mating success. Moreover, the fitness of the first generation progenies (percent parasitism, percent adult emergence and sex ratio) was not influenced by cold storage under fluctuating temperature treatment. Our results confirmed that cold storage of *L. fabarum* pupae under fluctuating regime is a protection method when compared to constant low temperature exposure.

Key words: *Aphis fabae*, Aphidiidae, mating behavior, odour recognition.

اثر ذخیره‌سازی در سرما روی رفتار جفت‌گیری حشرات بالغ نوظهور و شایستگی نتاج در زنبور پارازیتوئید *Lysiphlebus fabarum* در شرایط دمایی ثابت و نوسان‌دار

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چکیده

ذخیره‌سازی در سرما یک روش ارزشمند برای کاهش هزینه‌ها در تولید انبوه عوامل بیولوژیک می‌باشد. در بعضی از زنبورهای پارازیتوئید، رژیم دمایی نوسان‌دار (مواجهه‌ی طولانی با دمای پایین در ترکیب با دوره‌های کوتاه در دماهای بالاتر)، منجر به بهبود بقاء و باروری می‌شود. در این مطالعه شفیره‌های زنبور پارازیتوئید *Lysiphlebus fabarum* (Marshall) (Braconidae: Aphidiinae) روی شته سیاه باقلا، *Aphis fabae* به مدت دو هفته در شرایط سرمای شش درجه‌ی سلسیوس (رطوبت نسبی ۶۰-۵۰ درصد و دوره نوری ۱۴ ساعت روشنایی و ۱۰ ساعت تاریکی)، ذخیره‌سازی شدند تا رفتار جفت‌گیری حشرات بالغ نوظهور و همچنین شایستگی نتاج آن‌ها در دو شرایط دمایی نوسان‌دار (روزانه دو ساعت در دمای ۲۱ درجه سلسیوس) و ثابت مقایسه شود. نتایج نشان داد که حشرات نر ظاهر شده در شرایط دمایی نوسان‌دار برخلاف تیمار دمایی ثابت از نظر شناسایی بو، سرعت راه رفتن و جفت‌گیری موفق، تفاوت معنی‌داری با حشرات نر شاهد نداشت. همچنین نگهداری در سرما در شرایط نوسان‌دار تاثیری روی شایستگی نتاج نسل اول (درصد پارازیتیسم، درصد ظهور حشرات کامل و نسبت جنسی) نداشت. در مجموع نتایج نشان داد که نگهداری شفیره‌های زنبور پارازیتوئید *L. fabarum* در شرایط دمایی نوسان‌دار یک روش حفاظتی مؤثر محسوب می‌شود.

واژه‌های کلیدی: *Aphis fabae*، Aphidiidae، رفتار جفتگیری، شناسایی بو.

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Introduction

Cold storage of parasitoids is used extensively before mass release, to produce simultaneously a large amount of the desired developmental stage (Leopold *et al*., 1998; Ismail *et al*., 2010), during pest outbreaks (McDonald and Kok, 1990). This is also a valuable method for reducing the cost of biological control by increasing its flexibility and efficiency by prolonging insect development time (Colinet and Boivin, 2011; Bourdais *et al*., 2012).

In various species of parasitic wasp, the detrimental consequences of prolonged cold storage have been documented, for example on the rate of mortality (Langer and Hance, 2000; Colinet *et al*., 2006a), life history traits such as longevity (Rundle *et al*., 2004; Colinet *et al*., 2006b; Amice *et al*., 2008), fecundity (Pitcher *et al*., 2002), sex ratio (Ismail *et al*., 2010; Levie *et al*., 2005), sperm production (Lacoume *et al*., 2007), female and male sterility (Levie *et al*., 2005; Amice *et al*., 2008), flight ability (Couillien and Gregoire, 1994), morphology of antennae and wings (Bourdais *et al*., 2006; Amice *et al*., 2008; Colinet and Hance, 2009), as well antenna asymmetry (Bourdais *et al*., 2006). For this reason, a cold storage protocol is expected to be designed to reduce the negative effects of these chill injuries (Chen *et al*., 2008). In this respect, a protocol should determine suitable conditions of storage including optimal temperature, the type of temperature regime, duration of cold exposure, and also the storage stage.

