RESEARCH ARTICLE

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Hatching asynchrony impacts cognition in male zebra finches

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Abstract

Conditions experienced early in life can shape brain development and later cognition. Altricial songbirds are particularly vulnerable to early environmental perturbations. Research on "Developmental Stress" in songbirds has addressed how early-life conditions may impair song learning and has been extended to consider other components of adult phenotype. Early-life challenges ranging from ectoparasites to competition with siblings have been shown to compromise song learning and other measures of cognition, as well as behavioral strategy. Here, we examined both the effects of hatching asynchrony and early-life immune system challenge with lipopolysaccharide (LPS) on neophobia, song learning, motoric learning, and spatial cognition in male zebra finches (*Taeniopygia guttata*). We found that hatch order had a significant impact on motoric and spatial learning, such that later hatched males performed better than first and second hatched birds. In contrast, LPS treatment only impacted motoric learning and neither hatch order nor immune system challenge impacted song quality, song learning accuracy, or neophobia. These results are consistent with a growing body of evidence that conditions early in life can improve cognitive performance at adulthood. Moreover, these findings indicate that hatch order is an important factor to consider in developmental studies in asynchronously hatching birds.

1 | INTRODUCTION

The effects of early-life conditions on brain development and cognition have been studied intensively in songbirds (Buchanan, Grindstaff, & Pravosudov, 2013; MacDougall-Shackleton, & Spencer, 2012; Nowicki, Searcy, & Peters, 2002; Peters, Searcy, & Nowicki, 2014). Nestling birds rely upon their parents for food, generating variation in their early nutrition and making studies of early-life diet ecologically relevant (Ricklefs, 1983). Additionally, nestling birds are vulnerable to environmental perturbations ranging from ectoparasites to temperature fluctuations (Ricklefs, 1983). Such perturbations were initially shown to impact song learning in studies of the "Developmental Stress" hypothesis and subsequently have been shown to have consequences for brain development, physiology, and cognition, although not all perturbations cause impairments (Buchanan et al., 2013; MacDougall-Shackleton, & Spencer, 2012; Nowicki et al., 2002; Peters et al., 2014; Schoech, Rensel, & Heiss, 2011). Cognition has consequences for fitness, making it essential to understand how early-life conditions could program this trait for later life (Boogert, Fawcett, & Lefebvre, 2011; Brust, Krüger, Naguib, & Krause, 2014; Cole & Quinn, 2012; Cole, Morand-Ferron, Hinks, & Quinn, 2012).

Nestling songbirds not only depend upon parents for food but must also compete with siblings for the resources that parents bring back to the nest (Cotton, Wright, Kacelnik, & Ricklefs, 1999; Glassey & Forbes, 2002; Ricklefs, 1993). Manipulations of brood size in birds have found significant impacts on cognition, song learning, physiology, and fitness (Holveck, de, Lachlan, ten, & Riebel, 2008; Naguib, Riebel, Marzal, & Gil, 2004; Riebel, Spierings, Holveck, & Verhulst, 2012; Spencer et al., 2005b; Tschirren, Rutstein, Postma, Mariette, & Griffith, 2009), possibly as a result of compensatory growth once fledglings leave the nest (Metcalfe & Monaghan, 2001). Like brood size, hatching asynchrony and resulting size hierarchy of nestlings can influence nestling competition and alter physiology, behavior, and growth strategies (Cotton et al., 1999; Mainwaring & Hartley, 2013; Mainwaring, Hartley, Gilby, & Griffith, 2010; Nilsson & Svensson, 1996; Ricklefs, 1993; Rokka, Pihlaja, Siitari, & Soulsbury, 2014). For example, later hatched nestling barn swallows (*Hirundo rustica*) are lighter than their siblings and also have higher circulating concentrations of immunoglobulins in their blood (Saino, Galeotti, Sacchi, & Møller, 1997). Similarly, later hatched ring dove (*Streptopilia capicola*) nestlings have higher baseline levels of corticosterone, the primary glucocorticoid in birds, than earlier hatched nestlings (Eraud, Trouvé, Dano, Chastel, & Faivre, 2008). Further, experimental manipulations of size hierarchy in songbirds and studies of brood parasites demonstrate that smaller nestlings within a brood have different behavioral patterns and growth strategies, such that later

hatched nestlings grow faster presumably to be prepared for dispersal (Cotton et al., 1999; Hauber, 2003; Nilsson & Svensson, 1996). Collectively, evidence that hatching asynchrony impacts nestling growth and physiology suggest that, like other early-life challenges, hatch order could impact song learning and cognition at adulthood (Peters et al., 2014).

