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Sex ratio variations with temperature in an egg parasitoid: 
behavioural adjustment and physiological constraint.

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Conflicts of interest: none
**Abstract.** Sex allocation in haplodiploid arthropods is a central fitness-related decision that received much attention in insect parasitoids. The effect of temperature on the reproductive strategy of female parasitoids has rarely been addressed, despite evidence of its influence on other fitness-related traits. We explored mechanisms inducing the higher production of males typically observed at low and high temperature in parasitic wasps, considering that this pattern may result from both behavioural adjustment and physiological constraint. By observing the oviposition behaviour of an egg parasitoid, *Trichogramma euproctidis*, we were able to distinguish the sex ratio intended by the female from the secondary sex ratio, thereby discriminating between a change in behaviour and a physiological constraint on egg fertilization. More males emerged from eggs laid at low (+45%) or high (+80%) temperature than at medium temperature but the underlying mechanisms were different between the two conditions. We observed a behavioural change in sex allocation at high temperature, suggesting that laying sons may be advantageous at high temperature. At low temperature, the females intended a sex ratio similar to that at medium temperature but physiological constraints prevented egg fertilization during oviposition, resulting in an increased number of males emerging from eggs expected to be females. To our knowledge, this is the first experimental evidence that temperature modulates both sex allocation and physiological constraints in egg fertilization in parasitoids.

**Keywords:** Sex allocation, climate change, haplodiploidy, egg fertilization
Introduction

Sex allocation is a central fitness-related decision that should be selected in response to environmental factors, as predicted by theory and confirmed in many empirical studies (Charnov 1982, West 2009). Despite the role of temperature on life history traits (e.g., Huey and Kingsolver 1989; Nylin & Gotthard 1998; Angilletta et al. 2004) and behaviours (e.g. Traniello et al. 1984; Le Lann et al. 2011), only a few studies proposed theoretical models or empirical approaches to understand how organisms should adjust their sex allocation in response to this environmental factor. Among them, Werren and Charnov (1978) developed a perturbation and a cyclical model, assuming that parents can adjust the sex ratio of their progeny. In their perturbation model, they predicted that if periods of exceptional mortality during cold or heat waves occur and that mortality of one sex is higher than the other, mothers would gain to bias sex allocation towards the sex with the highest mortality because of the expected reduced competition in the next generation. Alternatively, Roy et al. (2002) suggested that a change in sex allocation might be adaptive under such dramatic perturbations when one sex has better capacities than the other to disperse and survive under harsh conditions. The cyclical model developed by Werren and Charnov (1978) predicted that adjustment of sex allocation in response to seasonal factors such as temperature should be selected if the relative reproductive success of males and females differs seasonally (Werren and Charnov 1978). Based on this model, it is also likely that organisms may adjust their sex allocation according to microclimates encountered in their habitat.

Temperature can also physiologically modulate sex ratio. Temperature-dependent sex determination during embryonic development is known in species from the “reptiles” group
(e.g., Ewert et al. 1994, Lang & Andrews 2005). In some birds (Göth and Booth 2005) and insects (Wilkes 1959), developmental temperature is also known to cause differential mortality between males and females, resulting in biased sex ratio. In haplodiploid arthropods, the fertilization of an egg produces a female offspring while no fertilization results in a male (Flanders 1956). Temperature can thus physiologically influence sex ratio by sterilizing males or incapacitating sperm (Wilkes 1959; Wilkes 1963; King 1987; Nguyen et al. 2013). Such an effect of temperature on egg fertilization implies that the primary sex ratio (i.e., the sex ratio at oviposition) or the secondary sex ratio (i.e., the sex ratio at emergence of the adult) may differ from the intended sex ratio, the sex ratio “decided” by the mother, because a physiological constraint occurs between the decision of the female and the end of the oviposition process. If a physiological constraint prevents egg fertilization, a son may be produced from an egg expected to be a daughter by the mother.

Among animal taxa, parasitoid Hymenoptera is one of the most studied groups when investigating sex allocation variations with environment (Ode & Hardy 2008). This is mainly explained by their reproductive system: mated females store sperm in their spermathecae and are generally able to control the sex of their progeny at oviposition by releasing or retaining sperm (Flanders 1956). Typically, more males are produced when parents and/or eggs are exposed to low or high temperatures (for reviews see King 1987; Colinet and Boivin 2011), although some species produce a constant sex ratio across a large range of temperatures (e.g., Ichiki et al. 2003; Jacas et al. 2007). However, the mechanisms underlying sex ratio variations at low and high temperatures have rarely been investigated (King 1987). By comparing intended sex ratio with primary or secondary sex ratio across a
temperature gradient, one may discriminate between an ovipositional decision by the female and a physiological constraint on egg fertilization.

