# Nest sanitation as an effective defence against brood parasitism

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## ABSTRACT

Egg rejection is a crucial defence strategy against brood parasitism, that requires the host to correctly recognise the foreign egg. Rejection behaviour has thus evolved in many hosts, facilitated by the visual differences between the parasitic and host eggs, and driving hosts to rely on colour and pattern cues. On the other hand, the need to recognise non-egg-shaped objects to carry out nest sanitation led birds to evolve the ability to discriminate and eject objects using mainly shape cues. However, little is known regarding the evolutionary significance of rejection behaviour in general and the cognitive processes underlying it. Here, we investigated the response of the barn swallow (*Hirundo rustica*) during pre-laying and laying stages to four objects types that differed in shape (eggs vs stars) and colour/pattern (mimetic vs non-mimetic) to investigate 1) what cognitive mechanisms are involved in object discrimination and 2) whether egg rejection is a direct defence against brood parasitism, or simply a product of nest sanitation. We found that swallows ejected stars more often than eggs in both stages, indicating that swallows possess a template for the shape of their eggs. Since the effect of colour/pattern on ejection decisions was minor, we suggest that barn swallows have not evolved a direct defence against brood parasitism but instead, egg ejection might be a product of their well-developed nest sanitation behaviour. Nonetheless, the fact that mimetic eggs were ejected especially in the pre-laying stage shows that nest sanitation could be an effective defence against poorly timed brood parasitism.

**Keywords:** mimicry, nest cleaning, visual cues, sensory ecology, template image, illumination

## Introduction

Brood parasites lay their eggs in host nests and reduce their reproductive costs by exploiting the parental care of their hosts. Parasitic behaviour usually decreases the fitness of host parents because of its negative effect on the survival of their offspring (Spottiswoode et al. 2012; Soler 2017a). In birds, hosts have therefore evolved various defence strategies, of which the most studied is parasitic egg rejection (Honza and Cherry 2017). Since egg rejection usually involves egg recognition (but see Šulc et al. 2019), brood parasitism is an excellent system for studying animal cognition (Manna et al. 2017).

We distinguish between interspecific brood parasites that lay eggs in the nests of other species, and conspecific brood parasites that lay eggs in the nests of the same species (Soler 2017b). Egg rejection behaviour has evolved in many hosts of interspecific brood parasites and can be manifested in three ways: egg ejection, egg burial, and nest desertion (Soler 2017b). The evolution of rejection behaviour has been facilitated by the noticeable difference between parasitic and host eggs allowing hosts to use various visual cues for recognition (Honza and Cherry 2017; Samaš et al. 2021). Egg rejection has also been observed in some conspecific brood parasites (Møller 1987; Brown and Brown 1989; Peer and Sealy 2000; Lahti and Lahti 2002; Lyon 2003; de Hierro and Ryan 2008; Soler et al. 2011; Samaš et al. 2014), whose eggs are intrinsically more similar to the host eggs. All these studies indicate that hosts have at least a rudimentary ability to recognise and reject parasitic eggs. However, whether egg rejection is a specific defensive response against brood parasitism, or simply nest sanitation behaviour, may in some cases be unclear (Moskát et al. 2003; Moskát and Hauber 2007; Wang et al. 2015; Guigueno and Sealy 2017).

Nest sanitation refers to a behaviour exhibited by many bird species (especially passerines), which consists of cleaning the nest of debris. This behaviour holds several adaptive functions, e.g. to keep the nest dry and free of parasites, reduce predator attraction, and facilitate egg-turning during incubation (Guigueno and Sealy 2017). Several studies have suggested that egg recognition evolved from the need to be able to recognise non-egg-shape objects during nest sanitation, and was later co-opted as a defensive strategy against brood parasitism (Yang et al. 2015a, b). The idea was first proposed by Rothstein (Rothstein 1975a) because egg ejection uses motor patterns identical to the removal of non-egg-shape debris (Swynnerton 1918; Rothstein 1975a). While this hypothesis has generally received poor support (reviewed in Li et al. 2021), it must be noted that all studies to date have only investigated correlations between nest sanitation and egg removing behaviour (Peer and Sealy 2004; Yang et al. 2015b; Luro and Hauber 2017) or whether nest sanitation elicits egg ejection (Yang et al. 2015a; Luro and Hauber 2017; Peer 2017; Su et al. 2018; Stratton and Dearborn 2021) without an evolutionary perspective. Therefore, further investigation of the potential relationship between the evolution of nest sanitation behaviour and foreign egg ejection is needed (Guigueno and Sealy 2017; Yang 2021).

It has been shown that shape is a key cue that affects whether birds reject or accept an object (Ortega and Cruz 1988; Moskát et al. 2003; Underwood and Sealy 2006; Guigueno and Sealy 2009; Poláček et al. 2013; Yang et al. 2019; Hauber et al. 2021). The shape of an object is logically a much stronger cue for nest sanitation than for parasitic egg rejection because eggs are more limited in their shape variability than non-egg-shape objects. Accordingly, egg shape variation exerts a weak effect on parasitic egg rejection by hosts, while egg colour and pattern are much more important cues (Honza and Cherry 2017; Samaš et al. 2021). This may be particularly the case for hosts of interspecific brood parasites, because colour and patterning are usually what distinguishes host and parasitic eggs the most. In conspecific brood parasites, the difference may be subtler because the hosts and parasites are females of the same species and have eggs highly similar not only in shape, but also in colour and pattern. Thus, relying on object shape as the main cue for object rejection (*i.e.* higher rejection rate of non-egg-shaped objects irrespective of their colour/pattern) would provide evidence of nest sanitation behaviour. On the other hand, hosts that have evolved defence against brood parasitism should use colour and/or pattern to recognize parasitic eggs.

