

Costly sexual harassment in a beetle

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Abstract. The optimal number of mating partners for females rarely coincides with that for males, leading to sexual conflict over mating frequency. In the bruchid beetle *Callosobruchus maculatus*, the fitness consequences to females of engaging in multiple copulations are complex, with studies demonstrating both costs and benefits to multiple mating. However, females kept continuously with males have a lower lifetime egg production compared with females mated only once and then isolated from males. This reduction in fitness may be a result of damage caused by male genitalia, which bear spines that puncture the female's reproductive tract, and/or toxic elements in the ejaculate. However, male harassment rather than costs of matings themselves could also explain the results. In the present study, the fitness costs of male harassment for female *C. maculatus* are estimated. The natural refractory period of females immediately after their first mating is used to separate the cost of harassment from the cost of mating. Male harassment results in females laying fewer eggs and this results in a tendency to produce fewer offspring. The results are discussed in the context of mate choice and sexual selection.

Key words. *Callosobruchus maculatus*, Coleoptera, harassment, mating frequency, polyandry.

Introduction

The optimal number of mating partners for females rarely coincides with that for males (Bateman, 1998; Arnqvist & Nilsson, 2000). A single copulation often provides females with potentially enough sperm for full fertility, whereas any additional copulation may increase male fitness. Even when females benefit from polyandry (Hosken & Stockley, 2003), optimal female mating frequency tends to be lower than for males. As a result, sexual conflict over mating rate should be common (Parker, 1983). Male harassment of females, defined as males' repeated unsuccessful attempts to mate, is part of this conflict and females are expected to try to resist mating if mating is costly. The existence of harassment costs has interesting consequences because they impose costs on 'choosy' females who may suffer more harassment at the same time as rejecting potential mates. Under these circumstances, convenience polyandry could evolve (Thornhill & Alcock, 1983; Arnqvist & Rowe, 2005). Female reluctance to mate with

certain males can only evolve if the cost of avoiding mating does not exceed the cost of copulation. From the male perspective, harassment can be favoured if, by virtue of being costly to females, it induces them to mate more rapidly to reduce these costs.

Repeated harassment is likely to have costs to both sexes, including loss of feeding time (Magurran & Seghers, 1994; Dadda *et al.*, 2005), energy expenditure (Watson *et al.*, 1998) increased risk of predation (Rowe *et al.*, 1994; Croft *et al.*, 2006) or physical injuries (Leboeuf & Mesnick, 1991). Male harassment can also disturb females during oviposition, reducing their fitness (McLain & Pratt, 1999). However, few studies have investigated the effect of male harassment on female lifetime fitness (Sakurai & Kasuya, 2008). In gryllid crickets (*Gryllus bimaculatus*), Bateman *et al.* (2006) report the results obtained with males whose sub-genital plate had been removed to prevent them from mating without altering their courtship behaviour. Females' longevity is reduced when housed with one or more of these males, in agreement with the hypothesis of costly harassment. However, the study does not control for potential fecundity–longevity trade-offs. In the adzuki bean beetle, *Callosobruchus chinensis*, Sakurai & Kasuya (2008) show strong evidence for a cost of harassment by ablating the aedeagus, thus rendering males unable to copulate. In

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Drosophila melanogaster, a cost of courtship is suggested by the negative correlation between female fitness and male courtship frequency (Friberg & Arnqvist, 2003). However, in *Drosophila simulans*, although there are longevity costs of continual exposure to males, lifetime reproductive success is not significantly reduced (Taylor *et al.*, 2008).

In *Callosobruchus maculatus* (Fabricius), multiple copulations are shown to increase fecundity (Ofuya, 1995; Savalli & Fox, 1999) and female longevity (Fox, 1993; Messina & Slade, 1999; Rönn *et al.*, 2006), although Crudgington & Siva-Jothy (2000) find that multiple mating reduces female longevity, whereas Eady *et al.* (2007) find no effect of mating frequency on longevity. In Fox's study (1993), the benefits of re-mating are significant only when females are housed without males. Females continuously housed with males have the same lifetime reproductive success as singly-mated females. Similarly, Rönn *et al.* (2006) and Edvardsson (2007) show that females housed continuously with males have a lower lifetime egg production compared with once-mated females that are subsequently isolated from males. The mechanisms responsible for these costs have yet to be elucidated. Matings are certainly costly, male genitalia bear spines that puncture the female's reproductive tract (Crudgington & Siva-Jothy, 2000) and ejaculatory components may be toxic to females (Eady *et al.*, 2007). However, males can also be observed persistently pursuing females and attempting copulations, so harassment rather than a cost of mating could also explain the previous results. In the present study, the aim is to estimate the fitness costs of male harassment for female *C. maculatus*. The natural refractory period of females immediately after their first mating (Edvardsson & Tregenza, 2005; Edvardsson *et al.*, 2008) is used to separate the cost of harassment from the cost of mating. The experimental design allows testing of the hypothesis that male harassment reduces oviposition, lowering female fitness.

