Identification of different critical embryonic periods to modify egg incubation temperature in mule ducks

Charlotte Andrieux, S. Biasutti, Josette Barrieu, Philippe Morganx, Mireille Morisson, Vincent Coustham, Stéphane Panserat, Marianne Houssier

Identification of Different Critical Embryonic Periods to Modify Egg Incubation Temperature in Mule Ducks

C. Andrieux[1], S. Biasutti[2], J. Barrieu[3], P. Morganx[3], M. Morisson[4], V. Coustham[1], S. Panserat[1], M. Houssier[1]

[1]Univ Pau & Pays Adour, E2S UPPA, INRAE, UMR 1419, Nutrition, Métabolisme, Aquaculture, Saint Pée sur Nivelle, 64310, France

[2]Univ Pau & Pays Adour, E2S UPPA, IUT Génie Biologique, 40000 Mont de Marsan, France

[3]INRAE Bordeaux-Aquitaine, UEPFG (Unité Expérimentale Palmipèdes à Foie Gras), Domaine d'Artiguères 1076, route de Haut Mauco, 40280 Benquet, France.

[4]GenPhySE, Université de Toulouse, INRAE, ENVT, 31326 Castanet Tolosan, France

Corresponding author: Marianne Houssier, 371 rue du ruisseau 40000 Mont de Marsan, France *marianne.houssier@univ-pau.fr*

Abstract

Egg incubation of mule ducks, mainly used for fatty liver production, is one of the critical phases on this sector. Based on hatching rate, the best incubation parameters have already been well described for poultry, but the literature on ducks is lacking. In this study we tested different incubation conditions by varying two important factors, temperature and relative humidity, in mule ducks. These variations were applied at different periods during embryogenesis in order to measure the impact of environmental disturbances on different zootechnical performances. The temperature was increased by 1.5°C (16h/24) and the relative humidity was set up to 65 %, during 10 days. Six 10-day developmental windows were tested, from **embryonic day 9** (**E9**) to embryonic day 14 (E14). Our results are in line with previous reports showing that increasing incubation temperature, even when relative humidity is adjusted, can have a negative impact on duck embryonic mortality up to 24.5 % for the condition E10-E20 ($P < 10^{-5}$). However, the hatchability can be maintained at the level of the control groups when these modifications are applied on the latest windows (from the 11th embryonic day. Sex ratio, hatching body weight, and internal temperature are also sensitive to these incubation changes, and their modification could have a major impact on later zootechnical performance. These results should contribute to the development or embryonic temperature programming approaches, especially for the fatty liver production industry.

Keywords

Poultry, embryogenesis, incubating, mortality, hatchability

Implications

Egg incubation technology is constantly evolving, with high- precision machines allowing better control of environmental factors. One of the main environmental factors is the temperature inside the incubator and it has been observed that an increase in incubation temperature can improve performance at pre-hatch, hatch and post-hatch periods. However, we show in this study that this temperature increase cannot be made at any time during mule duck embryogenesis if a decrease in hatchability is to be avoided. These results could be of great use to the fatty liver duck industry, especially in projects wishing to use embryonic thermal programming.

Introduction

The technology of egg incubation is already well known in poultry field and is a key to obtain good performances at hatch and post-hatch (Wilson, 1991; Mueller et al., 2015). Conditions of incubation depend not only on the temperature and the relative humidity but also on gas exchanges and several others parameters to mimic the natural phenomena, such as rotation of eggs or light exposure. These conditions are the center of many studies to optimize zootechnical performances at birth (Landauer, 1961; Yahav and Tzschentke 2006; El-Hanoun et al., 2012; Boleli et al., 2016; Abd El-Hack et al., 2019; El-shater et al., 2021). The most published measures are weight, internal temperature, sex ratio and also hatchability, defined as the percentage of fertile eggs that produce a live animal (King'ori, 2011). The best conditions of incubation are based on the optimum of hatchability (Barott, 1937; Bruzual et al., 2000; Harun et al., 2001).

In recent decades, increasing incubation temperature has emerged as a promising way to program the physiology of avian species, resulting in improved thermal tolerance of adults (Piestun et al., 2008), and increased meat production in chickens (Piestun et al., 2013) or increased fatty liver production after a period of overfeeding in ducks (Massimino et al., 2019). However, since the temperature of incubation is the factor with the strongest impact on growth and development of embryos (Al Sardary et al., 2016; Maatjens et al., 2016), its modulation can easily impair hatchability. The **relative humidity (RH)** is a parameter that can be used to avoid egg dehydration during a rise in temperature and to improve hatching performances (Barott, 1937) but it is also one of the most difficult to control. Nevertheless, depending on the duration, period, level and direction of temperature change applied, the consequences of survival on the hatching rate are highly variable and difficult to predict (Halle et al., 2012; Loyau et al., 2013; Piestun et al., 2013; Al-Rukibat et al., 2016; Narinç et al., 2016; Massimino et al., 2019). In addition, the species studied may also greatly influence performance after a change in temperature during a certain incubation period, since opposite results were measured after a relatively similar short-term heat treatment during the last days of incubation between chickens (*Galliform*) (Halle and Tzschentke, 2011) and Pekin ducks (*Anseriform*) (Halle et al., 2012). These differences may be due to a different timing of the ontogeny of thermoregulatory mechanisms between these two species of different orders (Tzschentke and Basta, 2000; Halle et al., 2012), and could therefore also be seen within the same subspecies (animals of the same order and family) with different incubation times.

In fatty liver production, two species of male's duck are used: Muscovy and mule. Mule duck is a sterile hybrid from the crossbred between a female Pekin (*Anas*

platyrhynchos) and a male Muscovy (*Cairina moschata*) and is used for more than 99 % of the production with around 16 000 tons of fresh fatty liver per year (Litt et al., 2021). The hatchability of duck is lower than hatchability of other poultry species, ranging from 65 to 82 % against 79 to 85 % for the other birds (Hodgetts, 1991), but the literature is not as broad as for other species such as broilers. The period of incubation of crossbred eggs is around 32 days for the mule duck, compared to 28 days for the Pekin and 35 days for the Muscovy (Brun et al., 2005). Conditions of incubation of the three species are relatively close, with an optimal ambient temperature comprised between 37 to 38°C and a relative humidity between 60 to 80 %, according to the embryogenesis period (Abd El-Hack et al., 2019).

The purpose of this study is therefore to measure, for the first time, a set of zootechnical parameters around the birth of mule ducks after a discontinuous (16h/24h) increase of 1.5°C of the incubation temperature with an adjusted targeted RH of 65 %. Six 10-day developmental windows were tested, from **embryonic day 9 (E9)** to embryonic day 14 (E14), to assess the impact of temperature on these parameters by measuring zootechnical performance before, during and after hatching.