Avian brood parasitism research has focused on several major cognitive mechanisms used in the process of foreign egg recognition (Rothstein 1974; reviewed in Manna et al. 2017). Discordancy-based recognition is thought to be the simpler mechanism, used when eggs are present in the nest and predicting that hosts should systematically eject the egg most different from the rest of the clutch based on a minority rule, even in the case the minority egg were theirs (Yang et al. 2014). On the other hand, the premise of template-based recognition (also called true recognition) is that birds know the appearance of their own eggs (which may be an innate or learned ability) and use this information to distinguish them from foreign eggs (Victoria 1972; Rothstein 1975b; Moskát et al. 2010). This mechanism can be further divided into recognition by direct comparison, where birds recognize their own eggs when they see them and discriminate against differing eggs by comparing them with their own; and recognition from a memorized or innate template, where birds can reject foreign eggs even in the absence of their own (Lahti and Lahti 2002; Moskát and Hauber 2007; Wang et al. 2015). Both discordancy and template recognition are not mutually exclusive but many experiments over the past 40 years showed primarily in steadily mounting support for the template recognition hypothesis (Manna et al. 2017). Finally, it has been suggested that birds may be aware of the onset of their own laying (Friedmann 1963; Davies 2000). This knowledge could potentially allow hosts of brood parasites to use an even simpler rule for foreign egg rejection: they should reject any egg appearing in the nest in the pre-laying stage, regardless of colour and pattern. Yet, to the best of our knowledge, studies have not yet investigated the role of different cognitive signals (e.g. shape vs colour/pattern) in the recognition mechanisms employed by hosts of brood parasites. This is crucial to identify what traits are used for creating the template image or how template-based and other mechanisms interact.

Here, we aimed to investigate the recognition abilities of the barn swallow (*Hirundo rustica*), a conspecific brood parasite (Møller 1987; Petrželková et al. 2015) but also host of an interspecific brood parasite, the common cuckoo *Cuculus canorus* (Campobello and Sealy 2009; Liang et al. 2013). We inserted objects that differ in shape (eggs or star-shape) and colour/pattern (non-mimetic blue or mimicking the colour and pattern of swallow eggs) into swallow nests in two different breeding stages, the pre-laying stage (*i.e.* in the absence of the host eggs) and the laying stage (with the host eggs present), and measured the amount of light at all nests. Our experimental design thus allowed us to test the following: (1) *mechanisms and cues used in object recognition*: When there are no eggs in the nest, swallows have to rely on an innate or learned template image of their eggs. Thus, we expect them to use a template in the pre-laying stage, while the possibility to observe their own clutch during laying stage would allow them to decide whether to reject foreign objects based on appearance differences. In addition, swallows may use a recognition mechanism based on the onset of laying to eject parasitic eggs. This mechanism predicts that swallows should eject all foreign eggs before they start laying their own eggs, or at least eject them more before laying than after (Davies 2000; Moskát and Hauber 2007; Wang et al. 2015). Because several cognitive mechanisms can coexist, we can expect to observe both template and onset of laying types of mechanisms in pre-laying. If so, we expect both mechanisms to be additive and we predict that swallows should eject stars and non-mimetic objects more than eggs and mimetic objects, respectively. We did not aim here to test discordancy-based recognition as it would have required manipulating the ratio of experimental to own eggs in the clutch (e.g. Stevens et al. 2013). (2) *light affects recognition*: object recognition can be affected by nest light availability. Although evidence for the effect is currently weak, all studies to date (Honza et al. 2011, 2014; Avilés et al. 2015; Medina and Langmore 2019; Manna et al. 2020) have investigated species nesting in bright conditions where visual discrimination is not impaired. This is not necessarily the case for swallows as they nest in poorly illuminated conditions (Langmore et al. 2005). We expect individuals with lower light levels in their nests will recognize and reject objects at a lower rate than individuals with well illuminated nests. (3) *nest sanitation vs. brood parasitism defence behaviour*: swallows cleaning their nest will predominantly use shape as a cue and will reject star-shape objects more often than eggs, regardless of colour and pattern. Conversely, defence against parasitism will involve use of colour and pattern rather than shape, and birds will reject more non-mimetic than mimetic eggs.

Overall, this study will investigate mechanisms and cues during the recognition process (Manna et al. 2017) in a novel experiment which may help to disentangle the complicated relationship between nest sanitation and true defence against brood parasitism (as manifested by rejection of foreign eggs, see Guigueno and Sealy 2017). Finally, majority of experimental nests were video-recorded, which allowed us to distinguish between recognition and rejection behaviour, identify the method of egg ejection (puncture- vs grasp-ejection), and to consider the role of males in ejection behaviour. All these factors may have implications for the evolution of rejection behaviour (e.g. Sealy and Neudorf 1995).

## Material and Methods

### *Study area and general approach*

Swallows were studied during the 2021 breeding season in two farms located in the villages Stará Hlína (49°02′21.4″ N, 14°49′06.8″ E) and Břilice (49°01′14.4″ N, 14°44′15.3″ E) in southern Bohemia, Czech Republic. In these localities, swallows breed inside beef cattle barns by building their nests on walls, hanging fluorescent lamps, or in crevices, usually under the ceiling. Swallows start arriving to these breeding sites in late March and females usually start laying eggs at the turn of April and May (in 2021, the first egg was laid on May 1st). Both females and males participate in nest building (Soler et al. 1998) but only females incubate eggs in European populations (Smith and Montgomerie 1992; our unpublished data).

As we wanted to detect the start of each nesting attempt in its earliest stage, we checked the contents of all old nests (swallows usually reuse nests over multiple years, see also Barclay 1988) every third day and searched for newly built swallow nests throughout the whole breeding season. Altogether, we found 82 active nests (49 in Břilice, 33 in Stará Hlína) that swallows used for 112 breeding attempts (64 in Břilice, 48 in Stará Hlína). We checked nest contents every day from the first day of nest lining with straw (which precedes nest lining with feathers) and during the egg laying stage.

