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NOTE

Test of immunocompetence handicap hypothesis in mice (*Mus musculus*) infected with *Trichinella spiralis*

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Abstract: The immunocompetence handicap hypothesis (ICHH) proposes that testosterone enhances the expression of sexual traits but suppresses immune function. However, studies to test the hypothesis have shown mixed results. Alternatively, sexual traits, immune function, and parasite susceptibility may be mediated by the stress hormone corticosterone. Here, we report an experimental test of the ICHH that included the manipulation of both testosterone and parasites in male laboratory mice (*Mus musculus* L., 1758). We conducted a factorial experiment, injecting each individual mouse with testosterone or not and infecting them with the nematode parasite *Trichinella spiralis* (Owen, 1835) or not. As predicted, testosterone enhanced the scent attractiveness of male mice, whereas parasite infection reduced it, but only in male mice not injected with testosterone. However, we found no evidence that corticosterone is involved in mediating the effects of testosterone. These results confirm that maintaining high testosterone levels entails the cost of increased parasite abundance. This study provides direct evidence supporting the ICHH.

Key words: immunocompetence handicap hypothesis, infection, testosterone, corticosterone, scent attractiveness, *Trichinella spiralis*.

Résumé : L'hypothèse du handicap de l'immunocompétence (HHIC) veut que la testostérone rehausse l'expression des caractères sexuels, mais réduise la fonction immunitaire. Les études visant à vérifier cette hypothèse ont toutefois produit des résultats mitigés. Par ailleurs, la corticostérone, une hormone de stress, peut avoir une incidence sur les caractères sexuels, la fonction immunitaire et la susceptibilité aux parasites. Nous faisons état d'un test expérimental de la HHIC qui comprend la manipulation de la testostérone et de parasites chez des souris de laboratoire (*Mus musculus* L., 1758) mâles. Nous avons mené une expérience factorielle consistant à injecter ou non de la testostérone dans chacune des souris, et de les infecter ou non avec le nématode parasite *Trichinella spiralis* (Owen, 1835). Conformément aux prédictions, la testostérone rehaussait l'attirance par l'odeur des souris mâles, alors que l'infection par le parasite la réduisait, mais seulement chez les mâles n'ayant pas reçu d'injection de testostérone. Rien n'indique cependant que la corticostérone intervient dans la médiation des effets de la testostérone. Ces résultats confirment le fait que le maintien de fortes concentrations de testostérone entraîne un coût découlant d'une plus grande abondance de parasites. L'étude fournit des observations directes à l'appui de la HHIC. [Traduit par la Rédaction]

Mots-clés : hypothèse du handicap de l'immunocompétence, infection, testostérone, corticostérone, attirance par l'odeur, *Trichinella spiralis*.

Introduction

Numerous studies have shown that scent cues from the urine of male mice (*Mus musculus* L., 1758) provide honest indicators of an individual's health and infection status, which can be used to detect and avoid infected conspecifics (Penn and Potts 1998; Møller et al. 1999). A wide variety of infections alter male scent attractiveness and infected mice may therefore be less attractive to conspecific female mice (reviewed by Kavaliers et al. 2005). The reduced scent attractiveness of the infected male mice is probably driven by the evolutionary need for female mice to choose mates with heritable parasite resistance to protect their progeny (Hamilton and Zuk 1982) and (or) to avoid the sexual transmission of contagious diseases (Able 1996).

The mechanisms underlying these infection-induced changes in odor attractiveness have drawn considerable attention. The immunocompetence handicap hypothesis (ICHH) proposes that sexual traits honestly signal a male individual's health because testosterone (T) has dual effects, triggering the development of sexual traits but also suppressing the immune functions (Folstad and Karter 1992). To reduce the immunosuppressive effects of this steroid hormone, infected male individuals lower their T levels (e.g., Barthelemy et al. 2004; Greiner et al. 2010), which then reduces the development and expression of T-dependent sexual traits (Møller et al. 1999). Therefore, only "high-quality" individuals that can afford to suppress their immune functions, such as male individuals genetically resistant to infection (Zala et al. 2008), can maintain exaggerated sexual traits. Although the assumptions of the ICHH have been tested in many studies, a meta-analysis of the experimental manipulation of T found limited support for the immunosuppressive effects of T (Roberts et al. 2004).

