

# Evolution of eggshell structure during rapid range expansion in a passerine bird

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## Summary

**1.** Environmental factors such as temperature, humidity and partial oxygen pressure can affect avian eggshell structure because gas exchange across the shell must allow sufficient water loss while preventing dehydration of the embryo. Studies of species with known chronology of colonization of novel environments provide a powerful insight into the relative importance of ecological factors shaping the evolution of eggshell structure.

**2.** Here, we examined changes in eggshell structure that accompanied rapid range expansion of house finches (*Carpodacus mexicanus*) across North America. We analysed thickness and pore density in eggshells from three ecologically distinct populations: the native desert population in southwestern Arizona and two 30-year-old populations in the northwestern (north-west Montana) and southeastern (south-east Alabama) parts of the species' range. We also conducted cross-foster exchanges of freshly laid eggs within and between the northwestern and southeastern populations to examine consequences of population differences in eggshell structure on embryo development.

**3.** Eggshell structure was most distinct in a recently established population inhabiting higher humidity environment (southeastern Alabama), where eggs were the largest, eggshells the thickest and pore density the lowest. Populations that experienced highly distinct ambient temperatures (southwestern Arizona and northwestern Montana) nevertheless had similar eggshell structure. These results were corroborated by experiments where humidity differences between cross-fostered nests had twice the effect on embryo survival compared to the effect of change in ambient temperature. Correspondingly, experimental egg exchanges between southeastern Alabama and northwestern Montana populations were associated with fourfold increase in embryo mortality compared to within-population egg exchanges.

**4.** We document rapid evolution of eggshell structure in response to colonization of novel environments and establish the relative importance of environmental factors on avian eggshells. We discuss these results in relation to population variation in incubation behaviour and its ability to shield eggshell structure from the selection exerted by novel environments.

**Key-words:** *Carpodacus mexicanus*, eggshell structure, gas exchange, house finch, humidity, incubation, porosity, range expansion, temperature

## Introduction

In oviparous species, eggshells provide protection from the outside environment while allowing heat, water and gas transfer needed for embryonic development (Tullett 1975; Tullett & Deeming 1982; Ar & Rahn 1985; Davis & Ackerman 1987). Environmental factors can strongly affect the evolution of eggshell structure because gas exchange across the shell allows

sufficient water loss while preventing dehydration of the embryo (Ar *et al.* 1974; Rahn *et al.* 1977; Board & Scott 1980; Tullett 1984). Further, behavioural adaptations such as onset and rates of incubation can compensate for environmental variation, patterns likely to be most pronounced in species colonizing novel environments (Lack 1968; Lyon & Montgomerie 1987; Conway & Martin 2000; Martin 2002; Badyaev, Hill & Beck 2003; Martin *et al.* 2007; Stein, Oh & Badyaev 2010). The relative importance of modifications in incubation behaviours and evolution of eggshell structure in birds is a debated issue – some studies showed that incubation behaviours do not significantly modulate embryonic water balance (e.g. Walsberg 1983), such

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that adaptive responses to humidity variation must be accomplished by changes in eggshell structure. Other authors suggested that the rapid evolution of eggshell structure is unlikely (Simkiss 1980; Board 1982) and that effects of novel environment are instead compensated by behavioural modifications of incubation. What is needed, but currently lacking, are direct studies of eggshell structure in species colonizing distinct environments.

Theory of conductivity provides general predictions for eggshell structure. Eggshell pores are small holes that transfer respiratory gases (oxygen, carbon dioxide and water vapour) between the embryo and the outside environment. Amount of exchange can be altered via number of pores per area (pore density), pore length (determined by the thickness of the shell) and total pore area. For example, longer pores decrease the amount of water loss, but also decrease gas exchange, while greater pore area increases both water loss and gas exchange. Thus, if eggshell structure varies with environmental conditions, then species nesting in hot and humid environments should have a greater pore area than similarly sized eggs of species nesting in colder and drier environments, allowing greater water conductance into and out of the egg (e.g. Davis & Ackerman 1985, 1987). Ambient humidity seems to have a particularly strong effect – birds nesting at high altitudes where low ambient humidity co-occurs with low partial oxygen pressure nevertheless have thicker eggshells and longer and more dispersed pores – adaptations preventing water loss despite the need for more oxygen (Ar *et al.* 1974; Packard, Sotherland & Packard 1977; Rahn *et al.* 1977; Carey 1994).

