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Effect of incubation temperature on sex-dependent embryo mortality and morphological traits in Mallard

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ABSTRACT

Although birds have genetically determined sex, the sex ratio has been reported to deviate from parity in several studies. Temperature-dependent sex determination, which is common in reptiles, is absent in birds. However, females are able to adjust their investment into eggs according to the sex of the embryo, which may cause sex-specific embryonic mortality. Incubation temperature may also cause sex-biased embryonic mortality, and it may differentially affect the phenotype of male and female hatchlings. We aimed to investigate differences between male and female Mallard embryos regarding their egg size, mortality during incubation and hatchling phenotype in relation to incubation temperature. Mallard eggs were incubated under six constant incubation temperatures (ranging from 35.0 to 38.0 °C). Hatchlings were weighed, and their morphological traits were measured. We determined the sex of hatchlings and unhatched embryos by genetic analysis and found higher male embryonic mortality at 35.5 °C (44 males vs. 28 females) and a higher proportion of female hatchlings at 38 °C (24 males vs. 38 females); however, these results were not statistically significant. Our results suggest that Mallard females do not differentiate quantitatively between sexes during egg production. Male hatchlings were significantly larger but not heavier than females. The size difference between sexes was most pronounced at temperatures around 36 °C, which is the mean temperature of naturally incubated Mallard eggs.

1. Introduction

Birds have genetically determined sex (for review see Ellegren (2000)), and sex ratio distribution is usually even within a clutch (Atamian and Sedingeir, 2010; Harmsen and Cooke, 1983; Koenig and Dickinson, 1996). However, it has been reported to deviate from parity in several studies (Appleby et al., 1997; Dijkstra et al., 1990; Kosztolányi et al., 2011). A biased fledgling sex ratio can be generally caused by two mechanisms: (i) adjusting the primary sex ratio of the clutch by the female (Appleby et al., 1997; Dijkstra et al., 1990), (ii) sex-specific mortality of embryos (Eiby et al., 2008; Göth and Booth, 2005; Pérez et al., 2006) or hatchlings (Kilner, 1998; Nager et al., 1999; Székely et al., 2004). Differential sex allocation can be related to female quality (Trivers and Willard, 1973) and occurs in species where one sex shows a higher variance in reproductive success than the other. This hypothesis has been supported in some bird species (Ankney, 1982; Dijkstra et al., 1990; Kilner, 1998; Mead et al., 1987) but rejected in others (Atamian and Sedingeir, 2010; Lislevand et al., 2005). In nidicolous species (i.e., species whose young remain in the nest for a period after hatching), the sex-specific mortality of hatchlings can be facilitated by sex-related differences in parental feeding rates (Clotfelter, 1996). This is, however, not applicable to nidifugous species (i.e., species whose young leave the nest shortly after hatching), in which active parental manipulation after hatching is highly restricted (Cooch et al., 1997). In such species, manipulation may still occur during incubation via variation in incubation temperatures.

Although temperature-dependent sex determination is a common phenomenon in some phylogenetic groups of reptiles (Booth, 2006), it seems to be limited in birds because of genetic sex determination. Still, there may be an impact of the interaction between sex and temperature on embryonic mortality. However, it has been considered to be non-adaptive in birds and therefore has not attracted much attention (Pike and Petrie, 2003). In consequence, even though many studies have documented increased embryonic mortality due to suboptimal incubation temperatures (Hassan et al., 2004; Hepp et al., 2006; Koláčková et al., 2015; Prince et al., 1969), information on the interaction between sex and incubation temperature is rare in birds (but see Batt and Cornwell (1972); Collins et al. (2013); DuRant et al. (2016) and studies

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investigating the Australian brush-turkey (*Alectura lathami*) – Göth and Booth (2005), Göth (2007) and Eiby et al. (2008)). This topic thus deserves more attention.

Apart from sex-related embryonic mortality, differential sex allocation might result in variation of young quality between sexes (Blanco et al., 2003; Howe, 1977; Nager et al., 1999). In nidifugous species, the investment into reproduction can be divided into two parts: (i) clutch formation and (ii) its incubation. Females primarily adjust their investment by manipulation of egg mass (Ankney, 1982; Cordero et al., 2000; Mead et al., 1987; Rubolini et al., 2009), enabling sex-related egg mass variation. Secondly, incubating parents invest effort to maintain the incubation temperature.

It has been shown that incubation temperature can influence phenotypic traits (Eiby and Booth, 2009; Hepp et al., 2006; Koláčková et al., 2015; Nord and Nilsson, 2011). Interaction between incubation temperature and sex may then modify survival-related traits, such as body mass and structural size (Kilner, 1998; Mead et al., 1987).

