Short periods of incubation during egg storage increase hatchability and chick quality in long-stored broiler eggs[1,2]

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ABSTRACT It is recognized that cool egg storage for 8 d or longer, commonly employed in broiler parent and commercial layer production, reduces hatchability. In this study, we investigated the efficacy of short periods of incubation during egg storage (SPIDES) in the restoration of hatchability of broiler hatching eggs stored for 21 d. Prolonged cool storage reduced hatchability of untreated eggs from 92 to 71%. The SPIDES treatment, which consisted of four 4-h preincubations at 4- to 5-d intervals during storage, reduced the incubation time and restored hatchability to 84% by lowering both early and late embryo mortality ($P = 0.0002$). The SPIDES-treated embryos exhibited higher proportions of viable cells after each preincubation ($P = 0.02$), potentially alleviating the negative effects of storage-induced cell death on embryo development. After completion of 4 preincubations, SPIDES embryos were advanced to intermediate primitive streak formation, a developmental stage previously associated with embryo mortality during storage. In contrast to reported preincubation methods imposed on-farm immediately before the eggs are first cooled, the SPIDES technique permits 4 d of cool storage before the initial preincubation treatment, introducing flexibility in the incubation protocol and enabling cool storage up to 3 wk with much improved hatch rates than would usually be expected. Although SPIDES chicks exhibited a BW equivalent to that of embryos derived from unstored eggs at hatch, the initial relative growth was increased as a result of SPIDES, generating a higher BW over the first 4 wk post-hatch ($P < 0.05$). Single preincubations of 6 and 12 h at 4 d of storage caused similar advances in embryo stage to the SPIDES treatment, but the hatchability was worse than in the untreated controls, suggesting small multiple preincubations during storage have a greater benefit than a single incubation performed on d 4 of storage. Future research regarding the cellular and molecular basis of physiological stress reduction in SPIDES embryos will yield new insights into the alleviation of early embryo mortality associated with egg storage.

Key words: broiler, hatchability, egg storage, blastoderm, early embryo mortality

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INTRODUCTION

Egg storage is a common practice in broiler parent and grandparent hatcheries to coordinate hatchery activities and anticipate demand. When in cool storage, eggs are maintained at a temperature well below physiological zero, defined as 21°C by Edwards (1902) and 27°C by Funk and Biellier (1944). In this state, a subset of blastodermal cells may be capable of mitotic activity (Arora and Kosin, 1968), yet embryonic development is halted (Bakst and Gupta, 1997; Fasenko et al., 2001a). Cool egg storage for up to 7 d is widely regarded as having little to no effect on hatchability. However, prolonged storage periods are well documented in decreasing hatchability (Merritt, 1964; Mather and Laughlin, 1976; Mather and Laughlin, 1977; Mather and Laughlin, 1979; Fasenko et al., 2001b; Fasenko, 2007) and chick quality (Becker, 1960; Byng and Nash, 1962; Tona et al., 2003, 2004), and increasing incubation times (Mather and Laughlin, 1976; Kirk et al., 1980; Christensen et al., 2002; Reijrink et al., 2010). It has been suggested that prolonged egg storage may induce embryonic stress, manifested in increased embryonic necrotic and apoptotic cell death, depressed embryonic metabolism, and developmental delays; as a result, irreparable damage to the embryo may occur, thus resulting in increased embryonic mortality and decreased chick performance (Fasenko, 1996; Bloom et al., 1998; Christensen et al., 2001; Fasenko, 2007; Hamidu et al., 2010).