During the breeding season, we performed four catching sessions in both localities to catch all breeding birds (one in May, two in June and one in July). Swallows were caught using mist nests and all adult birds were marked with a unique combination of rings (one standard aluminium and up to three plastic coloured rings). All birds were measured and their sex was determined by presence/absence of the brood patch and cloacal protuberance. We also took photos of every individual, and feather and blood samples for future studies. We photographed breeding pairs at their respective nests during all breeding attempts using digital cameras with long 400mm lenses. Photographs were then used for identification of the nest owners. In Břilice, we equipped all caught adults with RFID (radio-frequency identification) tags and used RFID readers at the majority of nests (33 of 49) to confirm the identity of breeding pairs at their nests. Moreover, we continuously video-recorded the majority of breeding attempts (56 of 64) in Břilice and several (9 of 48) in Stará Hlína using Mini Color CCTV cameras (Shenzhen MYYOU Co. Ltd, Shenzhen, China) and a digital video-recorder (DVR 4616A ELN AHD lite, Shenzhen DIGIT Co. Ltd, Shenzhen, China). Finally, we measured light availability (in lux) at every nest from 11:00 to 13:00 using a SpectraPen mini (Photon System Instruments, Drásov, Czech Republic) from the 15th to the 17th of June when the weather was clear. During these measurements, the device was placed next to the nest at the height of the nest rim.

*Experimental objects*

To investigate swallow rejection abilities, we inserted one of four types of plastic objects in their nests. Contrary to interspecific parasites (see e.g. Šulc et al. 2016), it seems that barn swallows do not remove any host eggs during or prior to parasitism (Møller 1987), therefore we did not remove any host eggs during our experiments. In this study, we used mimetic (in terms of colour and spotting) and non-mimetic (blue) egg-shape objects and the same mimetic and non-mimetic star-shape objects (Figure 1). The shape of the star was chosen to be very distinct from the egg shape and may be reminiscent of debris or shell remnants which elicit nest cleaning behaviour in birds (Guigueno and Sealy 2017). Moreover, star-shaped objects have been also used in previous experimental studies investigating nest sanitation (Ortega and Cruz 1988; Ortega et al. 1993; Guigueno and Sealy 2009). Objects were shaped using wooden moulds and white thermoplastic sheets. After heating, the softened thermoplastic sheet was gently pressed into the mould to create half a shell of a desired shape. After cooling, all half shells were taken out of the mould and edges were trimmed using manicure scissors. Then, two half shells of a given shape were filled with adhesive putty (Pritt Multi Tack, Henkel, Düsseldorf, Germany) and glued together using additional polyurethane glue (Pattex, Henkel, Düsseldorf, Germany). The size and shape of the experimental egg mimicked a real swallow egg, and both eggs and stars were designed to have a volume of 1865 $mm^3$, corresponding to the average volume of swallow eggs from our population (*mean* ± *SD*: 1843 ± 175$mm^3$, *n* = 190; Bayesian model testing whether the volume of experimental objects was different from the volume of the swallow eggs: estimate [credible interval] = -21.63 [−47.03, 3.13]). The weight of all objects was adjusted to correspond to the average weight of real swallow eggs (*mean* ± *SD*: 1.88 ± 0.07g for experimental objects, *n* = 40; and 1.89 ± 0.15g for swallow eggs, *n* = 128; Bayesian unequal variances model: estimate = 0.01 [−0.03, 0.04]).

The pattern and colour of mimetic eggs and stars were developed by printing a thin film that resembled the colour and spotting pattern of real swallow eggs (adapting the method by the study of Heathcote et al. 2020). The printed film was attached to the thermoplastic sheet during the heating process. An example egg that had 11.8% of the eggshell surface covered by spots was chosen, corresponding to the approximate average degree of spotting coverage of swallow eggs in our population (*mean* ± *SD:* 12.0 ± 4.5%, *n* = 190 eggs; Bayesian model testing whether the spotting coverage of experimental objects was different from the spotting coverage of the swallow eggs: estimate = 0.00 [−0.00, 0.01]). Before printing, the photograph of this swallow egg was calibrated and transformed into the bird visual system to resemble what swallow eggs would look like to our study species. We used the visual system of the blue tit (*Cyanistes caeruleus*) as this is the closest relative bird species with known spectral sensitivities (Hart et al. 2000). Since the printer (HP LaserJet Pro

M252dw, Palo Alto, California, USA) could not reproduce also the ultraviolet colour (UV, 300–400nm), mimetic experimental objects resembled the colour in the human-visible spectrum (400–700nm) only. However, all swallow nests used in the experiments were located inside barns under the ceiling where UV light could barely reach (Figure 2), therefore, we believe that the absence of UV matching did not influence the results. Finally, the printed film was attached to the thermoplastic sheet during the heating process. Non-mimetic objects were painted matt blue (Dupli-Color in Sofia matt shade corresponding to Pantone 7459C, Motip Dupli, Hassmersheim, Germany) for easy comparison with other studies that used a similar blue colour (Liang et al. 2013; Yang et al. 2015a; Su et al. 2018). Colour (ΔS) and luminance (ΔL) differences between the real swallow egg that was used as a model and experimental mimetic and non-mimetic objects are shown in Figure 1. Image calibration, egg volume, spotting, colour and luminance analyses were performed in ImageJ (Schneider et al. 2012) using the Multispectral Image Calibration and Analysis Toolbox (van den Berg et al. 2020).

