Cooperative nest defence in red-winged blackbirds: reciprocal altruism, kinship or by-product mutualism?

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Male red-winged blackbirds (*Agelaius phoeniceus*) often cooperate with their neighbours in defending nests against predators. Some studies have suggested that this is an example of by-product mutualism, whereas others have suggested the possibility of reciprocal altruism. No study has addressed the possibility of kin-selected cooperation in nest defence in this species. Reciprocal altruism, kin selection and by-product mutualism are not mutually exclusive alternatives, but few studies of territorial neighbours have tested for multiple mechanisms simultaneously. We test for these three possibilities in a population of red-winged blackbirds. We used simulated defections to test for reciprocal altruism. We used analysis of microsatellite loci to test for kin selection between adult male neighbours. We also used microsatellite loci to test for by-product mutualism resulting from nest defence of offspring sired on neighbouring territories. We found that male red-winged blackbirds cooperate in nest defence primarily as a form of reciprocal altruism. Experimental males reduced their level of nest defence relative to controls following simulated defection by a neighbour. In contrast to some earlier studies, we found no evidence for by-product mutualism: males did not defend nests where they had sired extra-pair offspring. We also found no evidence for kin selection: males were no more cooperative with more closely related neighbours. Considered alongside the results from other studies, our study suggests that mechanisms stabilizing cooperation in red-winged blackbirds may vary among populations.

Keywords: altruism; cooperation; tit-for-tat; mutualism; red-winged blackbirds; *Agelaius phoeniceus*

1. INTRODUCTION

Male red-winged blackbirds (*Agelaius phoeniceus*) often help defend the nests and territories of neighbouring males from potential nest predators (e.g. crows *Corvus brachyrhynchos*) (Beletsky & Orians 1989). By cooperating in nest defence, two males may be able to drive off a potential nest predator more effectively than can a lone male. If nest defence is costly, however, there may be a temptation to cheat, thereby avoiding the costs of nest defence. This creates a social dilemma for pairs of neighbours akin to the well-known Prisoner's Dilemma (Axelrod & Hamilton 1981): although both neighbours would do better with cooperative action, the temptation to cheat tends to destabilize cooperation. A fundamental problem in evolutionary biology is understanding how populations of cooperators are stabilized against invasion by cheaters (Darwin 1859; Dugatkin 1997).

One potential stabilizing mechanism is by-product mutualism, where the cooperative donor benefits by helping his own offspring on the cuckolded neighbour's territory and the recipient benefits as a by-product of the helper's selfish parental behaviour (Connor 1986, 1995). Previous studies of red-winged blackbirds suggest that males defend nests on other territories if they have sired offspring in those nests (Weatherhead *et al.* 1994; Gray 1997). This suggests that what appears to be an altruistic act is in fact a male's selfish defence of his own offspring. If cooperative

nest defence is a by-product of cuckoldry and parental care, there should be no temptation to cheat and no reciprocal punishment of perceived cheaters.

A second possible explanation for cooperative nest defence is kinship between neighbouring males (Hamilton 1964*a*,*b*). Although red-winged blackbirds are thought to disperse widely and not preferentially settle near relatives (Orians 1961), to our knowledge, this has not been tested using genetic data. This hypothesis predicts that males should be more cooperative towards particular neighbours to whom they are more closely related.

If there are no mutualistic or kin-selected benefits to the helper, other explanations are required. One interesting alternative is reciprocal altruism (Trivers 1971) stabilized by repeated play in a meta-game between neighbours (Axelrod & Hamilton 1981). There are several reciprocal strategies explored in the literature, the most prominent being tit-for-tat (TFT) (Axelrod & Hamilton 1981; Axelrod & Dion 1988). A TFT strategist plays a backward-looking Golden Rule, doing unto its partner what its partner did unto it in the preceding play. Reciprocity makes the pay-off from cooperation frequency dependent and stabilizes a cooperative population against invasion by cheaters (Getty 1987; Eshel *et al.* 1998). Despite numerous examples of the apparent use of TFT-like strategies in a variety of species (Packer 1977; Wilkinson 1984; Milinski 1987, 1990; Dugatkin 1991; Godard 1993), there is still controversy over the importance of reciprocal strategies in the maintenance of cooperation in non-human vertebrates (Masters & Waite 1990; Connor 1992; Milinski 1996).