Further, although birds can maintain the temperature of their incubated eggs under wide fluctuations in ambient temperature, the opportunity to compensate for changes in ambient humidity is limited (Walsberg 1980, 1983), leading to the suggestion that ambient humidity is the strongest selection pressure on eggshell structure (Kern & Cowie 2000; Deeming 2002). Both high and low humidity exert selection pressures on eggshell structure – low relative humidity can lead to embryo desiccation, while high humidity increases the risk of mechanical restriction of the embryo, hindering the initiation of pulmonary respiration and restricting the range of movements needed for successful hatching (Simkiss 1980). Indeed, studies of chickens (*Gallus gallus*) showed that experimental increase in eggshell porosity and associated water loss results in low hatching success (e.g. Snyder & Birchard 1982).

Less is known about the effect of ambient humidity on eggshell structure in wild birds. Experimental studies that eliminated the effects of incubation behaviours by artificially incubating eggs under variable humidity conditions showed that, although development can proceed, the highest hatching success is observed when experimental humidity treatment matches the population-specific ambient conditions, highlighting the importance of eggshell structure in mediating water loss of the developing embryo (Carey 1986; Walsberg & Schmidt 1992).

Detailed assessment of eggshell structure in wild birds has been hampered by the lack of methods to nondestructively assess porosity metrics that do not require whole, large and fresh eggshells. Tyler's (1953) method of assessing eggshell

porosity was originally developed for freshly laid whole chicken eggs. This method was successfully used in studies of raptors, large waterfowl and pelagic seabirds, all species with large and robust eggshells. This method, however, cannot be used for eggs of small passerines because small fragments of these eggs dissolve easily when placed in nitric acid, required by the method, even for a short amount of time. Blankespoor (1987) proposed a procedure for counting pores in which whole eggshells were submersed in hot sodium hydroxide (NaOH) followed by a wash in acid fuchsin to reveal location and relative size of pores. While more suitable for smaller passerine eggs than the Tyler method, this procedure still requires whole eggshells. To nondestructively assess eggshell porosity in eggshell fragments for this study, we proposed and tested a technique modified from Tyler (1953).

We studied the eggshell structure in three ecologically distinct populations of the house finch (*Carpodacus mexicanus*). This species is native to hot and dry deserts of western North America, but since 1940s, as a result of introductions and natural expansion, has colonized most of the continent, eventually occupying the widest ecological range of any extant bird species (Hill 1993; Badyaev 2009). The range expansion was accompanied by behavioural adjustments in incubation regime and greatly facilitated by maternal effects on morphology and development (Badyaev 2009). However, it is not known whether this ecological expansion was associated with changes in eggshell structure across newly established populations, particularly at the climatic extremes of species' distribution.

Thus, we examined eggshell structure in a native population in the Sonoran desert (southwestern Arizona) and in two 30-generation-old (i.e. 30 years old) populations at the northwestern (north-west Montana) and southeastern (south-east Alabama) parts of the species range. Finches in these environments experience distinct ecological conditions during egg laying and early incubation: from hot and dry environments in the Arizona population to cold and dry environments in the Montana population to humid and hot environments of the Alabama population. We examined changes in eggshell thickness and pore density across the populations. Additionally, we conducted reciprocal cross-fostering of freshly laid eggs between and within the Montana and Alabama populations. We predicted that as birds are less effective at controlling the effects of ambient humidity vs. temperature on embryo development, humidity will have a dominant effect on both eggshell structure and hatching probability of cross-fostered eggs – eggshells of Montana and Arizona house finches are predicted to have smaller pores and thicker eggshells to better retain water in lower humidity, whilst the birds in the higher humidity environment of Alabama will have thinner eggshells and greater pore area. Moreover, cross-fostering of eggs between environments of different humidity should be associated with greater embryonic mortality compared to exchanges between the environments of different ambient temperature.

We discuss our results in relation to the adaptive significance of eggshell structure, the importance of eggshell structure in relation to behavioural modifications of incubation and selective pressures associated with colonizing novel environments.

## Materials and methods

### STUDY POPULATIONS AND EGG COLLECTION

The three study populations were the native population in southwestern Arizona (altitude 759 m, ambient temperature ( $T$ ): mean  $24.6 \text{ }^\circ\text{C} \pm 0.5$  (SEM), relative humidity (RH):  $33.4\% \pm 1.5$ ,  $n = 146$  days during egg laying in the last 4 years), where finches have bred for at least 10 000 years, and in two recently established populations – in north-western Montana (altitude 978 m,  $T = 6.8 \text{ }^\circ\text{C} \pm 0.4$ , RH =  $51.0\% \pm 1.3$ ,  $n = 178$ ), where this species started breeding in the late 1970s and at the southeastern edge of their introduced range in Alabama (altitude 214 m,  $T = 18.3 \text{ }^\circ\text{C} \pm 0.4$ , RH =  $68.7\% \pm 1.0$ ,  $n = 167$ ), where breeding started in 1983 (Badyaev & Hill 2000). The study sites in Arizona, Montana and Alabama have been maintained since 2002, 1994 and 1993, respectively.

