



A novel function of egg burial: burying material prevents eggs rolling out of wind-swayed nests

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Egg burial behaviour, that is, when parents bury the eggs with a layer of nest material during the egg-laying stage, has been described in various egg-laying animals. Several functions of egg burial have been described in animals with different life histories and breeding traits, but rarely reveal distinctive functions between sister species. In the polygamous Eurasian penduline tit, *Remiz pendulinus*, sexual conflict over care has been proposed to drive egg burial since, during egg laying, females hide eggs from males to prevent them abandoning the nest. Females then have the option to desert the clutch themselves and leave parenting to the male. However, in a congeneric species, the Chinese penduline tit, *Remiz consobrinus*, males have been seen with females in the nest at night, which indicates males know of the eggs' existence. In this study, we investigated egg burial function in Chinese penduline tits and experimentally tested four hypothesized functions of egg-burying behaviour. (1) We found that egg burial is unlikely to play a role in sexual conflict resolution, as both males and females appeared to bury eggs during egg laying and both freely entered and roosted in the nests with eggs exposed at night. (2) Egg burial does not prevent nest parasitism, as no egg rejection or clutch abandonment was observed in clutches with model parasitic eggs. (3) Our results do not support the temperature regulation hypothesis since the temperature difference between buried and experimentally unburied eggs did not affect hatching success. (4) Notably, our results support the novel egg protection against wind hypothesis since the burying layer efficiently prevented the eggs from rolling out of wind-swayed nests. The difference our study found between the two *Remiz* species highlights that one behaviour, egg burial, can serve different evolutionary functions between closely related species.

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To maximize reproductive fitness in a breeding season, selective pressures lead parents to adapt various behaviours to protect their clutch and improve the survival of the offspring (Clutton-Brock, 1991; Badyaev, 2005; Grant et al., 2017). In egg-laying animals, egg development often relies on parental behaviours. For instance, reptiles select laying sites to ensure that embryos develop under a suitable temperature range (Iverson et al., 2004; Gunderson et al., 2020), frogs started providing care for eggs after evolving to live in an unstable terrestrial environment (Vági et al., 2019) and in many birds parents take turns to incubate throughout the day to protect eggs from overheating, excessive cold and predation (Gillis

et al., 2012; Troscianko et al., 2016). Parents may also deposit preen oils on the eggs to protect the eggs (and the developing embryos) from harmful microbes (Shawkey et al., 2008). However, parents need to balance behaviours that improve offspring survival with their own needs to feed, preen and socialize with other conspecifics. Therefore, various types of behaviours have evolved to deal with the temporary absence of the parent from the nest (Royle et al., 2012; Guerra-Grenier, 2019; Litman et al., 2019).

Egg burial is one of the parenting behaviours observed in many oviparous species to protect the eggs during this parental absence. Parents cover or bury the eggs with a layer of nest material or soil before leaving the nest at the egg-laying stage (Shine, 1999; Amat et al., 2012; Loukola et al., 2014). As a breeding behaviour, egg burial occurs in a wide range of taxa and may have evolved under

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multiple selection pressures that uniformly promote the reproductive fitness of parents. For species with simple ground nests, the burying layer acts as camouflage, preserving eggs from predation in crickets, turtles, water birds and gamebirds (Réale & Roff, 2002; Leighton et al., 2009; Troscianko et al., 2016; Černý et al., 2018). It can also hold fish eggs in place, preventing them from being washed away by fast-flowing river streams (DeVries, 1997), and protect insect eggs against moisture loss during drought (Réale & Roff, 2002). Owing to sympatric competition, great tits, *Parus major*, cover eggs to hide information on resource availability from pied flycatchers, *Ficedula hypoleuca*, to prevent them from usurping the same nestboxes (Loukola et al., 2014; Slagsvold & Wiebe, 2021).

Another important function of egg burial is to avoid the constraints imposed by ambient temperature during embryo development. Eggs are generally sensitive to temperature fluctuations since exposure to extreme weather for a certain period can harm the embryo (Beissinger et al., 2005; Pike et al., 2014). To avoid damage to the clutch caused by extremely low temperatures, some bird species improve the microhabitat by covering eggs with nest material (Gillis et al., 2012; Lambrechts et al., 2020) while fish ectoparasites bury eggs under sediment (Mikheev et al., 2001). In lizards, skinks, gamebirds and tits, parents have evolved to adjust the depth of egg burial across geographical populations to reach optimal temperatures for clutch development (Shine, 1999; Iverson et al., 2004; Göth and Booth, 2005; Loukola et al., 2020).

Egg burial may also have evolved as an important strategy to prevent brood parasitism. During the arms races between hosts and parasites, certain host species have evolved the ability to identify alien eggs (Lyon, 2003). Some of these host species can reject the alien eggs by burying them below the nest material instead of incubating them during the early stage of egg laying (Sealy, 1995; Jamieson et al., 2000). Female American coots, *Fulica americana*, are known to be able to accurately count and recognize conspecific parasitic eggs, which they either eject or bury as an expression of rejection (Jamieson et al., 2000; Lyon, 2003).

Finally, egg burial, like other aspects of parenting, can be involved in sexual conflict: since care is costly to the parent, it is often advantageous to entice the mate to provide more care than it would ideally do (Székely et al., 1996; Royle et al., 2012; Székely, 2014). Eurasian penduline tits, *Remiz pendulinus*, provide strictly uniparental care, where both male and female parents intend to pursue a new mating opportunity, such that they compete to desert the current clutch during egg laying and leave the parenting workload to the reproductive partner (Persson & Öhrström, 1989; Pogány et al., 2008). Valera et al. (1997) argued that, under this intense sexual conflict during the egg-laying stage, females bury their eggs, aggressively prevent the male partner entering the nest, and roost in the nest alone overnight. By doing this, females can hide the presence of eggs from the male since it may desert the nest once the eggs are detected. Therefore, by burying them, females potentially provide themselves with the option to abandon the offspring first.

In a congeneric species, the Chinese penduline tit, *Remiz consobrinus*, parents bury all eggs beneath nest material when they are absent from the nest (Zheng et al., 2018). Uniparental care is also the main parenting strategy (female-only care: 77%; male-only care: 7%), although biparental care exists as well (16%). However, eggs have been found buried only during the daytime, whereas they are uncovered at night when both male and female roost in the nest (Zheng et al., 2018). This suggests that male Chinese penduline tits may be aware of the presence of eggs in the nest. Thus, in this species, egg burial behaviour is likely to fulfil alternative functions to deceiving the reproductive partner. Penduline tits, *Remiz* spp., build closed pendulum-shaped nests suspended on the outer sides of branches (Bot & Van Dijk, 2009; Lloyd et al., 2017). The nests are

commonly distributed in regions with windy climates, which may cause eggs in suspended nests to easily be disturbed. Therefore, it is important to investigate the function of egg burial behaviour in Chinese penduline tits by looking at both nest characteristics and ecological conditions.

Although various functions of egg burial have been suggested from animals with distinct biological and ecological backgrounds, many studies have not tested the proposed functions experimentally (Valera et al., 1997; Jamieson et al., 2000; Réale & Roff, 2002; Iverson et al., 2004; Amat et al., 2012; Troscianko et al., 2016; Gómez et al., 2018). Some other behavioural functions that may not be intuitive from observation would be ignored without experimental studies. Here, we experimentally investigated four hypothesized functions of egg-burying behaviour in a wild population of Chinese penduline tits. First, we tested whether sexual conflict plays a role in the evolution of egg burial behaviour. We predicted that both males and females would bury eggs and females would not be aggressive to their male partners during egg laying. Second, we tested whether egg burial behaviour is an antiparasitism strategy. Intraspecific parasitism has been reported in penduline tits (Ball et al., 2017). Thus, we predicted that the burying layer could stop the parasitic bird from laying eggs or that the parents would easily recognize and thereafter reject the parasitic eggs on the burying layer (Petrie & Møller, 1991; Schleicher et al., 1997). Third, we tested whether the burying layer improves the temperature regulation of the clutch. We predicted egg burial would reduce temperature loss during the absence of the breeding pair from the nest, thereby optimizing egg development and hatching rate (Bochenski, 1961; Martin et al., 2007; Amat et al., 2012). Fourth, penduline tit nests sometimes sway in winds (J. Zheng & E. Zuidema, personal observations), but we have not witnessed any eggs falling out of nests. We hypothesized that the burying layer might prevent the eggs from crashing against each other inside the wind-swayed nest and/or prevent them from falling out of the nest during strong winds. Specifically, we predicted that eggs could fall out of the wind-swayed nests when burying material was experimentally removed but not when it was present.