Materials and methods

Study species

Callosobruchus maculatus is a widely distributed pest of legumes. Females attach their eggs to the surface of beans. After hatching, the larvae burrow into the seed and develop inside. The beetles used in this experiment were derived from Niamey, Niger, and have been in culture on black-eyed-beans (*Vigna unguiculata*) for 90 generations at 27 °C, 32% RH and under a LD 16:8h photoperiod at the University of Lincoln. Briefly, 250 males and 250 females had been housed together on 2000 beans for their entire lifespan. To retain a constant population size and ratio of resources to beetles, the newly-emerging adults were removed and weighed at each generation and another 500 individuals (estimated by mass) were placed on 2000 new beans. A black population was created by selecting on body-size colour in the Niamey population. Crossings showed that body-size colour can be used as a co-dominant phenotypic marker (Eady, 1991). Once the population size of the purely black population reached 500 individuals, it was

also maintained at a constant density of 500 beetles per 2000 beans for twelve generations prior to the experiments. Three generations prior to the experiment, the population sizes of both the black and the wild-type populations were increased to 2500 individuals.

Experimental treatments

To standardize for larval competition, individuals were selected from beans carrying one egg only (a single bean provides excess food for a single larva). Before emergence, 1600 beans were isolated in 'virgin chambers' (48-well cell culture plates; VWR International Ltd, The Netherlands). Beans were checked every 12h for emerging, virgin adults, which were then housed separately. Two-day-old females and males were placed together in a 40-mm Petri dish and observed until copulation had ceased. Males were discarded. Singly-mated females were then allocated to one of three experimental treatments: (i) females housed with five virgin males for 6h (male harassment treatment); (ii) females housed with five other virgin females for 6h (female treatment); and (iii) females housed alone (alone treatment). To ensure that females did not copulate a second time in the male treatment and that only potential costs of harassment were assessed and not confounded by additional copulations, re-mating was assessed using the co-dominant phenotypic marker (black body colour): singly-mated females were black and had mated with black males before subsequently being housed with wild-type males. If, as expected, females refuse to re-mate, their offspring should be entirely black. However, any female re-mating within the 6h spent with the five males (male treatment) could be detected by the appearance of dark tan individuals within their offspring (Eady, 1991). Five re-mated females were excluded from the analysis. In the female treatment (ii), nonfocal females were also from the wild-type line, which enabled recognition of the focal female. This protocol was repeated twice, with a sample size of 22 females per treatment in block 1 and 19 females per treatment in block 2 to give a total of 123 focal females (although final sample sizes vary somewhat because not all data were collected for all females). All focal females were housed with 20 fresh black-eyed-beans in a 90-mm Petri dish at 27 °C for 6h after the first copulation and allowed to oviposit. After this initial period of oviposition, females were transferred to another 90-mm Petri dish, again containing 20 fresh black-eyed-beans, for another 18h. After this, all females were transferred onto new beans (40 beans) where they remained until death. The density in the Petri dish was lower than during the culture to avoid exposing females to levels of harassment higher than they experienced in the laboratory for generations.

Measurements

In a pilot study prior to the main experiment, harassment was monitored together with copulation frequency of normal males. When a once-mated female was kept with a virgin male

for her entire lifespan (with beans available and in a 90-mm Petri dish), approximately 20% of harassment events lead to successful copulation. The rate of harassment was also high (four harassment events per hour on average). In the experiment proper, harassment frequency was estimated by observing each focal female every second minute during 30-min observation windows twice over the 6-h male-exposure period. Every time a male touched or chased a female, it was recorded as a harassment event. The number of eggs laid during the initial 6 h and the subsequent 18 h were recorded. Any female mortality was recorded every 24 h. Finally, the number of offspring emerging from the three sets of beans (6 h, 18 h and lifetime) were recorded. Because fecundity and longevity covary with body size (Fox, 1993), the elytra length of all focal females was also measured.

