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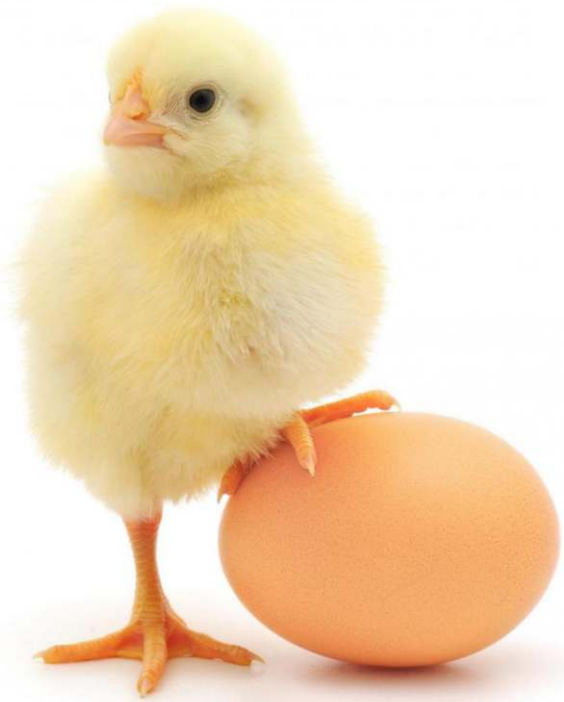

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Changes During Incubation Within Double-Yolked Duck (*Anas platyrhynchos domesticus*) Eggs: Yolk Position, Mortality, Hatchability and the Importance of an Optimal Egg Size

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Abstract: Double-yolked (DY) and single-yolked (SY) duck eggs (n = 1318 for both) were candled and weighed on Days (d) 2, 8, 12, 15, 19, 22 and 25 of incubation and yolk/embryo position was recorded on d2, d8 and at post-mortem. From d8 only eggs with live fertile yolk(s) remained in the incubator. On d2, 99.39% of yolks in DY eggs were in the adjacent position and by d8 yolk positions changed to parallel position in DY1F (one fertile yolk) and DY2F (two fertile yolks) eggs, 14.1 and 88.62%, respectively (p<0.001). Thus yolk position is associated with yolk fertility. In eggs with two infertile yolks yolk position remained adjacent in 88.21% of the eggs. Early (d1-d8), mid (d9-d21) and late (≥d22) embryonic mortality was significantly higher in DY compared to SY eggs (p<0.001). Early mortality was higher in DY1F eggs (31.58, vs. 12.03% DY2F eggs; p<0.001). Late mortality was higher in DY2F eggs (70.38, vs. 51.03% DY1F eggs; p<0.001). Hatchability of fertile SY eggs was 87.2% (1001/1148). Single ducklings hatched from 11 of 437 DY1F eggs (2.52%) and from two of 449 DY2F eggs (0.44%). The egg weight of hatched DY eggs tended towards the SY egg norm suggesting an optimal DY egg size and associated yolk volume for the successful production of viable ducklings.

Key words: Double-yolked egg, duck, hatchability, incubation, mortality, yolk position

INTRODUCTION

A small number of studies have examined developmental changes during incubation in double-yolked (DY) eggs (Jeffrey *et al*., 1953; Monkman, 1963; Burke *et al*., 1997; Fasenko *et al*., 2000). In broiler breeders, early (Day (d) 1-d7), mid (d8-d14) and late (d15-d21) mortality rates were significantly greater in DY than in SY eggs (Jeffrey *et al*., 1953; Fasenko *et al*., 2000).

In avian species there are two 'critical' periods during incubation when mortality rates peak: one in early embryonic life and the other shortly before hatching (Romanoff, 1949; Bogenfurst, 2004). Early embryonic mortality coincides with the development of the blood circulatory system and diet change from simple carbohydrates to more complex proteins and lipids in the yolk and albumen (Bogenfurst, 2004). Peak mortality before hatching in single-yolked (SY) eggs is attributed to failure to make proper transition to pulmonary respiration (Romanoff, 1949; Bogenfurst, 2004). However, in DY eggs immaturity or differential development (Bateson, 1976), or malposition of the embryos, or space constraints in the egg for two embryos to position themselves correctly for pipping and hatching and inadequate shell surface area necessary for embryo respiration were all suggested as contributing factors for the high mortality before and during hatching (Hollander and Levi, 1940; Monkman, 1963; Fasenko *et al*., 2000).