METHODS

Study Site

Fieldwork was conducted during the breeding season of Chinese penduline tits (1 May to 31 July) from 2017 to 2019 in the northwest part of the Liaohe Delta National Nature Reserve, China (40°45′–41°05′N, 120°28′–121°58′E, 44 km²). The area consists of nine reed ponds separated by roads. The main vegetation consists of reed, *Phragmites communis*, and cattail, *Imperata cylindrica*, whereas the roads are bordered by elm, *Ulmus pulmilla*, locust, *Robinia pseudoacacia*, willow, *Salix babylonica*, and poplar, *Populus tomentosa*, trees. The Chinese penduline tits selected sites on the outer branches of these trees to build their nests.

Study Species and Fieldwork

The Chinese penduline tit is a migratory passerine species that winters in the south of China, Japan and South Korea and breeds in the northeast of China and far eastern Russia (Tong, 1985; Gluschenko et al., 2014). Penduline tits are known for their penduline-shaped nests made from soft plant materials, mainly reed and cattails (Persson & Öhrström, 1989; Bot & Van Dijk, 2009; Zheng et al., 2018). The male penduline tit initiates nest building and simultaneously tries to attract a mate by singing constantly. The female joins in building the nest once it pairs up with a male. If a male does not find a mate, it builds the nest until stage D (for the

nest stages, see Fig. 1), when a clear basket has been created and then stops building activities until a female partner is found. Egg laying is initiated when nest stage D or E is reached (Fig. 1), which was an indication for us to start checking for the presence of eggs. The female lays one small, white egg early in the morning every day and has a final clutch size of five to eight (6.8 ± 0.6 eggs, Zheng et al., 2018). Parental care strategies of this species are varied because of high desertion rates of one partner after egg laying is completed. Female-only care, male-only care and biparental care co-exist in one population (Zheng et al., 2021).

We searched the study area for newly built nests every day by inspecting tree branches and tracking the songs of male penduline tits. The nest stage was recorded once a nest was found, and the development of that nest was checked every 2 days. We selected the nests lower than 4 m from the ground for conducting experiments (75.8% of nests were lower than 4 m) since they were easy to reach using a step ladder. When a nest was at the D stage, we started checking for the presence of eggs every day in 2016–2020 by carefully touching the inside of the nest. In this way, we were able to feel the egg underneath the burying material without disturbing this material. The start date of egg laying was recorded.

In Chinese penduline tit nests, eggs are often buried during the day (Fig. 1), while at night they are uncovered and one or both parents sit inside the nest. When egg laying started, we recorded the extent of egg burial and numbered the newly laid eggs with a waterproof marker every morning to record the laying order. The extent of egg burial was categorized into four levels: '0': no burying layer and eggs were exposed; '1': a thin layer of material covered the eggs and the researcher could easily feel the eggs underneath the material; '3': deep burial where the eggs were hardly detectable by touch; '2': between '1' and '3' where the researcher could feel the egg shape despite a sturdy layer of material over the eggs. We confirmed the clutch size 10 days after the first egg was laid and took the day that the last egg was laid as the first day of incubation (Zheng et al., 2018). Hatching was checked from the 11th day of incubation onwards. If we found hatchlings in the morning, we noted the number of hatched eggs and their laying order numbers. In the afternoon, we repeated the checks. The recording of hatching order lasted for 3 days due to the asynchronous hatching of Chinese

penduline tit chicks. We did not observe any eggs hatching more than 3 days after hatching initiation. Hatching success was calculated as the number of hatchlings divided by the clutch size.

Experimental Manipulations

We conducted four experiments. The first was to test the sexual conflict strategy found in Eurasian penduline tits (Valera et al., 1997) and was conducted in both 2018 and 2019. The other three experiments were to test our hypotheses: the antiparasitism experiment was conducted from 2017 to 2019, the temperature regulation experiment in 2018 and 2019 and the egg protection against wind experiment in 2019 and 2021. The sample sizes of the experiments are shown in Appendix Table A1.

Sexual Conflict Experiment

We investigated whether Chinese penduline tits bury eggs for the same function as in Eurasian penduline tits to resolve sexual conflict (Valera et al., 1997; Royle et al., 2012). We used two methods. (1) We identified the egg-burier through a 'material uncovering experiment'. We randomly selected one morning between day 2 and day 4 of egg laying to remove the layer of material that covered the eggs in the nest. We filmed the nests for 1.5 h with a clear close-up shot of the nest entrance to investigate the frequency of a specific sex conducting egg burial. After filming, we checked whether the eggs were buried with material by touching the inside of the nest. If the eggs were buried, we checked the video and identified egg burial when we saw a parent entering the nest with a big clump of material in its beak, and then built the layer (which causes the nest to shake) at the bottom of the nest with its tail up at the entrance. Male and female Chinese penduline tits are sexually dimorphic. From the video, we could distinguish a male from its grey head and black eye mask and a female from its light-brown head and brown eye mask (Zheng et al., 2021). Parents could take several bouts and minutes to complete the burial of the eggs. Therefore, we determined the frequencies and durations of both male and female egg burial from the videos. We also recorded aggressive interactions between the male and female of the

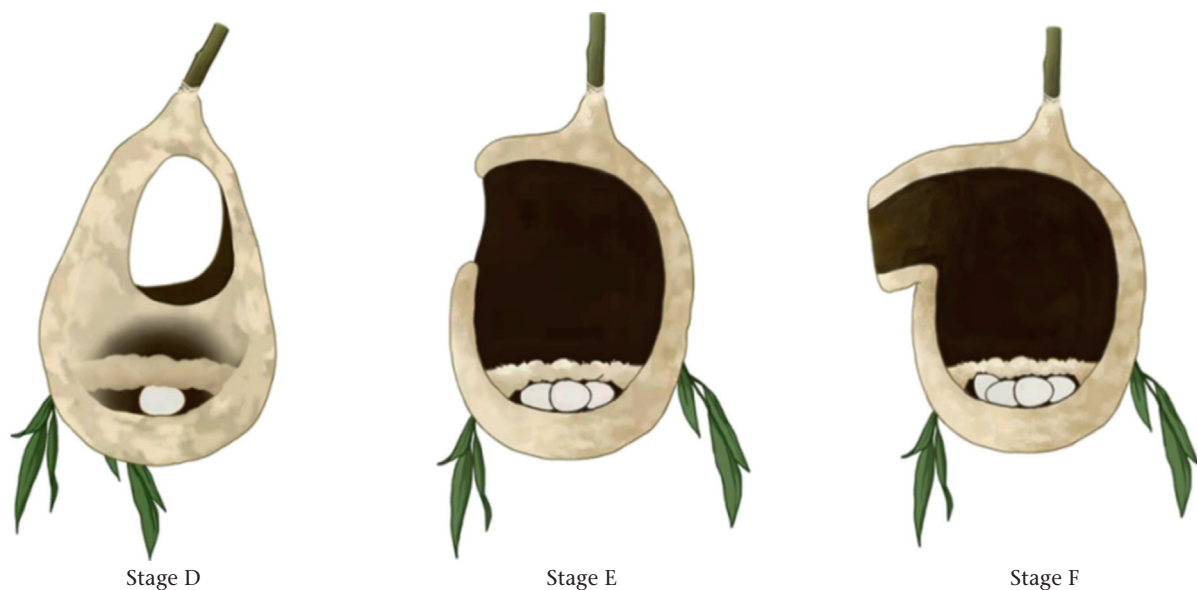


Figure 1. Schematic illustration of egg burial in Chinese penduline tits. The male and female build a layer of soft nest material above the eggs during the egg-laying period. Egg laying starts while the nest looks like a basket and the first one or two eggs are buried by a layer of nest material (stage D), then one side of the basket is closed and more eggs are laid (stage E); finally the entrance funnel is built on the open side (stage F). © Yuqi Guo.

breeding pairs, since female Eurasian penduline tits show aggressive behaviour at the nest entrance to stop the male partner from entering the nest (possibly to avoid the male from detecting the presence of eggs in the nest, Valera et al., 1997). Aggressive interactions were defined as one bird chasing another or blocking the entrance to the nest and attacking the other bird (Valera et al., 1997). These manipulations did not cause any nest abandonment ($N = 46$ nests). (2) Nest checks were conducted after 2100 hours when the sun had set and the parents were roosting. We lit up the nest with a torch and gently touched it with a long pole to chase out the birds. The number of birds that flew out of the nest was recorded. We also checked whether the clutch was covered with nest material by touching the inside of the nest. The whole night nest check was conducted within 1–2 min to minimize disturbance. All parents were observed at their nests on the next day and no nests were abandoned after night nest checks ($N = 46$ nests).

