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**Feeding on grains containing pesticide residues is detrimental to offspring development through parental effects in Grey partridge**

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## **Abstract**

Numerous toxicological studies have shown that ingestion of pesticides can induce physiological stress in breeding birds, with adverse consequences on egg laying parameters and offspring quality through parental effects. However, previous studies do not mimic current levels of pesticide residues in typical landscapes, and they do not consider potential cocktail effects of pesticides as they occur in the wild. Herein, we explored whether realistic pesticide exposure affected reproduction parameters and offspring condition through parental effects in Grey partridge. We fed 24 breeding pairs with either seeds from conventional agriculture crops treated with various pesticides during cropping, or organic grains without pesticide residues as controls. The conventional and organic grain diets mimicked food options potentially encountered by wild birds in the field. The results showed that ingesting low pesticide doses over a long period had consequences on reproduction and offspring quality without altering mortality in parents or chicks. Compared with organic pairs, conventional pairs yielded smaller chicks at hatching that had a lower body mass index at 24 days old. Additionally, these chicks displayed lower haematocrit when body mass index was higher. Therefore, ingestion of conventional grains by parents resulted in chronic exposure to pesticide residues, even at low doses, and this had detrimental consequences on offspring. These results demonstrate a sublethal effect of pesticide residues through parental effects. The consequences of parental exposure on chicks might partly explain the decline in wild Grey partridge populations, which raises questions for avian conservation and demography if current agrosystem approaches are continued.

**Keywords:** pesticide residues, cocktail effect, parental effects, offspring growth, farmland birds

## **Introduction**

To increase food productivity for humans, farmland landscapes have experienced major changes over recent decades that have affected ecosystem functions and biodiversity (Stanton et al. 2018). Agricultural intensification involving massive use of pesticides (DiBartolomeis et al. 2019; Malaj et al. 2020) has altered population dynamics of farmland birds (Mineau and Whiteside 2013; Hallmann et al. 2014; Gibbons et al. 2015; Stanton et al. 2018). Numerous toxicological studies on farmland birds showed that exposure to pesticides can impact reproduction by altering endocrine, neurological and immunological systems, and thereby adversely affect the health and behaviour of breeding birds and offspring (Mitra et al. 2011; Pandey et al. 2017). For example, pesticides have been shown to alter carotenoid-based secondary sexual traits involved in sexual selection (Lopez-Antia et al. 2015a; Pandey et al. 2017; Moreau et al. 2021), testicular histology, testosterone levels and sperm production (Grote et al. 2008; Mohanty et al. 2017), as well as acetylcholinesterase (AChE) activity, leading to decreased activity and body weight loss (Eng et al. 2017, 2019). Regarding effects on reproduction, pesticides can reduce clutch size (Lopez-Antia et al. 2021b), delay laying date of the first egg (Lopez-Antia et al. 2015b), induce egg infertility (Lopez-Antia et al. 2013), alter egg shape (Lopez-Antia et al. 2015b), decrease egg volume (Lopez-Antia et al. 2015b; Moreau et al. 2021), and more generally diminish hatchability, ultimately affecting the number of fledglings (Bennett et al. 1991; Hernández et al. 2008). Pesticides can also affect chick survival by lowering body weight, haematocrit and AChE activity in blood (Ahmad et al. 2015), altering lipid metabolism (Liu et al. 2019) and skeletal growth (Garg et al. 2004), and causing hypoactivity (Albadrany and Mohammad 2007).

In birds, egg quality and consequently chick survival are influenced by the body condition and health status of the laying female (Blount et al. 2002; Cucco et al. 2010).

Indeed, mating behaviour and hormones transferred from mother to egg are known to influence the development, physiology, morphology, immunity, behaviour and survival of her progeny through parental effects (Groothuis et al. 2005; Wolf and Wade 2009; Ruuskanen et al. 2015; Ruuskanen and Hsu 2018). For example, egg shape (e.g., Lopez-Antia et al. 2015b) and volume (e.g., Hernández et al. 2008) can be impacted by exposure of the mother to pesticides, with consequences for the quantity of nutritive resources available to the embryo during its development (Lopez-Antia et al. 2015b). Females can also deposit more resources in their eggs to prepare their offspring for the toxic environment that awaits them (Lopez-Antia et al. 2015a, 2015b). Another pathway, that is not mutually exclusive from that described above, involves the transfer of pesticide residues from mother to egg due to the typically lipophilic nature of such contaminants (Donoghue and Myers 2000; Bro et al. 2016), thereby exposing chicks to these substances during embryonic development (Liu et al. 2019; Ruuskanen et al. 2020).

Embryonic exposure to environmental pollutants can have severe consequences on chick development, affecting a wide range of life-history traits (Ottinger et al. 2008, 2009). Elimination of pesticides requires activation of various detoxification systems in an organism (Walker 1983), and energy and resources allocated to biodegradation of pesticides cannot be allocated to other functions such as growth (Liu et al. 2019). In addition, the detoxification capacities of embryos and chicks are lower than those of adults (Liu et al. 2019). Consequently, the presence of toxins in eggs may slow down embryonic growth. Unfortunately, such results are usually reported from laboratory experiments involving exposure to a single pesticide, often at high dose directly in the parent's food. Such experimental data are not representative of reality because they do not mimic (i) the current levels of pesticide residues in conventional landscapes, (ii) the potential cocktail effects of pesticides, and consequently (iii) the parental transfer of molecules as may occur in the wild.

Indeed, in agroecosystems, the majority of grains consumed by birds during autumn and winter are not directly treated (i.e., pesticides applied to crops), in contrast to ‘coated’ sowing seeds (i.e., directly treated with pesticides) which are mostly avoided by birds given the choice (Mineau and Palmer 2013; Lopez-Antia et al. 2014). Thus, levels of pesticide residues in avian food such as grains are believed to be low (Moreau et al. 2021). In addition, many pesticides are rarely applied alone; they are typically applied as complex formulations (i.e., pesticide cocktails), simultaneously or sequentially, potentially influencing interactions among molecules and their consequences in organisms (Hernández et al. 2017). Co-exposure to several pesticides has the potential to produce additive and/or synergistic cocktail effects (Mayne et al. 2005; Cedergreen 2014; Pandey et al. 2017). For example, an experiment using environmentally realistic pesticide concentrations showed that a combination of three organophosphates and two carbamates, applied at sublethal concentrations based on individual application, was lethal to Coho Salmon *Oncorhynchus kisutch* (Laetz et al. 2009). To date, studies assessing chronic and realistic exposure to pesticide cocktails have mainly been conducted in aquatic species, and few studies have investigated this risk in birds (Moreau et al. 2021).

Our knowledge on the consequences of exposing parents to environmentally realistic pesticide cocktails on the life-history traits of chicks is limited. In this study, we explored whether the reproductive parameters of adult grey partridge (*Perdix perdix*) were affected by realistic pesticide exposure (low dose and cocktail effects) and assessed the consequences on offspring condition. This flagship European farmland bird species is still declining despite many reinforcement population programs (Bro 2016). Currently, less than 10% of land use in France is organically farmed (AGRESTE 2020), thus grey partridges are more likely exposed to conventional than organic grains, with certainly most individuals confronted all their lives with seeds containing pesticides as their home range is limited (i.e., 7 to 15 ha per bird

depending on their life cycle; Bro 2016). We used an original experimental procedure involving 24 breeding pairs fed for 4 months with grain, with half of the pairs being fed with grains purchased from producers that grew crops under conventional agriculture with various pesticide applications during cropping, while the other pairs received organic grains (without pesticide residues). The health condition of parents was monitored individually throughout captivity and assessed by (i) haematocrit reflecting metabolic activity and nutritional state (Ots et al. 1998), (ii) body mass index reflecting the energy capital accumulated by feeding (Peig and Green 2009) and (iii) AChE activity as a proxy of nerve impulse transmission. Egg volume and hatching date were compared between the two groups. Finally, chick condition (all fed equally to test parental effects) was monitored for 1 month using skeletal growth and body mass index. At 1 month old, behavioural traits (i.e., exploration, general activity level and escape ability), general nervous system (AChE activity) and general condition (haematocrit) were also quantified as a proxy of the likelihood of survival. We predicted that adults fed with conventional grains for 4 months would experience physiological stress (i.e., weight loss, lower haematocrit, decreased AChE activity) due to chronic ingestion of a cocktail of pesticides. Furthermore, we predicted that this physiological stress would alter reproduction success, resulting in smaller eggs, delayed laying date, and smaller and weaker chicks.

## **Materials & Methods**

All experiments complied with French laws on animal experimentation, and all experimental protocols were approved by the Committee of Animal Experimentations of the Deux-Sèvres French Department (APA FIS#9465-201703101551625).