In this study, we aimed to test the effect of incubation temperature on sex ratio of both successfully hatched and unhatched embryos. Moreover, we investigated the differences between male and female embryos regarding their egg size, mortality during incubation and newborn hatchling phenotype. We used Mallard (*Anas platyrhynchos*) as a model species. It has precocial and nidifugous young, so the energy allocated to eggs is crucial for hatching success and body mass, and it is tightly linked to the probability of survival during the early post hatching period (Anderson and Alisauskas, 2001; Bolton, 1991; Pelayo and Clark, 2003). Only the female takes part in clutch incubation (Abraham, 1974; Caldwell and Cornwell, 1975). The female's ability to maintain an incubation temperature near optimum depends on her body condition and experience (Aldrich and Raveling, 1983). Mallards are seasonally monogamous birds (Bossema and Roemers, 1985; McKinney et al., 1983) with a slightly skewed adult sex ratio in favour of males (Owen and Dix, 1986). Males are the sex with more variable reproductive success in Mallard (Cunningham, 2003; Gauthier, 1988; McKinney et al., 1983). Previous research documented higher susceptibility of male Mallard embryos to short-term clutch cooling to 0 °C prior to the onset of incubation (Batt and Cornwell, 1972). However, to our knowledge, the interaction between sex and incubation temperature in their effect on embryonic mortality has not been investigated in this species before. Specifically, we aimed to find out whether (i) incubation temperature affects hatchling sex ratio, (ii) Mallard females generally allocate more energy to eggs of a particular sex, (iii) male and female hatchlings differ in their mass, structural size and incubation period.

2. Methods

2.1. Incubation experiments

We used Mallard eggs obtained from a commercial duck-raising producer, where the animals were kept under semi-wild conditions. Thus, it was impossible to control explicitly for female identities. However, the eggs were collected from a large flock of ducks over a period of several days. Given the fact that duck females lay one egg per day, the probability of sampling one individual multiple times was relatively low. Eggs were then assigned randomly to particular incubators, which further reduces the possibility of systematic bias in the analysis. We numbered all the eggs and determined their fresh mass to the nearest 0.1 g using electronic scales (1479V, Tanita, Japan). The length and width of all eggs were measured to the nearest 0.01 mm using digital callipers. Incubators with automatic egg turning (Mono 48, Bioska, Czech Republic) were used for incubation. We used four incubators that were used for varying incubation temperatures in the course of the work in order to rule out potential differences among them. We incubated the eggs in two seasons. In the first season, we sexed only the hatchlings; in the second season, we also took DNA samples from unhatched embryos. Some eggs were either infertile or stopped developing at an early stage and thus remained unsampled. We hypothesised that the possible effects of incubation temperature would be more pronounced at its extremes. Therefore, we incubated eggs over a relatively wide range of incubation temperatures (total number of eggs for each temperature is given in the parentheses): 35.0 °C (46 eggs), 35.5 °C (184 eggs), 36.0 °C (184 eggs), 37 .0°C (184 eggs) and 38.0 °C (92 eggs). This range of temperatures reflects temperatures observed in nests of Mallards breeding in the Czech Republic (P. Klvaňa, T. Albrecht and P. Musil, unpublished data).

Eggs were turned automatically every 3 h. We added water to the incubator water reservoir every third day, and we sprinkled the eggs with lukewarm water every day from day 25 of incubation. The average air humidity ranged from 42% to 52% at all incubation temperatures and did not significantly differ among treatments ($p > 0.05$). Eggs were cooled for 30 min at laboratory temperature (~20 °C) daily from day 9 of incubation. The cooling period was set based on the recommendations of the incubator producer, so that the negative effect of artificial conditions on hatching success is reduced to minimum. After 14 days of incubation, we candled all eggs (Weller, 1956) and excluded undeveloping eggs from the experiment. We took tissue samples from dead embryos (when possible) and stored them in 96% ethanol. From day 25 of incubation, the eggs were checked every 12 h to estimate the start of hatching. Pipped eggs were placed in net-sacks (Hořák and Albrecht, 2007) together with a numbered piece of paper for further identification. Dry hatchlings were collected every 12 h, and they were immediately euthanized with chloroform. After that, their fresh body mass was determined to the nearest 0.01 g using electronic scales (VIBRA AJ-2200CE, Shinko Denshi, Japan). Further, we determined the following structural measurements using digital callipers: length of *tarsometatarsus* (hereafter *tarsus*), bill length (from the front rim of nostrils to the tip of bill), bill width (at the level of front rim of nostrils) and total skull length (from the bill tip to the end of *os occipitale*). We subtracted the bill length from total skull length to get an approximate length of the cranial part of the skull (hereafter skull length). Then, we took tissue samples (one segment of the first finger) and stored them in 96% ethanol. After measurements, we stored hatchlings in a deep freezer at −80 °C for further analyses. To determine the residual yolk sac mass, we used 40 hatchlings from each of three different temperatures: 35.5; 36.0 and 37.0 °C. Out of our temperature range, we chose these temperatures because they most likely occur under natural conditions (Caldwell and Cornwell, 1975; P. Klvaňa, T. Albrecht and P. Musil, unpublished data). From these hatchlings, we extracted the residual yolk sac (hereafter yolk sac) and determined its wet mass using digital scales to nearest 0.0001 g (FR-200 MK II, A&D Company, Limited, Japan).