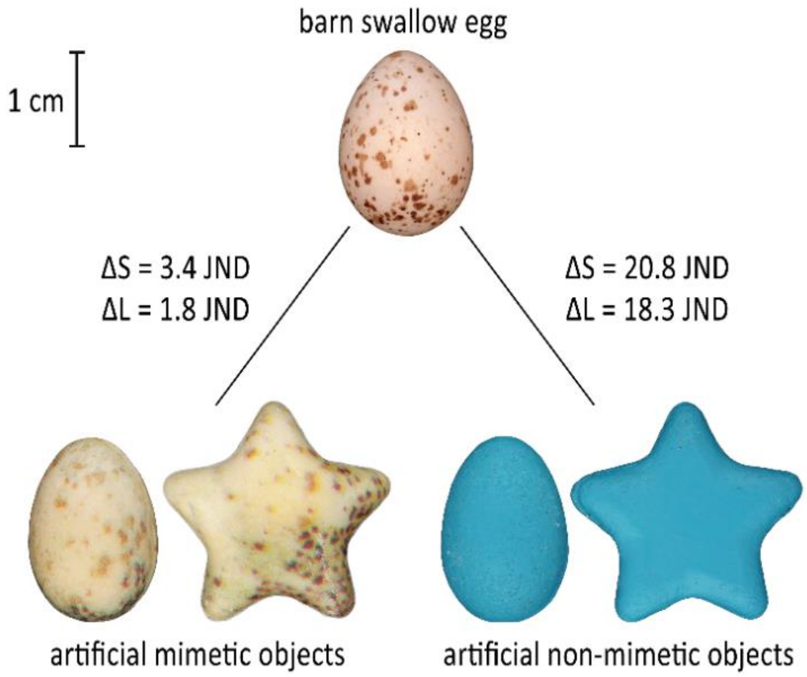

**Figure 1** *Artificial objects used for rejection experiments and a real barn swallow egg that was used as a model for making mimetic artificial eggs and stars. The difference in colour between the real egg and mimetic objects is due to the calibration and transformation of the real egg photograph to match for blue tit visual system. Chromatic (ΔS) and achromatic (ΔL) differences between the real egg and mimetic and non-mimetic objects were calculated. The JND unit denotes the "just noticeable difference" that predicts whether two colours are likely to be discriminable based on the signal to noise ratios of the channels of the blue tit visual system (Hart et al. 2000). Long-, medium-, and shortwave cone sensitivities and double cone sensitivities were used for calculation of ΔS and ΔL, respectively* (Hart et al. 2000).

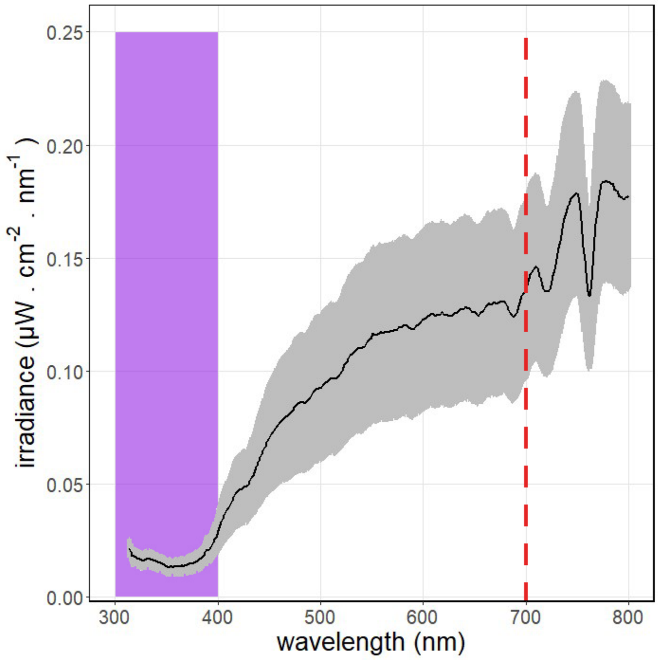

**Figure 2** *Mean irradiance spectrum measured at 72 swallow nests. The grey area indicates standard error of the mean. Red dashed line denotes the upper end of the spectrum perceived by birds* (Hart et al. 2000)*. Note the low values in the UV part of the spectrum below 400 nm (coloured in purple).*

*Experimental procedures*

To compare rejection behaviour in the presence or absence of a swallow's own eggs, we inserted an experimental object into swallow nests during one breeding attempt at two different stages of breeding cycle. First, we inserted one of the objects in the swallow nest during the pre-laying stage when swallows started to line their nest cup with feathers. The experiment ended when swallows rejected the object or when the object was still in the nest but the first egg was laid (in which case we removed the object from the nest). Since we could not know when swallows would lay their first egg, experimental objects did not have the exact same presentation duration (i.e., the difference between the time when the experimental object was inserted in the nest and the time when swallows laid the first egg). The average presentation duration of all experimental objects in the pre-laying stage was 4.5 ± 3.2 (*mean* ± *SD*) and did not differ between the different types of objects (for all pairwise comparisons, the highest posterior densities include zero). Second, we inserted the object into the nest in the laying stage on the day when the swallow female laid the third egg. In total, we carried out 165 experiments in active nests, among which 67 were in the pre-laying stage and 98 in the laying stage. In 65 breeding attempts, we conducted experiments in both stages (N = 130 experiments) inserting the same type of object into the nest. In 35 remaining experiments, we inserted an object either in the pre-laying stage (N = 2) or the laying stage (N = 33) of a given breeding attempt. Fewer experiments were conducted in the pre-laying stage mainly because swallow females did not always line their nests with feathers sufficiently in advance for us to time the start of the experiment correctly. Since swallow females often nest twice a year in our study area, we also performed the experiment in their second breeding attempt following the same procedure as in the first breeding attempt. The objects inserted in these second attempts were of the same colour and pattern (mimetic or non-mimetic) as in the first breeding attempt, but with a different shape (*i.e.* if a mimetic egg was used in the first attempt, a mimetic star was used in the second, and vice versa).