The hatchability of fertile SY domestic ducks eggs is approximately 62-73.6% (Ksiazkiewicz, 2002, 2003). However, DY eggs have a low percentage of live hatch (Jeffrey *et al*., 1953; Monkman, 1963; Burke *et al*., 1997; Fasenko *et al*., 2000) and the positioning of the embryos is regarded as of key importance, especially at the end of incubation (Monkman, 1963). In pigeons (*Columba livia*), all embryos developed to hatching stage in DY eggs (n = 5), but none hatched (Hollander and Levi, 1940), while both embryos died in the last stage of incubation in a DY ostrich (*Struthio camelus*) egg (n = 1) (Horbanczuk *et al*., 2003). In contrast, hatching was more successful in DY eggs of domestic fowl (Table 1). However, live twins only emerged when assisted at hatching (Jeffrey *et al*., 1953; Monkman, 1963).

It is now known that in DY duck eggs yolk position and size facilitate albumen secretion (Salamon and Kent, 2013). Further, yolk position has an influence on fertility with a marked primacy effect, i.e., the first yolk ovulated has a higher probability of being fertile (Salamon and Kent, 2014a). Here, DY and SY duck eggs were incubated and mortality rates were recorded. Yolk positions were determined during candling at d2

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and d8 of incubation and embryo position was determined post-mortem to examine the importance of yolk/embryo positioning during incubation and hatching.

MATERIALS AND METHODS

Eggs from a flock of Aylesbury ducks over a two year period at Ballyrichard (Arklow, Ireland; 52°50'5" N, 6°7'49" W) were studied. The flock and eggs were managed as described by Salamon and Kent (2014a). Briefly, eggs were collected at 9:30 am and again at 11:00 am daily, washed and stored until setting at fortnightly intervals. Eggs were set pointed end down in a Western incubator (Suffolk, UK) with temperature and relative humidity set in accord with the manufacturer's instructions and prior experience with automatically turning 45° from the vertical at hourly intervals.

A total of 1318 DY and 1318 SY eggs from 42 batches were identified before setting by individual candling. 316 DY eggs with one fertile and one infertile yolk (DY1F eggs), 403 DY eggs with two fertile yolks (DY2F eggs; two alive or one alive and one dead) and 1143 SY eggs containing live embryos remained in the incubator after d8 (see Salamon and Kent, 2014a). Batch 23 (17 DY1F eggs, eight DY2F eggs and 38 SY eggs) was excluded from the analysis due to an incomplete dataset.

The eggs were weighed on a digital scale (±0.1 g) on d2. Yolk position was recorded on d2 and d8 at candling and the yolk next to the airspace was termed Yolk 1 (Salamon and Kent, 2013). Yolk 1 has higher fertility, which suggests it was ovulated first (Salamon and Kent, 2014a) and probably passing down the oviduct first too, as large DY eggs are suggested to be laid blunt end first (Salamon and Kent, 2014b).

Mortality was recorded on d8, d12, d15, d19, d22, d25. On d26, eggs were candled again and transferred to the hatcher. Dead eggs were removed when identified, stored overnight and broken open the next day to estimate the day of embryonic death using Kaltofen (1971) as a guide. When the death of the embryo could not be determined, due to infection or advanced degradation of egg contents, the eggs were discarded (17 SY eggs, 152 DY1F eggs and 246 DY2F eggs). Dead embryos were divided into three categories: death in early (d1-d8), mid (d9-d21) and late (d22 and later) developmental periods.

On examining dead in eggs after hatching (d30/31), three post-mortem classifications were used: 1. died prior to entering airspace, 2. entered airspace, 3. pipped. The post-mortem embryo position (adjacent, semi parallel, parallel) and the head direction of the embryo(s) (normal towards air space, upside down, curled around long axis) were examined in DY eggs that had at least one live embryo on d26 (DY1F, n = 164; DY2F, n = 259). When there was doubt about embryo position at post-mortem the egg was discarded (DY1F, n = 47; DY2F, n = 94). In determining head direction only embryos that finished tucking of bill under the wing (latest by d25; Oppenheim, 1970; 1972; Kaltofen, 1971) were used, the remaining eggs were discarded (DY1F, n = 49; DY2F, n = 97).

The effect of DY egg shape on yolk positioning at d2 and the effects of DY egg shape, yolk positioning at d2 and fertility on yolk positioning at d8 were examined using GLM. Two-sample t-tests were used to compare egg weights (d2) of DY eggs with different fertility status. McNemar's chi-squared test was employed to examine the change in yolk positioning in DY eggs between d2, d8 and post-mortem embryo positioning using the statistical software R (version 2.15.3, R Development Core Team, 2013). Proportion tests were employed to compare early, mid and late mortalities within and between SY, DY1F and DY2F eggs.

