Intensity of *Haemoproteus* spp. blood infection differs between wild birds captured using different trapping methods

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Abstract

Infection with haemoparasites (*Haemosporida* spp.) is common within wild birds' populations and has been associated with adverse effects on birds' fitness and life performance. Existing literature suggests a hypothesis that birds infected with haemosporidians and exhibiting high parasitemia levels may demonstrate reduced activity, potentially leading to their underrepresentation in captures which use stationary traps, such as mist nets. However, there are limited studies demonstrating this phenomenon in wild birds. To study the possible underrepresentation of highly parasitized birds, captured with commonly used stationary mist nets, we compared parasitemia levels of haemosporidian parasites in wild birds captured during spring migration using both actively startling of birds and birds trapped passively into the mist nets. Utilizing cumulative link mixed models, we assessed the influence of trapping methods on parasitemia scores, controlling for factors such as species identity, season progression, and migration distance. Our dataset comprised 1815 individuals of 60 species, including 142 actively captured and 1673 passively captured ones. Although the number of infected individuals was higher in passively caught birds (24 % in active vs 43 % in passive), the study revealed a significant difference between infected birds within two capture methods: despite challenges in balancing sample sizes, and trapping dates, the findings affirm the underestimation of birds having high parasitemia levels with prevalent mist net techniques. We highlight the importance of considering potential biases in trapping methods when studying haemosporidian parasites in avian hosts during migration.

Keywords: avian haemosporidiosis, *Haemoproteus*, parasitemia, spring migration, chronic haemosporidiosis.

Introduction

Haemosporidian parasites, such as *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, are widespread avian blood parasites that are transmitted by dipteran vectors (Valkiūnas, 2005). Formerly, they were considered relatively benign for birds (Bennett, Peirce, and Ashford, 1993), but the modern view is that pathogenicity depends on numerous factors, ranging from host and parasite species to individual host reactions to the parasite (Cornet, Nicot, Rivero, and Gandon, 2014; Videvall et al., 2017). Experimental studies have shown that birds infected with haemosporidian parasites may suffer from anemia and hemolysis during erythrocytic stages (Palinauskas, Žiegytė, Šengaut, and Bernotienė, 2018). Exo-erythrocytic, or tissue stages, are less studied; however, there is growing evidence that *Haemoproteus* and *Plasmodium* exo-erythrocytic stages are also detrimental

Citation: Erokhina, M., Bushuev, A., Palinauskas, V., Platonova, E., and Mukhin, A. 2024. Intensity of *Haemoproteus* spp. blood infection differs between wild birds captured using different trapping methods. Bio. Comm. 69(3): 192–199. https://doi.org/10.21638/spbu03.2024.307

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Manuscript Editor: Pavel Skutschas, Department of Vertebrate Zoology, Faculty of Biology, Saint Petersburg State University, Saint Petersburg, Russia

Received: December 12, 2023;

Revised: March 21, 2024;

Accepted: March 27, 2024.

Supplementary information: Supplemental material to the article is available at https://doi.org/10.21638/spbu03.2024.307. Supplementary files are published as submitted by the authors, and are not copyedited.

Ethics statement: This paper does not contain any studies involving human participants or animals performed by any of the authors.

Competing interests: The authors have declared that no competing interests exist.

and, in some cases, can be lethal (Ilgūnas et al., 2019; Hernández-Lara, Duc, Ilgūnas, and Valkiūnas, 2021).

The most severe stage of primary haemosporidiosis is the acute phase — a brief period when the number of parasites in the blood reaches its maximum (Atkinson and Van Riper III, 1991; Valkiūnas, 2005). If the bird survives this critical period, the infection develops into the chronic stage with periodic relapses (Valkiūnas, 2005; Asghar et al., 2012). During the acute phase, birds may suffer from anemia, hemolysis, visceral tissue damage, weight loss, and other detrimental effects (Atkinson and Van Riper III, 1991; Valkiūnas, 2005; Atkinson, Thomas, and Hunter, 2008). This acute stage poses the highest risk of death for the infected bird. The elevated mortality risk during this crisis corresponds with the observation that the intensity of parasitemia in birds captured by stationary mist nets rarely exceeds 1 %. Such low parasitemia levels imply that the majority of birds in the population likely reside in the chronic stage of infection, characterized by persisting, albeit lower, levels of the parasites in their blood (Valkiūnas, 2005; Bensch et al., 2007).

