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**Faculty of Science**

**Red-backed shrike (*Lanius collurio*) versus Common  
cuckoo (*Cuculus canorus*): Clutch variability and egg  
recognition**

Master's thesis

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## **Annotation**

In this work, using photography, I tested the red-backed shrike (*Lanius collurio*) intraclutch egg variability and compared it with the interclutch egg variability. The effect of colour, size, volume and maculation as well as chromatic and achromatic contrasts were evaluated. Moreover, I tested ability of the red-backed shrike to recognize artificial cuckoo eggs and artificial shrike eggs, evaluate the rejection rate of these eggs, and compare it with the rejection rate of conspecific eggs. I showed that shrikes have low intraclutch variability and high interclutch variability in all measured parameters. Such variability might be an adaptation against brood parasitism. In addition, I found out that the shrikes' ability to recognize and reject a parasitic egg is significantly higher than expected.

## **Declaration**

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

In České Budějovice, December 6<sup>th</sup>, 2023

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Bc. Ladislava Krausová

## **Poděkování**

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# 1 Literature review

The decline in parasitism in the red-backed shrike (*Lanius collurio*) has been occurring since about the 1960s (Lovászi & Moskát, 2004). Adamík et al. (2009) compared ringing data in the Czech Republic and Slovakia and found a rapid decline between 1964 and 2006, from 2.19% to 0.37% of parasitism. This was probably the last evidence of parasitism in the Czech Republic. The red-backed shrike is known for its aggressive behaviour in defending the nest against nest predators and brood parasites, which it can reliably discriminate and respond to them appropriately. Cuckoo-hawk mimicry as an adaptation of the common cuckoo against physical attacking by its host is in shrike ineffective (Krausová et al., 2022), which may be discouraging for the cuckoo. As a frequent cuckoo host, the shrike had to find a solution to tackle the common cuckoo (*Cuculus canorus*). Recognition, rejection of the parasitic egg and high interclutch variability and low intraclutch variability are other strategies how to defend against nest parasitism (Øien et al., 1995; Karcza et al., 2003; Lovászi & Moskát, 2004).

## 1.1 Eggshell composition

The eggshell consists mainly of inorganic material (95%) with an organic matrix (4%) and water. The calcareous eggshell is formed mainly of calcite, a form of calcium carbonate, and of the minor magnesium, phosphate, and aragonite components (Board & Scott, 1980; Nys et al., 1999; Nys et al., 2004). The eggshell color may considerably differ and possibly reflects the physiological processes (breeding condition of the female or order of the laid eggs) (Mikšík et al. 1994, 1996). The coloration of the shells is influenced mainly by protoporphyrin, which causes the brown and black pigments, and by biliverdin and his zinc chelate, which causes the blue and green pigments of the eggshell (Kenedy & Vevers, 1976; Mikšík et al., 1996; Kilner, 2006). Eggs of different species may differ significantly in the content of these substances, for example Mikšík et al. (1994) compared colouring and contents of the protoporphyrin at seven bird species and found that the blackbird (*Turdus merula*) had the highest compound of the protoporphyrin, while the lowest amount was found in the eggs of the swift (*Apus apus*). Furthermore, the study showed that there could be high differences even within a species, as it is in the red-backed shrike, which eggs showed high variability of protoporphyrin content, which may reflect the physiological influences, as was mentioned above.

## 1.2 Egg variability

Among bird species, there is a wide variability of eggs, especially in background colour and patterning (maculation). The colour spectrum can range from white to brown, red, turquoise

or emerald green. In addition to the coloration of egg background, which can probably be a key factor in surviving in different types of landscapes (Castilla et al., 2007), the eggshell may be covered by spotting pattern. The spots may be arranged in a ring, clustered in patches or may be all around the eggshell in dense net.

Due to the remarkable variability, the distribution among bird taxa is still uneven (Kilner, 2006; Cherry & Gosler, 2010) – e.g., white eggs are laid not only by cavity-nesting kingfishers (, but also by completely unrelated hummingbirds nesting in open nests. In contrast, species in the tinamou family each lay completely different eggs. An extreme example are the members of a single species of wailing cisticola (*Cisticola lais*) laying white, pale blue, spotted, immaculate, or streaked eggs.

There are several hypotheses explaining the wide variation in eggs appearance, not only phylogeny and type of nesting but also crypsis (mimicry), selection by predators or brood parasites and their hosts, or mate selection, thermoregulation (Götmark, 1992), etc. Meanwhile, in species the egg spotting is probably an adaptation to parasitism; in species not parasitized by the cuckoo the spotting is more dependent on habitat conditions and diet (Gosler et al., 2005). Although patterning has several ecological functions, physiological factors have also significant impact. Gosler et al. (2005) showed that individuals of the great tit (*Parus major*) have different maculated eggs depending on the amount of calcium during laying. Patterning may act to compensate for calcium deficiency during this period. Similarly, level of biliverdin (antioxidant) in females (Navarro et al., 2011) or females' condition (Reynolds et al., 2009) could also affect the patterning of the eggs. Probably none of the hypotheses is better, but rather they reflect alternative potential evolutionary directions (Spottiswoode & Stevens, 2011).

### **1.2.1 Egg mimicry**

Markings, which provide camouflage not only against nest predators but also against brood parasites, are also known as egg mimicry. Götmark (1992) showed better camouflage of the dark speckled eggs on the ground than blue or white eggs. However, when the eggs were in nests, there were no significant differences in the predation rates of differently coloured eggs. Predators probably orient themselves by the nest rather than by the eggs in the nest, which may also be related to parasitism. Ancestrally, prior to the emergence of parasitism, it is conceivable that the development of the coloration and patterning of host eggs had an evolutionary purpose in terms of camouflage or thermoregulation – tendency to lay darker eggs in cold habitats to quickly heat up and slowly cool down the eggs.

In mimicking the characteristics of the host eggs, the parasites initially mimicked the characteristics (cue mimicry) of the model. This led to a subsequent coevolutionary competition (Jamie, 2017). Evolving mimetic eggs, the parasite reached the point of arms race. Possible defence strategies of hosts to decrease level of parasitism are either improving their level of egg discrimination or changing the appearance of its eggs to be sufficiently different from those of other hosts and parasites (Spottiswoode & Stevens, 2011). Moreover, parasite species show, but not necessarily, low intraclutch and higher interclutch variation to make it harder for the parasite to mimic the host eggs (Øien et al., 1995; Karcza et al., 2003; Avilés et al., 2007). Lower intraclutch variation is well known in species with a longer relation to the cuckoo (Avilés & Garamszegi, 2007). One of the common cuckoo's host, the red-backed shrike, has very variable egg with high variability of the porphyrin content. Eggs are typically maculated with brown or grey spots, forming a band around the larger end of the egg (Mikšik et al., 1994).

### 1.2.2 Cuckoo mimetic eggs

The egg discrimination strategy is known for cuckoos and their hosts. Moreover, in species that have higher egg recognition ability, the cuckoo lays more mimetic eggs. Although the common cuckoo has many host species, each female always specialises in one or few related host species. Cuckoo eggs are thus always matched to the specific egg type of the species, utilizing the most common host egg phenotype (Yang et al., 2010). The high variability between clutches could cause the division of cuckoos into specialized lineages, called gentes (Yang et al., 2010; Spottiswoode & Stevens, 2011). Although cuckoo eggs are always larger than the host, they are quite small for a bird the size of an adult cuckoo. Another special feature of the parasitic egg in cowbirds and many brood-parasitic cuckoo is thicker eggshell unexpected for their size (Moksnes & Roskaft, 1995; Antonov et al., 2006). Although the shell is strong, there must be some compromises for the young to hatch (Picman & Pribil, 1997). Brooker & Brooker (1991) compared the strength of eggs within the parasitic family Cuculinae.

Firstly, the strength and shape of the eggs were compared between subfamily *Clamator* and *Cuculus/Cacomatis/Chrysococcyx*. *Clamator* cuckoos have stronger and rounded eggs than the host compared to the other three groups. *Cuculus/Cacomatis/Chrysococcyx* cuckoos have still more strength eggs than host but are less rounded, which may be related to the strength (sphere is the strongest shape). Comparing the egg strength and shape of Cuculinae and the non-parasitic species, *Clamator* has significantly thicker eggshells than non-parasitic species. Other parasitic cuckoos lay much smaller eggs than the non-parasitic one; the comparisons are not

valid in this case. However, comparing eggs of the non-passerine species with *Cuculus/Cacomatis/Chrysococcyx* group is possible. However, no significant differences were found, even in a common cuckoo, whose eggs are considered to be thick. The possible importance in breaking strength of the egg may also be important in cowbird.

### **1.2.3 Eggshell patterning and type of nesting**

The patterning is often associated with the type of the nesting. Cavity-nesting species usually have either white or blue spotless eggs, while species nesting on more exposure places (on the ground, on a ledge, shallow niche, or cup-shaped nest) have either slightly or densely spotted eggs (Lack, 1958; Götmark, 1992; Kilner, 2006). Lack (1958) compared the coloration and patterning of the eggs through Turnidae. Confirming this statement, he found that the cavity-nesting pied flycatcher (*Muscicapa hypoleuca*) has spotless pale blue eggs, while the spotted flycatcher (*Muscicapa striata*), nesting on a ledge or in a shallow niche, has densely spotted white eggs. Moreover, three species of wagtails have also different type of eggs depending on the type of the nesting – cavity or niche-nester, the pied wagtail (*Motacilla alba*) has white speckled eggs; more open niches or ledge nester, the grey wagtail (*Motacilla cinerea*) has greyish buff eggs, and the yellow wagtail (*Motacilla flava*) nesting on the ground has very densely spotted eggs appearing uniform brown. In addition, the study by Oniki (1985) confirms the hypothesis that unconcealed eggs are more speckled than hidden eggs. Moreover, blue eggs occur more often in species nesting outside the forest shade and in a thick cup nest.