Antiparasitism Experiment

Intrabrood parasitism has been reported in penduline tits (Ball et al., 2017; also in Chinese penduline tits, Wang, n.d.). To examine the hypothesis that egg burial behaviour protects against brood parasitism, we added one dummy egg or one deserted penduline tit egg from another nest on the layer of material that the parent used to bury the first egg that was laid in the nest. Chinese penduline tits produce purely white eggs weighing about 1 g (Zheng et al., 2018). We made dummy eggs from white polymer clay (to simulate the egg colour) or blue polymer clay (to simulate a parasitic egg from a different species with a different egg colour or a novel object). The dummy eggs had the same weight and shape as Chinese penduline tit eggs. The deserted eggs were collected from biparentally abandoned clutches in the population and marked. We used the first egg-laying day for the experiment, because the fewer eggs are in the nest, the easier it generally is for birds to recognize parasitic eggs (Lyon, 2003; Wang et al., 2020). Female Chinese penduline tits lay eggs during the early morning (normally before 0500 hours). Since parents actively attend the nest during daytime (Zheng et al., 2018), we added the egg on top of the burying material in the morning (0800–0900 hours) and checked the nest again after 1800 hours (before parents roost in the nest) to see whether the dummy/deserted egg was buried or ejected. In this way, we ensured parents had enough time to react to the added egg and avoided disturbing the egg laying of the female. While adding the egg, we left the burying material untouched so that the added eggs would be easily recognized by the parents. We recorded the extent of egg burial again, collected the added dummy/deserted egg, and marked the egg that was laid on that day for our other experiments. None of the nests were abandoned because of the experiment ($N = 65$ nests).

Temperature Regulation Experiment

In this experiment, we compared the temperature difference between buried and unburied eggs to understand its effect on hatching outcomes. To accomplish this goal, we hung an old nest that had been collected in earlier seasons, 2–4 m from the breeding nest on the first day of egg laying. To ensure that the two nests experienced similar weather conditions, i.e. wind direction, wind speed and warmth from sunshine, the nest entrance was set in the same direction as the entrance of the breeding nest and the height difference between the two nests were less than 1 m. We also filmed both the old and the breeding nests in the same video frame for 0.5 h and compared the amplitude of the nest movements to confirm they moved due to wind at the same rhythm.

Since the clutch size of Chinese penduline tits is six to eight eggs, we conducted the experiment with the first-laid six eggs, from egg-laying day 2 until egg-laying day 6. The first six eggs were separated into two groups: we randomly selected egg numbers 2, 4, 5 or 1, 3, 5 as an experimental group, and the other three eggs (1, 3, 6 or 2, 4, 6 respectively) as a control group. As Chinese penduline tits bury their eggs during the daytime during the egg-laying stage (Zheng et al., 2018), the eggs belonging to the experimental group were moved from the breeding nest to the old nest before 0900 hours and returned to the breeding nest after 1800 hours to maximize the duration of the treatment before the parents returned to the nest to roost, around sunset (UTC+8, sunset time is around 1900 hours). The eggs belonging to the control group were kept in the breeding nest without any manipulation. Therefore, the moved eggs in the experimental group were unburied, whereas the eggs in the control group were naturally buried by the parents.

To compare the temperature difference between buried and unburied eggs, we set up iButtons (Model DS1922L, Maxim Integrated Company, San Jose, CA, U.S.A.) at four positions on day 3 or day 4 of the egg-laying stage (see Fig. 2a). (1) Under the burying layer (UB): we inserted the iButton under the covering material, so it could measure the temperature around the buried eggs. (2) Above the burying layer (AB): we placed the iButton on the side of the breeding nest and fixed it with a lapel pin to stop it from moving in the nest. It was used to measure the temperature above the burying material. (3) In the old nest (IO): we placed the iButton on the side of the old nest and fixed it with a lapel pin to prevent the iButton from moving inside the wind-swayed nest. In this way, we recorded the temperature of the experimentally unburied eggs. These temperatures were expected to be consistent with the temperatures measured by the AB iButton, above the burying layer. (4) Outside the nest: we added another iButton on a branch near the breeding nest to measure the ambient temperature. All the iButtons recorded temperatures every 3 min for 48 h. Since Chinese penduline tits do not bury eggs at night, we only selected the temperature data between 0600 and 1800 hours for statistical analyses. To investigate the effects of temperature differences caused by egg burial on fitness in Chinese penduline tits, the hatching success of eggs between the control group and the experimental group were compared.

Egg Protection Against Wind Experiment

To test the hypothesis that parents cover the eggs to protect them against wind throwing them out of the nest, we did an egg protection experiment. This was conducted simultaneously with the temperature regulation experiment. We used the old nests with uncovered eggs that hung beside the breeding nests as the experimental group. To test whether winds would hurl out the eggs, every morning after moving the eggs in the experimental group for the temperature regulation experiment, we blocked the entrance of the old nest with an ink-soaked stamper (Fig. 2b). To prevent the stamper from falling into the nest and damaging the eggs, we fixed it with a small clip. If winds moved the nest such that the eggs could be thrown out, the stamper would block and mark the eggs instead. The surface of the stamper was soft and we did not find any cracked or broken eggs in the nest after the experiment. In the evening, we checked the eggs for stamp marks before returning them to their breeding nest and recorded the egg number(s) of the marked egg(s). The date, nest ID and number of eggs were also recorded. Since the breeding nests served as a control group, where eggs were naturally covered with material by the parents, we also checked whether egg(s) had been hurled out of the breeding nest before returning the experimental eggs from the old nest in the evening. Maximum wind speed (m/s) data on the experimental

