Toe-clipping does not affect toad's short-term locomotor performance

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In this work, I assessed the effects of toe-clipping on sprint speed and run rate (number of runs per meter) of *Epidalea calamita* toads from agricultural land and their natural habitat. I videotaped toads in individual trials along a linear runway. Afterwards, I randomly assigned toads to two groups: control toads, which were not clipped, and experimental toads, whose third toe in each hind limb was immediately clipped. One hour later, I videotaped a second trial. Sprint speed in the first and second trials was similar in both groups. Likewise, run rate was not affected by toe-clipping. Sex and habitat had no effect on the effect of toe-clipping on locomotor performance. These findings suggest that toe-clipping should not increase toads' risk to become prey or hinder searching for prey or mates.

Introduction

Toe-clipping is widely used in research for different purposes. In demography, toe-clipping can be used as a marking technique in mark-recapture studies (Phillott *et al.* 2007), or to estimate age using skeletochronology (Comas *et al.* 2016, Zamora-Camacho & Comas 2017). Also, clipped toes provide suitable tissue samples for genetic studies (Gonser & Collura 1996), as well as for pathogen (Hyatt *et al.* 2007, St-Amour & Lesbarrères 2007) and contaminant detection (Todd *et al.* 2012, Pflieger *et al.* 2016). Toe-clipping is a non-lethal procedure to mark animals and obtain a tissue sample. It can be conducted in the field without need to transport animals to a laboratory. Therefore, this marking method is particularly convenient in declining populations or species.

There is, however, no consensus on whether or not toe-clipping is in line with ethical animal treatment (Minteer & Collins 2005) or affects individual fitness, animal welfare, or population viability (Castle *et al.* 2016). This issue is being debated (Phillott *et al.* 2007, Parris & McCarthy 2008, Phillott *et al.* 2008), since some studies have highlighted ethical concerns regarding toe-clipping (May 2004, Parris *et al.* 2010), while others considered toe-clipping an ethical technique to be used when required (Funk *et al.* 2005, Perry *et al.* 2011). Toe-clipping could inflict pain, which can be an ethical concern. Although pain is difficult to assess in animals (Rutherford 2002), corticosterone levels are positively related with pain-triggered stress (Benedetti *et al.* 2012). Narayan *et al.* (2011) found greater urinary corticosterone levels in toe-clipped than in handled-

only *Rhinella marina* toads, which indicates that toe-clipping induces stress. Nevertheless, a study by Fisher *et al.* (2013) on the same species contradicted those results, as they detected similar plasma corticosterone levels in toe-clipped and in handled-only individuals. Langkilde and Shine (2006) detected little effect of toe-clipping on plasma corticosterone levels in *Eulamprus heatwolei* lizards. Kinkead *et al.* (2006) found no differences in stress hormone levels between salamanders that had been toe-clipped with and without anaesthesia. Thus, anaesthesia does not reduce stress caused by toe-clipping. In addition, a recent review found little evidence for discomfort in toe-clipped laboratory rodents (Wever *et al.* 2017). In fact, from a physiological point of view, toe-clipping has been seen as an acceptable marking method in laboratory mice (Schaefer *et al.* 2010).

Toe-clipping may reduce return rates, which may bias estimates of population parameters, calling into question the validity of toe-clipping in mark-recapture studies (Parris & McCarthy 2001). Toe-clipped *Plethodon vehiculum* salamanders were less often recaptured than fluorescent-tagged conspecifics (Davis & Ovaska 2001). A negative effect of toe-clipping on frog return rates increases with the number of toes clipped (McCarthy & Parris 2004, Beard *et al.* 2008). However, such correspondence between survival and the number of toes clipped is not always proven (Borremans *et al.* 2015, Hoehn *et al.* 2015). In contrast, another study found that marking by toe-clipping had less effect on recapture probability than PIT (passive integrated transponder) tagging in *Hypsiboas faber* frogs (Guimarães *et al.* 2014). Similarly, in *Oligosoma maccanni* skinks recapture probabilities of toe-clipped and pen-marked (non-invasive method) individuals did not differ (Jones & Bell 2010).