### **(a) Experimental design**

The study took place in a commercial game farm in south-western France using a wild genetic strain (the F3 generation of wild trapped birds) on 7-month-old captive-born birds (n = 64) and lasted 8 months, from the 2<sup>nd</sup> of November 2020 to the 30<sup>th</sup> of June 2021 (Fig. 1). Before experimentation, all birds lived in the same pen and were fed commercial poultry food.

On the 2<sup>nd</sup> of November, each adult was sexed (according to the presence of secondary sexual characters), fitted with an alphanumeric metal ring, random pairs were established and placed in outdoor reproduction cages (80 cm × 50 cm × 50 cm) equipped with feeders, drinkers and metal sheet for shelter. Two experimental food treatments were given to 16 pairs selected randomly. In the first treatment, individuals were fed *ad libitum* with grains harvested from certified organic crops (i.e., purchased from organic producers), composed of 25% wheat, corn, pea and faba bean (organic pairs; Fig. 1). In the second treatment, birds were fed *ad libitum* with the same mixture of grains purchased from producers using conventional agriculture in which various pesticides were applied during the cropping season (conventional pairs; Fig. 1, ESM 1). Pesticide residues were quantified by GIRPA (Beaucouze, Loire Atlantique, France) with a limit of quantification at 0.010 mg/kg (ESM 2). Pesticide residues were found in the four conventional seeds: 2 organophosphate insecticides (chlorpyrifos-methyl and pirimiphos-methyl) were found in wheat and pea, 1 insecticides synergist (piperonyl butoxide) in corn and 1 triazole fungicide (tebuconazole) in faba bean (see ESM 2 for concentration details). Chlorpyrifos-methyl and pirimiphos-methyl act as AChE inhibitors and are usually applied with synergist like piperonyl butoxide, a cytochrome P450s inhibitors that delay the detoxification process (Jones 1998; Narváez et al. 2016). In the same way, tebuconazole is known as an endocrine disruptor for vertebrates (Zarn, Brüschweiler and Schlatter 2003), decreasing the levels of cholesterol and triglycerides (Lopez-Antia et al. 2018; Fernández-Vizcaíno et al. 2020) and is also involved in bone



demineralization (Wilson and Thorp 1998; Ortiz-Santaliestra et al. 2020). Conversely, a previous study (Moreau et al. 2021) showed that the energetic contents for both types of seed (organic and conventional grains) were the same.

On the 20<sup>th</sup> of January, pairs were exposed to light to trigger reproduction (Woodard et al. 1986). On the 15<sup>th</sup> of February, food treatments (i.e., mixtures of organic and conventional grains) were stopped and all pairs were then fed organic poultry (GAMM VERT Bio) enriched with proteins to support reproductive effort (Fig. 1). During winter, 14 partridges inadvertently died (5 in organic and 9 in conventional treatments); this mortality rate is typical for a breeding farm. Single individuals were re-paired randomly by food treatment when possible. Finally, 24 pairs were monitored up to egg laying (13 pairs in organic and 11 in conventional treatments).

Once laying began (first egg laid on the 26<sup>th</sup> of February by a conventional pair; Fig. 1), eggs were collected daily and stored in the dark at 15°C (Gómez-de-Travedo et al. 2014). Although captive Grey partridges can lay up to 40 eggs per season, wild birds lay on average 15–20 eggs (Bro 2016). To better approximate natural conditions for reproduction, we stopped collecting eggs when each pair had laid 20 eggs. Overall, 267 eggs were laid by the 13 organic pairs and 198 eggs were laid by the 11 conventional pairs. Eggs were placed in an incubator (37.8°C, 40% humidity) and candled (i.e., observation by transparency of the internal egg structure to confirm fertilisation) after 21 days of incubation. Only fertilised eggs were placed in a hatcher (37.7°C, 80% humidity) for 3 days. Overall, 107 eggs hatched successfully from organic pairs compared with 67 from conventional pairs.

Immediately after hatching (less than 5 h), chicks were removed from the hatcher and placed in a building (5 m × 5.5 m × 1.50 m, 37°C, 30.5 lux) divided into density-adapted compartments (50 cm × 100 cm × 50 cm) to separate chicks based on their parent's food treatment (i.e., parents that had been fed organic or conventional treatment, hereafter 'organic

chicks' and 'conventional chicks'). Compartments were covered by sawdust, and water and special feed for Grey partridge chicks (STARGIB G111 1er age) were provided *ad libitum* regardless of parent's food treatment. From day 3 to 24, each chick was monitored to assess morphological characteristics (Fig. 1). Behavioural and physiological variables were assessed when chicks reached 24 days old (Fig. 1). Sixty-nine chicks died and 105 survived during this period, including 64 organic and 41 conventional chicks. Grey partridge chicks are fragile, and this mortality rate is typical for a game farm (Blandin, pers. com.).

### **(b) Monitored parameters**

#### *Parents*

During the experiment, parents were monitored before food treatment on the 2<sup>nd</sup> of November and after having laid 20 eggs, or when they stopped laying, to assess their individual condition through body mass index, haematocrit and AChE activity. No sampling or measurement was carried out during partridge breeding to avoid disturbance (Fig. 1). Right and left tarsus lengths were measured twice with callipers (accuracy 0.1 mm), before food treatment and once in adulthood, and tarsus lengths remained stable in all birds. Body mass was recorded before food treatment and after laying using a spring scale (MS500 Pesola, accuracy 0.1 g). From body mass and mean tarsus lengths, we assessed body mass index (BMI) using the scaled mass index (Peig and Green 2009; Moreau et al. 2021). To assess haematocrit and AChE activity, blood samples were extracted from the brachial vein using a sterile needle and heparinised micro-capillary tubes. Blood samples were centrifuged immediately afterwards to determine haematocrit levels using the proportion of the tube filled with red blood cells divided by the total sample volume in the capillary tube, as a ratio of respective tube length (Biard et al. 2015). Length was measured with electronic callipers to the nearest 0.1 mm. The remaining blood was stored in Eppendorf tubes at -80°C until

analysis. For AChE activity assessment, a 50  $\mu\text{L}$  aliquot of blood was diluted in sodium phosphate buffer containing 1% Triton X-100 and AChE activity was measured at 324 nm at 37°C using a SpectraMax iD3 instrument (Molecular Devices Inc., Sunnyvale, CA, USA). Results were expressed in milliunits per minute (mU/min).

### *Eggs*

From the beginning of laying until each pair had laid 20 eggs or stopped laying, eggs were collected daily, and labelled with the pair number, laying date and laying rank. The mass (MS500 Pesola, accuracy 0.1 g), length and width (callipers, accuracy 0.1 mm) of each egg was measured before incubation to calculate the volume ( $V = 0.51 \times \text{length} \times \text{width}^2$ ; Hoyt 1979)

### *Chicks*

First, hatching rate (i.e., number of chicks hatched compared to the total number of laid eggs) was calculated, and 3-day-old chicks that were fit enough were ringed with an alphanumeric coloured metal ring.

#### Skeletal growth and body mass index

Skeletal growth and body mass index of chicks were monitored each week at 3, 10, 17 and 24 days old. Skeletal growth was assessed by measuring head-beak, tarsus and wing length (left and right) twice with callipers (accuracy 0.1 mm). Body mass was also recorded (MS500 Pesola, accuracy 0.1 g). From body mass and mean tarsus length (right and left), we evaluated BMI using the same scaled mass index applied for adults.

#### Behaviour and physiology

The behaviour of chicks at 24 days old was assessed in an open field test in the same building where chicks lived to maintain identical conditions (temperature, luminosity, humidity and proximity to other chicks). The open field design included two wooden boxes (100 cm × 100 cm × 30 cm) with a grid set on the floor (100 squares of 10 cm × 10 cm each), a removable plexiglass cover pierced with holes (diameter 4 cm), and two cameras (Muvi Série VCC-007-K2PRO 4K) placed perpendicular at 180 cm above the two boxes (Fig. 1). Chicks were caught 30 min before the open field test and separated based on parent's treatment (organic vs. conventional) into a dark cardboard box (66 cm × 38 cm × 17.5 cm). One chick per treatment was randomly introduced into the open field, with one chick per box. Once the chick was in the box, a camera began recording for 4 min. The experimenter then left the building and waited outside to avoid disturbing the chick. After 4 min, the experimenter returned to the building to catch the two chicks and release them into their compartments with food and water. Next, another two randomly caught chicks were subjected to the same treatment, and this was repeated until all chicks were tested (n = 105). Camera footage was then analysed by the same person *a posteriori*. Exploration, activity and escape attempts were assessed for each chick after the first minute, which was considered an acclimatisation period. Exploration was assessed by counting the number of visited squares during the 3-min period, activity was assessed by the time spent moving/in motion or walking, and escape attempts were assessed by counting jump and flight attempts. The same day, blood was sampled from all chicks to assess haematocrit and AChE activity, as described above for adults.