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The natural history of red-winged blackbirds is consistent with the conditions that favour the evolution of reciprocal altruism. Red-winged blackbirds are colonial, and neighbouring males often have relationships that last beyond a single breeding season (Beletsky & Orians 1989). Long-term relationships can favour the evolution of 'dear enemy' relationships through reciprocal altruism (Trivers 1971; Getty 1987). Beletsky & Orians (1989) have shown that males with familiar neighbours enjoy higher nesting success than males with unfamiliar neighbours, possibly because familiarity facilitates mutual defence. Weatherhead (1995), however, found the reverse pattern: males with at least one unfamiliar neighbour had reduced nest predation.

In this study, we tested for mutualism, kin selection and reciprocal altruism in a single population of red-winged blackbirds. We tested for by-product mutualism and kin selection by combining molecular paternity analysis of males and chicks on neighbouring territories with observations of nest defence against a stuffed crow. If nest defence is a form of by-product mutualism, then males should preferentially defend nests where they have sired extra-pair offspring. In addition, nests that contain extra-pair offspring should attract more neighbouring males to aid in defence since neighbours are the most likely sires of extra-pair offspring (Gibbs *et al.* 1990). If cooperative nest defence is a form of kin selection, then males should cooperate more with males with whom they share a greater genetic relatedness. An alternative form of by-product mutualism, which we cannot address in this study, might occur if the donor benefits directly by removing the potential nest predator from the neighbourhood, benefiting all of its neighbours indirectly. Defection should not elicit retaliation in any mutualistic or kin-selected interactions but it should when the individuals are using TFT-like reciprocal strategies (Rothstein & Pierotti 1988). We therefore use simulated defections to test for retaliation in response to defection.

2. MATERIAL AND METHODS

(a) *General methods*

We conducted this study at the Kellogg Biological Station Experimental Pond Facility, Hickory Corners, MI, USA (42°24′ N, 85°24′ W) in 1998 and 1999. The site consists of 18 ponds, which are *ca.* 30 m in diameter and 3 m deep. The ponds are arranged in three rows of six. Within rows, ponds are *ca.* 5 m apart, and rows are spaced *ca.* 10 m apart. The margin of each pond is densely planted with cattails (*Typha latifolia*). Between one and four males settled on each pond and each male held a harem of between one and six females. All territorial males were captured prior to the onset of nesting and given a unique combination of three coloured leg rings plus a numbered aluminium leg ring. We mapped each territory as it was established by observing the resident male's behaviour (Beletsky & Orians 1987).

(b) *Observations of cooperative nest defence on neighbouring territories*

To test for by-product mutualism, we determined each male's willingness to defend nests in other territories. In 1998, we presented stuffed crows at each of 98 nests 1–4 days after hatching. Red-winged blackbirds increase their level of nest defence

substantially after hatching and we were therefore more likely to observe helping in nest defence at this time (Knight & Temple 1988). To minimize the effects of repeated exposure to the stuffed crow (Knight & Temple 1986), we did not present the crow to nests on the same pond on the same day. Similarly, we did not present the crow to males neighbouring any location where we had already made a presentation that day. All presentations were performed between 06.00 and 10.00, when males were most likely to be on their territories. If not all neighbours were present for a presentation, we returned the next day to perform a replacement presentation.

For each trial, we positioned a stuffed crow in a perched position within 1 m of the nest. The crow was initially covered with a cloth. We then observed any reaction to the covered crow for 3 min from a hide 10 m from the crow. There was never any reaction to the covered crow. The cloth was removed by using an attached string, and we observed any reaction to the crow by all neighbours for 3 min.

We recorded the identity of each responding male and its latency to approach, closest approach, number of vocalizations of each type, hovers, dives and strikes. Red-winged blackbirds are known to use as many as seven defence calls (Orians 1961; Knight & Temple 1988) but for analyses we kept a tally of only the most common calls, the 'Teer' and the 'Titi'. We combined all other calls excluding the ubiquitous 'Chit' call, which red-winged blackbirds use constantly and which is probably a contact call between males and females (Yasukawa 1989).