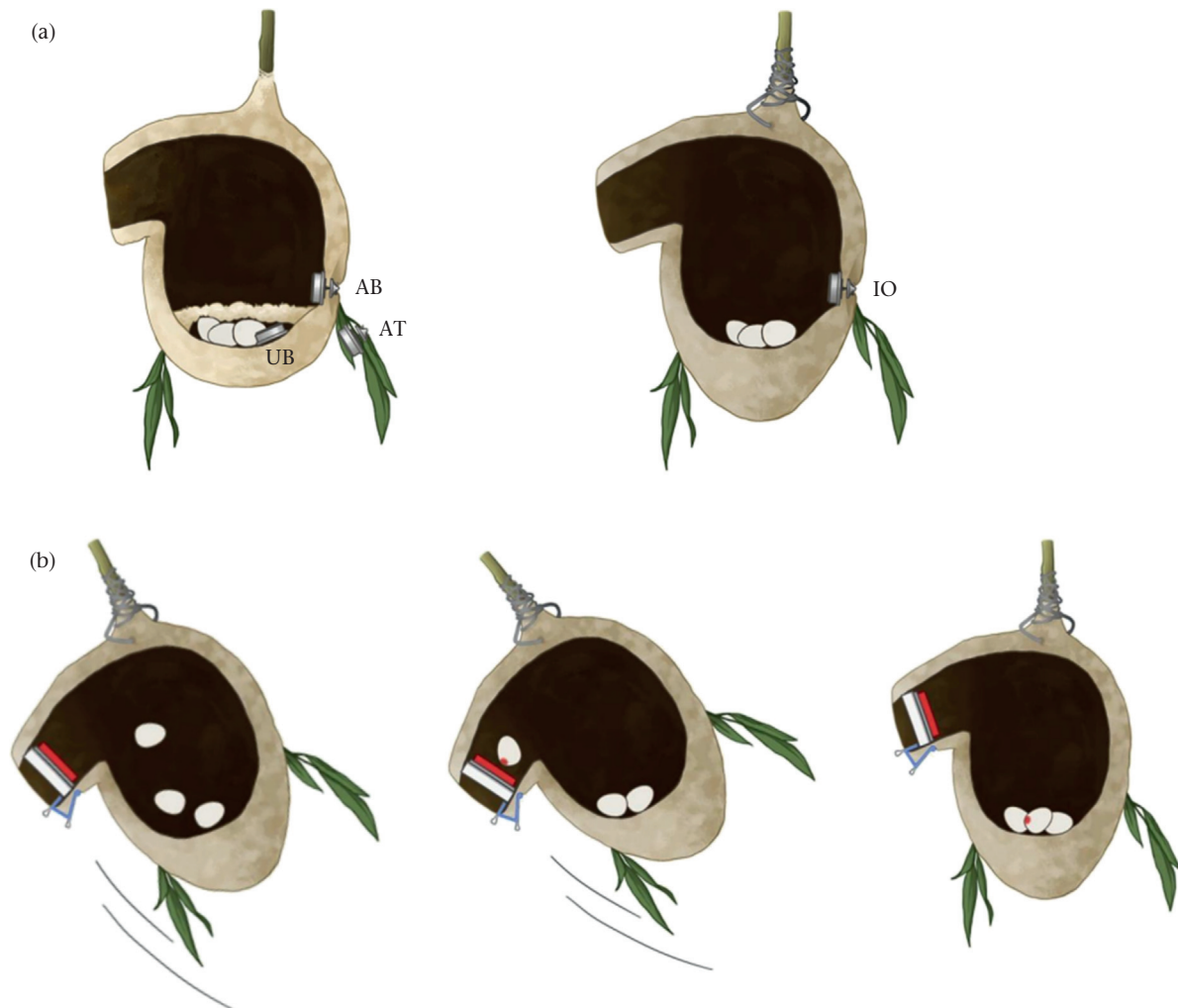


Figure 2. Schematic illustration of the egg burial experiment in Chinese penduline tits. (a) A breeding nest with naturally covered eggs is on the left, and an old nest with experimentally uncovered eggs is on the right. Labels refer to the temperature recorded by iButtons: above the burying material (AB), under the burying material (UB), the ambient temperature (AT) and the temperature in the old nest (IO). (b) Testing the effect of wind on egg movements in the experimental nest. The stamp pad with red ink blocks the entrance of the nest. The three drawings (from left to right) show the hypothetical positions of the eggs inside the experimentally uncovered nest under windy conditions. If an egg is about to be hurled out of the nest, it will be marked with red ink. © Yuqi Guo.

date were collected from a local meteorological station (10 km from the field site) and were extracted from <http://lishi.tianqi.com/dawa/index.html>. With these data, we could analyse the effects of wind speed on egg protection by the covering material.

Additionally, in 2018, when we solely conducted the temperature regulation experiment (we did not simultaneously do the egg protection against wind experiment in 2018), we accidentally found some eggs broken on the ground under some experimental nests when we checked these nests in the evening. These eggs fell out of the nests since there was no stamper blocking the nest entrance and the eggs were uncovered during the temperature regulation experiment. We recorded the number of fallen eggs from each of these nests and compared these results with the data collected from our egg protection against wind experiment that we did in 2019 and 2021.

Statistical Analyses

Statistical analyses were performed using R version 4.1.1 (R Core Team, 2019). The null hypotheses were rejected at $P < 0.05$. Mean \pm SD are provided.

(1) We calculated the proportion of nests that had a covering layer from egg burial. Since egg burial extent was an ordinal response variable, we used cumulative link mixed models (CLMM) from the 'ordinal' R package to analyse the variation of egg burial extent across the egg-laying stage. Time of day (morning, afternoon), date and egg-laying day were explanatory variables with nest ID as a random factor.

(2) For the sexual conflict experiment, videos were analysed with the behavioural observation research interactive software (BORIS v.7.7.5. Friard & Gamba, 2016). We recorded the frequencies (times/h) of males' and females' egg burial behaviour and counted acts of aggression by the female towards the male. We also recorded the duration of nest attendance (s/h) for the male and female of each pair during egg laying. The frequency of egg burial and the duration of nest attendance were both compared between males and females using Wilcoxon signed-rank tests.

(3) For the temperature regulation experiment, we calculated the average temperature/h at different positions. Aiming at testing the temperature differences of the iButton at three positions (IO, AB, UB) over different ambient temperatures, we created a generalized additive model (GAM) from the 'gam4' R package with

ambient temperature integrating as the smooth. The position of iButtons, ambient temperature and their interaction were explanatory variables and nest ID was included as a random factor. To compare the temperature difference between iButtons over ambient temperatures, we used the `difference_smooths` function from the 'gratia' R package to test whether the temperatures measured by iButtons at the three positions differed over ambient temperatures.

(4) For the experiment of egg protection against wind, we calculated the proportion of clutches with marked eggs and compared the probability of eggs being marked in the experimental group (unburied) with the probability of being thrown out in the control group (buried). Of the nests with egg(s) being marked, we calculated the average number of marked eggs. We constructed a generalized linear model (GLM) with Poisson distribution using the number of marked eggs as the response variable and the number of eggs in the experimental nests as the explanatory variable. For the eggs accidentally falling out of the nests in the 'temperature regulation' experiment in 2018, we repeated the analysis above and compared the percentage of nests with fallen eggs (chi-square test) and the average number of fallen eggs per nest (GLM, Poisson distribution; response variable: number of fallen eggs; predictor: nests in 2018 or nests for egg protection experiment) with the results obtained from our 'egg protection experiment'. We also created a generalized linear mixed model (GLMM) from the 'lme4' R package with binomial distribution using the clutch containing marked egg(s) or not as the response variable, wind speed and the number of eggs in the nest as the explanatory variables and the nest ID as the random factor.

(5) To investigate the effect of egg burial on eggs' hatching success, we created a GLMM with a binomial distribution. Hatching outcome of eggs (hatched and unhatched) was taken as the response variable. Egg burial (experimental group (unburied) and control group (buried)), laying order and clutch size were explanatory variables and nest ID was the random factor. For the hatching order, we created a GLMM with Poisson distribution using hatching order as the response variable and egg burial and laying order as predictor variables.

Ethical Note

Our study was carried out in a nature reserve located in the Liaohe Delta, China. Permissions for field experiments were obtained from the national reserve administration, ethic and welfare committee College of Life Science, Beijing Normal University (CLS-EAW-2020-002), and all research complied with local ethical guidelines and regulations. Our study did not involve any housing of animals. We monitored the behaviour of the birds with binoculars 10 m away and using video cameras (SONY HDR-XR160E) below nesting trees; the cameras were camouflaged with green cloth to match the environment to limit disturbance. We did not trap or take blood from birds for the experiments. We did experimental manipulations with old nests that we collected in the previous year after all breeding was completed. The old nests were hung 2–3 m from the breeding nests to reduce disturbance for the breeding pairs. We moved eggs regularly to experimental nests during the egg-laying stage; no eggs were broken during the experiments. The night nest checking did no harm to any birds since they all returned to the nests after the disturbance. Our study was approved by the Institutional animal care and use committee of Beijing Normal University and MOE KEY Laboratory for Biodiversity Science and Ecological Engineering, Beijing Normal University. The field work was approved by the Forestry Department of Liaoning Province, China.

RESULTS

Egg Burial Behaviour

Egg burial was a widespread behaviour recorded in 97.9% of nests ($N = 95$ nests). Penduline tits brought material into the nest and weaved it neatly to form a thin layer above all the eggs. The edge of the burying layer was tightly fixed along the side of the nest wall, so it would hardly move when it was touched. The extent of egg burial varied during egg laying (Table 1, Fig. 3a), becoming shallower from the fourth egg onwards, and it ceased on the last day of egg laying (-0.45 ± 0.89 days to the clutch completion day, $N = 37$ nests).