The number of dead chicks was monitored daily to calculate survival rate for each treatment (from parents fed organic or conventional feed). After behaviour and physiology measurements, rings were removed, and chicks were released in an outside aviary with other chicks of the same age.

### **(c) Statistical analyses**

#### *Parents*

We analysed the effects of food treatment (organic vs. conventional) on parent's BMI, haematocrit and Ache activity at three different times: (i) at the onset of the trial to check potential sampling bias; (ii) at the end of the laying period (i.e., when each pair had laid their first 20 eggs) to test for differences in individual condition); and (iii) between the onset and the end of the experiment to investigate potential differences in adjustment between food treatment. We performed analysis of variance (ANOVA) to investigate the effects of food treatment (organic vs. conventional), sex (male vs. female), and two-way interaction on BMI, haematocrit and AChE activity.

#### *Eggs*

We compared the number of eggs laid and the hatching rate between organic and conventional pairs using Wilcoxon rank sum tests. The effects of parent's food treatment on egg volume, egg-laying rank, and two-way interactions were analysed using linear mixed-effects models (LMM) and likelihood ratio tests (LRT), including the identity of pairs as a random factor. We chose to use a Cox mixed-effects model using pair identity as a random effect to test the effect of parent's food treatment on egg-laying dynamics because it is a robust model for testing the probability of occurrence of binary events such as laying. The proportional hazards assumptions was assessed *a posteriori* (Moreau et al. 2021).

#### *Chicks*

To test the effects of parent's food treatment on chick survival at 3, 10, 17 and 24 days old, we performed a Chi-squared test comparing the number of organic and conventional dead chicks. To assess skeletal growth, we conducted four principal component analysis (PCA)

tests at each age of monitoring (3, 10, 17 and 24 days old; Fig. 1) from tarsus, head-beak and wing length, given their high correlations. Scores of individuals on the first axes of the four PCs (PC1) represented synthetic variables accounting for morphology at each age. We then explored the effects of parent's food treatment on PC1 scores using LMM and LRT including parent's food treatment, age (3, 10, 17 and 24 days old), and two-way interactions with chick identity as a random effect. The same model was used to test variation in BMI. Finally, pairwise post hoc comparison tests between the two food treatments at each age were tested using Bonferroni correction.

We tested the effect of parent's food treatment on chick behaviour and physiological condition at 24 days old. Given the number of behavioural traits and their potential correlations, we conducted PCA including activity (time), exploration (number of visited squares) and escape attempts of chicks (number of attempts) to generate a variable for behaviour. We then explored the effects of parent's food treatment on PC1 behavioural scores, haematocrit and AChE activity with chick's BMI at week 4 as a covariable, and two-way interactions between parent's food treatment and BMI, using linear regression models (LM) with *F*-ratio tests.

Normality of residuals and distribution of residuals against fitted values were checked for each model. All statistical tests were performed using R software v. 3.6.1 (R Core Team, 2020) using the *lme4* package for LMM, *car* for deviance analysis, *smatr* for scale mass index computation (Peig and Green 2009), and *coxme* for the Cox mixed-effects model.

## Results

### (a) Adults

The effects of food treatment on parent's physiological condition are presented in Table 1. Before food treatment, female BMI was  $369.3 \pm 24.3$  g (mean  $\pm$  standard error [SE]),

significantly (4.3%) higher than male BMI ( $354.2 \pm 23.4$  g), while after laying, the difference reached 24.4% ( $452.1 \pm 39.1$ g for females,  $363.3 \pm 51.7$  g for males). During the 4-month food treatment experiment, BMI increased for all birds, but more for females than males (Fig. 2a). No effect of food treatment on BMI was detected.

At the onset of the experiment, haematocrit was similar for both food treatment groups and sexes, but after laying haematocrit in females was significantly lower (-16.7%) than in males (females  $35.9 \pm 4.3\%$ , males  $43.1 \pm 3.6\%$ ). While haematocrit decreased in all birds during the experiment, it decreased more in females than males (Fig. 2b). No effect of food treatment on haematocrit was detected.

Before food treatment, AChE activity was 2.2% higher for conventional pairs (females  $26.55 \pm 9.12$  mU/min, males  $31.9 \pm 10.42$  mU/min) than for organic pairs (females  $22.37 \pm 4.75$  mU/min, males  $25.9 \pm 9.26$  mU/min), regardless of sex, highlighting a sampling bias (Fig. 2c). After laying, AChE activity was significantly higher (31.1%) in females than males (females  $39.6 \pm 11.7$  mU/min, males  $30.2 \pm 10.3$  mU/min). During the 4-month food treatment, AChE activity increased for all birds in all groups, but more in females than males, except for one of the conventional males which remained stable (Fig. 2c).

### **(b) Eggs**

The number of eggs laid by females did not differ with parent's food treatment. Indeed, organic pairs ( $n = 13$  pairs) laid 267 eggs while conventional pairs ( $n = 11$  pairs) laid 198 eggs (Wilcoxon rank sum test:  $W = 112.5$ ,  $P = 0.13$ , Table 2). Hatching rate (% eggs hatched) did not vary between parent's food treatment, with 45.3% for organic pairs vs. 34.5% for conventional pairs ( $W = 88$ ,  $P = 0.86$ , Table 2). Egg volume of organic pairs did not differ from conventional pairs (organic eggs  $12.11 \pm 0.91$  mm<sup>3</sup>, conventional eggs  $11.77 \pm 0.86$  mm<sup>3</sup>; LMM,  $\chi^2 = 0.15$ ,  $df = 1$ ,  $P = 0.71$ ; Fig. 3). Although egg volume increased with egg-

laying rank ( $\chi^2 = 8.73$ ,  $df = 1$ ,  $P = 0.003$ ), the slope was not affected by parent's food treatment as the two-way interaction treatment  $\times$  rank was not significant ( $\chi^2 = 2.25$ ,  $df = 1$ ,  $P = 0.13$ ; Fig. 3). Egg-laying dynamics was also not affected by parent's food treatment (Cox mixed-effects model, parent's food treatment; coef = -0.23, se [coef] = 0.22,  $P = 0.31$ ).

### (c) Chicks

Chick survival was not affected by parent's food treatment at 3 days old (Chi-squared test,  $\chi^2 = 0.02$ ,  $df = 1$ ,  $P = 0.89$ ), nor at 10 days old (Chi-squared test,  $\chi^2 = 0.24$ ,  $df = 1$ ,  $P = 0.62$ ), 17 days old (Chi-squared test,  $\chi^2 = 0.75$ ,  $df = 1$ ,  $P = 0.39$ ) or 24 days old (Chi-squared test,  $\chi^2 = 0.15$ ,  $df = 1$ ,  $P = 0.7$ ; see Table 2 for survival rates). Morphological and physiological parameters of chicks are summarised in ESM 3. The first axis for each skeletal growth PCA was consistently highly and positively correlated with the three variables (correlation values in ESM 4). Skeletal growth PCA scores showed that parent's food treatment affected chick's skeletal growth at 3 days old only for conventional chicks larger than organic chicks (Table 3; Fig. 4a). BMI increased during growth, and organic chicks were 4.4% heavier than conventional chicks (organic chicks  $66.3 \pm 8.3$  g, conventional chicks  $63.5 \pm 6.7$  g) at 24 days old (Fig. 4b).

PC2 values (behavioural parameters) assessed at 24 days were high and positively correlated with PC1 scores for activity, exploration and escape attempts (correlation values in ESM 5). The effects of parent's food treatment on PC1 behavioural score and physiological condition of chicks are presented in Table 4. We did not detect any differences in behaviour or AChE activity between chicks from the two parent's food treatment groups (Table 4). Interestingly, conventional chicks displayed a trade-off between haematocrit and BMI (i.e., lower haematocrit with higher BMI) that was not observed for organic chicks (Fig. 5, Table 4).



## Discussion

In this study, we explored the sublethal effects of pesticide cocktail ingestion (organic vs. conventional grains) on adult Grey partridge by assessing reproduction and the consequences on chick growth and health through parental effects. Parents' condition (BMI, haematocrit and AChE activity) and laying parameters (number of eggs laid, hatching rate, egg volume and egg-laying sequence) were unaffected by food treatment. Conversely, chick skeletal growth was affected by parent's food treatment at 3 days old, with conventional chicks smaller than organic chicks, and although they caught up over time, their BMI was lower than that of organic chicks at 24 days old. Finally, it appears that only 24-day-old conventional chicks displayed a trade-off between BMI and haematocrit; larger chicks exhibited lower haematocrit. These results suggest the existence of a parental effect following exposure to sublethal but environmentally realistic pesticide doses.