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Experimentally elevated T may act on some parasites by altering the behaviors of the host (e.g., more aggressive behavior, greater space use, increased contact rates), enhancing the exposure to and transmission of the parasites (Martínez-Sanchis et al. 2003; Seivwright et al. 2005; Grear et al. 2009). Moreover, T may not suppress the immune functions in a way that causes a universal increase in infection intensity. Fuxjager et al. (2011) demonstrated that T acts differently on different types of parasites within the same system.

Because other biologically active substances that interact with T might also mediate the ICHH (Folstad and Karter 1992), we cannot ignore the hypothalamic–pituitary–adrenal (HPA) axis, which is activated in response to environmental stressors (Wingfield et al. 1998; Buchanan 2000). Infections have considerable potential to induce a chronically stressed state. In this case, the glucocorticoid corticosterone (CORT) secreted by the HPA axis can affect the immune and reproduction functions (McEwen et al. 1997; Sapolsky et al. 2000; Tilbrook et al. 2000). According to the stress-linked ICHH (Evans et al. 2000), the immunosuppressive effects of T act indirectly, through the positive covariance with circulating CORT levels. There is some evidence for the role of CORT in the ICHH (e.g., Evans et al. 2000; Owen-Ashley et al. 2004; Mateos 2005), although no positive link has been established between T and CORT (e.g., Bortolotti et al. 2009). In another study, Ezenwa et al. (2012) showed that fecal T levels correlated positively with cortisol in Grant's gazelle (*Nanger granti* (Brooke, 1872)), but there was no evidence that the association between T and immune functions is influenced by cortisol.

Therefore, the mechanism by which T and CORT interact to affect the immune functions is too complicated to allow us to draw conclusions yet, and different adaptive solutions have been proposed across different species (both host and parasite) and different contexts (e.g., Alonso-Alvarez et al. 2007; Mougeot et al. 2007; Bortolotti et al. 2009). In this study, we tested the potential roles of T in mice infected with the nematode parasite *Trichinella spiralis* (Owen, 1835), in both the expression of sexual traits and the response to infection. We conducted a factorial experiment and manipulated both the mice's T levels (by injection; hormonal treatment or HTREAT) and parasite infection (by artificial inoculation; parasite treatment or PTREAT) in male laboratory mice (outbred ICR strain), and determined their serum CORT levels as a measure of their responses to stress. To understand whether T mediates the association between expression of sexual traits and parasite infection, we tested the effects of T on male scent attractiveness and parasite abundance, while simultaneously considering the potential effects of CORT. We predicted that experimentally elevated T would enhance the expression of sexual traits (increased male scent attractiveness) and increase parasite abundance after infection, whereas parasite infection would reduce scent attractiveness. We also predicted that the experimental treatments, as stressors, would induce increased CORT levels.

Materials and methods

We conducted our experiment with 40 males and 60 females of outbred ICR mice (8–10 weeks old). After acclimation for 2 weeks, the male mice were randomly assigned to one of four groups (10 subjects/group, day 0): control T injection, no parasite infection (T0P0); control T injection, orally inoculated with *T. spiralis* muscle larvae (T0P+); T injection, no parasite infection (T+P0); and T injection, inoculated with *T. spiralis* larvae (T+P+).

The T0 male mice were injected subcutaneously with 200 μL of pitch oil and the T+ male mice with 200 μL of 10 mg Omnadren-250 (Jelfa, Jelenia Góra, Lower Silesia, Poland) diluted in pitch oil. Omnadren-250 consists of four testosterone ethers, which at this dose produce a stable increase in serum T levels within the physiological limits, over a 4 week period (Litvinova et al. 2005).