Despite the fact that cavity-nesting birds generally have spotless eggs, the variability across bird taxa is neither ecologically nor phylogenetically even (Gosler et al., 2005).

### **1.2.4 Predation**

It is generally believed that the high variability in spotting of species nesting on the ground is the result of the high level of predation on eggs. Markings are adapted for concealment from predators (Götmark, 1992; Gosler et al., 2005). Experiments by Castilla et al. (2007) conducted on red-legged partridge (*Alectoris rufa*) showed that brown and brown-spotted eggs have lower predation rates than white/white-spotted eggs in fallow landscapes. While brown-spotted eggs had the lowest predation in the forest in contrast to brown, white and white-spotted eggs. Predation rates were also influenced by predator type, while Rodentia was not selective based on egg colour, brown eggs with spots received the least predation in birds compared to the other three egg types. At the same time, for all predators combined, spotting had no effect on predation rates, but white eggs were still predated up to twice more than brown eggs. Weidinger (2001) showed that egg color did not affect nest survival, however,



there was less predation of brown eggs in yellowhammer compared to white eggs (antipredation strategy of the yellowhammer, ground/near-ground nester).

### **1.2.5 Parasitism**

Mimicry can also be the result of coevolution or an arms race between the nest parasite and its host. Cuckoo eggs may not always be perfect, the explanation may be that the host does not reject the parasitic eggs or that cuckoos are forced by lack of suitable host nests to lay their eggs in nests of other species that they do not normally parasitize. If the parasitic egg has reached the stage of "perfect" mimicry, the next counteradaptation of the host is to evolve a new egg type with more complicated egg patterns or egg signatures to escape the mimetic eggs of the parasite (Rothstein, 1990; Stokke et al., 2017). This may further lead to the production of more diversified eggs between individual females, i.e., higher interclutch variation or even lower intraspecific egg variation (Karcza et al., 2003; Lovászi & Moskát, 2004; Stokke et al., 2017). If such variability is created in the host, it becomes difficult for the cuckoo to mimic the host eggs (Cherry et al., 2007).

## **1.3 Egg recognition**

The ability to defend against nest parasitism is not only determined by altering the appearance of host's own eggs but also in the ability to recognize the parasitic egg. This capability is one of the key defences against the brood parasite (Samaš et al., 2021). Egg discrimination involves two phases, starting with the perceptual phase (egg recognition) and then the operational phase (egg rejection).

### **1.3.1 Key feature for recognition**

What are the key features to recognize objects (eggs)? Spottiswoode & Stevens (2010) published a simultaneous study quantifying visual cues to recognize a foreign egg. Clear results show that the hosts' decision whether to accept or reject parasitic eggs does not depend on one, but on a combination of several clues. There may be several distinguishing features, such as colour, weight and size, maculation, shape, or odour. The recognition process may also be influenced by other factors, such as the risk of parasitism or the costs associated with egg rejection (Honza & Cherry, 2017; Abolins-Abols et al., 2019; Samaš et al., 2021). Colour and maculation are recognized as crucial clues in discrimination. In addition, the greater the disparity between host and parasitic eggs, the increased likelihood of rejection (Spottiswoode & Stevens, 2010; Samaš et al., 2021). However, these two main clues are not used by all

parasitized species, as is the american robin (*Turdus migratorius*) (Hauber et al., 2020) or the great reed warbler (*Acrocephalus arundinaceus*) (Abolins-Abols et al., 2019).

By perceiving chromatic contrast, birds can compare the contrast between their own and parasitic eggs. Chromatic contrast (colour mimicry) is given in unit JND (Just-Noticeable Difference). As a rule, the lower the number, the better the mimicry, and at the same time, the higher the number, the better the bird perceives the difference. Values  $<1$  are indistinguishable even under perfect viewing conditions (Siddiqi et al., 2004; Hauber et al., 2020). Despite many studies investigating key egg recognition cues, there is still a lack of quantitative studies investigating overall eggshell characteristics and their impact on host behaviour (Spottiswoode & Stevens, 2010; Samaš et al., 2021).

#### **1.4 Egg rejection**

The second phase of the discrimination is the rejection of the parasitic egg by the hosts. There are several methods of rejection, including ejection, desertion of the nest, puncture-ejection, or building a new nest over the old one. The most effective way of rejection is ejection because some species are not able to grasp the egg into their bill. Firstly, they puncture the egg and then remove it from the nest (puncture-ejection). This ability often requires special pecking effort, due to the risk of breakage or soiling of host eggs (Davies & Brooke, 1989; Antonov et al., 2006; Honza et al., 2007; Šulc et al., 2019). In some cases, errors in recognizing host's own eggs may occur, particularly among young and inexperienced breeders (Lotem et al., 1995) or the false imprinting of foreign eggs during the laying phase (Moskát & Hauber, 2007).

High interspecific or low intraspecific variability of eggs may also influence the recognition rate following by rejection. While intraclutch variability has no effect on egg rejection, interclutch variability significantly affects the rejection rate of parasitic eggs (Øien et al., 1995; Soler & Møller, 1996; Honza et al., 2004). The study by Øien et al. (1995) describes the effect of intra- and interclutch variability on the rate of parasitic egg rejection in 75 species, 47 of which are suitable hosts for the common cuckoo and 28 unsuitable hosts. Rejection rates were used from previous experiments and for only 34 species. Despite intraclutch variability had no effect on rejection, rejection rates were assessed based on the average of all suitable hosts (47 species) rather than for each species separately.

Davies & Brooke (1989) investigated host egg discrimination after parasitisation with model cuckoo eggs. These eggs were made from gel coat resin and painted with acrylic paint to

demonstrate the colour of four cuckoo gentes ( *pied wagtail type, meadow pipit type, reed warbler type, and redstart type*). Two Silastic rubber moulds were used to create the eggs, both of which were close to the average size of a cuckoo egg ( $23.05 \times 17.23$  mm). The weight of the eggs was similar to the real cuckoo eggs (range 2.9 – 3.8 g). Moksnes et al. (1991) used real conspecific eggs and artificial cuckoo eggs made from a hard plastic (araldite) with the addition of a small amount of fiberglass powder and ground colour. The eggs were filled with mixture of albumen and glycerol and painted to simulate cuckoo eggs. The egg were the same size and weight as a real cuckoo egg. Experiments were conducted on 19 hosts of common cuckoo (including – song trush (*Turdus philomelos*) or meadow pipit (*Anthus pratensis*)). In comparison Procházka & Honza (2003) used only real eggs in discrimination experiments on common whitethroat (*Sylvia communis*). They had non-mimetic real eggs, resembled cuckoo eggs, from deserted clutches of the red-backed shrike or yellowhammer, and real conspecific eggs as mimetic eggs. Moskát & Fuisz (1999) were conducting experiments on the red-backed shrike, using plastic (eupol polyester poured into acrylic paint) egg of the same size and weight as a real cuckoo egg. The eggs were of two painted types: red-backed shrike (maculate) and redstart (*Phoenicurus phoenicurus*) (immaculate pale blue). Other studies have used artificial eggs made of plaster of Paris (Rothstein, 1975) or 3D printed eggs (Hauber et al., 2021).

## 1.5 Aims

- Using photography to test the red-backed shrike intraclutch egg variability and compare it with the interclutch egg variability. The effects of colour, volume, maculation, chromatic and achromatic contrasts will be evaluated.
- To test a reaction of the red-backed shrike to artificial cuckoo eggs and artificial shrike eggs, evaluate the rejection rate of these eggs and compare it with the rejection rate of conspecific eggs.

## 2 Materials and methods

### 2.1 Study area

The study took place in Znojmo (48°50'51.8"N 16°02'25.4"E) and part of the Podyjí National Park (48°48'51.8"N 16°00'02.3"E) in the South Moravia, the Czech Republic. The experiment was carried out mainly during the laying and early incubation phase of the nesting from May to June in 2022 and 2023. The main habitat is extensive pastures, old meadows, heathlands, and vineyards ecotone with many shrubs.

### 2.2 Study species

The red-backed shrike is a medium size passerine bird, occupies half-open habitat with scattered bushes and scrub. It prefers shrubs with thrones and spikes, like the wild rose (*Rosa canina*), blackthorn (*Prunus spinosa*), or hawthorn (*Crataegus spp.*). Its food consists mainly of insects, such as beetles (*Coleoptera*) or hymenopterous insects (*Hymenoptera*), but also lizards, small mammals (*Mus*, *Sorex*, etc.), and even birds (Hagemeijer & Blair, 1997; Šťastný & Hudec, 2011).

The open cup nest is often lined with moss or sheep wool. The clutch contains on average six eggs (personal observation); after the loss of the first clutch, they are able to nest again. The replacement clutch is in a new nest close to the original one. The female incubates eggs for 14-16 days, after hatching the juvenile birds remain in the nest on average for 14-16 days. At around 25 days of age, fledglings are able to actively hunt for insects, and by approximately 42 days old, they achieve independency (Hagemeijer & Blair, 1997; Lefranc & Worfolk, 1997; Lovászi & Moskát, 2004; Šťastný & Hudec, 2011).