There are some reports about the positive effects of fluctuating temperature regimes in contrast to constant low temperature on reducing child mortality (Renault *et al*., 2004; Colinet *et al*., 2007a), female longevity and the sex ratio of the progeny (Ismail *et al*., 2010), survival and reproductive potential of males (Colinet and Hance, 2009). Subsequently, the use of fluctuating thermal regime instead of constant condition has been proven to be beneficial in many biological features in different Aphidiinae species (Colinet and Hance, 2009), but limited information is available on F_1 progeny fitness (percent parasitism, percent adult emergence and sex ratio) after cold storage of their parents under fluctuating or constant thermal regimes. Indeed, this study is part of a program in order to determine a cold storage protocol of *Lysiphlebus fabarum* (Hym., Braconidae, Aphidiinae) as a biological control agent in field and greenhouses. *L. fabarum* attacks more than 70 aphid species (Stary, 1986), especially on black bean aphid, *Aphis fabae* (Hemiptera: Aphididae), and is considered as the most important aphid parasitoids in northern Iran (Stary *et al*., 2000) and central Europe (Volkl, 1992). While sexual (arrhenotokous) (Mossadegh *et al*., 2011) and asexual (thelytokous) (Rasekh *et al*., 2011) strains of *L. fabarum* have been reported in Iran, the former strain is used in this study.

The aim of this study was to determine the impact of cold storage on behavioral consequences of emerging adults and fitness of their progeny under fluctuating and constant thermal regimes. In fact, this investigation continues and completes our previous work, which has been performed on morphological and reproduction features of adult wasps when late instar larvae or pupae exposed to 6.0 or 8.0°C, for various periods (Mahi *et al*., 2014).

Materials and methods

Insect rearing: The black bean aphid, *A. fabae* Scopoli (Hemiptera: Aphididae), and the mummies of the parasitoid wasp, *L. fabarum* were obtained from bean fields in Khuzestan province (31°19′N, 48°41′E), Iran, in spring 2012. Stock colony of host aphids and parasitoids, was established on potted broad bean, *Vicia faba* L. (Fabaceae), in climate-controlled growth chambers at 21±1°C, 65–75% RH, and L16:D8 photoperiod.

Parasitoid used in cold storage experiment was obtained from a single generation. For this purpose, excised broad bean shoots were each infested with 100 adult aphids, in a plastic cylinder (8.0 cm × 15.0 cm). After 12 hours, the adult aphids were removed and the nymphs left *in situ* to develop to the second instar (54±6 h). The results of previous experiment indicated that the storage of pupae for two weeks at 6°C under fluctuating (PF_2) or constant temperature regimes (PC_2) yielded the highest survival rate, sex ratio, egg size as well as the largest adults in terms of hind tibia (Mahi *et al*., 2014). So, these treatments were recruited to be evaluated the aspects of mating behavior and fitness of progenies.

Based on our previous studies, second instar is the most suitable stage for parasitism by *L. fabarum* (Mohammadi *et al*., 2016). To produce synchronous wasp cohorts, 30 of two-days old mated females were introduced into each excised bean shoots bearing ca. 200 second instar aphids, in a ventilated plastic cylinders (20.0 × 8.0 cm). These emerging females have been maintaining in mixed-sex (1:1 sex ratio) in the plastic cylinders and supplied with diluted honey (30%) and water on cotton rolls. After 10 h, wasps were removed and the parasitized aphids were left *in situ* to reach appreciate developmental stage (newly formed pupae in mummies = 144±6 h post-parasitism). The mummies were transferred to cooling conditions at 6°C and 50–60% RH. In storage, all insects were held in a 10:14 L:D photoperiod to correspond to late-season conditions that would be consistent with cold weather. At this storage temperature, parasitoids were subjected for two weeks to one of two further treatments, either a constant temperature regime, or fluctuating temperature regime (a daily exposure to 21±1ºC for two hours). In control treatment, the mummies developed directly at 21±1ºC. After cold storage periods, these immature stages were transferred back to 21ºC to emerge the adult wasps. In all subsequent experiments, virgin males and females were used in 8±2 h after emergence.