The extent of egg burial differed over the day: the burial material was thicker in the morning than in the afternoon (extent of burial score: morning: 1.82 ± 0.86 ; afternoon: 1.00 ± 1.05 ; Table 1, Fig. 3b). Egg burial was absent at night ($N = 46$ nests).

Sexual Conflict Experiment

After removing the burying material, we observed both male and female Chinese penduline tits exhibiting egg burial behaviour at all experimental nests ($N = 15$ nests). During the egg-laying stage, parents spent $25.8 \pm 0.15\%$ of the time attending nests during observations based on videos ($N = 15$ nests). The time attending the nest was similar for females and males (males: $12.9 \pm 11.2\%$; females: $12.9 \pm 12.1\%$; Wilcoxon test: $v = 59.5$, $P = 0.34$). The frequency of egg burial behaviour was similar in males and females (males: 1.26 ± 1.03 times/h; females: 1.03 ± 0.98 times/h; Wilcoxon test: $v = 87$, $P = 0.97$, $N = 15$ nests; Fig. 4). Furthermore, female aggression was rare. Only one female was observed on one occasion showing aggressive behaviour to the male when it tried to enter the nest ($N = 15$ nests, observation length > 20 h in total). Both male and female of the breeding pairs roosted inside the nest together at night in 93.5% of the nests ($N = 46$ nests); in the remaining 6.5% of nests, only one parent (the male or the female) roosted inside the nest at night.

Antiparasitism Experiment

None of the dummy eggs ($N = 30$ nests, 22 nests with one white egg and eight with one blue egg) or abandoned penduline tit eggs ($N = 35$ nests) were rejected or removed by the parents. Instead, all added eggs were accepted and buried together with the original clutch of eggs.

Temperature Regulation Experiment

The temperatures at the three different positions within the nests were correlated with the ambient temperature ($F = 7970$, $P < 0.001$, $N = 14$ nests; Fig. 5a). The temperatures differed

Table 1
Extent of egg burial in Chinese penduline tits

Fixed factor	Estimate	SE	Z	P
Time of day (afternoon, morning)	2.24	0.53	4.25	<0.001
Egg-laying day	-0.48	0.20	-2.44	0.01
Date	-0.02	0.03	-0.53	0.60

An ordinal logistic regression model is used with nest ID as random factor ($N = 30$ nests). Four extents of egg burial depths were distinguished. Morning and afternoon refer to nests checked between 0700 and 0900 and between 1600 and 1800 hours, respectively. P values in bold indicate that the factors were significantly correlated with the response variable.

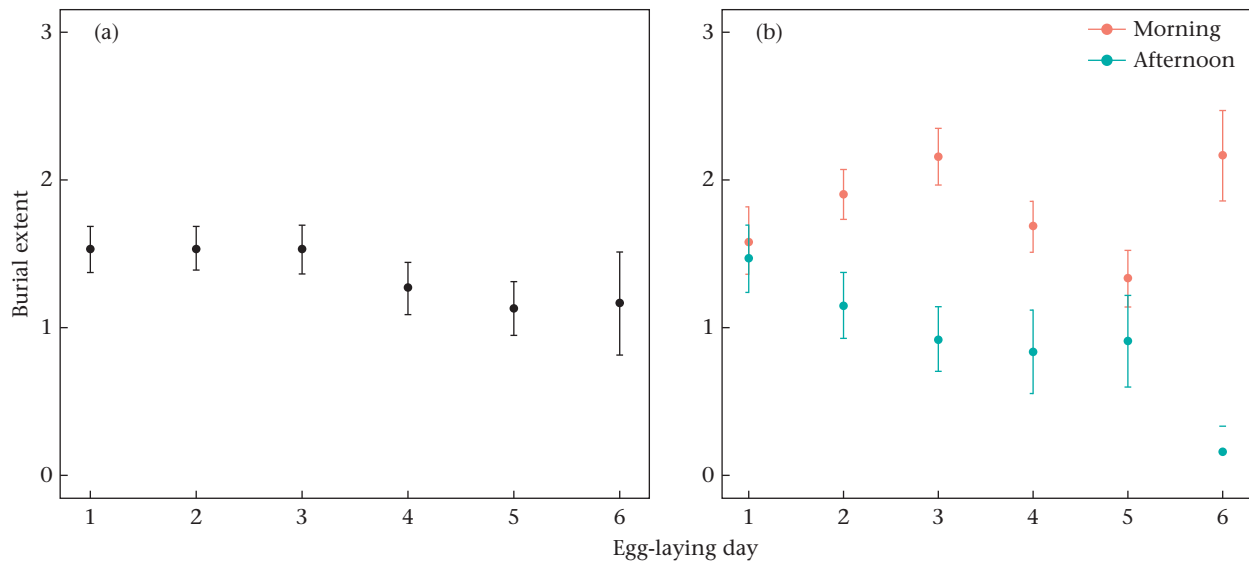


Figure 3. Extent of egg burial in Chinese penduline tits. (a) Egg burial during daytime from day 1 to day 6 (mean \pm SE, see definition of egg burial scores in Methods) of egg laying. (b) Egg burial in the morning and afternoon in relation to egg-laying days 1–6 (mean \pm SE, $N = 19$ nests).

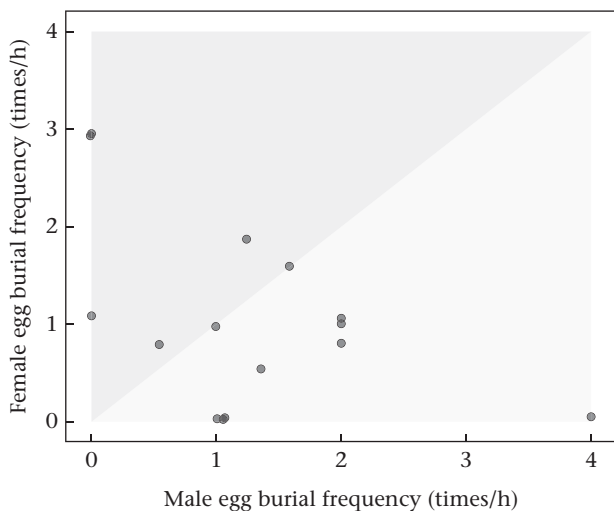


Figure 4. The frequency of egg burial behaviour by male and female Chinese penduline tits ($N = 15$ breeding pairs). The dark grey area indicates samples where the female attended the nest more than the male; the light grey area indicates where the male attended the nest more than the female. The border indicates equal attendance by the female and male.

significantly between positions when we controlled for the effect of ambient temperature, i.e. the interaction of position with ambient temperature (GAM, position * ambient temperature: $F = 60.3$, $P < 0.001$). The temperature under the burying material was higher than the temperature above it when the ambient temperature was between 18 and 22 °C, but lower when the ambient temperature was between 24 and 31 °C (Fig. 5b, UB–AB). The temperature difference ‘under the burying material’ and ‘in the old nest’ (UB–IO) had a similar pattern to UB–AB, and the temperature in the old nest was consistent with the temperature above the burying material across ambient temperatures (Fig. 5b, AB–IO). This indicates that our experimental groups (in the old nests) well simulated the temperature of the eggs’ environment without burying material in the breeding nest. The mean values for ambient temperature and temperatures measured at the three locations inside the control and experimental nests in the morning

were significantly higher than those in the afternoon (Appendix Table A2).

Egg Protection Against Wind Experiment

We found stamp marks on eggs in 45.2% of experimentally uncovered clutches ($N = 31$ nests; Fig. 6a). The number of marked eggs (mean \pm SD: 1.57 ± 0.75 eggs) tended to increase with clutch size ($z = 1.87$, $P = 0.06$). Actually, during our temperature regulation experiment in 2018 when we did not conduct the egg protection experiment (i.e. no stamper blocked the entrance), we found that at nine of 17 nests (52.94%, mean \pm SD: 1.33 ± 0.50 eggs) at least one egg from the uncovered experimental nests fell on the ground due to the wind sway. The percentage of nests with fallen eggs in 2018 was similar to the percentage of nests with marked eggs in the experimental nests ($\chi^2 = 0.05$, $P = 0.83$). Also, the number of marked eggs from the experimental nests and the number of eggs that accidentally fell out of the experimental nests in 2018 did not differ ($z = 0.70$, $P = 0.49$). This indicates that the marked eggs would have fallen out of the wind-swayed nests if the stamper was not blocking the nest entrance (Fig. 2b). However, none of the eggs were lost in the control nests where the eggs were naturally buried by the parents ($N = 31$ nests).

