Male gametocyte fecundity and sex ratio of a malaria parasite, *Plasmodium mexicanum*

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SUMMARY

Evolutionary theory predicts that the sex ratio of *Plasmodium* gametocytes will be determined by the number of gametes produced per male gametocyte (male fecundity), parasite clonal diversity and any factor that reduces male gametes’ ability to find and combine with female gametes. Despite the importance of male gametocyte fecundity for sex ratio theory as applied to malaria parasites, few data are available on gamete production by male gametocytes. In this study, exflagellating gametes, a measure of male fecundity, were counted for 866 gametocytes from 26 natural infections of the lizard malaria parasite, *Plasmodium mexicanum*. The maximum male fecundity observed was 8, but most gametocytes produced 2–3 gametes, a value consistent with the typical sex ratio observed for *P. mexicanum*. Male gametocytes in infections with higher gametocytaemia had lower fecundity. Male fecundity was not correlated with gametocyte size, but differed among infections, suggesting genetic variation for fecundity. Fecundity and sex ratio were correlated (more female gametocytes with higher fecundity) as predicted by theory. Results agree with evolutionary theory, but also suggest a possible tradeoff between production time and fecundity, which could explain the low fecundity of this species, the variation among infections, and the correlation with gametocytaemia.

Key words: malaria, sex ratio, male fecundity, *Plasmodium*, gamete production.

INTRODUCTION

Sex ratio theory is a venerable focus in evolutionary biology originating with Darwin’s verbal treatment and a mathematical model presented by Düssing (reviewed by Edwards, 1998). The application of the theory to sexually reproducing protists such as malaria parasites (*Plasmodium* and related genera, Martinsen et al. 2008), though, is much more recent (Godfray and Werren, 1996; Read et al. 1992; Schall, 2008, 2009). Read et al. (1992) recognized that one model in sex ratio theory, Local Mate Competition (LMC, Hamilton, 1967), fits the breeding structure of malaria parasites and can account for the variation in gametocyte sex ratio seen among malaria parasite species, among geographical populations within a species, and even within individual infections over time (Schall, 2009). This model concludes that the unbeatable sex ratio of populations with little genetic diversity will be strongly female-biased (with the specific bias dependent on the degree of inbreeding), but that this bias may be limited by male fecundity. The proportion of males in a population should have a minimum of $1/(1 + c)$, where $c$ is male fecundity, to ensure that all females are mated. This minimum is particularly important in predicting the sex ratio of clonal populations, such as single-genotype infections of *Plasmodium*, because there should be just enough males to mate with all females when inbreeding is complete. Despite the importance of fecundity for sex ratio theory, detailed data on gamete production in *Plasmodium* parasites are scarce.

The sexual cells (gametocytes) of malaria parasites are produced following bouts of asexual replication in the vertebrate host and remain in the host’s erythrocytes undergoing no further development until they are taken up by a blood-feeding insect vector (Garnham, 1966). Within the insect midgut, these sexually dimorphic gametocytes begin rapid gametogenesis. Each female gametocyte produces a single large gamete. The haploid male genome replicates 3 times to produce a maximum of 8 gametes (Alano and Billker, 2005), although gamete production can be lower if the process is truncated or if anucleate gametes are produced (Sinden, 1983; Sinden et al. 1978). The number of gametes produced by a male gametocyte (male fecundity) must be a life-history trait that is critical for fertilization and transmission success in addition to its predicted importance in shaping gametocyte sex ratio.

Although there are few data on male gametocyte fecundity related to sex ratio, several perplexing relevant observations emerge from data on the sex ratios of various *Plasmodium* species. First, the gametocyte sex ratios of *P. mexicanum* and other lizard malaria parasites tend to be far less female-biased than seen in...
the malaria parasites of birds and mammals (Schall, 2009), even for single-clone infections (Neal and Schall, 2010). Low male gametocyte fecundity in *P. mexicanum* and other reptilian malaria parasites is one possible explanation for less female-biased sex ratios. If variation in fecundity among taxa exists, why should such an important life-history trait vary, especially in a way that could reduce fertilization and transmission success? Second, gametocyte sex ratio appears to differ among parasite genotypes for *P. mexicanum* (Neal and Schall, 2010), for the rodent malaria parasite *P. chabaudi* (Reece et al. 2008) and for *P. falciparum* (Burkot et al. 1984; Ranford-Cartwright et al. 1993). Variation in fecundity within a species, like variation among taxa, is a possible cause of variation in sex ratio; if so, this again begs the question as to why there would be a genetic polymorphism for such an important life-history trait.

