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The odour of pyrazine increases the egg mass of domestic chickens (*Gallus gallus domesticus* L.)

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Abstract

Forty leghorn chickens at the commencement of egg laying, were divided into two groups, each with 10 females and 10 males. One group was exposed to the odour of synthetic pyrazine (2-methoxy-3-isobutylpyrazine) for 16 weeks while the other acted as a control. During the first 4 weeks the hens exposed to pyrazine odour laid significantly fewer eggs (unfertilized) than the controls, but thereafter both groups laid a similar number. Mean egg mass of the pyrazine exposed hens was significantly ($P = 0.012$) greater (5.5%) than that of the control group. No significant difference was found in body mass or eggshell thickness. Similarly, there were no consistent significant differences between groups in oestrogen and testosterone concentrations in the blood of females and males, respectively. No pyrazine could be detected in cloacal extracts. The experiment shows that an external odour can affect the internal reproductive system of the chicken. It is suggested that the pyrazine-engendered increase in egg mass involves neuroendocrine regulation within the hypothalamus rather than hormonal interactions 'downstream' of the brain.

Key words: pyrazine odour, egg mass increase, domestic chicken, *Gallus gallus domesticus*

INTRODUCTION

In the life of many wild birds, pyrazine, which is widely distributed in plants and certain insects (Woolfson & Rothschild, 1990), must play a significant role since its odour, like the colour red, signals the presence of both edible and inedible food. Thus, excreted by aposematic moths and beetles it functions as an alarm signal but when present in various ripe fruits, often combined with red coloration it attracts frugivorous birds. The fact that this volatile compound improves recall of past events for birds (Barnea, Gvarayhu & Rothschild, in press) adds to the success of pyrazine as an alerting signal.

It has been shown (Jemiolo, Androlini & Novotny, 1986; Jemiolo & Novotny, 1994) that 2,5-dimethyl pyrazine which is present in the urine of female house mice inhibits the onset of puberty in both sexes. They also demonstrate that the external application of this synthetic pyrazine produced a similar result.

We were therefore encouraged to study its possible effects on the various aspects of the breeding system of birds. Chickens were considered suitable experimental subjects because the Phasianidae frequently encounter pyrazines in nature as they feed on both plants and insects. Our original experiment was planned to investigate whether or not exposure to an external odour of pyrazine could influence and prolong the period of egg laying and increase egg numbers (clutch size). This plan was altered considerably (see later) to answer to a single question: Can an external odouriferous volatile influence the internal organs of a bird's reproductive system?

MATERIALS AND METHODS

Forty leghorn chickens to be used for exposure to pyrazine odour (20 males and 20 females) were obtained from a commercial hatchery. They were reared in outdoor cages until 4 months old, at the Faculty of Agriculture, Hebrew University, Israel. During this period, 2 males died. The birds were then brought to the Meier Segal, Garden for Zoological Research at Tel-Aviv University, Israel, where numbered wing tags were inserted for individual identification.

Using these numbered tags and a statistical table of random digits, the birds were divided randomly into 2 groups: control (C) and experimental (P). Each group contained 10 males and 10 females, individually housed

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out of doors, in cages 33 × 38 × 46 cm. In order that all females faced a male in the cage opposite, another was added to each group; these male birds were not included in the experiment.

The same commercial ration (18% protein) and water was provided *ad libitum* in identical containers throughout the experiment to both groups. Since methoxypyrazine has such a remarkably powerful and persistent odour, the 2 groups of birds were in cages 100 m apart, a distance that was necessary to ensure no contamination of the control birds with the volatile pyrazine. Also, the control birds were always visited before the experimental birds for cleaning, feeding and egg counting. All tools and assistants' protective clothing were kept separate for each group. The 2 groups were similar in every respect apart from the presence of the volatile pyrazine in the cages of the experimental birds.

Odour exposure began 2 weeks after the chickens were housed in outdoor cages, and was continued for 16 weeks.

Experimental group (P)

Four perforated plastic boxes were suspended 1 m above and in front of the cages. The tested odour consisted of synthetic 2-methoxy-3-isobutylpyrazine (Pyrazine Specialities Inc., Atlanta, GA, U.S.A.), diluted by dissolving 10 µl in 99 ml distilled water and 1 ml ethanol. At 24-h intervals, 10 ml of this solution was added to the plastic boxes, thus ensuring a continuous exposure of the chickens to the tested odour.