Several lines of evidence, both direct and indirect, support the assumption that there is an undercount of birds with high levels of parasitemia. Mukhin et al. (2016) demonstrated that experimentally infected siskins (*Spinus spinus*) significantly reduced their locomotor activity during the acute phase of primary malaria compared to uninfected control birds. Additionally, less mobile and alerted birds should be more attractive prey for a predator, and positive correlations between susceptibility to predation and the intensity of blood parasites were demonstrated using examples of the European sparrowhawk (*Accipiter nisus*) and the Eurasian goshawk (*A. gentilis*) and their avian prey (Møller and Nielsen, 2007). Holmstad, Jensen, and Skorping (2006) found an association between the intensity of *Leucocytozoon lavati* and the freezing behavior of willow ptarmigans (*Lagopus lagopus*). Valkiūnas (2005) compared blood samples of juvenile chaffinches (*Fringilla coelebs*) obtained through traditional trapping techniques with those from birds shot nearby. It turned out that among the shot young finches, 3 out of 36 infected with *Haemoproteus fringillae* exhibited parasitemia levels comparable to those seen during the acute phase in artificially infected experimental birds. In contrast, among over 400 finches captured passively, such high parasitemia values were not observed (Valkiūnas, 2005).

The hypothesis was formulated suggesting that birds undergoing a peak of malarial or other haemosporidian-caused pathological processes, and therefore unable to exhibit the same level of activity and exploratory behavior as healthy ones, could potentially be underrepresented in passive captures (Valkiūnas, 2005; Mukhin et al., 2016). In this context, stationary bird trapping methods can be categorized as "passive", implying that only actively flying or moving birds can become trapped. Our study aimed to compare the levels of parasitemia caused by haemosporidian blood parasites in wild birds captured during spring migration, utilizing two distinct methods — active and passive trapping. We assumed that intentionally inducing birds to fly into the nets (for instance, by startling them) would enable us to observe a greater number of birds with high intensity of parasitemia levels in comparison to those captured using passive techniques.

Materials and methods

Three mist nets (Ecotone, Poland), each six meters long, were set up amidst the reeds and bushes. Our active trapping approach involved deliberately startling the birds present within the vegetation. Several individuals participated in scaring the birds towards the mist nets that were positioned nearby. The process was initiated approximately forty meters away from the line of nets and was repeated twice from both sides. At the same time, noise was generated using improvised objects and vocalizations. The active trapping area was located at a distance of 0.5 km from the Rybachy field site, where the stationary mist nets were deployed (55°05′ N, 20°44′ E). Both sites were located along the shores of the Curonian Lagoon, within reeds and willow undergrowth.

Blood samples were collected from all captured birds through a wing vein (ulnar) venipuncture. Two drops of blood were used to prepare two thin blood smears. These smears were subsequently air-dried, fixed with absolute methanol, and stained using Giemsa according to the standard protocol (Valkiūnas et al., 2008). The prepared smears were examined under a light microscope at 1000 × magnification, employing oil immersion. The relative intensity of parasitemia was evaluated as recommended in Valkiūnas (2005). We used a scoring system to assess the degree of parasitemia caused by *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites in the birds' blood. The scoring interpretation was as follows: a score of 1 represents less than 0.01 % of infected erythrocytes (< 0.01 %); a score of 2 corresponds to ≥ 0.01 — < 0.1 %; a score of 3 indicates ≥ 0.1 — < 1 %; and a score of 4 corresponds to the parasitemia level of equal or greater than 1 % (≥ 1 %) (Valkiūnas, 2005).

A total of 1815 trapped birds were analyzed, representing 60 different species. However, there was a significant disparity between the samples of actively and passively captured birds, with 142 individuals in the active group and 1673 individuals in the passive group. Active trapping occurred during the spring months of May and early June 2015; April and May 2016. A total of 29 attempts were made, with each attempt corresponding to a day of capturing.