Aspects of mating behavior

1. Recognition of females' odour and walking speed toward them: The ability of males to detect virgin control females and direct motion toward them was tested using a wind tunnel (30 cm length × 1 cm diameter). Wind flow (1 m/s) was generated by a fan, to transfer the smell of females to virgin males from different treatments (PF_2, PC_2 and control, with the number of 25 replicates). With continuous observation of each male, the time spent to get males to females was determined. The males that did not travel the tube length for 10 minutes were considered as non-responding and were excluded. The experiment was performed in a growth chamber (21±1°C, 65–75% RH), and homogenous illumination of 450 lux. Data from this test were abnormally distributed, so the nonparametric Kruskal-Wallis H and Mann-Whiney U test were used.

2. Competitive ability in mating: Competitive mating ability of each treated male (PF_2 or PC_2) was assayed though competition with a control virgin male, in a Petri dish (8.0 cm diameter × 1.0 cm height), while both males had access to a control virgin female. Continuous observation was performed to determine which male successfully mated with the female. Moreover, pre-mating and copulation periods were recorded in all replicates (n = 25). Differences in the frequency of mating success were analyzed using a χ^2 goodness of fit test, based on the probability of each mating (50% treated male, 50% control male).

3. Post storage fitness of progeny: In this experiment, 8 hours old (±2 h) males from different treatments (PF_2, PC_2 or control; n = 20) were singly transferred into a ventilated plastic cylinders (20.0 x 8.0 cm) containing a virgin female coming from different treatments (PF_2, PC_2 or control), and also an excised bean shoot bearing 35 second-instar aphids. After 24 h, parasitoids were removed and aphids were reared (in a growth chamber at 21°C) to determine the number of mummies formed, percentage of adult emergence and sex ratio of progenies. Data from progeny fitness were analyzed using a two-way ANOVA with paternal and maternal effects as independent fixed factor. Data for parasitism rate, emergence rate and sex ratio were arcsine-transformed before analysis. SPSS software (SPSS, 2007) was used for statistical analysis and also the figures were drawn by Microsoft Excel 2003.

Results and Discussion

1. Recognition of females and walking speed toward them: Cold storing mummies of *L. fabarum* under constant regimes, made detrimental effects on the movement ability of male wasps toward females (120±21.4 sec.) relative to control males (55.9±2.4 sec.) ($U_{1,38}$= 33.0; P<0.001) and males treated under fluctuating regime (56.3±2 sec.) ($U_{1,38}$= 35.0; P<0.001). There was no significant difference between control and fluctuating temperature treatments males ($U_{1,38}$= 198.0; P= 0.968). The results revealed that cold storage of males under fluctuating temperature treatment (PF_2) did not affect odour recognition and their walking toward females. Other studies also confirm the positive effects of fluctuating temperature regime on the mobility of males (Tezze and Botto, 2004; Colinet and Hance, 2009), and their capacity to detect female odours (Herard *et al*., 1988). It seems that in addition to a physiological recovery in fluctuating regime (Colinet *et al*., 2007b), exposure to constant low temperature can reduce the running speed of males by physiologically damaging the neuromuscular system (Yocum *et al*., 1994).

2. Competitive ability in mating: There was no significant difference in pre-mating period of control female with fluctuating male ($F_{1,23}$= 0.01, P= 0.92) or constant male ($F_{1,23}$= 0.11, P= 0.74), compared to pre-mating period of a pair of control (Figure 1a).

The copulation period of males treated under fluctuating temperature regime with control females was not significantly different compared with control treatment ($U_{1,23}$= 0.74, P= 0.89). Whereas, copulation period of males stored under constant regime was significantly lower than control males ($F_{1,23}$= 17.0, P= 0.04) (Figure 1b).