Several interventions have been developed to compensate for decreased hatchability and chick quality due to

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prolonged storage. Early observations that preincubating eggs before storage increased hatchability (Kosin, 1956; Kan et al., 1962; Coleman and Siegel, 1966) were later expanded upon by the observation that older hens tended to produce more developmentally advanced embryos at oviposition that were more resistant to storage than less developed embryos (Mather and Laughlin, 1979; Fasenko et al., 1992). The ability of the slightly developmentally advanced embryo to resist storage-induced effects is proposed to be at least partially due to the increase in total cell number and maintenance of the developmental plasticity of the pregastrulation embryo, thus providing a larger reservoir of available cells to compensate for increased cell death induced by prolonged storage (Hamburger and Hamilton, 1951; Eyal-Giladi and Kochav, 1976; Bloom et al., 1998; Fasenko et al., 2001a; Hamidu et al., 2011). Further work by Fasenko and colleagues revealed that advancing embryos to complete hypoblast formation via a single preincubation (**PI**) before egg storage restored hatchability to varying degrees over a 14-d storage period (Fasenko et al., 2001a,b). However, in commercial settings, eggs frequently must be stored at the commercial farm for several days before transport to a hatchery, and an immediate PI following collection is rarely feasible. A PI delivered during the storage period is preferable.

In this study, we expanded upon a technique introduced by Meir and Ar (1998) that consisted of several short PI delivered after an initial storage period. We have adapted this scheme in a regimen named **SPIDES** (short periods of incubation during egg storage), to include four 4-h PI delivered at 4- to 5-d intervals during 21 d of storage (Dymond et al., 2012). The SPIDES treatment reported here was employed because it gave the best restoration of hatchability in a series of trials investigating the optimal frequency and duration of the PI treatments (French et al., 2011; Nicholson et al., 2011). Preliminary commercial trials have since been successfully carried out using SPIDES (Nicholson et al., 2012). In this manuscript, we describe the developmental ramifications of the SPIDES treatment, as well as its effect on hatchability and chick quality.

MATERIALS AND METHODS

Experimental Design

Ross 308 eggs were obtained from a commercial broiler parent stock farm with breeders maintained under standard husbandry conditions. Three independent trials were conducted using eggs produced by 35-, 36-, and 33-wk-old flocks that were used in trials 1 (December 2011 to January 2012), 2 (January to February 2012), and 3 (April to May 2012), respectively. Hens were mated naturally with a male:female ratio of 1:10. The number of eggs/chicks used in each trial is listed in Table 1.

Egg Requisition, Transport, and Storage

The eggs were stored on farm for approximately 2 d postoviposition in a 16°C cool room at 75% RH. They were then placed in egg cartons, and transported (about 10–12 h) to the Beltsville Agricultural Research Center (**BARC**) hatchery in a temperature-controlled vehicle. To monitor possible blastoderm development during transport to BARC hatchery, in trial 3 a random subset of 30 embryos was examined before transport and the developmental stage of the blastoderm was determined according to the staging method of Eyal-Giladi and Kochav (**EGK**; 1976). The remaining trial 3 eggs were placed in egg cartons with TinyTag 2 thermal sensors (Gemini Data Loggers, West Sussex, UK) to monitor surface temperature throughout

Table 1. Sample sizes for each experiment described in the manuscript[1]

		Treatment												
					NSP				SPIDES					
Trial	Trait	Pretransit	IS	COI	1 PI	2 PI	3 PI	4 PI	1 PI	2 PI	3 PI	4 PI	6 h	12 h
1	Staging		20		23	22	27	22	23	26	25	24		
	BD				19	17	26	12	22	25	22	22		
	CC				9	11	12	7	11	13	15	12		
	Hatch			100			130				134			
2	Staging		19		15	15	13	19	14	13	11	13		
	BD				15	13	11	17	14	13	10	12		
	CC				10	11	7	11	10	10	8	10		
	Hatch			100			52				53			
3	Staging	30	25		26	16	27	27	21	23	22	24	23	19
	BD				19	14	21	20	20	19	19	23		
	CC				11	10	9	9	15	10	9	11		
	Hatch			130			165				155		125	125
	Wk 0			46			66				59		34	22
	Wk 4			45			65				59		34	21