In the experimental nests, the probability of eggs being marked increased with wind speed (Table 2, Fig. 6b), but was not associated with the number of eggs present in the experimental nests (Table 2). No egg was damaged or cracked by other eggs in the experimentally uncovered and naturally covered nests ($N_1 = N_2 = 31$ nests).

Hatching Success

After the two simultaneously conducted experiments of temperature regulation and egg protection against wind, we found that hatching success did not differ between the experimental and control eggs (hatching success: experimental group (unburied): 0.90 ± 0.15 ; $N_1 = 45$ eggs; control group (buried): 0.91 ± 0.14 , $N_2 = 56$ eggs; $N = 17$ nests; Table 3). In addition, neither laying order nor clutch size was associated with hatching outcome (Table 3). Hatching order was positively associated with laying

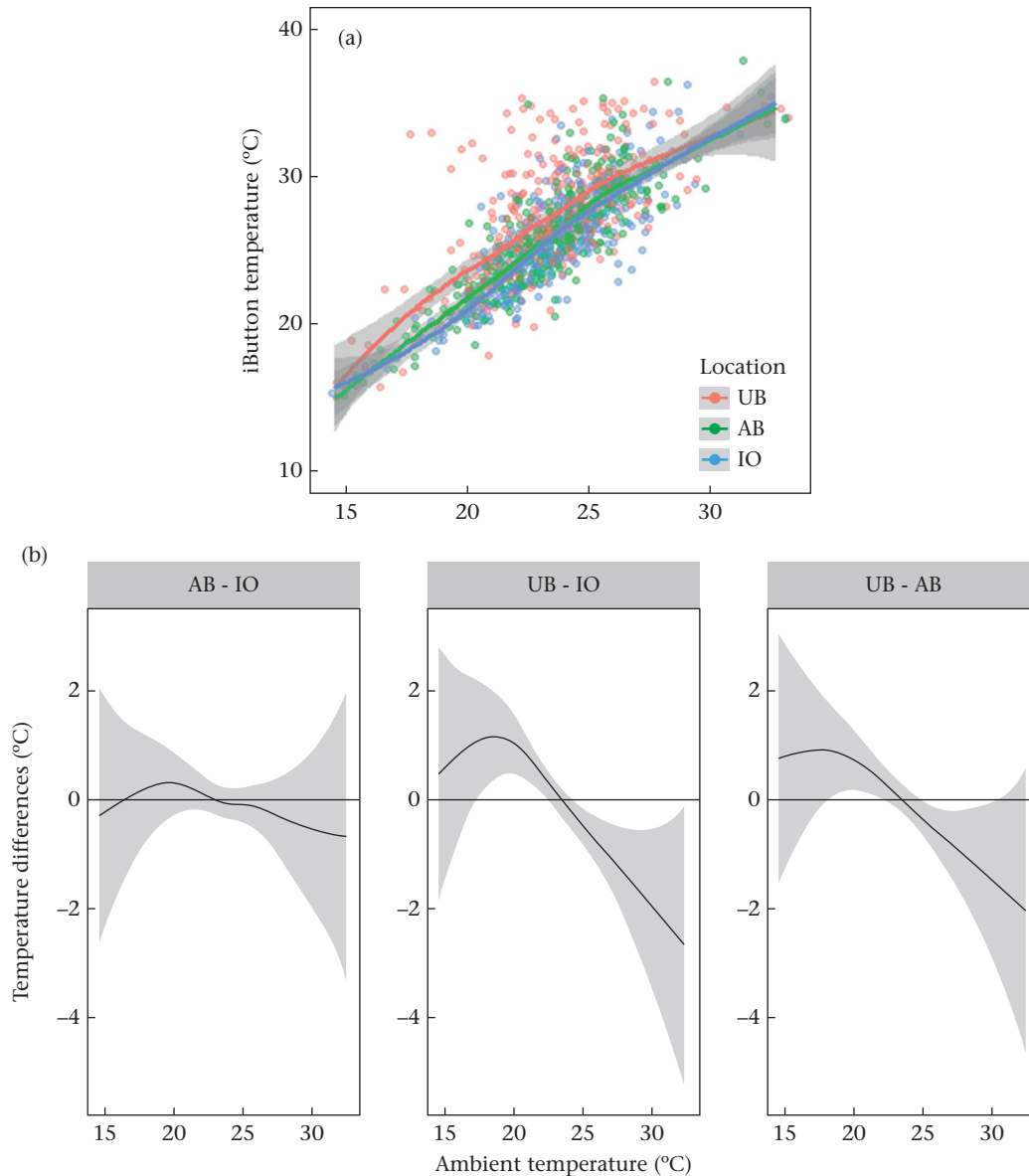


Figure 5. The relationship between internal nest temperature and ambient temperature. (a) Temperature under the egg-burying material (UB), above the burying material (AB) and in the old nest (IO). The positions of temperature loggers (iButtons) are shown in Fig. 2. Each dot refers to the average temperature/h of an iButton ($N_{UB} = N_{AB} = N_{IO} = 14$ nests) and the grey shading indicates the 95% confidence intervals. (b) Temperature differences between three iButton positions. The curves indicate the difference between two iButtons and the grey shading indicates 95% confidence intervals ($N = 14$ nests). The areas not covered by the grey shading on the line $y = 0$ indicate significant temperature differences over the ambient temperatures.

order (i.e. the early laid eggs also hatched early), but not egg burial during egg laying (Table 3).

DISCUSSION

Egg burial is a common behaviour in various egg-producing animals and here we report the findings of four experimental manipulations in nests of Chinese penduline tits which frequently exhibit egg burial behaviour. Unlike studies on the closely related Eurasian penduline tits, our study does not support the sexual conflict hypothesis. Our results also did not support the anti-parasitism hypothesis. Instead, our results are most consistent with the hypothesis of egg protection against wind. Lastly, the burying material was found to regulate the clutch temperature; however, this seems not to affect hatching success.

Egg Burial Behaviour as Protection Against Wind

We found wind may be the ecological driver of egg burial behaviour evolving in Chinese penduline tits since the burying layer effectively stopped eggs from rolling out of the wind-swayed nests. Wind closely interacts with egg movement in various ways. On the one hand, wind helps insect eggs survive the dry season by assisting dispersal (Alvarez et al., 1999) and pushes the eggs of sardines, *Sardinops sagax*, towards the shore to keep them close to the spawning area (Stenevik et al., 2001). On the other hand, wind can decrease avian reproductive success by continuously reducing heat or destroying the whole clutch by strong gusts (Heenan et al., 2012; Zia et al., 2014). To prevent harm from wind, sandpipers, *Calidris melanotos*, construct lined nest scrapes to minimize heat loss from eggs, and auks (Alcidae) lay pyriform eggs to avoid them