The number of mating success in males treated under fluctuating temperature regime was not significantly different compared with control males (χ^2= 0.36, P= 0.55). Whereas, storage of males under constant regime, resulted in weaker individuals, in competition with control males (χ^2 = 4.84, P= 0.03) (Figure 1c).

In connection to acceptance rate of males, results showed that more males of appropriate treatment (PF_2) were accepted by control females, relative to males stored under constant temperature regime. The effects of cold stress on mating success have been previously reported (Shreve *et al*., 2004). It seems that behavioral alterations (performance of proper courtship behavior) (Mackauer, 1969), changes in the shape of antennae (Bourdais *et al*., 2006; Amice *et al*., 2008), and the difference in the size of rival males (the larger males of appropriate treatment) had impact on acceptance rate.

3. Progeny fitness:

3.1. Parasitism and emergence rate: The main effects of maternal, parental and their interaction term were all significant (Table 1). When control or fluctuating females were paired with control males, they significantly mummified more from a pair of constant female and control male (Both P<0.001), with no significant difference from each other (P= 0.171). Similarly, mating of fluctuating males with control females or fluctuating females resulted in higher parasitism rate than a pair of fluctuating male and constant female (Both P<0.001), with no significant difference from each other (P= 0.535). Interestingly, constant pairs produced more mummies than a pair of constant male with control female (P<0.001) or fluctuating female (P<0.001) (Figure 2a). When the control female was paired with control male or fluctuating male, with no significant difference from each other (P= 0.249), had a significant difference from constant male (Both P<0.001). This trend was also happened for fluctuating females when were paired with different males (Figure 2a).

Cold storage is known to reduce of fecundity, such as cold-stressed females parasitized fewer aphids than control ones (Bourdais *et al*., 2012). Base on our results, constant regime can reach the level of sterility where females do lay significantly less eggs than fluctuating or control females. There are several examples where female parasitoids sterility has been reported (Archer and Eikenbary, 1973; Foerster *et al*., 2004), although, it can also be due to a reduced capacity to recognize hosts' suability (Bourdais *et al*., 2012). The male sterility has been also proven in our study, when males were exposed to constant temperature regime, in contrast to fluctuating condition. Decrease in maturation rate of oocytes or malformation of ovarioles can be the reason of female sterility (Colinet and Boivin, 2011). Cold stress may cause alteration of reproductive organs or sperm production (Lacoume *et al*., 2007), which ends to male sterility (Amice *et al*., 2008; Colinet and Hance, 2009).

In situation that males in different treatments were paired with a constant female, a pair of constant produced significantly more mummies than control male/constant female (P<0.001) or fluctuating male/constant female (P<0.001) (Figure 2a). The result that shows that sterility of males and females is not the only reason of reduced fecundity in constant treatment, suggesting that constant cold stress may cause alteration of reproductive organs or mating behavior such as it makes them a better couple, compared with other treated mates.

Several studies have shown that males were more susceptible to cold exposure than female parasitoids (Ali *et al*., 1997; Colinet *et al*., 2006b). Two main factors have been mentioned as these differences; one is haplodiploidy (Clarke *et al*., 1986), and the second factor is sexual size dimorphism (Godfray, 1994). Despite these reports, Jarry and

Tremblay (1989) reported that cold-exposure of mummies does not affect sexes differentially. Our results also showed that constant cold stress had deleterious effects on both sexes, and females were not more cold-tolerant than males.

The emergence rate of progenies that their mothers as control females or fluctuating females were paired with control males, was significantly higher than a pair of constant female and control male (P= 0.002; P= 0.004, respectively). This trend was also observed for fluctuating males when they were paired with control females or fluctuating females, in comparison with constant females (Both P<0.001) (Figure 2b). When a pair of control were compared with constant male/control female, emergence rate of the control pair was significantly higher (P= 0.013). This trend was almost happened for fluctuating female when was paired with different males. When constant females were paired with different males, emergence rate was not significantly different between treatments ($F_{2,57}$= 2.645; P= 0.08) (Figure 2b).