Although toe-clipping reduces return rates in some species, it remains unclear whether this is because of a reduction in recapture rate, survival rate, or both (McCarthy *et al.* 2009). The effect of toe-clipping on recapture and survival probabilities differs even among frog species with similar habitat requirements (Waddle *et al.* 2008). When return rates are diminished by toe-clipping, failure to find a marked individual might be due to behavioural changes that affect

recapture probability but do not necessarily reduce fitness (Phillott *et al.* 2007, Guimarães *et al.* 2014). However, amphibians do not seem to display altered behaviour following toe-clipping (Lüddecke & Amézquita 1999, Kinkead *et al.* 2006).

Survival can be jeopardized by infections due to toe-clipping. Nevertheless, infection rates due this reason are usually low (Lemckert 1996, Reaser & Dexter 1996), although they can increase without appropriate prophylactic measures (Golay & Durrer 1994). Survival can also be reduced owing to limitations in food acquisition capability. Indeed, free-ranging toe-clipped *Plethodon vehiculum* salamanders gained less body mass than fluorescent-tagged individuals in the same area, which suggests their lower ability to capture prey (Davis & Ovaska 2001). In contrast, toe-clipping had no effect on body condition of *Bombina variegata* toads (Hartel & Nemes 2006). Survival of *Pseudacris maculata* frogs that had one toe in two different feet clipped was similar to that of elastomer-marked conspecifics (Swanson *et al.* 2013). Moreover, toe-clipping had no effect on survival of *Rana pipiens* frogs (Ginnan *et al.* 2014).

Toe-clipping could also affect survival by reducing locomotor performance. The effects of toe-clipping on short-term locomotion of *Carlia pectoralis* skinks and *Litoria nasuta* frogs were worse than those of elastomer-tagging, although full locomotion capability was restored in two weeks (Schmidt & Schwarzkopf 2010). However, toe-clipping did not affect average or maximum running speed of *Eulamprus quoyii* lizards tested within 30 minutes after toe-clipping (Borges-Landáez & Shine 2003). Similarly, toe-clipping did not affect locomotor performance of *Cnemidophorus sexlineatus* lizards (Dodd 1993). Also, sprint speed was not affected by the number of toes lost in *Sceloporus merriami* lizards (Huey *et al.* 1990). Nevertheless, clipping toes whose morphology plays a particular role in locomotion may reduce locomotion performance. Such is the case of pad-bearing *Anolis carolinensis* lizards, whose clinging performance was negatively affected by toe-clipping (Bloch & Irschick 2004). Yet, the capacity of vertical locomotion of *Hemidactylus turcicus* geckos, which have adhesive pads in their toes, was not

affected by toe-clipping (Paulissen & Meyer 2000). Also, toe-clipping did not affect locomotor performance of adhesive-disc-bearing *Dendropsophus labialis* frogs (Lüddecke & Amézquita 1999).

These contrasting results reveal that the effects of toe-clipping on vertebrates are highly variable among species. Therefore, the effects of toe-clipping should be assessed for each species or, at least, for each type of locomotion among related species. In my experiment, I evaluated the effects of toe-clipping on locomotor performance of natterjack toads (*Epidalea calamita*). Unlike most anurans, which move by jumping, these toads move by means of intermittent runs of variable length and duration (Zamora-Camacho 2018). Hence, I studied the effects of toe-clipping on both sprint speed and run rate (measured as the number of runs that toads use to cover one meter). Also, I captured toads from agricultural land and natural habitat to check if animal origin modifies the effect of toe-clipping on locomotor performance, since such performance varies between both habitats in this species (Zamora-Camacho 2018).

Material and methods

Epidalea calamita toads are medium-sized bufonids (snout-vent length range is 49–86 mm) inhabiting vast regions in central and western Europe (cf. <http://www.vertebradosibericos.org/anfibios/epical.html> [in Spanish]). These toads can be found in various habitats, including pristine and human-altered environments (cf. <http://www.vertebradosibericos.org/anfibios/epical.html> [in Spanish]). Toads are terrestrial, except during amplexus, and are mainly active during warm, rainy or very humid nights (cf. <http://www.vertebradosibericos.org/anfibios/epical.html> [in Spanish]). While inactive, toads remain under rocks or logs, or in holes they burrow with their forelimbs (cf. <http://www.vertebradosibericos.org/anfibios/epical.html> [in Spanish]). Their activity period varies geographically: in central and northern, cold habitats, toads hibernate in winter and reproduce during spring and summer, whilst in southern, hot habitats toads aestivate instead, and mate during autumn, winter, or

early spring (cf. <http://www.vertebradosibericos.org/anfibios/epical.html> [in Spanish]). *Epidalea calamita* prefers small, shallow, ephemeral ponds to lay eggs, and tadpoles complete metamorphosis in very short periods, often shorter than 6 weeks (Tejedo & Reques 1994).