2.2. Sex determination

For sex determination, we used genetic analysis as described in Griffiths et al. (1998). In brief, DNA was extracted using Qiagen DNAeasy kit and manufacturer's protocol for tissue samples. The sex-linked CHD gene was amplified using primers P2 and P8 following PCR conditions described in Griffiths et al. (1998). PCR products were stained with ethidium bromide and separated by electrophoresis on 2% agarose gel. In birds, females are heterogametic (ZW) and males are homogametic (ZZ). CHD-W and CHD-Z genes have introns of different size, which leads to two bands appearing in the case of a female and one band in the case of a male.

2.3. Statistical analyses

All analyses were performed in R 3.5.0 (R Core Team, 2018). We used Chi-square goodness of fit test to assess whether the sex ratio deviated from parity and chi-square test based on contingency tables to assess whether the sex ratios varied among incubation temperatures.

The effect of egg mass, incubation temperature and sex on several body traits of hatchlings was assessed based on general linear models with normal distribution of errors. Egg mass was a continuous variable, while incubation temperature and sex were categorical variables. We used general linear models to test the effects of egg mass, incubation temperature and sex on several body traits of hatchlings and on incubation period. The initial model included these main effects and all two- and three-way interactions. We used the stepwise deletion procedure to eliminate insignificant terms from the initial model to get a minimal adequate model (MAM). The MAM did not include any interactions ($p > 0.05$). Residuals were tested for normality by Kolmogorov-Smirnov test.

In addition, we estimated the overall hatchling structural size using the first Principle Component (PC1) from Principle Components Analysis (PCA) of the following measurements: bill length (factor loading = −0.60), bill width (factor loading = −0.85), *tarsus* length (factor loading = −0.75) and skull length (factor loading = −0.75). Because the factor loading values were below zero, we used the reverted value of PC1, which showed a positive correlation with all measurements under analysis. The first axis explained 54.92% of variation.

3. Results

We did not find any effect of incubation temperature on sex ratio ($\chi^2 = 3.920$, df = 4, p = 0.417), when comparing the proportion of males and females hatched under different incubation temperatures. The largest deviation from parity occurred at 38.0 °C with 38 female and 24 male hatchlings, but it was not statistically significant (Table 1). In dead embryos, the largest deviation from parity occurred at 35.5 °C with 28 female and 44 male dead embryos, but it was also insignificant (Table 1). In total, we identified sex in 412 hatchlings, 207 of which were classified as females and 205 as males. This sex ratio did not significantly differ from 1:1 sex ratio ($\chi^2 = 0.01$, df = 1, p = 0.92). Out of 173 dead embryos, 79 were females and 94 were males. This ratio also did not significantly differ from parity ($\chi^2 = 1.30$, df = 1, p = 0.25). Finally, we compared the sex ratios of hatchlings and dead embryos, and we did not find a significant difference ($\chi^2 = 0.85$, df = 1, p = 0.36).

Hatching success was highest at 37.0 °C and decreased at both higher and lower temperatures (Table 1).

We investigated whether the female energy allocation into egg formation relates to the sex of a hatchling it produces; however, we did not find any relationship between sex and egg mass, length or width (Table 2).

We tested the effects of egg mass, incubation temperature and sex on several body traits of hatchlings. Egg mass significantly and positively affected all body traits we studied (MAM: Body mass: $F_{1, 405} = 459.09$, p « 0.001, b = 0.58, SE = 0.03; Skull length: $F_{1, 405} = 42.25$, p « 0.001, b = 0.07, SE = 0.01; Bill length: $F_{1, 405} = 12.74$, p < 0.001, b = 0.03, SE = 0.01; Bill width: $F_{1, 405} = 47.96$, p « 0.001, b = 0.04, SE = 0.01; *Tarsus* length: $F_{1, 405} = 32.03$, p « 0.001, b = 0.07, SE = 0.01; Overall structural size: $F_{1, 405} = 61.93$, p « 0.001, b = 0.08, SE = 0.01). We did not find any significant effect of incubation temperature or sex on hatchling body mass (MAM: $F_{4, 405} = 2.04$, p = 0.09; $F_{1, 405} = 2.80$, p = 0.10, respectively, Fig. 1A). However, all structural measurements studied were significantly affected by incubation temperature (MAM: Skull length: $F_{4, 405} = 4.04$, p = 0.003, Fig. 2; Bill length: $F_{4, 405} = 8.07$, p « 0.001, Fig. 3; Bill width: $F_{4, 405} = 7.54$, p « 0.001, Fig. 4; *Tarsus* length: $F_{4, 405} = 19.28$, p « 0.001, Fig. 5). The overall structural size was also significantly and negatively affected by incubation temperature (MAM: $F_{4, 405} = 5.00$, p = 0.001, Fig. 1B). We did Tukey HSD post-hoc tests for all variables affected by incubation temperature; significant differences between temperatures are denoted by superscript letters in Figs. 1–5.