RESULTS

Yolk positioning: Three yolk positions were identified on d2 in DY eggs: adjacent, semi parallel and parallel (Fig. 1), but in 43 eggs yolks were either floating (n = 5 at d2, n = 34 at d8) or of an unidentified position (n = 4 at d8; Table 2). DY egg shape did not influence yolk position at d2 (F = 1.54, p = 0.216, df = 926, adj R^2 = 0.001%). 99.39% of yolks in DY eggs were in adjacent position at d2 and 51.44% remained in adjacent position at d8. However overall in DY eggs yolk position changed from d2 to d8; 9.56% of yolks changed to semi parallel (McNemar's χ^2 = 670.04, df = 1, p<0.001) and 36.12% changed to parallel position (McNemar's χ^2 = 673.01, df = 1, p<0.001) and these changes were associated with fertility of yolks.

Yolk fertility (using the data of Salamon and Kent, 2014a) was significantly associated with yolk positioning at d8 (t = 23.1, p<0.001) according to the GLM (F = 178.8, p<0.001, df = 924, adj R^2 = 36.52%), but DY egg shape (t = 1.57, p = 0.116) and yolk positioning at d2 (t = 0.56, p = 0.576) were not. On d8, 88.21% of yolks in DY0F eggs (i.e., two infertile yolks) remained in adjacent position. In DY1F eggs, 63.66% remained adjacent position at d8, while 20.93% changed to semi parallel (McNemar's χ^2 = 281.09, df = 1, p<0.001) and 14.1% changed to parallel (McNemar's χ^2 = 284.03, df = 1, p<0.001). In DY2F eggs, 88.62% of yolks changed from adjacent to parallel by d8 (McNemar's χ^2 = 25.29, df = 1, p<0.001; Table 3).

Mortality during incubation: In SY eggs early (d1-d8, 3.75%, 43/1148), mid (d9-d21, 3.75%, 43/1148) and late mortality (≥d22, 5.31%, 61/1148) were all low (Table 4; Fig. 2, 3). Mortality in DY eggs was higher than in SY eggs in early, mid and late mortality periods (all p<0.001; Fig. 2, 3). Early embryonic mortality was higher in DY1F eggs (31.58%, 138/437 vs. DY2F eggs, 12.03%, 54/449; p<0.001), while late embryonic mortality (≥d22) was higher in DY2F eggs (70.38%, 316/449 vs. DY1F eggs, 51.03%, 223/437; p<0.001; Fig. 3).

Table 1: Studies showing domestic fowl hatching from DY eggs with one (DY1F) or two fertile yolks (DY2F)

Study	No. of DY eggs	Hatched live chicks (from No. of DY eggs)
Jeffrey *et al.* (1953)[1]	152	1 twin (1DY2F)
Monkman (1963)[1]	220	7 twins (7 DY2F), 37 single (10 DY2F, 27 DY1F)
Burke *et al.* (1997) exp. 4[2]	895	38 single (38 DY1F)
Burke *et al.* (1997) exp. 5[2]	778	17 single (17 DY1F)
Fasenko *et al.* (2000)	128	6 single (6 DY1F)

Table 2: Yolk positions in DY eggs at d2 and d8 of incubation

Yolk position	d2 (%)	d8 (%)
Adjacent	1310 (99.39)	678 (51.44)
Semi parallel	2 (0.15)	126 (9.56)
Parallel	1 (0.08)	476 (36.12)
Floating	5 (0.38)	34 (2.58)
Unidentified	-	4 (0.30)
Total	1318 (100)	1318 (100)

Table 3: Yolk positions in DY eggs at d8 relative to fertility status (DY0F-two infertile yolks, DY1F-one fertile and one infertile yolk, DY2F-two fertile yolks)

Yolk position at d8	DY0F (%)	DY1F (%)	DY2F (%)
Adjacent	359 (88.21)	289 (63.66)	30 (6.56)
Semi parallel	10 (2.46)	95 (20.93)	21 (4.60)
Parallel	7 (1.72)	64 (14.10)	405 (88.62)
Floating	28 (6.88)	5 (1.10)	1 (0.22)
Unidentified	3 (0.74)	1 (0.22)	-
Total	407 (100)	454 (100)	457 (100)

Of the 1148 SY eggs, 147 died and the day of death is known for 130 of these eggs (88.44% of dead SY eggs). Of these 33.08% (43/130) were early embryonic mortality, 29.23% (38/130) were mid embryonic mortality and 37.69% (49/130) were late embryonic mortality.

