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Prenatal learning in an Australian songbird: habituation and individual discrimination in superb fairy-wren embryos

Diane Colombelli-Négrel1, Mark E. Hauber2 and Sonia Kleindorfer1

1School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide 5001, Australia
2Department of Psychology, Hunter College and The Graduate Center, City University of New York, New York, NY 10065, USA

Embryos were traditionally considered to possess limited learning abilities because of the immaturity of their developing brains. By contrast, neonates from diverse species show behaviours dependent on prior embryonic experience. Stimulus discrimination is a key component of learning and has been shown by a handful of studies in non-human embryos. Superb fairy-wren embryos (Malurus cyaneus) learn a vocal password that has been taught to them by the attending female during incubation. The fairy-wren embryos use the learned element as their begging call after hatching to solicit more parental feeding. In this study, we test whether superb fairy-wren embryos have the capacity to discriminate between acoustical stimuli and whether they show non-associative learning. We measured embryonic heart rate response using a habituation/dishabituation paradigm with eggs sourced from nests in the wild. Fairy-wren embryos lowered their heart rate in response to the broadcasts of conspecific versus heterospecific calls, and in response to the calls of novel conspecific individuals. Thus, fairy-wrens join humans as vocal-learning species with known prenatal learning and individual discrimination.

1. Introduction

Learning at different life stages may bring age-specific benefits for survival (reviewed in [1–3]). There are many forms of learning, including perceptual learning (the ability to learn to recognize and respond to stimuli previously experienced [4,5]), social learning (the acquisition of skills or behaviours through social observation or interaction between peers [6]) and sensorimotor learning (the acquisition of sensory-guided motor behaviour through imitation and practice; reviewed in [7]). Despite increasing empirical evidence that prenatal experience influences postnatal perception, cognitive performance and behavioural displays [8–11], few studies have tested explicitly for learning in embryos. In fact, most studies showing evidence for embryonic learning exposed individuals to a stimulus prenatally, but only tested their discriminatory capacity towards the stimuli after birth, hatching or eclosure [10,12,13]. Such examples suggest that discrimination, a key component of different types of learning, is already present at the embryonic stage [14,15], including in humans [16–18].

There is now growing evidence that embryonic learning exists in animals. No study as yet has shown evidence for embryonic social learning. But embryonic perceptual learning, tested in neonates, has been demonstrated across many taxa, including insects [19], amphibians [20,21], fish [22], rats [13], dogs [12], chickens [23], chimpanzees [24,25] and humans [10,26]. Recently, Colombelli-Négrel et al. [27] showed the first example of non-human sensorimotor embryonic learning; superb fairy-wren (Malurus cyaneus) females use an incubation call to teach their embryos a vocal password during the last stage of
incubation [27]. After hatching, nestlings must emit this password in order to be recognized by their parents as true genetic offspring, instead of being rejected as foreign, parasitic cuckoo chicks [27]. However, even that study paralleled most previous comparative work on embryonic learning in that the evidence was assessed after the embryos had hatched, and was based on vocal behaviours and displays during the nestling stage.

To date, prenatal acoustic discrimination has been confirmed only in humans ([16,17], reviewed in [28,29]) and birds [15,30,31]. Human fetuses start responding to airborne sounds at about 30 ± 1 weeks of gestational age [32], and, specifically, to their mother’s voice at 32–34 weeks [33]. From this stage onwards, fetuses can discriminate between vowel sounds [29], male and female voice [17], mother versus stranger voice [16,18] and the mother’s language versus unfamiliar language [34]. In birds, the behavioural work on the role of auditory imprinting by embryos in ducks and chickens showed that avian embryos are able to respond to acoustical stimulation during the last 20% of the incubation period [35,36]. Pekin duck (Aix galericulata) embryos responded to maternal calls with bill clapping sounds inside the egg up to 7 days before hatching and started to vocalize within the shell 4 days prior to hatching [15]. Sudden heart rate changes occurred in response to maternal calls and music (but rarely to another species’ calls) from 1 to 8 days before hatching [15,37]. In addition, neurochemical data from live chicken (Gallus gallus domesticus) embryos showed that unhatched chicks had adult-like auditory responses in the telencephalon following exposure to conspecific calls but not to artificial sounds [38].

Birds, and oscine birds in particular, are a model system to study human language because of the shared ontogenetic paths to acquire imitatively learned vocal perceptual and motor skills during a sensitive period of exposure to species-typical vocalizations [39–41]. Songbirds and humans also have parallel neural organization and developmental paths to acquire song or language, including hemispheric lateralization and motor–auditory rehearsal systems [42]. Like humans, songbirds are vocal learners as they have the ability to not only make associations with the sounds that they perceive, but also to modify and imitate the acoustic and/or syntactic structure of the sounds they hear and produce (see [43–45]). Yet, until recently, most research on prenatal auditory discrimination in birds has been carried out on non-visual-learning species (including ducks and chickens), with very little or no evidence of and focus on embryonic learning or prenatal cognitive abilities in vocal-learning species (but see [27]). The major advantage of studying prenatal response in avian embryos is that the eggs can be easily housed under different experimental conditions. The use of eggs of vocal-learning bird species is especially informative because of the many neurological similarities in auditory processing across avian and mammalian lineages that show vocal learning.