All structural measurements were affected by sex, with males being larger than females (MAM: Skull length: $F_{1, 405} = 28.14$, p « 0.001, Fig. 2; Bill length: $F_{1, 405} = 4.60$, p = 0.03, Fig. 3; Bill width: $F_{1, 405} = 3.82$, p = 0.05, Fig. 4; *Tarsus* length: $F_{1, 405} = 4.82$, p = 0.03, Fig. 5). Similarly, the overall structural size was significantly affected by sex (MAM: $F_{1, 405} = 15.61$, p « 0.001, Fig. 1B). We transformed the structural measurements to z-scores to compare the standardized effect size of sex on morphometric traits. The effect size of sex on skull length was 0.438, on bill length 0.165, on bill width 0.191 and on *tarsus* length 0.237. We did separate ANCOVAs for particular temperature levels to determine the temperatures at which the structural measurements were most affected by sex. We found significant differences between sexes at the middle incubation temperatures (35.5, 36.0 and 37.0 °C) in the case of skull length and at 36.0 °C in the case of bill width, *tarsus* length and overall structural size (Table 3).

Because our results suggest that male hatchlings are structurally larger but not significantly heavier, we decided to perform some additional analyses in order to explain this discrepancy. First, we suggested that the larger structural size of male hatchlings may be caused by their tendency to hatch later (and therefore to have more time for development). We tested the effects of egg mass, incubation temperature and sex on the incubation period. The incubation period was significantly and negatively affected only by incubation temperature (MAM: $F_{4, 405} = 249.56$, p « 0.001). Neither egg mass nor sex had any significant effect on incubation period (MAM: $F_{1, 405} = 0.60$, p = 0.44; $F_{1, 405} = 1.23$, p = 0.27, respectively). Second, we suggested that material conversion from the yolk to the body may be enhanced in male hatchlings. Then, males would have a smaller yolk sac than females at hatch, while their overall body mass would not differ. We tested the effects of egg mass, incubation temperature and sex on mass of yolk sac. Yolk sac mass was significantly and positively affected by egg mass and negatively affected by incubation temperature (MAM: $F_{1, 115} = 4.41$, p = 0.04; $F_{2, 115} = 9.79$, p < 0.001, respectively) but it was not affected by sex (MAM: $F_{1, 115} = 0.60$, p = 0.44, Fig. 6).

Table 1
Sex ratio of Mallard hatchlings and dead Mallard embryos incubated under different temperatures tested against equal sex ratio. Chi square goodness of fit test, df = 1.

Incubation temperature (°C)	35.0	35.5	36.0	37.0	38.0
Number of incubated eggs	46	184	184	184	92
Hatching success (%)	15.2	47.3	65.8	73.9	68.5
Number of sampled eggs	41	158	156	162	68
Female hatchlings	3	40	58	68	38
Male hatchlings	4	46	63	68	24
χ^2	0.14	0.42	0.21	0	3.16
p value	0.71	0.52	0.65	1	0.08
Dead female embryos	20	28	15	13	3
Dead male embryos	14	44	20	13	3
X^2	1.06	3.56	0.71	0	0
p value	0.30	0.06	0.40	1	1

Table 2
Egg mass, egg length and egg width of embryos of different sex in Mallard. 286 female and 299 male embryos were included into analyses. One-way ANOVA.

	Sex	Mean	Minimum	Maximum	SE	F (1,583)	p-value
Egg mass (g)	F	62.78	50.00	75.79	0.27	0.02	0.90
	M	62.83	51.00	77.33	0.27		
Egg length (mm)	F	59.42	52.80	67.93	0.14	0.01	0.94
	M	59.44	53.26	68.23	0.14		
Egg width (mm)	F	43.55	39.90	47.19	0.07	0.003	0.96
	M	43.55	40.01	46.77	0.07		