Of the 437 DY1F eggs, 426 died and the day of death could be determined in 274 eggs (64.32% of dead DY1F eggs). Of these 44.52% (122/274) were early embryonic mortality, 22.63% (62/274) were mid embryonic mortality and 32.85% (90/274) were late embryonic mortality, all differing from each other significantly (all p<0.01).

There were 164 DY1F eggs examined post-mortem; in 20.12% (33/164) of these eggs the embryos were bathed in watery fluid (Type I embryos; Burke *et al.*, 1997) and in 50% (82/164) of these eggs the embryos internalized the whole or some of Yolk 2 (i.e., Type II embryos; Burke *et al.*, 1997). Type II DY1F eggs tended to be heavier than Type I DY1F eggs, though the difference was not significant (112.63±17.32 g vs. 106.35±18.02 g; p = 0.085). In 0.6% (1/164) of eggs, Yolk 2 was not utilized, while the remaining 29.27% (48/164) eggs are unknown.

Of the 449 DY2F eggs, 447 died and the day of death is known for both yolks in 201 eggs (44.97% of dead DY2F eggs). In 53.23% of those eggs (107/201) both embryos died on the same day (early mortality: 30.84%, 33/107; mid mortality: 18.69%, 20/107; late mortality: 50.47%, 54/107). However, in the remaining 94 eggs (46.77%) there were 1-8 days (48.94%, 46/94), 9-16 days (26.6%, 25/94) and 17-24 days (24.46%, 23/94) between the deaths of the two embryos. Thus in approximately half of these DY2F eggs, the death of one embryo did not necessarily mean the relatively sudden death of the other embryo. In DY2F eggs it was not possible to distinguish the embryos of Yolk 1 and Yolk 2 after d8, especially in parallel position; therefore it is difficult to determine precisely, which of the two embryos died first.

Hatchability: Here data from eggs post d26 of incubation is considered. Fertile SY eggs had a hatchability rate of 87.2% (1001/1148). In DY eggs, 2.52% (11/437) of DY1F eggs hatched and 0.44% (2/449) of DY2F eggs produced a single duckling. Further, 2.52% (11/437) of DY1F and 1.78% (8/449) of DY2F eggs were pipped, embryos in 1.37% (6/437) of DY1F and 1.78% (8/449) of DY2F eggs entered airspace, while embryos in 33.64% (147/437) of DY1F and 54.12% (243/449) of DY2F eggs died prior to entering airspace.

There was no significant difference in the egg weights at d2 between the died prior to entering airspace (113.1±18.71 g), entered airspace (118.21±23.85 g), pipped (111.58±24.86 g) or hatched (103.58±11.82 g) DY egg groups (all p>0.05). However, DY eggs that hatched tended to be lighter than those that died prior to entering airspace, entered airspace or pipped. Further, the weight of hatched DY eggs tended towards the weight of SY eggs at d2 (91.7±6.74 g).

Embryo positioning: The post-mortem embryo positions were recorded and are based on the yolk positions (adjacent, semi parallel, parallel; Fig. 1). Of the 100 DY1F eggs with known post-mortem embryo position that died prior to entering airspace, the embryo and the infertile yolk were in adjacent position (embryo next to the airspace, infertile yolk distant to the airspace) in 99% (99/100) of the eggs, while the embryo and the infertile yolk were in parallel position (embryo and infertile yolk both next to the airspace) in 1% (1/100) of the eggs (Table 5). In DY1F eggs, all eggs that entered airspace (n = 6) and all eggs that pipped (n = 11), the embryo and the infertile yolk were in adjacent position (Table 5).

In DY2F eggs, of the 149 eggs with known post-mortem embryo position that died prior to entering airspace, the two embryos were in adjacent position in 17.45% (26/149), semi parallel position in 1.34% (2/149) and parallel position in 81.21% (121/149) of those eggs (Table 6). In DY2F eggs that entered airspace the embryos were in adjacent position in 12.5% of eggs (1/8) and were in parallel position in 87.5% (7/8) of eggs, while the embryos were in parallel position in all DY2F eggs that pipped (n = 8; Table 6). There was a significant positive association between yolk position on d8 and post-mortem embryo position (McNemar's χ^2 = 42.15, df = 3, p<0.001), i.e., 82.27% (232/282) of embryos at post-mortem were in the same position as the yolks were at d8 in those DY eggs (died prior to entering airspace, entered airspace, pipped).