Fieldwork was carried out during the reproductive season between January and April 2015, in pristine pine grove (Pinares de Cartaya, SW Spain: 37°20'N, 07°09'W), and surrounding agricultural land. In this region, winters are rather wet and relatively warm, commonly without frosts, while summers are hot and very dry. I sampled in stone pine (*Pinus pinea*) grove, with an underbrush consisting of diverse Mediterranean bushes such as *Cistus ladanifer*, *Rosmarinus officinalis*, and *Pistacea lentiscus*. Even though there is no certainty that such vegetation is autochthonous or anthropic, it has dominated the landscape for at least 4000 years, so it can be considered a natural habitat (Martínez & Montero 2004). On the other hand, agricultural land (about 5 km away from the pine grove) traditionally consisted of diverse extensive crops, but they have recently been replaced with plantations where strawberries, raspberries, and oranges are grown. Agricultural area is regularly watered during the summer, and landowners use manure, fertilizers, herbicides, fungicides, and pesticides at their discretion. During mating season, small temporary puddles where toads mate are widespread in both habitats.

I captured toads when they were active during rainy nights, or searched for them when they were resting in their shelters. I searched for toads randomly in both habitats in order to avoid biased results. However, agricultural land consists of private properties that could not be accessed. Thus, in this habitat I only sampled public areas such as paths, ditches, meadows, or areas of empty ground. Males were identified by their purple vocal sacs in their throats and blackish nuptial pads in their forelimbs (cf. <http://www.vertebradosibericos.org/anfibios/epical.html> [in Spanish]). Within two or three hours after capture, I took toads to the laboratory in small individual plastic terraria with wet peat. There, I housed toads in larger, individual plastic terraria (20 × 13 × 9 cm) containing with wet-peat substrate and an opaque piece of plastic

to serve as a shelter, and assigned them an identification number (ID).

The day after capture, the first trial testing locomotor performance of toads was carried out on a linear runway (200 × 15 × 15 cm) made of brown cardboard. Cardboard’s rugged surface provided appropriate traction, which is important because substrate texture might affect locomotor performance (Vanhooydonck *et al.* 2015). The runway was divided into 10-cm stretches, delimited with transversal white stripes. I released toads at one end of the runway, and stimulated them constantly and gently by hand during each trial. Also, a dark background at the end of the runway resembled a shelter encouraged toads to move forward. Then, for the second trial, toads were randomly assigned to one of two groups. In the ‘toe-clipped’ group, immediately after locomotion trials the third toe in each hind limb of each individual was clipped. I chose that toe to avoid clipping the longest (fourth) toe, but also because third toes are long enough to obtain appropriate sections for skeletochronology analyses for another study (Zamora-Camacho & Comas 2017). The wounds were properly disinfected with 3M Vetbond™ tissue adhesive no. 1469. In the control group, I handled toads for one minute but did not toe-clip them. After an hour, locomotor performance of the toads from both groups was carried out in the same setting as during the first trial. In total, I used 118 toads (cf. Table 1). Because temperature may affect amphibian locomotor performance (Preest & Pough 2003), I ensured that toads were at room temperature (approx. 19 °C), measured with a thermometer Hibok 18 (to the nearest 0.1 °C) connected to a 1-mm-diameter thermocouple that I inserted 8 mm inside toads’ cloacas just before each trial. Toads performed trials individually. All trials were videotaped with a

video camera Canon EOS 550D (25 frames per second), after the sunset to coincide with toad daily activity period.