In all three populations, to enable egg collection at appropriate times, the onset of full incubation was determined by daily monitoring of female presence on the nest during egg laying and by obtaining nest temperature data from thermocouples (iButton-TMEX, Dallas Semiconductor and HOBO ProSeries; Onset Computer Corporation, Cape Cod, MA, USA), which were installed in each nest at the time of nest-building and were set to record egg temperature every 5 min (Badyaev *et al.* 2003; Badyaev, Hill & Beck 2003). All females laid one egg per day between 06.30 and 11.00 until the clutch was complete, eggs were numbered sequentially on the day of laying, and in nests where incubation began with the first egg, the first egg was collected on the morning of laying and replaced with an egg from a different house finch nest as a part of egg cross-fostering experiment (below) or with a dummy egg if the replaced egg was used for eggshell analysis. All subsequent eggs in these nests with early onset of full incubation were removed within 18 h after laying and replaced with other eggs (protocol in Young & Badyaev 2004). After the female laid her last egg (fourth or fifth), dummy eggs were either removed to enable rapid renesting or foster eggs were allowed to develop as a part of the cross-fostering experiment. In nests where incubation started with the last or penultimate egg, all eggs were removed simultaneously 18–20 h after the last egg was laid. Immediately after collection, eggs were photographed on a specially designed photostand (Badyaev, Oh & Mui 2006), measured and stored at  $-20 \text{ }^\circ\text{C}$  until further analysis. For this study, we analysed eggshell structure from  $n = 20$  eggs (five clutches) in the Arizona population,  $n = 21$  eggs (five clutches) from the Montana population and  $n = 18$  eggs (five clutches) from the Alabama population. Because nest identity was entered as a covariate in all models (see Statistical Methods, below) and because clutch sizes showed the same range across populations, clutch size was not entered into statistical analyses as a separate covariate. Further details of the dataset are in Badyaev *et al.* (2008).

Four cross-fostering treatments were conducted in 2000–2003: 36 eggs from 10 additional clutches and 32 eggs from nine clutches were transferred between nests within the Montana population and Alabama populations, respectively, and 87 eggs from 19 nests and 49 eggs from 10 nests were transferred from Montana to Alabama and from Alabama to Montana, respectively. All freshly laid cross-fostered eggs were stored at  $4 \text{ }^\circ\text{C}$  for up to 48 h post-laying and then either used to replace eggs within a population or carried in a double-sided cooler container with installed thermoprobes on a commercial aviation flight (approximate transport duration 10 h) to a different population. Eggs that did not survive to hatching were opened on day 15 of incubation and embryo age (in days) was assessed using the atlas of embryonic developmental stages (Hamburger & Hamilton 1951; Bellairs & Osmond 2005).

Daily (24 h average) ambient temperature ( $^\circ\text{C}$ ) and relative humidity (%) were recorded by permanent weather stations at the Missoula

Airport 1.2 km from the Montana population and on the campus of Auburn University in Alabama (Badyaev *et al.* 2003). For each cross-fostered egg for within- and between-population exchanges, we recorded: mean ambient temperature and humidity in the original location during the week following egg laying and mean ambient temperature and humidity during the week following egg-deposition into a foster nest. Nest identity was retained as a covariate in subsequent analyses. For embryo survival analyses, changes in humidity between the origin and foster nest were partitioned into three categories – ‘humidity same’ (within 5% of original location) and ‘increased’ and ‘decreased’ (more than 5% change). Ambient temperature categories were ‘temperature same’ (within  $3 \text{ }^\circ\text{C}$  of original location) and ‘increased’ and ‘decreased’ (more than  $3 \text{ }^\circ\text{C}$  change).

### EGGSHELL PORE ENLARGING AND COUNTING

We developed a procedure for enlarging and counting eggshell pores in small (mean area  $12.87 \text{ mm}^2$ ) fragments of avian eggshells. This procedure consistently produced visible and identifiable pores for easy counting and allows for the use of shell fragments rather than whole eggs, the use of thin or fragile eggs that would otherwise be destroyed by other methods using NaOH and the use of previously collected and frozen or stored eggshell samples, including those left in nests after hatching.

Samples were placed flat on an absorbent surface and allowed to thaw at room temperature. For each fragment and before membrane removal, we measured thickness from five different areas of the fragment using a micrometre (to  $0.001 \text{ mm}$  resolution with an accuracy  $\pm 0.0001 \text{ mm}$ ). To remove membranes and other organic material, we placed fragments in a biopsy cassette with NaOH, which allowed NaOH to permeate the shell while preventing damage from bumping. The biopsy cage was transferred to 40 mL of hot ( $85\text{--}90 \text{ }^\circ\text{C}$ ) 5% NaOH solution for 1 min, with shells removed after each 1-min period and the structural integrity of eggshells and the presence of shell membranes recorded to determine the optimum time of submergence in NaOH.