Table 4: Cumulative number and proportion (%) of live and dead DY1F, DY2F and SY eggs on each measurement day. DY2F eggs were only considered as dead when both embryos were dead

	------- d2- ------		--------- d8 ---------		------- d12 -------		------- d15 --------		------- d19 -------		------- d22 -------		------- d25 -------	
	live	dead	live	dead	live	dead	live	dead	live	dead	live	dead	live	dead
DYF1	437	0	299	138	289	148	267	170	244	193	234	203	208	229
DYF2	449	0	395	54	370	79	351	98	330	119	318	131	293	156
SY	1148	0	1105	43	1099	49	1088	60	1075	73	1062	86	1045	103
	-- d2 (%) --		------ d8 (%) -------		----- d12 (%) ------		----- d15 (%) -----		----- d19 (%) -----		---- d22 (%) -----		----- d25 (%) ----	
DYF1	100	0	68.42	31.58	66.13	33.87	61.10	38.90	55.84	44.16	53.55	46.45	47.60	52.40
DYF2	100	0	87.97	12.03	82.14	17.59	78.17	21.83	73.50	26.50	70.82	29.18	65.26	34.74
SY	100	0	96.25	3.75	95.73	4.27	94.77	5.23	93.64	6.36	92.51	7.49	91.03	8.97

Table 5: Total number of DY1F eggs that died prior to entering airspace, entered airspace and pipped in relation to post-mortem embryo positions (PEP) and head direction of embryo (HE) within the egg

DY1F	Died prior to entering airspace	Entered airspace	Pipped
PEP			
Adjacent	99 (99%)	6 (100%)	11 (100%)
Semi parallel	-	-	-
Parallel	1 (1%)	-	-
Total	100 (100%)	6 (100%)	11 (100%)
HE			
'Normal' position	63 (64.29%)	6 (100%)	11 (100%)
Upside down	33 (33.67%)	-	-
Curled around axis	2 (2.04%)	-	-
Total	98 (100%)	6 (100%)	11 (100%)

Table 6: Total number of DY2F eggs that died prior to entering airspace, entered airspace and pipped in relation to post-mortem embryo positions (PEP) and head direction of embryos (HE) within the egg

DY2F	Died prior to entering airspace	Entered airspace	Pipped
PEP			
Adjacent	26 (17.45%)	1 (12.5%)	-
Semi parallel	2 (1.34%)	-	-
Parallel	121 (81.21%)	7 (87.5%)	8 (100%)
Total	149 (100%)	8 (100%)	8 (100%)
HE			
Both in 'normal' position	53 (36.3%)	5 (62.5%)	2 (25%)
One in 'normal' position, one upside down	49 (33.56%)	3 (37.5%)	5 (62.5%)
Both upside down	22 (15.07%)	-	1 (12.5%)
One in 'normal' position, one early dead	13 (8.9%)	-	-
One upside down, one early dead	4 (2.74%)	-	-
Both curled around axis	5 (3.43%)	-	-
Total	146 (100%)	8 (100%)	8 (100%)

In DY1F eggs, of the 98 eggs that died prior to entering airspace the embryo was in 'normal' position (head towards airspace) in 64.29% (63/98) of eggs, the embryo was upside down in 33.67% (33/98) of eggs, while in 2.04% (2/98) of eggs the embryo was curled around the long axis of the egg (Table 5). In DY1F eggs, all eggs that entered airspace (n = 6) and all eggs that pipped (n = 11), the embryo was in 'normal' position (Table 5).

In DY2F eggs, of the 146 eggs that died prior to entering airspace both embryos were in 'normal' position in 36.3% (53/146) of eggs, one embryo was in 'normal' position and the other was upside down in 33.56% (49/146) of eggs, both embryos were upside down in 15.07% (22/146) of eggs and in 3.42% (5/146) of eggs both embryos were curled around the long axis (Table 6). In 8.9% (13/146) of DY2F eggs that died prior to entering airspace one embryo was in 'normal' position and the other died early ($\geq$d4), while in 2.74% (4/146) of DY2F eggs that died prior to entering airspace the embryo was upside down and the other died early ($\geq$d4). From the DY2F eggs that entered airspace 62.5% (5/8) of eggs contained embryos both in 'normal' position, while in 37.5% (3/8) of eggs one embryo was in 'normal' position and the other was upside down. From the DY2F eggs that pipped 25% (2/8) of eggs contained embryos both in 'normal' position, in 62.5% (5/8) of eggs one embryo was in 'normal' position and one upside down, while in 12.5% (1/8) of eggs both embryos were upside down (Table 6).