(c) *Experimental tests for retaliation*

In 1999, we used simulated defections within pairs of neighbouring males to test for retaliation. We first recorded the territorial songs of all males during early April (two weeks prior to nesting but well after territories were established). All recordings were made from a hide between 06.00 and 10.00. We uploaded several examples of territorial song from each male into .aud files at 16 bit resolution. We then filtered as much noise as possible from the recordings without reducing the quality of the song and standardized all songs to equal amplitude using COOL EDIT sound-editing software. To avoid pseudoreplication (Kroodsma *et al.* 2001) we spliced a minimum of four song examples from each male together, separating each example with 10 s of silence. This resulted in a call rate of approximately six songs per minute (within the natural song rate in this population).

We performed simulated defection trials in late May when almost all nests contained nestlings from the first brood. All presentations were made between 06.00 and 10.00 on calm days. A simulated-defection trial consisted of pre-defection, defection and post-defection presentations of a crow. For each presentation, we placed a stuffed crow covered with a cloth at the territorial boundary (as opposed to observations of cooperative nest defence where we placed the crow 1 m from the nest). This ensured that, on average, both males should benefit equally from nest defence. After placing the covered crow, we retreated to a hide 10 m away and allowed 3 min for all males in the area to recover from the disturbance. The cover was removed with a string, and we observed each neighbour's behaviour for 3 min, recording the same measures as described in § 2b.

We first performed pre-defection presentations at all territorial boundaries in the population. We also used these data from pre-defection trials to test whether males cooperated more with more closely related neighbours. Following the pre-defection trials, we randomly assigned pairs of neighbouring males to experimental and control groups. To simulate defections in the experimental

group, we captured one of the two neighbours prior to the defection presentation and held it in a covered cage located in a nearby building for the duration of the presentation. We captured males using walk-in traps baited with corn. These traps were situated so that they were not associated with any particular territory and treatments were treated identically in this respect. It is therefore unlikely that the act of capturing the males influenced our results. The captured male (neighbouring male; the focal male being the non-captured male) was supplied with a generous amount of food and suffered no apparent ill effects from its short time in captivity. In the control group, neighbouring males were captured and taken to the same building but immediately released. We waited 30 min and in the case of the control group verified that the captured male had returned to his territory. We then performed a crow presentation as above except that, for experimental pairs, we played a recording of the captured male's song from within his territory. For pairs assigned to the control treatment, we played recorded ambient noise from within the captured male's territory during the defection presentation. The ambient noise was recorded from nearby areas that were not inhabited by red-winged blackbirds (each playback of ambient sound was a different recording made on a different day or at a different location). The captured male in experimental treatments was released immediately after the simulated defection and we verified that it had returned to its territory. The post-defection presentation was identical to the pre-defection presentation and was performed on the day following the simulated defection after we had verified that both males were present.

(d) Genetic analyses

When we captured birds for marking, we also obtained 50–80 μl of blood from the brachial vein of all territorial males and many females, and immediately placed each sample in 800 ml of 'Queen's' lysis buffer (Seutin *et al*. 1991). We obtained 50 μl blood samples from nestlings taken from the brachial vein at *ca*. 8 days after hatching. DNA was extracted from the blood samples using Proteinase K digestion followed by NH4A0C extraction and precipitation in isopropyl alcohol. The DNA was washed once more in 70% ethyl alcohol.

We determined the paternity of nestlings using six microsatellite loci: Qm 5, Qm 10, Qm 21, Qm 31, Dpμ 16 and Maμ10 (Dawson *et al*. 1997; Gibbs *et al*. 1997). We assayed genetic variation at these loci using PCR amplification in 25 μl reaction volumes. Two different reaction conditions were required for the six loci. Qm 10, Dpμ 16 and Maμ 10 were amplified using 250 ng of template DNA, 2 pmol of each primer (fluorescently labelled forward primer), 500 μM of dNTPs and 0.75 units of Taq polymerase. Qm 5, Qm 21 and Qm 31 were amplified using 125 ng of template DNA, 1.25 pmol of each primer (fluorescently labelled forward primer), 625 μM of dNTPs and 0.75 units of Taq polymerase. The PCR product was run on 6% polyacrylamide gels and visualized using an FMBIO gel scanner. All gels were scored manually by two authors (R.O. and K.S.) and verified using FMBIO image-analysis software.