The red-backed shrike is known for its aggressiveness against nest predators (e.g., Euroasian jay (*Garrulus glandarius*) – predator of nest contains (Strnad et al., 2012); or the common kestrel (*Falco tinnunculus*) – predator of the fledglings (Strnad et al., 2012). Moreover, shrikes are not only aggressive, but they have also high cognitive abilities as they are able to distinguish between harmless birds and predators and even between different types of predators and response adequately to them (Goławski & Mitrus, 2008; Srová et al., 2016; Strnadová et al. 2018, Krausová et al., 2022).

## **2.3 Experimental design**

To test the ability of the red-backed shrike to recognize the parasitic eggs, we conducted experimental nest parasitism. Experiments were conducted in the day, when the fifth egg was laid or in the beginning phase of the incubation. Phase of the nesting was tested using the simple egg water test (Westerskov, 1950). After adding the parasite egg, the nest was checked every day for a maximum of five days. Even though the cuckoo removes one egg when laying in the host nests, Davies & Brooke (1989) showed that it has no effect on the host response; therefore, there were not removed any host eggs in experiment. If the egg was ejected or the nest was abandoned (the eggs were cold), the experiment was scored as a rejection. If the egg was still present in the nest after five days, the experiment was scored as acceptance, and the model egg was taken from the nest.

### **2.3.1 Model eggs**

For the experiments, three types of model eggs were used (Fig. 1): 1) artificial cuckoo egg (hereafter ACE); 2) artificial shrike egg (hereafter ASE); and 3) conspecific red-backed shrike eggs (hereafter CSE) from abandoned clutches. The artificial eggs were made of thermoplastic sheets, moulded (see Fig. S1 in Supplements) to match the real size and weight (on average 3g) of the eggs of the shrike and cuckoo found in the shrike's nest. Cuckoo's eggs from the shrike nests as well as shrike eggs were collected in the Czech Republic, about a hundred years ago, originating from the collections of the Moravian Museum at the Budišov Castle. Both types of eggs were photographed and calibrated to match the bird's vision (Troscianko & Stevens, 2015). Photos were then printed on the PVC film to match the coloration and patterning of the real eggs. Eggs are not able to reflect the ultraviolet light (UV), however they are calibrated directly for avian visual system (according to the method by Heathcote et al. 2020).

Moreover, shrike eggs from abandoned nests were used to test the ability of shrikes to recognize conspecific eggs and reject them.



**Fig. 1:** Types of model eggs: (1) conspecific shrike egg, (2) artificial shrike egg, (3) artificial cuckoo egg.

### **2.3.2 Egg variability**

To test the interclutch and intraclutch variability of conspecific eggs, all experimental clutches were photographed. The photography of the eggs was carried out after the parasitism experiment and in the time of the complete clutch. Careful and fast manipulation with eggs was crucial to avoid any damage. After removing the eggs from the nest, a fabric cover was placed in the nest to prevent the birds from seeing the empty nest. The eggs were carefully arranged on a perforated white board to avoid slipping. A standard grey scale with known reflectance (18%) (X-Rite Colour Checker Grey Scale Chart) and a scale was always placed on the plate for facilitate further analysis (see Fig. S2 in Supplements). The entire plate was then covered with a photography light tent to avoid unwanted harsh shadows. The photographs were taken with a standard Nikon camera (D3100), in RAW (respectively NEF) format to preserve true colours.

#### *Import of images to ImageJ*

After photographing the clutches, image calibration and other analysis (pattern, chromatic/achromatic contrast, volume) were conducted in ImageJ using the Multispectral Image Calibration and Analysis micaToolbox (Troscianko & Stevens, 2015; Šulc et al., 2019). The first step was to generate the multispectral image. Then it was necessary to set the important and required camera parameters and filter combination. The camera type was set up to "visible" for standard red-green-blue (RGB) images, the image type set up to RAW (in which the photos were taken) to preserve all the colours, and the standard reflectance at 18%. After setting the required parameters, the area of the grey standard was marked in the image. Next, the scale was marked and then set up at 50 mm so that the size could be standardized for all eggs in future analyses (in all photos).

## *Size*

At this point, regions of the interest (ROIs) in the shape of eggs can be selected (Troscianko & Stevens, 2015). Once the curvature was created, the volume ( $\text{mm}^3$ ) could be automatically calculated by using a circular cross-section and splitting the eggs into a large number of long-axis slices. This analysis is called a least-squared function (Troscianko, 2014). Afterwards, the volume difference was calculated by comparing each egg with each other in R (R Core Team, 2023). Subsequently, the variability of eggs within and between clutches could be further calculated.

## *Pattern (maculation)*

Spotting pattern analysis was performed by using the Auto Local Treshold plugin of the ImageJ software, which converts color images into binary (black/white) format. White pixels – background egg colour, and black pixels – spots. The resulting black-and-white images were visually compared with the original colour images to check the accuracy of the thresholding. The percentage of spotting was calculated by dividing the pixels of spots by the total number of pixels of the selected egg (Landini, 2011; Schneider et al., 1012). Afterwards, the difference in pattern was calculated in the same way as for the size.

## *Hue and saturation*

To assess the egg coloration, the parameters of color hue and saturation were calculated. Under the term hue we can imagine a particular shade of color, while saturation represents a kind of purity of color, i.e., the proportion of impurities to a given hue of color, a fully saturated color is one in which there is no proportion of impurities (W-technika.cz, 2023). First, using the micaToolbox (Troscianko & Stevens, 2015), converted to the avian visual model (trichromatic, without UV), to calculate catches of three individual cones (short-, medium- and long-wavelength), and then the saturation and hue parameters (avian visual modelling) were calculated using the *pavo* version 2.9.0 (Maia et al., 2019) in program R version 4.3.1 (R Core Team, 2023). Using the *pavo* package, the RGB color is transformed into HSV (hue, saturation, value). Hue is represented as a theta angle, also giving the direction of vector  $r$ , where vector  $r$  is a measurement of saturation (chroma) – how different a color is from achromatic white/black (Stoddard & Prum, 2008).

## *Chromatic and achromatic contrast*

Another tested parameter was the chromatic contrast ( $\Delta S$ ), that describes the colour contrast between two eggs, and the achromatic contrast ( $\Delta L$ ), describing the difference between the two colors in their intensity (brightness) (Avilés, 2008). To compare the colour and brightness differences, the multispectral image was converted to the avian visual system. Afterwards, a model using information about the sensitivity of the cones of the species and the sensitivity of the camera sensor needs to be created. Although the micaToolbox contains this information for many cameras and animal species, information on the visual system of the red-backed shrike is missing. In the case of absent cone sensitivity data for a given species, data on its closest relative can be used (Šulc, 2022). There are two classes of colour vision in birds, UV-sensitive (UVS) and violet-sensitive (VS). In the family Laniidae, the type of vision has not yet been determined, however, there are close relatives of Corvidae that have VS (violet-sensitive) vision (Stoddard & Stevens, 2011). For the VS vision type (red-backed shrike), imageJ uses spectral sensitivity data of the indian peafowl (*Pavo cristatus*) (Hart, 2002). To compare the colours and brightness of the two objects, the relative excitation of the individual cones was first calculated using the Batch Multispectral Image Analysis (analysis of multiple images at the same time). The results are further analysed using function Colour JND Difference Calculator (Troscianko & Stevens, 2015).

Host eggs were compared with parasitic conspecific eggs and color and brightness in all possible pairs was calculated, using relative numbers of cones of *Pavo cristatus* (Hart et al., 2001): short-wavelength-sensitive (SWS) single cone = 1.9, medium-wavelength-sensitive (MWS) single cone = 2.2 and long-wavelength-sensitive (LWS) single cone = 2.1 (Honza et al., 2014) and the double cones (brightness) (Abernathy et al., 2017). Afterwards, the comparison of chromatic contrast between all three types of model eggs was made. While chromatic contrast is used by birds in nests with good light conditions, achromatic contrast is used by birds in places with poor light conditions (e.g., hole-nesters) (Avilés, 2008).



## 2.4 Statistical analyses

For statistical analysis, I used six parameters of egg to test the egg variability within and between clutches and three types of parasitic eggs compared with all host eggs. All of the six parameters were evaluated using the Analysis of Variance (ANOVA), command *aov()* in R version 4.3.1. Comparisons of the parasitic and host's egg were evaluated using the Welch's t-test in R, using *t.test()* function, in case of normal distribution of data, in other cases the Wilcox t-test, using *wilcox.test()* function in R. The graphs were created using the package *ggplot2()* (Wickham, 2016) with *geom\_boxplot()* function for creating boxplots.

