# Kinship reinforces cooperative predator inspection in a cichlid fish

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## Abstract

Kin selection theory predicts that cooperation is facilitated between genetic relatives, as by cooperating with kin an individual might increase its inclusive fitness. Although numerous theoretical papers support Hamilton's inclusive fitness theory, experimental evidence is still underrepresented, in particular in noncooperative breeders. Cooperative predator inspection is one of the most intriguing antipredator strategies, as it implies high costs on inspectors. During an inspection event, one or more individuals leave the safety of a group and approach a potential predator to gather information about the current predator risk. We investigated the effect of genetic relatedness on cooperative predator inspection in juveniles of the cichlid fish *Pelvicachromis taeniatus*, a species in which juveniles live in shoals under natural conditions. We show that relatedness significantly influenced predator inspection behaviour with kin dyads being significantly more cooperative. Thus, our results indicate a higher disposition for cooperative antipredator behaviour among kin as predicted by kin selection theory.

# Introduction

The ubiquitous occurrence of cooperation (i.e. acts that benefit others on own costs) among animals was already extensively described by Darwin's contemporaries (Kropotkin, 1902), and at that time considered as potential problem for Darwin's theory of natural selection (Darwin, 1859). Today, examples for cooperation range from bacteria and microbes (e.g. Diggle *et al.*, 2007; López-Villavicencio *et al.*, 2011; Rumbaugh *et al.*, 2012; Inglis *et al.*, 2014; Pollitt *et al.*, 2014) to social insects (Foster *et al.*, 2005; Tibbets & Injaian, 2013) and vertebrates (e.g. birds: Clutton-Brock, 2002; mammals: Eberle & Kappeler, 2006; Dechmann *et al.*, 2010; fish: Taborsky, 1984). Some cooperative interactions can be

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explained by an increase in direct fitness, for instance because cooperation provides mutual benefits to both actor and recipient (West et al., 2007), or because it is based on reciprocal cooperation (Trivers, 1971; Schneeberger et al., 2012). However, a major step towards a better understanding of social behaviour in general, and cooperation in particular, was provided by Hamilton's (1964) inclusive fitness theory, stating that individuals do not maximize direct fitness but their inclusive fitness, which can be indirectly achieved by increasing the fitness of genetic relatives (Bourke, 2011). This theory allows explaining extreme forms of altruism, which have, for example, evolved in eusocial insects, where individuals forsake their own reproduction to raise their queen's offspring (Hughes et al., 2008), but it is also applicable to any other form of social interaction (West & Gardner, 2010). Cooperative strategies like reciprocity may be prone to cheating (Kokko et al., 2001; Bergmüller et al., 2010; Jiricny et al., 2010), and thus, genetic relatedness between interacting individuals may further facilitate the evolution of cooperation.

On the occasion of the 50th anniversary of Hamilton's and Maynard Smith's papers (1963, 1964), numerous reviews and theoretical papers stress the importance of inclusive fitness theory (e.g. Van Dyken & Wade, 2012; Lehmann & Rousset, 2014; McGlothlin et al., 2014; Taylor & Maciejewski, 2014; Van Cleve & Akçay, 2014; Wild & Koykka, 2014). However, although results of numerous correlative studies on cooperation are in accordance with the predictions of kin selection theory (Pfennig & Collins, 1993; Höglund et al., 1999; Gerlach et al., 2007; Markman et al., 2009; Ruch et al., 2009; Chaine et al., 2010; Dobler & Koelliker, 2010), studies employing an experimental approach are still underrepresented (but see Schneider & Bilde, 2008; West et al., 2008; Rumbaugh et al., 2012; Ho et al., 2013; Carazo et al., 2014; Hatchwell et al., 2014). Most empirical studies address kin selection in terms of cooperative breeding, which has been studied in various vertebrate species (Clutton-Brock, 2002). For example, kin selected benefits explain the evolution of cooperation in birds (Komdeur, 1994; Russell & Hatchwell, 2001; Hatchwell, 2009; Wright et al., 2010), and mammals (Lukas & Clutton-Brock, 2012). Nevertheless, even in cooperative breeders, inclusive fitness benefits could be overestimated (Clutton-Brock, 2002), with alternative explanations being possible (e.g. Kokko et al., 2001; Clutton-Brock, 2009). For example, kinship is often correlated with familiarity, which may confound effects of relatedness when it is not controlled for (e.g. Penn & Frommen, 2010). In addition, nowadays there is convincing evidence that the evolution and maintenance of cooperative breeding can be independent from genetic relatedness among actors, and that it can be driven by direct fitness benefits among nonrelatives (Balshine-Earn et al., 1998; Queller et al., 2000; Stiver et al., 2005; Riehl, 2010).