Experimental nests were checked daily for object rejection. We observed egg ejection as the only type of rejection response and did not record any egg burial or nest desertion. Bird species – including swallows – have been shown to reject most parasitic eggs within the first five days after parasitism (e.g. Møller 1987; Brown and Brown 1989; Moskát and Fuisz 1999; Aidala et al. 2015; Yang and Feeney 2020). Hence in the experiments during the laying stage, we considered an object to be accepted when it remained in the nest at least throughout the first five days after insertion and the nest was still active (*i.e.* eggs were incubated and the swallow female visited the nest). Moreover, we used this five-day acceptance window to correspond to the average presentation duration of objects in the pre-laying stage (see above). Note that although we refer to this stage as the laying stage (because we inserted the object during the laying period), response towards the object often took place during the incubation period. Nine cases when the experimental object was inserted the day before swallows laid their first egg and thus had less than a day to reject it were not included into the final analysis. The final dataset therefore contained 156 experiments. During daily nest checks in the laying stage, we sometimes found that star-shaped object had slipped to the bottom of the nest, thus becoming hidden below swallow eggs. In these cases, we always moved the object up to be more visible for swallows.

### *Statistical analysis*

We used a mixed-effects Bayesian model, with rejection as the Bernoulli distributed dependent variable (accept = 0, reject = 1) and colour, shape and timing as factors, including all interactions between them (including the three-way interaction). We included these interactions to test whether the effects of colour and shape interact with each other, and whether any such interactions depend on timing (i.e. pre-laying or laying). We included scaled lux (lighting level at the nest) values as continuous fixed factor. Female ID and breeding attempt number (*i.e.* whether this was the first, second or third breeding attempt of a given female, with *n* = 95, *n* = 59 and *n* = 2 respectively) were included as random intercepts. Default priors were used, and convergence was assessed for each model parameter using the Rhat value (all potential scale reduction statistics ~1.00).

We answered our specific hypotheses using selected contrasts (e.g. all star conditions vs. all egg conditions). We first extracted posterior draws from the posterior distributions of conditional means for each possible combination of the experimental factors (colour, shape and timing), and used these to calculate the relevant contrast. If the 95% credible interval (CI) of the most credible difference (MCD) contained zero, we concluded that there was no evidence for a difference in rejection rates for the conditions in the contrast. All statistical analyses were performed in R 4.0.3 (R Development Core Team 2018) using the *brms* library version 2.15.0 (Bürkner 2017). Statistical code is provided in the Supplementary material 1.

## Results

We performed 156 successful rejection experiments and swallows ejected 38% of objects in total. Ejection rates of all individual objects for pre-laying and laying stages are shown in Figure 3. A summary of all tested hypotheses is presented in Table 1.

Our Bayesian model showed evidence that the ejection rate of foreign objects is influenced by the interaction between colour, shape and timing (estimate [CI] = 5.80 [0.39, 12.72]). We found a two-way interaction between shape and timing (estimate = −4.32 [−0.18, −10.06]). Stars were ejected more frequently than eggs in the pre-laying stage (MCD [CI] = 0.16 [0.0000005, 0.63]), suggesting evidence of a template for shape. In the laying stage, stars were also ejected more frequently than eggs (MCD = 0.20 [0.00001, 0.64]) supporting recognition based on shape differences. However, the interaction suggests that this preference to eject stars was even stronger in the laying stage which can be explained by very low ejection rate of mimetic eggs in the laying stage (see below).

There was no difference in ejection rate between mimetic and non-mimetic objects in the pre-laying stage (MCD = −0.007 [−0.39, 0.26]) suggesting no evidence for a colour template. Although there was no difference in ejection rates between mimetic and non-mimetic objects in the laying stage either (MCD = −0.01 [−0.48, 0.20]), mimetic eggs were accepted more often than non-mimetic eggs (MCD = −0.10 [−0.94, −0.000008]), suggesting that egg recognition might rely on colour/pattern differences. There was evidence for a two-way interaction between colour and timing (estimate = −4.56 [−0.23, -10.57]) which can be again explained by high acceptance of mimetic eggs in the laying stage.

Finally, the mechanism based on the onset of laying has been partially supported. In the pre-laying stage, swallows accepted 58% of all experimental eggs, which implies that swallows did not use this mechanism to simply get rid of all or majority of experimental eggs. However, overall ejection rate of experimental eggs (especially mimetic eggs) was higher in pre-laying than laying stage (MCD = 0.27 [0.00009, 0.72]).

Ejection of experimental objects was not influenced by the amount of light inside nests (estimate = -0.53 [−2.50, 0.69]). This indicates that light availability in swallow nests is not a crucial factor for object recognition.

Overall, we showed that 53% of all stars were ejected by swallows and that the shape of the objects was an important cue because they ejected stars more often than eggs (MCD = 0.23 [0.00003, 0.49]). This indicates strongly evolved nest sanitation behaviour in this species. Swallows ejected stars more often during pre-laying than laying stage (MCD = 0.22 [0.000081, 0.68]) showing sanitation behaviour was especially pronounced before egg laying. On the other hand, there was no overall difference in ejection rate between mimetic and non-mimetic eggs (MCD = −0.05 [−0.30, 0.18]), suggesting a lack of evolved defence against brood parasitism. Swallows, however, ejected more non-mimetic than mimetic eggs during laying stage. This may at first glance suggest defence against brood parasitism, but it is also possible that non-mimetic eggs were ejected in order to clean the nest. This may be supported by the comparable decrease in nest sanitation behaviour of all stars from pre-laying to laying stage (rejection rate decreased by 40%) with decrease in rejection of blue eggs from pre-laying to laying stage (rejection rate decreased by 35%).