Early life development is a strong determinant of survival in nature, with high selection pressure on growth rate, driving individuals to reach a large size and mass as quickly as possible (Metcalf and Monaghan 2001; Criscuolo et al. 2008; Monaghan and Hausmann 2015). Environmental conditions experienced during early development thus influence various life-history traits related to survival and/or reproductive success (see Lindström 1999 for a review). Indeed, numerous studies on birds show a link between juvenile growth and fitness such as pre- and post-independence survival (Stienen and Brenninkmeijer 2002; NaefDaenzer and Grübler 2016), adult reproductive success (Haywood and Perrins 1992) and territory acquisition (Verhulst et al. 1997). In birds, embryonic and post-hatching growth rely exclusively on resources contained in the egg, such as size and mass, yolk and albumen composition, water content, and shell thickness and structure (Blom and Lilja 2004). Many studies have reported a strong positive correlation between egg volume and hatching size (see Krist 2011 for a review), or between egg quality and BMI and health status of laying females

(Blount et al. 2002; Hayward and Wingfield 2004; Cucco et al. 2010). Surprisingly, we did not observe any influence of food treatment on parent's condition, in contrast to a previous study on Grey partridge (Moreau et al. 2021). We showed that female body mass increased as haematocrit decreased during the experiment, regardless of food treatment. This indicates that allocation of energy to body mass and/or egg production lowered haematocrit by impairing the production of red blood cells (Cox et al. 2010). Female AChE activity also increased after laying 20 eggs, supporting the physiological activity necessary for reproduction. We also found no difference in egg number, egg volume, egg-laying chronology or hatching rate between organic and conventional pairs, suggesting either a lack of statistical power (only 13 organic pairs and 11 conventional pairs) or the absence of an effect. By contrast, we observed differences in skeletal growth at 3 days old and body mass at 24 days old between organic and conventional chicks, suggesting that conventional females transferred pesticide residues into their eggs. Indeed, in birds, during egg formation, females transfer various substances to embryos, including essential components (water, minerals, proteins, lipids, vitamins, antibodies and hormones) and some lipophilic contaminants encountered during adult life (Donoghue and Myers 2000; Kitulagodage et al. 2011; Guo et al. 2018). Our current results suggest that conventional females may detoxify molecules in their eggs, as occurs for mercury (see Ackerman et al. 2020 for a review), perfluoroalkyl acids in seabirds (Lopez-Antia et al. 2021a), organochlorine pesticides in Great tit (Van den Steen et al. 2009) and glyphosate (Ruuskanen et al. 2020). A likely candidate is tebuconazole, a fungicide whose residues were present in our conventional grains (ESM 2), for which molecules were detected in eggs of contaminated parent's in Zebrafish (Li et al. 2019) and House sparrow (Bellot et al. 2022, in prep.), but we cannot exclude other molecules. However, to date, no non-lethal technique to assess pesticides residues in egg yolk without interfering with embryo viability

exists (Pacyna-Kuchta 2022), thus the only way to determine if eggs from organic pairs contain fewer pesticides than eggs from conventional pairs would be to sacrifice eggs.

The presence of such molecules in eggs could be responsible for the smaller size of conventional chicks compared with organic chicks at the beginning of their growth since egg volume did not differ significantly between parents receiving different food treatments. Tebuconazole is a well-known endocrine disruptor in birds, decreasing the levels of cholesterol and triglycerides (i.e., lipid reserves) in juvenile Japanese quails (Lopez-Antia et al. 2018; Fernández-Vizcaíno et al. 2020) and leading to alkaline phosphatase accumulation (i.e., an enzyme increasing in response to bone demineralization) in red-legged partridge chicks (Ortiz-Santaliestra et al. 2020). The delayed skeletal growth of the conventional chicks after hatching could therefore be possibly explained by tebuconazole residues in eggs. While they appeared to catch up with organic chicks in terms of skeletal growth as early as 10 days old, our results showed that BMI remained lower at 24 days old. Skeletal growth is one of the most energetically demanding stages in vertebrate life cycles, and it may lead to a trade-off in resource allocation (Garg et al. 2004). Indeed, embryonic exposure to environmental pollutants may have severe consequences on chick development, impacting a wide range of life-history traits through a balance between organism's maintenance for growth and detoxification processes (Ottinger et al. 2008, 2009). For example, broiler chicks contaminated with chlorpyrifos showed a decrease in body weight, haematocrit and AChE activity (Ahmad et al. 2015). Chlorpyrifos-methyl, and pirimiphos-methyl, both organophosphates (OPs hereafter) were found in our conventional grains, and act as AChE inhibitors. One way of detoxification to prevent from AChE inhibition consists in catalysing the dearylation of these molecules through cytochrome P450s to facilitate the excretion in water-soluble metabolites (Narváez et al. 2016). In addition to both OPs, piperonyl-butoxyde (i.e., an insecticide synergist found in our conventional grains, see ESM 2) may emphasize the

energy costs in the detoxification process as it is a cytochrome P450s inhibitor (Jones 1998). In our case, the synergist cocktail of chlorpyrifos-methyl, pirimifos-methyl and piperonyl-butoxide residues may have been transmitted into eggs, exhibiting conventional chicks to higher energetic costs than organic ones in order to reduce the impact of these pesticides through detoxification. Indeed, when maintenance costs essential to life (i.e., metabolic rate, tissue reparation, detoxification, etc.) become priority, energy spent on growth should be lowered (McNab 2002). This hypothesis is supported by a higher BMI after 1 month of growth associated with a lower haematocrit in conventional chicks, whereas BMI was not related to haematocrit in organic chicks. Similarly, conventional chicks appear to face another trade-off between fat storage and red blood cell count; studies suggest that the 'compensatory growth' that juveniles may exhibit under certain conditions inducing physiological stress (e.g., pesticide detoxification) is associated with varying long-term costs on many other life-history traits (Metcalf and Monaghan 2001; Honarmand et al. 2010; Krause and Naguib 2011), although no differences in immune or behavioural parameters were detected herein. It would have been useful to dose pesticides load in chick's blood, but the small amount of blood that could be collected did not allow us to perform both physiological and blood pesticide analyses.

## **Conclusion**

Overall, our study demonstrates that under environmental realistic conditions, ingestion of conventional grains containing low doses of pesticide cocktails by Grey partridge parents may have modest but significant negative consequences on their offspring through parental effects. This hypothesis is supported by the lack of alteration in physiological condition and reproduction parameters between parents fed either organic or conventional grains, although we could not measure pesticide levels in eggs. Conventional chicks suffered

from slightly delayed skeletal growth, assimilation issues, and physiological trade-off costs. Growth limitation can also have long-term consequences on physiology and life-history traits, and thus on adult fitness. Many farmland bird species that rely solely on grains to survive during winter may face such issues, which may have long-term consequences at a population level with certain pesticides and/or synergistic effects across generations. The two OPs (i.e., chlorpyrifos and pirimifos), the insecticide synergist (i.e., piperonyl-butoxyde) and the triazole fungicide (i.e., tebuconazole) found in the conventional grains that fed the parents are pesticides widely use worldwide (Sharma et al. 2019) and are thus likely to impact farmland birds since organic farming is still little spread in arable land in France and in other countries. We suggest that sublethal effects of such compounds on non-target species should be included in the regulation. Moreover, as agroecosystem pollution is not resulting only from pesticides, there is an urgent need to analyse cocktail effects, not only between molecules of pesticides but also between pesticides and other pollutants such as heavy metals. The consequences of parental exposure on chicks might partly explain the decline in wild Grey partridge populations, and it raises the question of pesticide use in relation to avian conservation and demography if current agroecosystem approaches persist.

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265 **Figure legends:**

266 **Fig. 1** Experimental design for testing the effects of food treatment (organic grains vs.  
267 conventional grains) on offspring development through parental effects in Grey partridge.