Fresh fragments of equal size from the same egg were then measured for optimal time (most pores visible without destroying shell) in nitric acid ( $\text{HNO}_3$ ). The nitric acid enlarges the pores and removes any residual organic material on the shell; submersion in distilled water must be done immediately after removal to neutralize the acid and stop the reaction. Fragments were transferred to hot 5% NaOH for 4 min, then rinsed in distilled water and dried in an incubator (Brinsea Octagon 20 Advance) at  $37.6 \text{ }^\circ\text{C}$ , and five thickness measurements were taken again. Once dry, each fragment was placed in a separate biopsy cassette and transferred to 5%  $\text{HNO}_3$ . Fragments were removed from NaOH, and pores were counted at 1 s intervals to determine the optimum time of submergence in nitric acid. Fragments were rinsed with distilled water between nitric acid trials. Pore number was determined by shining a light through the fragment under a dissecting microscope (Leica MZ125, NJ, USA). This procedure was repeated for fragments from three different eggs in each sample. Random fragments from each egg were additionally painted with either acid fuchsin or methylene blue to determine whether dye rendered pores more visible and whether circular holes in shells seen without dye corresponded to actual pores and not other shell artifacts.

The finalized procedure (described below and in Appendix S1) was used to determine pore counts. Twenty shells from 6 to 8 clutches from each population were measured. For each shell, two to three fragments were selected at random. To count the pores, we examined fragments under a dissecting microscope. We placed fragments convex side up and shone a light from underneath until the pores became visible. We took a

digital image of each fragment with a ruler for subsequent pore measurements.

#### EFFECTIVENESS OF PORE ENLARGING METHOD

Membranes were retained on the eggshell fragments for up to 3 min in the 5% NaOH solution; eggshells were either crushed or too delicate for transfer to nitric acid after 5–6 min in the solution. The optimal time for immersion in NaOH (most membrane removal with least damage to eggshell) was 4 min. Pores first became visible after mean = 3 s in 5% nitric acid, with all pores visible after 7 s. Eggshells dissolved or broke after 10 s in the acid. We were able to accurately count pores without dye. Neither acid fuchsin nor methylene blue produced consistent results due to variation in the size and shape of shell fragments; dye often did not evaporate or diffuse through fragments. When methylene blue was applied to larger (>10 mm<sup>2</sup>) fragments, darker spots corresponded to pores visible without dye. Further, it was not possible to successfully apply acid fuchsin to small (<10 mm<sup>2</sup>) eggshell fragments because the dye could not be applied in a thin enough coat. Our recommended protocol for assessing eggshell porosity metrics is in Appendix S1.

#### MEASUREMENTS OF PORE DENSITY

We used SIGMA SCAN 5.0 (SPSS Inc. 2005 Somers, NY, USA) to measure the area of each shell fragment. Pore density was calculated as pore number divided by fragment area. Pore density on fragments, especially those from the acute and obtuse ends of the egg, is an effective method for the assessment of overall pore density for whole eggshells (Peebles & Brake 1987).