The main challenge when studying sex allocation is to discriminate between sex allocation and progeny sex ratio. Several methods have been proposed over the last decades, including genetic analyses (e.g., Ratnieks & Keller 1998; Khidr et al. 2013) or the observation of female behaviour during oviposition in species where the oviposition behaviour differs with the sex of the progeny deposited (e.g., Cole 1981, Suzuki et al. 1984, van Dijken & Waage 1987). In the genus *Trichogramma* (Hymenoptera: Trichogrammatidae), when a female offspring is deposited, a pause in abdominal movements during oviposition, which corresponds to the fertilization of the egg by a spermatozoid, is observed. When a male progeny is deposited, no such pause is observed and the abdominal movements are continuous (Suzuki et al. 1984, Martel & Boivin 2007). It is thus possible in these species to calculate an intended sex ratio by observing the oviposition sequence of a female and subsequently to compare it to the primary or secondary sex ratio.

We investigated the effect of temperature during female oviposition on offspring sex ratio in the egg parasitoid *Trichogramma euproctidis* Girault. We compared the intended sex ratio to the secondary sex ratio of progeny laid at three temperatures to explore the behavioural and physiological mechanisms involved.

**Material & Methods**
Trichogrammatidae are minute endoparasitoids that usually parasitize eggs of a wide range of Lepidoptera species. The *Trichogramma euproctidis* (Girault) strain used in this study originates from Egypt and was maintained at 24 ±1°C, 50% RH, and LD 16:8 h on cold-killed eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). Using cold-killed eggs is common in studies on *Trichogramma* and does not affect oviposition behaviour of females nor quality of their progeny (Daumal & Boinel 1994, Smith 1996). In *Ephestia kuehniella* eggs, *T. euproctidis* only lays one egg per oviposition sequence. Superparasitism may occur but it requires a second oviposition sequence.

**Sex ratio experiment.** We measured intended and secondary sex ratio of *T. euproctidis* at three temperatures during oviposition: 14, 24 or 34°C. We considered that a 10-degree variation above and below the average temperature (24°C in our colony) is likely to occur during a cold or a heat wave, as well as across a day or across microhabitats. Such a variation did not induce any short-term lethal effects on parasitoids and females remained active despite changes in walking speed (unpublished data). Parasitized eggs were withdrawn from the colony and isolated in 300μL Beem® polyethylene capsules with a drop of honey. The next morning, freshly emerged females were mated once with a virgin male, stored at 24°C during one to five hours and transferred to a climate room at 14, 24 or 34°C one hour before observation. After this acclimation period, 20 cold-killed *E. kuehniella* eggs, placed side by side in 4 lines of 5 eggs on a filter paper disc were offered to each female. The experimental arena was delimited by the plastic end piece of a camera (Ø20 mm) covered with Fluon® to
prevent females from climbing. Ovipositions were recorded for 30 females per temperature with a camera (Dino-lite Pro AM413ZT, x 60) and analyzed using The Observer XT software (Noldus, Wageningen, The Netherlands). Oviposition sequences were divided into three components: host drilling, egg deposition (indicated by abdominal movements) and, if any, pause in abdominal movements during egg deposition (i.e., when an egg was fertilized). During these pauses, the whole body typically stopped moving for a few seconds (Video 1). Sex of each egg deposited was determined, based on the absence or presence of a pause in abdominal movements, and the intended sex ratio was calculated. This protocol was successfully used in several Trichogramma species (e.g. Suzuki et al. 1984), including T. euproctidis (e.g. Martel & Boivin 2007 –previously named T. turkestanica in this study-, Martel et al. 2010). Duration of pauses in the oviposition sequence was also recorded to make sure that they were long enough, especially at 34˚C, to be accurately observed. Superparasitism was quantified when a second oviposition sequence was observed on a parasitized host. Observation stopped when a female walked off the patch for more than 3 minutes. Wasps were thus exposed to 14, 24 or 34˚C for one hour before observation plus duration of patch exploitation, which increased as temperature decreased. At the end of an observation, the wasp was removed; eggs were isolated in 300μL Beem® polyethylene capsules and incubated at 24 ±1˚C until emergence to determine the sex of the emerging adult. Emerging sex was then compared to intended sex and secondary sex ratio was compared to intended sex ratio for each mother. Superparasitized hosts were not included in the calculation of intended and secondary sex ratios as we were not able to directly compare intended and emerging sex of a given egg. Hatching success was measured for
both sexes to examine if temperature during oviposition had a differential effect on males and females egg survival, which may explain differences in secondary sex-ratio.