Control group (C)

An identical procedure was used for the control group (group C) as for the pyrazine exposed group, except that no pyrazine was added to the solution containing 99 ml of distilled water and 1 ml ethanol.

Examination of cloacal contents for pyrazine

Since Jemiolo & Novotny (1994) had found pyrazine in the urine of the house mouse, the cloacal content of birds that had been exposed to pyrazine were collected at Ashton Wold from chickens and geese using Rhode Island reds and farmyard geese. The samples were examined on our behalf by Professor John Pickett by coupled GC MS. Samples of the faeces were soaked in freshly distilled dichloromethane (30 ml each) at 4 °C for 48 h. The solvent was removed and collected along with washings (10 ml). The extracts were evaporated to *c.* 5 ml and subjected to vacuum distillation (0.06 Torr, 25 °C, 4 h). The purified extracts were concentrated to 20 µl before analysis by coupled GC–MS.

Coupled GC-mass spectrometry

A capillary GC column (30 m × 0.3 HP-5 column) was directly coupled to a mass spectrometer and integrated data system (70–250 VG analytical). Ionization was by electron impact at 70 eV, 230 °C. The oven temperature was programmed at 30 °C/5 min, then 5 C/min to 250 °C.

The presence or absence of alkylmethoxypyrazines was determined by selection ion monitoring. Selected masses were: 124 (base peak for 2-isaobutyl-3-methoxypyrazine), 137 (M-151; base peak for 2-isopropyl-3-methoxypyrazine), 138 (base peak for 2-sec-butyl-3-methoxypyrazine), 151 (M-15), 152(M*) and 166 (M*).

Statistical analysis and parameters tested of the chicken

For the statistical analysis, the 16 weeks were divided into 8 periods (see Figs 1 & 2) of 2 weeks each. Some mortality occurred during the study: 5(C) males and 2(C) females died during period IV, and 1(P) female died in period VI. This mortality sometimes affected the number of individuals included in each statistical test. Presented means are expressed as mean ± SE.

Egg numbers and egg mass

Laying of both experimental and control groups started simultaneously aged 4 months, a few days before odour exposure began. Eggs laid by each female in both groups were collected and counted daily, marked and weighed. The relative amount of yolk and albumin was not assessed. Statistical analysis of egg mass and egg numbers was only carried out using data for females that had survived the whole experimental period, i.e. 9(P) females and 8(C) females. A *t*-test was carried out to compare the overall mean egg mass and egg number per female between the 2 groups for the entire experimental period. In addition, a repeated measure analysis of variance was carried out to test for time and exposure effects on these variables. Means of egg mass and egg numbers per hen for each period were calculated in both groups before the analysis. Since these variables are expressed as ratios, they are not normally distributed. Therefore in all tests, before the statistical analysis, the reciprocal transformation was applied (Sokal & Rohlf, 1981).

Eggshell thickness

Eggshell thickness was recorded for each female in both groups using a Peacock calliper (accuracy of 0.01 mm) at 5 points across the eggshell. Measurements were taken 4 weeks after the onset of odour exposure and twice again at 2-week intervals (periods II, III and IV, before any mortality occurred). A repeated measures analysis of variance (ANOVA) was used to test for time and exposure effects.

Body mass

Measurements were taken: (1) before the onset of odour treatment (date 1); (2) 4 weeks after treatment began (date 2); (3) 16 weeks after the onset of odour treatment (date 3).

Because of the mortality in both groups, data were analysed by a 2-way ANOVA, for each date separately, testing for gender and treatment differences between the 2 experimental groups.

Hormonal blood concentrations

Blood was prepared and assayed using testosterone/oestradiol double antibody kits (Diagnostic Products Cooperation). Blood concentrations (ng/dl) of oestrogens (in females) and of testosterone (in both males and females) were analysed from samples obtained on the same 3 dates as the body mass measurements. For reasons explained earlier (see egg numbers and egg mass), before the statistical analysis the reciprocal transformation was applied. Any possible effect of pyrazine odour on hormonal blood concentrations was tested by repeated measures ANOVA, where date 1 was used as a covariate. This enabled us to test for pyrazine effect on hormonal blood concentrations after adjustment for pre-existing differences in the 2 groups. Because of the mortality losses and the difficulty of obtaining blood samples from all individual birds, sample sizes for each test vary and therefore, are given specifically, for each case in the Results.