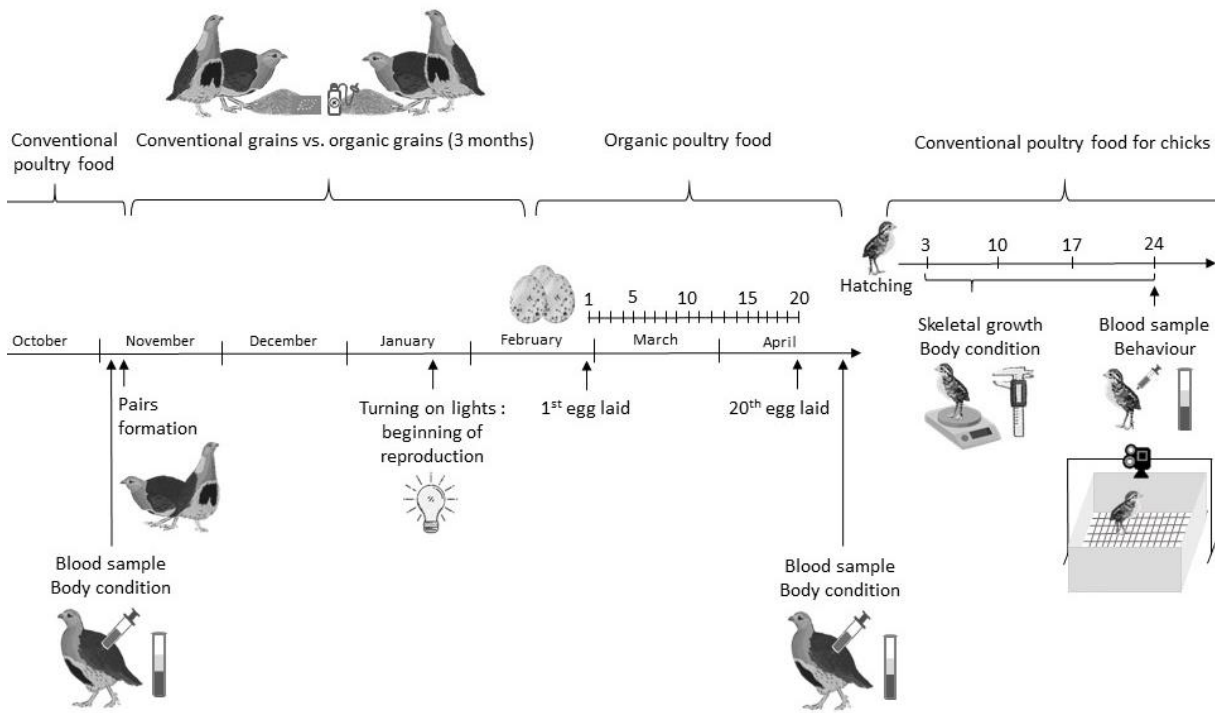
268 **Fig. 2** Variation in (a) body mass index, (b) haematocrit and (c) AChE activity before food  
269 treatment and after egg laying according to food treatment (organic vs. conventional) and sex.  
270 The horizontal line corresponds to the median, the top and bottom of boxes are the first and  
271 third quartiles, and whiskers delineate the lower and higher values included in the 95%  
272 confidence interval. Black points correspond to the mean and open dots correspond to  
273 outliers. Sample size is in brackets.

274 **Fig. 3** Variation in egg volume according to egg-laying rank and parent's food treatment  
275 (organic vs. conventional). The line corresponds to the predicted values for each group, and  
276 shading represents the 95% confidence interval.

277 **Fig. 4** Variation in chick's (a) skeletal growth (PC1 score) and (b) body mass index (BMI)  
278 according to parent's food treatment (organic vs. conventional) and age (3, 10, 17 and 24 days  
279 old). The horizontal line corresponds to the median, the top and bottom of boxes are the first  
280 and third quartiles, and whiskers delineate the lower and higher values included in the 95%  
281 confidence interval. Black points correspond to means and open dots correspond to outliers.  
282 Sample size is in brackets. Probabilities correspond to post hoc tests and Bonferroni  
283 correction for multiple comparisons, with significant effects in bold.

284 **Fig. 5** Variation in haematocrit according to BMI and parent's food treatment (organic vs.  
285 conventional) in 24-day-old chicks. The line corresponds to the predicted value for each  
286 group, and shading represents the 95% confidence interval.

