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Abstract

In chickens, food consumption can be altered by exposing the chicks to scents as embryos. We exposed eggs to an orange-scented food additive in the final days of incubation. Following hatching, we tested these exposed chicks' ability to detect this scent at a variety of concentrations. We found that orange-exposed chicks responded to an orange-scented solution at lower concentrations than control chicks. This sensitization may allow chicks to be more effective at locating acceptable food items but requires further testing to determine its significance. Orange-exposed and control chicks were also tested with the scent of raspberry. Orange-exposed chicks responded to the raspberry presentation significantly more than the control chicks did, suggesting that the embryonic exposure to orange may have influenced how the chicks responded towards another fruity smell. This result suggests that chicks may be learning general characteristics of exposed scents while in the egg, though this needs further research.

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**Embryonic exposure of chicken chicks (*Gallus gallus domesticus*) leads to heightened
sensitivities towards the exposed scent**

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Short title: Exposure to scents heightens chicken sensitivities

Keywords: olfaction; chicken; imprinting; sensitivity

Summary

In chickens, food consumption can be altered by exposing the chicks to scents as embryos. We exposed eggs to an orange-scented food additive in the final days of incubation. Following hatching, we tested these exposed chicks' ability to detect this scent at a variety of concentrations. We found that orange-exposed chicks responded to an orange-scented solution at lower concentrations than control chicks. This sensitization may allow chicks to be more effective at locating acceptable food items but requires further testing to determine its significance. Orange-exposed and control chicks were also tested with the scent of raspberry. Orange-exposed chicks responded to the raspberry presentation significantly more than the control chicks did, suggesting that the embryonic exposure to orange may have influenced how the chicks responded towards another fruity smell. This result suggests that chicks may be learning general characteristics of exposed scents while in the egg, though this needs further research.

Introduction

Many early studies (for example Grubb, 1972; Hutchison & Wenzel, 1980) investigating the sense of smell of birds focused on Procellariiform seabirds while more recent studies of avian olfaction have expanded to include other wild birds including songbirds (Mennerat, 2008; Amo et al., 2013), penguins (Culik, 2001; Cunningham et al., 2008; Cunningham & Bonadonna, 2015), and vultures (Owre & Northington, 1961; Graves, 1992). While studies on wild birds have significantly advanced our understanding of how birds use odours, they are often difficult to conduct, repeat, and control. For these reasons, laboratory experiments, which allow for controlled conditions and ease of repeatability, have been conducted and have led to significant contributions to this field of knowledge.

In 1929, August Krogh coined the Krogh principle: “For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied”. Despite the fact that they rank among the lower third of birds with respect to their relative olfactory bulb size (Bang & Cobb, 1968; Bang, 1971), in terms of studies on avian olfaction the most convenient of birds to study has been the chicken (*Gallus gallus domesticus*; see reviews in Jones & Roper, 1997; Krause et al., 2016) since chickens are easy to raise in captivity and to manipulate both *in ovo* and after hatch. For example, chickens have been used in neurophysiological investigations to examine the adaptation and sensitization of olfactory bulb neurons (McKeegan & Lippens, 2003), to study the concentration of odour needed to stimulate electrical activity in the olfactory bulb (McKeegan et al., 2002), and to explore the development of

the olfactory nerve (Ayer-Le Lievre et al., 1995; Drapkin & Silverman, 1999). Behavioural studies have demonstrated that the blocking of the nares decreases weight gain in chicks (Porter et al., 2002), that birds may identify predators by scent (Fluck et al., 1996), that chicks can associate specific scented food with illness and adjust their food choices accordingly (Turro et al., 1994; Porter et al., 2002) and that novel odours can decrease the likelihood that a chick forages (Jones, 1987; Bertin et al., 2010).