Passive trapping, on the other hand, was conducted nearly daily, commencing from mid-May (although the specific start date varied across different years). Data on bird haemosporidian infection from passive captures are available for the years 2011, 2012, 2013, 2015, and 2016. Unfortunately, the discrepancy in trapping dates between the active and passive methods underscores the need for cautious interpretation of our results. Notably, our dataset is heavily skewed towards passive captures, as the number of passive captures significantly outweighs that of active captures. Additionally, the majority of active trapping occurred in April 2016, whereas collecting data for passive captures generally commenced in mid-May. In order to compare the prevalence of haemosporidian parasites among birds caught using different methods, we applied a binomial generalized linear mixed effect model (GLMM) using '*glmer*' function from the *lme4* R package (Bates, Mächler, Bolker, and Walker, 2015). The binary variable 'infection status' was set as a dependent variable.

To study the potential differences in parasitemia scores between the birds captured with active and passive methods, we used a cumulative link mixed model (CLMM), which is an ordinal regression model incorporating random effects. The CLMM was built using '*clmm*' function from the *ordinal* R package (Christensen, 2022). We developed two models to analyze our data: one included all infected bird species captured using both trapping approaches, while the other focused on a reduced subset of infected bird species present in both trapping methods, ensuring uniformity between the two approaches. In our statistical analysis, we exclusively considered parasitemia scores attributed to *Haemoproteus* spp. due to the limited prevalence of other blood parasites.

In the CLMM analysis, the ordinal dependent variable was the parasitemia score. The initial set of fixed factors for both CLMM and GLMM models included Type, DayN, interaction between Type and DayN, *Leucocytozoon*, *Plasmodium* and Migration distance. Species identity and Year were included as random effects. The final models were selected using Bayesian information criterion (BIC). The "Type" variable indicated whether birds were captured passively or actively; "DayN" represented a so-called April-days, effectively accounting for the progression of the season from April to June (e.g., 1st April corresponds to April-day 1st, and 24th May corresponds to April-day 54th). This variable aimed to determine if the parasitemia score exhibited alterations as the season advanced. Interaction between Type and DayN mean that the direction of odds is different for different types of capture, if significant; *Leucocytozoon* and *Plasmodium* — binary factor, indicating the presence or absence of these parasites. Migration distance predictor stood for the short- and long-distance migrants. We considered long migrants to be those avian species whose wintering grounds are in Africa and short migrants to be those that remain within or near Europe during winter, according to Birds of the World (https://birdsoftheworld.org/bow/home). Conditional and marginal coefficients of determination (R^2) for CLMM and GLMM were estimated using '*r2*' function from the R package *performance*. All statistical analysis was performed in R v. 4.2.2 (R Core Team, 2022). We assumed statistical significance at the $P < 0.05$.

Results

Out of the 1815 birds, 142 were caught actively, while 1673 were caught with stationary mist nets. Out of them 757 were infected with *Haemoproteus* spp.: 34 among actively captured individuals (24 % of all actively caught birds) and 723 among the passively caught ones (43 % of all passively caught birds). We observed only three cases (2 %) of *Plasmodium* parasites among the birds captured using active methods and 68 cases (4 %) among the birds captured using stationary traps. Regarding the parasite belonging to genus *Leucocytozoon*, there were four cases (3 %) detected in the actively caught birds and 99 instances (6 %) in the birds captured with the passive methods. Mean *Haemoproteus* parasitemia score in the actively caught infected birds was 3.8 and all infected birds had parasitemia higher than 1 score, which means that 100 % of the infected birds had an elevated parasitemia level. In the passive captures mean parasitemia score was 2.9 and 432 out of 723 infected birds, or 60 %, had parasitemia score higher than one.

According to the BIC, the best GLMM included only one fixed effect (type of trapping) and two random effects (species identity and year) (Table S1). The probability of catching an infected bird was significantly lower within the active catches than within the passive ones ($P = 0.016$) (Table S2). The fixed effect alone explained 0.7 % of variance in the infection status (marginal $R^2 = 0.007$), whereas the full model explained 32.4 % of model's variance (conditional $R^2 = 0.324$) (Table S2).

The CLMM that encompassed the infected birds from all species combined yielded significant differences between the two types of birds captures ($P \leq 0.001$) (Table S3). The final model included, again, only one fixed predictor — "Type" and one random predictor — "Species" (Table S4). In this model "Type" factor had a negative effect on parasitemia score, suggesting that within the passive catches it was less likely for birds to have high parasitemia score.