To cast light on this issue, I gathered data on the male fecundity and gametocyte sex ratio, size and density from natural infections of the lizard malaria parasite *P. mexicanum*. My goals were as follows. (1) To determine the fecundity of *P. mexicanum*. The sex ratios of single-clone infections of *P. mexicanum* are generally around 40% male (Neal and Schall, 2010), suggesting that the fecundity of this parasite is low. If the parasite conforms to the LMC model, a sex ratio of 40% male would predict a male fecundity of only 1–2. (2) To determine whether fecundity is related to other infection traits such as gametocyte size, the clonal diversity of the infection, asexual parasitaemia, or gametocytaemia. Schall (1989) found evidence that gametocytes in natural infections increase in size over time, so larger gametocytes may have more energy to invest in gamete production, or their large size might be due to swelling associated with senescence. Also, variation in fecundity might provide an alternative approach to fertility insurance (West et al. 2001). Fertility insurance predicts an increase in the proportion of male gametocytes in response to conditions that would reduce male gamete success to insure fertilization. A similar increase in the total number of male gametes could be achieved by increasing the number of gametes produced per male gametocyte. This could lead to variation in fecundity due to competition with other clones, interactions with the immune system (possibly related to asexual parasitaemia), or male gametes’ ease of finding females (related to gametocytaemia). (3) To test whether there is variation among infections for male gametocyte fecundity and whether it is correlated with sex ratio in single-clone infections. Neal and Schall (2010) found evidence of genetic variation for sex ratio, which could be caused by genetic variation for male fecundity. If this is the case, fecundity should vary among infections and be correlated with the sex ratios of single-clone infections.

**Materials and Methods**

**Infected lizards**

The infections of *P. mexicanum* used for this study were all sampled from naturally infected western fence lizards (*Sceloporus occidentalis*) captured during July–August 2009 at the University of California Hopland Research and Extension Center, located in Mendocino County, California, USA (Fricke et al. 2010). Drops of blood were taken from each lizard to produce a thin blood smear for Giemsa staining and dried and frozen drops on filter paper. Lizards harbouring gametocytes were identified by scanning the stained smears at 1000×. Infected lizards were housed outside in vector-proof cages for 3 days during which time 2 μl of blood were drawn daily for exflagellation experiments. The lizards were subsequently released at their site of capture.

**Male gametogenesis**

Lizards were kept in an incubator at 34 °C (the lizards’ preferred body temperature, Schall, 1990) for at least 30 min prior to inducing gametogenesis. Exflagellation was induced by mixing 2 μl of blood with 6 μl of exflagellation medium (50 mM NaHCO3) on a slide and covering it with a cover-slip. Other exflagellation media were tested in preliminary experiments, most of which contained combinations of glucose, Na+, and Cl–, and some additionally contained xanthurenic acid (Alano and Billker, 2005). Most combinations were not successful at inducing exflagellation, and none were as effective as the simple NaHCO3 solution (data not shown). Within minutes after mixing blood with the medium, the gametocytes rounded up into spheres similar to that seen in other *Plasmodium* species (Carter and Graves, 1988). Exflagellating gametocytes were first observed 20 min after the blood was combined with exflagellation medium and the highest proportion of gametocytes exflagellating occurred after about 25–35 min. Therefore slides were kept on a damp filter paper in a Petri dish for 25 min before observations began for counting gametes. The preparations were examined at 1000× for 10 min and, after locating each exflagellating gametocyte, I counted the number of flagella emerging and assumed that any gametocyte that was exflagellating was mature. The exflagellation rate of each infection was estimated by dividing the number of exflagellating gametocytes observed during this time-period by the total number of gametocytes observed during this time. This procedure was repeated on 3 consecutive days for each infected lizard and the time of day that the gamete counts were performed was varied to ensure that there was no variation in fecundity on a small time-scale.