Due to the ease of manipulation, chickens have also been broadly studied for how exposure to odours early in life alters behaviours going forward. Multiple studies (Jones & Gentle, 1985; Jones, 1987; Jones & Carmichael, 1999) have showed that chicks that are exposed to odours immediately following hatch show increased food consumption when given the appropriately scented food, or a preference to stand near litter scented with this scent, days later. These sorts of early exposures are known to decrease a chick's fear response in novel situations (Jones et al., 2002), presumably due to the chicks' recognition of a known element of the environment. Interestingly, because scents readily cross the egg shell (Board, 1982), and the olfactory receptor neurons are responsive to scents by embryonic day 13 (E13; Lalloue et al., 2003), and the entire olfactory system (epithelium and the olfactory bulbs) is reactive to odours by E18 (Gomez & Celii, 2008), chickens have also been used to explore how exposure to odours *in ovo* affects behaviours after hatching. Sneddon et al. (1998) painted eggs with strawberry scent in the days prior to hatch and later these chicks drank more strawberry-flavoured water, and spent more time in strawberry-scented areas, than unexposed controls. This work has been confirmed by other studies, which showed that

embryological exposure to scents changes behaviour in the egg (Hagelin et al., 2013) and alters food consumption post-hatch (Bertin et al., 2010; Bertin et al., 2012). Aigueperse et al. (2013) found that even feeding a hen a scented food can impact the food choice of their chicks, suggesting that maternal signals regarding preferred diet are transferred into the egg to alter the neurophysiology of the developing embryo.

Despite these studies which have investigated how embryonic exposure to scents affects behaviour, it has yet to be determined if this early exposure alters a bird's sensitivity towards detecting the scent. In this study, we exposed eggs to a novel orange scent and then tested whether this exposure allowed birds to detect the scent at lower concentrations than unexposed birds. We used a methodology, the Porter method, that has been successfully employed to assess olfactory capabilities in chickens and other species (Porter et al., 1999; Cunningham et al., 2003; Bonadonna et al., 2006). Based upon previous studies that showed that embryonic exposure to scents in mammals caused heightened sensitivities towards the particular scent after birth (for example, Yee & Wysocki, 2001), we predicted that exposure to orange scent in the egg would produce chicks that were capable of detecting the orange scent at lower concentrations than unexposed chicks. We also tested whether orange-exposed birds responded more strongly towards another fruit-related scent, raspberry, as opposed to a nut-derived scent, almond.

Material and methods

Subjects

One hundred and sixty-five eggs of white leghorn chickens (Charles River Company, North Franklin, CT, USA) were used. Once the eggs were received, they were placed in a High Hatch incubator maintained at approximately 37.5°C with a relative humidity of 48-55%. Eggs were automatically rotated. The use of these chicks was approved by St. John Fisher College's Institutional Animal Care and Use Committee.

Treatment

On day E15 the eggs were placed in one of two Hovabator styrofoam incubators, to allow for exposure to the odour. Eighty-two eggs were exposed to the orange stimulus (McCormick imitation orange extract) by adding a full transfer pipette of orange extract (5 mL) to the bottom water reservoir (100 mL) of the incubator twice daily on days E15-20, following established protocols (Sneddon et al., 1998). A fan on the top of the incubator and the electric heater helped with the circulation of the odour throughout the incubator. On day E20 the orange extract along with the water in the bottom reservoir were removed and replaced with fresh water, thereby decreasing the likelihood that the exposure to the orange scent occurred during the hatching or post-hatch phase. To our noses, there was no detectable scent of orange in the incubator by the time the chicks hatched. For the control eggs, eighty-three eggs were placed in a separate incubator. The water reservoir was filled with water, but nothing else, throughout development. Water was added to each incubator at the same times each day to ensure that the conditions in the incubators were similar.

The Porter method

As the chicks began to hatch, on day E21-22, plastic colour bands were placed upon their legs to allow for identification and the chicks were placed in a common brooder where food (Blue Seal Starter Crumble) and water was provided *ad libitum*. Chicks remained in the brooder until testing. Chicks were tested with the Porter method (Porter et al., 1999) approximately 12 hours after they hatched.

To test a chick's response to an odour, a chick was held in the hand of one experimenter with the ventral side facing up. To induce a sleep-like state a second experimenter placed a 40-W incandescent light bulb approximately 3 cm from the posterior of the body to warm the bird. We classified a chick as “asleep” when its eyes closed and its body ceased movement. Once the chick was considered to be “asleep”, we waited approximately 1 minute to ensure that the chick was truly “asleep”. If the bird awoke during this time, we waited until the bird fell back to into its sleep-like state and then waited another minute. All birds eventually slept and were tested with the complete array of presentations. Each chick was tested only once.