Material and Methods

Experimental design

Three experimental trials were done to test seven conditions of egg incubation (Figure 1), including the control condition. Each condition was performed using 299, 300 and 289 eggs per incubator for trials 1, 2 and 3 respectively. In each trial, a control

group was added, with an average temperature of 37.6°C and an average relative humidity (RH) of 61.5 %, 62.8 % and 63.6 % for the trial 1, 2 and 3 respectively. The six others conditions differed from control groups by the temperature (+1.5°C, 16h/24) and RH (+5 % targeted) during 10 days, but at different developmental stages during the incubation. In the trial 1, we applied the changes in conditions from embryonic day 11 (E11) until E21, first window chosen according to the high expression level of genes involved in lipid and carbohydrate metabolism measured during this period (Massimino et al., 2020). In the trial 2, windows E10 to E20 and E12 to E22 were tested. In the trial 3, windows E9 to E19, E13 to E23 and E14 to E24 were tested in order to cover a wide incubation period and to see if the periods are more or less traumatic for the embryos. The target of temperature has been reached for all the conditions but the targeted RH was more difficult to attain, and most of the time remained close to the control group (E9-E19: 62.5 %, E10-E20: 64.3 %, E11-E21: 59.8 %, E12-E22: 61.9 %, E13-E23: 63.0 % and E14-24: 62.3 %).

For the experimental trials 1, 2 and 3, eggs obtained from the same females, aged of 36, 42 and 49 weeks respectively. The genotype H85 was provided by Grimaud Frères Selection company (Roussay, France). Eggs were collected on a day and stored at ambient temperature three days before incubation. Eggs were dispatched randomly between treatments to obtain an equal number of eggs in each group with 299, 300 and 289 eggs in trial 1, 2 and 3 respectively. In each trial, eggs were incubated in identical automated commercial incubators with regulation of temperature, humidity and ventilation (SOLOGNE model of LA NATIONALE, reconditioned model). Incubation parameters were recorded during the whole incubation period and temperature and RH were confirmed by two independent sensors (LoRa® SPY U). Each incubator had its

own pair of sensors fixed on incubator inner wall closed to the doors. All eggs were subjected to an automatic rotation of 90° every three hours and were sprayed manually the afternoon before the changing of conditions of incubation.

Two light controls, called candling, were done during the incubation period in order to estimate the viability of the egg. The first was done at the 7th embryonic day (E7) and the second, after conditions of incubation change, at the 28th embryonic day (E28). Unfertile eggs were removed from incubators and the remaining eggs were pooled to avoid local temperature disturbance.

At the 28th day of incubation, all eggs were placed in the same hatchery (BRETAGNE model of LA NATIONALE, reconditioned model) at 37.3°C and 80 % of RH. During four days, the number of newly hatched ducks was recorded once a day.

Egg incubation measurements

The temperature and the relative humidity, in incubators, were recorded every 15 minutes by sensors (LoRa® SPY U), and results were available on a software. Targeted at 60 % for control conditions and 65 % for treated conditions, means of RH throughout the 10-days incubation period were 61.5 % for control and 59.8 % for changes conditions in trial 1; 62.8 % for control, 64.3 % for E10-E20 and 61.9 % for E12-E22 conditions in trial 2; 63.6 % for control, 65.5 % for E9-E19, 63.0 % for E13-E23 and 62.3 % for E14-E24 in trial 3.

At the first candling (E7), the number of non-fertile eggs and dead embryos were recorded. At the second candling (E28), only the number of dead embryos was recorded. Fertility, early (up to E7), medium (between E7 and E28) and late (in hatchery) mortality rates were therefore measured. Fertility was the ratio of the number of fertilized

158 eggs, observed at E7, to the number of eggs in the incubator. Mortalities were the ratio
159 of the number of died eggs, recorded at E7, E28 and at hatch, to the number of eggs
160 fertilized in the incubator.
161
162 ***Hatch and post-hatch measurements***
163 Once a day, the number and gender of hatched ducklings were recorded. The
164 identification of the sex was based on two criteria: black spots on the duckling head and
165 black eyes for males and no black spot and red eyes for females, completed by vent
166 sexing when it is not clear. The weight of male and female ducklings was recorded using
167 a scale (SARTORIUS Signum 1, model SIWRDCP.1.3.1) during the peak hatching day
168 corresponding to the day with the highest proportion of hatched ducklings (n = 129 to
169 214 excepted trial 1 n = 10). For each group, hatchability, sex ratio, mean weight and
170 internal temperature, by using digital express thermometers (Digital Express
171 Thermometer from Gilbert), were measured.
172 Around 50 male ducklings and 50 female ducklings, born at the hatching peak, were
173 arbitrary kept to do measurements of weight (Automatic weighing system) and internal
174 temperature after six days of life. Internal temperatures were measured using digital
175 express thermometers (Digital Express Thermometer from Gilbert), inserted into the
176 cloaca. During the rearing period, water and food were supplied *ad libitum*.
177 Because of the health crisis related to COVID19, the trial 1 (March 2020 to April 2020)
178 had to be stopped at D1. Only the weight and internal temperature of 10 animals at
179 hatching were recorded due to staff limitations.
180
181 ***Statistical analysis***

Within a single trial, statistical tests were done to compare the control group and groups with a change in incubation conditions. Hatchability, mortalities and sex ratio were compared by using a Chi-squared test. Weights and internal temperatures were compared by using one-way Anova or student and Wilcoxon tests depending on normality and homoscedasticity. For these tests, the individual was used as the experimental unit, and the variable response was studied according to the treatment. All statistical analyses were performed with R software version 3.6.2. Statistical testing was performed on datasets at a statistical significance of 5%.

Results

In each trial, an incubation control was done with constant conditions of incubation. Even if conditions were similar, we observed a variation between controls of the different trials, named the inter-trial dispersion (Figure 2). Inter-trial dispersions were estimated by the difference between the highest value and each value of the two other trials. Regarding mid-embryonic mortalities, the maximum inter-trial dispersions were 2.0 % between trial 1 and 2 and 3.8 % between trial 3 and 2, although not significant (Figure 2a). In contrast, for the late embryonic mortalities, differences were significant (Figure 2b) and these maximal inter-trial dispersions were 3.5 % between trial 1 and 2 and 5.7 % between trial 3 and 2. Interestingly, the most variable factor also proved to be the most critical: hatchability. The maximum inter-trial dispersions were as high as 8.0 % between trial 2 and 3, thus becoming significant with a p-value lower than 0.01 (Figure 2c). Between trials 1 and 3, the dispersion was 3.7 % and was not significantly different.

205 Similarly, we did not measure a significant difference between the three trials for the sex
206 ratio (Figure 2d), despite a maximum inter-trial dispersion of 3.8 % between trials 2 and
207 3.