Statistical analysis

Analysis of covariance with elytra length and experimental block as covariates was used to compare the different components of female fitness (early egg laying, early number of offspring, lifetime reproductive success and longevity) between the three treatments. For longevity, lifetime reproductive success (LRS) was also included as a covariate. Analyses were performed in R software (from 'R Development Core Team', R Foundation for Statistical Computing, Vienna, Austria). All traits except longevity were normally distributed (Kolmogorov–Smirnov test, all $P > 0.05$ except longevity: $Z = 1.54$; $P = 0.018$). Residuals from all analyses were checked and found to be normally distributed (Kolmogorov–Smirnov test, all $P > 0.05$). The results are presented as the mean \pm SE.

Results

Housing a nonvirgin female with five virgin males resulted in a high rate of harassment (mean \pm SE = 18.0 ± 2.9) harassment events per hour, $n = 31$ females). Compared with the preliminary study, five males harassed just under five times more than one single male (4.6 ± 0.6 , $n = 30$ females).

The effect of female presence (females with five other females versus females alone) on female fitness was first tested using a complete model with elytra length, experimental block and LRS (for longevity only) as covariates. Female presence had no effect on any of the components of female fitness (eggs laid in 6 h: $F_{4,49} = 0.03$ $P = 0.998$; eggs laid in 18 h: $F_{4,59} = 0.37$ $P = 0.827$; offspring emerging after an oviposition period of 6 h: $F_{4,45} = 0.21$ $P = 0.930$; offspring emerging after an oviposition period of 18 h: $F_{4,58} = 0.44$ $P = 0.780$; LRS: $F_{4,45} = 0.67$ $P = 0.617$; longevity: $F_{7,41} = 1.46$ $P = 0.216$). Hence we pooled these two control treatments.

All components of fitness were positively affected by size, measured by female average elytra length (Table 1), although there was no significant interaction between elytra length and treatment for any of the traits analysed. Size was therefore included as an explanatory variable in all the models presented below. Similarly, there was a significant effect of experimental block on all variables except the total LRS (Table 1) but

no significant interaction between block and treatment (eggs laid in 6 h: $F_{5,78} = 0.41$ $P = 0.839$; eggs laid in 18 h: $F_{5,86} = 0.52$ $P = 0.757$; offspring emerging after an oviposition period of 6 h: $F_{5,73} = 0.32$ $P = 0.897$; offspring emerging after an oviposition period of 18 h: $F_{5,85} = 0.39$ $P = 0.857$; total LRS: $F_{5,71} = 0.45$ $P = 0.812$). Results of the minimal statistical model (Crawley, 2002) are presented in Table 1. In comparison to the control treatments, male harassment resulted in fewer eggs laid during the 6 h of exposure to harassment (harassment: 18 ± 2.3 eggs; control: 24 ± 1.4 eggs; Fig. 1 and Table 1). This reduction in egg laying tended to continue during the subsequent 18 h after the males were removed but the effect was not significant (harassment: 5 ± 0.9 eggs; control: 7 ± 0.6 eggs; Fig. 1 and Table 1).

As a consequence of the lower early fecundity for the male-harassment treatment, harassed females tended to produce a lower number of offspring in the first 6 h and the subsequent 18 h (harassment = 16 ± 2.1 offspring in 6 h; control: 20 ± 1.2 offspring in 6 h; harassment = 4 ± 0.7 offspring in the subsequent 18 h; control: 6 ± 0.6 offspring in the subsequent 18 h; Fig. 1 and Table 1). The hatching success tended to be higher in the control treatment and for eggs laid during the next 18 h but these differences were not significant (harassment: $82.7 \pm 1.6\%$ for eggs laid during 6 h; control: $85.7 \pm 5.2\%$; $F_{1,71} = 0.09$ $P = 0.759$; harassment: $83.4 \pm 2.8\%$ for eggs laid during 18 h; control: $88.2 \pm 2.6\%$; $F_{1,70} = 0.79$ $P = 0.376$; hatching 6 h versus hatching 18 h: $t_{147} = -1.02$ $P = 0.307$). Overall, male harassment tended to decrease lifetime reproductive success for females by almost 19% (harassment: LRS = 43 ± 5.2 offspring; control: LRS = 53 ± 3.4 offspring; Fig. 2 and Table 1).