In addition to nutritional conditions, parasites can also impact developing songbirds' physiology, song learning, and cognition (Grindstaff, 2016). Studies of early-life parasitic infection have found impaired song learning (Bischoff, Tschirren, & Richner, 2009; Buchanan, Catchpole, Lewis, & Lodge, 1999; Spencer, Buchanan, Leitner, Goldsmith, & Catchpole, 2005a), while a study of immune system challenge with lipopolysaccharide (LPS) reported that immune system activation compromised adult performance in a novel foraging task (Grindstaff, Hunsaker, & Cox, 2012). Thus, as has been demonstrated in other taxa, early-life infection or immune system activation can have consequences for cognitive performance in birds (Bilbo & Schwarz, 2009).

Collectively, both hatch order and early-life immune system challenge have potential to impact later cognition. In the present study, we tested the hypotheses that both immune system challenge and hatch order impact behavioral phenotype (neophobia), song learning, motoric learning, and spatial cognition in zebra finches by assigning juvenile males of different hatch order status to either complete control, vehicle control (phosphate buffered saline [PBS]), or LPS injection treatments. Then, at adulthood, we compared subjects using three behavioral assays, and measures of both the quality and accuracy with which they learned their songs (Table 1). We predicted that later hatched birds would experience greater competition with siblings and this early-life challenge would lead to greater neophobia, a measure of behavioral phenotype that can be associated with cognitive performance (Boogert, Reader, & Laland, 2006; Carere & Locurto, 2011). Further, we expected later hatched birds would show impaired cognitive performance and song learning based on the assumption that later hatched nestlings face greater challenge and experience higher glucocorticoid levels (Eraud et al., 2008; Spencer & Verhulst, 2007; Spencer, Buchanan, Goldsmith a., & Catchpole, 2003). Additionally, we expected LPS treatment to compromise cognitive performance and song learning and increase neophobia based on prior studies (Grindstaff, 2008, 2016). We chose to complete this work in zebra finches because they exhibit hatching asynchrony, resulting in a size hierarchy which may place later hatched nestlings at a competitive disadvantage (Mainwaring et al., 2010; Rutkowska & Cichon, 2005; Zann, 1994, 1996). We chose to treat males with LPS twice during development, once during the sensory phase of song learning (day 30 posthatch) and once during the sensory-motor phase (day 50 posthatch; Immelmann, 1969; Table 1), because interruptions of either early memorization of tutor song, or the practice of song production, could result in impaired song at adulthood. We chose LPS to induce an immune system challenge because it produces a well-characterized inflammatory and sickness response (Grindstaff, 2008; Hart, 1988; Wang, Rousset, Hagberg, & Mallard, 2006).

2 | METHODS

2.1 | Subjects and housing

All methods were approved by the Virginia Tech Institutional Animal Care and Use Committee (BIOL15-015). Study subjects were reared by 11 zebra finch pairs housed in divided flight cages (48 × 47 × 52 cm; Prevue Pet Products, Chicago, IL). Birds were supplied with ad libitum access to finch seed mix (Kaytee Milwaukee, WI), water, grit, and cuttlebone and maintained on a 14:10 light–dark cycle throughout the study. Once laying began, nest boxes were checked daily for eggs, and the date each new egg was found was recorded. Upon hatching, young were uniquely color marked with a nontoxic marker (Crayola Easton, PA) for individual identification and in order to keep track of nestling hatch order. Birds received uniquely numbered and colored leg bands upon fledging. For the purposes of data analysis nestlings were categorized as either first hatched (n = 10), second hatched (n = 9), and third or later hatched (n = 12). All nestlings hatched third or later were combined into one group due to smaller sample size and increasing synchrony of hatching later in a clutch. Because the experimental timeline required the identification of males before molt into sex-specific adult plumage, once nestlings had fledged (day 20–22 posthatch) approximately 10 μL of blood was collected from the brachial vein for genotyping. The DNA was extracted using a Qaigen DNEasy kit (Qiagen INC, Valencia, CA) and sex determination was made by amplification of P2 and P8 sex-linked CHD genes following Griffiths, Double, Orr, and Dawson (1998). Young were weighed to the nearest 0.01 g on day 10 posthatch. On day 35 posthatch, juvenile males were separated from their family and moved to group housing with a genetically unrelated tutor male. Two males per treatment group were assigned to a tutor for a total of six unrelated juveniles per tutor. Siblings were never assigned to the same tutor.