In the present study, we examine kin-biased cooperation in a noncooperatively breeding fish. Fishes are a major group in the study of the evolution of group living (Krause & Ruxton, 2002). Several studies demonstrated kin-biased shoaling preferences (e.g. Ward & Hart, 2003; Gerlach & Lysiak, 2006; Frommen et al., 2007) or kin-structured populations (e.g. Gerlach et al., 2001; Piyapong et al., 2011; but see Croft et al., 2012). Still, the adaptive significance of kin structuring often remains unclear. Kin selection has been suggested as an evolutionary force promoting and maintaining shoaling with related individuals (Smith, 1986; Alfieri & Dugatkin, 2006). Especially, predator inspection offers an excellent opportunity to study kin-biased cooperative behaviour, because it has clear benefits and costs. Kin selection has been postulated as a means to maintain cooperation in predator inspection visits (for a detailed discussion, see Wilson & Dugatkin, 1997; Thomas et al., 2008). During predator inspection, one or more individuals leave the safety of a group and inspect a potential predator (Milinski, 1987; Dugatkin, 1988). By doing so, they gain, on the one hand, information about the current predation risk; on the other hand, they face high costs in terms of an increase in predation risk (Dugatkin, 1992; Milinski et al., 1997). Predator inspection is often carried out in pairs or small groups (Pitcher et al., 1986), which is thought of as being beneficial, as companions dilute the risk when staying close enough to the leader (Milinski et al., 1997). Inspecting in groups has been shown to follow complex behavioural rules (e.g. Dugatkin, 1988; Dugatkin & Alfieri, 1991; Pitcher, 1992). Cooperative predator inspection has been explained using different theoretical approaches, including reciprocal cooperation, group selection or indirect genetic effects (e.g. Milinski, 1987; Wilson & Dugatkin, 1997; Bleakley & Brodie, 2009). Milinski (1987), for example, suggested that three-spined sticklebacks (Gasterosteus aculeatus) play 'tit for tat' when confronted with a predator. In contrast, Thomas et al. (2008) found no evidence for 'tit-for-tat' behaviour in guppies (Poecilia reticulata).

The aim of our study was to investigate to what extent cooperative predator inspection is influenced by relatedness and whether kin selection can maintain cooperation between related individuals, which has been postulated, but until now, seldom been tested experimentally. Thus, we tested whether dyads of juvenile cichlid fish composed of either unfamiliar kin or unfamiliar nonkin differed in their predator inspection behaviour. Our study animal, Pelvicachromis taeniatus, is a small biparental cichlid fish capable of recognizing kin through phenotype matching (Thünken et al., 2007; Hesse et al., 2012; Thünken et al., 2014). Juveniles form loose shoals in nature (Lamboj, 2006) and under laboratory conditions, and kin forms denser shoals than nonkin (Hesse & Thünken, 2014). Furthermore, juveniles engage in predator inspection behaviour (Hesse et al., 2015). Thus, juvenile P. taeniatus are a suitable model organism to study the potential for kin selection acting on the evolution of cooperative behaviour.

# **Materials and methods**

# Study animal

*Pelvicachromis taeniatus* is a small, cave-breeding cichlid from Western Africa. Our study population originated from the Moliwe River in Cameroon (Langen *et al.*, 2011). *Pelvicachromis taeniatus* forms monogamous pairs, and males and females prefer close kin as breeding partners (Thünken *et al.*, 2007, 2012). *Pelvicachromis taeniatus* performs biparental brood care and free-swimming fry are guarded by both parents for several weeks (Thünken *et al.*, 2010). Hereafter, juveniles live in shoals until they reach sexual maturity (Lamboj, 2006). When given the choice between a shoal of related and unrelated individuals, juvenile *P. taeniatus* prefer shoaling with their kin (Thünken *et al.,* 2015).