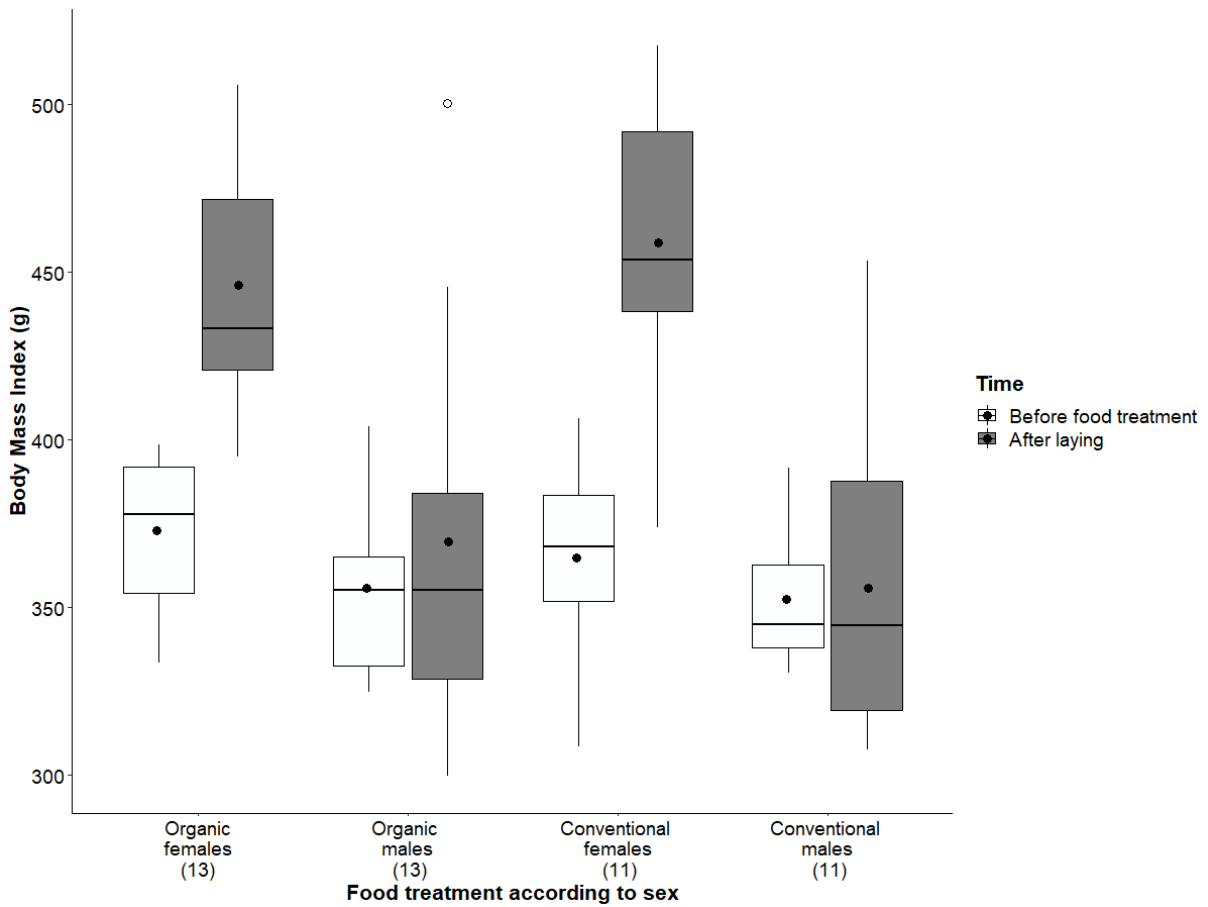
Fig 1



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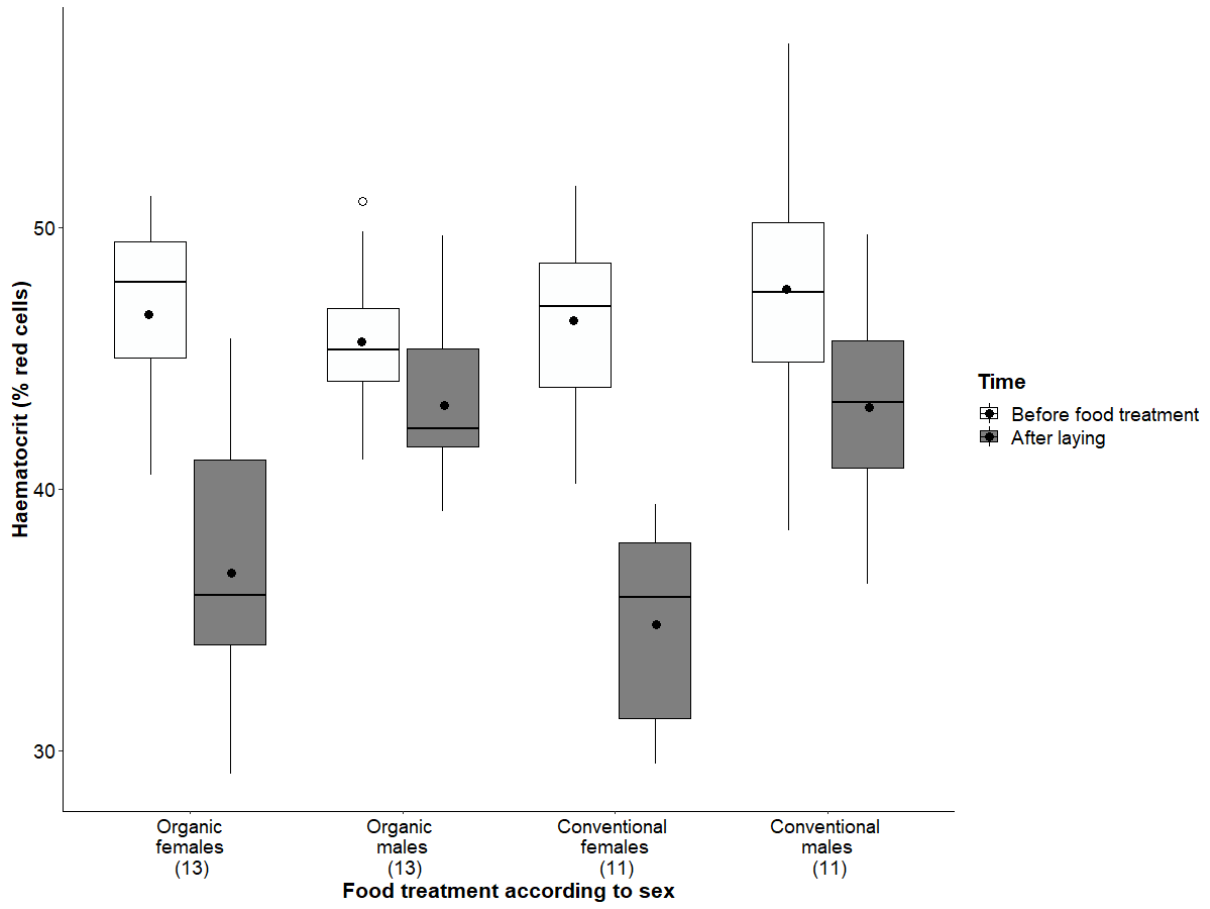
Fig 2.a



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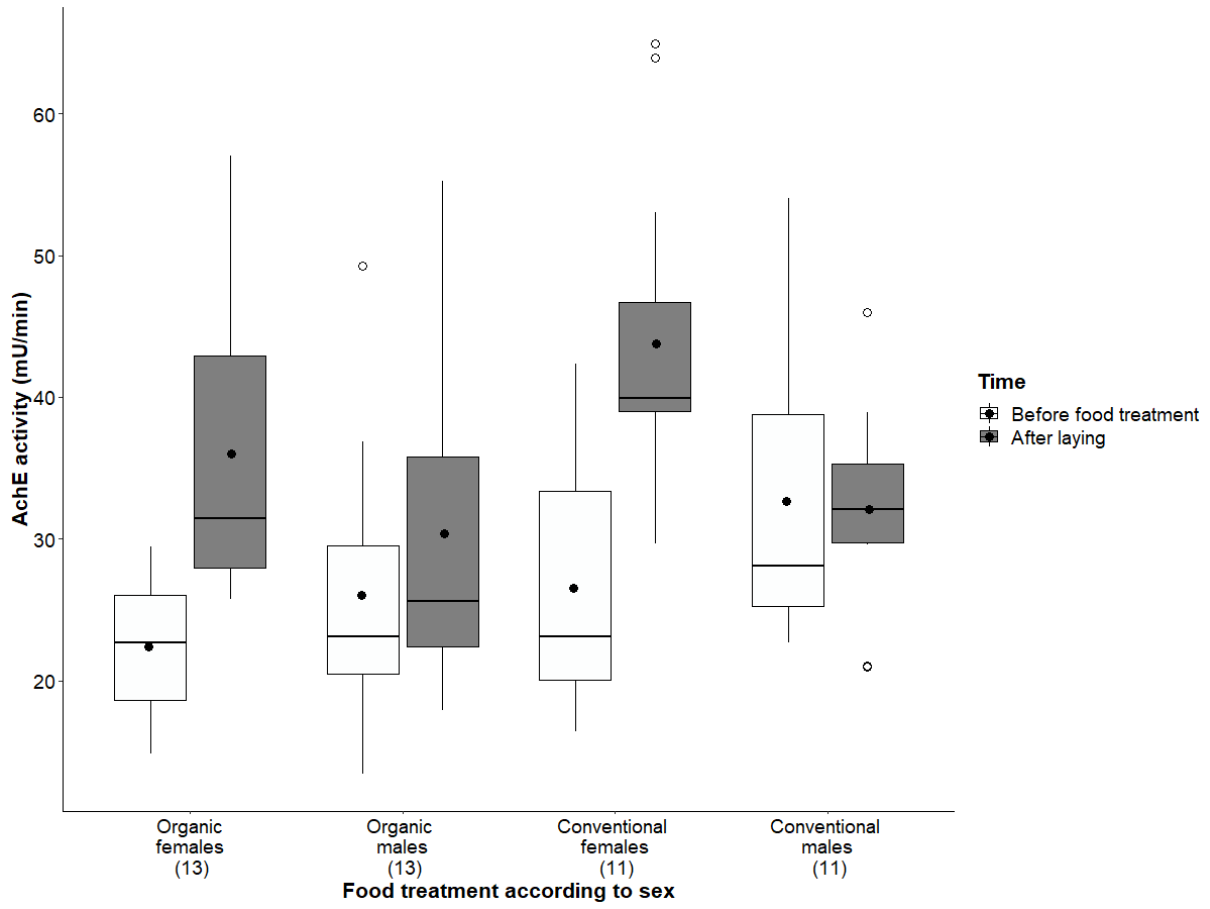
Fig 2.b