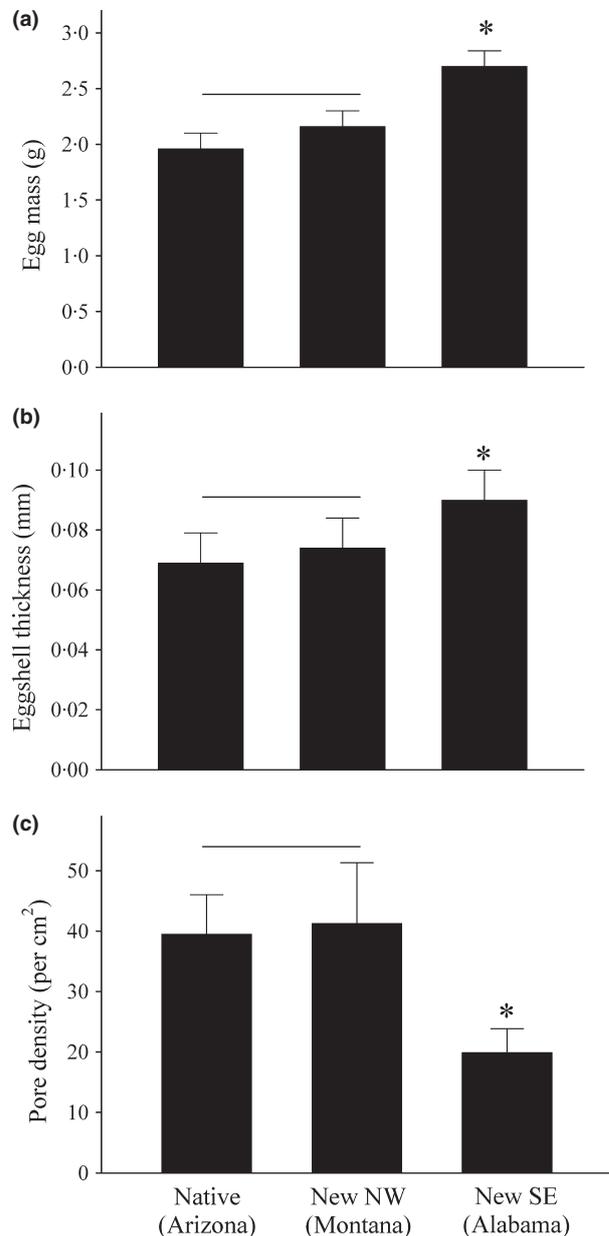
#### STATISTICAL ANALYSIS

Pore density was square root transformed prior to statistical analyses. Population differences in shell thickness and pore density were assessed with a Waller–Duncan multiple comparison test with corrections for uneven samples sizes. We analysed embryo survival of cross-fostered eggs with the log-rank test of equality [for categorical variables, i.e. population and nest identity (that includes year as a category)] (PROC LIFETEST in SAS 9.11 Cary, NC, USA) and with the Cox proportional hazard regression model for continuous variables (i.e. relative humidity and temperature). The full model was assessed with PROC PHREG, and the significance of each of the four treatments (transfer within Alabama, within Montana, from Alabama to Montana and from Montana to Alabama) was assessed by creating a four-level dummy variable with within Alabama transfer as a reference group. Survival functions are presented as Kaplan–Meier curves for treatment categories, and associated hazard ratios  $H$  (interpreted as an effect on survival probability with other parameters in the model kept constant) were tested with  $\chi^2$  tests.

## Results

#### POROSITY AND THICKNESS OF EGGSHELLS ACROSS POPULATIONS

House finches in the recently established Alabama population had larger egg sizes than finches in either the native Arizona population or the recently established Montana population ( $F_{2,48} = 8.04$ ,  $P = 0.001$ ; Fig. 1a). Egg size differences between populations were partially reflected in changes in eggshell structure – eggshells from the Alabama population were thicker than



**Fig. 1.** Population differences in (a) egg mass, (b) eggshell thickness and (c) pore density in native (Arizona) and two recently established populations in northwestern (Montana) and southeastern (Alabama) North America. Line connects means not different with Waller–Duncan  $K$ -ratio test. Asterisk indicates significant difference from other populations at  $\alpha = 0.05$ .

either Arizona or Montana shells ( $F_{2,58} = 10.09$ ,  $P < 0.001$ ; Waller–Duncan  $t = 1.87$ , Fig. 1b) and had lowest relative pore density ( $19.92 \pm 3.94$  pores cm) than either the Arizona ( $39.5 \pm 7.43$ ) or the Montana ( $41.30 \pm 10.42$ ) populations ( $F_{2,32} = 3.27$ ,  $P = 0.05$ ; Waller–Duncan  $t = 2.17$ ; Fig. 1c).

#### POPULATION DIFFERENCES IN COMPONENTS OF EGG SHELL STRUCTURE

Pore density covaried with eggshell thickness in all populations (Fig. 2, overall  $F_{14,49} = 3.25$ ,  $P = 0.02$ ,  $F_{\text{pore density}} = 14.65$ ,

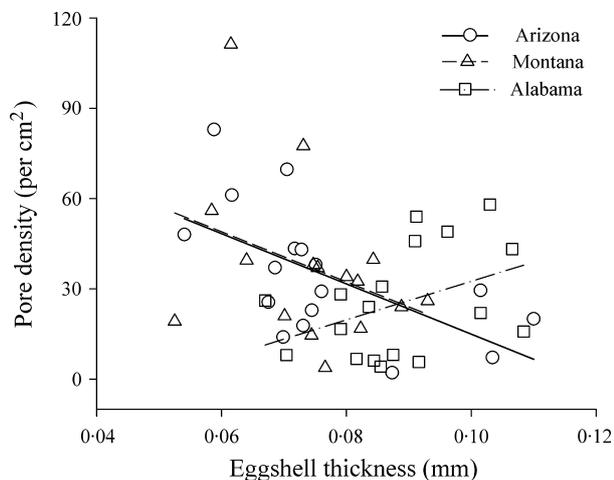


Fig. 2. Eggshell thickness in relation to pore density in the native and recently established populations. Lines are linear partial regressions.