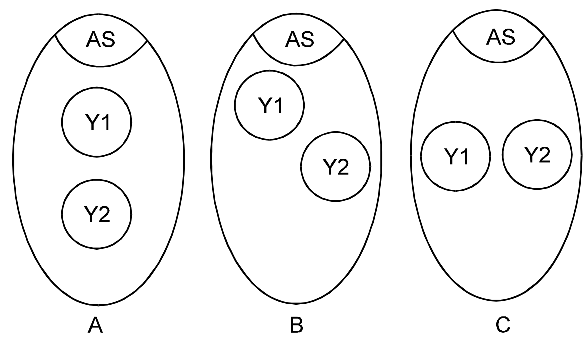

Fig. 1: Yolk positions within DY eggs: adjacent (A), semi parallel (B) and parallel (C). When candled on Day 2 of incubation, the yolk next to the airspace (AS) is termed Yolk 1 (Y1), the other Yolk 2 (Y2)

DISCUSSION

Yolk positioning: Here, in DY eggs three yolk positions were identified: adjacent, semi parallel and parallel (Fig. 1). Initially yolks were in adjacent position in 99.39% of DY eggs on d2. However yolk position changed over the course of incubation, especially in fertile DY eggs.

In the infertile DY eggs 88.21% of yolks remained in the adjacent position, but these eggs were removed on identification at d8. Yolk positioning at d8 was associated with yolk fertility (p<0.001; Table 3) and in DY1F eggs 14.1% of yolks changed to parallel position

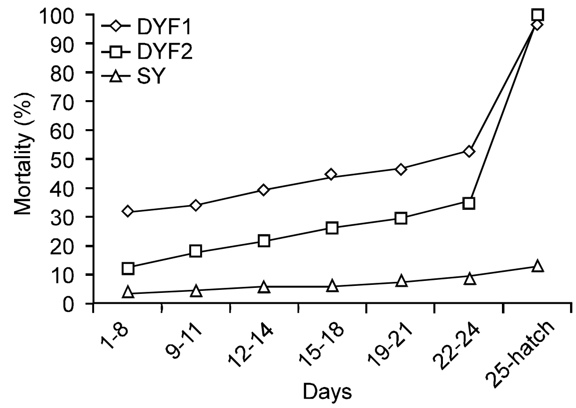

Fig. 2: Cumulative mortality (%) of DY1F, DY2F and SY eggs between measurement days over the incubation period

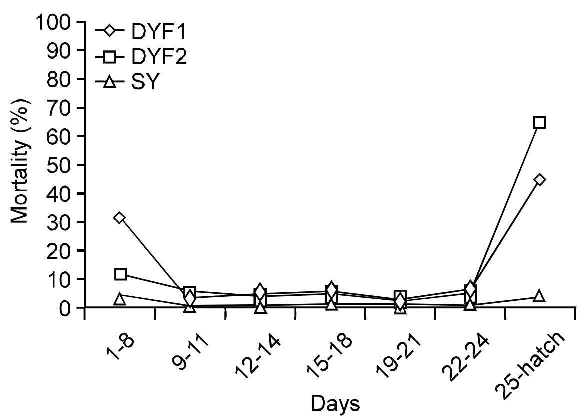

Fig. 3: Non-cumulative mortality (%) of DY1F, DY2F and SY eggs between measurement days over the incubation period

by d8 (p<0.001). In DY2F eggs 88.62% of yolks changed to parallel position by d8 (p<0.001), i.e., having a yolk position such that the two embryo were equidistant to the airspace at the round end with its greater pore density (Mazanowski *et al.*, 2005) facilitating higher rates of gas exchange (Ohsawa *et al.*, 1985), facilitating gaseous exchange in similar measures for each embryo.

Mortality during incubation: In avian species there are two 'critical' periods during incubation with mortality peaks: first early in development and then shortly before hatching (Romanoff, 1949; Bogenfurst, 2004). The early mortality in hen eggs is attributed to physiological changes at d4: the development and start of blood circulation (Romanoff, 1949; Kaltofen, 1971; Bogenfurst, 2004) and the change from simple carbohydrates to more complex protein and lipid diet (Bogenfurst, 2004). No early embryonic mortality peak was found in SY eggs in contrast to previous studies (Romanoff, 1949; Bogenfurst, 2004); in fact it was the same as mid embryonic mortality at 3.75%. In DY eggs higher mortality rates were found at the early embryonic mortality period (d1-d8) consistent with earlier work on DY eggs (Jeffrey *et al.*, 1953; Fasenko *et al.*, 2000).