[1]Blank cells indicate no eggs were assigned to a given experimental class. BD: blastoderm diameter; CC: cell count; hatch: % hatch of fertile eggs; IS: initial staging; COI: control incubation, unstored controls; NSP: not treated with short periods of incubation during egg storage (SPIDES); 6 h: single 6 h preincubation (PI); 12 h: single 12 h PI. NSP PI indicates the equivalent time period at which NSP eggs were sampled relative to the PI treatment administered to SPIDES eggs; no PI was delivered to NSP eggs at any time.

transport; temperature during transit was maintained at 16 to 18°C. Upon delivery to the BARC hatchery in all trials, a random subset of embryos was staged according to the EGK staging procedure. The remaining eggs were immediately arrayed in setter trays with no space between adjacent eggs, then placed in a walk-in cool room maintained at 16°C, 75% RH, and with a total capacity of approximately 2,000 eggs. Eggs were not turned during storage.

Incubation Profiles and Experimental Classes

For each trial, upon arrival at the BARC hatchery, a randomly chosen subset of eggs were designated as an initial staging (**IS**) group and analyzed to determine the developmental stage of embryos upon receipt. The remaining eggs were then randomly assigned to 1 of 3 treatment groups: control incubation (**COI**) treatment were eggs stored 4 d (postoviposition) and then incubated; non-SPIDES control eggs (**NSP**) were stored for 21 d with no PI before incubation; PI eggs (SPIDES) were treated with four 4-h PI at 37.5°C, delivered at 4- to 6-d intervals over the course of 21 d of cool storage: PI occurred on d 4, 8, 14, and 18 postoviposition in trial 1, and d 5, 9, 15, and 19 in trials 2 and 3. In the third trial, we evaluated 2 additional treatments where 6- or 12-h periods of incubation were given on d 4 postoviposition, after which the eggs were returned to cool storage and set at 21 d postoviposition. For each treatment, setter trays were transferred from the cool room directly to the center of a 37.5°C incubator with a total capacity of 4,224 eggs on 32 trays, each of which accommodates 114 eggs (Buckeye Incubator Company USA, Lenexa, KS) at 60% humidity; CO_2 levels were not monitored. No equilibration period was provided when transitioning between cool storage and the incubator. Up to 4 setter trays were present in the incubator, depending on the treatment; no additional trays were present in the incubator. After each PI, eggs were immediately returned to the cool room. A subset of eggs was removed 24 h following each PI and embryos were isolated and characterized as below. Internal egg temperature was measured immediately upon arrival at the BARC hatchery through incubation to hatch by inserting a TinyTag 2 sensor (Gemini Data Loggers, West Sussex, UK) into an egg's interior through a 2-mm hole, which was then sealed with parafilm; a minimum of 2 sensors was used for each treatment group. Eggs used for temperature monitoring failed to hatch and were discarded at the completion of the study.

On d 21 postoviposition, all eggs were transferred to the incubator under identical conditions used for PI. Eggs were candled at 10 d of incubation: clear eggs were opened and the germinal disc region identified as being unfertilized or fertilized with a dead embryo; dead embryos were identified as early mortalities. After 19 d of incubation, eggs were transferred to a hatcher (Buckeye Incubator Company USA, Lenexa, KS) with a total capacity of 1,000 eggs, maintained at 37°C dry-bulb and 29.5°C wet-bulb temperature. Beginning 12 h before the predicted hatch time of 504 h (492 h incubation, excluding any PI), the hatcher was monitored every 2 h. Chicks were removed from the hatcher only when completely dry and were weighed at that time; time of hatch was recorded at the time of weighing and thus is an estimate, up to several hours after the actual hatch time depending on the rate at which each chick dried. Chicks were euthanized immediately following weighing. The hatch was concluded 48 h after the predicted hatch time of 504 h (552 h incubation, excluding any PI); eggs that had failed to hatch were opened to classify as unfertilized or late mortalities. Hatchability of fertile eggs was calculated. In trial 3, hatched chicks were divided into 2 groups: those to be euthanized for body composition analysis by dual energy x-ray absorptiometry (**DEXA**; Mitchell et al., 1997), and those to be retained for 4 wk for growth analysis and DEXA analysis 24 d posthatch. Upon weighing, retained chicks were immediately placed into a battery brooder at a density of 10 chicks per pen. This density was maintained for the first week then lowered to 6 chicks per pen for the second week posthatch, and finally to 4 chicks per pen for the remaining weeks of the trial. Chicks from each treatment were randomly assigned different pens; sex was not determined. The temperature of the brooder was maintained at 35°C for the first week, then reduced by approximately 5°C per week for each remaining week of the trial. Chicks were fed ad libitum from immediately after hatch and acclimated from a 24L:0D to a 16L:8D regimen over the course of 1 wk, then maintained on a 16L:8D schedule. Chicks were fed a commercially produced corn soy diet (11.7 MJ of ME/kg, 19% protein). Each chick was tagged at hatch with a specific identifier and weighed on a weekly basis. No vaccines were administered.