**Clonal diversity within infections**

To determine the genetic diversity of each infection, DNA was extracted from the blood dots on filter
paper using a Qiagen DNEasy blood and tissue kit (Qiagen Sciences, Germantown, Maryland, USA). Samples were subjected to PCR for 4 microsatellite loci (Pmx 306, 732, 747, and 839) using primers and conditions presented by Schall and Vardo (2007). Anderson et al. (2010) showed that 4 loci could differentiate approximately 90% of genetic clones for Plasmodium falciparum. The primers used amplify DNA from both asexual and sexual stage parasites, but Vardo-Zalik (2009) showed that a high proportion of alleles (and thus clones) and their relative proportions detected using this method can later be found in the DNA of oocysts in the insect vector, suggesting that this method is useful for estimating the genetic diversity of the sexual cells alone. The PCR product was analysed using an ABI prism genetic analyzer and the results were viewed using the program GeneMapper (ABI, Carlsbad, California, USA). Because all stages of P. mexicanum found in the lizard are haploid, I scored an infection as having a single clone if only 1 allele was seen at each of the 4 loci. Multi-clone infections had at least 2 alleles for at least 1 of the loci.

Gametocyte sex ratio

To determine the sex ratio of each single-clone infection, stained slides were viewed at 1000× on a light microscope. Male and female gametocytes can be readily distinguished based on differences in staining as described by Schall (1989). Mature gametocytes were scored as male or female until 200 gametocytes were scored. Care was taken to view many fields from all areas of each slide to reduce the effect of possible aggregation of gametocytes. When there were >30 gametocytes per 1000 erythrocytes, avoiding possible aggregates was problematic, so these sex ratio counts \((n=2)\) were excluded from sex ratio analysis, although the infections were included in all other analyses.

The equation \(1/(1+fe\) where \(e\) is male fecundity, represents the minimum sex ratio (proportion of males) expected by LMC for any infection, and therefore the predicted sex ratio of single-clone infections. This equation was used to calculate the predicted sex ratio of each single-clone infection by using a summary statistic describing the average fecundity of each infection \((\lambda,\text{ described in more detail below})\) as an estimate of \(e\) in the equation above. Since previous studies have shown that a high proportion of gametes produced may be dysfunctional (Sinden, 1983; Sinden et al. 1978), I repeated this calculation assuming that on average only 40% or 77% of gametes were viable (the values observed by Sinden (60% anucleate, 1983) and Sinden et al. (23% anucleate, 1978)). Linear regression analysis was used to compare predicted sex ratios with the sex ratio counts for all single-clone infections.

Male gametocyte size and gametocytaemia

The size of gametocytes prior to the onset of gametogenesis was determined by photographing 30 male gametocytes per infection from stained slides using a Motic 1000 microscope camera. The area of both the gametocyte and the red blood cell it was infecting was measured using the software Motic Images Plus 2.0 (Motic China Group Co.). Gametocyte size was analysed in 2 ways: as the raw gametocyte area and as gametocyte area divided by red blood cell area. The ratio was meant to control for differences in gametocyte size that might have arisen from unknown variation in making smears. Gametocytaemia and asexual parasitaemia were determined by counting 1000 erythrocytes on a stained slide and noting the number of gametocytes or asexual parasites observed therein. Both are expressed here as parasites per 1000 erythrocytes. Slides were viewed at 1000× on a light microscope and care was taken to sample a wide range of locations on the slide.

Statistical analysis

All statistical analysis was performed using JMP 8 (SAS Institute, Cary, NC, USA) except the multiple regression analysis, which was performed using SAS 9.2 (SAS Institute, Cary, NC, USA). When 1.0 was subtracted from the number of gametes produced by each gametocyte, these counts did not differ significantly from a Poisson distribution \((P=0.298)\), so this transformation was used for all analyses.

To compare fecundity with other infection traits, a Poisson distribution was fitted to the gamete counts from each infection and \(\lambda\), the measure of central tendency for a Poisson distribution, was measured. A multiple regression with backward selection (terms were sequentially removed from the maximal model) tested the effect of logarithmically transformed gametocytaemia, logarithmically transformed asexual parasitaemia, and gametocyte size (corrected and uncorrected, 2 separate models) on fecundity \((\lambda)\). A second multiple regression using the same selection method examined the effect of logarithmically transformed gametocytaemia and fecundity \((\lambda)\) on sex ratio.