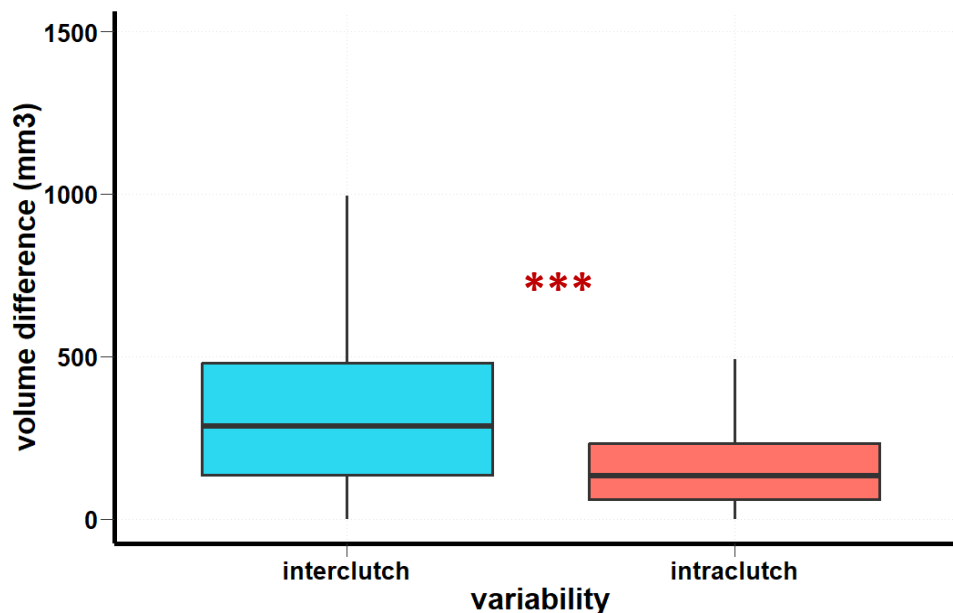
## 2.5 Ethical note

The survival of the birds was monitored during the experiments and no effect on their success rate was observed. The experiments taking place in the Podyjí National Park were authorized by the Podyjí National Park Administration (NPP 0795/2022 and 0375/2023). Permissions for studies wild red-backed shrikes was granted by the Regional Authority of the South Moravian Region (JMK/50180/2023) and Ministry of the Environment of the Czech Republic (MZP/2020/630/113) and the license permitting experimentation with animals no. CZ01629 was offered by the Ministry of the Agriculture of the Czech Republic. All experiments were in accordance with international, national, and institutional guidelines for the care and use of animals.

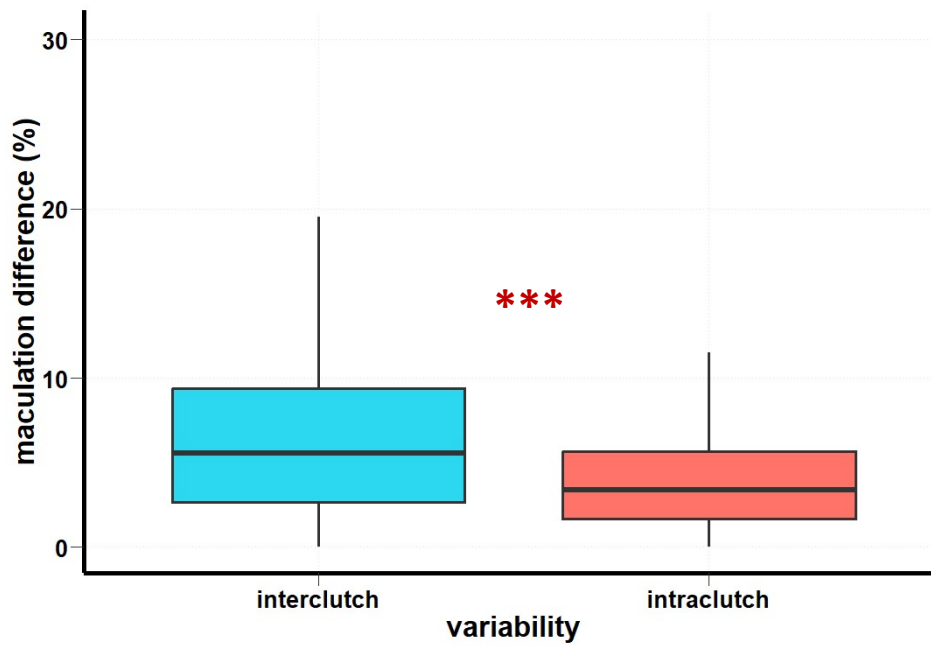
### 3 Results

#### 3.1 Variability of shrikes' clutches

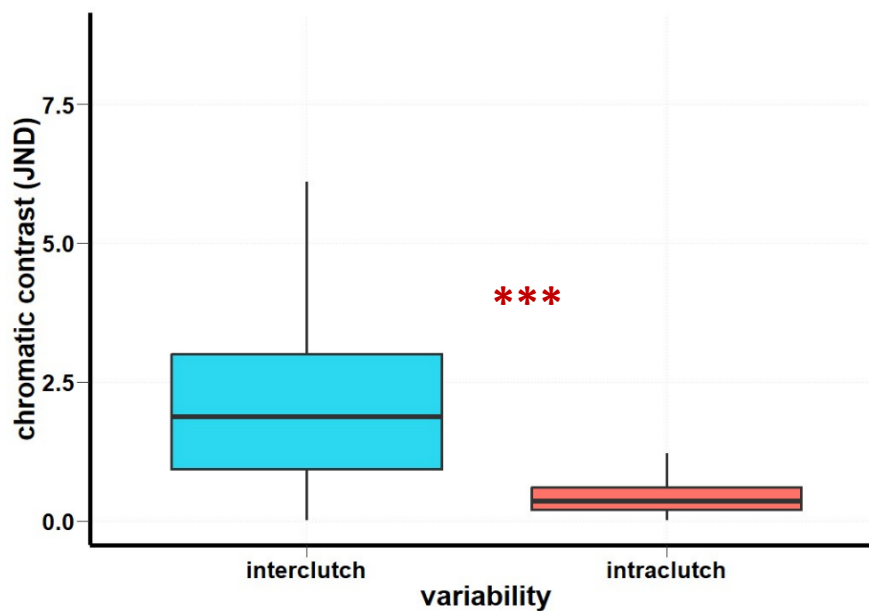
A total of 75 nests, comprising 388 eggs, were recorded during the years 2022 and 2023. To measure egg variability, six egg parameters were used - colour saturation (see Fig. S3 in Supplements), hue (see Supplements Fig. S4), volume (Fig. 2; see Fig. S5 in Supplements), pattern (Fig. 3; see Fig. S6 in Supplements), chromatic (Fig. 4) and achromatic contrast (Fig. 5). Six parameters were tested within and between the clutches. Interclutch variability was significantly higher than the intraclutch variability of eggs for all tested parameters (ANOVA,  $p < 0.001$ ) (Fig. 2-5).



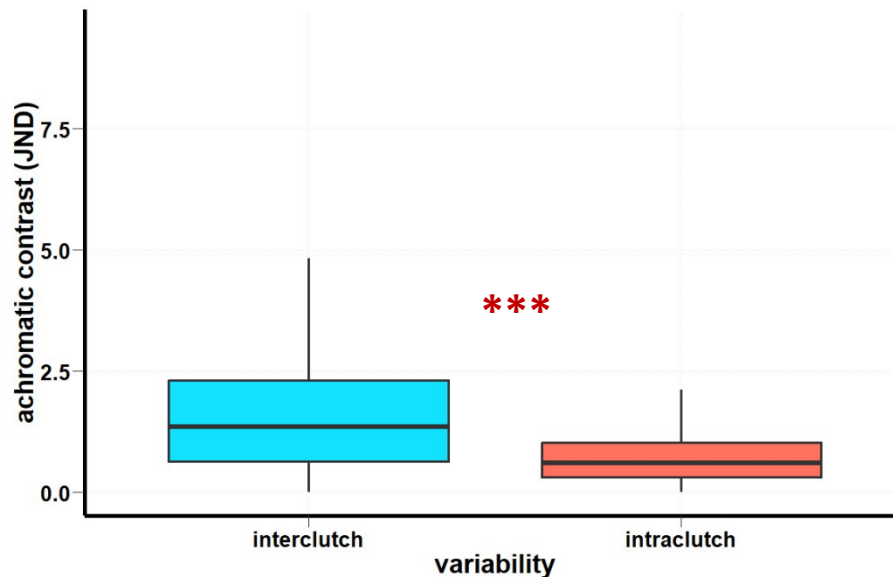
**Fig. 2** - Variability of eggs within and between clutches in differences in egg volumes ( $\text{mm}^3$ ) (N=388 eggs; N=75 nests). The bold line refers to the median, the bottom and top of the box represent the first and third quartiles, whiskers show 10–90% of data



**Fig. 3** - Variability of eggs within and between clutches in differences in egg maculation (%) (N=388 eggs; N=75 nests). The bold line refers to the median, the bottom and top of the box represent the first and third quartiles, whiskers show 10–90% of data.