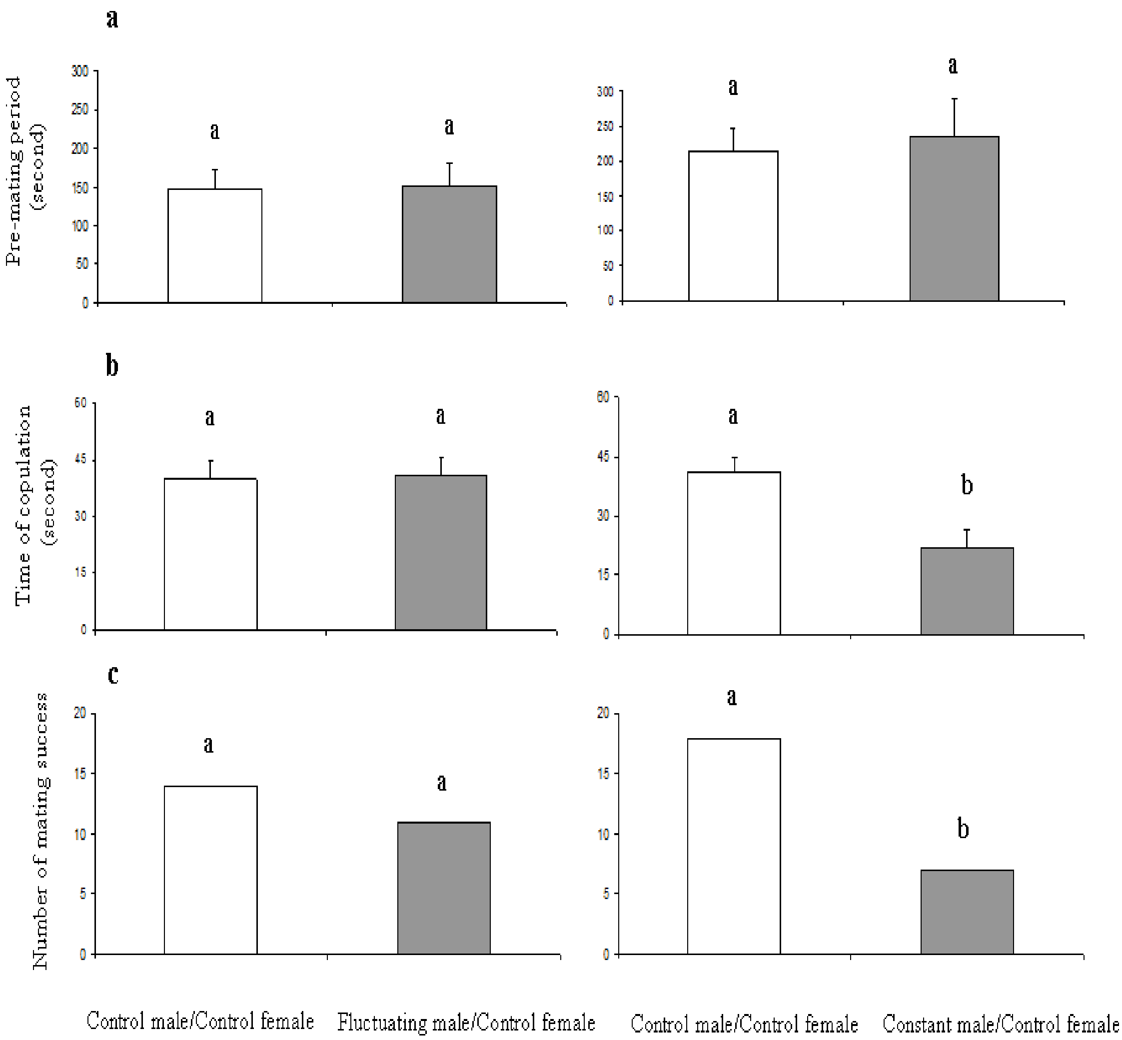

Fig. 1. Mean (± SE) pre-mating period (a), time of copulation (b) and the number of mating success (c) of *Lysiphlebus fabarum* males reared on *Aphis fabae* at 21ºC and then stored (as either constant or fluctuating temperature regime in pupae stage at 6.0ºC for two weeks), when had access to female control. Columns bearing the same letters were not significantly different when different cold treated males were coupled with control females (ANOVA one-way, α = 0.05).