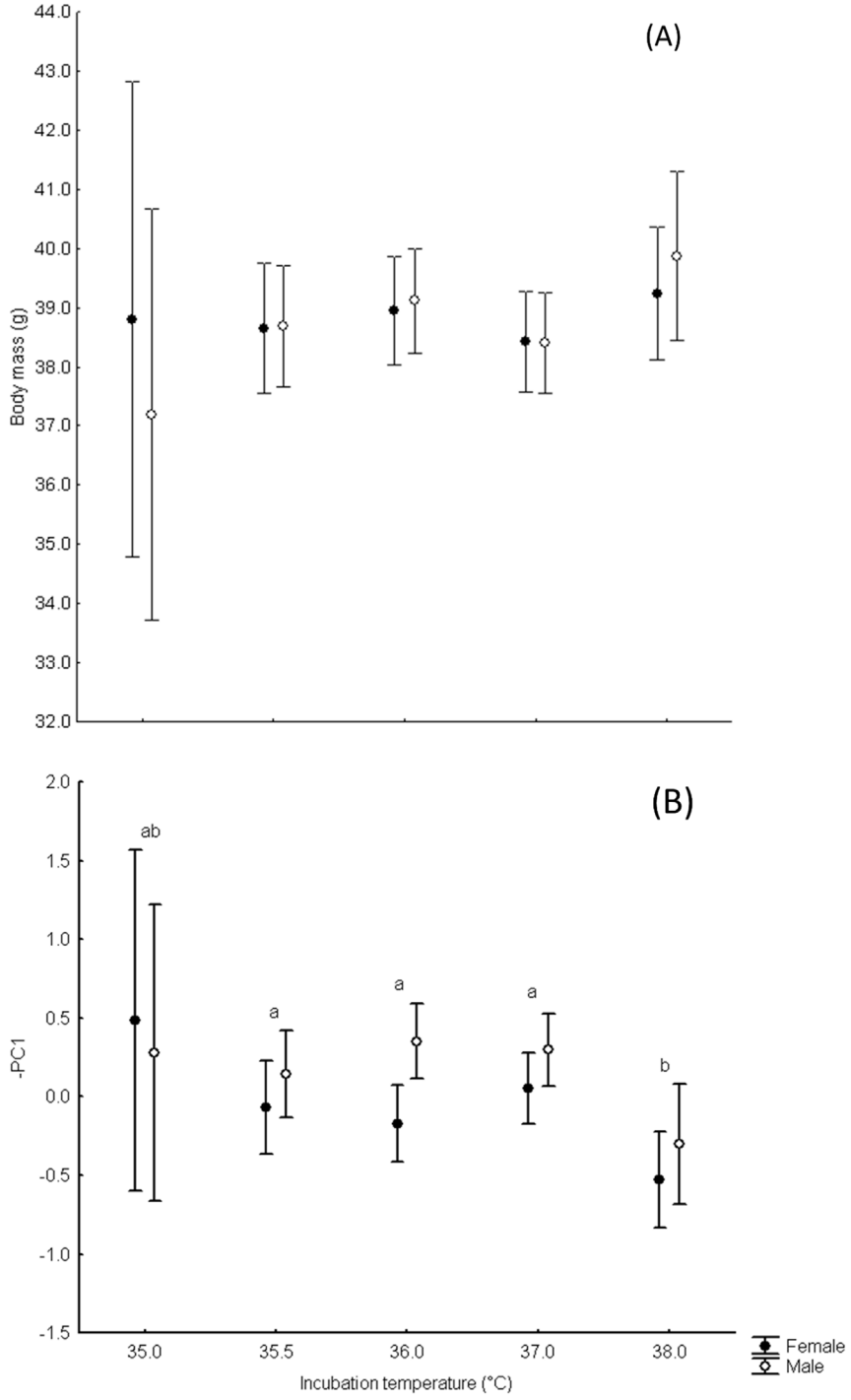

Fig. 1. (A) Body mass of male and female Mallard hatchlings at different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. **(B)** Sex differences in overall structural size (-PC1) of Mallard hatchlings among different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. Superscript letters indicate significant differences between incubation temperatures (Tukey HSD test, $p < 0.05$).

4. Discussion

We searched for possible manipulation of the sex ratio during incubation via variation in the incubation temperature. Suboptimal incubation temperature is usually caused by low body condition or insufficient experience of the incubating female (Aldrich and Raveling, 1983). Thus, a clutch incubated by a low-quality female may produce hatchlings with skewed sex ratio. We found lower hatching success at both high and low incubation temperatures (See our previous study, Koláčková et al. (2015) for more details.). In the dead embryos, the largest deviation from parity in favour of males occurred at 35.5 °C; however, it was not statistically significant. Batt and Cornwell (1972) showed that male Mallard embryos are more susceptible to cold exposure prior to incubation. In contrast, Eiby et al. (2008) found female-biased embryonic mortality at low incubation temperatures in the Australian Brush-turkey, and DuRant et al. (2016) found the same effect in the Wood Duck (*Aix sponsa*). In hatchlings, the largest deviation from parity in favour of females occurred at 38.0 °C. This result suggests a possible existence of male-biased embryonic mortality at high temperatures, as was found by Eiby et al. (2008) in the Australian Brush-

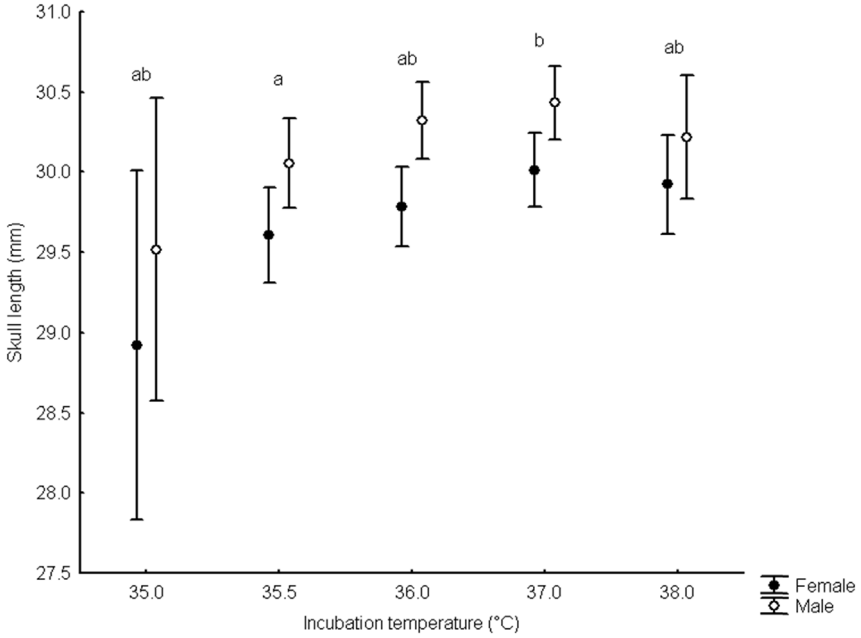

Fig. 2. Sex differences in skull length of Mallard hatchlings among different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. Superscript letters indicate significant differences between incubation temperatures (Tukey HSD test, $p < 0.05$).