Locomotion was analysed using software Tracker ver. 4.92. This software allows frame-by-frame video analysis with 1-ms precision. I calculated the time that toads needed to cover each stretch by counting the time elapsed since each toad’s snout crossed one transversal strip until it crossed the next one. Then, I divided 10 cm (the length of each stretch) by the time needed to cover each stretch, thus obtaining speed in cm s⁻¹ (Zamora-Camacho 2018). Because sprint speed was what I was interested in, I made calculations using the speed of the fastest stretch. Besides, toads frequently stopped during the trials, so I counted the times that each toad stopped (i.e., the number of runs that toads used to cover the runway length) during each trial. I divided this number by the length of the runway in meters, and obtained run rate (runs m⁻¹). After the trials, toads were released at their capture sites. Toad capture and management was conducted in agreement with permits for Junta de Andalucía (Reference AWG/MGD/MGM/CB) and the Bioethics Committee of Granada University (reference 18/07/2017/098).

Data were normally distributed, and residuals were homoscedastic, so I applied parametric statistics (Quinn & Keough 2002). First, I conducted three-way ANOVA to test whether sprint speed differed between the toads selected for toe-clipping and control prior to toe-clipping, with sex and habitat included as factors. Second, I conducted three-way ANOVA to test the effect of toe-clipping or lack thereof (control) on sprint speed in the second trial, with sex and habitat included as factors. Finally, due to unexpected results in the first trial (toads that were not to be toe-clipped were faster than toads whose toes were clipped

Table 1. Sample sizes of toads of each sex and from each habitat assigned to each group. Total sample sizes by groups are in Figs. 1 and 2.

Habitat	Control		Experimental		Total
	females	males	females	males	
Agricultural land	15	12	21	11	59
Pine grove	15	15	15	14	59
Total	30	27	36	25	118

for the second trial), I used a linear mixed model of restricted maximum likelihood (REML-LMM; Zuur *et al.* 2009) to include sprint speed and run rate from the first trial as covariates when testing the effect of toe-clipping or lack thereof on sprint speed and run rate, respectively, in the second trial, with sex and habitat included as factors, and ID as a random factor to avoid any possible pseudo-replication. Sum of squares was type III. Moreover, for the final models including sprint speed and run rate from the first trials as covariates, I calculated effect sizes as a measure of the magnitude of between-treatment differences (Cohen 1988), as well as evaluated statistical power, i.e., the probability that the null hypothesis (non-significant differences between treatments) is rejected when the alternative hypothesis (significant differences between treatments) is true (Quesada & Figuerola 2010). Finally, I calculated sample sizes that would be needed to obtain statistically significant results for the effect size obtained and statistical power of 0.95. Statistical analyses were performed in R, using the *nlme* package (<https://cran.r-project.org/web/packages/nlme/index.html>) except for the analyses of statistical power and sample sizes, which were carried out in G*Power 3 (Faul *et al.* 2007).

Results

Sprint speed

In the first trial, sprint speed (mean $\pm$ SE) in males was greater than in females (67.17 ± 1.97 *versus* 47.42 ± 1.76 cm s⁻¹; cf. Table 2),

Table 2. Results of testing the effects of sex, habitat, treatment, and their interactions on sprint speed in the first trial. Significant results are set in boldface.

Effect	$F_{1,110}$	p
Sex	55.791	< 0.001
Habitat	0.573	0.451
Allocation to treatment*	5.148	0.025
Sex $\times$ habitat	0.309	0.579
Sex $\times$ treatment	0.523	0.471
Habitat $\times$ treatment	2.671	0.105
Sex $\times$ habitat $\times$ treatment	0.433	0.512

* To be clipped *versus* not to be clipped.

and, surprisingly, in toads that were not to be toe-clipped (60.30 ± 1.89 *versus* 54.34 ± 1.85 cm s⁻¹; cf. Table 2). All other effects and interactions were not significant in this model (cf. Table 2). Removing non-significant interactions had no effect on the results (results not shown).

In the second trial, sprint speed (mean $\pm$ SE) in males was greater than in females (67.49 ± 2.22 *versus* 45.87 ± 1.98 cm s⁻¹; cf. Table 3) and in toads that were not toe-clipped (59.87 ± 2.12 *versus* 53.49 ± 2.09 cm s⁻¹; cf. Table 3). The interaction habitat $\times$ treatment was significant in this model while all other effects and interactions were not significant in this model (Table 3). Removing non-significant interactions had no effect on the results (results not shown).