To maximize our ability to assign paternity, we included only territorial males as potential fathers. Previous work has shown that floating males and non-neighbours rarely, if ever, gain fertilizations (Gibbs *et al*. 1990). We calculated maximum-likelihood estimates of paternity using the program CERVUS (Marshall *et al*. 1998). We were able to establish paternity for all but two out of 250 offspring for which we obtained DNA. Out of the offspring for which we were able to determine paternity, 217 had

Table 1. Component-variable loadings and percentage of variance explained by each of the first two corresponding dimensions are shown for the correspondence analysis performed on the defence data recorded over both years of this study.

	CD1	CD2
approach	−0.90	0.03
latency	−0.87	0.06
hover	0.64	0.11
teer	0.63	−0.42
strikes	0.52	−0.90
dives	0.48	0.10
titi	0.35	0.23
calls	0.30	0.79
percentage variance	43.05	17.55

only one candidate father that matched for all loci. For the offspring with more than one non-excluded father, 26 were assigned paternity at the 95% confidence level and seven were assigned paternity at the 80% confidence level. The two offspring for which no male could be assigned paternity were mismatched at two or more loci for all candidate fathers. We estimated the pairwise and average relatednesses among territorial males using the program RELATEDNESS (Queller & Goodnight 1989; Goodnight & Queller 2001).

(e) Statistical analysis

Since our behavioural observations consisted of measures of several nest-defence behaviour variables for each individual, we used correspondence analysis (CA) to reduce the number of variables to two orthogonal variables. CA is similar to principal component analysis (PCA) in that it reduces the number of dimensions in a multivariate dataset. CA, however, is more appropriate than PCA when the component variables are not linearly related or are composed of counts (ter Braak 1985). In the case of nest defence, CA is more appropriate because some component behaviours may be expressed only at low levels of aggression, whereas others may be expressed only at higher levels of aggression. For instance, closeness of approach must reach zero before any strikes can occur. In addition, except for closest approach, each component variable is a count rather than a continuous metric.

We performed a single CA on all nest-defence data from the study. The component variables correlated with the first correspondent dimension (CD1) such that an increase in CD1 was associated with an increase in all components nest defence (table 1). For clarity, we therefore refer to CD1 as nest defence. The second correspondent dimension (CD2) appeared to distinguish individuals that favoured the less common calls over 'Teer' calls and strikes from those that favoured strikes and 'Teer' calls (table 1). However, analyses on CD2 yielded no significant results and therefore are not discussed further.

3. RESULTS

(a) Relatedness among neighbouring males

Males were no more related to neighbours than they were to non-neighbours (two-sample *t*-test, separate variances: $t_{89.5} = -1.462$, $p > 0.10$). However, males may still be more cooperative with neighbours who are closely related to them. We therefore tested for a relationship between defence behaviour directed towards a crow

presented on a territorial boundary and the relatedness between the male and its neighbour. We found no significant relationship between genetic relatedness and defence behaviour directed towards a crow at a territorial boundary (ANCOVA: neighbour $F_{18,39} = 0.443$, $p > 0.95$; relatedness $F_{1,39} = 0.713$, $p > 0.25$).

(b) Nest defence in response to cuckoldry

To determine whether neighbouring males were more likely to defend nests in which they had sired offspring, we compared the genetic father of cuckolded offspring with another randomly chosen unrelated neighbour whose territory was the same distance from the nest. We included only nests with one cuckolding male. There was no significant difference in nest defence between males with offspring in the nest and those without (paired *t*-test: $t_{24} = 0.654$, $p > 0.995$).

It is possible that males are able to gain copulations with more than one female on a territory and only a few of those copulations result in fertilizations. We therefore compared the average level of defence at a nest for all nests on a territory (data from 1998) by a male that gained fertilizations on that territory and by a randomly chosen neighbour that had not gained any fertilizations. There was again no significant difference in nest defence between males that had gained fertilizations on a territory and those that had not (paired *t*-test: $t_{20} = 0.323$, $p > 0.995$).

If females solicit copulations from several males to gain additional parental care in the form of increased nest defence, then nests with cuckolded young might receive more helpers than those without cuckolded young. We found a significant association between the number of males attacking a crow and cuckoldry (nested ANOVA: territory $F_{13,19} = 1.708$, $p > 0.10$, cuckoldry within territory $F_{19,32} = 2.498$, $p < 0.05$). However, the difference is in the opposite direction to that predicted: nests without extra-pair fertilizations had slightly more helpers than those with extra-pair fertilizations.