#### Breeding of experimental fish

All experimental fish were bred under standardized conditions between April and October 2011 at the laboratory of the Institute of Evolutionary Biology and Ecology of the University of Bonn. Breeding pairs of unrelated *P. taeniatus* (F1 generation of wild-caught fish) were individually introduced into a breeding tank  $(length \times width \times height:$ 45 cm  $\times$  40 cm  $\times$  30 cm, one breeding pair per tank), which was equipped with a breeding cave, an aquarium heater, an internal filter, gravel and java moss (Taxiphyllum barbieri). The water temperature was kept at  $24 \pm 1$  °C and the light: dark regime was 12:12 h. They were fed daily with a mixture of defrosted Chironomus larvae, Artemia and black mosquito larvae. Until spawning occurred, approx. 30% of the water was exchanged weekly to increase spawning probability. Breeding caves were checked for eggs daily.

## Rearing conditions of experimental fish

After spawning, eggs of 15 breeding pairs were removed from the parents and raised artificially in small tanks (30 cm  $\times$  20 cm  $\times$  20 cm). To examine the effect of relatedness on cooperation independent from familiarity, sibling groups were split  $14 \pm 1$  days after hatching into two subgroups of 10 to 15 fish. By doing so, we created similar sized groups of unfamiliar kin. Fish were split after  $14 \pm 1$  days as mortality rates at early larval stages (i.e. egg and wriggler stage) are unpredictable and vary greatly between clutches. Sibling groups were split shortly after individuals reached the free-swimming stage. Test fish spent only the egg (approx. 2 days) and larval stage (approx. 12 days) together. Trials took place at least 4 months after splitting the groups. It is highly unlikely that fish can individually recognize other fish with whom they spent a few days as larvae in a group consisting of more than 20 larvae months ago, and adjust their current behaviour based on those prior experiences and interactions with them (see also Utne-Palm & Hart, 2000). Thus, confounding effects based on familiarity are extremely unlikely. Each sibling group was housed in a tank  $(45 \text{ cm} \times 40 \text{ cm} \times 30 \text{ cm})$  equipped with sand, java moss and an internal filter. All tanks were surrounded by opaque plastic sheets to prevent visual contact between inhabitants of different tanks. The water temperature was kept at 23  $\pm$  1 °C, and the experimental subjects were held under a light: dark regime of 12:12 h. Free-swimming fry were first fed with living Artemia nauplii provided in a standardized, highly concentrated suspension (10  $\mu$ l per fish). Later on, fish were fed daily in excess with a mixture of defrosted Chironimus larvae, Artemia and black mosquito larvae.

# Predators

We used five snakeheads (Parachanna obscura, mean total length =  $13.25 \pm SD = 1.37$  cm) as predators. P. obscura is a sit-and-wait predator, which occurs in the same habitat as P. taeniatus (Bonou & Teugels, 1985). Snakeheads are an established predator model for the study of antipredator behaviour (e.g. Kelley & Magurran, 2003; Botham et al., 2006; Hesse et al., 2015). They were obtained from a commercial fish trader (PantaRhei Aquaristik, Wedemark) and housed individually in tanks (45 cm  $\times$  40 cm  $\times$  30 cm) equipped with an internal filter, gravel, plants (java moss (Taxiphyllum barbieri), java fern (Microsorum pteropus)) and rocks to provide shelter. The water temperature was kept at  $23 \pm 1$  °C, and the light: dark regime was set to 12:12 h. Snakeheads were fed every 3 days with a freshly killed P. taeniatus.

## **Experimental set-up**

The experimental tank (70 cm  $\times$  35 cm  $\times$  35 cm, water level 12 cm) was divided into three compartments: a predator compartment (15 cm), an experimental compartment (38.5 cm) and an acclimatization compartment containing a plastic plant as refuge (16.5 cm) (Fig. 1) (cf. Frommen et al., 2009). The acclimatization compartment was separated from the rest of the tank by a removable, opaque plastic partition to ensure an undisturbed acclimatization period. A transparent perforated plastic partition (permitting visual as well as olfactory contact between prey and predator) separated the predator compartment from the experimental compartment. The experimental compartment contained an inspection zone (22 cm) directly in front of the predator compartment. The size of the inspection zone was based on the size of the predators and the highest predation risk found in the literature based on fast start performance of teleost fish (Webb, 1978; Domenici & Blake, 1997) and on an experiment on risk allocation (Milinski et al., 1997). Pretests revealed that the behaviour in the inspection zone was different from normal shoaling behaviour. Within this distance to the predator, fish showed inspection behaviour, that is they approached the predator purposefully, stopped near the predator and then slowly departed it again (Pitcher et al., 1986). All compartments were marked on the bottom by black lines.