$P < 0.05$ ), but in different directions ( $F_{\text{population}} = 3.22$ ,  $P = 0.05$ ). Pore density decreased with eggshell thickness in the Arizona and Montana populations (standardized regression coefficient (in SD)  $b_{ST} = -0.59$ ,  $t = -2.89$ ,  $P < 0.01$  and  $b_{ST} = -0.34$ ,  $t = -2.09$ ,  $P = 0.04$ , respectively), but increased in the Alabama population ( $b_{ST} = 0.40$ ,  $t = 2.01$ ,  $P = 0.05$ ; Fig. 2). The relationship between pore density and eggshell thickness tended to covary with egg-laying order ( $F = 2.68$ ,  $P = 0.06$ ), but the effect of egg-laying order differed among the populations ( $F_{\text{population} \times \text{laying order}} = 2.84$ ,  $P = 0.02$ ; Fig. 3).

#### SURVIVAL ANALYSIS OF CROSS-FOSTERED EGGS

Within-population transfer of eggs did not affect the survival of embryos in either the Montana ( $H = 0.88$ , i.e. 88% survival compared to control, Fig. 4a) or Alabama populations ( $H = 0.76$ , Fig. 4a). Transfer from the Alabama to Montana population was associated with 3.69-fold increase in mortality (Fig. 4a), while the transfer from the Montana to Alabama population was associated with more than fourfold increase in mortality risk ( $H = 4.18$ , Fig. 4a). Overall, treatment (within vs. among populations and the directions of transfer) was a highly significant predictor of survival probability (Wald's  $\chi^2 = 71.79$ ,  $P < 0.001$ ). Humidity difference between the original and foster nest locations was associated with twofold variation in survival probability ( $H = 2.076$ , Wald's  $\chi^2 = 6.96$ ,  $P = 0.008$ , Fig. 4b), while even large temperature differences between the Montana and Alabama populations did not affect survival probability ( $H = 1.0$ , Wald's  $\chi^2 = 0.09$ ,  $P = 0.76$ , Fig. 4c).

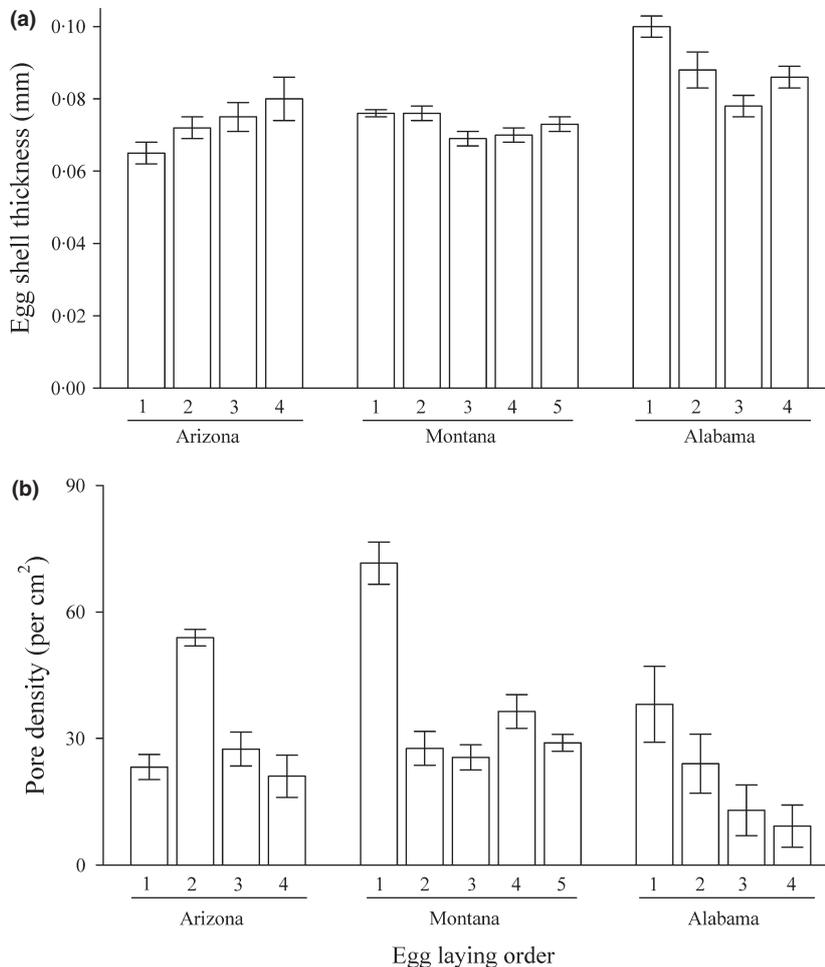
#### Discussion

The eggshell not only protects developing embryos from the environment but also facilitates embryo-environment interactions in the form of heat transfer, water loss and gas exchange (Rahn, Paganelli & Ar 1987). Consequently, eggshell structure is expected to vary across environments that differ in humidity, temperature and oxygen concentration (e.g. Davis, Platterreiger