We video-recorded 26 ejections (eight eggs and 18 stars; examples are shown in Supplementary material 2) and identified the sex of the ejecting individual in 24 cases. Males ejected three objects (two mimetic eggs and one non-mimetic star) exclusively in the pre-laying stage, although in two of these cases females also attempted ejection. The handling time birds needed to eject did not differ between eggs and stars (*mean* ± *SD*: 22.5 ± 17.7s for eggs and 23.9 ± 19.8s for stars, Bayesian unequal variances model, estimate = 1.55 [−14.71, 18.73]). We also video-recorded 39 cases of acceptance and did not observe any response towards experimental objects in 36 of them. Additionally, we observed three possible but unsuccessful ejection attempts by females pecking mimetic objects (two eggs and one star) during the pre-laying stage (Supplementary material 3).

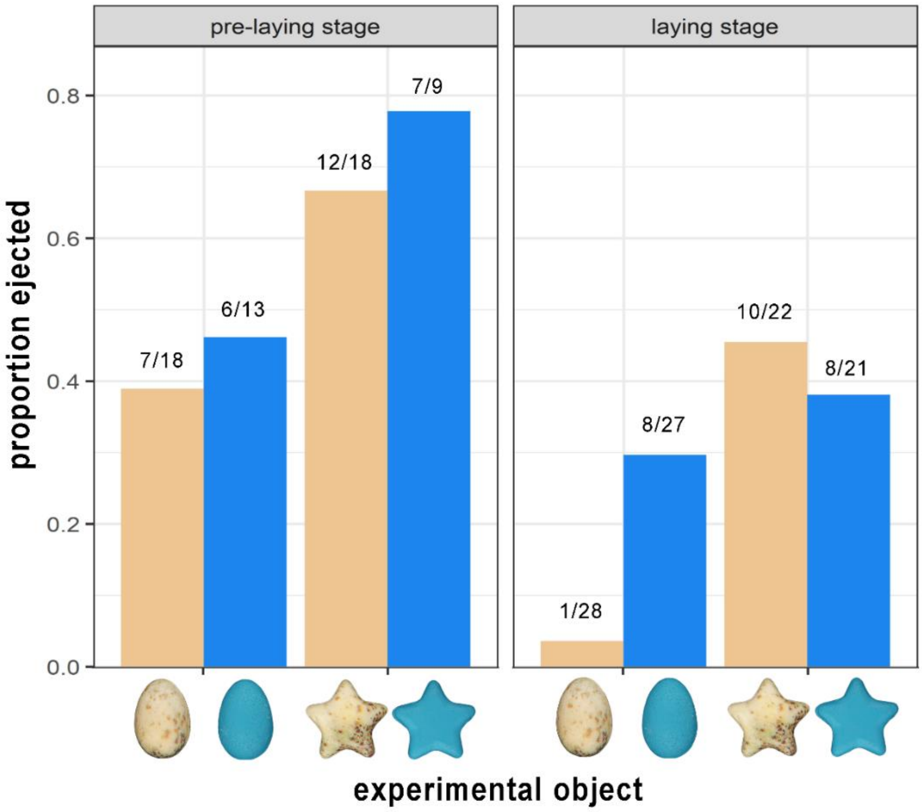

**Figure 3** *Ejection rates of the four object types in pre-laying and laying stage. Numbers above bars indicate the proportion of ejected objects out of total number of objects of the same type used in a given stage.*

**Table 1** *Summary of the tested hypotheses.*

| Question | Hypotheses | Expected cue | Prediction | Comparison | Supported |
|---|---|---|---|---|---|
| 1. What mechanisms and cues are used in object recognition? | H1: template for shape | shape | stars ejected more than eggs in the pre-laying stage | stars vs eggs in pre-laying | yes |
| | H2: template for colour/pattern | colour/pattern | non-mimetic objects ejected more than mimetic objects in pre-laying | non-mimetic objects vs mimetic objects in pre-laying | no |
| | H3: ejection based on shape difference | shape | stars ejected more than eggs in laying | stars vs eggs in laying | yes |
| | H4: ejection based on colour/pattern difference | colour/pattern | non-mimetic objects ejected more than mimetic objects in laying | non-mimetic vs mimetic objects in laying, mimetic vs non-mimetic eggs | only for eggs |
| | H5: onset of laying | presence of own eggs in nest | eggs ejected more in pre-laying than laying | eggs in pre-laying vs all eggs in laying | yes |
| 2. Does the amount of light in nests affect object recognition? | light at nests affects object recognition | light level at nest | lower ejection rate in dim nests than in well-illuminated nests | light at nests included in model as continuous fixed factor | no |
| 3. What is the purpose of egg ejection? | H1: defence against brood parasitism | colour/pattern | non-mimetic eggs ejected more than mimetic eggs | all non-mimetic eggs vs all mimetic eggs | no |
| | H2: nest sanitation | shape | stars ejected more than eggs | all stars vs all eggs | yes |

## Discussion

Swallows ejected stars more often than eggs, confirming that shape is an important cue for birds to recognize and remove foreign objects (see also e.g. Yang et al. 2019; Hauber et al. 2021). The use of shape differences is not surprising because it allows effective nest sanitation, which increases offspring survival by e.g. keeping the nest dry and free of parasites (Guigueno and Sealy 2012). Moreover, we found that nest sanitation was stronger during pre-laying (70% of all stars ejected) compared to the laying stage (49% of stars ejected), which highlights the critical significance of this behaviour in swallows, particularly during nest cup preparation. Therefore, we suggest that nest sanitation may also be an important part of the nest preparation in the barn swallow and other species that tend to reuse old nests from previous years that first need to be cleaned before egg-laying.