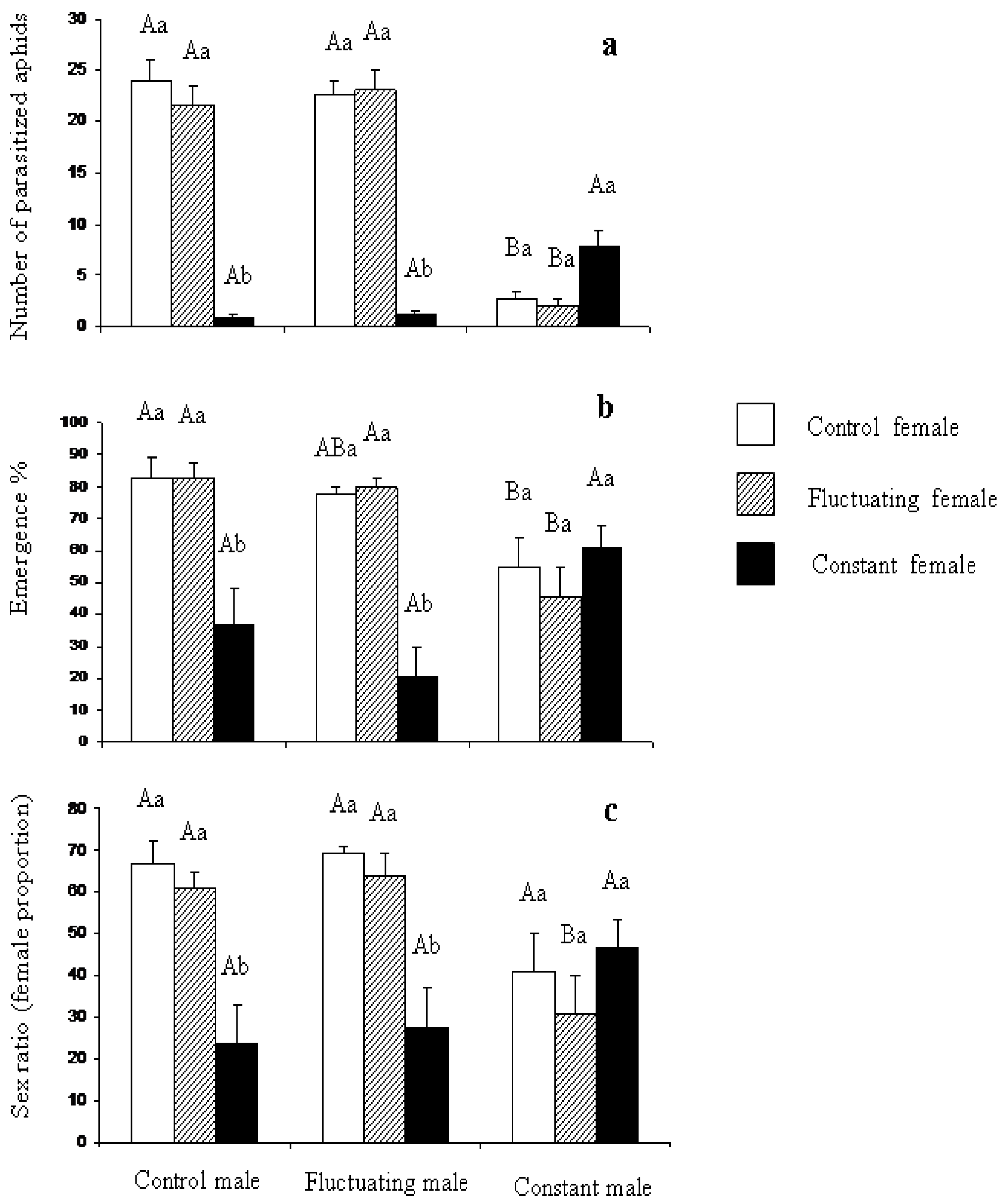

Fig. 2. Mean (± SE) percent parasitism (a), percent offspring emergence (b) and sex ratio (c) of *Lysiphlebus fabarum* in different couples, that males and females were reared on *Aphis fabae* at 21.0ºC, L:D 16:8 photoperiod and then stored as pupae at 6.0ºC with either constant or fluctuating temperature regimes for two weeks. Columns bearing the same upper case letters were not significantly different when the same females were coupled with different treated males (One-way ANOVA for normally distributed data; Mann Whitney U-test for abnormally distributed data, α = 0.05). Columns bearing the same lower case letters were not significantly different when different treated females were coupled with the same males.

Table 1. Two-way ANOVA of effects of cold storage on percent parasitism, percent offspring emergence and sex ratio of *Lysiphlebus fabarum* in different couples, that males and females were reared on *Aphis fabae* at 21.0ºC, L:D 16:8 photoperiod and then stored as pupae at 6.0ºC with either constant or fluctuating temperature regimes for two weeks

Source of variation	% parasitism			% offspring emergence			sex ratio		
	df	F	P	df	F	P	df	F	P
Parental effect	2	58.53	<0.001	2	3.88	0.022	2	1.06	0.348
Maternal effect	2	76.39	<0.001	2	9.45	<0.001	2	6.7	0.001
Interaction	4	44.17	<0.001	4	2.95	<0.021	4	2.01	0.095
Residual d.f.	123			117			113		

As the first report on the effects of parents' cold storage on progenies fitness, pairing of constant males with fluctuating or control females greatly reduced the parasitism and emergence rate. A similar result was observed when constant females were paired with fluctuating or control females. These observations showed that the constant temperature regime has deleterious effects on the reproductive success of *L. fabarum* males and females.

3.2. Sex ratio: The main effect of parental effect was not significant, but that of maternal effect was, although their interaction term was not (Table 1). Fewer female progeny emerged when control males were paired with constant females, in comparison to control (P= 0.001) and fluctuating females (P= 0.008). Similar results occurred for constant females in comparison to control (P= 0.001) and fluctuating females (P= 0.004), when had access to fluctuating males (Figure 2c).