Territorial males may reduce their defence of a nest on their territory if they are able to determine that the nest contains cuckolded offspring. We again used a nested ANOVA to account for the fact that males have multiple nests on each territory. Territorial males did not significantly alter their level of nest defence in response to cuckoldry (nested ANOVA: territory $F_{13,19} = 1.659$, $p > 0.10$, cuckoldry within territory $F_{19,32} = 1.751$, $p > 0.10$).

(c) Simulated defection experiment

The results of the defection experiment showed that experimental males reduce their level of nest defence compared with control males in response to their neighbour's lack of defence in the defection presentation (repeated-measures ANOVA: treatment $F_{1,11} = 2.571$, $p > 0.10$, trial $F_{2,22} = 0.429$, $p > 0.50$, treatment × trial $F_{2,22} = 4.132$, $p < 0.05$; figure 1*a*.) Nest defence by experimental and control males was almost identical during the pre-defection presentation of the crow. Focal males in the experimental group significantly decreased their level of nest defence during the defection presentation and the day after defection at the common territorial border. Focal males in the control group increased their level of defence slightly over the same period.
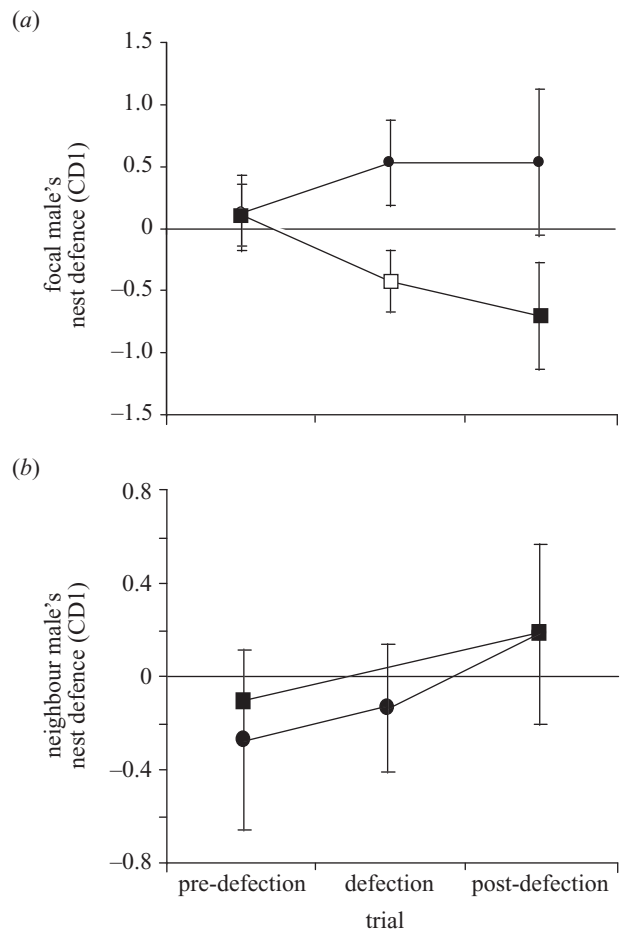
Figure 1. Nest-defence behaviour towards a mounted crow by (*a*) the focal male and (*b*) the neighbouring male. Experimental trials ($n = 6$) are indicated by squares and control trials by circles. An open symbol indicates that the neighbouring male was temporarily removed and a playback of his territorial song played instead. Filled symbols indicate that both males were free to participate in nest defence. Error bars: ± 1 s.e.

The decrease in defence observed for focal males in the experimental treatment might result from differences in the behaviour of neighbouring males between control and experimental treatments resulting from their differing lengths of captivity. We therefore compared the defence behaviour of neighbouring males between treatments in both pre- and post-defection presentations. We found no significant difference in the defence behaviour of neighbouring males in response to the treatment or between trials (repeated-measures ANOVA: treatment $F_{1,11} = 0.047$, $p > 0.75$, trial $F_{1,11} = 0.882$, $p > 0.25$, treatment × trial $F_{1,11} = 0.044$, $p > 0.75$; figure 1*b*). This implies that neighbouring males did not respond to the focal males' reduced nest defence. This may be because of the difference between reduced defence and out-of-sight no defence. It is likely that the reduction in defence by the neighbouring males is small compared with the initial reduction by the focal male when the neighbour was entirely absent.