The preference for accepting eggs over stars in the pre-laying stage suggests that swallows possess a template for the shape of their eggs (sensu Moskát et al. 2010; Wang et al. 2015). By contrast, swallows ejected mimetic and non-mimetic objects at a similar rate in the pre-laying stage, suggesting that they did not use a colour and/or pattern template image of their eggs during the recognition process. These results contradict experimental studies showing that birds primarily form very accurate memory of colours (Sakai et al. 2000), while non-colour cues are memorized secondarily (Aoki et al. 2000) and with relatively low accuracy (Ono et al. 2002). Our results are also in conflict with many experimental studies showing that hosts of brood parasites have usually evolved true recognition of parasitic eggs, i.e. use a template image of colour and/or spotting pattern of their eggs (reviewed in Manna et al. 2017). However, these studies mostly used different approaches to support the use of template, e.g. by observing that foreign eggs are ejected even when host eggs are a minority in the clutch (Moskát et al. 2010; Stevens et al. 2013) or when foreign eggs are the only eggs in the clutch and direct comparison with host eggs is not possible (Moskát et al. 2010; Bán et al. 2013). As far as we know there is only one study (Wang et al. 2015) that investigated the presence of template image as we did, by comparing rejection rates of mimetic and non-mimetic objects during the pre-laying stage. The authors found that yellow-bellied prinia (*Prinia flaviventris*) ejected non-mimetic more often than mimetic eggs (Wang et al. 2015), which we consider to be strong evidence that prinias possess an innate or learned template for the colour and/or pattern of their eggs, and suggests evolution of direct defence against brood parasitism. On the other hand, our study shows that the swallows of our study population lack such a template, indicating that direct defence against brood parasitism did not evolve in the pre-laying stage. Moreover, the template image for egg colour and pattern may not be inherited but instead acquired by observational learning, as evidenced by older females rejecting parasitic eggs more often than naïve first-time breeders (Lotem et al. 1992; Moskát et al. 2014). Whether female breeding experience also influences template acquisition and egg recognition in the barn swallow, however, requires further investigation.

Although we did not find evidence for the use of a colour template in the pre-laying stage, swallows nonetheless ejected 46% of non-mimetic and 39% of mimetic eggs. The average ejection rate of both egg types was higher in the pre-laying stage (42%) than in the laying stage (16%), with the difference being largest for mimetic eggs. This suggests that although swallows did not eject all parasitic eggs before they started laying their own, a recognition mechanism based on the onset of laying increased the chance of ejecting the foreign egg. Previous studies struggled to disentangle whether egg ejection in the pre-laying stage serves as a direct defence against brood parasitism or simply for nest sanitation (Moskát et al. 2003; Moskát and Hauber 2007; Wang et al. 2015). Here, it is likely that swallows ejected experimental eggs in the pre-laying stage to clean their nests, because mimetic and non-mimetic eggs were ejected at similar rates (MCD = −0.007 [−0.53, 0.33]). Otherwise, we would expect to observe an additive effect of both recognition mechanisms (template for colour and/or pattern and onset of laying), resulting in an even higher ejection of non-mimetic eggs (as observed in prinias by Wang et al. 2015). Two other studies showed that barn swallows and cliff swallows can eject the majority of conspecific eggs from empty nests (Møller 1987; Brown and Brown 1989), also implying the existence of a recognition mechanism based on the onset of laying. Additionally, swallows commonly reuse the same nests not only between but also within a breeding season (Barclay 1988; this study). In light of these previous findings and our results, we suggest that egg ejection in the pre-laying stage is part of nest sanitation behaviour that allows swallows to get rid of unhatched eggs from a previous breeding attempt. Such a strong sense of nest cleaning can also effectively serve as a defence strategy against poorly timed brood parasitism occurring before the host starts laying its own eggs.

Egg ejection in the pre-laying stage may also be beneficial for hosts because it is free from ejection costs and recognition errors that hosts sometimes have to pay when their own eggs are present in the nest (Moskát and Hauber 2007). These costs, together with the inability of swallows to distinguish between mimetic eggs from their own, were probably the main reason why we observed very low rejection of mimetic eggs (4**%**) during the laying stage (for similar results, see also Møller 1987; Liang et al. 2013). On the other hand, non-mimetic eggs differed substantially from host eggs and were therefore ejected more often (30%), which is consistent with recent studies on European (Yang et al. 2015b) and Asian (Liang et al. 2013; Yang et al. 2015a, b) swallow populations. Our experimental design did not allow us to distinguish whether swallows ejected blue eggs based on a discordancy mechanism or a template-based direct comparison. Hence, future studies should investigate this question by directly testing discordancy-based recognition, i.e. whether barn swallows reject their own egg(s) when in minority in a clutch.

An interesting question is whether the ejection of non-mimetic eggs in the laying stage is an evidence of direct defence against brood parasitism or a product of nest sanitation as in the pre-laying stage. The ejection rate of blue eggs decreased from the pre-laying to the laying stage, in a trend similar to the decline of nest sanitation behaviour, as inferred from ejection rates of both star types. These comparable declines (of 35% for blue eggs and 40% for stars, see Results and Figure 3) suggest that blue eggs were rejected by swallows for the same reason as stars, i.e. to clean the nest, rather than to defend the nest against brood parasitism. Several studies already speculated whether egg rejection is a product of nest sanitation behaviour only (Moskát et al. 2003; Moskát and Hauber 2007; Wang et al. 2015) but only dealt with egg ejection during the pre-laying stage. Here, we present a novel way to disentangle brood parasitism defence from nest sanitation when host nests contain eggs. In addition, we provide evidence that nest sanitation relies not only on shape but also on colour and/or pattern.