To avoid interactions of test fish with their reflections, the experimental tank was covered with grey plastic sheets on the inner sides. Additionally, the experimental tank was surrounded with white Styrofoam to minimize disturbance. The tank was filled with aged, substrate-treated tap water  $(23 \pm 1 \text{ °C})$  (for a detailed explanation, see Meuthen *et al.*, 2011) to a height of 12 cm. After each experiment, it was cleaned, rinsed with hot water and refilled. Behaviours of test



**Fig. 1** Experimental set-up viewed from the side. The experimental tank (70 cm  $\times$  35 cm  $\times$  35 cm) was divided into three compartments: predator compartment (15 cm), experimental compartment (38.5 cm) containing the inspection zone (22 cm) and an acclimatization compartment (16.5 cm). The predator compartment was separated from the rest of the tank through a transparent perforated plastic sheet. The acclimatization compartment containing a plastic plant as refuge was separated by a removable opaque plastic sheet.

fish were recorded by a webcam (Logitech Webcam, Pro 9000) attached to a wooden frame placed 70 cm above the centre of the tank. The experimental tank was illuminated from above by a fluorescent tube (Osram Lumilux L, 58W).

## **Experimental procedure**

To investigate cooperative predator inspection, a pair of unfamiliar juvenile fish differing in relatedness was tested (full-sibling pairs vs. nonkin pairs). Fish were carefully netted and each was placed in a small plastic tank (17 cm  $\times$  10 cm  $\times$  10 cm, water level 5 cm). Characteristic fin patterns (dots) were recorded to recognize individual test fish, so fish could be placed back into their corresponding home tanks after the experiment. Test fish were immature, so their sex could not be determined definitely. Each fish was only tested once.

A snakehead was carefully netted and introduced into the predator compartment. No predator was used more than twice each day. Test fish were then transferred into the experimental tank by gently and simultaneously pouring them from the plastic tanks into the acclimatization compartment. They were allowed to acclimatize for 45 min. Subsequently, the opaque partition separating the acclimatization compartment from the experimental compartment was lifted using a pulley system. As our experimental fish were predator naïve individuals, we used a conspecific alarm cue (1 ml) that was added at the centre of the tank just before the trial started to elicit a stronger antipredator response, to increase the vigilance of test fish and stimulate the predator (Alemadi & Wisenden, 2002; Ferrari et al., 2009). Individuals used for alarm cue extraction were unrelated adult fish and the whole fish was used (for further details, see Meuthen et al., 2014). Each experiment lasted 45 min. Afterwards, the standard body length (SL = distance from the tip of the snout to the beginning of the caudal fin) of the test fish was measured. There was no significant size difference between fish of the two treatment groups (linear mixed-effects model (LME), LRT:  $\chi^2 = 1.739$ , d.f. = 1, *P* = 0.187, mean difference in SL<sub>unfamiliar</sub> kin  $\pm$  SD = 0.51  $\pm$  0.41 cm, mean difference in SL<sub>unfamiliar</sub> nonkin  $\pm$  SD = 0.39  $\pm$  0.2 cm).