The experimental design was approved by the Beltsville Area Animal Care and Use Committee.

Determination of Embryonic Characteristics

Embryos were isolated as described in Gupta and Bakst (1993). Briefly, albumen was removed from the yolk and a filter ring was positioned firmly on the perivitelline layer (**PL**) such that the blastoderm was centered in the ring. The PL was then cut around the outside perimeter of the ring and lifted off the yolk, the ring supporting the PL. The ring, PL, and blastoderm were transferred to a shallow dish of PBS. Using a stereomicroscope (Nikon SMZ1500, Melville, NY), the blastoderm was dissected free of the accompanying yolk and PL, and classified by its stage of development according to EGK or Hamburger Hamilton (**HH**; 1951). Embryo diameters were measured along the longest axis using NIS-Elements digital image analysis software (Nikon). Measurements of embryos at or below stage

(**st**) XIV (EGK) included the area opaca; measurements of embryos at st 2 (HH) and above only included the embryo proper and excluded extraembryonic tissue.

Total blastodermal cell numbers and viable cell counts were determined exclusively in intact embryos isolated for embryonic staging; partial or torn embryos were discarded. Cell numbers and viability were determined as follows: approximately 10 intact embryos isolated after a given treatment were individually dissociated by incubation in 0.0006% trypsin and 0.02% EDTA for 5 min at 37°C; cells were then dispersed by aspirating with a micropipette; trypsin was neutralized by addition of newborn calf serum to a final concentration of 0.5×; dissociated cells were stained with 4 μM calcein and 4 μM ethidium bromide using the Live/Dead Viability/Cytotoxicity Cell Kit (Life Technologies, Grand Island, NY), loaded onto a hemocytometer, and visualized by fluorescence microscopy (Zeiss Axioskop, Jena, Germany); green cells were recorded as viable, red cells as dead, and both categories were summed to determine the total cell number.

DEXA Analysis

A DEXA analysis was performed with a GE Lunar model 7635 (Waukesha, WI). Chicken carcasses were placed on their dorsal side on the scanner bed; up to forty 1-d-old chicks and six 4-wk-old birds were placed on the scanner bed at once; feathers were not removed from the birds before scanning. Analysis was performed using the small animal setting of the manufacturer's software, and data obtained were bone mineral content (g), bone mineral density (g/cm^3), fat (g), and lean mass (g). Total tissue mass for the determination of fat, lean, and bone proportion was determined by adding fat, lean, and bone content.

Statistical Analysis

All characteristics measured in this study were modeled using generalized linear mixed-effects ANOVA models (**GLMM**; Gbur et al., 2012; Stroup, 2012), using the appropriate statistical distribution to directly and accurately model each characteristic without transforming data values to approximate normality. Percentages or proportions calculated as the number of one specific outcome observed out of a total number of events, such as proportion of viable cells, percentage of hatched eggs, percentage of clears, and percentage of late mortalities, were modeled using a binomial distribution. The DEXA-inferred body composition proportions of bone, fat, or lean were modeled using a beta distribution. The large counts of total cells, naturally exhibiting a skewed distribution of data values, were modeled using a lognormal distribution.