Our study therefore suggests that swallows did not evolve direct defence against brood parasitism, which may be surprising but could be explained by the rarity of interspecific brood parasitism in European swallow populations (highest parasitism rate reported – 1.2%; Campobello and Sealy 2009; Liang et al. 2013). On the other hand, conspecific brood parasitism seems to be relatively common in this species, and has been linked to lower hatching and fledging success of offspring (Møller 1987; Petrželková et al. 2015). However, the costs that hosts pay for being parasitized by conspecific brood parasites are not necessarily as high as the costs incurred by interspecific parasites (Lyon and Eadie 2008; Soler 2017a). Considering that conspecific parasitic eggs are inherently excellent mimics, the costs of rejection and recognition errors may exceed the benefits of ejecting the parasitic egg, and overall prevent the evolution of ejection behaviour (Takasu 2017). Future studies should focus on quantifying experimentally the exact costs of conspecific brood parasitism in host swallows. In addition, the barn swallow was probably originally a solitary breeding species, with colonial breeding evolving later (Snapp 1976). As conspecific brood parasitism has been associated with colonial breeding and nest proximity (Lyon and Eadie 2008), it may be a relatively recent reproduction strategy in this species, against which colonial swallows may not have had enough time to evolve defence behaviour in the form of egg recognition.

Compared to the previous study where swallows ejected almost all conspecific eggs inserted in the pre-laying stage (Møller 1987), we recorded a much lower ejection rate (39% and 46% for mimetic and non-mimetic blue eggs, respectively). One possible explanation for our substantially lower egg ejection rate is that we used artificial plastic eggs that were impossible to puncture, as opposed to real conspecific eggs used in other studies. This may potentially influence the type of rejection (Šulc et al. 2016b; Roncalli et al. 2017) or even decrease the motivation for rejection (Ruiz-Raya et al. 2015). However, we find this explanation unlikely for two reasons. First, despite swallows sometimes showing intense pecking behaviour towards objects, they did not seem to struggle for a long time and were able to eject eggs relatively quickly (Supplementary material 2). Moreover, most swallows (36 of 39) that accepted experimental objects showed no signs of unsuccessful ejection attempts (e.g. pecking) on our video-recordings. In only three cases, we observed females briefly pecking experimental objects (two eggs and one star) immediately after insertion during the pre-laying stage (Supplementary material 3), but then losing interest in them. This indicates that if swallows recognize a foreign object in their nest, they usually grasp-eject it, and shows only a small difference between recognition and ejection behaviour. In addition, we suggest that swallows are capable of ejecting foreign objects (including eggs) by grasping, which is considered less costly than puncture ejection in terms of energetic and ejection costs (Rohwer et al. 1989; Soler et al. 2002). However, experiments with real eggs and video recordings are needed to confirm whether grasp-ejection is the preferred method over puncture-ejection in this species.

According to previous studies, the availability of light at the nest does not influence the recognition and rejection of parasitic eggs in several species (Honza et al. 2011, 2014; Avilés et al. 2015; Medina and Langmore 2019; Manna et al. 2020). Our study supports these findings because nest light availability was not a crucial factor impairing swallows' ability to recognize and eject foreign objects. However, we cannot exclude the possibility that an illumination threshold for object recognition exists, because we always observed acceptance of the objects (N = 9) below the illuminance of 5 lx (four nests). However, this potential threshold for discrimination should be considered with caution because of our limited sample size.

Finally, our video-recordings showed that females were predominantly responsible for object ejection. Since only swallow females incubate their eggs in Europe (Smith and Montgomerie 1992; our unpublished data), our observations support the hypothesis that the sex incubating eggs is generally responsible for egg recognition and ejection (Sealy and Neudorf 1995). Surprisingly, we also recorded three males ejecting objects. These cases took place always in the pre-laying stage and since males participate in nest building (Soler et al. 1998), we suggest that the males who ejected the object were also contributing to nest cleaning. Interestingly, two of these males ejected mimetic eggs, which suggests that not only females but also males are aware of the onset of females' egg-laying.

## Conclusion

It seems that conspecific brood parasitism is not a strong enough selective pressure in our barn swallow study population for them to have evolved a direct defence strategy to recognize and reject parasitic eggs. Instead, egg ejection appears to be a product of nest sanitation behaviour, which is well developed in this species, especially in the pre-laying stage. Since nest sanitation may be an opportunistic behaviour in swallows (Spencer 2005), it would be interesting to test whether the actual condition of birds may influence the response towards parasitic eggs. We show evidence that individuals of a single population can rely on both true recognition and onset of laying-based mechanisms, and further propose that shape plays a major role in object discrimination, with colour and pattern only being employed when comparison with an individual's own eggs is possible. Although swallows nest in very dark conditions, it seems that light availability is not a limiting factor affecting their ability to recognize foreign objects. On video-recordings, we observed that swallows are capable of grasp-ejecting experimental eggs. We demonstrated that swallow males are able to eject foreign objects from their nests, though this occurs very rarely. This is again most likely the demonstration of nest sanitation during nest building, to which the males also contribute (Soler et al. 1998). We suggest that future studies should perform similar experiments in hosts exposed to stronger parasitism pressure to test whether the ultimate and proximate mechanisms of recognition vary depending on the strength of selection.

## Data availability

All data for these analyses can be found in the Supplementary information.

## Code availability

The code employed in these analyses can be found in the Supplementary information.

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## Contributions

M.Š., A.E.H., T.A. and V.J. conceived the ideas and designed methodology; M.Š., V.J., L.M., O.T and T.A. collected data; M.Š. and L.M. analysed video-recordings; A.E.H. performed statistical analyses, J.T. designed experimental objects and M.Š. led the writing of the manuscript. All authors contributed to the drafts and gave final approval for publication.

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## Conflict of interest

Authors declare no conflict of interests.

## Ethical approval

We declare that all experiments performed for this study were approved by the animal and ethics representatives of The Czech Academy of Sciences and nature conservation authorities (62065/2017-MZE-17214 and MZP/2020/630/964). The fieldwork adhered to the Czech Law on the Protection of Animals against Mistreatment (licence no. CZ03971 and CZ04122).

## Supplementary Information

Supplementary material 1: Code used for statistical analyses in R software

Supplementary material 2: Four cases of object ejections by swallow females

Supplementary material 3: Three cases of swallow females pecking experimental objects without ejecting them

Supplementary material 4: Raw data used in the study