**Survival experiment.** A behavioural change in sex allocation in response to temperature may have been selected for by sex-dependent mortality during a cold or a heat wave (Werren & Charnov 1978). To test this hypothesis, we measured survival to emergence of female and male parasitoids developing at 14, 24 or 32 °C (*T. euproctidis* failed to emerge at 34°C). Using only mated females would have limited the number of sons produced, since sex ratios were close to 0.13 at 24°C. We thus used virgin females to produce sons. Sixty mated and virgin females were allowed to parasitize twenty eggs each at 24°C. The number of ovipositions was noted for virgin females, since only sons could be produced, while the predicted number of sons and daughters was noted for mated females using the method described above. Eggs were then incubated at 14, 24 or 32°C (twenty replicates per temperature), and the number of emerging sons and daughters was compared to the number of ovipositions. Hatching success was calculated for each sex at each temperature.

**Statistical analyses.**

We performed generalized linear models (GLMs) with temperature included as a factor to test for differences in the number of ovipositions, the number of intended and emerging males, and intended sex ratio and secondary sex ratio. We considered a Poisson error structure for the number of ovipositions and the number of expected and emerging males,
and a binomial error structure for intended and secondary sex ratios. The “multcomp” package, available in R software, was used to perform post-hoc tests after a GLM. The numbers of intended and emerging males were compared with a Wilcoxon signed-rank test for each temperature, as well as the intended and secondary sex ratios. Mean durations of the pause in abdominal movements (indicating fertilization) was compared using an ANOVA with temperature as factor, with a Tukey test used as post-hoc test. The influence of sex, temperature and the interaction between these factors on hatching success of eggs laid at the three temperatures (reared at 24˚C) was analyzed using a GLM considering a binomial error structure. The number of superparasitized hosts was compared using a GLM with temperature as factor, considering a Poisson error structure. The influence of sex, temperature and the interaction between these two factors on hatching success of parasitoids laid at 24˚C and reared at the three temperatures was analyzed using a GLM, considering a binomial error structure.

All statistical analyses were carried out using R software version 2.14.1 (R Development Core Team, 2011).

Results

**Sex ratio experiment.** After removing superparasitized hosts, the number of hosts considered for analyses was respectively 549, 558 and 554 at 14, 24 and 34 ºC. Observation of a pause appears to be a reliable method to predict sex of the progeny in *T. euproctidis* since 98% of
intended females resulted in the emergence of a female at 24°C while 96% of intended males resulted in the emergence of a male.

Temperature did not influence the number of ovipositions by *T. euproctidis* (Table 1), but females laid more sons at 34°C than at 14°C (p < 0.001) and 24°C (p < 0.001), the last two treatments being similar (p = 0.541) (Figure 1). The intended sex ratio was thus higher at the highest temperature (GLM, df = 2, 89, z = 4.12, p < 0.001). Secondary sex ratio was also influenced by temperature (GLM, df = 2, 89, z = 3.74, p<0.001) but the pattern was different. It was significantly higher at 34°C than at 14°C (p = 0.005) and 24°C (p < 0.001), intermediary at 14°C and the lowest at 24°C (Figure 1). The number of intended and emerging males were similar at 34°C (V =114, p = 0.737) and 24°C (V = 69, p = 0.315) while more males than expected emerged at 14°C (V = 9, p < 0.001) (Figure 2), resulting in a secondary sex ratio higher than the intended sex ratio (V = 11, p < 0.001).

The pause duration in abdominal movements associated with egg fertilization significantly decreased when temperature increased (ANOVA, $F_{2,89} = 178.5, p < 0.001$) (Figure 3). The pause lasted a minimum of 2.4 seconds at 34°C, a duration long enough to be accurately quantified.