To mitigate the potential impact of species affiliation, a revised analysis was conducted on a subset of species represented in both active and passive captures (Table S5). For GLMM analysis, the final sample consisted of 17 species shared between both trapping techniques

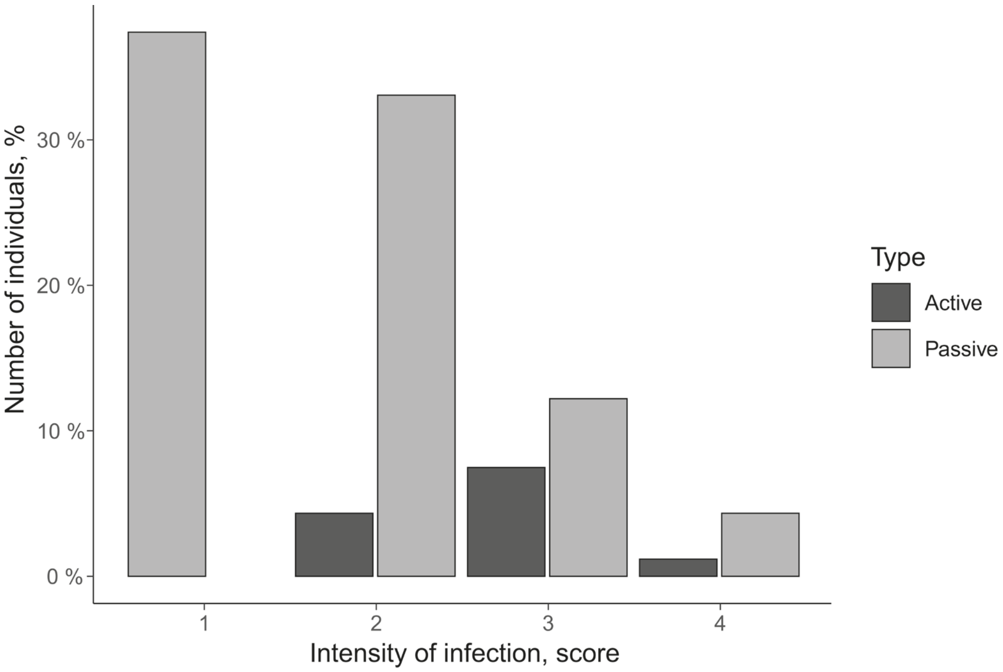

Fig. 1. Percentage of birds with the same parasitemia scores of *Haemoproteus* spp. for actively and passively trapped birds from the same species sample.

(Table S6). For CLMM, only the infected birds could be used, resulting in a sample of 10 species shared among the infected birds from both capture methods. The list of shared species of birds is provided in Table S6.

The set of predictors in the best GLMM based on the reduced sample was the same as in the GLMM for all species (Table S7). However, the single fixed predictor for the type of capture showed only a trend towards significance ($P = 0.076$) (Table S8). That means that in the reduced sample which consisted of only shared between the two trapping methods species of birds, the chance to catch an infected bird was not significantly different between the actively and passively caught birds. Notably, that, as in the GLMM with all species sample, the full model for shared species explained a much larger proportion of the variance (conditional $R^2 = 0.338$) than the fixed effect alone (marginal $R^2 = 0.014$) (Table S8).

The subset reduced the bird count from 757 individuals with *Haemoproteus* spp. to 254, comprising 33 actively captured and 221 passively captured birds (Fig. 1). The CLMM based on this reduced sample, again, included only one fixed predictor — “Type” and one random effect — “Species” (Table S9). The model revealed strong significance of the “Type” predictor ($P \leq 0.001$) (Table 1). Similar to the model based on the full sample of species, the model built using a reduced sample showed that within the passive catches, it was less likely to catch a bird with a higher parasitemia score. Interestingly, the second-best model (Table S9) also included the significant predictor for migration status ($P = 0.01$), which suggests that birds with long migration distances tended to have higher parasitemia scores of *Haemoproteus* spp. compared to those with short distances.