208 For all the conditions, fertility of eggs was around 93 % and the embryonic
209 mortality at the first candling varied around 1 % (data not shown). The second candling
210 was done after the change of incubation conditions and reflected the impact of this
211 change on the embryo mortality. The results of three trials conducted at different time
212 with their own variability can only be compared statistically with their internal control
213 (within the same row, Table 1). However, even considering the **maximal inter-trial**
214 **dispersion (ITD)**, it is possible to distinguish two groups among the experimental the
215 early change groups (with E9-E19 and E10-E20) and the late change groups (with E11-
216 E21, E12-E22, E123-E23, and E14-E24) that appeared to respond differently to
217 incubation changes. The average mid-embryonic mortality of the control groups was 8.3
218 % while the increase of 1.5°C (16h/24, with the adjusted RH) in the early stages of
219 development (from the 9th or 10th embryonic day), resulted in a strong increase of this
220 rate in the second candling, reaching 24.5 % for trial 2. This observation was then
221 confirmed by the statistical tests performed within each trial (table 1, first panel). In trial
222 3, the mid-embryonic mortality was significantly higher for the condition E9-E19
223 (reaching 20.5 %) compared to the control of the trial 3, and conditions E13-E23 and
224 E14-E24 (6.4 %, 13.0 % and 13.1 % respectively; $P < 0.05$). In trial 2, the mid-embryonic
225 mortality was significantly higher for the condition E10-E20 (24.5 % mortality) compared
226 to the control of the trial 2 (10.2%), and the condition E12-E22 (15.9 %) ($P < 0.05$).
227 Changes in condition from E11 to E14 led to a significantly lower increase in mortality

than earlier stages and one condition (E12-E22) did not show a significant difference compared to its control from trial 2.

The measurement of late mortality was done inside the hatchery after the incubation period and all results were grouped in the second panel of the Table 1. However, no statistical difference has been detected at this stage.

For all the conditions, the hatching peak occurred on embryonic day 30 (E30), with an average hatchability of 84.2% for control groups (Table 1, third panel). Again, the two earliest groups (trial 2 and 3) presented the strongest impact on hatchability, with a significant decrease compared to their own control ($P < 0.001$), dropping below 70 %. On the other hand, among the four late conditions changes, only the E14-E24 differed significantly from its control.

In the last panel of the table 1, the proportion of total males born appeared to be higher with earlier temperature change. If we looked at groups with early condition changes, we observed a significantly higher sex ratio in favor of males, compared to their own control. Groups E9-E19 and E10-E20 raised indeed a rate of 67.6 % and 65.5 % of males compared to controls with an average around 52 % ($P < 0.05$). Three out of the four other experimental groups have the same sex ratio as the controls.

Post-hatch body weight and internal temperature measurements are listed in Table 2 all groups with condition of incubation changes presented lower body weights than their control group on the day of hatching (Table 2, first panel). The condition E11-E21 appeared to have a lower average birth weight than the other groups but this result was not statistically different, due to the fact that only 10 animals were sampled. However, six days later (D6), most of the experimental groups had caught up to the weight of their control group (Table 2, second panel). At this stage, only the condition

E12-E22 remained significantly below its own control ($P < 0.001$). At D6, the internal temperature was also lower for all groups with changes in incubation conditions, compared to their own controls (Table 2, third panel) and these differences were significant ($P < 0.01$).

Discussion

In ducks, the incubation is one of the most delicate periods in terms of survival (Wilson, 1991; Mueller et al., 2015). In recent years, the concept of embryonic thermal programming has implied that changes in egg incubation conditions could optimize animal resistance and even production (Yahav et al., 2004; Piestun et al., 2011; Massimino et al., 2019; Carvalho et al., 2020). In this study, we tested six embryonic periods during which we increased temperature and relative humidity (RH), in a discontinuous way (16h/24h) to measure the consequences of such variations on early performances.

Evidence of variability between trials

Many external parameters may explain differences in hatchability within the same species, such as age of females, quality of transport, storage of eggs before incubation, incubation conditions (El-Hanoun et al., 2012)… However, the contribution of these parameters should be minimal in your trials since we were careful to reproduce the same control conditions between trials. Besides, the same incubator was used for all the control conditions in the three trials to avoid an incubator effect, but an inter-trial dispersion was still observed. The age of the females providing the eggs changed

between trials, but the results do not show a correlation between this factor and hatching, since the oldest females were not the ones that gave the worst yields. Only the relative humidity was difficult to control and varied between 61.5 % to 63.6 % despite a targeted of 60 %. This difference could be at least partially responsible for the variability in hatching measured at the end of the trial, since RH was a very important parameter for the proper conduct of embryogenesis (Barott, 1937; Bruzual et al., 2000; Peebles et al., 2001). The RH variation could be related to the manual part of its control by spraying, and therefore to the spraying duration, the amount of water sprayed or the duration of door opening… Finally, the observed inter-trial dispersion could also be due to random but significantly different inter-individual variability between groups in different trials.

Consequently, the actual impact of the treatment was tempered by this inter-trial dispersion which was allowed by the realization of a control in each of the trials.

Early embryonic stages are more sensitive to temperature changes

Three critical periods have been described in poultry to explain embryonic mortality (Romanoff, 1949; Sauveur, 1988). The early mortality was linked to difficulties of setting up the first embryonic structures. The mid-mortality was mainly due to the start of essential functions as the respiration or the kidney function. The mortality in the hatchery, also called late mortality, involved the difficulty of getting out of the eggshell. In consequence, a change of environmental factors during these critical periods could be lethal for embryos (Romanoff, 1949; Ono et al., 1994; Loyau et al., 2015).

Here, the changes in incubation conditions were applied for 10 days, over six different periods from E9 to E14, thus over a fairly wide range of time corresponding to

300 the middle of the incubation and known to have high plasticity at a molecular level in
301 duck (Massimino et al., 2020). We measured early embryonic mortality (E7), mid-
302 mortality (E28) and late mortality in the hatchery. The early embryonic mortalities were
303 identical between all groups, due to the lack of change in incubation conditions at this
304 time. In contrast, we showed an increase in mid-mortality in almost all treated groups
305 compared to their own control. These results confirmed that incubation must respect
306 very strict parameters to obtain good hatch performance (Wilson, 1991; Mueller et al.,
307 2015) and that the application of new incubation conditions for the purpose of embryonic
308 programming requires careful analysis of its impact on survival.

309 However, we also found an interesting result regarding the period of change, with
310 two groups standing out. Indeed, trials 2 and 3 allowed us to statistically compare early
311 change conditions (starting at E9 and E10) with later changes (starting at E12, E13 and
312 E14), and these comparisons showed us that the earlier the changes, the greater the
313 impact on embryonic mortality.

314 These results suggest that our incubation parameter changes were more likely to
315 disrupt the early settlement of embryonic structures, than the later functionality of
316 essential organs. Because several systems are organized during the first two weeks of
317 embryogenesis, (the digestive system, the body temperature regulation system, the
318 organization of embryonic membranes, the immune system, the nervous system...), this
319 period appeared to be more critical and the embryo appeared to be more vulnerable to
320 external factors (Dibner et al., 1998; King et al., 2000; Jenkins et al., 2004; Baarendse et
321 al., 2007; De Oliveira et al., 2008; Baggott, 2009). Consistent with previous studies in
322 poultry, these results in ducks confirm that early embryonic stages are more sensitive to

changes in environmental factors, and that a modification in condition can therefore become lethal (Yan et al., 2015; Al Sardary et al., 2016; Carvalho et al., 2020).