Characteristics whose observed values range from very near zero to very large positive values, such as number of hours until 50% of the eggs hatched or DEXA-inferred bone mineral content, bone mineral density, fat, and lean were modeled using a gamma distribution, with typically skewed distributions with small variance when the mean is closer to zero and more symmetric distributions with larger variance when the mean increases away from zero. Similarly, proportion (i.e., binomial or beta-distributed) data exhibit larger variances when the treatment's mean proportion falls in the middle of the 0 to 100% range, with the variances becoming smaller as the mean proportion approaches either 0 or 100%. The asymmetric shape of these nonnormal distributions renders inaccurate the typical practice of reporting the mean proportion ± SE, whether graphically or in tabular form. Instead, 95% CI were reported for each estimated treatment mean; CI can be accurately and readily obtained, regardless of the distribution of the data; intervals will be asymmetric when appropriate, with lower and upper limits always within the range of the distribution (e.g., 0 to 100% for binomial or beta distributions).

Pairwise comparisons of treatment means, using letters to indicate statistically different means, rely heavily on normal distribution theory and are not accurately or universally applicable to all distributions used in GLMM. For purposes of consistency, no pairwise comparisons are reported; rather, treatment mean comparisons were inferred by examination of CI.

The collection of eggs receiving a specific incubation treatment during a trial was a large-sized experimental unit. Each egg, embryo, and chick were small-sized experimental units. Regardless of data distribution, the experimental units were specified as random effects, in the structure appropriate for the ANOVA model of each characteristic. When repeated measures of a characteristic were recorded on the same experimental unit, correlation often existed among these measurements. Weekly chick BW, at hatch to 4 wk, was modeled using a first-order auto-regressive among-weeks covariance structure. Similarly, within-chick correlation among weekly relative growth values was modeled allowing a unique covariance for each pair of weeks, with the covariance matrix having no specific structure. When measures are taken on different, independent experimental units, the among-replicates, within-treatment variability can have a different magnitude for different treatments. Models specify groups of treatments such that all treatments in the same group exhibit similar among-replicates variability. These variance groups were specified for estimating blastoderm diameter means for each embryonic stage and incubation treatment and for estimating embryonic stage at each PI treatment.

For each trial and treatment, the hours required to attain 50% hatched eggs was estimated by fitting a probit regression model to number hatched of the total number of eggs onto hours of incubation. Subsequently, a GLMM ANOVA was fit to these hour data using a gamma distribution, to obtain estimates of treatment means and CI. A GLMM analysis of covariance (AN-

Table 2. Thermal profile of control and treated eggs, and resulting developmental progression[1]

Treatment	Total 37°C PI time during storage (h)	Average time (h) above >21°C		Average time (h) above >37°C		Average stage after treatment	
IS	0	0.0		0.0		EGK 10.5	(10.3, 10.7)
NSP	0	0.0		0.0		EGK 10.4	(10.2, 10.5)
SPIDES	16	25.1	(21.7, 28.4)	6.4	(5.2, 7.7)	HH 3	(2.8, 3.3)
6 h	6	8.5	(3.7, 13.2)	5.2	(3.5, 6.9)	EGK 14	(13.6, 14.3)
12 h	12	14.1	(9.3, 18.8)	10.1	(8.4, 11.9)	HH 3.5	(3.1, 3.8)

[1]The average total time (h) experienced by each egg with a thermal sensor above physiological zero (21°C) and standard incubation temperature (37°C). The average stage resulting from the treatments are indicated: EGK (Eyal-Giladi and Kochav, 1976) and HH (Hamburger and Hamilton, 1951). The 95% CI lower and upper limits are shown in parentheses. NSP: not treated with short periods of incubation during egg storage (SPIDES); 6 h: single 6 h preincubation (PI); 12 h: single 12 h PI; IS: initial staging.

COVA) was fit to estimate and compare among SPIDES, NSP, and single-PI treatments, the rate of change in embryonic stage in relation to the number of PI.