When sprint speed from the first trial was included in the analysis as a covariate, sprint speed in the second trial was still greater in males than in females (59.83 ± 1.96 *versus* 52.15 ± 1.72 cm s⁻¹; cf. Table 4). Habitat differences in sprint speed were non-significant (Table 4). Notably, the effect of treatment on sprint speed became non-significant in this model (Table 4 and Fig. 1). The relationship between sprint speed in the first and second trials was positive and significant ($F_{1,109} = 70.761$, $p < 0.001$, $\beta = 0.636$). All other effects and interactions were not significant in this model (Table 4). Removing non-significant interactions had no effect on the results (results not shown).

Run rate

In the first trial, run rate (mean $\pm$ SE) was greater in agricultural-land than in pine-grove

Table 3. Results of testing the effects of sex, habitat, treatment, and their interactions on sprint speed in the second trial. Significant results are set in boldface.

Effect	$F_{1,110}$	p
Sex	52.849	< 0.001
Habitat	1.216	0.272
Treatment	4.607	0.034
Sex $\times$ habitat	0.937	0.335
Sex $\times$ treatment	0.012	0.912
Habitat $\times$ treatment	4.307	0.040
Sex $\times$ habitat $\times$ treatment	0.109	0.742

Fig. 1. Sprint speed in the second trial when sprint speed from the first trial was included in the analysis as a covariate, and toad ID was included as a random factor. Vertical bars represent standard errors; n = sample size.

toads (5.28 ± 0.29 versus 4.09 ± 0.28 runs m^{-1} ; Table 5). Any other effect and interaction were not significant in this model (Table 5). Removing non-significant interactions had no effect on the results (results not shown). Run rate in the second trial was not significantly affected by treatment, sex, habitat, or their interactions (Table 6). Removing non-significant interactions had no effect on the results (results not shown).

When run rate from the first trial was included in the analysis as a covariate, run rate in the second trial was not affected by treatment (Table 7 and Fig. 2), sex, habitat or their interac-

tions (Table 7). The relationship between run rate in the first and second trials was positive and significant ($F_{1,109} = 124.410$, $p < 0.001$, $\beta = 0.758$). All other effects and interactions were not significant in this model (Table 7). Removing non-significant interactions had no effect on the results (results not shown).

Effect sizes and statistical power

Effect size in the sprint speed analysis was small ($d = 0.029$), and its statistical power low ($1 - \beta = 0.05$). Therefore, to detect significant differences between treatments, sample size for the effect size obtained and statistical power of 0.95 should be at least 61 808 toads.

Effect size in the run rate analysis was also small ($d = 0.172$), and its statistical power ($1 - \beta = 0.15$) low. Therefore, to obtain statistical differences between groups, sample size for the effect size obtained and statistical power of 0.95 should be at least 1759 toads.

Table 5. Results of testing the effects of sex, habitat, treatment, and their interactions on run rate in the first trial. Significant results are set in boldface.

Effect	$F_{1,110}$	p
Sex	0.426	0.515
Habitat	8.834	0.004
Allocation to treatment*	0.448	0.505
Sex $\times$ habitat	0.300	0.585
Sex $\times$ treatment	1.394	0.240
Habitat $\times$ treatment	0.020	0.889
Sex $\times$ habitat $\times$ treatment	2.380	0.126

* To be clipped *versus* not to be clipped.

Table 4. Results of testing the effects of sex, habitat, treatment, and their interactions on sprint speed in the second trial, when sprint speed from the first trial was included in the analysis as a covariate, and ID was included as a random factor. Significant results are set in boldface.

Effect	$F_{1,110}$	p
Sex	7.218	0.007
Habitat	0.746	0.388
Treatment	0.005	0.945
Sex $\times$ habitat	1.609	0.205
Sex $\times$ treatment	0.142	0.706
Habitat $\times$ treatment	0.842	0.359
Sex $\times$ habitat $\times$ treatment	0.011	0.916

Table 6. Results of testing the effects of sex, habitat, treatment, and their interactions on run rate in the second trial.

Effect	$F_{1,110}$	p
Sex	1.116	0.293
Habitat	0.527	0.469
Treatment	0.001	0.970
Sex $\times$ habitat	0.063	0.803
Sex $\times$ treatment	0.006	0.939
Habitat $\times$ treatment	0.371	0.544
Sex $\times$ habitat $\times$ treatment	2.513	0.116

Table 7. Results of testing the effects of sex, habitat, treatment, and their interactions on run rate in the second trial, when run rate from the first trial was included in the analysis as a covariate, and ID was included as a random factor.