Male harassment also reduced female longevity slightly but significantly, independent of female fecundity (male harassment treatment: 13.7 ± 0.65 days; control: 13.9 ± 0.53 days; females: 14.1 ± 0.62 days; Table 1).

Discussion

Females housed with males for 6 h suffer from decreased fecundity, which results in lower offspring production in the short term (minus 19% in the lifetime reproductive success). Because the rate of reproduction (the equivalent of the Malthusian parameter r) is a close fitness surrogate, it appears that there are fitness costs to harassment. Additionally, there is a tendency for harassed females to produce fewer offspring over their lifetime consistent with fitness costs to harassment. In this population, individuals live for an average of 14 days, and females are only exposed to male harassment for 6 h. This probably represents less than 3% of the natural level of harassment, assuming that males are active for 16 h a day (LD 16:8 h photoperiod) during their entire lifespan and that the harassment rate is constant. This is an approximation and it is sensible to assume that the harassment frequency will decrease with the age of the males (Sakurai & Kasuya, 2008). Nevertheless, the observed tendency towards decreased fitness in the presence of males is expected to be amplified when females are kept with males over their entire lifespan. It is concluded that

Table 1. Analysis of covariance of the effects of male harassment on six components of female fitness.

| | Sum of squares | d.f. | F | P |
|--------------------------------------|----------------|------|-------|---------|
| Eggs in 6 h | | | | |
| Treatment | 435 | 1 | 4.34 | 0.040 |
| Size | 1133 | 1 | 11.04 | 0.001 |
| Block | 1102 | 1 | 10.97 | 0.001 |
| Error | 8134 | 81 | | |
| Eggs 18 h | | | | |
| Treatment | 59 | 1 | 3.06 | 0.084 |
| Size | 103 | 1 | 5.31 | 0.024 |
| Block | 276 | 1 | 14.25 | 0.0003 |
| Error | 1726 | 89 | | |
| Offspring 6 h | | | | |
| Treatment | 241 | 1 | 3.23 | 0.076 |
| Size | 911 | 1 | 12.22 | 0.0008 |
| Block | 810 | 1 | 10.86 | 0.001 |
| Error | 5666 | 76 | | |
| Offspring 18 h | | | | |
| Treatment | 76 | 1 | 4.58 | 0.035 |
| Size | 132 | 1 | 7.95 | 0.006 |
| Block | 127 | 1 | 7.66 | 0.007 |
| Error | 1463 | 88 | | |
| Lifetime reproductive success | | | | |
| Treatment | 1918 | 1 | 3.68 | 0.059 |
| Size | 10517 | 1 | 20.18 | 0.00002 |
| Error | 39090 | 75 | | |
| Longevity | | | | |
| Lifetime reproductive success | 9.4 | 1 | 1.16 | 0.285 |
| Treatment | 56 | 1 | 6.90 | 0.011 |
| Size | 33 | 1 | 4.01 | 0.049 |
| Block | 146 | 1 | 18.03 | 0.00006 |
| Error | 551 | 68 | | |

d.f., degrees of freedom.

the effect of male harassment is not negligible in *C. maculatus* and could be a major cost of reproduction. Previous studies where female *C. maculatus* are housed with males for their entire lifespan also suggest a cost of harassment (Rönn *et al.*, 2006; Edvardsson, 2007). In particular, Rönn *et al.* (2006) show a decrease of female lifespan (−1 or 2 days depending on the population) and fecundity (−13 eggs). Similarly, Edvardsson (2007) observes a decreased lifespan (−1.5 day) and fitness (−21 offspring) for females housed with males and without water. In these two studies, a cost of re-mating cannot be ruled out, but other studies with this species (Fox, 1993; Arnqvist *et al.*, 2005) suggest that the cost observed exceeds the cost of mating and is likely due to male harassment. This cost of lifetime cohabitation with males is either similar to (−14%, Rönn *et al.*, 2006, but no data on offspring production are available) or bigger (−24%, Edvardsson, 2007) than the harassment cost estimated in the present study.