During the mid-embryonic period mortality rates were relatively low and constant (d9-d21; DY1F eggs: 2.29-5.26%; DY2F eggs: 2.67-5.57%; SY eggs: 0.52-1.13%; Fig. 3). Cumulative mortality showed a steady increase with slower increase in SY eggs (Fig. 2). When DY1F and DY2F were compared cumulative mortality was parallel, but approximately 20% higher until d24 in DY1F eggs (Fig. 2) consistent with the possibility that the infertile yolk or its albumen may have been a source of bacterial challenge to the living embryo, consistent with the study of Fasenko *et al.* (2000). In DY1F eggs early embryonic mortality was higher than mid embryonic mortality (31.58 vs. 14.87%, respectively; p<0.001), while early mortality was about ten times higher than mid mortality in DY broiler breeder eggs (Jeffrey *et al.*, 1953; Fasenko *et al.*, 2000). In DY2F eggs early embryonic mortality was lower than mid embryonic mortality (12.03 vs. 17.37%, respectively; p = 0.03).

DY eggs have more albumen than SY eggs, but it forms a significantly smaller proportion of their weight in pheasants (Deeming, 2011) and ducks (Salamon and Kent, 2013) and only 24.5% additional albumen is secreted for Yolk 2 in DY duck eggs (Salamon and Kent, 2013).

In DY1F eggs, the single embryo may also utilize all or some of Yolk 2 and the study of Burke *et al.* (1997) termed such embryos Type II. Here, 50% of DY1F eggs were Type II, while 15.63% (20/128) of DY broiler breeder eggs contained Type II embryos (Fasenko *et al.*, 2000). Further, Type II DY eggs tended to be heavier than the Type I DY eggs (containing embryos not utilizing Yolk 2) consistent with the finding of Burke *et al.* (1997) in broiler breeders. These results and the finding that small DY eggs have small yolks (Salamon and Kent, 2013) show that the utilization of Yolk 2 in (especially small) DY1F eggs may be crucial for the survival of the embryo if its own yolk is small and the evidence in this study discussed below suggest this. Further, before hatching in DY1F eggs the embryo utilizing Yolk 2 tends to be larger than a single embryo from a SY egg (Jeffrey *et al.*, 1953; Burke *et al.*, 1997; Fasenko *et al.*, 2000).

In DY2F eggs when two yolks are fertile a relatively limited amount of albumen is available for each embryo (Salamon and Kent, 2013) constraining the development/growth and explains why twin chicks from DY eggs are lighter (Jeffrey *et al.*, 1953). Experimental studies with domestic fowl showed that the removal of 1-20% of the egg's albumen content resulted in lower embryo and chick weight at hatch (Deeming, 1989; Hill, 1993; Finkler *et al.*, 1998; Everaert *et al.*, 2013; reviewed in Willems *et al*., 2014) and decreased tibiotarsus length (Finkler *et al*., 1998). Though in Yellow-legged gulls (*Larus michahellis*) the removal of 2.29% of the egg's albumen content did not affect hatchling mass or tarsus length (Bonisoli Alquati *et al*., 2007). However, the removal of 15-20% of the egg's yolk content did not affect body size, but decreased the amount of residual yolk after hatching (Finkler *et al*., 1998). Similarly in comparative studies in breeder hens, embryos from smaller eggs with smaller yolks had lower residual yolk left after hatching than embryos from larger eggs with larger yolks (Nangsuay *et al*., 2011; Sahan *et al*., 2014). These findings all show the nutritional challenges that developing embryos have in DY eggs, especially in DY2F eggs.

During the later embryonic period ($\geq$d22) 32.85% of DY1F eggs died. From these eggs 18.89% died on d22 that coincide with the increased activity of the duck embryo before tucking (Oppenheim, 1970, 1972), 41.11% died on d25 and 27.78% died on d26, which two days coincide with the internal and external pipping (Oppenheim, 1970; Kaltofen, 1971). Late mortality was 50.47% in DY2F eggs, where the embryos died on the same day. The mortality rates in DY1F and DY2F eggs peak at the end of the incubation period consistent with the second 'critical' period (Romanoff, 1949; Bogenfurst, 2004). During this 'critical' period, from d22, the duck embryo tucks its head under the right wing and the bill is pushed up to the airspace by d25 (Oppenheim, 1970, 1972; Kaltofen, 1971) and then makes the transition to breathe with lungs, at which point mortality may be higher (Bogenfurst, 2004). Further, in DY2F eggs more embryos would be expected to die due to crowding or malpositioning (see below) at the end of the incubation period, which may be supported by our late mortality results (DY2F eggs, 70.38%; DY1F eggs, 51.03%; SY eggs, 5.31%; Fig. 2).