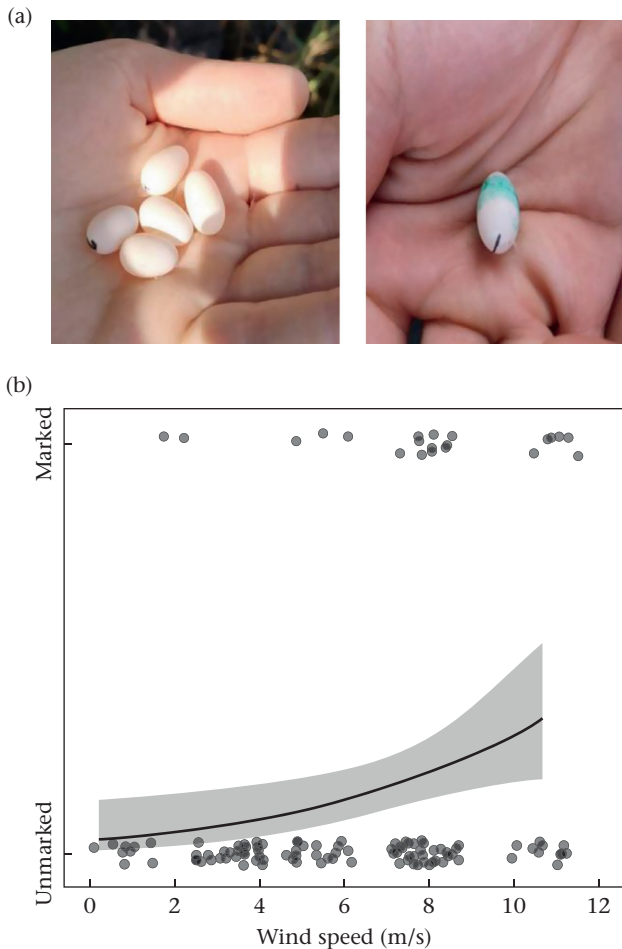


Figure 6. (a) Penduline tit eggs before the experiment on egg protection against the wind (left) and one egg marked by a green stamper after the experiment (right). Photo ©Jia Zheng. (b) The relationship between wind speed and ink marks on eggs. Each dot depicts an egg(s) marked in a nest on a certain day. The grey shading indicates the 95% confidence intervals ($N = 31$ nests).

Table 2

The presence of ink-marked eggs in Chinese penduline tit nests in relation to wind speed (m/s) and number of eggs in the nest ($N = 31$ nests)

Fixed factor	Estimate	SE	Z	P	R ²
Intercept	-4.19	1.33	-3.16	0.002	0.35
Wind speed	0.26	0.11	2.25	0.02	
No. of eggs in the nest	0.20	0.48	0.42	0.68	

A GLMM with binomial distribution was used with nest ID as random factor. The *P* value in bold indicates that the factor was significantly correlated with the response variable.

rolling off the cliff in the wind (Reid et al., 2002; Hays & Hauber, 2018). However, egg burial behaviour has never been examined as a functional behaviour against strong winds in any animal. We found Chinese penduline tits only bury eggs during the egg-laying stage and that during this time parents seldom stay inside the nests. Through our experimental design, we found that in over 40% of the experimental nests at least one unbury egg would have fallen out, whereas no eggs were lost in the breeding nest where eggs were buried by parents. These findings imply egg burial is a strategy that can efficiently enhance reproductive fitness when the parents are absent from the nest. To our knowledge, this evolutionary implication of egg burial behaviour has not been suggested and verified in any other species.

The clade comprising penduline tits (Remizidae) generally breeds in coastal areas, valleys and around lakes, where winds are present, and builds suspended nests (Persson & Öhrström, 1989; Bot & Van Dijk, 2009; Lloyd et al., 2017; Zheng et al., 2018; except for verdin, *Auriparus flaviceps*, Taylor, 1971). These nests, built on the outer branches of trees, are predated at low rates (Persson & Öhrström, 1989; Lloyd et al., 2017). However, even though penduline tits evolved to build closed, domed nests with only one small nest entrance, the suspended nests are easily swayed by wind such that fitness could be reduced by eggs rolling out when the parents are absent (Sidis, et al., 1994; Valera et al., 1997; this study). Long-tailed tits, *Aegithalos caudatus*, and verdins are two passerine species that also build closed, ball-shaped nests with only one small entrance like penduline tits, but their nests are fixed in place in bushes or cacti and egg burial behaviour is absent (Taylor, 1971; Hatchwell et al., 1999). The windy climate and the unstable nest position probably jointly drove the unique function of egg burial to evolve in penduline tits.

No study has reported egg burial behaviour in suspended nests of other species (e.g. Victoria, 1972; Fraga & Kreft, 2007; Quader, 2006; Lloyd et al., 2017). There may be two reasons for this. First, eggs are often only investigated during incubation, but not during egg laying, to monitor final clutch size in studies of breeding biology (e.g. Sankamethawe et al., 2008). Second, egg burial is most frequently described in species with stabilized ground nests or cavity nests (e.g. Jamieson et al., 2000; Guigueno & Sealy, 2010; Amat et al., 2012; Loukola et al., 2014). Species with suspended nests are generally rare. The limited reachability and complexity of the nests might make them difficult to investigate (Rakotomanana & Nakamura, 2012). Third, suspended nests from different species vary greatly in structure. Deep nest chambers or long pouch sizes may help keep eggs and young from falling out (Oniki, 1985; Winkler, 2016). In a study of Baya weavers, *Ploceus philippinus*, wind blew eggs or nestlings out of 18% of nests (Quader, 2006). This relatively low proportion of egg loss (compared to 45% in uncovered Chinese penduline tit nests) suggests that the deep nest chamber might alleviate the selection pressure of wind, so that egg burial does not have to evolve. Therefore, we suggest that egg protection from the wind during egg laying could also be a potential driver for egg burial in other species with suspended nests where it has not yet been described.

Evolutionary Implications of Egg Burial in Other Species

Valera et al. (1997) suggested that females independently bury eggs to resolve sexual conflict over parental care in Eurasian penduline tits. In this species, clutches are cared for by either the male or the female. This means both males and females are willing to desert the clutch so that they can leave the work of parenting to their partner. Under this sexual conflict over care, females evolved to hide eggs by burying them under nest material and aggressively stop males from entering the nest, such that the male has no clue of the presence of eggs. In this way, females have the option of deserting or caring for the clutch before the male partner. However, we reject this hypothesis in the congeneric Chinese penduline tit. In our study, both males and females buried eggs at a similar frequency, females were rarely aggressive towards males and the pair roosted together on the uncovered eggs at night. This indicates that males and females shared information about the eggs and cooperatively attended the clutch during the egg-laying stage. Therefore, although egg burial behaviour is exhibited in these two congeneric species, it may serve different functions (i.e. sexual conflict and protection against wind-sways).

A phylogenetic study showed that in the *Remiz* genus the Chinese penduline tit lineage diverged earlier than that of the Eurasian

Table 3
Hatching success and hatching order in relation to egg burial, laying order and clutch size in Chinese penduline tits ($N = 17$ nests)

	Fixed factor	Estimate	SE	Z	P	R ²
Hatching outcome (hatched or unhatched)	Intercept	-0.87	3.43	-0.25	0.80	0.10
	Buried or unburied	-0.22	0.74	-0.29	0.77	
	Laying order	-0.04	0.19	-0.21	0.83	
	Clutch size	0.59	0.53	1.10	0.27	
Hatching order	Intercept	-0.22	0.30	-0.73	0.47	0.17
	Buried or unburied	0.13	0.21	0.61	0.54	
	Laying order	0.17	0.06	3.38	<0.001	

Two GLMMs were used with nest ID as random factor. Hatching success was modelled with a binomial distribution and hatching order was modelled with a Poisson distribution. The *P* value in bold indicates that the factor was significantly correlated with the response variable.

penduline tit (Barani-Beiranvand et al., 2017). Because the two species encounter similar wind speeds when egg laying takes place in May and June (average monthly maximum wind speed: Eurasian penduline tit population, in 1997, Vienna, Austria 7.77 m/s, Valera et al., 1997; our Chinese penduline tit population, Liaohe delta, China 7.62 m/s), we suggest that egg burial behaviour probably originally evolved with the function of protecting eggs from falling out of the nest in windy conditions as we found in Chinese penduline tits. Subsequently, owing to the escalated conflict over parental care between the sexes in the later lineage of Eurasian penduline tits, egg burial probably evolved as a manipulative female behaviour, functioning to hide eggs from males and giving females the option to desert first. Egg burial is a behaviour that can be adjusted under conditional selection pressures in closely related species or adapted to fulfil various evolutionary purposes (DeVries, 1997; Shine, 1999; Amat et al., 2012; Slagsvold & Wiebe, 2021). Therefore, it is worth conducting more phylogenetic investigations to reveal the evolutionary process of egg burial within certain clades.