#### Data acquisition

Videos were examined naively with regard to the identity of the test fish. After one fish entered the inspection zone, the trial started and the consecutive 400s were analysed. We chose this time frame since test fish habituated to the presence of predators with elapsed time (S. Hesse, personal observation). Snapshots were taken of each video every 5 s, that is 80 snapshots/trial. If both focal fish had not entered the inspection zone after 45 min, experiments were excluded from analysis (N = 5). We distinguish between two different types of inspections behaviour: (1) cooperative inspections or (2) solitary inspections. In a cooperative inspection, both fish entered the inspection zone - either simultaneously or time-delayed (i.e. the snapshot shows two fish in the inspection zone) - and a cooperative inspection ended when one fish left the inspection zone (i.e. the snapshot of a previously cooperatively inspecting dyad shows only one fish in the inspection zone). A solitary inspection was defined as only one fish entering the inspection zone (i.e. snapshot showing only one fish in the inspection zone) or a fish being abandoned by its companion fish (thus remaining in the inspection zone alone; only one fish present in the snapshot of a previously cooperatively inspecting dyad) and number of defections (number of events when one test fish was abandoned during predator inspection for each pair) for each trial was noted. A single inspection event ended either when the inspector left the inspection zone, or when the other fish entered the inspection zone (i.e.

time-delayed cooperative inspection). All inspection events were measured per dyad and not per individual fish as fish could not be individually distinguished from the snapshots. We recorded the time fish spent inspecting the predator either cooperatively or solitarily (time (s) inferred by the 5-sec snapshots spent in either cooperative or solitary inspection). To analyse the data, median values of each dyad were used.

#### Data analysis

In total, 46 valid trials were performed (unfamiliar kin: N = 21, unfamiliar nonkin: N = 25). Analyses were performed with the R. 2.9.1 statistical software package R-Development-Core-Team (2009). Data were normally distributed according to Lilliefors test and showed homogeneous variances according to Bartlett tests so linear mixed-effect models (LMEs) were run. Reported P-values of models refer to the increase in deviance when the respective variable was removed. Tests of statistical significance were based on likelihood ratio tests (LRT), which follow a chi-square distribution. These routines use maximum-likelihood parameter estimation. Nonsignificant factors were removed from the models. P-values are two-tailed throughout. Time spent in cooperative inspections was the dependent variable, kinship and body size difference were the explanatory variables, and family combination was entered as random factor and never removed to correct for multiple use of families. Time spent in solitary inspections and number of defections was also included as explanatory variables to test whether they affected time spent in cooperation. Additionally, the impact of relatedness on the time spent in solitary inspections using family means was examined using Wilcoxon rank sum test. Family means were used to analyse time spent in solitary inspection as data failed normal distribution after transformation. Number of defections was also examined using Wilcoxon rank sum test. Family means were used as data showed overdispersion.

# Results

Kinship explained differences in time test fish spent in cooperative inspections (LME, LRT:  $\chi^2 = 10.614$ , d.f. = 1, P = 0.001, Fig. 2). Time spent in cooperative inspections was negatively related to time spent in solitary inspection (LME, LRT:  $\chi^2 = 15.305$ , d.f. = 1, P < 0.001), whereas body size difference (LME, LRT:  $\chi^2 = 1.783$ , d.f. = 1, P = 0.182) and number of defections (LME, LRT:  $\chi^2 = 0.305$ , d.f. = 1, P = 0.861) did not significantly explain variation in time spent in cooperative inspections. Dyads consisting of nonkin spent significantly more time involved in solitary inspections events compared to dyads consisting of unfamiliar kin (mean\_{kin} \pm SD = 40.385s \pm 61.134, mean<sub>nonkin</sub>  $\pm$  SD = 112.115s  $\pm$  103.237; Wilcoxon rank

sum test, W = 33.500, P = 0.001, Fig. 3). Number of defections did not differ significantly between kin and nonkin (mean<sub>kin</sub>  $\pm$  SD = 0.885  $\pm$  0.860, mean<sub>non kin</sub>  $\pm$  SD = 1.192  $\pm$  1.182; Wilcoxon rank sum test, W = 72.5, P = 0.542).

# **Discussion**

Predation is among the strongest selective forces affecting the fitness of an individual (Lima & Dill, 1990). By inspecting a potential predator, valuable information may be gained upon the identity of the predator, its hunger status and intentions (Dugatkin, 1992). Cooperative predator inspection is a strategy to deal with predation. Here, we investigated differences in cooperative predator inspection behaviour between kin and nonkin dyads to evaluate the influence of kinship on this dangerous behaviour. Dvads of related P. taeniatus were significantly more often involved in cooperative predator inspections than nonkin dyads. The time test fish spent in solitary inspections also significantly explained the time test fish spent in cooperative inspection, indicating that cooperating individuals spent less time inspecting alone. Nonkin dyads performed significantly more solitary inspections, indicating a lower disposition to cooperate compared to kin dyads. In accordance with the predictions of kin selection theory (Hamilton, 1964), the results of the present study reveal that kinship enforces cooperation, in our case cooperative predator inspections in a fish.