2.2 | LPS treatment

Once males were identified, they were assigned to one of three immune challenge treatment groups: LPS (n = 11), vehicle (sterilized PBS; n = 10), or complete control (no injection; n = 10). Sibling males were assigned to different treatments and hatch order was balanced across treatments. On day 30, during the sensory phase of song learning, juvenile males received either an initial injection of 1 mg/kg of LPS derived from *Escherichia coli* (Sigma L3012, St. Louis, MO) in 40 μL of a 1:1 solution of sterile PBS and presterilized Freund's Incomplete Adjuvant (Sigma F5506, St. Louis, MO; Grindstaff et al., 2012; Owen-Ashley, Turner, Hahn, & Wingfield, 2006), 40 μL filter-sterilized PBS, or received no injection. All injections were done subcutaneously near the keel, after cleansing the skin with ethanol. A secondary injection was administered on day 50, during the sensory-motor phase of song learning.

2.3 | Cognition and neophobia assays

Motoric and spatial learning tasks were adapted from assays designed specifically for zebra finches (Boogert, Giraldeau, & Lefebvre, 2008;

TABLE 1 Experimental timeline

Days Posthatch	30	35	40	50	60	100
Developmental Stage	<---Sensory phase (0–60 days)----------------------------					Crystalized song produced
	|---Sensory-motor phase (30–90 days)--|				
Event	First injection	Moved to group housing	Motoric learning task	Second injection	Spatial memory task	Song recording Neophobia assay

Hodgson et al., 2007). Motoric learning was tested on day 40 posthatch, spatial cognition was tested on day 60 posthatch, and neophobia was assayed on day 100 posthatch (Table 1). This testing timeline was designed to evaluate as many cognitive metrics as possible while avoiding any confounding effects of acute response to LPS treatment (i.e., birds were tested 5 days after treatments). For all trials, birds were moved from group housing 24 hr before testing began and were individually housed in a custom built cage (50 cm × 38 cm × 30 cm) placed inside a sound attenuation chamber (IAC minibooths, Hillside, IL). Birds were always fasted for 6 hr before any cognition or behavioral testing and a maximum of 12, 2-min trials were conducted per day, with 6 minutes between consecutive trials.

For the motoric task, two 2.5 cm × 2.5 cm × 2 cm blocks with a center hole of 1.2 cm diameter and 1 cm depth were placed in each subject's cage to allow the birds to habituate to the testing apparatus for 24 hr. During trials, the holes were covered with lids consisting of a blue plastic disc (diameter 1.5 cm) with a rubber bumper affixed to one side to weigh the lid down. The motoric learning task consisted of a shaping procedure in which birds were trained to pull the lids off of the wells in order to retrieve a food reward. This was done at three levels: level 1, with the lid next to the well; level 2, with the lid half-covering the well; and level 3, with the lid covering the well. Birds advanced to the next level when they successfully completed three consecutive trials at each level. The total number of trials required for each bird to successfully complete all three levels of the motoric learning task was summed across days. One bird from the PBS vehicle treatment group was removed from the study after failing to learn the motoric task after 5 days (60 trials). Birds were returned to group housing with their tutor after completing the motoric learning task (a maximum of 3 days).