We only observed 14 cases of superparasitism out of the 1675 parasitized eggs and temperature did not influence superparasitism frequency (GLM, df = 2, 89, z = -0.53, p = 0.644). Every host on which two oviposition sequences were observed resulted in the emergence of two parasitoids while only one parasitoid emerged from host attacked only one time.
Hatching success was very high and was not affected by temperature, sex (Table 1) or the interaction between these two factors (Table 1).

**Survival experiment.** Hatching success was significantly influenced by developmental temperature \((p = 0.036)\), but we did not observe a significant effect of sex \((p = 0.60)\) or interaction between both factors \((p = 0.78)\). Hatching success was significantly lower at 32°C (Mean [95% CI], ♂: 43% [33-52%], ♀: 51% [46-59%]) than at 14°C (♂: 81% [75-88%], ♀: 86% [80-91%]) and 24°C (♂: 90% [87-94%], ♀: 93% [90-96%]).

**Discussion**

This study explored the mechanisms inducing the higher production of males typically observed at low and high temperature in parasitoids, which can vary from a slight increase to the production of only males according to the species and the protocol used (King 1987; Colinet and Boivin 2011). Our main results are that (1) female *T. euproctidis* laid more sons at high temperature and (2) the secondary sex ratio was higher than the intended sex ratio at low temperature. These results indicate that temperature influences the sex allocation by the ovipositing female and physiologically constrains the secondary sex ratio.

**Intended sex ratio.** Variation in temperature can be a reliable indicator of environmental quality for arthropods and trigger the production of individuals with different capacities to
develop, reproduce or disperse (Wellington et al. 1999). The increase in the intended proportion of *T. euproctidis* males at high temperature is likely an evolutionary response to these environmental conditions. In the field, parasitoid females may encounter not only temporal variations in temperature, during a heat wave or along seasonal variations, but also spatial variations, when foraging in habitats with different microclimates.

The influence of extreme variations in temperature on sex allocation has mainly been investigated in the perturbation model developed by Werren and Charnov (1978), who predicted that if mortality is male-biased during a cold or a heat wave, parasitoid females would gain by laying more sons than under optimal weather conditions as competition would be reduced between sons. Hatching success indeed decreased when *T. euproctidis* was reared at high temperature, but mortality was not biased towards one sex under this climatic condition. The perturbation model thus does not explain the higher production of males at high temperature. Alternatively, Roy et al. (2003) proposed that a change in sex allocation may be an adaptive response to such perturbations if one sex has better capacities to disperse than the other. However, females are generally more prone to initiate flight and disperse than males (Jones et al. 1996) in parasitic wasps, including a closely related species, *Trichogramma minutum* (Forsse et al. 1992). The cyclical model on seasonal variations developed by Werren and Charnov (1978) is also unlikely to explain the variations in sex allocation observed in our study as it implies that both sexes should live long enough to mate with a partner that developed during a different season, while *Trichogramma* only lives for a few days. Moreover, temperature is not a reliable cue of seasonality in comparison to photoperiod because it fluctuates widely from day to day (Danilevskii 1965;
Danks 2006; Bradshaw and Holzapfel 2008). If seasonal variations in sex allocation occur, parasitoids should thus rely on photoperiod and not temperature.

It is thus likely that thermal variation in sex allocation has been selected in response to spatial thermal variations. *Trichogramma* are polyphagous species that parasitize eggs of a wide range of Lepidoptera species that are known to select their oviposition site according to microclimate, species laying their eggs in either shady habitats or sunny habitats (Rausher 1979). *Trichogramma* females are thus likely to exploit different microclimates at a small spatial scale, for example within a crop or along a hedgerow, and may vary offspring sex ratios accordingly if the relative reproductive success of males and females differs. This hypothesis would hold if *Trichogramma* females forage during a short period of time per day as observed in *Drosophila* parasitoids (Fleury et al. 2000) -otherwise the hourly variations in temperature would alter the reliability of microclimate as an estimate of the average temperature for progeny development- or if they can integrate both temperature and time of the day. In insects, a strong correlation exists between body size and several life history traits, especially fecundity (Honěk 1993), even though this relationship is not always true in the field (e.g. Kazmer and Luck 1995). Moreover female fecundity is more closely correlated with body size than the components of male reproductive success (Honěk 1993; Jarošík and Honěk 2007). As body size generally decreases when temperature increases in ectotherms (Nylin and Gotthard 1998; Angilletta et al. 2004), it is likely that female fitness would be more affected by high temperature than male fitness. Laying more males may thus be adaptive under hot microclimate.
The increased male-biased sex ratio at high temperature might also be not adaptive but rather reflects a constraint. High temperature may alter the neural system of parasitoid females and interfere with oviposition decision. However, the high temperature (34°C) used in our experiment did not result in alteration of locomotor activity or foraging behaviours, it is thus likely that sex allocation variation was adaptive.