RESULTS

Egg mass

A comparison of overall mean egg mass, for the entire experimental period, between the two groups, showed that (P) females ($n=9$) laid significantly ($t_{15}=-2.84$, $P=0.012$) heavier eggs than those of (C) ($n=8$), with respective mean values of $60.2+2$ *vs* $56.9+2.8$ g, and a mean difference of 3.3 g. When testing the data in eight periods (Fig.1), we found that both time and treatment significantly affected egg mass ($F_{7,91}=105.89$, $P=0.0001$ and $F_{1,13}=5.82$, $P=0.031$, respectively). These effects can be seen in Fig.1 by the gradual mass increase that occurred in both groups over time, as well as by the egg mass differences between the two groups that occurred in seven out of eight cases (period II and onwards), with heavier eggs laid by (P) females. In addition, the interaction between time and treatment was also found to be significant ($F_{7,91}=2.90$, $P=0.009$), indicating that the effect of time on egg mass was different for the two groups. Logarithmic regression lines which were calculated for the data and their slope values (Fig.1) describe the nature of this differential effect, showing that the increase in egg mass over time was steeper in the (P) group. For example, in period I, mean egg mass per female was very similar in the two groups, whereas in period VIII this difference was significant (4.7 g). Although the mean overall egg production values per hen of both groups are nearly similar (5875 g for an experimental bird and 5804 g for a control bird) this does not provide a full picture, since at the end of the experimental period the difference in egg mass not only increases with time but exceeds that of the average difference.

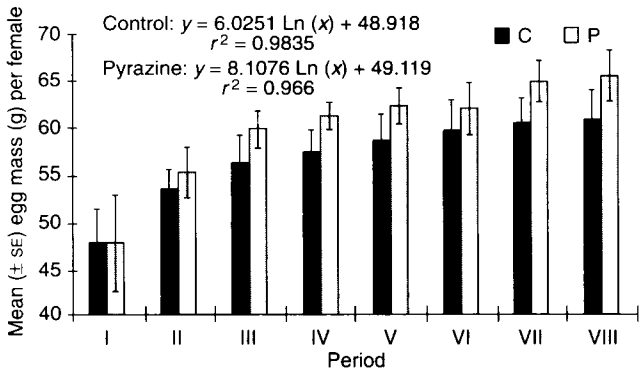

Fig. 1. Mean (± SE) egg mass (g) per female, in the two experimental groups, in eight periods of 2 weeks each, from the onset of odour treatment until the end of the experiment. C, control; P, experiment.

Egg numbers

A comparison of the mean number of eggs laid by a female for the entire experimental period showed that overall, (C) females ($n=8$) laid significantly more eggs than (P) females; 102.0 ± 4.9 *vs* 96.1 ± 6.07 eggs respectively, ($T_{15}=2.17$, $P=0.047$). When the data were considered with respect for the eight periods (Figs 1 & 2), it was seen that the difference occurred only during the periods I and II (the first 4 weeks). From then until the end of the experiments (periods III to VIII, 12 weeks) both groups laid a similar number of eggs.

The analysis showed that time had a significant effect on the number of eggs laid ($F_{7,105}=14.68$, $P=0.001$), which is demonstrated in Fig. 2 by a gradual increase in numbers of eggs laid at the beginning of the experiment, in both groups. In addition, the interaction between time and treatment was also significant ($F_{7,105}=2.13$, $P=0.047$), indicating that the effect of time on the number of eggs laid was different for the two groups. This differential effect existed only during the first 4 weeks (periods I and II) of pyrazine exposure, and disappeared later.

Body mass

No significant differences were found between the two experimental groups, for either gender, on any of the three tested dates (Table 1).

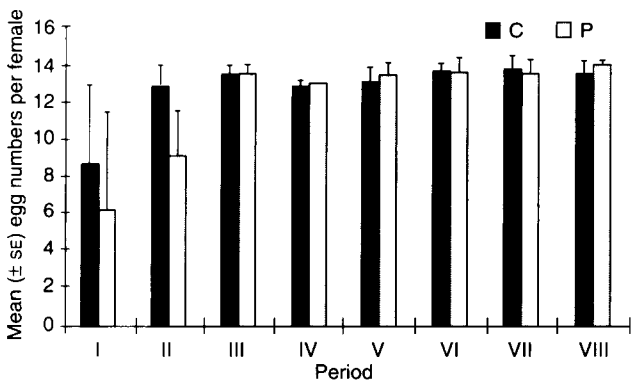

Fig. 2. Mean (± SE) egg numbers per female, in the two experimental groups, in eight periods of 2 weeks each, from the onset of odour treatment until the end of the experiment. C, control; P, pyrazine.