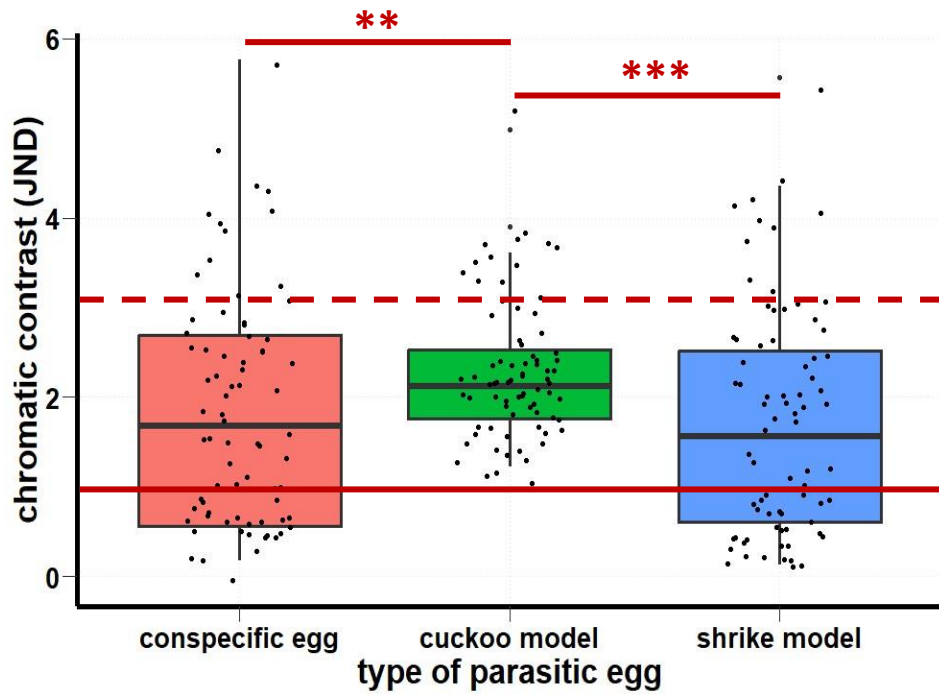
**Fig. 4** - Variability of eggs within and between clutches in chromatic contrast (color, JND) (N=388 eggs; N=75 nests). The bold line refers to the median, the bottom and top of the box represent the first and third quartiles, whiskers show 10–90% of data.



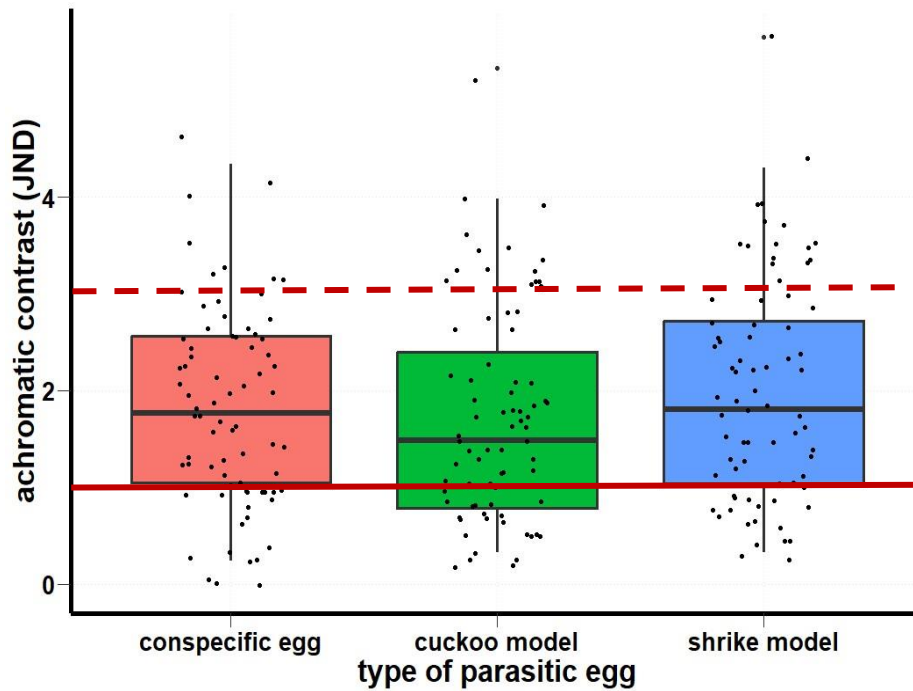
**Fig. 5** - Variability of eggs within and between clutches in achromatic contrast (brightness, JND) (N=388 eggs; N=75 nests). The bold line refers to the median, the bottom and top of the box represent the first and third quartiles, whiskers show 10–90% of data.

### 3.2 Measurements of tested eggs

Comparing the chromatic contrast of the three types of parasitic eggs with the host eggs (Fig. 6), the ACE seemed to be the most different from both the ASE (Welch's *t*-test,  $p=0.001$ ) and the CSE (Welch's *t*-test,  $p=0.011$ ). The difference between the ASE and the CSE was not significant (Welch's *t*-test,  $p=0.605$ ). Comparing the achromatic contrast of the eggs (Fig. 7), no significant differences were found between ACE and ASE (Welch's *t*-test,  $p=0.929$ ); ACE and CSE (Welch's *t*-test,  $p=0.651$ ), neither between CSE and ASE (Welch's *t*-test,  $p=0.118$ ). Most values were higher than 1 JND, below this value birds should not be able to distinguish between two objects. However, most values were below 3 JND (red lines in the graph), below which color differences are still considered hardly distinguishable.



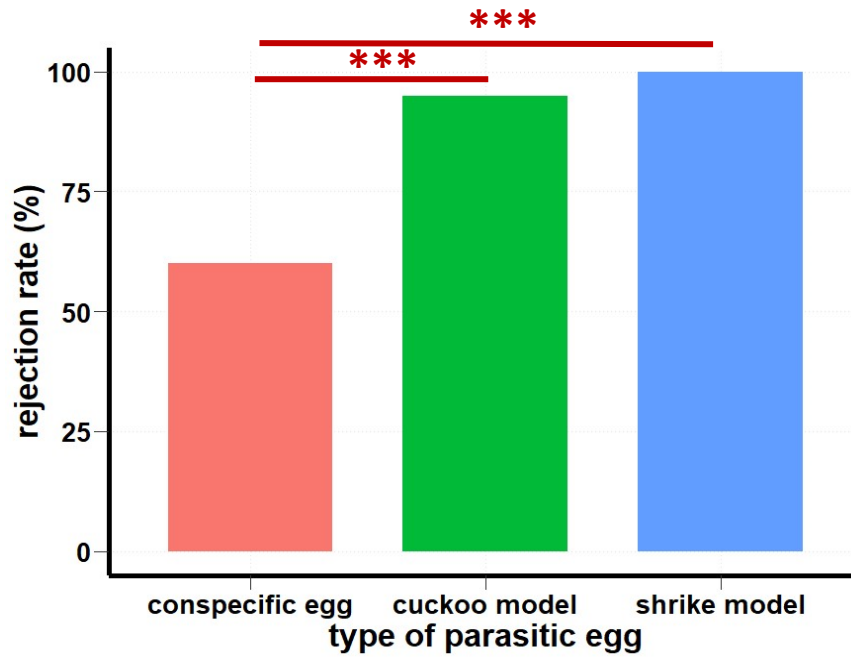
**Fig. 6** – Comparison of the chromatic contrast (JND) between host eggs and three types of parasitic eggs (N=75). The red solid line shows the boundary (1 JND) below which the two objects are indistinguishable to birds. The red dashed line shows the boundary (3 JND) below which the two objects are still very difficult for birds to distinguish. Red lines and asterisks indicate significant differences between three types of parasitic eggs. The bold line refers to the median, the bottom and top of the box represent the first and third quartiles, whiskers show 10–90% of data and dots are outliers.



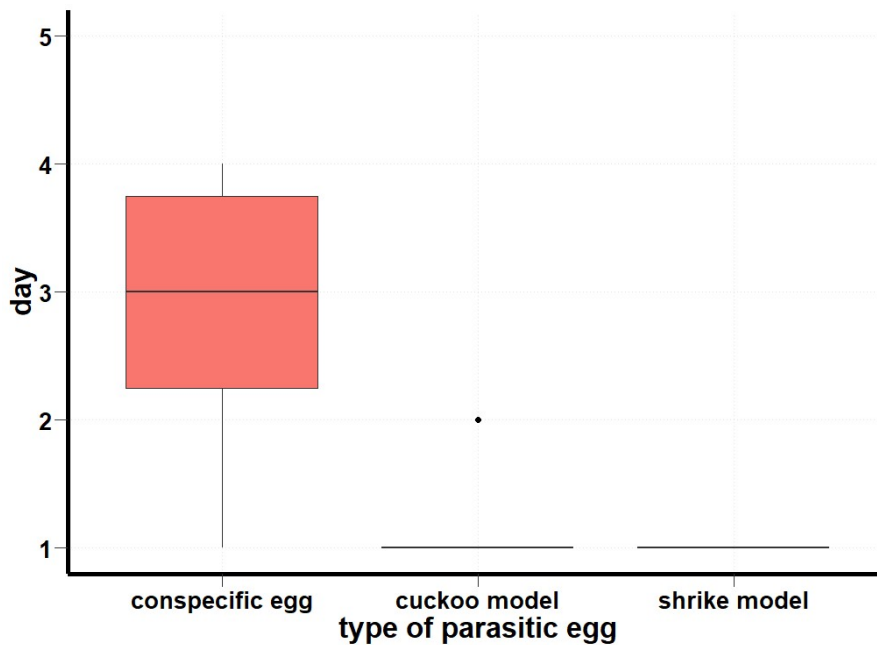
**Fig. 7** - Comparison of the achromatic contrast (JND) between host eggs and three types of parasitic eggs (N=75). The red solid line shows the boundary (1 JND) below which the two objects are indistinguishable to birds. The red dashed line shows the boundary (3 JND) below which the two objects are still very difficult for birds to distinguish. Red lines and asterisks indicate significant differences between three types of parasitic eggs. The bold line refers to the median, the bottom and top of the box represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.