Changes in incubation conditions provide a first challenge before hatching

A few days later, almost all late change conditions (after E11) showed no significant decrease in hatchability compared to their own control, unlike the two earliest conditions. These data demonstrated that although mid-term mortality was increased by the later changes in incubation condition, hatching performance was the same as the control groups. Finally, this assessment indicated that total mortality during embryogenesis was less impacted by a discontinuous temperature increase of 1.5°C and 5 % RH, when applied from the 11th embryonic day, confirming previous study in chicken (Collin et al., 2005; Piestun et al., 2008). On the contrary, when these increases in temperature and RH were applied from E9 or E10, hatchability was definitely lower compared to the control condition. This decrease, visible even considering the theoretical maximum dispersion, was probably directly related to the high mortality measured at the second candling. This information could be a valuable aid for the design of new embryonic thermal programming conditions in mule ducks.

These results led us to hypothesize a pre-elimination of weak embryos during the late changes of incubation conditions, and a proportional reduction in hatchery mortality. Unfortunately, we did not measure a significant decrease in late mortality, certainly because of the low number of deaths at this period for all groups. However, it can be assumed that in the control groups, mortality spread over time because the weakest embryos were not exposed to environmental changes until hatching, but ultimately the final survival rate was the same as for the late treated groups. This observation also

suggests that there was no selection of animals despite the high mid-mortality observed with these new incubation conditions, but potentially a time lag, which could therefore be worth further investigating in the context of embryonic programming. On the contrary, the earlier changes (starting from E9 or E10) actually induced a higher overall mortality, and not a time lag.

An unbalanced sex ratio linked to the crossbred but also influenced by environmental factors

The unbalance in the sex ratio of mule duck is well known with a preponderance of males at hatching (Batellier et al., 2004 a), explained by an alteration of female embryo development (Byerly et al., 1935). This unbalanced sex ratio follows the rule of Haldane (Haldane, 1922), explaining the greater fragility of the heterogamous sex (the female in ducks) during cellular division.

In contrast to a recent study that showed no effect of the incubation temperature on sex ratio in Mallard ducks (Koláčková et al., 2019), our results suggest that increasing temperature and RH at the earliest stages of embryogenesis may further increase female mortality in mule ducks. In chicken, last days of incubation seem to be also critical and impact the sex ratio in favor of males (Tzschentke and Halle., 2009; Elmehdawi et al., 2015). These data tend to confirm the greater fragility of heterogamous sex in hybrid species, and could be investigated as a precocious method of selection of males for the fatty liver production (Batellier et al., 2004 b).

The catch-up growth, a mechanism that could influence long-term physiology

At hatch, all groups with incubation conditions changes had lower weights than control groups. Numerous studies on chickens (Piestun et al., 2008; Al-Rukibat et al., 2016; Zaboli et al., 2016) and mule ducks (Massimino et al., 2019) have already shown that an increase in incubation temperature leads to a decrease in hatching body weight. These observations can be explained in part by an acceleration of embryonic development (Al Sardary et al., 2016; Boleli et al., 2016; Maatjens et al., 2016) resulting in increased yolk sac consumption prior to the first meal (Lamot et al., 2014). Although here all groups had their peak hatch on the same day (E30), hatcher surveys were only done once a day at 8:00 am, leaving a 24-hour window for the animals to emerge from their shells. It was therefore possible that animals treated with the new incubation conditions were born on average a few hours earlier than the control group resulting in a decreased hatch weight. To support this hypothesis, it has been shown in quail that when hatchings are synchronized taking into account the acceleration of development induced by a rise in temperature, the birth weight of thermally treated individuals is not different from controls (Carvalho et al., 2020).

However, this growth delay was totally recovered in only a few days by most of the treated groups, since at D6, only the E12-E22 condition still presented a significant difference with its control. This catch-up growth has already been observed in poultry exposed to increased temperature during embryogenesis (Piestun et al., 2013; Zaboli et al., 2016; Massimino et al., 2019), but also in mammals with delay of growth at birth for different reasons (McLaughlin et al., 2020). This phenomenon is not yet fully understood, but two main theories seem to emerge (Wit et al., 2002): the neuroendocrine (involving Growth Hormone, thyroid, Insulin-like Growth Factor-I axis) and the growth plate hypothesis. Whatever the mechanism, the long-term

394 consequences on metabolism are no longer to be demonstrated (Bieswal et al., 2006;
395 Singhal, 2017; Berends et al., 2018) and may be of interest to study in the context of
396 embryonic programming.

397 Finally, as previously shown in chicken (Loyau et al., 2015) and duck (Massimino
398 et al., 2019) all groups treated with increased incubation temperature had lower body
399 temperatures compared to their own control after birth. In chickens, this decrease in
400 body temperature is often associated with a better thermotolerance of the animals (Al-
401 Zghoul et al., 2015; Al-Rukibat et al., 2016), and can be explained by the application of a
402 temperature increase during the embryonic development of the axes responsible for
403 thermal regulation (Jenkins et al., 2004; Piestun et al., 2008). Our results in mule ducks
404 suggest that incubation changes from E9 to E14 during 10 days may alter the same
405 axes of thermal regulation, but no differences were measured between the earliest and
406 latest treated groups.

407

408 **Conclusion**

409 Although changes in temperature and RH during incubation might improve certain
410 animal performances. This study also confirms that incubation parameters are critical to
411 the zootechnical performance of male ducks, a sterile hybrid from the crossbred
412 between a female Pekin and a male Muscovy, at hatch and that the timing of changes is
413 an important factor in maintaining optimal hatchability.

414

415 **Ethic approval**

416

All experimental procedures were in accordance with the French national guidelines for the care of animals for research purposes. The protocols were approved by the committee of the Care and Use of Animals of the Grand Sud-Ouest (n°73) under the file reference APAFIS#25163-2020062311266761 v5.

The present study was carried out in the certified Experimental Station for Waterfowl Breeding (INRAE, UEPFG, France), which received the accreditation number C40–037-1.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

Author ORCIDs

Corresponding author- Marianne Houssier : 0000-0002-5329-4597

Mireille Morisson : 0000-0002-5279-6675, Vincent Coustham : 0000-0002-5399-2723, Stéphane Panserat : 0000-0002-4479-9868.

Author Contributions

Conceptualization and methodology: C. Andrieux, S. Panserat and M. Houssier; investigation: C. Andrieux, S. Biasutti, J. Barrieu, P. Morganx and M. Houssier ; data analysis and software: C. Andrieux; writing-original draft: C. Andrieux and M. Houssier; writing-review & editing: C. Andrieux, M. Morisson, V. Coustham, S. Panserat and M. Houssier; supervision: S. Panserat and M. Houssier

Declaration of interest

The authors state that this work was supported by a grant from the Comité Interprofessionnel des Palmipèdes à Foie Gras (CIFOG) but that these funders played no role in the design of the study and in the collection, analysis and interpretation of the results.

Acknowledgements

We thank all the technicians of the experimental station of Artigueres for the daily care given to the animals and the monitoring of the good conditions for the experiments.