No Support for the Antiparasitism Hypothesis

In species experiencing a high probability of brood parasitism, strategies have evolved to identify parasitic eggs and either remove them from the clutch (Gochfeld, 1979; Moksnes et al., 1991; Martín-Gálvez et al., 2006; Luro & Hauber, 2017), bury them and lay new eggs on top of the burying layer or abandon the nest (Jamieson et al., 2000; Guigueno & Sealy, 2010). However, our results did not support this hypothesis since parents accepted the eggs we added and buried them along with their own clutch.

The common cuckoo, *Cuculus canorus*, an obligate interspecific brood parasite, occurs frequently at our field site. It frequently parasitizes the nest of the sympatric oriental reed warbler, *Acrocephalus orientalis*, which builds open cup nests on reed stems (Li et al., 2016). However, we did not find any common cuckoo eggs in Chinese penduline tit nests over 4 years of study, although small passerines with closed nests, such as cavity-breeders, can also be a suitable host for cuckoos (Grim et al., 2014; Zhang et al., 2021). In addition, conspecific brood parasitism has been reported in some penduline tit species at around 6% (Schleicher et al., 1997; Ball et al., 2017; Chinese penduline tits: about 6.5%, Wang, n.d.). However, this rate is low compared to that in other species that have evolved the ability to recognize parasitic eggs (parasitism rates around 20–40%, Jamieson et al., 2000; Sealy, 1995; Guigueno & Sealy, 2010). Therefore, the low rate of conspecific brood parasitism may not be strong enough to force a mechanism against brood parasitism to evolve in Chinese penduline tits.

Temperature Differences Cannot Explain Egg Burial Behaviour

Our results did not support the hypothesis that egg burial behaviour functions as a temperature regulation mechanism in Chinese penduline tits. Although the temperature around buried

eggs differed significantly from that of unburied eggs, hatching success of both were similar. These findings suggest that there was a temperature difference caused by the burying layer before the incubation period, but this may not have influenced the viability of embryos.

The eggs of passerine birds can endure short periods of low temperatures before incubation (Morton & Pereyra, 1985), but not extreme ambient temperatures where the lethal threshold is reached (Zerba & Morton, 1983; Gillis et al., 2012). In contrast to our results, experimental studies have found a positive relationship between minimum temperature before incubation and hatching success in the pearly-eyed thrasher, *Margarops fuscatus* (Beissinger et al., 2005) and the house sparrow, *Passer domesticus* (Veiga, 1992). Notably, the eggs in these two studies experienced a relatively low temperature over the entire night. However, in our study, the experimental eggs were returned to the breeding nests where the parents roosted at night and provided a warm environment generated from their body temperature. Also, the experimental eggs never experienced long-term exposure to extremely low temperatures during the daytime. Therefore, although a significant temperature difference was found between the buried and unburied eggs, the lack of change in hatching success implies that the temperature difference caused by egg burial was not extreme enough to affect embryo viability in Chinese penduline tits.

Besides, compared to the temperature above the burying layer, the temperature under this material was found to be warmer in cold weather but cooler in hot weather. The burying material probably provides insulation from the surroundings. In a large-scale geographical study on blue tits, *Cyanistes caeruleus*, egg coverage was more extensive when mean spring temperatures were lower, since the thicker covering material may provide better insulation to ensure the viability of eggs (Loukola et al., 2020). We found that, in penduline tits, the burying material was thicker in the morning, when the weather was colder, than in the afternoon. This implies that the low temperature in the morning might threaten egg viability. Our results did not find temperature effects on hatching success, but whether temperature influences nestling development, physiological condition or survival remains unmeasured (DuRant et al., 2013; Gullett et al., 2015; Martin et al., 2017). Future studies should measure these comprehensive effects of temperature on eggs. More investigations should also be carried out across populations in colder climates or in the laboratory to explore the temperature threshold of egg viability. Furthermore, under the background of climate change, tracking the historical climate that penduline tits experienced might also assist in revealing a previous relation between egg burial and temperature.

Does Predation Also Drive Egg Burial?

Egg burial is a strategy commonly used to decrease the risk of predation in fishes, sea turtles and waders (Amat et al., 2012; Grégoir et al., 2018; Lei & Booth, 2018). We did not experimentally test whether egg burial can prevent or reduce predation while

parents are absent. In 2016 and 2017, the predation rate in the study population was low at 7.4% (eight of 108 nests), whereas it increased to 23.9% in 2019 (11 of 46 nests). Snakes were assumed to be the main predators of eggs and nestlings of Chinese penduline tits in 2019 since no debris was left in the nest and nests were not damaged (Davison & Bollinger, 2000); we also filmed predation by a snake once. Notably, 10 of 11 egg predation cases in 2019 occurred during the incubation period, when egg burial had stopped. Additionally, during the temperature regulation experiment, there were two cases where the eggs we moved to the old nest were predated by snakes due to a small hole on the nest roof. However, the eggs buried in the breeding nests, which were next to the old nests, were not predated. Therefore, protection against predation might be a secondary motivation for egg burial in Chinese penduline tits. This function of egg burial has been shown in other studies on ground-nesting bird species, such as Kittlitz's plovers, *Charadrius pecuarius* (Troscianko et al., 2016) and great crested grebes, *Podiceps cristatus* (Keller, 1989), which investigated the effects of vision-based predators. However, snakes use olfaction to locate prey from a distance (Kutsuma et al., 2018). The burying layer may block odours from the eggs and thus prevent snakes from finding them. Also, the burying layer is tightly woven into the nest chamber, making the eggs difficult to reach for snakes, which lack the ability to dig. The relationship between olfactory-based predators and egg burial behaviour in the Chinese penduline tit warrants further investigation.

Conclusion

Certain behaviour can be adopted by different species while fulfilling various functions. Our study found a novel function of egg burial in that it serves to protect eggs from falling out of the nests in windy weather. This function may have been ignored in other species with unstable or open nests and needs more experimental examination. We also verified that Chinese penduline tits did not adopt egg burial for resolving sexual conflict as in Eurasian penduline tits. We found that egg burial can help regulate temperature around the eggs, but hatching success was not affected. Future investigations across multiple populations located in various climatic regions are needed to reveal the existence of a temperature regulation function of egg burial behaviour. In addition, a broader phylogenetic investigation of egg burial would add new insights to the evolution of this behaviour.

Author Contributions

J.Z. designed the experiments, took charge of doing the fieldwork, executing the field experiments from 2016 to 2019, analysing data and writing the manuscript. E.Z. executed the field experiments in 2019, assisted in analysing data and editing the manuscript. Z.Z. assisted in editing the manuscript and provided financial support. M.G. executed the field experiments in 2021. T.S. assisted in editing the manuscript. J.K. supervised this project, edited the manuscript and provided financial support.

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Appendix

Table A1
Number of nests used in the four experiments and egg burial monitoring in different years

	2016	2017	2018	2019	2020	Total
Checking the existence of egg burial	15	25	23	24	8	95
Variation in burial extent	–	–	16	21	–	37
Sexual conflict experiment	–	–	6	9	–	15
Antiparasitism experiment	–	8 with blue dummy eggs 12 with white dummy eggs	10 with white dummy eggs 11 with abandoned eggs	24 with abandoned eggs	–	65
Temperature regulation experiment	–	–	3	11	–	14
Egg protection against wind experiment	–	–	–	23	8	31

Table A2
The average temperature (\pm SD; °C) of different locations outside and inside the nest of Chinese penduline tits in the morning and afternoon

	Ambient temperature	Temperature under the burying layer (UB)	Temperature above the burying layer (AB)	Temperature in the old nest (IO)	Sample size
Morning (°C)	22.65 \pm 2.63	25.26 \pm 3.87	25.12 \pm 3.65	24.58 \pm 3.81	182
Afternoon (°C)	24.13 \pm 2.40	27.25 \pm 3.60	26.74 \pm 3.47	26.54 \pm 3.39	140
Temperature difference (paired <i>t</i> test)	$t = -9.14, P < \mathbf{0.001}$	$t = -8.23, P < \mathbf{0.001}$	$t = -8.26, P < \mathbf{0.001}$	$t = -4.88, P < \mathbf{0.001}$	

The *P* values in bold indicate that the temperatures in the morning and in the afternoon at certain spots were significantly different.