### 3.3 Rejection results

We tested the ability of the shrikes to recognize and reject parasitic eggs (N=60). Results of the rejection experiment (Fig. 8) show that in the case of the addition of an ASE, shrikes rejected it in 100% (20 of 20 cases). After adding ACE, they rejected it in 95% (19 of 20 cases). Shrikes also showed remarkable cognitive ability in recognizing and rejecting CSE, with 60% (12 from 20 cases). Model eggs (ACE, ASE) were rejected within the first day after addition to the clutch, while CSE were rejected mostly on the third day after addition (Fig. 9).

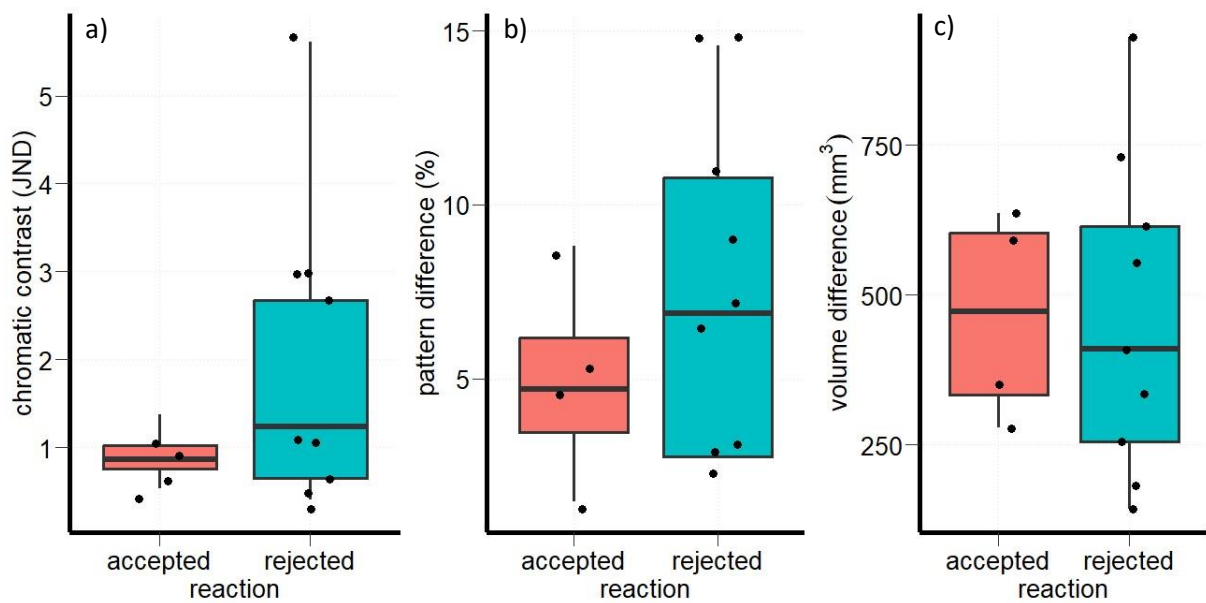


**Fig. 8** – The red-backed shrike responses (rejection rate) to the three types of parasitic egg (N=60). Red lines and asterisks indicate significant differences between CSE (conspecific egg) and ACE (cuckoo model) ( $p < 0.001$ ) and between CSE and ASE (shrike model) ( $p < 0.001$ ).



**Fig. 9** - Day of rejection (ejection only) of parasitic eggs after addition to the clutch (N=46). The bold line refers to the median, the bottom and top of the box represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.

To test whether conspecific egg mimicry influenced a host rejection, I measured level of mimicry in 13 nests where the reaction to conspecific egg was tested. The conspecific egg was compared with each egg from one clutch and then the average value per nest was calculated (for all three parameters). The reaction to conspecific eggs by host pairs was 4 times acceptance and 9 times rejection. Firstly, we focused on the colour difference (chromatic contrast) between host and conspecific eggs, where we did not find any significant results (Wilcox.test,  $p=0.302$ ; Fig. 10a). Moreover, we found out that the reaction was not further affected by either pattern difference (Welch's t.test,  $p=0.139$ ; Fig. 10b) or volume difference (Welch's t.test,  $p=0.509$ ; Fig. 10c).



**Fig. 10** – Conspecific eggs mimicry characteristics in parasited nest, where the parasitic egg was accepted or rejected (N=13). (a) chromatic contrast (Just Noticeable Difference, JND), (b) pattern difference (%), (c) volume difference (mm<sup>3</sup>). The bold line refers to the median, the bottom and top of the box represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.



## 4 Discussion

Egg variability is one of the adaptations to defend against brood parasitism (Øien et al., 1995; Karcza et al., 2003; Avilés et al., 2007; Spottiswoode & Stevens, 2011). Our results show that shrikes have low intraclutch and high interclutch variability of the eggs, in all tested parameters (chromatic and achromatic contrast, color hue, color saturation, volume, and patterning). The ImageJ program used in this study is a unique way to measure eggs, where the vision parameters of a specific or closely related bird species can be set and these parameters are reflected in the egg analysis. Additionally, thanks to the results from ImageJ, it is possible to quantify the differences between the eggs. Pattern comparison with ImageJ and micaToolbox was previously successfully used to find identity signals in four alcid species - common murre (*Uria aalge*), thick-billed murre (*Uria lomvia*), razorbill (*Alca torda*), and dovekie (*Alle alle*) (Quach et al., 2019), or in great reed warbler (Šulc et al., 2021).

Shrike eggs variability was previously tested in several studies with human evaluators but never with program ImageJ (Øien et al., 1995; Soler & Moller, 1996). In the study by Øien et al. (1995), clutches of 75 bird species from museum collection (Denmark) were photographed. Photos were evaluated using 1–5 scale, where 1 is for low and 5 for high variability. The variability of eggs of the red-backed shrike between clutches was rated as high/within clutches as low (4.2/2.0). Such difference is interesting in comparison to other suitable host species for the common cuckoo, which have higher intraclutch and lower interclutch variability than red-backed shrike, like the marsh warble (*Acrocephalus palustris*), the crested lark (*Galerida cristata*), the snow bunting (*Plectrophenax nivalis*), or even the great reed warbler – still a highly parasitized species (Šulc et al., 2016). In addition, when comparing egg variability with species that are considered unsuitable hosts for the common cuckoo, e.g., common starling (*Sturnus vulgaris*), the European pied flycatcher (*Ficedula hypoleuca*) or the blue tit (*Cyanistes caeruleus*), the analysis shows that parasitized species generally exhibit higher interclutch and lower intraclutch variability than unparasitized species (Øien et al., 1995). Soler & Møller (1996) are consistent with results from Øien et al. (1995), even the shrike is one of the three (*Anthus pratensis* and *Fringilla coelebs*) species with the highest interclutch variability.

The low intraclutch egg variability may make it easier for the host to recognize the parasitic egg, and at the same time with high interclutch variability it is difficult for the cuckoo to mimic the egg perfectly (Freeman, 1988). Stokke et al. (1999) investigated the rejection of artificial cuckoo eggs in relation with intraclutch egg variability in the reed warbler (*Acrocephalus*

*scirpaceus*). The eggs were scored using the same scale as in the study by Øien et al. (1995). Individuals that rejected artificial eggs had lower intraclutch variability (on average 2.69) than individuals that did not reject eggs (on average 3.41). This low intraclutch variability probably helped the tested birds to recognize a foreign egg.

The results show high ability of the shrikes to recognize and reject parasitic eggs: ACEs were rejected in 95% while, ASEs in 100%. Egg rejection has already been tested in several studies with different methodological approaches. Experiments were conducted on a common reed warble – *Acrocephalus scirpaceus* (Davies & Brooke, 1989), the whitethroat (Procházka & Honza, 2003), red-backed shrike (Moskát & Fuisz, 1999) etc.) or occasional hosts (yellowhammer – *Emberiza cintrinella*) (Procházka & Honza, 2004) of the common cuckoo. The apparently high ability to reject parasitic eggs was demonstrated by the great reed warblers, which rejected 61.5% of true cuckoo eggs, 74.2% of artificial cuckoo eggs (synthetic clay) (Lotem et al., 1995). Comparing this with the experiments conducted by Moskát & Hauber (2007), great reed warbler rejected true cuckoo eggs at 75% when a host egg was replaced by a cuckoo egg in a nest, however, when parasitizing a clutch containing 2-5 eggs, the rejection rate decreased to only 37.5%. Both experiments were conducted during the egg laying phase of the nesting. Another species that has demonstrated high rejection ability is the blackcap (*Sylvia atricapilla*) in the study by Honza et al. (2004), where blackcap nests were parasitized by: (1) artificial mimetic cuckoo egg (hard plastic), (2) real non-mimetic egg painted light blue (yellowhammer, house sparrow *Passer domesticus*, cockatiel *Nymphicus hollandicus* and Bourke's parrot *Neophema bourkii*). Artificial cuckoo egg was rejected in 87.5%, real non-mimetic egg in 95.7%. Despite the fact that some cuckoo host species have learned to reject parasitic eggs, a relatively large proportion of hosts (36%) accept the eggs (Soler, 2014). One such species is the meadow pipit. This common host of the common cuckoo shows almost no ability to reject parasitic eggs. Rejection in the meadow pipit was tested in a study by Moksnes et al. (1993), using either mimetic model cuckoo egg or non-mimetic egg (white-willow warble type). Mimetic eggs were rejected in only 4.8% and non-mimetic egg in 8.3%. At the very extreme, swallows were presented four different objects, shape of an egg/star and colour of mimetic/non-mimetic. At the laying phase, the swallows rejected the mimetic egg in only 1%, the non-mimetic egg in 29.6%, the mimetic star in 45.5% and the non-mimetic star in 38%. Thus, swallows showed a high rate of acceptance of foreign objects (Šulc et al., 2022).