A generalized linear model (GLM) with Poisson distribution and Log link function was used to determine whether there was variation among infections for the number of gametes produced by each male gametocyte (male fecundity). A second GLM tested whether the clonal diversity of an infection (single vs multiple clones) affected gamete production. The day on which counts were performed (1, 2 or 3) was included as a model effect in both GLM to account for replication. ANOVA tested for variation in gametocyte size (corrected and uncorrected by erythrocyte area) among infections. The variation in fecundity within infections for single- and
multi-clone infections was examined using range (maximum minus minimum male fecundity) and median absolute deviation (MAD, a robust measure of spread, Hoaglin et al. 1983). When 3 was subtracted from the range, the values did not differ from a Poisson distribution, and thus a GLM analysis was performed.

RESULTS

Distribution of male gametocyte fecundities
Gamete counts were performed for 26 naturally infected lizards for a total of 866 exflagellating gametocytes examined. For one infection, only 1 exflagellating gametocyte was observed and the infection and its gamete count were therefore excluded from further analysis. The number of exflagellating gametocytes observed for each infection over the course of 3 days ranged from 7 to 91 with a mean of 34·6. The day on which counts were performed did not affect fecundity (P > 0·4, GLM). The median and mode of all gamete counts combined was 3 (range=1–8, λ = 2·69; Fig. 1). The median gamete counts and λ (shown in Fig. 2 with confidence intervals) for each infection ranged from 2 to 4.

Factors affecting male fecundity
Out of 25 infections, there were 23 unique genotypes based on the 4 microsatellite markers. Two pairs of infections had identical genotypes. There was significant variation among infections for male fecundity (P < 0·0001, GLM), but it did not differ for the 2 pairs of infections with the same genotype (P > 0·60, GLM). Fig. 2 shows fecundity (λ) values and their confidence intervals for each infection. Gametocytaemia was the only significant predictor of fecundity in the multiple regression model (R² = 0·209, P = 0·022). While asexual parasitaemia (range <1–272) and gametocytaemia (range 1–176) were significantly correlated (R = 0·675, P = 0·0002), simple correlation confirms the lack of significant correlation between asexual parasitaemia and fecundity (R = −0·180, P = 0·387). Male gametocytes in infections with higher gametocytaemia produced fewer gametes (Fig. 3).

When tested separately, both single- and multi-clone infections showed significant variation in fecundity among infections (single: P < 0·0001, multi: P = 0·048, GLM), although there was no difference in fecundity between the single- and multi-clone infections overall (P = 0·603, GLM). Also, the range of individual male gametocyte fecundities observed in single- and multi-clone infections did not differ (P = 0·959, GLM). The MAD for all infections except 2 (1 single- and 1 multi-clone infection) was 1·0. Because almost no variation existed in MAD...
of gamete production, no statistical analysis was performed on these values.

There was no relationship between gametocyte size and the exflagellation rate (range 10–30%) of the infections \((P > 0.30, R^2 < 0.05, \text{linear regression})\), and although both gamete production \((\text{above})\) and gametocyte size \((P < 0.0001, \text{ANOVA})\) varied significantly among infections, gametocyte size was not a significant predictor of fecundity \((\text{above, multiple regression})\).

**Sex ratio and fecundity**

The average sex ratio of single-clone infections in this study was 43.8% male and ranged from 34 to 58% male. Fecundity was the only significant predictor of sex ratio in the multiple regression model \((R^2 = 0.371, P = 0.036)\). While gametocytaemia and fecundity are correlated \((R = -0.457, P = 0.022)\), simple correlation confirms the lack of significant correlation between gametocytaemia and sex ratio \((R = -0.073, P = 0.804)\).

Linear regression showed a significant correlation between predicted and observed sex ratio for all percentages of successful gametes tested \((40, 77, 100\% \text{ successful, } R^2 > 0.34, P < 0.05)\). The slope of the regression line is similar to the slope of \(y = x\) (as expected if observed = predicted) over a range of values for the percentage of successful gametes. If 40% of gametes are successful, there is a close concordance between observed and predicted values (Fig. 4).