Data from single PI treatments, although observed in only one of the 3 trials, were included in the ANOVA models, with the assumption that had these treatments been replicated, the among-replicate variability would have been similar to that observed for the treatments observed in all 3 trials. Any characteristic without mention of a specific distribution was modeled using a normal distribution. A 95% level of significance ($\alpha = 0.05$) was used for all hypothesis tests and CI. All statistical analysis was performed using SAS PROC GLIMMIX (SAS Institute Inc., 2011).

RESULTS AND DISCUSSION

Thermal Profile of Stored and Treated Eggs and Embryonic Developmental Progression

To determine the cumulative effect of each treatment, internal egg temperature was monitored during cool storage, PI, and the final incubation to hatch; a representative thermal profile of a 4-h SPIDES PI is shown in Figure 1. We speculated the warming and cooling profiles of the eggs used in this study would reflect developmental progression of the embryos. Blastoderms staged upon receipt at the BARC hatchery (initial stage; Table 2) were on average st 10.5 (EGK), the stage expected at oviposition (Eyal-Giladi and Kochav, 1976). Eggs obtained in trial 3 were maintained at 17°C ± 1°C throughout transit. The initial stage of embryos in trial 3 did not differ from the blastoderms examined before shipment to BARC hatchery in the same trial.

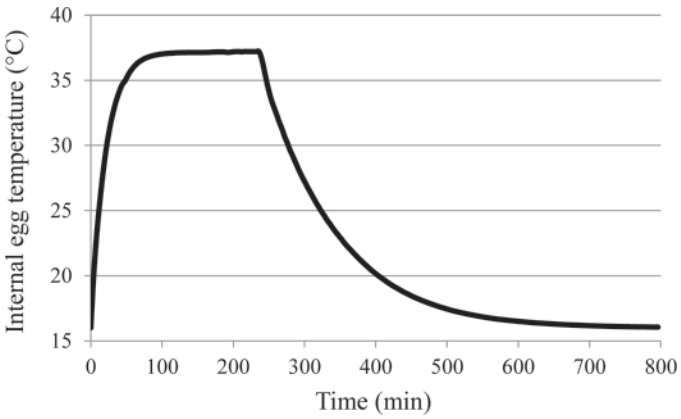

Figure 1. Representative thermal profile of a 4-h short periods of incubation during egg storage (SPIDES) preincubation as measured by a TinyTag thermal sensor (Gemini Data Loggers, West Sussex, UK). Temperature readings were recorded every 4 min.

Whereas the NSP eggs showed no developmental advancement through the storage period (Figure 2A, Table 2), the SPIDES and 12 h treatments resulted in developmental advancement to st 3 (HH) and st 3.5 (HH), respectively ($P < 0.0001$; Table 2), characterized by formation of an intermediate primitive streak (Table 2; Hamburger and Hamilton, 1951). Embryos subjected to a single 6-h PI treatment were advanced to st XIV (EGK), at which hypoblast formation is complete and a bridge is formed between the hypoblast and area opaca (Eyal-Giladi and Kochav, 1976). The relationship between stage progression and number of PI in SPIDES embryos or total incubation time in 6- and 12-h embryos (single PI) was highly linear (both $P < 0.0001$).

A slight discrepancy exists between total time at the standard incubation temperature of 37.5°C and observed developmental stage compared with that predicted by Hamburger and Hamilton (1951): progression to st 3 (HH) generally requires 12 to 13 h. However, both SPIDES and 12 h embryos reached an average st 3 (HH) with a total of 6.4 and 10.1 h internal egg temperature at 37.5°C, respectively. If the total time above physiological zero (21°C; Edwards, 1902) is considered, SPIDES embryos exceeded the 12-h treatment embryos by an average of 11 h due to the multiple cycles of heating and cooling, as opposed to the single heating and cooling cycle during the 12-h incubation. Thus, it is likely a combination of time at near-incubation temperatures and total time above physiological zero that determines the degree of developmental progression.