Each “sleeping” chick was exposed to six stimuli: (1) 100% pure orange extract solution, (2) 50% pure orange extract solution, (3) 25% pure orange extract solution, (4) 100% pure raspberry extract (McCormick), (5) 100% pure almond extract (McCormick), and (6) distilled water. Dilutions were made by mixing distilled water with the pure orange extract to create the required dilution. Odour stimuli (10mL) were prepared and placed in a 100-mL soft squeeze bottle (VWR® wash bottle). During testing, the tip of the bottle was held approximately 2-3 cm from the “sleeping” bird’s nostrils and the

bottle was squeezed 15 times over 10 seconds. For each bird, to ensure a blind study, the order in which the stimuli were administered was changed and the experimenter deploying the odour and scoring the behaviour did not know, and could not smell, which scent was being expressed. With regard to the dilution deployments, however, the order always went from low to high concentration, to prevent the responses to a higher concentration from affecting the subsequent response to a lower concentration. The water presentation was randomly inserted into the dilution portion of the test. All possible orders of the dilution series were evenly and randomly selected. The order of raspberry and almond was also randomly selected.

The Porter method uses a four-point scale to quantify the chick's response to the deployment: 0 = no response; 1 = slight movements; 2 = head shaking and more drastic movements than response 1; and 3 = larger head movements, vocalizations and waking up.

Statistics

Results collected via the Porter method are categorical and not normally distributed, hence we used non-parametric statistical analyses. For the dilution experiment, we first tested for an overall difference within the control birds and within the exposed birds using a Friedman's test. Since we found significant differences in each group, we used a Wilcoxon signed-rank test to compare the response to the three dilutions (100%, 50%, 25%) against the water for each group. To compare the response

of the orange-exposed birds to the dilutions directly against the response of the control birds to the dilutions we used using a Mann-Whitney U test.

Additionally, using ordinary least squares, we ran a multiple regression model in an attempt to determine whether the response to the odour was dependent upon the order of presentation, the exposed odour (orange or water), or an interaction between the order and the score by each chick to the presented odour. The model was run in R 3.1.2 (R Development Core Team).

We also investigated whether there were significant differences between the two groups in the chick's responses to raspberry or almond by using a Mann-Whitney U test. Finally, we tested whether the responses of orange-exposed or control birds were significantly different than their responses to water using a Wilcoxon signed-rank test.

Results

Of the 82 orange-exposed chicks, only 19 hatched (23%). Hatching success was similarly low in the control chicks (18%), where only 15/83 chicks hatched. Although both hatch rates are low compared to normal (70 - 80% range), they were not statistically different from each other (Chi square = 0.61, d.f. = 1, $P = 0.43$), suggesting that our orange exposure had no impact on hatch rates. This low hatch-rate was likely due to issues with transport, as many eggs, upon candling, did not show viable embryos.

Table 1 shows the number of chicks from each treatment group (orange-exposed or control) that responded to each stimulus. When looking at how orange-exposed chicks behaved in the dilution experiment, we found an overall significant difference in

their responses to the three orange scents and the water (Figure 1; Friedman test statistic = 28.44, d.f = 3, $P = 0.00003$). We found that the response to the 100%, 50% and 25% were all significantly greater than the response to water (Wilcoxon signed-rank test: $Z_{100\% \text{ orange } \neq \text{water}} = 3.72$, $P = 0.0002$; $Z_{50\% \text{ orange } \neq \text{water}} = 3.62$, $P = 0.0003$; $Z_{25\% \text{ orange } \neq \text{water}} = 3.41$, $P = 0.0007$). We also found significant differences, by control chicks, in their response to the three orange scents and the water (Figure 1; Friedman test statistic = 16.36, d.f = 3, $P = 0.001$). When looking at the pairwise comparisons, we found that the scores by the control chicks for the 100% and 50% orange were significantly greater than their response to water ($Z_{100\% \text{ orange } \neq \text{water}} = 3.05$; $P = 0.0022$; $Z_{50\% \text{ orange } \neq \text{water}} = 2.67$; $P = 0.008$). The response to the 25% orange solution, however, was not significantly different than the response to the water ($Z_{25\% \text{ orange } \neq \text{water}} = 1.19$; $P = 0.24$). The orange-exposed chicks, therefore, were able to respond to the orange scent at a lower dilution, 25%, than the control chicks.

We also directly compared the response of the orange-exposed and control birds to each deployment. The two groups responded similarly to the water (Mann-Whitney U test: $Z = 0.29$, $P = 0.77$) and to the 100% orange deployment ($Z = 1.40$, $P = 0.16$). However, for both the 50% deployment ($Z = 2.08$, $P = 0.037$) and the 25% deployment ($Z = 2.69$, $P = 0.007$), the orange-exposed chicks responded significantly more to the orange scent than did the control chicks.