In a related species (*C. chinensis*), Sakurai & Kasuya (2008) show a large fitness cost of the male harassment: the fitness of

females that cohabit with males that can harass but not copulate is 18.6% lower than that of the females that receive neither harassment nor additional copulations. In gryllid crickets (*Gryllus bimaculatus*), the visual and olfactory contact with a male, as well as the presence of one to three males that can harass but not copulate, results in an approximately 30–35% decrease in longevity (Bateman *et al.*, 2006). In these two experiments, males are manipulated to prevent copulation but still allow harassment. Because this manipulation of males is not trivial, there are few examples of such experiments in the literature. It is nevertheless to be emphasized that every one of these experiments specifically designed to study harassment finds that it is costly.

A large block effect is seen on most of the fitness components measured (except LRS). Various factors could explain why some components of fitness are higher in the first experiment. First, female size varies between the two experiments (on average, by 4% smaller in the second experiment). Behavioural factors such as the lower harassment frequency in experiment 1 could also contribute. Both these morphological and behavioural differences might be mediated by the environment, including social environment. Beetles are maintained in an incubator at a fixed temperature, so this is constant, but there might be variation in the nutritional quality of beans from different sources. Other apparently minor differences in the experimental setting may have contributed; for example, the amount of time females have to wait with the first male after the first copulation before they are transferred onto beans might differ slightly between the two blocks. Overall, the lower fitness in experiment 1 does not affect the conclusions because the effect of harassment is in the same direction in both experiments and statistical analyses account for the block effect.

Examples in the literature show that a high density of female neighbours can decrease female fitness as well as male harassment (Bateman *et al.*, 2006; Smith, 2007). In the present experiment, the presence of five other virgin females has no influence on any of the fitness estimates, indicating that focal females in the female-only groups do not suffer from intra-sexual aggression or disturbance. This rules out the possibility that the costs of male harassment are equivalent to the costs of intra-sexual interactions. Similar results are found in *Drosophila simulans* (Taylor *et al.*, 2008), but not in Amarillo fish (Valero *et al.*, 2005). Had the other females been nonvirgin, it is possible that female presence might disrupt the oviposition rate of focal females (and consequently fitness) as females may compete for oviposition resources.

Harassment primarily affects the number of eggs laid, suggesting that male harassment interferes with or discourages oviposition. It should be noted that, despite this reduction, all females lay approximately one-third of their eggs during the initial 6 h, which is in agreement with previously published results (Wilson & Hill, 1989; Eady, 1991). Two mechanisms (not mutually exclusive) could be involved: either physical disturbance because the presence of males constrains females to keep moving and take evasive action to avoid mating, or metabolic cost due to the increased energy expenditure of

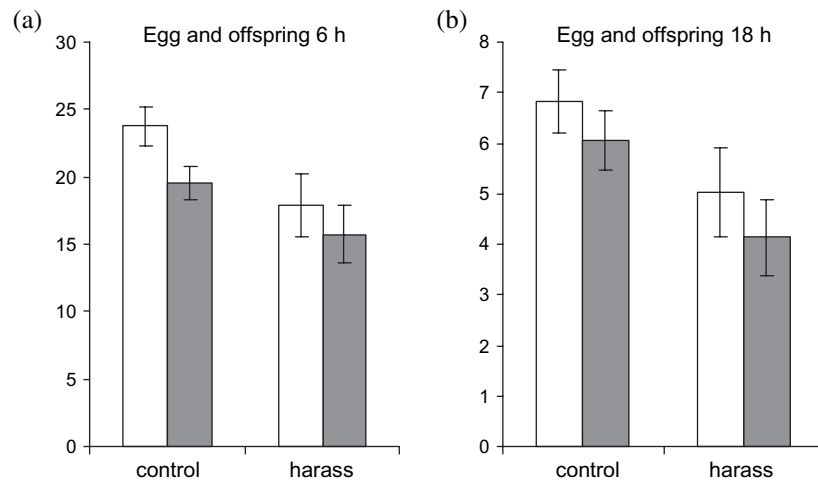


Fig. 1. Effects of male harassment on female egg and offspring production during the first 24 h: (a) number of eggs laid (white bars) and number of offspring produced (grey bars) from eggs laid during the initial 6 h, in presence of the males (for the harassment treatment); (b) number of eggs laid (white bars) and number of offspring produced (grey bars) from eggs laid during the next 18 h. The 'control' treatment pools the females kept alone and the females kept with five other virgin females (no significant difference between these two groups, see text). Error bars indicate standard errors.