**DISCUSSION**

Although sex ratio theory originated with studies by Darwin and Düsing about the same time that the malaria parasite’s life cycle was being revealed (Edwards, 1998; Schall, 2009), the usefulness of selectionist thinking to explain the within- and among-species variation in gametocyte sex ratios was only recognized over the past 2 decades (Paul et al. 1999; Read et al. 1992; Schall, 1989). In particular, the model of Local Mate Competition has suggested that parasite clonal diversity in the vertebrate host blood (and thus the degree of inbreeding) and male fecundity are central in moulding the proportion of male and female gametocytes produced in an infection (Reece et al. 2008, 2003). This present study centred on male gametocyte fecundity of the lizard malaria parasite, *P. mexicanum*, to pursue several questions. Is the male fecundity of *P. mexicanum* low (1–2) as expected under the LMC model based on the only slightly female-biased gametocyte sex ratio seen in single-clone infections of this species? Is there genetic variation for male fecundity as suggested by the apparent genetic variation in gametocyte sex ratio for this species? Does male fecundity vary based on gametocyte density, asexual parasite density, gametocyte size, or infection clonal diversity? Do male fecundity and gametocyte sex ratio co-vary among infections as predicted by sex ratio theory? The results reported here suggest that fecundity is low, as expected, and varies among infections. Further, fecundity was found to be correlated with gametocytaemia, and the relationship between the fecundity and sex ratio of single-clone infections is very similar to the prediction of LMC.

**Male gametocyte fecundity**

Studies on *P. falciparum* show that the parasite undergoes 3 nuclear divisions within the male gametocyte, leading to a maximum of 8 male gametes (Alano and Carter, 1990; Lobo and Kumar, 1998; Sinden, 1985). *P. mexicanum* produced a maximum of 8 male gametes, but this was uncommon. Most male gametocytes produced 2–3 gametes, suggesting that a large proportion of male gametocytes do not achieve their maximal potential fecundity. Reece et al. (2008) found that male gametocytes of a clone of *P. berghei* produce a mean of 2.03 viable gametes and Ranford-Cartwright (1995) estimated that 2 clones of *P. falciparum* both produced an average of 4–5 gametes per gametocyte. The data from *P. mexicanum* presented here is additional support for the possibility that male gametocytes often fail to achieve their maximal potential fecundity and, combined with data from other species referenced above, suggests that there may be variation in fecundity among species.
Also, variation in fecundity was found among the infections used in this study. While it is unclear from the data presented here whether this variation was caused by primarily genetic or environmental factors, Neal and Schall’s (2010) study found a donor effect on sex ratio in replicate single-clone infections suggestive of genetic variation for gametocyte sex ratio, which could be explained if there is genetic variation for fecundity.

**Fecundity and other infection traits**

The only factor measured in this study that appears to affect male fecundity is gametocytaemia: infections with a lower density of gametocytes tend to have higher male fecundity. If there is a tradeoff between fecundity and speed of gamete production (discussed below), low gametocytaemia may select for higher fecundity at the expense of speed to ensure that there are sufficient male gametes to locate the rare female gametes in the mating environment. Adjusting fecundity in response to low gametocytaemia could be an alternative to the sex ratio shift traditionally predicted by Fertility Insurance, and would result in a similar increase in the proportion of male gametes available for mating.

If fecundity adjustment is a means of Fertility Insurance, fecundity might also be predicted to correlate with asexual parasitaemia, since asexual parasitaemia may often be related to an immune response. It is asexual parasites that often trigger an immune response, and immune factors carried over from the lizard could kill male gametes in the mating environment. However, I found no evidence of such a relationship. It is possible that an immune response, if mounted, would affect gamete success more than gamete production and would therefore not be detectable in a study that measures only gamete production. Also, little is known about the response of the lizard immune system to *P. mexicanum*, but data (summarized by Neal and Schall, 2010) suggest the immune response is weak or nonexistent.

Competition for mating success might also be predicted to cause differences in fecundity between single- and multi-clone infections if fecundity is plastic, or a greater range in fecundity in multi-clone infections if fecundity is fixed but varies among genotypes. However, no difference in fecundity or range of fecundity between single- and multi-clone infections was observed. Gametocyte size, which varies among infections, was also found to be unrelated to both fecundity and exflagellation rate. Schall (1989) observed that gametocytes of *P. mexicanum* increase in size over the course of natural infection, but larger gametocytes have no increased mating potential based on the factors measured here.