Table 1. Mean (± SE) body mass (g) of control and experimental chickens, on the three dates when tested. Numbers in parentheses, sample size

Date	Gender	Control body mass (g)	Experimental body mass (g)
1	Males	1553 ± 134 (9)	1624 ± 29 (9)
	Females	1160 ± 52 (10)	1174 ± 94 (10)
2	Males	1902 ± 145 (9)	1900 ± 102 (9)
	Females	1410 ± 70 (10)	1520 ± 136 (10)
3	Males	2215 ± 187 (4)	2104 ± 105 (9)
	Females	1553 ± 126 (8)	1576 ± 118 (10)

Eggshell thickness

Time, as well as exposure to pyrazine odour, during the first 8 weeks (periods II, III and IV) had no effect on eggshell thickness, in either group, (10 females in each). Values of mean eggshell thickness were in period II, 0.42 ± 0.03 mm for control eggs *vs* 0.40 ± 0.03 mm for pyrazine-exposed eggs; and respectively, in periods III and IV, 0.40 ± 0.03 mm *vs* 0.41 ± 0.03 mm and 0.43 ± 0.05 *vs* 0.41 ± 0.02 mm ($n = 50$ for both groups).

Hormonal blood concentrations

For the statistical analysis of oestrogen and testosterone concentrations, only birds for which there was data for all three test dates were included, i.e. 10(P) and 7(C) females for oestrogen; 6(P) and 4(C) males, 8(P) and 6(C) females for testosterone. There were no consistent significant differences in blood hormone concentrations between exposed and control birds (Fig. 3). In females, no significant differences were found in oestrogen concentrations and testosterone was significantly lower only in period III ($F_{1,17} = 19.75$, $P = 0.001$). However, the difference was no greater than before exposure. Values in birds following exposure were no different from

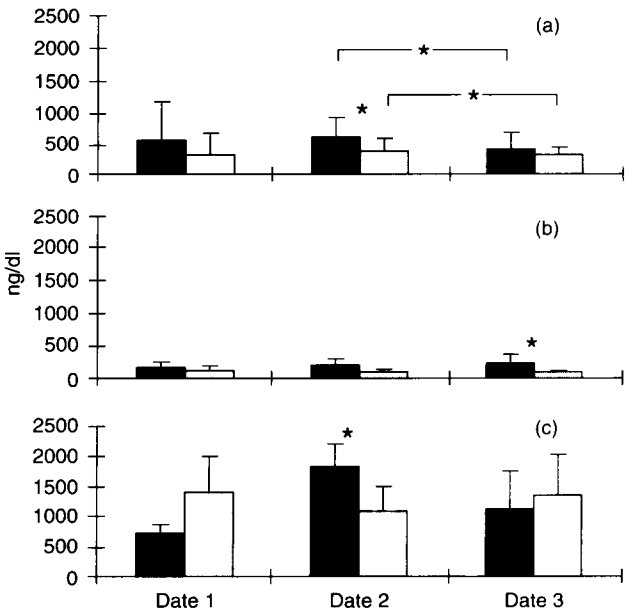

Fig. 3. Mean (± SE) hormonal blood levels (ng/dl) in the two experimental groups on three dates: prior to the onset of the odour treatment (date 1); 4 weeks after treatment began (date 2); 16 weeks after the onset of the odour treatment (date 3). (a) Oestrogen in females; (b) testosterone in females; (c) testosterone in males. *, significant difference; ■, control; ■, pyrazine.

values before exposure. Similarly, testosterone was significantly lower in exposed males in period II ($F_{1,11} = 8.54$, $P = 0.022$), but testosterone values in exposed males were not significantly different before or after exposure.

Examination of cloacal contents for pyrazine

No trace of alkylmethoxypyrazines were detected.

DISCUSSION

In his review of olfaction in birds Roper (1999) writes 'anatomical and physiological studies show that the Avian peripheral and central olfactory apparatus is similar in structure and function at both a macroscopic and microscopic level to that of other vertebrates'. The earlier view that birds possessed a poor sense of smell has been abandoned and 'the extent of olfactory development in birds is thought to be on a par with that found in mammals' (Keverne, 1999; Mason Russell & Clark, 2000).