When investigating a potential for an order effect on the responses to the odours, we found both the order of presentation, and the interaction between the order of presentation and the score given by chicks to the odour were not significant (p

> 0.05). These results suggest that overall there was no effect of the order of presentation on the responses to the odours, and are summarised in Table 2.

We compared whether the responses of orange-exposed and control birds to raspberry or almond were significantly different than their response to water. We found that the orange-exposed bird's response to raspberry was significantly greater than their response to water (Figure 2; Wilcoxon signed rank test: $Z_{\text{raspberry} \neq \text{water}} = 3.29$, $P = 0.001$) but the control exposed bird's responses were not ($Z_{\text{raspberry} \neq \text{water}} = 1.54$, $P = 0.12$). The response of orange-exposed birds to almond was not significantly different from water ($Z_{\text{almond} \neq \text{water}} = 0.86$, $P = 0.39$), nor was the response of control birds ($Z_{\text{almond} \neq \text{water}} = 1.57$, $P = 0.11$). We also tested the responses of orange-exposed and control chicks to the raspberry deployment and also to the almond deployment. The orange-exposed chicks responded significantly more than the control chicks to the raspberry deployment (Mann-Whitney U test: $Z = 2.23$, $P = 0.025$). The responses given by the two groups to almond, however, were not significantly different ($Z = 1.04$, $P = 0.30$).

Discussion

In this study, we attempted to determine whether embryonic exposure of chickens to an orange scent affected the sensitivity at which chicks could respond to this odour. We also tested whether being exposed during development to one fruit-derived odour would impact how the chicks responded to another fruit-derived odour. We found that chicks that were exposed to an orange scent on days E15-20 demonstrated greater sensitivity to this scent than control chicks, as exposed chicks responded to the

25% orange dilution while the control chicks did not (Figure 1). The order that the scents were deployed had no effect on the response (Table 2). Furthermore, the orange-exposed chicks responded more towards another fruit-related scent (raspberry) than the control chicks (Figure 2), suggesting that orange-exposed chicks may have recognized some similarities in these two fruity scents. Interestingly, the early exposure to the orange scent appears to have only impacted the response to the fruit-related scent, since orange-exposed and control birds demonstrated a lack of response to the almond-derived odour (Figure 2). Although chickens have long been studied with respect to olfaction, and how embryonic exposure affects behaviour later in life (for example Bertin et al., 2010) this is the first study that shows that this exposure heightens the sensitivity of the exposed chick towards the scent. This heightened sensitivity may be one of the bases for explaining how olfactory imprinting, where exposure of an animal to an odour during a sensitive period increases the significance of the scent and alters behaviours later in life, occurs in birds (Bateson, 1966). Our results mirror studies in mice which show that exposure to an odourant prior to birth causes an increase in sensitivity towards that odourant (Voznessenskaya et al., 1994; Yee & Wysocki, 2001). The increased sensitivity towards an exposed scent could be due to neurological changes in the olfactory epithelium (see O'Neill et al., 2016) or in the olfactory bulb (see Todrank et al., 2010; Liu et al., 2016), the site of the first synapse in the olfactory system, or in both. Further research should be conducted on chickens which would differentiate between the effects of the periphery and central structures (Yee & Wysocki, 2001) in the imprinting process.

Generally, possessing heightened sensitivities towards odours experienced in the egg may have broad implications for how chicks behave following hatching. For example, by developing heightened sensitivities, a chick may more effectively choose appropriate locations to position themselves once hatched since it is known that imprinted chicks elect to stand in bedding that has a familiar scent (Jones & Gentle, 1985; Jones & Carmichael, 1999). Heightened sensitivities may also improve the ability to recognize relatives by scent (reviewed in Hagelin, 2007) since the chick would be exposed to odours associated with their parents throughout incubation. Finally, a chick may forage more effectively if it is exposed to food-related scents while in the egg. The heightened sensitivities towards the exposed scent could help to explain how chicks use their imprinted memories to appropriately choose food (Sneddon et al., 1998; Bertin et al., 2010). In a generalist forager such as the chicken, however, multiple types of food are consumed. Our finding that orange-exposed chicks respond significantly more than control chicks towards another fruity scent, raspberry (Figure 2), suggests that embryonic exposure to a scent might tune a chick, or build in preferences, to the broader category of food to which the imprinted scent belongs, though this needs further testing with more fruit-related scents to confirm the phenomenon. Broad imprinting may facilitate the finding of food that is similar to the original imprinted odour thereby allowing for a broader diet to be consumed. However, the potential for imprinting does not extend to all odours, as we found that orange-exposed and control chicks did not respond to the novel scent of almond (Figure 2). Thus, food scented with imprinted odours, and perhaps scented with a similar odour, may be selected, but not