Superb fairy-wren embryos learn a vocal element from their mothers that they produce as an imitative nestling begging call after hatching [27]. We use the fairy-wren embryos as an experimental system to test the prenatal discriminative abilities of a non-human vocal-learning species. Our main question is whether fairy-wren embryos discriminate between different acoustical stimuli and if they show non-associative learning. If the embryos of vocal-learning species are able to acoustically discriminate between calls in general, then they should show the same pattern of discrimination towards conspecific and heterospecific calls in vivo. We first tested the embryos with conspecific fairy-wren female incubation calls, heterospecific calls or white noise stimuli. We predicted greater changes in heart rate response to the more salient conspecific versus the heterospecific calls, and greater response to bird calls versus white noise. To directly test whether embryos can learn to discriminate between vocalizations of superb fairy-wren individuals, we also used a classic paradigm to test for habituation–dishabituation [46–48]—a particular type of learning, in which habituation is characterized by a consistent directional pattern in response to a repeated stimulus (different call exemplars from the same conspecific) followed by a dishabituation phase (control: another different set of calls from the same individual) or test: a set of calls from a novel individual. This allowed us to test for experience-dependent responses by embryos to vocalizations of different superb fairy-wren individuals. Based on heart rate patterns seen in humans and adult birds, where lower heart rate is considered to be a physiological mechanism for orientating and attention [34,48–50], we predicted that fairy-wren heart rate measures would be lower in the test group (novel individual) than in the control group (same individual) if the fairy-wren embryo has the capacity to discriminate between acoustical stimuli.

2. Material and methods

(a) Study sites

We conducted this study during the austral breeding season of 2012–2013 at two study sites: (i) Cleland Wildlife Sanctuary (34°58’ S, 138°41’ E) and (ii) Newland Head Conservation Park (35°37’ S, 138°29’ E), South Australia. We carried out all experiments between 06.00 and 11.00 h local time.

(b) Experiment 1: heart rate response to conspecific versus heterospecific calls

We quantified heart rate variation of 43 superb fairy-wren embryos (one egg per nest; n = 43 nests; 19 in 2012 and 24 in 2013) in response to the following stimuli: (i) superb fairy-wren female incubation calls (conspecific calls; n = 22), (ii) contact calls of the allopatric winter wren (Troglodytes hiemalis; heterospecific calls; n = 11; figure 1) and (iii) white noise (control; n = 10). We generated different stimulus tracks with 1 min of pre-playback silence (pre), 1 min of playback (trial) and 1 min of silence post-playback (post). For the conspecific call stimuli, we used 15 sets of incubation calls from 15 different fairy-wren females recorded at 15 nests other than the focal nest in 2012 and 2013. For the heterospecific call stimuli, we used five sets of contact calls from five different winter wrens sourced from the internet: http://www.xeno-canto.org/species/Troglodytes-hiemalis. We generated four different tracks for the white noise stimuli using AMADEUS PRO 1.5 (Hairersoft Inc, Switzerland). The 1 min of playback (trial) consisted of six evenly spaced fairy-wren incubation calls or winter wren contact calls from the same individual or 1 s white noise. The amplitude of the playsbacks was normalized at −15 dB and saved as uncompressed 16 bit 44.1 kHz wave files using AMADEUS PRO 1.5.

We measured heart rates in the field using a digital egg monitor (Buddy, Vetronics Services, UK). This device generates heart rate data by tracking light absorption changes owing to embryonic blood flow, and is able to detect heart rate from the first week of the incubation period, especially in species with thin eggshells [51]. We broadcast the playback stimuli as uncompressed files from an Apple iPod (Apple Inc., USA) connected to a yo-yo...
speaker placed 5–10 cm from the monitor. For all eggs, we measured the embryo’s heart rate every 10 s for the 1 min of pre, trial and post (total 3 min per egg). Each egg was only presented with one stimulus track, which was selected randomly. To avoid a decrease in heart rate values owing to dropping egg temperatures, the egg monitor was placed on a small portable heat pack. We tested only one egg per nest with this setting and each egg was tested only once, between day 9 and 13 of incubation. At the completion of the experiments, each egg was returned to its natal nest; we detected no decrease in hatching success nor direct parental rejection of these eggs subsequent to the experimentation.