& Ackerman 1984; Arad, Gavrielilevin & Marder 1988). Indeed, empirical studies often find remarkable plasticity in eggshell architecture in relation to variation in ambient humidity and partial oxygen pressure (Rahn *et al.* 1977; Carey 1994; LeonVelarde, Monge & Carey 1997). However, how rapidly eggshell structure can change within a species in response to colonization of novel environments is unknown. Instead, greater attention has been paid to behavioural adjustments, such as incubation onset and dynamics and associated incubation provisioning (Lyon & Montgomerie 1987; Martin & Ghalambor 1999; Conway & Martin 2000; Martin 2002; Badyaev *et al.* 2003; Martin *et al.* 2007; Stein, Oh & Badyaev 2010). Understanding whether eggshells are subject to selective pressures exerted by novel environments in a species with well-understood chronology of colonization events might shed light on the adaptive significance of variation in eggshell structure and clarify constraints on and relative importance of incubation behaviours during the colonization process.

We documented significant divergence in eggshell structure between the recently established population in Alabama, where finches are breeding in conditions of higher humidity than both the Montana and Arizona populations. The finding that eggshell thickness and pore density are different in the Alabama population (Fig. 1), beyond that expected from variation in egg size (Fig. 2), supports the prediction that humidity is the driving selective pressure on eggshell structure. Because birds are better at behaviourally modifying temperature rather than humidity at their nests, it is likely that such selection is decoupled from behavioural adjustment of incubation. The strong effect of ambient humidity on eggshell structure is further supported by the results of cross-fostering experiments. Differences in relative humidity between original and cross-fostering nests were associated with more than a twofold increase in risk of mortality, while even large changes in temperature did not carry a significant mortality risk (Fig. 4). Pore density in the Alabama population increased with eggshell thickness, while decreasing with thickness in the Arizona and Montana populations (Fig. 2). This finding is expected, as thicker shells increase the length of pores, decreasing the rate of water conductance and overall effective pore area (e.g. Ar *et al.* 1974).

Thicker shells not only increase pore length and decrease conductance but may also increase chances of mechanical restriction of the embryo, particularly in humid environments (Walsberg & Schmidt 1992). However, eggshells in Alabama finches are thicker than in either in the Arizona or Montana populations; this runs counter to the expectation that birds nesting in high humidity environments should show thinning of the shell. To some extent, this is because of larger eggs in house finches nesting in Alabama (Fig. 1), but another potential explanation is the elevated risk of trans-shell bacterial infection. Because the risk of trans-shell bacterial infection is greater under high humidity conditions (Beissinger 1999; Cook *et al.* 2005), thickening of the shell beyond that expected from egg size variation may help offset the increased chances of infection. Thus, greater pore number compensating for decrease in conductance because of thickening of the eggshell, which in turn may be under selection to decrease risk of bacterial infection,



**Fig. 3.** Variation in (a) eggshell thickness and (b) pore density across egg-laying order in  $n = 5$  clutches in the native population in southwestern Arizona and two 30-year-old populations – northwestern Montana and southeastern Alabama. Shown are means  $\pm$  SE.

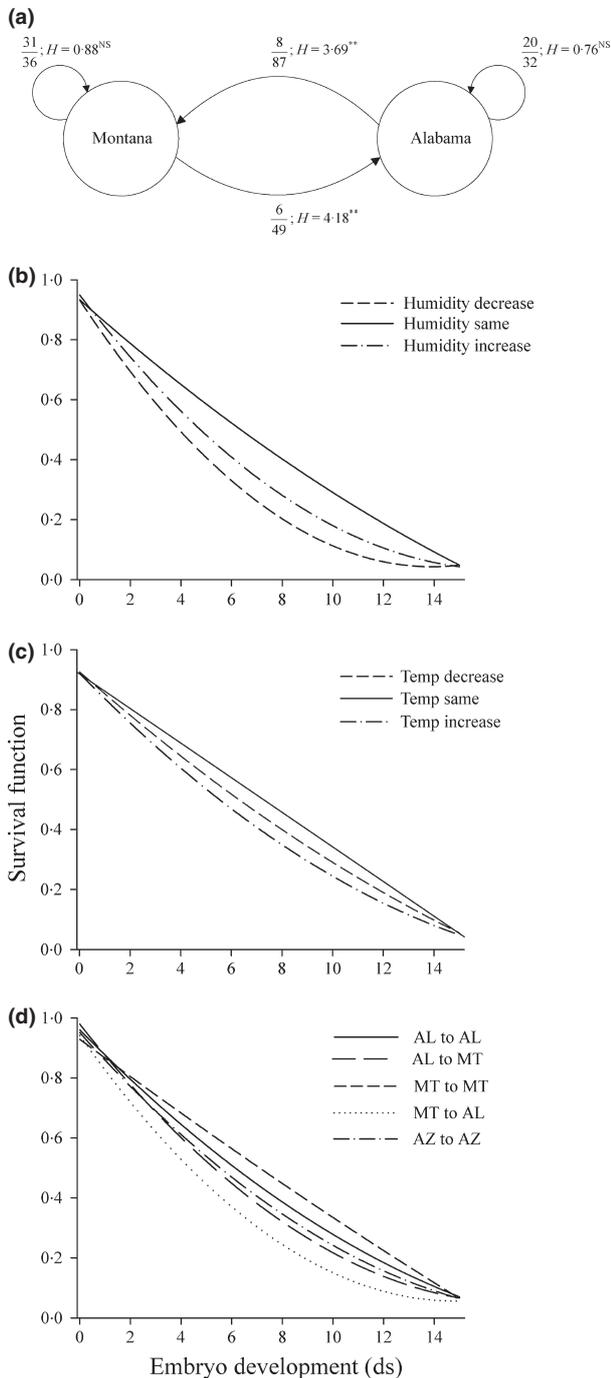
may represent a trade-off associated with this originally desert species breeding in environments with much elevated humidity (>35%) compared to their native range.