To make the experiments more difficult for the red-backed shrikes, we added conspecific shrike eggs in clutches. Shrikes surprisingly showed a high cognitive ability to recognize them

and rejected them in 60%. These results are in accordance with Lovász & Moskát (2004) where shrike rejected 58% of conspecific eggs. Rejection of conspecific eggs has also been tested in the studies mentioned above, but none of the species showed such high cognitive abilities as observed shrikes. Rejection rate of conspecific eggs in blackcap was only 37.1% (Honza et al., 2004), in whitethroat 53,8% (Procházka & Honza, 2003). Great reed warblers rejected conspecific eggs in 35% when a host egg was replaced by a cuckoo egg in a nest, however, when parasitizing a clutch containing 2-5 eggs, the rejection rate decreased to 0% (Moskát & Hauber, 2007). In comparison with Lotem et al. (1995), the great reed warblers reject 78.5% of conspecific eggs painted light brown, and 94% of conspecific eggs painted dark brown. However, these conspecific eggs were painted compared to other conspecific eggs.

Rejection experiments have been tested using eggs made of different materials such as plaster (Rothstein, 1975), hard plastic (Tryjanowski et al., 2021), 3D printed egg (Hauber et al., 2020), polymer clay (Li et al., 2020), or from thermoplastic sheets covered by thin printed film calibrated to bird vision (Šulc et al., 2022). Our eggs were produced according to the methods of Šulc et al. (2022) from thermoplastic with a special film calibrated directly to the bird's vision, which made them significantly more authentic than other egg models. This is also confirmed by my results comparing chromatic contrast (JND) of eggs which showed no significant difference between CSE and ASE, only the ACE seemed to be the most different from the others.

In literature, the value of 1 JND is often given as the limit below which two objects are indistinguishable in good light conditions (Siddiqi et al., 2004; Hauber et al., 2020), but shrike is a species that nests in the darkest places within the bush (Syrová, unpublished data), where light conditions are not ideal. Therefore, it was expected that the JND value is higher for discriminating objects in such nesting species. My results show that the cuckoo egg differed in chromatic contrast from the other two, most of the measured values were below the value of 3 JND, which is a threshold value when it is very difficult to distinguish two objects (Dell'Aglio et al., 2018). Based on the nesting of shrikes in the darkest parts of the bush, the achromatic contrast (or brightness), that birds use to compare objects in areas with poor light conditions, was analysed. No significant differences were found among the three types of parasitic eggs, and most of the measured values remained below the threshold of 3 JND.

In addition, the premise of this thesis was that shrikes, like their close relatives, corvids, rely solely on violet sensitivity and not the UV spectrum. However, shrikes may use UV to some

extent, although this has not yet been tested (Stoddard & Stevens, 2011). The methods of model egg design are still imperfect and it is possible that shrikes recognize the material (plastic) as a clue for rejecting model eggs. Conversely, they have shown high cognitive ability in recognizing even conspecific eggs.

Shrikes have learned to recognize and reject parasitic eggs from its nests, even in the case of conspecific eggs. Its high interclutch and low intraclutch variability is a good counter-adaptation against the cuckoo mimic host eggs. Such egg variability may be difficult for the cuckoo to mimic, and the parasitic egg may be more easily detected and removed from the nest. The combination of these factors may have caused a significant decline in parasitism.

Another possible factor could have been the sudden decline of shrike's population in Europe. Records coming from the 1950-1960s, for example from Britain (Ash, 1970) or the Czech Republic (Holáň, 1993), mainly due to the development of large-scale agriculture (Holáň & Formánek, 1996). However, from the 1980s, populations began to stabilise again, but there was no increase in parasitism. The question is whether the cuckoo will be or is no longer reparasitized the red-backed shrike in the Czech Republic. In 2022 and 2023, parasitization of four nests by cuckoos was observed in Podyjí National Park (none of these tries were successful for the cuckoo), however, according to the appearance of the eggs, the cuckoo line is from a different host species and thus either a confusion of host or an attempt to reparasitize shrike could have occurred.

## **5 Conclusion**

By being able to recognize and reject parasitic eggs, the red-backed shrike has demonstrated high cognitive abilities, even more so when rejecting conspecific eggs. Moreover, the results show that the variability of the eggs of the shrike is low within clutches and high between clutches. Such variability is a suitable adaptation to prevent nest parasitism and may contribute significantly to its decline. Mimicking the host eggs is almost impossible in such a case, and so it appears that the shrike has reached the peak of the arms race with the common cuckoo. The red-backed shrike is a good example of a species that has been abandoned by the cuckoo, not only in the Czech Republic but also in Hungary. There may be several reasons, except high aggression against cuckoo and ability to recognize parasitic eggs, there may be the sudden decline of the shrike's population during the 1950 and 1960 years, populations may have become too small for the cuckoo to be profitable anymore. Not only the effect of population density but also key cues on eggs for recognition may play an important role in decreasing parasitization. Validating the importance of these factors requires further research.

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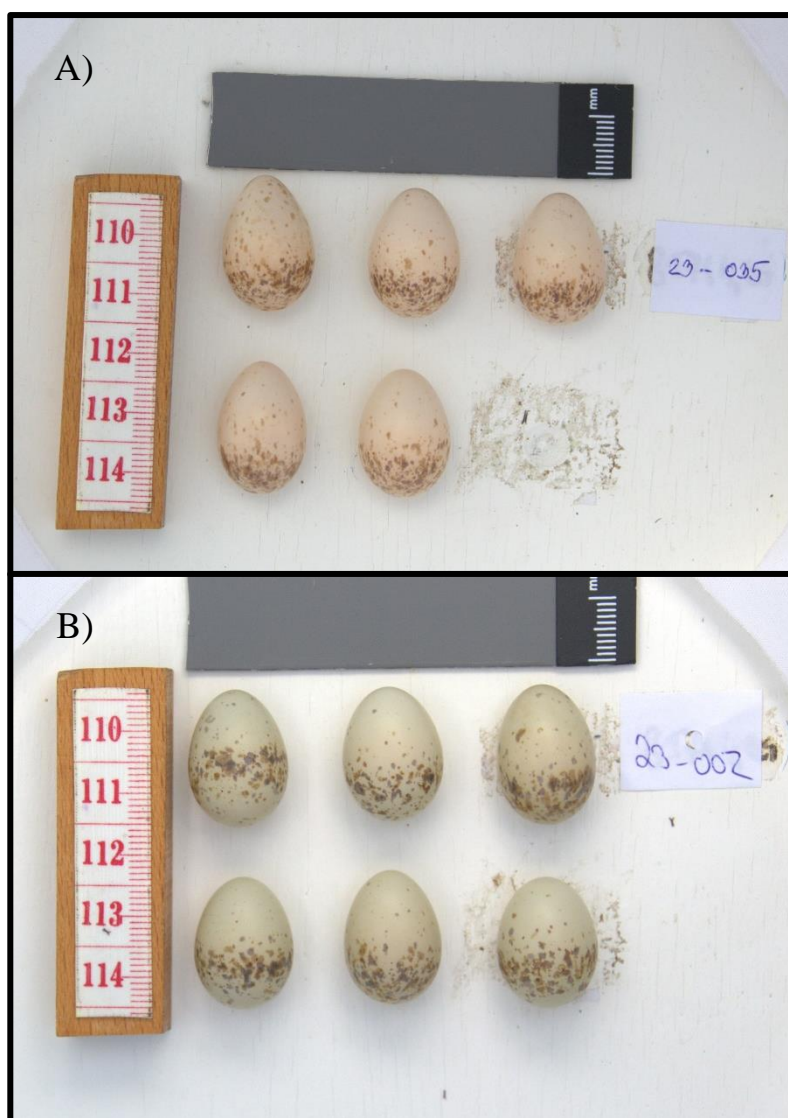
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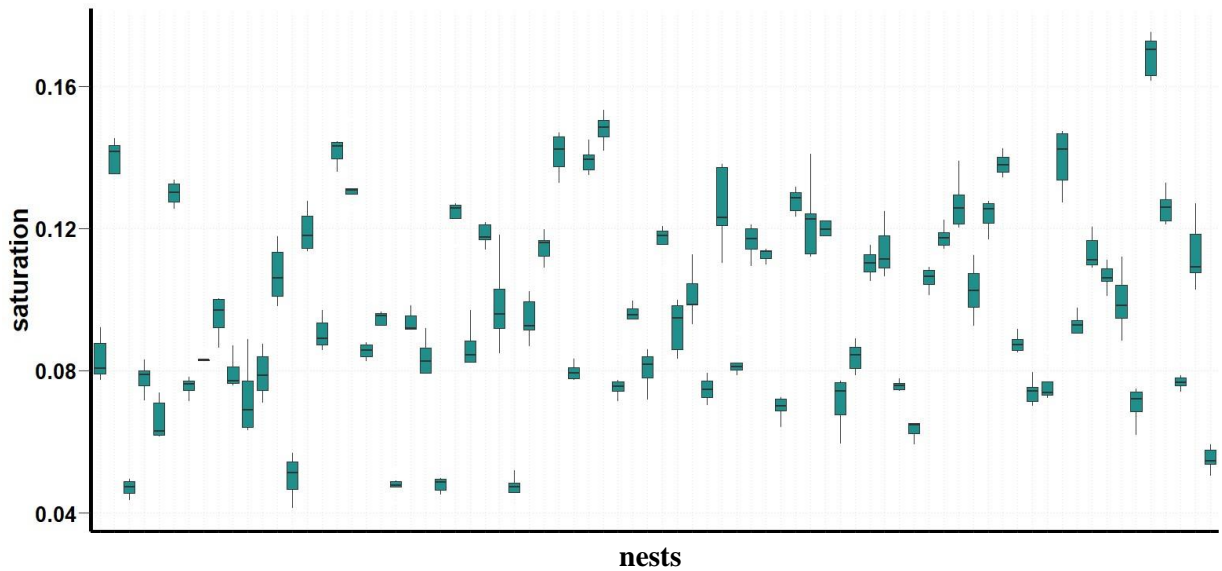
## 7 Supplements