females to avoid males. Although the present data cannot rule out the hypothesis of a metabolic cost, it is unlikely that elevated metabolism would generate such an immediate effect. The hypothesis of physical disturbance appears to be more likely. This could be tested by comparing the post-treatment oviposition rates of females exposed to harassment or no harassment in the absence of oviposition sites during the period of exposure. Any subsequent variation in oviposition rate could then be attributable to metabolic costs.

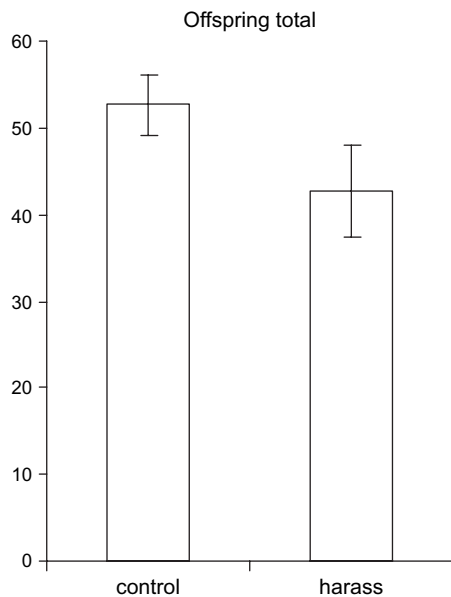


Fig. 2. Effects of male harassment on female's lifetime reproductive success (total number of offspring). Error bars indicate standard errors.

More generally, male harassment is expected to strongly affect female behaviour. Females can mitigate the cost of harassment by increasing their movements, as is suggested in the case in *C. maculatus*. Female movements to avoid harassment are reported in a variety of organisms. In garter snakes (*Thamnophis sirtalis parietalis*), for example, females disperse from the den as rapidly as possible and minimize contact with males (Shine *et al.*, 2005). Females can also diminish the cost of harassment by accepting mating attempts more readily. This behaviour is referred to as convenience polyandry (Thornhill & Alcock, 1983; Arnqvist & Rowe, 2005). Convenience polyandry is plausible in *C. maculatus* where last-male sperm precedence (P2) is high (80–90%; Eady *et al.*, 2004) and males do not appear to be any less inclined to harass females that have recently mated. On an evolutionary time scale, harassment can lead to the evolution of morphological adaptations to reduce the cost. Andromorphs in damselflies are an extreme example, where mimicking males helps these morphs to avoid harassment (Sirot & Brockmann, 2001). Size could also play a role because, where the sexes are of similar size, it may generally be less energetically expensive for a female to refuse mating than for a male to achieve it against female resistance (Clutton Brock & Parker, 1995). Harassment cost therefore is expected to create positive selection on body size. In *C. maculatus*, males appear to impose a high harassment pressure on females despite their significantly smaller size. This might reflect an arms race where female size raises the cost of males' continuing attempts to mate, whereas males counteract by increasing the cost to enhance the probability that females will re-mate.

Most studies of sexual conflict over mating frequency focus on the benefits of re-mating for females, but few are interested in costs. The cost of mating can be separated into the cost of copulation (e.g. genital damage; Crudgington &

Siva-Jothy, 2000, Edvardsson & Tregenza, 2005), the cost of egg production (life-history trade offs; Rönn *et al.*, 2006) and the cost of harassment, which has often been neglected. In *C. maculatus*, recent results show that females tend to accept re-mating more easily when kept without water (Edvardsson, 2007): Females appear to adjust their re-mating frequency according to the balance between the benefit in terms of gained resources (mainly water) and the costs of mating, including the cost of harassment (Crudgington & Siva-Jothy, 2000; Edvardsson & Tregenza, 2005). The present results illustrate that a fuller understanding of the costs and benefits of re-mating requires separating and measuring the effect of exposure to males in terms of sexual harassment independently from that due to matings (genital damage).

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