On day 58 posthatch, 48 hr before spatial testing began, birds were again moved to individual housing for a four corner spatial learning task (modified from Hodgson et al., 2007). For the spatial task, birds' preferences for specific corners of their testing cages were first determined and then their ability to learn and remember the location of a food reward hidden in an unpreferred corner was assessed. To test for corner preferences, on day 59 posthatch, four blocks were baited with food, the wells covered with lids, and the blocks placed in the corners of the cage for 10 consecutive trials. The corners that were visited first, the most often, and the least were not chosen to be baited during the spatial learning task. During the spatial task a specific corner of the cage was selected to be baited in every trial, requiring the bird to remember the location of the food reward. A bird passed a trial if the first well it uncovered was the well containing the food reward. If the bird failed the trial, the bird was given until the end of the 2-min trial period to visit other corners in order to find the baited corner. A bird passed the spatial learning task when it visited the baited corner first

in six of seven consecutive trials. The number of trials required to finish the spatial learning task was summed across days. No birds required greater than the predetermined maximum of 60 total trials (5 days) to complete the spatial assay.

In the neophobia assay, which has been interpreted as a measure of habituation (Roth, LaDage, & Pravosudov, 2010) as well as behavioral phenotype (Carere, Drent, Privitera, Koolhaas, & Groothuis, 2005), birds were presented with a novel food cup in the center of their cage and the latency in seconds for a bird to eat from the novel cup was recorded. "Novel" cups were 5 cm in diameter, spray-painted yellow, and had three brightly colored plastic disks (2.5 cm diameter red, blue, and green) affixed to the sides; birds had previously been exposed to larger colored food cups but had most recently been fed from large white food cups. After a 5 min time out, birds were presented with their familiar cup, again placed in the middle of the cage, and the latency to eat was recorded. The difference in time to eat from the novel cup relative to the familiar cup was used as a measure of neophobia. All trials were video recorded and food cups were removed immediately after a bird began to eat. Birds were returned to their tutor flocks as soon as this one-trial assay was completed.

2.4 | Song recordings and scoring

Recordings of male subjects were made after mature song was produced around day 100 posthatch and the songs of each male tutor were recorded at the end of the study. All recordings were made by transferring birds individually to custom cages within sound attenuation chambers equipped with a Shure S57 cardiod microphone (Radioshack, Fort Worth, TX) connected to an AudioBox 1818VSL mixer (Prosonus, Baton Rouge, LA) run by a laptop computer (Dell Latitude 3440, Round Rock, TX). The computer was running Sound Analysis Pro software set to automatically record and store songs (Tchernichovski et al., 2000; Tchernichovski, & Mitra, 2001). We failed to obtain song recordings from two males from the LPS group (recorded $n = 9$), one male from the vehicle control group (recorded $n = 9$), and two males from the complete control group (recorded $n = 8$) because they did not sing during the recording period. After recording at least 10 songs from each remaining male, we used Syrinx software (Syrinx Software, V2.6h; J. Burt, syrinxpc.com) to generate spectrograms of each recording. One motif per recording was randomly selected and three measurements of song quality were made. Duration was measured to the nearest tenth of a second and the total number of unique syllables (a measure of complexity; Airey & DeVoogd, 2000) was quantified. Both measures were averaged across the 10 song motifs from each male (Fig. 1; Airey, & DeVoogd, 2000). Finally, the motifs of each male subject were compared to 10 motifs
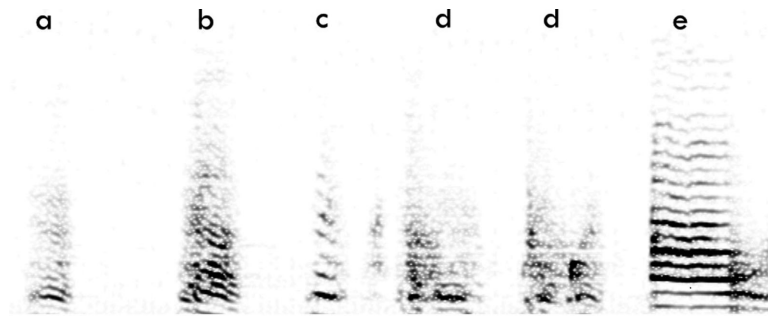
FIGURE 1 Example spectrogram of a song motif. Unique syllables are labeled with a single letter. In this motif, there are five unique syllable types and six total syllables

from his tutor to assess the accuracy of song learning, using the asymmetric comparison module in Sound Analysis Pro (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). Before analysis, all motifs were amplitude normalized using Audacity software (Audacity Team, http://www.audacityteam.org/). The amplitude in Sound Analysis Pro was set at 49 dB and all other settings were kept at default.