The experiment described supports this belief, although it is essential to repeat it with a larger number of birds. We have shown that if exposed to the odoriferous volatile of synthetic 2-methoxy-3-isobutyl pyrazine, chickens lay larger eggs than the unexposed controls. We do not know how or why this change

occurs, nor why the expected drop in the number of larger eggs is lacking.

It would be interesting to replace methoxypyrazine with hydroxypyrazine in a similar experiment, since the latter is almost or entirely odourless to humans. It is possible that chemesthesis as well as olfaction is involved in the chickens' reaction to pyrazine. The trigeminal nerve (VIth cranial nerve in birds) is a major component of this system that also responds to odours (Mason Russell & Clark, 2000), but at present this is only speculation.

Jemiolo & Novotny (1994) demonstrated that the odour of synthetic 2,5-dimethyl pyrazine, which occurs naturally in the urine of white house mice, inhibits puberty in both juvenile sexes. Thus, in birds and mammals, the pyrazines exert a systemic effect, but in the experimental chickens the pyrazines came from an outside source as it has not been recorded from the bodies or cloaca of birds. However, in the field, birds will frequently come into contact with pyrazine, both in edible and inedible plants and insect prey (Woolfson & Rothschild, 1990), but only briefly and in minute quantities compared with continuous exposure of our experimental dilutions.

In our experiment, body mass did not differ between the two groups, and from this we infer that all birds remained in good health. Nevertheless, we could not assess the more subtle affect to their well-being from prolonged exposure to pyrazine volatiles, but it is quite possible that the smaller egg numbers of the experimental birds, during the first few weeks of oviposition, was the result of a primary depressing or stressful effect of the odour. The egg mass was already, by the third week, larger than that of the controls and the expected drop in numbers was lacking. Only two laying birds were available for comparison before the treatment began and their eight eggs were then similar both in size and weight.

Egg size is at least partly hormonally controlled (Scanes, 2000; Williams, Reed & Walzem, 2001), but there is a host of factors that could affect egg size and number (Meijer, 1992; Christian & Williams, 1999) such as genetic characters, latitude, altitude, day length, temperature, availability of food, and the size, age and behaviour of the hens. Furthermore, domestic chickens have been selected for such characters as continuous oviposition and the production of more and larger eggs, which may have influenced the results of our experiments. It is also possible that time, temperature or selection may have affected both our experimental and control birds. Nevertheless, the most probable influences on egg size are the gonadotrophic hormones, luteinizing hormone, follicle stimulating hormone and oestrogen (Williams, 1999; Johnson, 2000). However, in our study there were no differences in oestrogen concentrations which could have accounted for the larger eggs in pyrazine-exposed birds.

One possible mechanism for the effect of pyrazine on egg size relates to the timing of each ovulation, which is determined by the surge in gonadotrophin-releasing hormones (GnRH) from the hypothalamus (Johnson & van Tienhoven, 1980). Normally, the interval between successive surges slightly exceeds 24 h so that ovulation occurs somewhat later each day until the timing is later than the 'open-window'. There is no ovulation on that day, and so no egg is laid during the following 24 h. Thereafter, ovulation resumes early in the 'open-window', and the sequence is repeated. Exposure to pyrazine could inhibit GnRH surges and delay successive ovulations. This would be consistent with the deferred puberty observed in mice after exposure to pyrazine (Jemiolo & Novotny, 1994). Theoretically, pyrazine could influence the inter-ovulation period without affecting the total number of eggs laid, and a longer inter-ovulation interval would allow more time for yolk and albumin to accumulate Meijer (1992) has demonstrated that this has resulted in a similiar development of larger eggs in starlings. However, as we have shown, in both groups of chickens, egg production increased to *c.* 13.5 eggs each 14-day period, allowing little scope for this scheme to operate

Such a potential mechanism would imply a direct neural or neuroendocrine effect of pyrazine odour (Curtet *et al.*, 2000; Sanchez & Arnt, 2000) within the hypothalamus. The odour of pyrazine could, within the hypothalamus and pituitary gland, also influence the avian antidiuretic hormone arginine vasotocin (AVT), which also controls oviposition in birds (Scanes, 2000). Pyrazine has been used in medicine as a diuretic for over 30 years (Barlin, 1982) and the odour, as we have shown experimentally, can influence the size of eggs in the chicken. This suggests there could be a link between the odoriferous pyrazine volatile and the action of AVT.