Financial support statement

This work was supported by grants from the “Comité Interprofessionnel des Palmipèdes à Foie Gras (CIFOG agreement 2020/05) and from “Comité Départemental des Landes” (CD40- CA salary for three years).

References

Abd El-Hack, M. E.; Hurtado, C. B.; Toro, D. M.; Alagawany, M.; Abdelfattah, E. M.; Elnesr, S. S., 2019: Impact of environmental and incubation factors on hatchability of duck eggs. *Biological Rhythm Research.,* **2019**, 1–10.

Al-Rukibat, R. K.; Al-Zghoul, M. B.; Hananeh, W. M.; Al-Natour, M. Q.; Abu-Basha, E. A., 2016: Thermal manipulation during late embryogenesis: Effect on body weight and temperature, thyroid hormones, and differential white blood cell counts in broiler chickens. *Poultry Science.*, **96**, 234–240.

Al-Zghoul, M. B.; Ismail, Z. B.; Dalab, A. E. S.; Al-Ramadan, A.; Althnaian, T. A.; Al-ramadan, S. Y.; Ali, A. M.; Albokhadaim, I. F.; Al Busadah, K. A.; Eljarah, A.; Jawasreh, K. I.; Hannon, K. M., 2015: Hsp90, Hsp60 and HSF-1 genes expression in muscle, heart and brain of thermally manipulated broiler chicken. *Research in Veterinary Science.*, **99**, 105–111.

Al Sardary, S.; Mohammad, S., 2016: Effect of Thermal Manipulation during Embryogenesis on Hatching Traits. *International Journal of Agricultural Science.*, **1**, 190–195.

Baarendse, P. J. J.; Debonne, M.; Decuypere, E.; Kemp, B.; Van Den Brand, H., 2007: Ontogeny of avian thermoregulation from a neural point of view. *World's Poultry Science Journal.*, **63**, 267–276.

Baggott, G. K., 2009: Development of extra-embryonic membranes and fluid compartments. *Avian Biology Research.*, **2**, 21–26.

Barott, H. G., 1937: Effect of temperature, humidity and other factors on the hatch of hens' eggs and on energy metabolism of chick embryos. *Technical Bulletin No 553, United States Department of Agriculture.*, **553**, 1–46.

Batellier, F.; Marchal, F.; Scheller, M. F.; Gautron, J.; Sellier, N.; Taouis, M.; Monbrun, C.;

Vignal, A.; Brillard, J. P., 2004a: Sex ratios in mule duck embryos at various stages of incubation. *Theriogenology*., **61**, 573–580.

Batellier, F.; Govoroun, M.; Brillard, J. P., 2004b: Sex-ratio chez les oiseaux sauvages et domestiques. *Productions Animales*., **17**, 365–372.

Berends, L. M.; Dearden, L.; Tung, Y. C. L.; Voshol, P.; Fernandez-Twinn, D. S.; Ozanne, S. E., 2018: Programming of central and peripheral insulin resistance by low birthweight and postnatal catch-up growth in male mice. *Diabetologia*., **61**, 2225–2234.

Bieswal, F.; Ahn, M. T.; Reusens, B.; Holvoet, P.; Raes, M.; Rees, W. D.; Remacle, C., 2006: The importance of catch-up growth after early malnutrition for the programming of obesity in male rat. *Obesity*., **14**, 1330–1343.

Boleli, I. C.; Morita, V. S.; Matos, J. B.; Thimotheo, M.; Almeida, V. R., 2016: Poultry egg incubation: Integrating and optimizing production efficiency. *Brazilian Journal of Poultry Science*., **18**, 1–16.

Brun, J. M.; Richard, M. M.; Marie-Etancelin, C.; Rouvier, R.; Larzul, C., 2005: Le canrd mulard: déterminisme génétique d'un hybride intergénérique. *Productions Animales*., **18**, 295–308.

Bruzual, J. J.; Peak, S. D.; Brake, J.; Peebles, E. D., 2000: Effects of relative humidity during incubation on hatchability and body weight of broiler chicks from young breeder flocks. *Poultry Science*., **79**, 827–830.

Byerly, T. C.; Jull, M. A., 1935: Sex Ratio and Embryonic Mortality in the Domestic Fowl. *Poultry Science*., **14**, 217–220.

Carvalho, A. V.; Hennequet-Antier, C.; Crochet, S.; Bordeau, T.; Couroussé, N.; Cailleau-Audouin, E.; Chartrin, P.; Darras, V. M.; Zerjal, T.; Collin, A.; Coustham, V., 2020: Embryonic

505 thermal manipulation has short and long-term effects on the development and the physiology of
506 the Japanese quail. *PLoS ONE*., **15**, e0227700

507 Collin, A.; Picard, M.; Yahav, S., 2005: The effect of duration of thermal manipulation during
508 broiler chick embryogenesis on body weight and body temperature of post-hatched chicks.
509 *Animal Research*., **54**, 105–111.

510 De Oliveira, J. E.; Uni, Z.; Ferket, P. R., 2008: Important metabolic pathways in poultry embryos
511 prior to hatch. *World's Poultry Science Journal*., **64**, 488–499.

512 Dibner, J. J.; Knight, C. D.; Kitchell, M. L.; Atwell, C. A.; Downs, A. C.; Ivey, F. J., 1998: Early
513 feeding and development of the immune system in neonatal poultry. *Journal of Applied Poultry*
514 *Research*., **7**, 425–436.

515 El-Hanoun, A. M.; Rizk, R. E.; Shahein, E. H. A.; Hassan, N. S.; Brake, J., 2012: Effect of
516 incubation humidity and flock age on hatchability traits and posthatch growth in Pekin ducks.
517 *Poultry Science*., **91**, 2390–2397.

518 El-shater, S. N.; Rizk, H.; Abdelrahman, H. A.; Awad, M. A.; Khalifa, E. F.; Khalil, K. M.; Khalil,
519 K. M., 2021: Embryonic thermal manipulation of Japanese quail : effects on embryonic
520 development, hatchability, and post-hatch performance. *Tropical Animal Health and Production*.,
521 **53**, 1–10.

522 Elmehdawi, A.; Hall, M.; Skewes, P.; Wicker, D.; Maurice, D. V.; Smith, J.; Benton, R., 2015:
523 Low-intensity, short-duration thermal stimulation during the late phase of incubation alters
524 secondary sex ratio in favour of males. *British Poultry Science*., **56**, 381–388.

525 Haldane, J. B. S., 1922: Sex ratio and unisexual sterility in hybrid animals. *Journal of Genetics*.,
526 **XII**, 101–109.

527 Halle, I.; Tzschentke, B., 2011: Influence of temperature manipulation during the last 4 days of

528 incubation on hatching results, post-hatching performance and adaptability to warm growing
529 conditions in broiler chickens. *Journal of Poultry Science*., **48**, 97–105.

530 Halle, I.; Tzschentke, B.; Henning, M.; Köhler, P., 2012: Influence of temperature stimulation
531 during the last 6 days of incubation on hatching results and later performance in Pekin ducks.
532 *Archiv für Geflugelkunde*., **76**, 176–183.