**Sex ratio and fecundity**

The average sex ratio of single-clone infections in this study (43.8% male) was almost identical to the sex ratio reported by Neal and Schall (2010) for experimentally induced infections and Schall (1989) for natural infections. Sex ratio was associated with fecundity in single-clone infections, and the sex ratios predicted under LMC using these fecundities fit the observed values well. If only about 40% of gametes are successful on average (due to deformation or factors associated with fertility insurance), the regression closely approximates the line \( y = x \). This concordance with evolutionary theory is striking because the variation in both gametocyte sex ratio and fecundity was not great among infections and argues that selection must be intense on moulding gametocyte sex ratio in malaria parasites (Reece et al. 2008). Additionally, the assumption of 40% successful gametes is consistent with Sinden’s (1983) observation that up to 60% of gametes produced are not functional, suggesting that such low gamete success is plausible and perhaps even likely.

**Low fecundity: constrained or adaptive**

Male fecundity appears low for *P. mexicanum*, and may drive gametocyte sex ratio toward a higher proportion of male cells than observed in other *Plasmodium* species, including the human malaria parasites (Schall, 2009). Why should fecundity, which must be an important life-history trait, be so often reduced below the maximum possible of 8 gametes in this species? It seems that low fecundity must either be due to constraint, meaning that gametocytes are unable to produce more gametes given certain conditions, or be adaptive.

Possible constraints on gametogenesis include resource limitation, gametocyte maturity and time. Alano and Billker (2005) propose a trade-off between speed and quantity in gamete production such that gametocytes that rapidly produce gametes are not able to consistently or accurately produce a maximum number. Thus time constraints placed on gametogenesis by removal of water from the bloodmeal in the insect’s midgut (Alano and Billker, 2005) or the rapid clotting of blood seen in fence lizards infected with *P. mexicanum* (Vardo-Zalik and Schall, 2008) could select for faster gamete production and thus lower fecundity.

Adaptive reductions in fecundity may also be feasible, particularly if a trade-off between speed and quantity of gamete production exists. Some conditions might favour the quick production of fewer gametes (such as competition among clones), whereas others might favour slower production of more gametes (such as infections with low gametocytaemia, above). Reduction in fecundity could be facultative, allowing the parasite to respond to immediate
conditions, or fixed, for example if genes that tend to cause high gametocytaemia and low fecundity travel together.

The results presented here are contradictory: the correlation between gametocytaemia and fecundity found in this study suggests that low fecundity may be, at least in part, adaptive, but there was no relationship between the clonal diversity and fecundity. It is possible that low fecundity is adaptive but not plastic; however, further investigation is necessary before a more definitive conclusion can be reached. Knowing whether fecundity and timing of gametogenesis are in fact related, and how the length of gametogenesis relates to the time available to the parasite in the sand fly could help determine whether low fecundity is due to a time constraint. If low fecundity is adaptive, it would be interesting to vary environmental conditions such as gametocytaemia and determine whether the parasite shifts its fecundity in response.

Potential sources of error in measures of male fecundity

The number of flagella emerging during exflagellation was used here to estimate male gametocyte fecundity. Since the in vitro environment in which this study was carried out is quite different from a sand fly midgut, some care should be taken in interpreting these results. Also, several sources of error could bias these results. Sinden et al. (1978) reported that gametes of *P. falciparum* are not always released synchronously. If this is also true for *P. mexicanum*, some gametes may have already left a gametocyte when it was examined. Also, not every gamete was stained for a nucleus, and thus the proportion of gametes that were not nucleated but were counted would lead to an overestimate of fecundity, whereas gametes that had already escaped and consequently were not counted would lead to an underestimate of fecundity. For the study of variation among infections, all infections should have been affected by these factors randomly, and thus should not affect the interpretation of the results on variation in fecundity.

Conclusion

The observed patterns connecting male gametocyte fecundity with gametocyte density and sex ratio are striking, especially when several sources of error in counting male gametes and gametocyte sex ratio will result in some degree of experimental error. The results agree with sex ratio theory, both in a broad sense (the low fecundity of males was predicted based on the distribution of gametocyte sex ratios) and within infections (the higher production of male gametocytes in infections with lower fecundity).

These findings argue that the sexual stages in the life history of malaria parasites continue to offer intriguing problems for an evolutionary and ecological approach to the study of *Plasmodium* biology.

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