The P0 male mice received 200 μL of phosphate-buffered saline (PBS, pH 7.4) and the P+ male mice were orally inoculated with 200 ± 10 muscle larvae of *T. spiralis* suspended in 200 μL of PBS. *Trichinella spiralis* is a nematode parasite with a wide range of mammalian hosts and a direct life cycle. Newborn larvae, produced in the small intestine by adult female worms, travel throughout the bloodstream (intestinal phase) and enter muscle cells, where they grow to the infective stage of the nematode (chronic phase; Gottstein et al. 2009). Infection mainly occurs when raw muscle tissue contaminated with infective larvae is consumed (Gottstein et al. 2009). Wild animals are frequently infected with low numbers of larvae of *Trichinella* spp. However, high infective doses of larvae (about 200 muscle larvae per subject) are generally used to induce a strong immune response (e.g., Furze and Selkirk 2005; Kołodziej-Sobocińska et al. 2006). The larvae of *T. spiralis* (genotype T1) used for inoculation were obtained from previously infected Kunming mice. The muscles of the infected mice were artificially digested within 1% pepsin–HCL solution at 37 °C for 18 h, according to the standard method (Pozio 2007), and maintained in PBS until the inoculation procedure. At the end of the experiment, the male mice were killed by cervical dislocation. Their eviscerated carcasses were digested and parasite abundance was estimated with direct muscle larval counts under a microscope at 40× magnification.

On day 30 after T injection and parasite infection, we collected blood samples within 5 min via the tail vein for hormone assays. The blood samples were incubated at 37 °C for 1 h and then centrifuged at 3000*g* for 15 min. The resulting serum samples were each pipetted into a tube and stored at –20 °C until analysis. The T assays were performed with a commercially available enzyme immunoassay (EIA) kit (Testosterone EIA Kit #ADI-900-065; Assay Designs, Ann Arbor, Michigan, USA). The CORT assays were performed with the Corticosterone EIA Kit (ADI-900-097; Assay Designs). The samples were diluted 1:20 and assayed in duplicate. Hormone levels are expressed as nanogram/millilitre of serum.

We also collected urine samples with gentle abdominal massage at days 0 and 30 after treatment, which were immediately stored in tubes at –20 °C until analysis. A female olfactory test was conducted for the scent attractiveness assays. The preferences of virgin female mice for two urine samples were tested in their home cages during the dark phase as described previously (Zhang et al. 2007). Briefly, for each test, we kept one subject as the test female mouse in the home cage, while temporarily removing its cage mates into a clean mouse cage. The urine samples were presented to the female mouse with disposable glass capillaries, one end of which was manually injected with a 2 μL urine sample. The other end of the capillary was sealed to suspend the sample inside the capillary. Holding the sealed end, the two capillaries containing the before- and after-treatment male scents were presented simultaneously to the female mouse, separated by approximately 2 cm. We recorded the investigative behavior of the female mouse for 3 min after she first showed a sniffing response. The cumulative times (seconds) during which the test female mouse investigated the urine samples were recorded with two stopwatches. The differences between the times that the female mouse spent investigating the urine samples collected before and after treatment were calculated to estimate the changes in scent attractiveness. A total of 60 female mice were used randomly for all tests and each female mouse was used only once each day. Any mouse that did not respond to the scents within 3 min was excluded from the test that day.

All animals were cared for in accordance with the *Guide for the care and use of laboratory animals* (The National Research Council 1996). All animal procedures were reviewed and approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

All groups consisted of 10 mice and all experiments were repeated at least three times. Before all statistical analyses, the data

Table 1. Two-way ANOVA of the treatment effects on concentrations of serum testosterone and serum corticosterone and the changes in scent attractiveness of male mice (*Mus musculus*).

	Testosterone			Corticosterone			Changes in scent attractiveness		
Dependent variable	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
HTREAT	1, 36	35.27	<0.001	1, 36	0.37	0.55	1, 36	11.52	0.002
PTREAT	1, 36	0.00024	0.99	1, 36	2.93	0.095	1, 36	9.36	0.004
HTREAT × PTREAT	1, 36	0.017	0.9	1, 36	0.22	0.64	1, 36	0.22	0.64

Note: HTREAT is hormone treatment (T0, control injected; T+, testosterone injected) and PTREAT is parasite treatment (P0, control inoculated; P+, inoculated with the nematode parasite *Trichinella spiralis* muscle larvae).

were examined for normality of variance with the Kolmogorov–Smirnov test. We used two-way analysis of variance (ANOVA) to evaluate the effects of HTREAT, PTREAT, and their possible interaction (PTREAT × HTREAT) on *T. spiralis* abundance, hormones levels, and the changes in scent attractiveness. Comparisons of the parasite abundance between groups were made with the Student's *t* test. Because the behavioral data were not normally distributed, the Wilcoxon test was used to test the differences in male scent attractiveness before and after treatments. Pearson's correlation analysis was used to determine the correlations between CORT levels and T levels, *T. spiralis* abundance, or scent attractiveness. All analyses were performed with SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA). Data are shown as means ± SE unless otherwise stated and differences were considered statistically significant when $p \leq 0.05$.