Eggshells in the Montana population tended to be thicker than the eggshells in the native Arizona population and had greater pore density but not significantly so. As the Arizona and Montana populations differ primarily in ambient temperature and not relative humidity during breeding, this indicates that even wide variation in ambient temperature can be compensated for by incubation behaviour. In all three populations, incubation behaviour is closely linked to ambient temperature (Badyaev *et al.* 2003; Badyaev & Oh 2008), but the rapid evolution of eggshell structure in the humid environment of Alabama suggests that incubation behaviour does not fully compensate for changes in relative humidity in this recently established population. Interestingly, changes in eggshell porosity had been suggested as a potential mechanism by which incubation length can be adjusted in response to selection (Massaro & Davis 2005; Zimmermann & Hipfner 2007); it is therefore possible that incubation behaviour itself is influenced by changes in eggshell porosity in the Alabama population.

Pore density and shell thickness varied with egg-laying order in all populations, although the specific patterns differed (Fig. 3). The mechanisms behind these patterns require further study. Some researchers have interpreted lower pore density

and thinner eggshell of later laid eggs within a clutch as an adaptation associated with faster embryo development via increase in gas exchange (Mand 1996; Hipfner *et al.* 2001; Massaro & Davis 2004). Equally plausible, however, is that the observed variation is a nonadaptive consequence of within-clutch variation in duration of eggshell formation caused by variable within-clutch inter-ovulation intervals in all the three populations (Badyaev, Oh & Mui 2006).

These results suggest that eggshell structure can evolve rapidly under conditions where behaviour cannot readily modify the microclimate of the nest. Birds colonizing novel environments should show variation in eggshell structure within or between clutches, resulting in selection on optimum structure depending on nesting environment. Proximate mechanisms underlying pore formation are poorly understood, but thought to be influenced by the number and distribution of organic seeding sites during the formation of the shell (Tullett 1975), where unequal distribution causes gaps in mammillae and calcite columns that eventually create pores. Thus, functional changes associated with oogenesis, such as changes in oviduct secretion and absorption of bicarbonate, may influence pore number (Bebout & Hempleman 1994). However, observed changes in eggshell porosity between clutches in response to altitude suggest that birds may be capable of rapid physiological alteration of eggshell structure (Rahn *et al.* 1982; Hempleman, Adamson &



**Fig. 4.** Cross-fostering of freshly laid eggs within and between recently established populations and associated survival probabilities. (a) Number of eggs survived to hatching (numerator) out of total cross-fostered (denominator) and the Cox proportional hazard ratio ( $H$ ) associated with transfer. NS indicates lack of significance, \*\*indicates  $P < 0.01$ . Survival probability associated with egg cross-fostering (b) between environment with lower relative humidity (dashed line), same relative humidity (solid line) and higher relative humidity (dot-and-dash line), (c) between environments with lower ambient temperature (dashed line), same ambient temperature (solid line) and higher ambient temperature (dot-and-dash line). (d) Survival differences associated with egg cross-fostering between and within populations. Lines in (b–d) are the Cox proportional hazard functions. Abscissa is duration of embryo development after transfer and before developmental arrest.

Bebout 1993). Further work on the mechanisms proximately involved in producing modifications in eggshell structure, as well as variation within and between clutches, can provide a better understanding of rapid adaptive evolution of eggshell structure.

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## Supporting Information

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

### Appendix S1. Protocol for enlarging pores on eggshell fragments.

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