Hatchability: The hatchability of fertile SY eggs was high at 87.2%. In contrast very few DY eggs produced hatchlings (DY1F eggs, 2.52%; DY2F eggs, 0.44%) consistent with previous studies with domestic fowl (Jeffrey *et al*., 1953; Burke *et al*., 1997; Fasenko *et al*., 2000; Table 1). Further, Monkman (1963) found slightly higher hatchability in DY hen eggs: 3.18% (7/220) with live twins, 4.55% (10/220) with one live and one dead twin and 12.27% (27/220) with single chicks. However, Monkman (1963) did assist chicks at hatching to avoid suffocation and this would explain the higher hatching success rate. Here, two single ducklings hatched from two DY2F eggs where the second embryos died early (d4) where effectively the eggs were DY1F eggs from d4 showing that the early death of the sibling is not necessarily fatal to the remaining live embryo. Further, there were also 2.52% DY1F and 1.78% DY2F eggs that pipped, 1.37% DY1F and 1.78% DY2F eggs that entered airspace and 33.64% DY1F and 54.12% DY2F eggs that died prior to entering the airspace. Thus embryos can develop to an advanced stage in DY eggs, though they may fail to hatch. Hatchability appears constrained in DY eggs especially when two embryos develop to later stages of incubation as movement can be restricted within the egg (Hollander and Levi, 1940; Monkman, 1963; Fasenko *et al*., 2000).

Further, there were no DY egg weight differences on d2 between the different groups (hatched, 103.58±11.82 g; pipped, 111.58±24.86 g; entered airspace, 118.21±23.85 g; died prior to entering airspace, 113.1±18.71 g), though they were heavier than SY eggs at d2 (91.7±6.74 g; all $p<0.001$). DY eggs that successfully hatched tended to be lighter and closer to the weight of SY eggs suggesting an optimal DY egg size for the successful production of ducklings and if the lighter DY egg had only one fertile yolk its probability of successfully hatching was increased. Similarly, there was a higher chance of having two live embryos in DY hen eggs within the range of 80-90 g, but that chance decreased outside those weight limits (Monkman, 1963) consistent with an optimal egg size for successful development in DY eggs.

Post-mortem embryo positioning: Post-mortem position of embryos that were alive on d26 (when transferred to the hatcher) was associated with yolk position on d8 ($p<0.001$), showing that 82.27% of embryos in DY eggs were in the same yolk position at d8. While in DY1F eggs, all but one of the embryos with known position were in adjacent position (died prior to entering airspace, entered airspace, pipped; Table 5), DY2F eggs with known embryo positioning showed variation, especially in the eggs that died prior to entering airspace, with embryos in 81.21% of eggs in parallel position (Table 6). As far as we are aware, findings have not yet been presented in such detail in DY eggs as this study about embryo positioning during incubation and hatching.

Here, in DY1F eggs head direction of the embryos was 'normal' (i.e., towards airspace) in 64.29% of eggs that died prior to entering airspace and in 100% of the eggs that entered airspace or pipped. In DY2F eggs the head direction of both embryos were 'normal' in 36.3, 62.5 and 25% of eggs that died prior to entering airspace, entered airspace and pipped respectively. However, head towards the narrow end of the egg (i.e., upside down) is one form of malpositioning and is considered as a contributing factor for the high mortality before and during hatching (Hollander and Levi, 1940; Monkman, 1963; Fasenko *et al*., 2000). Here, in DY1F eggs the embryo was upside down in 33.67% of eggs, while in 2.04% of eggs the embryo was curled around the long axis of the egg that also proved to be fatal (Table 5). However, in DY broiler breeder eggs approximately 5%

of single embryos that died at later stages of incubation were upside down (Fasenko *et al*., 2000). In DY2F eggs (died prior to entering airspace; n = 146) 33.56% of eggs contained one embryo in 'normal' position and one upside down, in 15.07% of eggs both embryos were upside down and in 3.43% of eggs both embryos were curled around the long axis of the egg (Table 6). Further, in DY2F eggs that entered airspace and pipped, 37.5% (3/8) and 62.5% (5/8) of eggs respectively, had one embryo in 'normal' position and one upside down. In comparison, from only 12 DY broiler breeder eggs that had twins, in 75% (9/12) of eggs one embryo was in 'normal' position and one was upside down, while in 8.33% (1/12) of eggs both embryos were upside down (Fasenko *et al*., 2000). In pigeons, from five DY eggs, two eggs contained one embryo in 'normal' position and one upside down, while one contained embryos curled around the long axis of the egg (Hollander and Levi, 1940). Here the results show that the upside down malposition occurs in 33.67% of DY1F eggs (died prior to entering airspace) and 48.63% of DY2F eggs (died prior to entering airspace) with one or two embryos upside down. The malposition results presented here are higher for DY1F eggs and lower for DY2F eggs compared to findings in other DY studies (Hollander and Levi, 1940; Fasenko *et al*., 2000), however it has to be noted that those studies had lower sample sizes.

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