2.5 | Verification of LPS injections

To ensure that LPS injections were effective in causing an immune response, six adult male zebra finches were injected with LPS ($n = 3$) or PBS ($n = 3$) as described previously and sickness behavior was measured by an observer blind to treatment following Moyers, Kosarski, Adelman, and Hawley (2015). Briefly, birds were housed individually in custom-built cages within sound attenuation chambers and behavior was video recorded for 30 min using a Logitech web camera (Logitech, Newark, CA), beginning 8 hr after injection. Each bird was assigned one of five behaviors every 30 sec during the 30-min period (for a total of 60 time points): immobile, hopping, eating, preening, and drinking (Moyers et al., 2015). If a bird performed more than one behavior in the 30-sec period, the period would be coded as the behavior the bird spent a greater amount of time doing.

2.6 | Statistics

All response variables were checked for normality before analyses using Kolmogorov–Smirnov tests and normal probability plots, and log transformed when appropriate. Effects of hatch order and immune challenge treatment were included as fixed factors in all models, but we excluded the interaction between these terms due to small sample sizes within treatment combinations. In all models, we also included family as a random factor to account for the nonindependence of hatchlings from the same nest. Separate generalized linear models with a Poisson distribution and a log link function were used to assess performance in the motoric and spatial tasks (which were quantified as counts). When a fixed effect was statistically significant, we ran pairwise comparisons among groups to determine which groups differed significantly. Our neophobia measure, as well as all song performance measures, was log10 transformed and analysis of variance (ANOVAs) ANOVAs were used in these cases. We also examined the effects of hatch order (as a fixed factor) and family (as a random factor) on day

TABLE 2 Summary of the main effects of immune challenge treatment and hatch order on cognitive measures

Model	β	SE	χ^2	df	P
Motoric task					
Immune challenge treatment	−0.242	0.161	11.810	2	0.003
Hatch order	0.220	0.114	6.413	2	0.040
Family (random factor)	1.379	0.354	71.531	10	<0.001
Spatial task					
Immune challenge treatment	0.244	0.122	4.160	2	0.125
Hatch order	0.393	0.099	17.356	2	<0.001
Family (random factor)	−0.574	0.274	36.926	10	<0.001

Results from generalized linear models of count data using a Poisson distribution and log link function.

10 mass with an ANOVA. The immune challenge treatment was not included in this model because it did not occur until day 30. In the case of ANOVAs, we ran Tukey's post hoc tests to determine which groups differed significantly.

3 | RESULTS

We found three main effects of hatch order and one of immune challenge treatment (Tables 2 and 3). Specifically, we found that performance in the motoric task was influenced by both hatch order ($\chi^2 = 6.413$, df $= 2$, $P = 0.040$) and immune challenge treatment ($\chi^2 = 11.810$, df $= 2$, $P = 0.003$), while performance in the spatial task was related only to hatch order ($\chi^2 = 15.329$, df $= 2$, $P < 0.001$). Specifically, third or later hatched birds performed better than first or second hatched subjects in both the motoric (pairwise comparison, both $P \leq 0.05$, Fig. 2) and spatial tasks (pairwise comparison, $P \leq 0.003$; Fig. 3). Individuals who received the LPS injection did better on the motoric task than individuals that received the PBS injection ($P < 0.001$) and tended to do better than the total control group ($P = 0.11$; Fig. 4). All measures of song performance and neophobia were unrelated to the immune challenge treatment and hatch order (Table 3; Supplementary Table S1). Mass at 10 days of age was significantly influenced by hatch order ($F_{2, 17} = 4.390$, $P = 0.029$); first hatched nestlings were significantly lighter than those that hatched later (Tukey's post hoc test, both $P \leq 0.015$; Fig. 5) but second hatched nestlings did not differ from those hatched third or later ($P = 0.328$). Finally, as expected (e.g., Moyers

TABLE 3 Summary of the main effects of immune challenge treatment and hatch order on cognitive measures