533 Harun, M. A. S.; Veeneklaas, R. J.; Visser, G. H.; Van Kampen, M., 2001: Artificial incubation of
534 Muscovy duck eggs: Why some eggs hatch and others do not. *Poultry Science*., **80**, 219–224.

535 Hodgetts, B., 1991: Current hatchabilities in species of domestic importance and the scope for
536 improvement. Proceedings of the *Avian incubation* conference, *sine dato* 1990, *sine loco*, pp.
537 139–144.

538 Jenkins, S. A.; Porter, T. E., 2004: Ontogeny of the hypothalamo-pituitary-adrenocortical axis in
539 the chicken embryo: A review. *Domestic Animal Endocrinology*., **26**, 267–275.

540 King'ori, A. M., 2011: Review of the factors that influence egg fertility and hatchability in poultry.
541 *International Journal of Poultry Science*., **10**, 483–492.

542 King, D. E.; Asem, E. K.; Adeola, O., 2000: Ontogenetic development of intestinal digestive
543 functions in white pekin ducks. *Journal of Nutrition*., **130**, 57–62.

544 Koláčková, M.; Kreisinger, J.; Albrecht, T.; Hořák, D., 2019: Effect of incubation temperature on
545 sex-dependent embryo mortality and morphological traits in Mallard. *Journal of Thermal*
546 *Biology*., **83**, 95–102.

547 Lamot, D. M.; Van De Linde, I. B.; Molenaar, R.; Van Der Pol, C. W.; Wijtten, P. J. A.; Kemp, B.;
548 Van Den Brand, H., 2014: Effects of moment of hatch and feed access on chicken development.
549 *Poultry Science*., **93**, 2604–2614.

550 Landauer, W., 1961: The Hatchability of Chicken Eggs as Influenced by Environment and

551 Heredity. University of Connecticut, Storrs Agricultural Experiment Station, Storrs, CT, USA.

552 Litt, J.; Leterrier, C.; Fortun-Lamothe, L., 2021: Conditions d'élevage des palmipèdes à foie
553 gras : des demandes sociétales à une démarche de progrès. *INRAE Productions Animales.*, **33**,
554 203–222.

555 Loyau, T.; Berri, C.; Bedrani, L.; Praud, C.; Duelos, M. J., 2013: Thermal manipulation of the
556 embryo modifies the physiology and body compositiob of broiler chickens reared in floor pens
557 without affecting breast meat processing quality. *American Society of Animal Science.*, **91**,
558 3674–3685.

559 Loyau, T.; Bedrani, L.; Berri, C.; Métayer-Coustard, S.; Praud, C.; Coustham, V.; Mignon-
560 Grasteau, S.; Duclos, M. J.; Tesseraud, S.; Rideau, N.; Hennequet-Antier, C.; Everaert, N.;
561 Yahav, S.; Collin, A., 2015: Cyclic variations in incubation conditions induce adaptive responses
562 to later heat exposure in chickens: A review. *Animal.*, **9**, 76–85.

563 Maatjens, C. M.; Van Roovert-Reijrink, I. A. M.; Engel, B.; Van Der Pol, C. W.; Kemp, B.; Van
564 Den Brand, H., 2016: Temperature during the last week of incubation. I. Effects on hatching
565 pattern and broiler chicken embryonic organ development. *Poultry Science.*, **95**, 956–965.

566 Massimino, W.; Davail, S.; Bernadet, M. dominique; Pioche, T.; Ricaud, K.; Gontier, K.; Manse,
567 H.; Morisson, M.; Collin, A.; Panserat, S.; Houssier, M., 2019: Impact of thermal manipulation
568 during embryogenesis on hepatic metabolism in mule ducks. *Fontiers in Physiology.*, **10**, 1–12.

569 Massimino, W.; Davail, S.; Secula, A.; Andrieux, C.; Bernadet, M. dominique; Pioche, T.;
570 Ricaud, K.; Gontier, K.; Morisson, M.; Collin, A.; Panserat, S.; Houssier, M., 2020: Ontogeny of
571 hepatic metabolism in mule ducks highlights different gene expression profiles between
572 carbohydrate and lipid metabolic pathways. *BMC Genomics.*, **21**, 1–13.

McLaughlin, E. J.; Hiscock, R. J.; Robinson, A. J.; Hui, L.; Tong, S.; Dane, K. M.; Middleton, A. L.; Walker, S. P.; MacDonald, T. M., 2020: Appropriate-for-gestational-age infants who exhibit reduced antenatal growth velocity display postnatal catch-up growth. *PLoS ONE*., **15**, e0238700.

Mueller, C. A.; Burggren, W. W.; Tazawa, H., 2015: The Physiology of the Avian Embryo. In: *Scanes, C. G. (ed.*) *Sturkie's Avian Physiology*. Sixth Edition. Elsevier, Denton, TX, USA, pp. 739–766.

Narinç, D.; Erdo, S.; Tahtabiçen, E.; Aksoy, T., 2016: Effects of thermal manipulations during embryogenesis of broiler chickens on developmental stability , hatchability and chick quality. *Animal*., **10**, 1328–1335.

Ono, H.; Hou, P. C. L.; Tazawa, H., 1994: Responses of developing chicken embryos to acute changes in ambient temperature: Noninvasive study of heart rate. *Israel Journal of Zoology*., **40**, 467–479.

Peebles, E. D.; Burnham, M. R.; Gardner, C. W.; Brake, J.; Bruzual, J. J.; Gerard, P. D., 2001: Effects of incubator humidity and hen age on yolk composition in broiler hatching eggs from young breeders. *Poultry Science*., **80**, 1444–1450.

Piestun, Y.; Shinder, D.; Ruzal, M.; Halevy, O.; Brake, J.; Yahav, S., 2008: Thermal manipulations during broiler embryogenesis: Effect on the acquisition of thermotolerance. *Poultry Science*., **87**, 1516–1525.

Piestun, Y.; Halevy, O.; Shinder, D.; Ruzal, M.; Druyan, S.; Yahav, S., 2011: Thermal manipulations during broiler embryogenesis improves post-hatch performance under hot conditions. *Journal of Thermal Biology*., **36**, 469–474.

Piestun, Y.; Druyan, S.; Brake, J.; Yahav, S., 2013: Thermal treatments prior to and during the

596 beginning of incubation affect phenotypic characteristics of broiler chickens posthatching.
597 *Poultry Science*., **92**, 882–889.

598 Romanoff, A. L., 1949: Critical Periods and Causes of Death in Avian Embryonic Development.
599 *The Auk*., **66**, 264–270.

600 Sauveur, B., 1988: Développement embryonnaire et incubation. In: *Sauveur, B. (ed.)*
601 *Reproduction des volailles et production d'œufs*. Inra, Paris, France, pp. 229–265. ebook
602 00859NUM.

603 Singhal, A., 2017: Long-Term Adverse Effects of Early Growth Acceleration or Catch-Up
604 Growth. *Annals of Nutrition and Metabolism*., **70**, 236–240.