(c) Experiment 2: habituation/dishabituation procedures

To specifically test for physiological correlates of learning in fairy-wren embryos, we used a habituation/dishabituation procedure in 2013. We first presented the embryos with three different sets of incubation calls from the same female for three successive trials (habituation phase; figure 2). On the fourth trial (dishabituation phase), we presented the embryos with either a set of incubation calls from a novel female (test group) or yet another series of incubation calls from the same individual (control group; figure 2). For each trial, we used a different set of incubation calls, so that embryos would show habituation to a particular female and not to a specific incubation call from that female (figure 2; see [52]). Each stimulus track had 1 min of playback (consisting of six evenly spaced fairy-wren calls) followed by 1 min of silence, repeated four times in total. For the stimulus tracks, we used the incubation calls from 12 different females (eight different females for the tests, four different females for the controls). During the entire habituation/dishabituation procedure, we measured the heart rate of each embryo every 10 s as described in the previous experiment.

We exposed 17 fairy-wren embryos (one egg per nest; n = 17 nests) to our habituation/dishabituation protocol: eight were tested with a test set (the call of a different individual fairy-wren for the fourth trial) and nine with a control set (the call

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**Figure 1.** Spectrograms of the call types (conspecific, heterospecific) used in our experiments. The figure represents (a) two incubation calls from different superb fairy-wren females and (b) two contact calls from different winter wrens.
of the same individual for the fourth trial). We used the second egg for 10 of the 43 nests used in the conspecific versus heterospecific experiment (above), and the remaining seven eggs came from seven different nests that were not previously used. We tested only one egg per nest with this protocol and each egg was tested only once, between days 10 and 11 of incubation. At the completion of the experiments, each egg was returned to its nest of origin as described above.

### (d) Statistical analyses

For both experiments, heart rate data were averaged during each 1 min period across the six measurements (10 s); these data were then analysed with SPSS 22 for Windows (SPSS Inc., Chicago, IL). For experiment 1, we applied ANOVA to response variables during the 1 min of pre-playback silence, with year, study site and age of the eggs as fixed factors. We then analysed each playback type (conspecific, heterospecific and white noise) separately, and used paired t-tests across individuals to test for variation in heart rate (i) between the 1 min of pre-playback silence (pre) and the 1 min of playback (trial) and (ii) between 1 min of playback (trial) and the 1 min of post-playback silence (post). For experiment 2, we used a paired t-test to compare heart rate values during the 1 min of playback between the first and the third trials to test if habituation occurred within each subject. We then tested if there was an impact of treatment (test, control) or age on the habituation using an ANOVA. Finally, we compared the last heart rate response to the playback stimuli in trial 3 (habituation phase) and the first heart rate response to the playback stimuli in trial 4 (dishabituation phase) using an ANOVA.

### 3. Results

#### (a) Experiment 1: heart rate response to conspecific versus heterospecific calls

Average heart rate measures during the 1 min of pre-playback silence did not vary significantly between years (ANOVA: F_{1,43} = 2.24; p = 0.14), study sites (F_{1,43} = 1.19; p = 0.18) or age of the eggs (F_{1,43} = 0.15; p = 0.96). During the 1 min of playback (trial), fairy-wren embryos decreased their heart rate in response to both conspecific (paired t-test: t = 3.32; d.f. = 21; p = 0.003) and heterospecific calls (t = 2.60; d.f. = 10; p = 0.027), but not in response to the white noise (t = 0.58; d.f. = 9; p = 0.57; figure 3). Heart rates remained low in response to fairy-wren calls during the post period (compared with the trial values; paired t-test—conspecific calls: t = 3.38; d.f. = 21; p = 0.003), but not in response to the winter wren calls (heterospecific calls: t = 1.10; d.f. = 10; p = 0.30) or the white noise (t = 0.62; d.f. = 9; p = 0.55; figure 3).

#### (b) Experiment 2: habituation/dishabituation

All embryos showed habituation and lowered their average heart rate between the 1 min of playback in trials 1 and 3 and the 1 min of silence after the playback. After habituation (in trial 4), embryos were presented with either a set of incubation calls from a novel female (test group) or yet another series of incubation calls from the same individual (control group). All trials consisted of 1 min of playback followed by 1 min of silence.

![Design schematics of experiment 2](image)

**Figure 2.** Design schematics of experiment 2: all embryos were habituated to different sets of incubation calls from a randomly selected female (different for each embryo) between trials 1 and 3. After habituation (in trial 4), embryos were presented with either a set of incubation calls from a novel female (test group) or yet another series of incubation calls from the same individual (control group). All trials consisted of 1 min of playback followed by 1 min of silence.