When control females were paired with different males, sex ratio did not significantly change between treatments ($F_{2,57}$= 2.794; P= 0.07), similar to constant female ($F_{2,57}$= 0.63; P= 0.536). While in fluctuating females, sex ratio was significantly lower in constant male relative to other pairs (Figure 2c).

There are some reports about the impact of fluctuating thermal regimes on the survival and sex ratio of a cold-exposed parasitic wasp (Colinet *et al*., 2006b). These work show that fluctuating temperatures can improve cold survival in some parasitoid wasps (Leopold *et al*., 1998), but despite the necessity of studying parasitoids cold tolerance under fluctuating temperature regimes, the effect of parents' cold storage on F1 progeny fitness had not been studied yet. It is predicable that extreme temperatures can affect sex ratio via mating failure and physio-anatomical perturbations in sperm production (Colinet and Hance, 2009). Based on results, cold storage of females or males under constant temperature regimes decreased sex ratio of progenies relative to control, in contrast to fluctuating regime. Male sterility due to cold is also an important concern that has been reported in several parasitoid species (Levie *et al*., 2005). This is more important in the sense that female parasitoids generally mate only once, and mating with a sterile male would result in unsuccessful fertilization (Colinet and Hance, 2009).

To conclude, we found that a constant cold stress has deleterious effects on recognition of odours, mating behavior and fitness of progenies, while thermal fluctuating regimes could alleviate the disturbances of mating behaviors and physiological costs of progenies. The results of this study and our previous research (Mahi *et al*., 2014), confirm that cold storage of *L. fabarum* pupae under fluctuating regime is a protection method when compared to constant low temperature exposures. These results can be used to calibrate cold storage conditions for sexual strain of *L. fabarum* that are mass-reared as a biological control agent.

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