turkey. Unfortunately, we may fail to detect it because we did not sample dead embryos in the first season of incubation, and we sampled only six dead embryos at 38.0 °C in the second season. We suggest that a larger sample size, especially at the extreme temperatures, may show statistically significant results. Although we tried to mimic natural incubation behaviour of Mallard female by cooling of the eggs for 30 min per day, these incubation conditions do differ from natural ones. Caldwell and Cornwell (1975) found that the rest period, when the female Mallard is off the nest, averages 24 min (it is 77 min on average based on our unpublished data from the Czech Republic, P. Klvaňa et al.). However, they found several rest periods per day, so that the clutch is daily attended on average 22.7 h. Thus, the absence of significant sex-specific embryonic mortality in our case could be a consequence of incubating eggs under artificial conditions, as these presumably differ in their effect on sex determination from those found in nature, as shown by Paitz et al. (2010) in their study on the Red-eared Slider Turtle (*Trachemys scripta*). However, such effects could be less pronounced in homoiothermic birds compared to reptiles. The possible effect of incubation temperature on male embryonic mortality thus needs further investigation in Mallard.

Our results suggest that Mallard females do not differentiate quantitatively between sexes during egg production. Sex-dependent allocation of material in eggs has been studied in several avian species. For example, it has been found in Mountain White-crowned Sparrow (*Zonotrichia leucophrys oriantha*; Mead et al., 1987), House Sparrow (*Passer domesticus*; Cordero et al., 2000), and Yellow-legged Gull (*Larus*

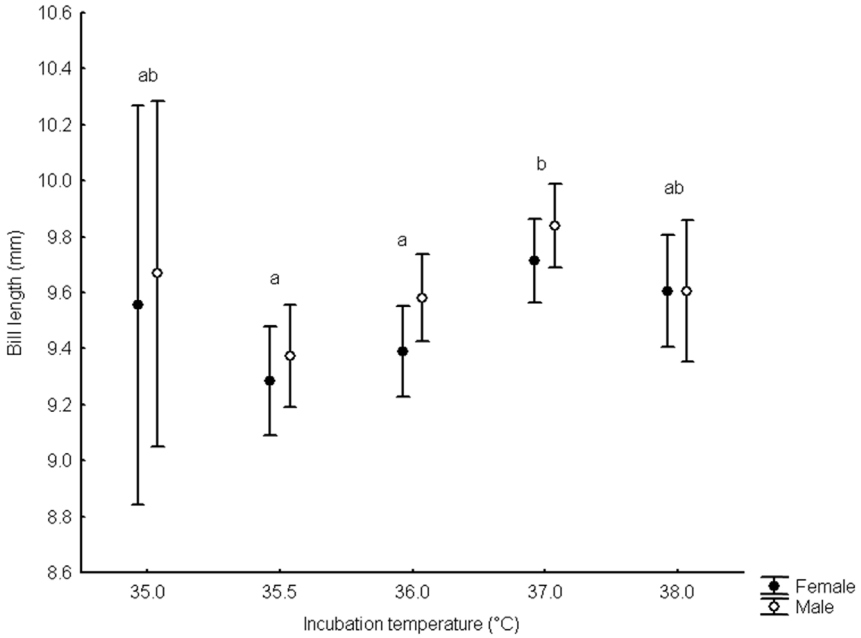

Fig. 3. Sex differences in bill length of Mallard hatchlings among different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. Superscript letters indicate significant differences between incubation temperatures (Tukey HSD test, $p < 0.05$).

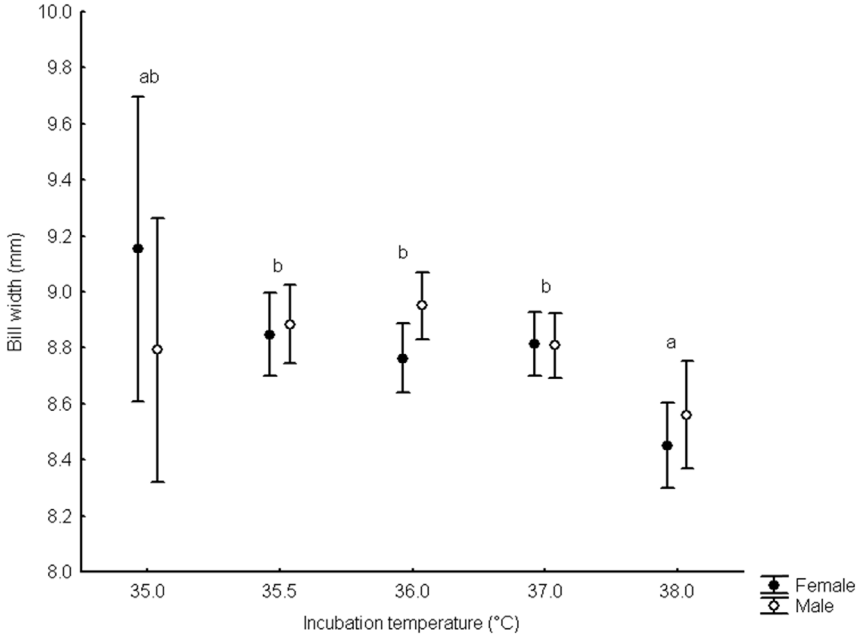

Fig. 4. Sex differences in bill width of Mallard hatchlings among different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. Superscript letters indicate significant differences between incubation temperatures (Tukey HSD test, $p < 0.05$).