Effect	$F_{1,110}$	p
Sex	1.278	0.258
Habitat	0.191	0.662
Treatment	1.535	0.215
Sex $\times$ habitat	0.038	0.845
Sex $\times$ treatment	0.107	0.744
Habitat $\times$ treatment	0.075	0.784
Sex $\times$ habitat $\times$ treatment	0.437	0.510

Given the small effect sizes, i.e., very small differences between treatments and groups, low statistical power was expected in both cases. Low statistical power suggests small probability of rejecting the null hypothesis when the alternative hypothesis is true. Therefore, I feel confident not to reject the null hypotheses that treatment (toe-clipping) had no effect on sprint speed and run rate. (For data dispersion see Fig. 3).

Discussion

In my experiment, toad sprint speed and run rate were not affected by toe-clipping. These results

Fig. 2. Run rate in the second trial when run rate from the first trial was included in the analysis as a covariate, and toad ID was included as a random factor. Vertical bars represent standard errors; n = sample size.

suggest that toe-clipping has no immediate effect on toad locomotion, and match outcomes of number of several earlier studies (Huey *et al.* 1990, Dodd 1993, Lüddecke & Amézquita 1999, Paulissen & Meyer 2000, Borges-Landáez & Shine 2003, Hudson *et al.* 2017, but see Bloch & Irschick 2004, Schmidt & Schwarzkopf 2010). Additionally, the interactions between treatment and either sex or habitat were not significant, which indicates that toe-clipping had no effect

Fig. 3. Data points of sprint speed and run rate in the second trial, for visual assessment of data dispersion.

on sprint speed and run rate of toads of both sexes and from both habitats.

Locomotor performance affect the ability of animals to escape predators (Watkins 1996, McGee *et al.* 2009), forage (Budick & O'Malley 2000, Higham 2007), and reproduce (Husak *et al.* 2006, Husak *et al.* 2008). Therefore, *E. calamita* toads can be toe-clipped in the field for different purposes (*see* Introduction), with no immediate effects on their probability to be preyed upon, or ability to compete for prey. Handling animals in the field can eliminate stress caused by transportation and housing in the laboratory (Marra *et al.* 1995, Morgan & Tromborg 2007). However, the fact that locomotor performance is not reduced by toe-clipping does not necessarily mean that return rates are not diminished, which may bias results of mark-recapture studies (McCarthy & Parris 2004). Nonetheless, this does not mean that survival is reduced as well (Waddle *et al.* 2008, McCarthy *et al.* 2009).

Digit regeneration my limit usefulness of toe-clipping as a marking method in amphibians (Campbell *et al.* 2009), but clipped toes do not always regrow, and when they do, lack of pigmentation of regenerated digits may help recognition (Ferner 2010). In fact, Brannelly *et al.* (2014) detected that toe-clipping is more reliable than PIT-tagging and elastomer-tagging in individual recognition of *Litoria verreauxii alpina* frogs. Therefore, toe-clipping seems to be a viable method for marking anurans (Grafe *et al.* 2011).

Results herein also highlight the convenience of measuring traits before and after treatment assignment. Without data on locomotor performance prior to treatment assignment, spurious differences in sprint speed between treatments would have been taken for reliable. However, because data on sprint speed prior to treatment assignment showed previous differences between groups, I could recognize that those were random differences in individual locomotor performance between groups (type I error; Smith *et al.* 2002). In fact, differences in sprint speed between non-toe-clipped and toe-clipped individuals in the second trial disappeared when sprint speed from the first trial was included in the analysis as a covariate. Furthermore, measuring locomotor performance twice allowed to detect high repeat-

ability of locomotor performance, mirrored by a high, significant and positive relationship of both sprint speed and run rate prior to and after treatment assignment. In their studies, also Austin and Shaffer (1992) and Watkins (1997) found high repeatability of locomotor performance.

In summary, neither sprint speed or run rate were affected by toe-clipping in the short term, which suggests that toe-clipping in the field should not increase toads' risk to become prey or hinder searching for prey or mates. Moreover, my results show the importance of measuring traits before and after the treatment is applied, to control for possible bias due to random errors. Also, my data support high repeatability of locomotor performance in this species.

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