605 Tzschentke, B.; Basta, D., 2000: Development of hypothalamic neuronal thermosensitivity in
606 birds during the perinatal period. *Journal of Thermal Biology*., **25**, 119–123.

607 Tzschentke, B.; Halle, I., 2009: Influence of temperature stimulation during the last 4 days of
608 incubation on secondary sex ratio and later performance in male and female broiler chicks.
609 *British Poultry Science*., **50**, 634–640.

610 Wilson, H. R., 1991: Physiological requirements of developing embryo: temperature and turning.
611 In: Tullett, S. G. (ed.), *Avian incubation*. Butterworth & Co (Publishers) Ltd, London, UK, pp.
612 145–156.

613 Wit, J. M.; Boersma, B., 2002: Catch-up growth : Definition , mechanism , and models. *Journal*
614 *of pediatric endocrinology & metabolism*., **15**, 1229–1241.

615 Yahav, S.; Collin, A.; Shinder, D.; Picard, M., 2004: Thermal manipulations during broiler chick
616 embryogenesis: Effects of timing and temperature. *Poultry Science*., **83**, 1959–1963.

617 Yahav, S.; Tzschentke, B., 2006: Perinatal thermal manipulations in poultry , does it cause long-
618 lasting thermoregulatory memory ? Proceedings of the *12th European Poultry Conference*., 10-

14 september 2006, Verona, Italy, pp. 1–6.

Yan, X. P.; Liu, H. H.; Liu, J. Y.; Zhang, R. P.; Wang, G. S.; Li, Q. Q.; Wang, D. M. C.; Li, L.; Wang, J. W., 2015: Evidence in duck for supporting alteration of incubation temperature may have influence on methylation of genomic DNA. *Poultry Science.*, **94**, 2537–2545.

Zaboli, G. R.; Rahimi, S.; Shariatmadari, F.; Torshizi, M. A. K.; Baghbanzadeh, A.; Mehri, M., 2016: Thermal manipulation during pre and post-hatch on thermotolerance of male broiler chickens exposed to chronic heat stress. *Poultry Science.*, **96**, 478–485.

Tables

Table 1. Impact of changing incubation conditions on embryonic mortality and birth parameters in mule ducks. The results of mid mortality (first panel), late mortality (second panel), hatchability (third panel) and proportion of males born (fourth panel) are listed according to the embryonic window (E). The maximum of inter-trial dispersion (ITD) and the average +/-SD of controls complete the table.

		+1.5°C 16h/24 65 % of relative humidity targeted					
Incubation results	Control	E9-E19	E10-E20	E11-E21	E12-E22	E13-E23	E14-E24
Mid embryonic mortality (%)							
Trial 1	8.2[b]			13.8[a]			
Trial 2	10.2[b]		24.5[a]		15.9[b]		
Trial 3	6.4[c]	20.5[a]				13.0[b]	13.1[b]
Estimation of max ITD		+3.8		+2.0		+3.8	+3.8
Average ± SD	8.3 ± 1.9						
Late mortality (%)							
Trial 1	7.0			5.5			
Trial 2	10.5		13.9		9.1		
Trial 3	4.8	9.0				4.7	4.0
Estimation of max ITD		+5.7		+2.5		+5.7	+5.7
Average ± SD	7.5 ± 2.9						
Hatchability (%)							
Trial 1	84.4			76.1			
Trial 2	80.1[a]		62.1[b]		76.1[a]		
Trial 3	88.1[a]	69.5[c]				82.7[a]	80.5[b]
Estimation of max ITD			+8.0	+3.7	+8.0		
Average ± SD	84.2 ± 4.0						
Proportion of males born (%)							
Trial 1	51.7			60.3			
Trial 2	53.8[b]		65.5[a]		60.5[ab]		
Trial 3	50.0[c]	67.6[a]				60.7[ab]	55.5[bc]
Estimation of max ITD		+3.8		+2.1		+3.8	+3.8
Average ± SD	51.8 ± 1.9						

[a-c] Within a row, means values without a common superscript letter differ significantly ($P < 0.05$).

636 **Table 2. Impact of changing incubation conditions on rearing parameters in mule ducks**.

637 The results of weight at hatch (first panel), weight at 6 days (D6) (second panel) and internal

638 temperature at 6 days (third panel) are represented according to the embryonic window (E). The

639 condition E11-E21 has no data at D6 for BW and internal temperature.

640

		+1.5°C 16h/24 65 % of relative humidity targeted					
Rearing parameters	Control	E9-E19	E10-E20	E11-E21	E12-E22	E13-E23	E14-E24
Weight at hatch (g)							
Trial 1	49.0			46.5			
Trial 2	53.7[a]		52.5[b]		51.8[b]		
Trial 3	54.7[a]	52.9[b]				51.8[b]	52.3[b]
Weight at D6 (g)							
Trial 2	174.2[a]		175.6[a]		158.6[b]		
Trial 3	215.7	251.0				209.5	212.3
Internal temperature at D6 (°C)							
Trial 2	41.8[a]		41.3[c]		41.5[b]		
Trial 3	41.9[a]	41.7[b]				41.8[b]	41.7[b]

641

642 [a-c] Within a row, means values without a common superscript letter differ significantly ($P < 0.05$).