4. DISCUSSION

We found that, in our population, reciprocity plays a significant role in stabilizing cooperative nest defence

among territorial males. We found no evidence for kin selection or by-product mutualism. Experimental males decreased their defence against a nest predator after their neighbours appeared to defect in the earlier defection trial. Control males maintained or increased their level of defence. There was no association between nest defence and cuckoldry, suggesting that by-product mutualism vis-à-vis parental care plays a minimal role in cooperative nest defence in this population. We also found no evidence for kin selection among adult male neighbours in this population. Males neither live with relatives nor cooperate more with males with whom they share a greater genetic relationship. We cannot rule out the hypothesis that nest defence by a male may also help its neighbours by driving potential nest predators from the area (by-product mutualism).

Our finding that males do not preferentially defend nests where they have sired extra-pair offspring contrasts with other studies. Gray (1997) found that males were more likely to defend at nests where they had cuckolded offspring, and Weatherhead *et al.* (1994) found that nests that contained extra-pair offspring enjoyed higher nesting success. In our population, nest success was almost 100% and the few nests that failed did so because of nestling starvation, not predation. We were therefore unable to examine the relationship between cooperative nest defence and nest success.

We were not able to address the hypothesis that cooperative nest defence is a form of by-product mutualism where 'donors' benefit immediately from efforts to drive the crow out of the neighbourhood, and the recipients also benefit from this. There is, however, some anecdotal evidence that this process may be occurring. Crows are abundant in the area and, when red-winged blackbirds are not breeding, frequently forage along the pond margins. However, when red-winged blackbirds are breeding crows are never seen near the ponds, although they are frequently heard in the trees nearby. When the breeding season is over, the crows immediately return. This suggests that nest defence may benefit all the males breeding in the area. This 'get-it-out-of-the-neighbourhood' or 'move-on' hypothesis may explain some of the mobbing behaviour, but it is not a mutually exclusive alternative to reciprocity.

A decrease in nest-defence intensity in response to perceived defection by their neighbour lends strong support to the hypothesis that males in this population of red-winged blackbirds use TFT-like strategies in their cooperative interactions. No other form of cooperation (i.e. mutualism or kin selection) predicts selection for retaliation in response to defection (Rothstein & Pierotti 1988). The threat of retaliation in response to defection helps preserve cooperative relationships since retaliation in the future would be costly for the defector (Axelrod & Hamilton 1981). Since red-winged blackbirds are neighbours for the entire breeding season and possibly future breeding seasons (Beletsky & Orians 1989), males should have an interest in perpetuating cooperative relationships with their neighbours.

The reduction in observed defence could have been in response to increased risk to the focal male when the defecting male did not participate in defence. By defending alone, the focal male was probably at greater risk of injury than when the neighbour aided in defence (Milinski 1987; Dugatkin 1988; Lazarus & Metcalfe 1990; Masters & Waite 1990; Milinski *et al.* 1990). Increased risk may have caused the focal male to decrease nest defence during the defection presentation. However, this does not explain why males continued to show decreased nest defence in the post-defection presentation, especially since the neighbouring male had returned and was defending at the same level as in the pre-defection presentation (figure 1*b*). The one plausible adaptive explanation therefore is that males reduced nest defence in the post-defection presentation because they were retaliating in response to the perceived defection by their neighbour.

The combined results of our study and others suggest that the selective forces responsible for cooperative nest defence may vary among populations. Why populations vary in the mechanisms that stabilize cooperation is an open question. Factors such as rate of extra-pair paternity, geometry of territories or density of territories could potentially alter the benefits of cooperating and cheating. Nesting success is known to vary with marsh geometry and predator composition (Weatherhead 1995). Weatherhead (1995) also found that females do better when nesting with males with unfamiliar neighbours, while Beletsky & Orians (1989) found that familiar neighbours enhance reproductive success. Comparative studies of populations with different densities or rates of cuckoldry could be useful in identifying ecological parameters that influence the evolution and maintenance of cooperative behaviours.

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