Considering the reaction of mice and other small mammals to the smell of pyrazine, it is not surprising to find that it can influence the reproductive system of birds, especially as there is a world-wide occurence of this alkaloid in both plants and animals (Brophy, 1989; Woolfson & Rothschild, 1990). It is likely to be encountered on every foraging sortie of a passerine or phasianid bird.

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REFERENCES

Barlin, G. B. (1982). The pyrazines. *The chemistry of heterocyclic compounds*: 1–10. Weissberger, A. & Taylor, E. C. (Eds). Chichester: Wiley.

Barnea, A., Gvaryahu, G. & Rothschild, M. (In press). The effect of the odour of pyrazine and colours on recall of past events and learning in domestic chicks (*Gallus gallus domesticus*). In *Insects, birds and interaction*. van Emden, H. & Rothschild, M. (Eds). Intercepts.

Brophy, J. J. (1989). Pyrazines obtained from insects, their source, identification, syntheses and function. In *Studies in natural products chemistry* **5** *Structure and elucidation (B)*: 221–273. Atta-ur-Rahmar (Ed.). Amsterdam: Elsevier.

Christian, J. K. & Williams, T. D. (1999). Effects of exogenous 17-β estradiol on the reproductive physiology and reproductive performance of European starlings (*Sturnus vulgaris*). *J. exp. Biol.* **202**: 2679–2685.

Curtet, S., Soulier, J. L., Zahradnik, I., Giner, M., Berque-Bestel, I., Mialet, J., Lezoualc'h, F., Donzeau-Gouge, P., Sicsic, S., Fischmeister, R. & Langlois, M. (2000). New arylpiperazine derivatives as antagonists of the human cloned 5-HT4 receptor isoforms. *J. med. Chem.* **43**(20): 3761–3769.

Jemiolo, B., Androlini, F. & Novotny, M. (1986). Chemical and biological investigations of female mouse pheromones. *Chem. Signals Vertebr.* No. 4: 79–85.

Jemiolo, B. & Novotny, M. (1994). Inhibition of sexual maturation in juvenile female and male mice by a chemosignal of female origin. *Physiol. Behav.* **55**(3): 519–522.

Johnson, A. L. & van Tienhoven, A. (1980). Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, *Gallus domesticus*, *Biol. Reprod.* **23**: 386–393.

Johnson, A. L. (2000). Reproduction in the female. In *Sturkie's avian physiology*: 569–596. 5th edn. Causey Whittow, G. (Ed.). San Diego, CA: Academic Press.

Keverne, E. B. (1999). The vomeronasal organ. *Science Olfaction* **286**(5440): 716–720.

Mason Russell, J. & Clark, L. (2000). The chemical senses in birds. In *Sturkie's avian physiology*: 39–56. 5th edn. Causey Whittow, G. (Ed.). San Diego, CA: Academic Press.

Meijer, T. (1992). Egg-laying patterns in captive starlings. *Ardea* **80**: 301–310.

Roper, T. J. (1999). Olfaction in birds. *Adv. Stud. Behav.* **28**: 247–332.

Sanchez, C. & Arnt, J. (2000). In-vivo assessment of 5-HT2A and 5-HT2C antagonistic properties of newer antipsychotics. *Behav. Pharmacol.* **11**: (3–4): 291–298.

Scanes, C. G. (2000). Introduction to endocrinology: pituitary gland. In *Starkie's avian physiology*: 437–460. 5th edn. Causey Whittow, G. (Ed.). San Diego, CA: Academic Press.

Sokal, R. R. & Rohlf, F. J. (1981). *Biometry. The principles and practice of statistics in biological research*. New York: Freemand.

Williams, T. D. (1999). Avian reproduction, overview. In *Encyclopedia of Reproduction* **1**: 325–336. London: Academic Press.

Williams, T. D., Reed, W. L. & Walzem, R. L. (2001). Egg size variation: mechanisms and hormonal control. In *Avian endocrinology*: 205–217. Dawson, A. & Chaturvedi, C. M. (Eds). New Delhi: Narosa.

Woolfson, A. & Rothschild, M. (1990). Speculating about pyrazines. *Proc. R. Soc. Lond. Biol. Sci.* **242**: 113–119.