Table 1. Result of the cumulative link mixed model (CLMM) for the sample with common species for both trapping methods

	Parasitemia score		
Predictors	*Odds Ratios*	*CI*	*p*
1 | 2	0.08	0.04–0.17	**< 0.001**
2 | 3	0.52	0.26–1.07	0.074
3 | 4	3.84	1.76–8.37	**0.001**
Type [Passive]	0.13	0.07–0.27	**< 0.001**
Random Effects			
σ^2	3.29		
$\tau_{00\ Species}$	0.26		
ICC	0.07		
$N_{Species}$	10		
Observations	254		
Marginal R^2 / Conditional R^2	0.116 / 0.181		

Notes: CI — 95 % confidence interval. Random effects: σ^2 = Residual variance, τ_{00} = random intercept variance, ICC = intraclass correlation coefficient; R^2 — coefficient of determination. Significant values are in bold.

Discussion

Haemoproteus parasites were prevalent in our samples, while *Leucocytozoon* spp. were rare, and *Plasmodium* spp. were observed only in a few cases. Unlike the *Plasmodium, Haemoproteus* species do not multiply in red blood cells, thus they typically induce light (less than 1 %) or moderate parasitemia during the acute phase (about 5 %) (Valkiūnas, 2005). Such peculiarity of these parasites' life cycle created a view that haemoproteosis is relatively benign to their avian hosts (Bennett, Peirce, and Ashford, 1993). However, as already mentioned above, recent studies have demonstrated that *Haemoproteus's* exo-erythrocytic meronts can damage different tissues and lead to numerous lesions in organs when they mature and rupture (Atkinson, Thomas, and Hunter, 2008; Valkiūnas and Iezhova, 2017; Ortiz-Catedral et al., 2019; Duc, Ilgūnas, Kubiliūnaitė, and Valkiūnas, 2021).

For our analysis, we only used score points for *Haemoproteus* spp. infection. That implies that we regarded the level of parasitemia during haemoproteosis as indicative of the parasite's pathogenicity, as it is usually the case for true malaria infection with *Plasmodium* spp. However, there is no clear evidence that this is true for *Haemoproteus* infection (Duc, Ilgūnas, Kubiliūnaitė, and Valkiūnas, 2021; Duc et al., 2023).

In our study, we observed higher parasitemia score of *Haemoproteus* infections in actively caught infected birds, which were hiding in the vegetation in comparison to passively caught flying infected birds. This is in accordance with a general assumption that blood parasites have a negative effect on their host, and that highly parasitized birds become less active and tend to hide and save their resources. However, little is known about the detrimental effect for birds with chronic haemoproteosis. Field studies have indicated that the intensity of chronic *Haemoproteus* spp. parasitemia tends to be higher in comparison to the chronic parasitemia caused by more virulent *Plasmodium* spp. in wild birds (Bensch et al., 2007; Asghar, Hasselquist, and Bensch, 2011; Emmenegger et al., 2021). It is assumed that this difference may stem from the life-cycle variations between the two parasites. *Haemoproteus* parasites might incur lower costs in hosting gametocytes in the peripheral blood and possibly sustain longer periods of circulating gametocytes (Emmenegger et al., 2023). Few studies involving chronically infected wild birds treated with anti-Haemosporida medications have shown that even low levels of chronic *Haemoproteus* spp. infections can have detrimental effects on their hosts (Merino, Moreno, Sanz, and Arriero, 2000; Puente et al., 2010). Asghar et al. (2011) documented that great reed warblers (*Acrocephalus arundinaceus*) infected with *H. payevskyi* (GRW1) tended to arrive later in spring at breeding sites, and they observed an inverse correlation between parasitemia levels and the reproductive success of the hosts.

Approximately half of the captured species winter in tropical Africa. These migrants face infection risks during their both spring and autumn migration and on wintering grounds (Waldenström et al., 2002; Sorensen et al., 2016). Surviving the initial infection, they encounter further challenges during late winter migration, including physical strain and immune changes, associated with previously acquired haemosporidian relapses (Applegate and Beaudoin, 1970; Peirce and Mead, 1978; Valkiūnas, 2005; Altizer, Bartel, and Han, 2011). Different lineages of haemosporidian parasites vary in their transmission locations, their range of parasitemia and moreover, parasitemia of the same lineage differs within different host species (Nilsson et al., 2016; Emmenegger et al., 2023). Our study did not analyze parasite lineages, meaning we cannot determine whether we are observing the relapse of a previously acquired haemoproteosis or whether the elevated parasitemia is due to the late stage of the acute phase of a recent infection.