Model	F	df	P
Unique syllables			
Immune challenge treatment	0.928	2, 14	0.418
Hatch order	0.525	2, 14	0.603
Family (random factor)	0.520	7, 14	0.805
Motif duration			
Immune challenge treatment	1.126	2, 12	0.352
Hatch order	0.445	2, 14	0.649
Family (random factor)	2.710	7, 14	0.053
% Similarity with tutor			
Immune challenge treatment	0.076	2, 9	0.927
Hatch Order	0.527	2, 9	0.607
Family (random factor)	0.395	7, 9	0.883
Tutor	0.696	5, 9	0.640
Neophobia			
Immune challenge treatment	0.484	2, 11	0.629
hatch order	0.003	2, 11	0.997
Family (random factor)	7.030	10, 11	0.002

ANOVA results for models with data that were normally distributed or log transformed to improve normality.

et al., 2015; Owen-Ashley et al., 2006), birds treated with LPS in the separate verification test spent significantly more time immobile than did PBS treated birds 8 hr after treatment ($t = 6.225$, $P = 0.006$).

4 | DISCUSSION

The goal of this research was to determine how hatching asynchrony and immune system challenge shape future behavior and cognition by examining their impacts on song quality and learning accuracy, neophobia, motoric learning, and spatial cognition. Later hatched nestlings could experience stressors including competition and resource limitation, resulting in altered growth strategies (Cotton et al., 1999; Eraud et al., 2008; Nilsson & Svensson, 1996). Although several studies have examined the impact of brood size on phenotypic outcomes because larger broods can induce altered growth patterns (Naguib et al., 2004), the impact of hatching asynchrony on cognition has not been as well described. Similarly, although several studies have addressed the effects of parasitic infection on later cognition (Bischoff et al., 2009; Buchanan et al., 1999; Spencer et al., 2005a), fewer studies have attempted to disentangle the effects of infection from those of immune system response (Grindstaff, 2016; Grindstaff et al., 2012).

Collectively, our findings suggest that hatch order could program cognition in a way that prepares an individual to be successful in adulthood, possibly as a component of a broader phenotypic strategy previously proposed in later-hatched nestlings (Cotton et al., 1999; Nilsson & Svensson, 1996; Ricklefs, 1993). Although the impact of hatch order effects on fitness will depend upon ecological factors such as resource availability, our findings are consistent with later hatched males having cognitive traits that may prepare them for future competition. Specifically, we found that males that were hatched later in a clutch were heavier than early hatched males of the same age (Fig. 5), and performed better on the motoric and spatial tasks, which could improve foraging success in the future (Table 2; Figs. 2 and 3). These findings are consistent with other studies of captive zebra finches showing faster growth of later hatched birds (Mainwaring, Blount, & Hartley, 2012; Rutkowska & Cichon, 2005; Skagen, 1988). Although we found no effect of hatch order on neophobia, another study reported that later hatched birds did explore a novel environment, although not a novel object, more quickly (Mainwaring & Hartley, 2013; Rokka et al., 2014). Thus, our results together with prior studies raise the hypothesis that later hatched zebra finch males grow more quickly and develop a behavioral strategy of enhanced motoric and spatial learning that could facilitate foraging efficiency in adulthood. Whether our finding of improved motoric and spatial performance results from factors such as greater social enrichment from older siblings or as part of "Environment Phenotype Matching,"
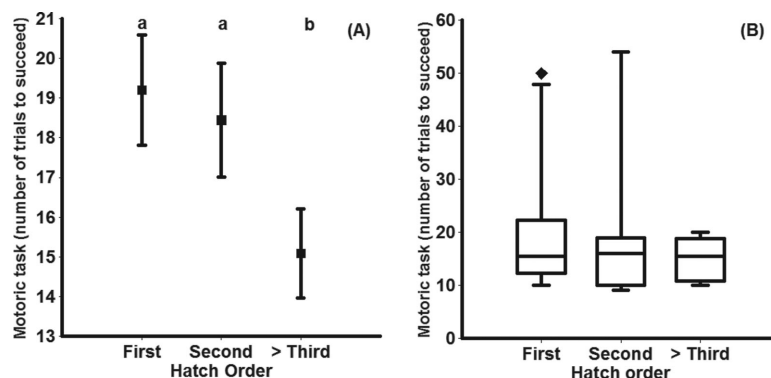