Results

Effects of treatments on *T. spiralis* abundance

At the end of the experiment, the P0 male mice contained no detectable *T. spiralis* larvae, whereas the T0P+ male mice contained 902 ± 102 larvae (mean ± SD; $n = 10$) and the T+P+ male mice contained 1186 ± 128 larvae (mean ± SD; $n = 10$). The variations in *T. spirals* abundance were explained by the parasite treatment (PTREAT: $F_{[1,36]} = 1627.26$, $p < 0.001$), the hormone treatment (HTREAT: $F_{[1,36]} = 30.11$, $p < 0.001$), and the PTREAT × HTREAT interaction ($F_{[1,36]} = 30.11$, $p < 0.001$). Among the P+ male mice, *T. spiralis* abundance was significantly higher in the T+ male mice than in the T0 male mice ($t_{[18]} = -5.49$, $p < 0.001$).

Effects of treatments on T, CORT, and scent attractiveness

T levels differed significantly between treatment groups (Table 1, Fig. 1*a*). T+ male mice had higher T concentrations than T0 male mice (Table 1: significant HTREAT effect; Fig. 1*a*). The parasite treatment had no significant effect on the T concentrations (Table 1: no significant PTREAT effect and HTREAT × PTREAT effect; Fig. 1*a*).

T injection and parasite infection did not alter CORT levels (Table 1: no significant HTREAT effect, PTREAT effect, and HTREAT × PTREAT effect; Fig. 1*b*). However, the parasite treatment had a marginally significant effect on CORT concentrations ($0.05 < p < 0.1$; Table 1, Fig. 1*b*).

The changes in the attractiveness of the male scent collected before and after treatments differed significantly between groups (Table 1, Fig. 1*c*). The hormone treatment caused a significant increase in male scent attractiveness (Table 1: significant HTREAT effect; Fig. 1*c*), whereas the parasite treatment had a negative effect on scent attractiveness (Table 1: significant PTREAT effect; Fig. 1*c*). In the T+P+ male mice, scent attractiveness returned to the before-treatment levels ($Z = -1.28$, $p = 0.20$; Fig. 1*c*).

Correlations between CORT and T, *T. spiralis* abundance, or scent attractiveness

There was no relationship between CORT and T levels. A partial correlation analysis, controlling for hormone treatment, revealed a marginally significant positive relationship between CORT levels and *T. spiralis* abundance ($r = 0.30$, $p = 0.064$; Fig. 2). However, we found no significant correlation between CORT levels and scent attractiveness.

Discussion

Our hormonal treatment successfully elevated T levels, but these levels did not exceed the natural variation measured in ICR mice (Moshkin et al. 2003; Litvinova et al. 2005). Because muscle larvae were detected in the P+ male mice at the end of the experiment, the infective dose used in this study successfully developed chronic *T. spiralis* infections in male mice. The experimentally elevated T levels in the male mice cause an increase in parasite intensity, as predicted under the T-induced immunosuppression paradigm. This is consistent with the results of Reddington et al. (1981), who showed that gonadectomized female mice injected with T shed more *T. spiralis* muscle larvae than male mice. T-induced increases in susceptibility to infection have also been demonstrated in several host–parasite systems (e.g., Hughes and Randolph 2001; Mougeot et al. 2006; Cox and John-Alder 2007). Because the number of newborn larvae produced and the number that survive depend on the host immune response (Pozio 2007; Gottstein et al. 2009), our results indicate that T exerts an immunosuppressive effect on the mouse response to *T. spiralis* infection. The immune response to *T. spiralis* infection appears to be life-long and has been shown to involve the synergistic actions of humoral and cell-mediated mechanisms (Fabre et al. 2009). Further studies should address the concrete mechanisms underlying the role of T in immunosuppression during *T. spiralis* infection.