All of these observations imply that specific *Haemoproteus* lineages, as well as their avian host species, can vary in their contribution to the final results. To mitigate the impact of this unaccounted effect, we excluded bird species unique to each trapping method. Yet, even upon reducing the sample to only 10 species shared between both trapping datasets, the overall findings on the influence of trapping techniques remained largely unchanged. Interestingly, the second-best model suggested that long-distance migrants had slightly higher scores of *Haemoproteus* parasitemia than short-distance migrants. This is in accordance with theoretical assumptions that those birds that undertake longer distances between breeding and wintering grounds might need to prioritize energy for flight over immune defense which could compromise their immunity, increase susceptibility to infection, and consequently lead to a higher level of parasitic infestation (Altizer, Bartel, and Han, 2011; Sorensen et al., 2019).

Our study was conducted during the climatic spring, spanning from April to early June. Studies on migrating birds have yielded conflicting results regarding the impact of parasites in general and haemosporidiosis in particular on bird migration patterns (reviewed in Poulin and de Angeli Dutra (2021)). For instance, a recent study found that the timing of spring migration in great reed warblers (Emmenegger et al., 2021) and dark-eyed juncos (*Junco hyemalis hyemalis*) (Byrd et al., 2023) was not affected by haemosporidian parasites, while previous studies indicated a delay in spring migration for haemosporidian-infected birds (Møller, de Lope, and Saino, 2004; Asghar, Hasselquist, and Bensch, 2011; López, Muñoz, Soriguer, and Figuerola, 2013; Emmenegger et al., 2018). In our models, we did not

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Some studies suggest that higher virulence and mortality occur in birds simultaneously infected with genetically different haemosporidian parasites, although this may vary depending on the specific host-parasite pairs (Pigeault et al., 2018). In our study, co-infection with *Plasmodium* or *Leucocytozoon* did not result in a higher parasitemia score for *Haemoproteus* infection. However, this effect may have been masked by our technique for microscopic examination of smears used, leading to an underestimation of co-infections.

Interestingly, our results also suggest that there were higher chances of catching infected birds within the passive captures compared to the active ones when all available birds were included in the analysis. However, the significance of this difference disappeared when we reduced the sample to only shared bird species. We assume that the first result reflects the uneven severity of infection by the same haemosporidian parasite, which may vary among avian host species, and also that some parasite species exhibit host specificity. Nevertheless, subsequent analysis of the infected birds alone, irrespective of the sample size and species variety, indicated that parasitemia score was higher within the actively caught infected birds. In summary, we approach our results with caution due to the highly imbalanced data. However, our findings are consistent with existing theoretical assumptions and a few field studies on the effect of *Haemoproteus* infection on the fitness of wild birds.

To date, this is the second study that reveals the effect of the used methodology on the outcomes derived from the study of haemosporidian parasites. Similar studies have demonstrated a relationship between the degree of haemosporidian infection and the method of data collection, often involving the shooting of actively flying wild birds. However, our approach, which is non-lethal to the captured birds, costs a lot of human resources.

Conclusion

Our study provides valuable insights into haemosporidian parasites and the methodological nuances that shape study outcomes. The results are consistent with the widely accepted idea that elevated levels of parasitemia, induced by haemosporidian parasites, influence the behavior of wild birds. However, it is important to note that our conclusion is specific to the haemosporidian parasites of the *Haemoproteus* genus. Currently, there is a lack of definitive data establishing a clear connection between pathogenicity and the degree of blood parasitemia for this particular parasite group. Although the GLMM suggested that there were more infected birds within the passive catches, the employed cumulative link models revealed a disparity in parasitemia scores between the actively and passively captured birds. The probability of capturing birds with high parasitemia scores was lower in the passive captures compared to the active ones, validating our initial hypothesis. We point out the importance of species-specificity of haemosporidian parasites, particularly for *Haemoproteus* genus. Thus, our study confirms the notion that there is an underestimation of birds with high parasitemia levels when using widespread mist net techniques.

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