FIGURE 2 Effect of hatch order on the number of trials required to complete the motoric task, in which birds were trained to remove lids covering wells to obtain a food reward. (A) The estimated marginal mean number of trials (±standard error) required to complete the task where significant differences are denoted by different letters above bars while bars that share the same letter do not differ significantly. (B) Boxplot of the data depicting the median number of trials with boxes extending to the 25th and 75th percentiles, whiskers extending to 90th and 10th percentiles, and outliers plotted as points. Birds hatched third or later required significantly fewer trials to complete the motoric task; fewer trials reflect superior performance
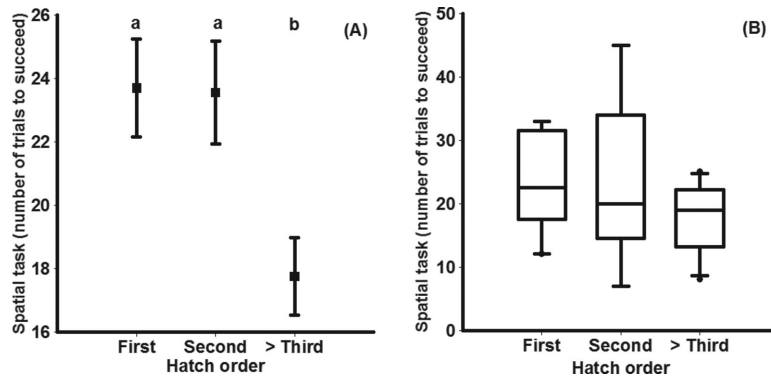
FIGURE 3 Effect of hatch order on the number of trials required to complete the spatial task, in which birds were trained to remember the location of a food reward. (A) The estimated marginal mean number of trials (±standard error) required to complete the task where significant differences are denoted by different letters above bars while bars that share the same letter do not differ significantly. (B) Boxplot of the data depicting the median number of trials with boxes extending to 25th and 75th percentiles, whiskers extending to 90th and 10th percentiles, and outliers plotted as points. Birds hatched third or later required significantly fewer trials to complete the spatial task; fewer trials reflect superior performance
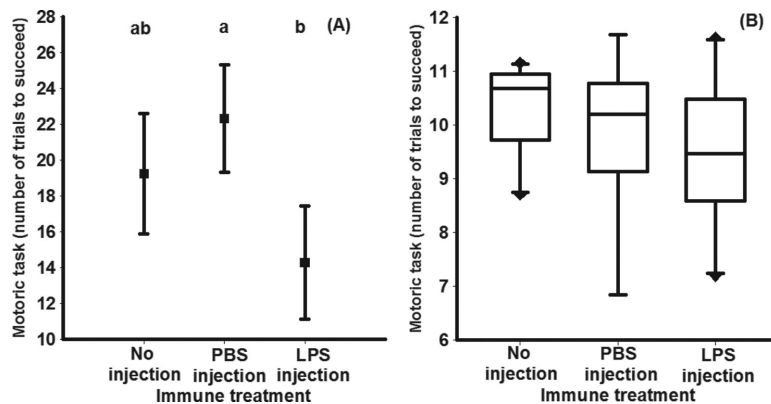

FIGURE 4 Effect of immune system challenge treatment on the number of trials required to complete the motoric task, in which birds were trained to remove lids covering wells to obtain a food reward. (A) The estimated marginal mean number of trials (±standard error) required to complete the task where significant differences are denoted by different letters above bars while bars that share the same letter do not differ significantly. (B) Box plot depicting the median number of trials with boxes extending to 25th and 75th percentiles, whiskers extending to 90th and 10th percentiles, and outliers plotted as points. Birds treated with LPS required significantly fewer trials to complete the motoric task than birds from either control group; fewer trials reflect superior performance

which is the hypothesis that stressors early in life may program animals so they can cope with the environments into which they are born (Chaby, Sheriff, Hirrlinger, & Braithwaite, 2015; Monaghan, 2008; Zimmer, Boogert, & Spencer, 2013), remains to be determined. However, the present findings join an increasing number of studies demonstrating that early-life stressors can improve performance in some tasks (Buchanan et al., 2013; Crino & Breuner, 2015; Crino, Driscoll, Ton & Breuner, 2014; Schoech et al., 2011). Importantly, our results highlight the potential for asynchronous hatching to influence later life cognition and encourage future studies examining the consequences of hatching asynchrony for birds' developmental trajectories and cognitive outcomes.