643

644

Figure captions

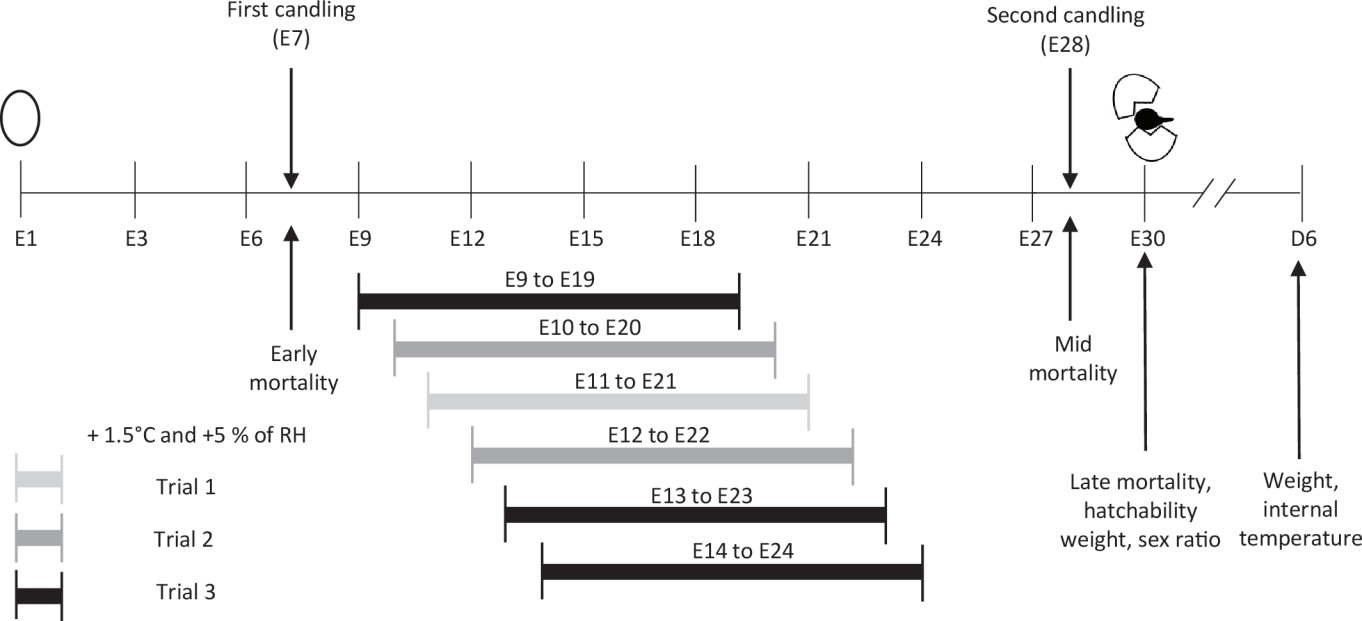
Figure 1. Experimental design

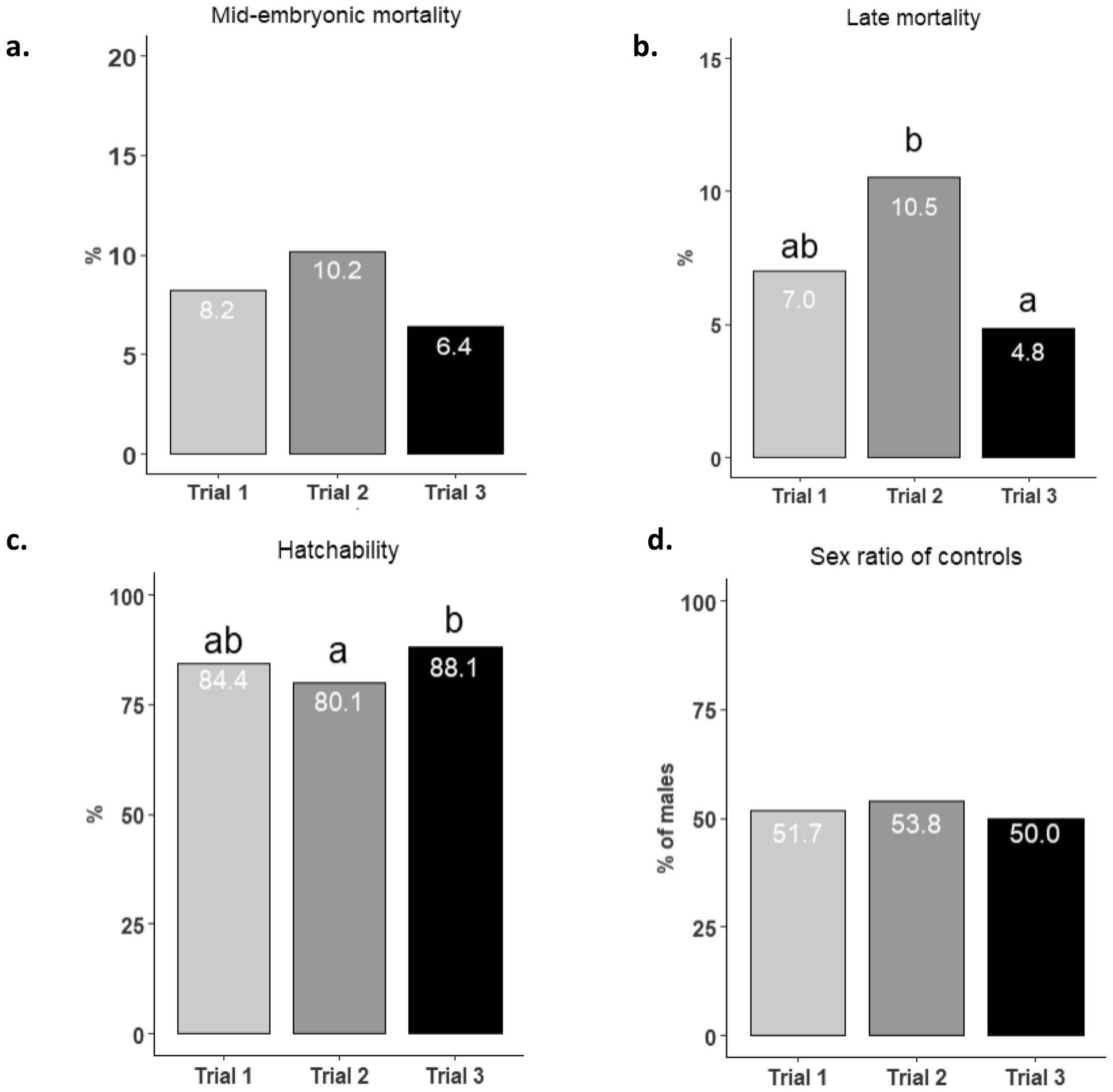
The chronology of the different trials is schematized on this frieze representing the incubation time of mule duck eggs.

Changes in conditions (+1.5°C 16h/24 and target +5 % RH) applied for 10 days are illustrated by the horizontal lines representing 10-day intervals. The period from E11 to E21 was tested on trial 1 (light gray line), periods E10-E20 and E12-E22 on trial 2 (dark gray lines) and periods E9-E19, E13-E23 and E14-E24 on trial 3 (black line). A control group was present for each trial with a temperature of 37.6°C and a mean relative humidity (RH) of 62.6 %.

Figure 2. The inter-trial dispersion between controls for 4 parameters: mid embryonic mortality, late embryonic mortality, hatchability and sex ratio in mule ducks

Measurement of mid-embryonic mortality (n = 265 to 279) (a), late embryonic mortality (n = 247 to 256) (b), hatching rate (c) (n = 265 to 279), and sex ratio (d) (n = 221 to 238) for the control conditions of trials 1, 2, and 3. Chi^2 statistical tests were used. Bars with different superscript letters are significantly different ($P < 0.05$).


First candling
(E7)
Second candling
(E28)
E1
E3
E6
E9
E12
E15
E18
E21
E24
E27
E30
D6
E9 to E19
E10 to E20
E11 to E21
E12 to E22
E13 to E23
E14 to E24
Early
mortality
Mid
mortality
Late mortality,
hatchability
weight, sex ratio
Weight,
internal
temperature
+ 1.5°C and +5 % of RH
Trial 1
Trial 2
Trial 3


a.
Mid-embryonic mortality
%
20
15
10
5
0
8.2
10.2
6.4
Trial 1
Trial 2
Trial 3
b.
Late mortality
%
15
10
5
0
ab
7.0
b
10.5
a
4.8
Trial 1
Trial 2
Trial 3
c.
Hatchability
%
100
75
50
25
0
ab
84.4
a
80.1
b
88.1
Trial 1
Trial 2
Trial 3
d.
Sex ratio of controls
% of males
100
75
50
25
0
51.7
53.8
50.0
Trial 1
Trial 2
Trial 3