all scented foods. A future study should evaluate whether exposure to a fruity scent leads to increased food consumption of a similar, but not identical food, as our research suggests. We ran the orange and the raspberry blends through a gas chromatograph and found that their peaks did not align (see Supplemental data). However, the response of animals towards blended scents like the food additives used here, and their component odours, is complicated and is just beginning to be studied. How birds treat blended scents and their constituents should be further investigated, particularly in light of work by Coureaud et al. (2008; 2009) with European rabbits (*Oryctolagus cuniculus*) which showed that how rabbits respond to blended scents and their constituents differs based upon whether the constituent or the blend is learned.

One might expect that imprinting while in the egg might be more critical to the development of appropriate behaviours in precocial chicks, such as the chicken, as compared to an altricial chick. Recent work by Caspers et al. (2015, 2017), however, demonstrates that this may not be the case as Zebra finch (*Taeniopygia guttata*) chicks can imprint on the scent of their parents and to nesting material while still in the egg. Despite these recent studies, research should continue to assess the relative importance of learning from maternal cues (Aigueperse et al., 2013), while in the egg, or following hatch, to all types of salient olfactory cues in precocial and altricial species as developmental differences may play a role in the importance of each.

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Figure 1. Responses of orange-exposed birds (black bars) and control birds (gray bars) to different concentrations of McCormick imitation orange extract, or water. Figure a) shows that orange-exposed birds had significantly greater responses to 100%, 50% and 25% orange extract, as compared to their response to water (* $p < 0.05$; Wilcoxon signed-rank test). Figure b) shows that control chicks had significantly greater responses to 100% and 50% orange extract as compared to their response to water (* $p < 0.05$) See Results for details.

Figure 2. Responses of (a) orange-exposed birds (black bars) and (b) control birds (gray bars) to McCormick imitation raspberry or almond extract, or water. Compared to the response to water, the response to raspberry was higher for orange-exposed chicks (* $p < 0.05$, Mann-Whitney U test), but not for control chicks. Both groups responded similarly to the almond presentation as compared to water. See Results for details.

Table 1. The number of orange-exposed and control chicks responding to each presentation.

	100% Orange	50% Orange	25% Orange	Water	Raspberry	Almond
Orange-exposed chicks (n = 19)	19	19	17	9	18	11
Control chicks (n = 15)	15	15	12	8	12	12

Table 2. Multiple regression model testing whether the order of the presentation of the odour, the presentation (orange dilution series or water), or the interaction between order and the presentation affect the response given by the chicks. The italics show a significant effect.

	Estimate	Standard Error	p-value
Intercept	1.65	1.32	0.21
Presentation: 100% orange	-0.78	1.36	0.56
Presentation: 50% orange	-0.04	1.38	0.98
Presentation: 25% orange	0.48	1.50	0.75
Exposure	-0.51	0.14	0.00044
Order	0.24	0.35	0.49
Presentation: 100% * Order	-0.24	0.37	0.52
Presentation: 50% * Order	-0.06	0.46	0.89
Presentation: 25% * Order	-0.26	0.44	0.61

Supplemental data. Data collected from Gas Chromatograph for (a) orange and (b) raspberry extract. Data was collected on a Thermo Scientific Trace 1300 Series Gas Chromatograph-ISQ Single Quadrupole Mass Spectrometer using a 30m x 0.25 mm i.d. Capillary column with a 0.25 mm coating of 5%phenyl/95% methyl silicone (Crossbond™) [Restek catalog #12223] with a carrier flow of 1.5 mL He/min and collected as EI mass spectra. The temperature program was 40°C for 1 min, ramped at 20°C/min to 250°C and then held at 250°C for 1 minute. Samples were injected neat as 1 uL samples with a split ratio of 33.

a)

b)