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Fig 2.c

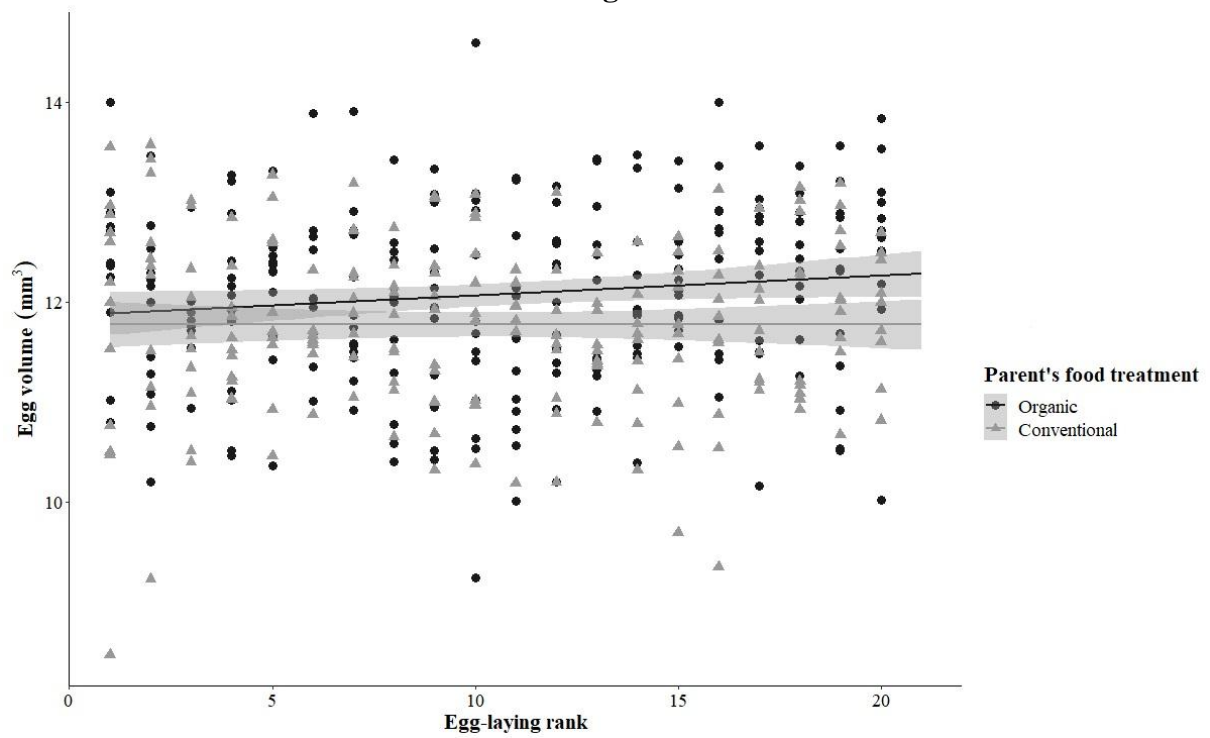


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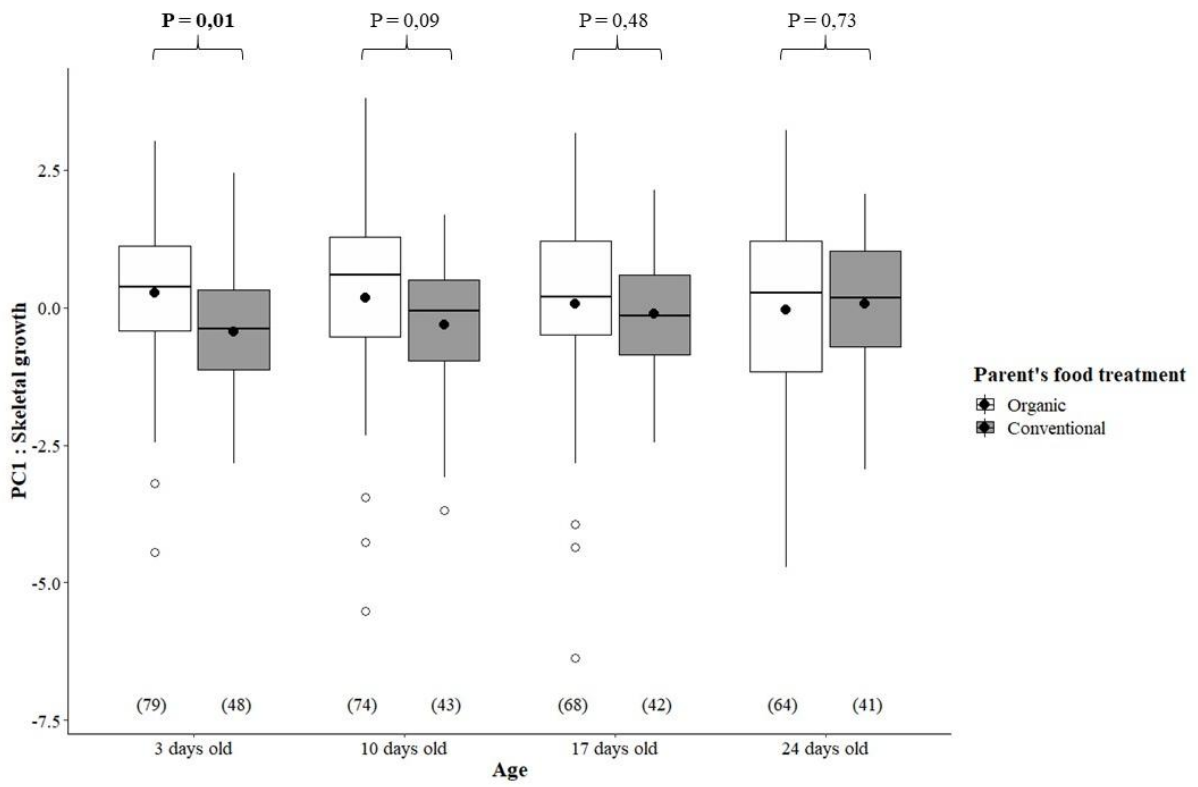
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Fig 3



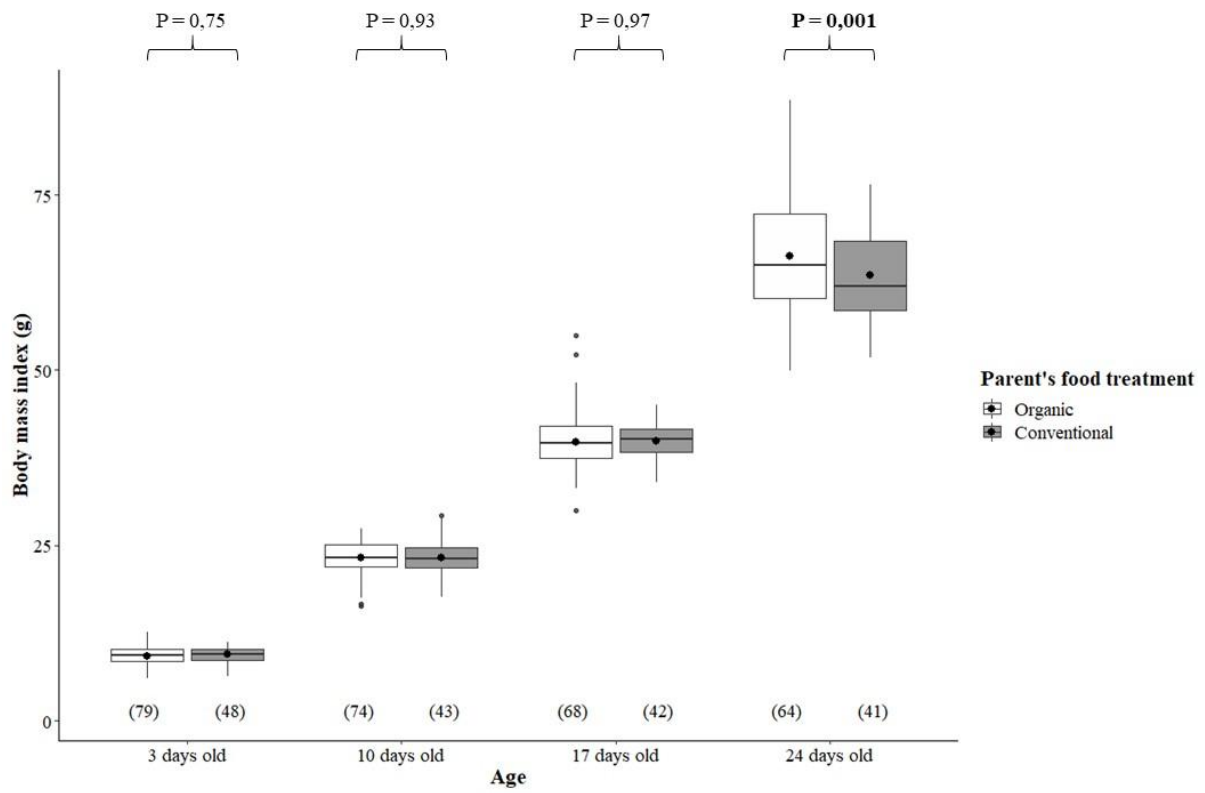
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Fig 4.a



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**Fig 4.b**

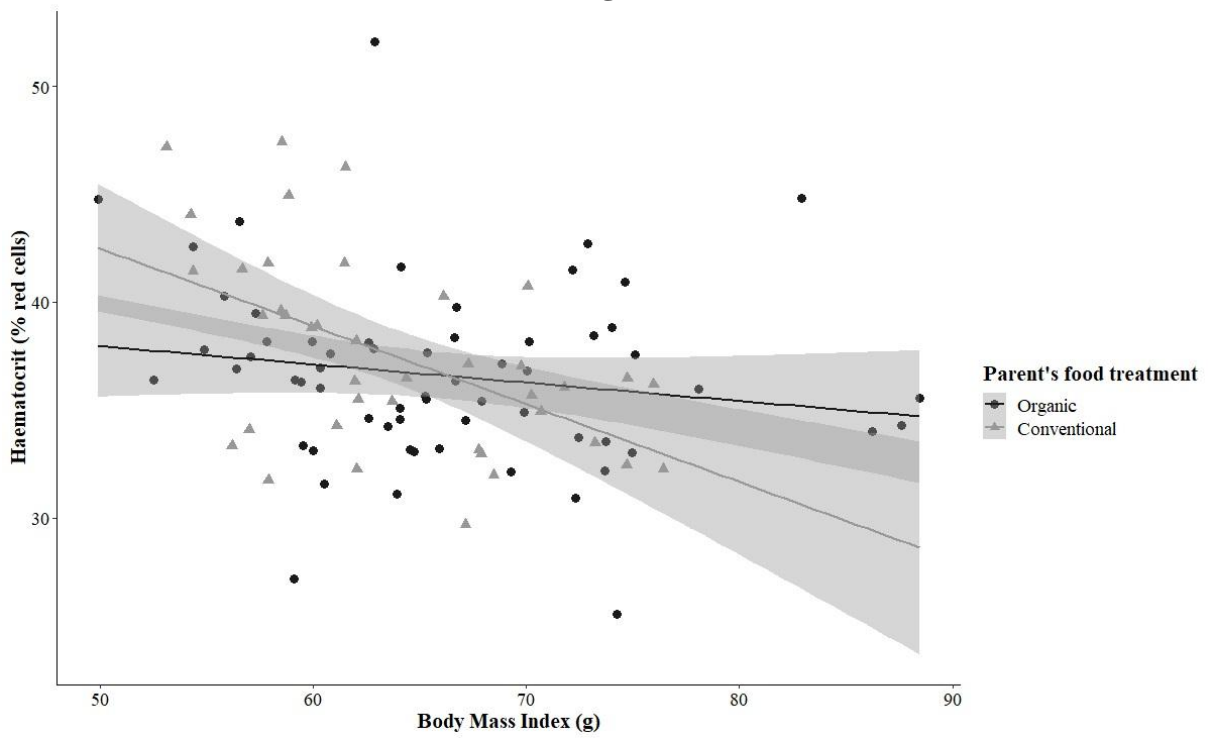


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**Fig 5**



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307 **Table 1: Effects of food treatment (organic vs. conventional), sex, and their interaction on the physiological condition of Grey partridge**  
 308 **(BMI, haematocrit and AchE activity) before food treatment and after egg-laying.**