Alternatively, the mouse's susceptibility to infection may be increased by behavioral changes that increase their exposure to and transmission of the parasite. Elevated T levels have been shown to correlate with typical changes in the host's activities and territorial behaviors (Martínez-Sanchis et al. 2003; Grear et al. 2009). However, because the male mice were housed individually in this study, we believe that their susceptibility to *T. spiralis* infection was not strongly influenced by their behavior in this experiment, although the infection of a natural host population may be influenced by elevated T-induced behavioral changes.

As expected, elevated T enhanced the attractiveness of male scent, whereas inoculation with the parasite reduced male scent attractiveness, but only in T0P+ male mice. Parasite inoculation did not suppress scent attractiveness in T+ male mice. Because the scent attractiveness of the male rodent is T-dependent, we infer that the T treatment allowed the individual P+ males to develop and maintain sexual traits. However, we found no difference in the T levels of P+ and P0 male mice, which suggests that other factors are responsible for the influence of parasite infection on scent attractiveness in male mice. One possible reason for the decline in scent attractiveness in infected male mice is the modulation of the activity of the HPA axis by stressors, here *T. spiralis* infection. However, inconsistent with previous studies that have reported elevated CORT release in *T. spiralis*-infected rodents (Furze and Selkirk 2005; Tumkiratiwong et al. 2006), we found that CORT levels increased only marginally in the parasite-infected male mice in this study. This may be attributed to the infective dose of parasite used in our experiment, because

Fig. 1. Effects of testosterone and parasite treatments on (*a*) serum testosterone concentrations (ng/mL), (*b*) serum corticosterone concentrations (ng/mL), and (*c*) changes in scent attractiveness (s) in male mice (*Mus musculus*). Values are means ± SE. T0, control injected; T+, testosterone injected; P0, control inoculated; P+, inoculated with the nematode parasite *Trichinella spiralis* muscle larvae.