**Secondary sex ratio.** The low temperature treatment did not induce a strong thermal stress on parasitoid females since they remained active and parasitized as many hosts as did females at higher temperatures. However more males were obtained than what the female intended to deposit at low temperature whereas no difference between intended and secondary sex ratio was observed at other temperatures. Low temperature thus acted as a physiological sex ratio distorter during oviposition, as mating and rearing temperatures were kept constant at 24°C. This pattern does not result from a difference in mortality between sexes as hatching success was similar and very high (>95%) for males and females laid at low temperature and reared at 24°C. Moreover, this pattern cannot arise from misleading behavioural observations when assessing egg fertilization because the pause in abdominal movements lasted longer (> 15 sec) at low temperature and was easily noticeable. A physiological constraint on egg fertilization likely occurred during oviposition at low temperature. Richards (1963) observed that sperm velocity in the American cockroach *Periplaneta americana* decreased with temperature. At low temperature, *T. euproctidis* spermatozoids probably did not move fast enough from the spermatheca to the egg despite the long pause in abdominal movements occurring during oviposition. A reduction in
spermatozoid speed may thus affect fertilization, and consequently distort intended sex ratio. In insects, fertilization of eggs is induced by muscular contractions of the spermathecal sac that are controlled by a neural loop (Clarke and Lange 2001). It may also be that this neural loop was affected at low temperature or that the frequency or amplitude of the muscular contractions of the spermatheca and associated ducts decreased to the point that spermatozoid transfer was reduced.

**Conclusion.** Models of climate change predict an increase in mean global temperature and in its variance in the next decades (IPCC 2007). Our study underlines the influence of such variations in temperature on insect behaviour, especially the allocation of sex. These climatic variations have significant demographic impacts, as a drop in temperature resulted in a 50% increase in secondary sex ratio while a rise in temperature resulted in a 65% increase in intended sex ratio, in comparison to females foraging under constant thermal conditions. In the next decades, sex ratio may thus be distorted under extreme climatic events either through a physiological constraint on egg fertilization or a behavioural adjustment in response to temperature. Our results suggest, together with other studies (e.g., Petchey et al. 2010; Rall et al. 2010; Sentis et al. 2013), that models predicting the evolution of insect populations and communities in response to climate change should integrate the effect of temperature on behaviour and physiology, and that more empirical studies are needed to understand how organisms will cope with changing environment.
Literature cited


Table 1. Number of ovipositions and hatching success, measured on *T. euproctidis* females allowed to parasitize 20 cold-killed *E. kuehniella* eggs at three temperatures. Mean ± s.e.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Oviposition</th>
<th>♂ hatching success</th>
<th>♀ hatching success</th>
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<tbody>
<tr>
<td>14</td>
<td>18.60 ± 0.23</td>
<td>0.96</td>
<td>0.95</td>
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<tr>
<td>24</td>
<td>18.63 ± 0.33</td>
<td>0.96</td>
<td>0.96</td>
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<tr>
<td>34</td>
<td>18.57 ± 0.33</td>
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<td></td>
<td>0.13</td>
<td>0.913</td>
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<tr>
<td></td>
<td>0.48</td>
<td>0.810</td>
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Fig 1. Box plots of intended sex ratio (white) and secondary sex ratio (grey) measured at three temperatures for *T. euproctidis*. Sex ratio was measured as the proportion of males in the progeny.

Fig 2. Box plots of the number of males that emerged from hosts in which the female *T. euproctidis* deposited a female as indicated by the duration of the pause in abdominal movements (unintended males). The hosts were cold-killed *E. kuehniella* eggs and the observations were done at three temperatures.

Fig. 3. Box plots of pause duration in abdominal movements during oviposition by *T. euproctidis* at three temperatures.
Figure 1
Figure 2
Figure 3