michahellis; Rubolini et al., 2009). In contrast, studies on Northern Lapwing (*Vanellus vanellus*; Lislevand et al., 2005) and Greater Sage-grouse (*Centrocercus urophasianus*; Atamian and Sedingeir, 2010) did not find any support for this phenomenon. Among Anseriformes, Ankney (1982) found sex-dependent allocation of egg material in the Lesser Snow Goose (*Chen caerulescens caerulescens*), but Leblanc (1987) could not find it in Canada Goose (*Branta canadensis*) and similarly Blums and Mednis (1996) found no evidence of sex-dependent allocation of egg material in three species of *Anatinae*. The Trivers-Willard hypothesis (Trivers and Willard, 1973) states that females in good condition should invest more resources into the sex with more variable reproductive success. In case of Mallards, reproductive success is assumed to be more variable in males (Cunningham, 2003; Gauthier, 1988; McKinney et al., 1983), which are both heavier and structurally larger than females as adults (Hudec, 1994). Females under our study were fed *ad libitum*, i.e. in a good body condition. Therefore, we supposed that they should have invested more material into male eggs. However, we did not investigate other possible differences in egg quality, such as yolk mass or chemical composition.

Male hatchlings were slightly heavier than females; however, the difference was not significant. Body mass is the main factor positively affecting the growth and survival of a hatchling (Anderson and Alisauskas, 2001; Williams, 1994). However, larger body mass may cause higher energy demands, which could be disadvantageous in the case of food restriction. For example, Cooch et al. (1997) found greater mortality of male hatchlings in the Lesser Snow Goose, where males are

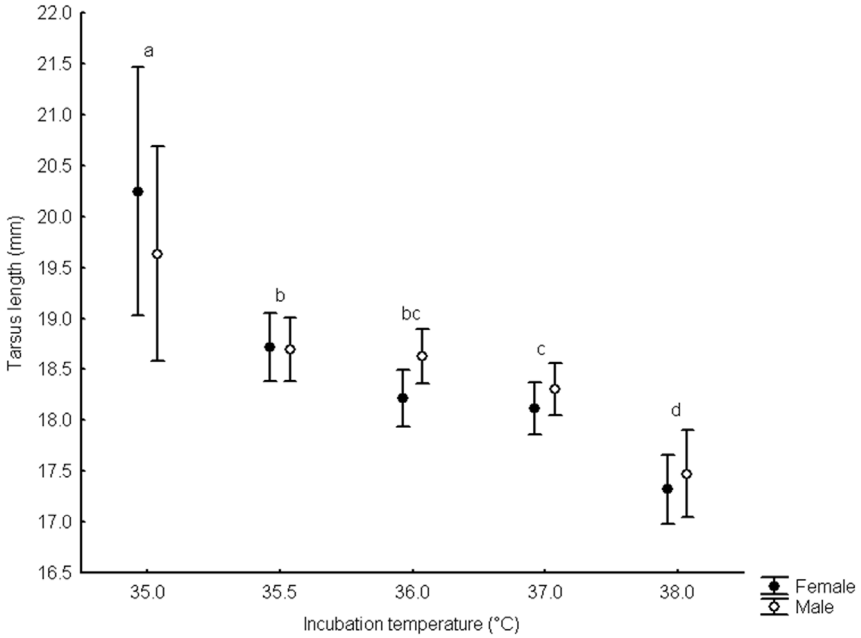

Fig. 5. Sex differences in *tarsus* length of Mallard hatchlings among different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. Superscript letters indicate significant differences between incubation temperatures (Tukey HSD test, $p < 0.05$).

Table 3
Effect of sex on structural measurements and overall structural size (-PC1) of Mallard hatchlings. Separate ANCOVAs for particular temperature levels.

Incubation temperature (°C)	35.0	35.5	36.0	37.0	38.0
Number of hatchlings	7	86	121	136	62
Skull length					
F-value	1.98	6.45	11.94	8.21	1.81
p-value	0.23	0.01	0.001	0.005	0.18
Bill length					
F-value	0.06	0.83	2.77	1.95	0.01
p-value	0.82	0.37	0.10	0.17	0.94
Bill width					
F-value	1.35	0.87	5.33	0.03	0.87
p-value	0.31	0.36	0.02	0.87	0.35
Tarsus length					
F-value	0.33	< 0.01	5.63	1.38	0.77
p-value	0.60	0.95	0.02	0.24	0.39
-PC1					
F-value	0.05	1.92	11.79	3.39	1.31
p-value	0.83	0.17	0.001	0.07	0.26
Degrees of freedom	1,4	1,83	1118	1133	1,59

the larger sex. Similarly, Benito and González-Solís (2007) reported greater mortality of the larger sex hatchlings in various avian species. In contrast, males showed better survival to adult age than female Mallards (Owen and Dix, 1986).