Inspecting a predator also provides valuable direct fitness benefits for inspecting fish (Dugatkin & Godin, 1992; Pitcher, 1992) and explains why test fish engaged frequently in this highly risky behaviour alone. Direct information on type and hunger status of the predator allows prey to adjust their behaviour to the current predation threat, for example whether to stop foraging. However, a study performed with guppies by Dugatkin (1992) demonstrated how dangerous inspection visits



**Fig. 2** Mean time  $\pm$  SE (s) test fish spent in cooperative inspections during the trial (total time: 400 s). \*\*\*Indicates P = 0.001.

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**Fig. 3** Mean time (% of 400s trials) test fish spent in solitary inspections during the trial. Whiskers indicate standard error, **\*\***Indicates P < 0.01.

are. In that study, the tendency to approach a predator predicted mortality risk of individual guppies: fish engaging frequently in predator inspections were more likely to die (Dugatkin, 1992). Therefore, a cooperative predator inspection strategy like 'tit for tat' relying on frequent interactions between cooperating individuals is less beneficial if mortality risk is high, thus resulting in the death of one (or more) familiar players. In such a high-risk scenario, kin selection may facilitate cooperation between relatives and provide direct as well as indirect fitness benefits for inspectors and noninspectors (i.e. the rest of the shoal as information is transmitted, e.g Dugatkin & Godin, 1992). In shoals, small fish and especially juveniles are highly exposed to predation, as small prey is preferred by predators (see Sogard, 1997 for a review). If kin is more willing to cooperate, reciprocation between familiar individuals is no longer a prerequisite for cooperative predator inspection, especially if shoals (or populations) are kin-structured. Consequently, our results suggest that apart from cooperation triggered by reciprocal altruism, kin selection facilitates cooperation among unfamiliar individuals.

Besides kin selection, cooperation during predator inspection may be maintained by several mechanisms including by-product mutualism (Connor, 1995, Stephens *et al.*, 1997) and reciprocity (Milinski, 1987). The number of defections did not negatively affect the degree of cooperative behaviour (i.e. time spent in cooperative inspections). A negative correlation between number of defections and cooperation would be expected especially in a 'tit-for-tat'-like scenario. Our result is consistent with a study performed in guppies, showing that defection during predator inspection did not affect subsequent cooperative behaviour of defected individuals (Thomas *et al.*, 2008).

Several laboratory studies showed kin shoaling preferences in fishes, including cichlids (e.g. reviewed in Ward & Hart, 2003; Frommen et al., 2007; Lee-Jenkins & Godin, 2013). Still, the adaptive significance is often ambigous. Benefits of grouping with kin are suggested to include improved responses to predators (Hain & Neff, 2009) and increased shoal cohesion (Hesse & Thünken, 2014), increased growth rates (Gerlach et al., 2007; Thünken et al., 2015) and less aggression (Olsen et al., 1996). Still, other studies did not describe such advantages (e.g. Mehlis et al., 2009). Interestingly, Pivapong et al. (2011) found kin aggregation only in predator rich habitats, pointing towards a link between predatory environment and kin-triggered social aggregations. In our study, we provide evidence how kin recognition leads to kin-directed antipredator benefits in such a risky environment.

In summary, our results indicate that kinship influences and shapes cooperative behaviour in a predation context. As predicted by kin selection theory, risk sharing and cooperation was kin-biased; related dyads of juvenile *P. taeniatus* were more willing to cooperate. Although predator inspection scenarios have traditionally been used to investigate the evolution of cooperation based on reciprocity, our experiment demonstrates for the first time that cooperation during predator inspection may also be based on kinship. Therefore, our study increases our understanding of how kin-directed benefits facilitate the evolution of cooperation.

## **Ethical statement**

The experiments comply with the current laws in the country in which they were performed.

# **Conflict of interest**

The authors declare that they have no conflict of interest.

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# **Supporting information**

Additional Supporting Information may be found in the online version of this article:

Data S1 Supporting information.

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