Immune challenge with LPS during the sensory and sensory-motor periods of song learning (i.e., days 30 and 50 posthatch) did not produce consistent impacts on song learning or cognition, although it was associated with improved motoric learning (Tables 2 and 3; Fig. 4). It is likely that the timing of exposure to LPS influences its effect on pheno-

type; in contrast to our findings, Grindstaff et al. (2012) treated zebra finches at days 5 and 28 posthatch and found that males challenged with LPS showed impaired motoric learning in the same foraging task used in the present study. Thus, it seems that immune challenge during very early postnatal brain development has greater consequences for these forms of adult cognition and behavior than during the period of song learning targeted by the present study. Although neural proliferation within the song control system continues through the sensory and sensory-motor periods of song learning, when we administered LPS, overall brain architecture is established, which could explain why we failed to find an impact on song learning (Bottjer, 1993; Nowicki et al., 2002). Whether aspects of cognition not measured in this or previous studies are impacted by early immune system challenge remains unclear.

In addition to the timing of stressors, the underlying mechanisms by which particular environmental conditions impact the brain and subsequent cognition likely differ. While LPS challenge could impact
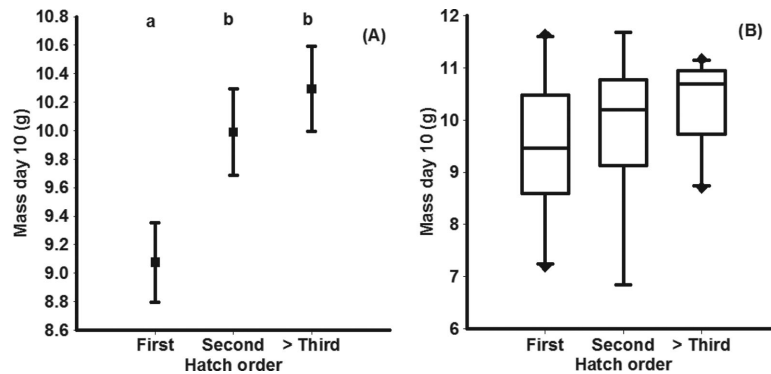
FIGURE 5 Effect of immune system challenge treatment on nestling mass on day 10 posthatch. (A) The estimated marginal mean number of mass (± standard error) where significant differences are denoted by different letters above bars while bars that share the same letter do not differ significantly. (B) Box plot depicting the median mass with boxes extending to 25th and 75th percentiles, whiskers extending to 90th and 10th percentiles, and outliers plotted as points. Later hatched birds were significantly heavier on day 10 posthatch than were first hatched male nestlings

cognition either through indirect effects, such as elevated glucocorticoids and reduced food intake, or direct impacts of immune activation on the brain (Bilbo, & Schwarz, 2009; Grindstaff, 2016), hatching asynchrony is linked to growth trajectory, immune function, and circulating glucocorticoids (Eraud et al., 2008; Nilsson & Svensson, 1996; Ricklefs, 1993; Saino et al., 1997). Disentangling the mechanisms by which early-life stressors act on phenotypic outcomes is challenging (Schoech et al., 2011), but elevated corticosterone should be evaluated as a possible mediator of the effects of hatching asynchrony on phenotypic outcomes for two reasons. First, later hatched birds have been reported to have higher baseline corticosterone levels (Eraud et al., 2008). Second, two separate studies in zebra finches found that treatment with corticosterone during development improved performance in a novel foraging task (Crino et al., 2014; Spencer et al., 2003). Thus, hatch order could impact the glucocorticoid-mediated stress response system to influence subsequent cognition, perhaps as a component of a broader behavioral strategy previously described in later hatched nestlings (Cotton et al., 1999; Nilsson & Svensson, 1996; Ricklefs, 1993). The next step in this study is to manipulate hatching asynchrony and/or nestling size hierarchy and measure sibling competition and corticosterone levels to determine how these variables predict cognition at adulthood. Further, to fully evaluate the "Environment Matching" hypothesis, future research should examine the relationship between hatching asynchrony and cognitive performance across a range of contexts to determine if later hatched birds perform better under competitive conditions like those in which they were raised, or in all contexts. Finally, future work on developmental conditions in songbirds should include hatch order as a covariate in analyses.

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