Interestingly, male hatchlings were structurally larger in all the measurements. Importantly, we did not find any interaction between sex and temperature, indicating that the difference in structural parameters between males and females is not dependent on incubation temperature. The effect size of sex was largest in the case of skull length, and the difference between sexes was significant at the middle incubation temperatures (35.5, 36.0 and 37.0 °C). These temperatures are close to optimum in terms of hatching success (Koláčková et al., 2015; Prince et al., 1969). However, these were also the temperatures with the largest sample size in our study. We suggest that a larger sample size at the extreme temperatures may lead to more solid results. We tested the hypothesis that the larger structural size of males could be caused by longer incubation period and thus more time for development of male embryos, but we did not find any difference in incubation period between sexes.

Unfortunately, the effect of incubation temperature interacting with sex in their influence on phenotype remains under-explored. Göth and Booth (2005) found male-biased sex ratio and low body mass of hatchlings at low incubation temperature and female-biased sex ratio and higher body mass at high incubation temperature. They suggested that the difference in the mean hatchling body mass cannot be attributed to sex because male and female hatchlings in the medium temperature did not differ in mass. Unfortunately, they did not compare masses of males and females either at the low or high incubation temperatures.

In the current study, we found that the only significant difference in phenotypic traits between male and female hatchlings was the difference in structural size. We suggest that the larger structural size of male hatchlings may not be directly influenced by maternal investment in terms of energy and material deposited in the egg. More likely, it is a result of different responses of their endocrine systems to incubation temperature, as was suggested for different metabolic rates of sexes in Painted Turtle (*Chrysemys picta*; Spencer and Janzen, 2014).

Finally, we found no difference in yolk sac mass between sexes. The relationship between sex and yolk sac mass has not been studied very often in birds, but, for example, in a freshwater turtle, the study of Rhen and Lang (1999) found no effect of sex on yolk sac mass, which corroborate our results. The dependence of yolk sack mass on incubation temperature has been reported in some previous studies involving birds and reptiles, where lower temperatures cause longer incubation times and more yolk material is converted to hatchling tissue (Booth et al., 2004; Gutzke et al., 1987; Koláčková et al., 2015; Rhen and Lang, 1999). We found lower yolk sac mass at 35.5 °C here. For more details about relationship between incubation temperature and hatchling phenotype, see our previous study, Koláčková et al. (2015).

5. Conclusions

We found no sex differences in incubation period and embryo mortality during incubation at different incubation temperatures in Mallard. We also found no sex differences in egg mass and hatchling mass; however, we found that male hatchlings were structurally larger in comparison to females and that this difference tended to be most

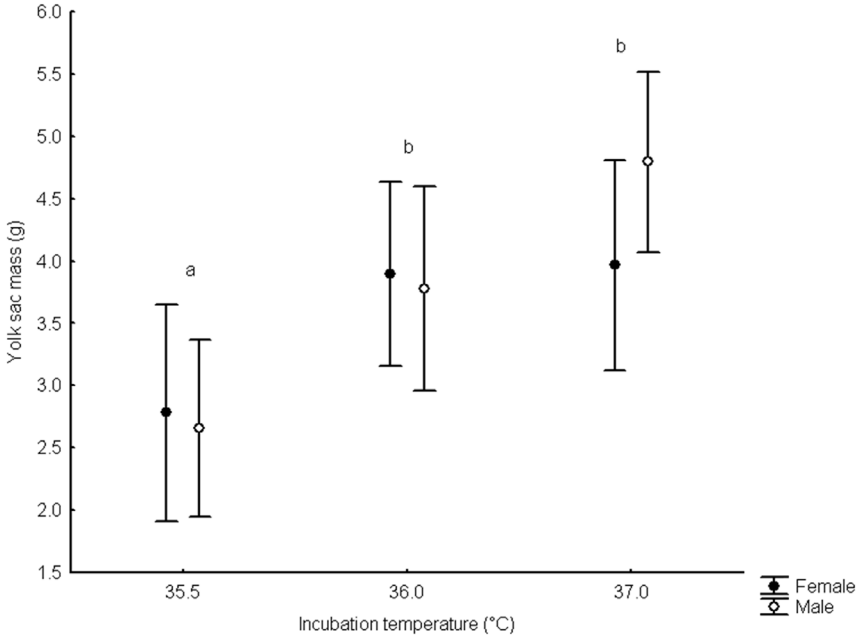

Fig. 6. Yolk sac mass of male and female Mallard hatchlings among different incubation temperatures. Least square means, vertical bars denote 95% confidence interval. Superscript letters indicate significant differences between incubation temperatures (Tukey HSD test, $p < 0.05$).

pronounced at temperatures around 36.0 °C, which is close to optimum in terms of hatching success and reflects a natural incubation temperature in Mallards.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.05.007.

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