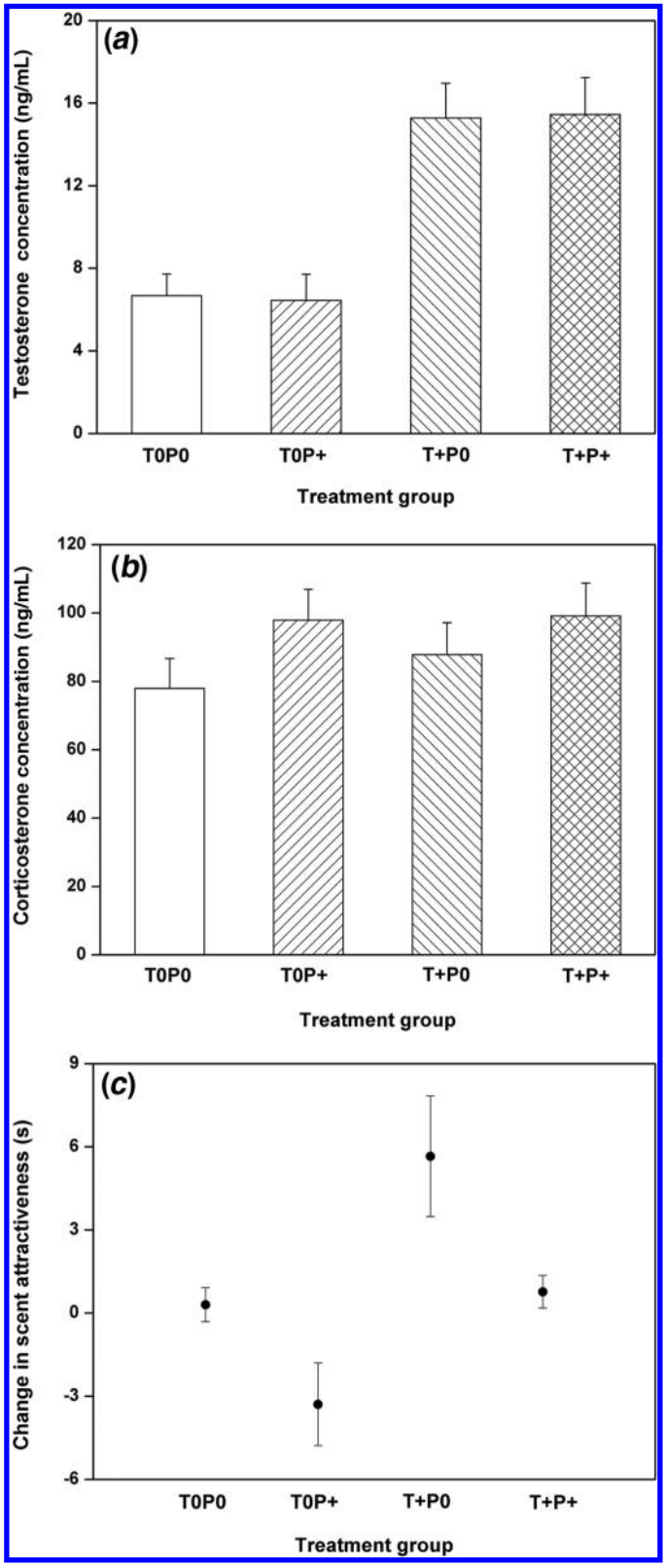
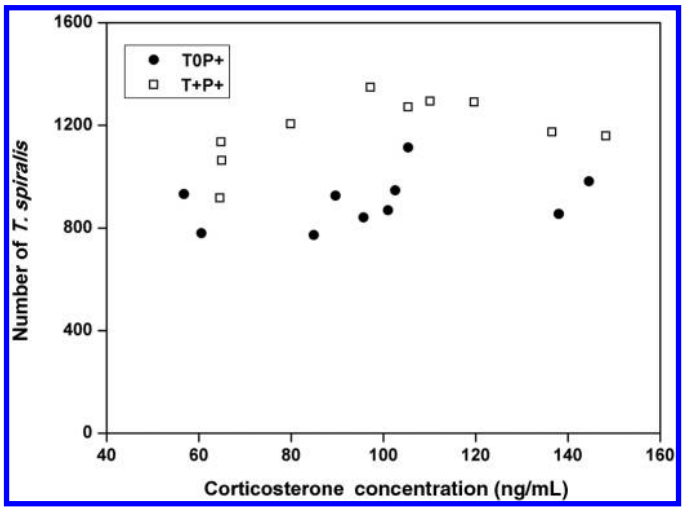


Fig. 2. Relationships between serum corticosterone levels (ng/mL) and numbers of the nematode parasite *Trichinella spiralis* muscle larvae in male mice (*Mus musculus*). T0, control injected; T+, testosterone injected; P+, inoculated with *T. spiralis* muscle larvae.



changes in circulating CORT levels are related to the intensity of the infection of *Trichinella* spp. (Bailenger et al. 1985; Furze and Selkirk 2005). This was confirmed by the marginally positive correlation between CORT levels and parasite abundance in our study. Despite the nonsignificant increase in CORT levels, the CORT secreted by the stimulated HPA axis has previously correlated negatively with scent attractiveness in male mice (Moshkin et al. 2002). Therefore, the reduced scent attractiveness of *T. spiralis* infected male mice might have been partly affected by changes in CORT levels.

In conclusion, our results strongly support the ICHH, because male mice that were infected with *T. spiralis* displayed scent attractiveness to female mice equivalent to that of healthy individuals when their T was elevated experimentally and tolerated a greater parasite burden. Although we failed to find potent evidence that CORT is involved in the immunosuppressive effects of T, we cannot rule out the possibility that CORT plays important roles in the host–parasite system and in parasite-mediated sexual selection because the activity of the HPA axis seemed to depend on the infective dose of parasite used in our study. The ICHH predicts that elevated T levels entail the physiological cost of suppressed immunity (Folstad and Karter 1992). Our study demonstrates that male mice benefit from high levels of T but that elevated T entails a fitness cost in terms of increased parasite susceptibility. Thus, we have shown that T is an important mediator in the life-history trade-offs in male mice.

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