309 Organic females served as a reference in models.  $\beta \pm SE$  were extracted from minimal models including only significant factors. Int., intercept;  
 310 Conv., conventional. Significant effects are in bold.  
 311

Response variables	Fixed factors	Before food treatment		After egg-laying		Difference between before food treatment and after egg-laying	
		<i>F</i> ratio test	$\beta \pm SE$	<i>F</i> ratio test	$\beta \pm SE$	<i>F</i> ratio test	$\beta \pm SE$
BMI (g)			Int. = 372.93 $\pm$ 6.70		Int. = 446.19 $\pm$ 12.86		Int. = 73.26 $\pm$ 0.84
	Food	<i>F</i> <sub>1,44</sub> = 0.64, <i>P</i> = 0.43		<i>F</i> <sub>1,44</sub> = 0.01, <i>P</i> = 0.97		<i>F</i> <sub>1,44</sub> = 0.26, <i>P</i> = 0.60	
	Sex	<i>F</i> <sub>1,44</sub> = <b>4.68</b> , <i>P</i> = <b>0.03</b>	<b>Male = -17.21 <math>\pm</math> 9.5</b>	<i>F</i> <sub>1,44</sub> = <b>43.98</b> , <i>P</i> < <b>0.001</b>	<b>Male = -76.56 <math>\pm</math> 18.19</b>	<i>F</i> <sub>1,44</sub> = <b>58.01</b> , <i>P</i> < <b>0.001</b>	<b>Male = -59.35 <math>\pm</math> 13.14</b>
	Food x Sex	<i>F</i> <sub>1,44</sub> = 0.11, <i>P</i> = 0.74		<i>F</i> <sub>1,44</sub> = 0.98, <i>P</i> = 0.33		<i>F</i> <sub>1,44</sub> = 2.59, <i>P</i> = 0.11	
Haematocrit (%)			Int. = 46.66 $\pm$ 1.07		Int. = 36.78 $\pm$ 1.11		Int. = -9.88 $\pm$ 1.17
	Food	<i>F</i> <sub>1,44</sub> = 0.63, <i>P</i> = 0.43		<i>F</i> <sub>1,44</sub> = 0.76, <i>P</i> = 0.38		<i>F</i> <sub>1,44</sub> = 2.42, <i>P</i> = 0.15	
	Sex	<i>F</i> <sub>1,44</sub> = 0.01, <i>P</i> = 0.98		<i>F</i> <sub>1,44</sub> = <b>39.48</b> , <i>P</i> < <b>0.001</b>	<b>Male = 6.39 <math>\pm</math> 1.57</b>	<i>F</i> <sub>1,44</sub> = <b>36.03</b> , <i>P</i> < <b>0.001</b>	<b>Male = 7.45 <math>\pm</math> 1.65</b>
	Food x Sex	<i>F</i> <sub>1,44</sub> = 1.01, <i>P</i> = 0.32		<i>F</i> <sub>1,44</sub> = 0.66, <i>P</i> = 0.41		<i>F</i> <sub>1,44</sub> = 0.02, <i>P</i> = 0.35	
AchE (mU/min)			Int. = 22.37 $\pm$ 2.37		Int. = 36.02 $\pm$ 2.92		Int. = 13.64 $\pm$ 1.85
	Food	<i>F</i> <sub>1,44</sub> = <b>4.42</b> , <i>P</i> = <b>0.04</b>	<b>Conv. = 4.18 <math>\pm</math> 3.51</b>	<i>F</i> <sub>1,44</sub> = 2.64, <i>P</i> = 0.11		<i>F</i> <sub>1,44</sub> = 0.02, <i>P</i> = 0.89	
	Sex	<i>F</i> <sub>1,44</sub> = 3.58, <i>P</i> = 0.06		<i>F</i> <sub>1,44</sub> = <b>7.39</b> , <i>P</i> < <b>0.01</b>	<b>Male = -5.66 <math>\pm</math> 4.13</b>	<i>F</i> <sub>1,44</sub> = <b>44.97</b> , <i>P</i> < <b>0.001</b>	<b>Male = -9.28 <math>\pm</math> 2.62</b>
	Food x Sex	<i>F</i> <sub>1,44</sub> = 0.25, <i>P</i> = 0.62		<i>F</i> <sub>1,44</sub> = 0.95, <i>P</i> = 0.33		<i>F</i> <sub>1,44</sub> = <b>4.72</b> , <i>P</i> = <b>0.03</b>	<b>Male conv. = -8.53 <math>\pm</math> 3.92</b>

312 **Table 2: Summary of reproduction settings depending on parent's food treatment (organic**  
 313 **vs. conventional).**

314 Data are presented as a number (n) or a percentage (%). H is Hatching day.

Reproduction settings	Age (days)	Organic pairs	Conventional pairs
Pairs (n)		13	11
Eggs laid (n)		267	198
Hatching rate (%)		45.3	34.5
Chicks (n)	H	107	67
	3	79	48
	10	74	43
	17	68	42
	24	64	41
Chicks' survival rate (%)			
	From H to 3 days old	73.8	71.6
	From 3 to 10 days old	93.6	89.6
	From 10 to 17 days old	91.9	97.7
	From 17 to 24 days old	94.1	97.6
	From H to 24 days old	59.8	61.1

315

316 **Table 3: Effects of parent's food treatment (organic vs. conventional) and age (3, 10, 17 and**  
 317 **24 days old) and their interactions on skeletal growth (PC1) and BM1 in chicks determined**  
 318 **using LMM.**

319  $\beta \pm SE$  were extracted from minimal models including only significant factors. Organic chicks at 3  
 320 days old served as the reference in models. Significant effects are in bold. Food corresponds to  
 321 parent's food treatment. Int., intercept; Conv., conventional.

Response variables	Fixed factors	Chi-squared test	$\beta \pm SE$
Skeletal growth (PC1)			Int. = $0.26 \pm 0.16$
	Food	$\chi_1 = \mathbf{6.56}, P = \mathbf{0.01}$	<b>Conv. = <math>-0.69 \pm 0.27</math></b>
	Age	$\chi_3 = 5.57, P = 0.13$	
	Food x Age	$\chi_3 = \mathbf{8.49}, P = \mathbf{0.03}$	<b>Conv. week 4 = <math>0.79 \pm 0.29</math></b>
BMI			Int. = $9.23 \pm 0.49$
	Food	$\chi_1 = 0.10, P = 0.75$	
	Age	$\chi_3 = \mathbf{7635.82}, P < \mathbf{0.001}$	
	Food x Age	$\chi_3 = \mathbf{10.36}, P = \mathbf{0.02}$	<b>Conv. week 4 = <math>-3.08 \pm 1.09</math></b>

322 **Table 4: Effects of parent's food treatment (organic vs. conventional) on behaviour (PC1 score) and physiological condition (haematocrit**  
 323 **and AchE activity) of 24-day-old chicks determined using LM.**

324 Organic chicks served as the reference in models.  $\beta \pm SE$  were extracted from minimal models including only significant factors. Significant  
 325 effects are in bold. Food corresponds to parent's food treatment. BMI is for chick's body mass index. Int., intercept; Conv., conventional.  
 326

Response variables	Fixed factors	<i>F</i> ratio test	$\beta \pm SE$
Behaviour (PC1)			Int. = $0.16 \pm 1.52$
	Food	$F_{1,101} = 0.03, P = 0.91$	
	BMI	$F_{1,101} = 0.09, P = 0.83$	
	Food x BMI	$F_{1,101} = 0.65, P = 0.59$	
Haematocrit			Int. = $42.19 \pm 4.18$
	Food	$F_{1,101} = \mathbf{95.33}, P = \mathbf{0.02}$	<b>Conv. = <math>18.28 \pm 7.66</math></b>
	BMI	$F_{1,101} = 30.48, P = 0.18$	
	Food x BMI	$F_{1,101} = \mathbf{90.98}, P = \mathbf{0.02}$	<b>Conv. = <math>-0.27 \pm 0.12</math></b>
AchE activity			Int. = $50.62 \pm 11.24$
	Food	$F_{1,101} = 1.6, P = 0.91$	
	BMI	$F_{1,101} = 6.1, P = 0.82$	
	Food x BMI	$F_{1,101} = 4.5, P = 0.84$	

327