<table>
<thead>
<tr>
<th>Control group</th>
<th>Test group</th>
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<tbody>
<tr>
<td>set A set B set C set D</td>
<td>set E set F set G set H</td>
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<td>trial 1</td>
<td>trial 2</td>
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<td>habituation phase</td>
<td>dishabituation phase</td>
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![Heart rate response](image)

**Figure 3.** Heart rate response to conspecific versus heterospecific calls (experiment 1). Data are shown as embryonic heart rate response differences (beats per minute; mean ± s.e.) to the playback of (a) superb fairy-wren incubation calls (conspecific calls; n = 22), (b) contact calls of the allopatric winter wren (heterospecific calls; n = 11) and (c) white noise (control; n = 10). The data presented are the differences in heart rate between the 1 min of pre-playback silence (pre) and the 1 min of playback (trial) in black, and the difference in heart rate between the 1 min of playback (trial) and the 1 min of post-playback silence (post) in grey.
between trials 1 and 3 (paired $t$-test: $t = 4.39$; d.f. = 16; $p < 0.001$). We found no effect of treatment (test, control) or age (and their interaction terms) on the extent of habituation (ANOVA: all $p > 0.37$). We then compared the difference in heart rates between the last response with the stimuli in trial 3 (habituation phase) and the first response with the stimuli in trial 4 (dishabituation phase): we found that embryos presented with the test stimuli (novel individual) had lower heart rates in trial 4 relative to those presented with the control stimuli (same individual; ANOVA: $F_{1,16} = 6.70$; $p = 0.021$; figure 5).

4. Discussion

The embryos of a vocal-learning songbird species, the superb fairy-wren, have functional and complex discriminative abilities, and acoustically driven learning, in ovo. Fairy-wren eggs hatched their heart rate when we broadcast conspecific and heterospecific calls, but not in response to white noise. In addition, heart rate remained lower after hearing conspecific calls, but not after hearing heterospecific calls. Similar to human fetuses [16,18], fairy-wren embryos were able to learn to discriminate between the vocalizations of individual conspecifics. In our habituation/dishabituation experiments, embryos presented in the fourth and final trial with calls from the same individual as the preceding third trial did not change their heart rate, whereas embryos presented with the call from a novel individual lowered their heart rate. Lower heart rate is a physiological correlate of attention [34,48–50]. Thus, the fairy-wren embryos showed a physiological response to a salient auditory-cue for vocal learning; they responded more to the call of a novel individual than to another call of a familiar individual.

Fairy-wren embryos showed fine-tuned discrimination between different calls, as indicated by heart rate values remaining low after hearing conspecific calls but not after hearing heterospecific calls. We did not identify the proximate basis for the prolonged embryonic responses to conspecific versus heterospecific calls in fairy-wrens, which parallel prior results from non-vocal-learning bird species [31]. Embryos may have responded based on call structure or call duration, and future work should experimentally manipulate the many acoustic features by which fairy-wren and winter wren calls differ (figure 1). Irrespective of the cue, the prolonged decrease in heart rate after exposure to conspecific calls is suggestive of greater cognitive attention to the more salient (conspecific) stimuli [34,49,50], perhaps, because the fairy-wren embryos have learned generalized aspects of their own mother’s calls to allow them to respond to other, unfamiliar conspecific calls, too. Future studies could test the extent of heart rate modulation during conspecific call exposure and the accuracy of begging call similarity with the maternal password, after hatching [27]. We predict that different embryonic physiological states would affect the quantity and quality of imitative call learning.

To the best of our knowledge, while the ability to discriminate between different individuals of the same species has been shown extensively in adults and offspring in many taxa [46,53–56], only humans are known to be capable of such discrimination at the embryonic stage [16–18]. Specifically, previous studies have shown that human fetuses can discriminate prenatally between male and female voices [17] as well as mother versus stranger voices [16,18]. In our work, we have argued previously that fairy-wrens may benefit from advanced discriminative capacity in order to thwart the successful fledging of their primary brood parasite, the Horsfield’s bronze-cuckoo (Chalcites basalis) [27]. Indeed, there is no evidence so far that Horsfield’s bronze-cuckoo chicks (a non-vocal-learning species) learn their foster parental calls as embryos, and instead they use trial-and-error call matching, after hatching, to produce the mimetic begging calls [57,58] that will elicit the parental feeding by hosts [59]. Therefore, further studies are needed to test if embryonic vocal learning extends to other vocal-learning species (i.e. parrots, hummingbirds, cetaceans) and, beyond, to vocal non-learning species (i.e. fowl, ducks, cuckoos, most mammals; see [60]).

In summary, these experiments have demonstrated that fairy-wren embryos can discriminate between the calls of conspecific versus heterospecific individuals. The finding that the embryos had different heart rate responses in the habituation learning trials shows that the physiological capacity for some aspects of attention, learning and perhaps memory, are already formed and functional well before hatching. Considering that non-vocal-learning species lack
the special brain regions that are necessary for imitative acoustical learning (reviewed in [43,44]), these results open new windows of opportunity to understand the neural organization and development of imitative vocal learning (including human speech) during embryonic stages.

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