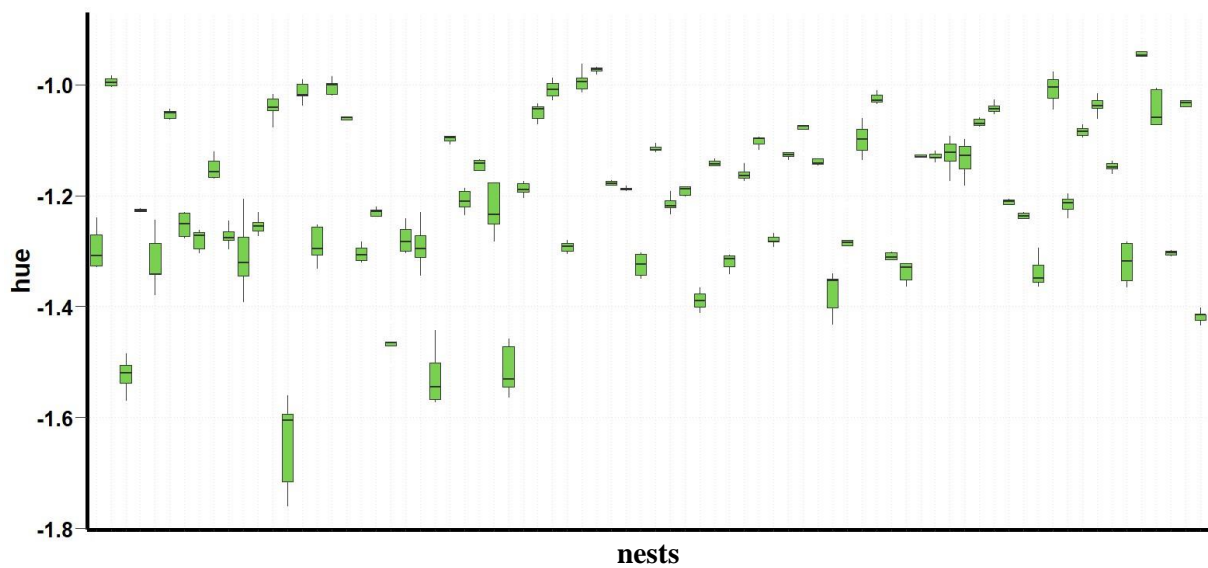
**Fig. S1** – Photos of shrikes clutches on the white board with scale and standard grey scale with known reflectance (18%) (X-Rite Colour Checker Grey Scale Chart). A) – clutch from the nest 23-035; B) – clutch from the nest 23-002.



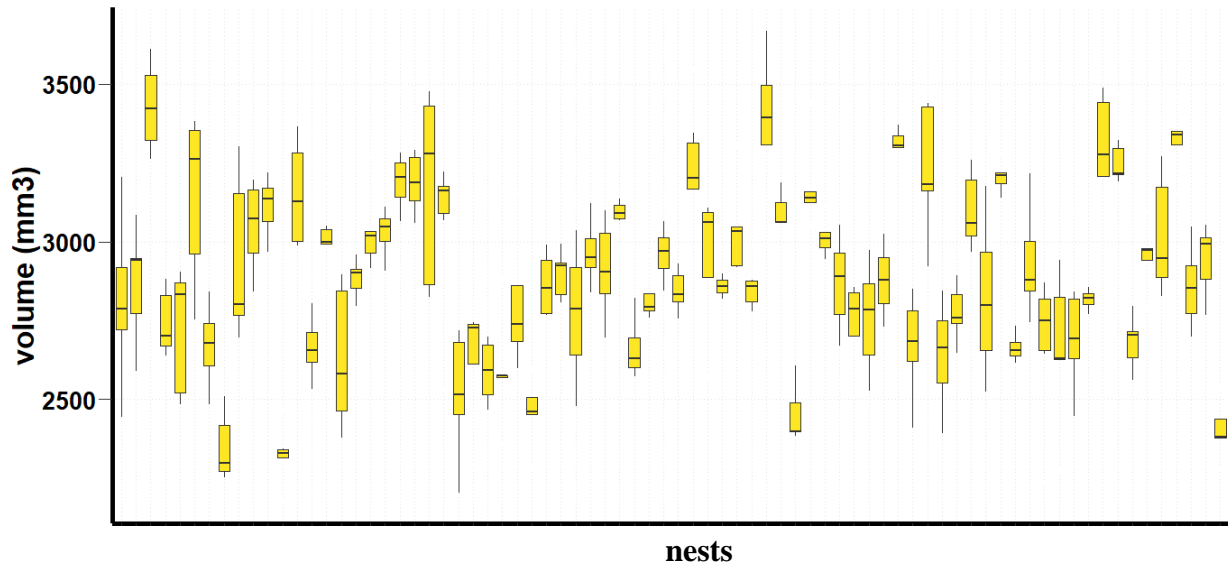
**Fig. S2** – The process of creating artificial cuckoo and artificial shrike eggs. 1. – printed PVC film with two types of egg; 2. – thermoplastic sheet with printed film; 3. - egg moulds; 4. - finished artificial eggs.



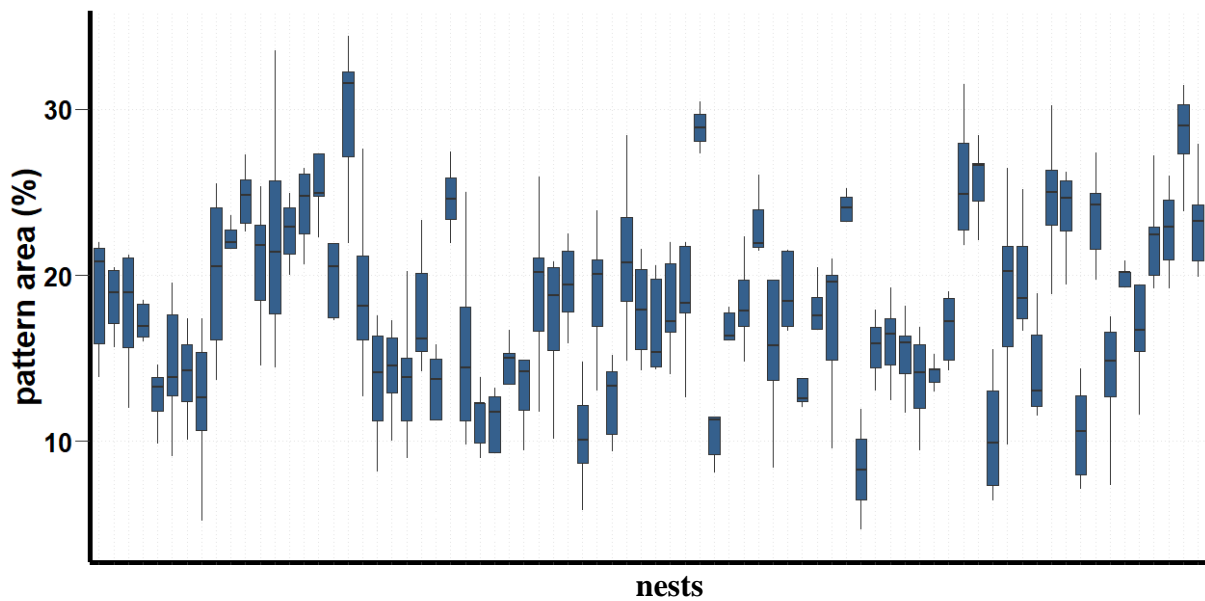
**Fig. S3** - The difference in color saturation between clutches (nests) (N=75). The bold line refers to the median, the bottom and top of the boxes represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.



**Fig. S4** - The difference in color hue between clutches (nests) (N=75). The bold line refers to the median, the bottom and top of the boxes represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.



**Fig. S5** - The difference in volume ( $\text{mm}^3$ ) between clutches (nests) ( $N=75$ ). The bold line refers to the median, the bottom and top of the boxes represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.



**Fig. S6** - The difference in pattern area (% maculation) between clutches (nests) ( $N=75$ ). The bold line refers to the median, the bottom and top of the boxes represents the first and third quartiles, whiskers show 10-90% of data and dots are outliers.