Embryo Characteristics

To determine the effects of the different egg storage treatments and their respective developmental progression on embryo characteristics, we examined blastoderm diameter, total cell number, and proportion of viable cells. Blastoderm diameter showed a strong over-

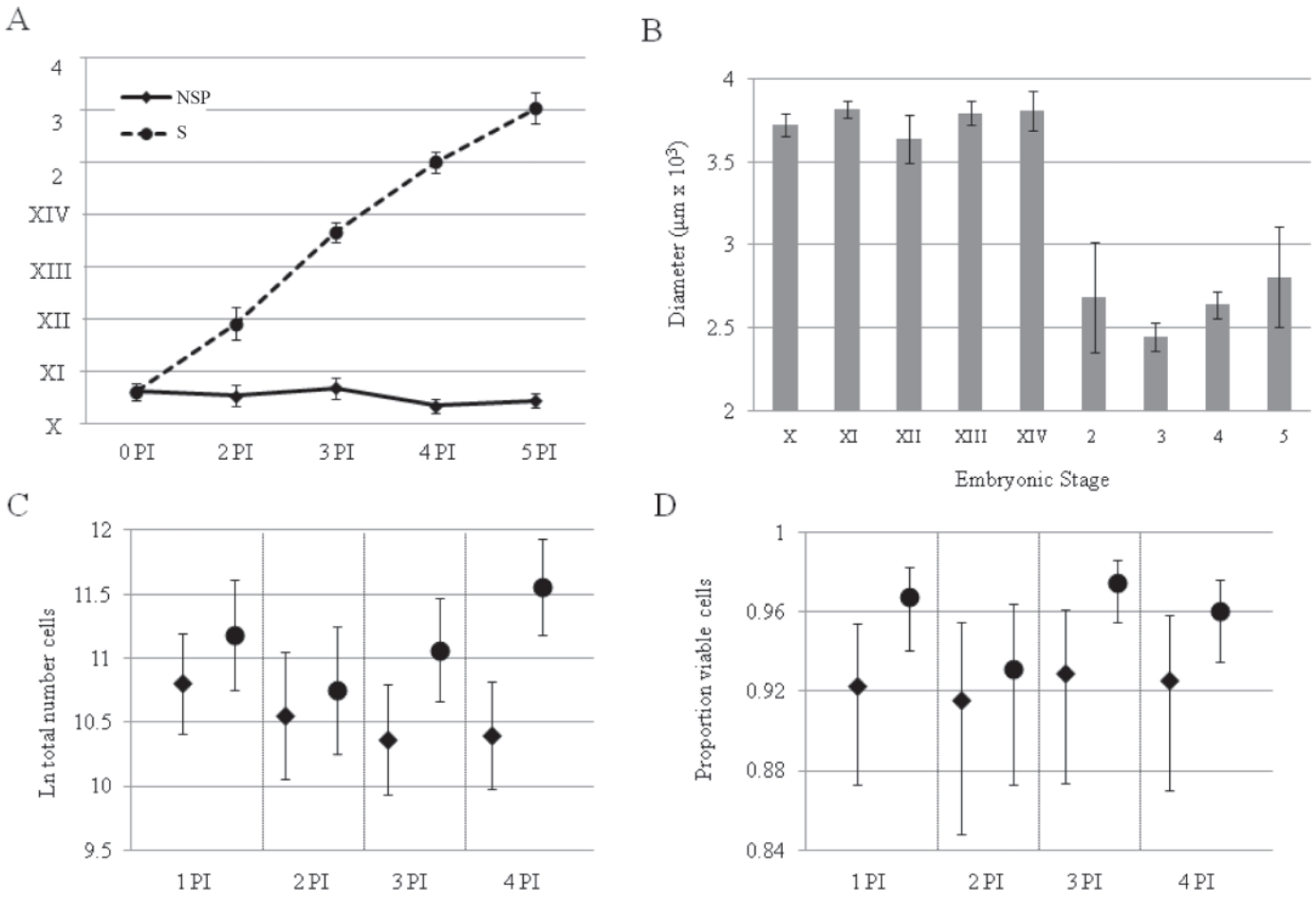

Figure 2. Embryo characteristics are influenced by short periods of incubation during egg storage (SPIDES) treatment. A) Stage progression in SPIDES (dashed line) and NSP embryos (solid line) after each of 4 preincubation (PI) or equivalent lengths of cool storage. B) Blastoderm diameter as a function of embryonic stage for all treatments. C) Total cell number in blastoderms in NSP (diamond) and SPIDES (circle) embryos over the course of 4 PI. D) The proportion of viable blastoderm cells in NSP (diamond) and SPIDES (circle) embryos over the course of 4 PI. Roman numerals in A and B indicate EYG stages (Eyal-Giladi and Kochav, 1976); Arabic numerals indicate HH stages (Hamburger and Hamilton, 1951). Error bars represent the 95% CI. PI: preincubation; NSP: non-SPIDES; S: SPIDES.

all stage effect ($P < 0.0001$). Diameter was relatively stable across st X-XIV (EGK), showed a reduction at st 2 (HH) due to a shift in measurements to include only the embryo proper and not extraembryonic tissue, then subsequent gradual increase in later stages (Figure 2B). The effect of treatment on embryo diameter was minor and inconsistent with multiple treatments sporadically giving rise to a significant change in diameter of the same stage embryos, suggesting these differences may be of little biological relevance.

In agreement with previously reported results suggesting limited mitotic activity may restore lost embryonic cells during storage (Arora and Kosin, 1968; Bakst and Akuffo, 2002; Bakst et al., 2012), the total number of blastodermal cells continued to decrease through the duration of storage in NSP embryos, yet appeared to stabilize between 14 to 18 d postoviposition (Figure 2C). The SPIDES treatment increased the total number of cells after each PI, although the overall treatment effect did not reach significance ($P = 0.06$); the effect was most pronounced following PI 3 and 4 when embryos exhibited the highest level of developmental progression (Figure 2C). Cell viability exhibited a significant overall treatment effect ($P = 0.02$) between SPIDES and NSP treatments, with cell viability increasing following each SPIDES PI treatment relative to NSP (Figure 2D). The increase in the number of viable cells following each PI suggests the SPIDES treatment most likely reduces storage-induced cell death, either via advancement of the embryo through the developmental progression or an overall increase in viable cells at any given developmental stage.

Hatchability Characteristics

Control, unstored embryos (COI) were stored only 4 d, and then incubated to hatch. These eggs exhibited a hatch rate of 92% and low early and late embryonic mortality (Table 3). In contrast, NSP eggs, stored for 21 d, showed a decreased hatch rate of 71%. This was partially rescued by the SPIDES treatment, restoring the hatch rate to 84% (Table 3). The 6- and 12-h PI were detrimental to hatchability, greatly increasing early embryonic mortality (Table 3). The overall treat-

Table 3. Hatchability characteristics of short periods of incubation during egg storage (SPIDES), 6 h preincubation (PI), 12 h PI, and nontreated controls[1]

Treatment	Hatchability (%)		Early mortality (%)		Late mortality (%)		Incubation time (h)	
COI	92	(88, 95)	4	(2, 8)	3	(1, 7)	511	(506, 516)
NSP	71	(65, 77)	14	(10, 19)	14	(9, 21)	519	(514, 524)
SPIDES	84	(79, 88)	11	(7, 16)	5	(3, 10)	504	(499, 508)
6 h	58		35		7		508	
12 h	35		63		2		507	

[1]Incubation time was defined as the number of hours of incubation, excluding PI, required to reach a 50% hatch rate. Hatchability and mortality was calculated as a percent of fertile eggs. COI: control incubation, unstored controls; NSP: non-SPIDES; 6 h: single 6 h PI; 12 h: single 12 h PI. The 95% CI lower and upper limits are shown in parentheses. Confidence intervals are not shown for the 6- or 12-h treatments because a single trial was conducted.

ment effect on hatchability was highly significant ($P = 0.0002$).

Early and late mortality was increased in NSP eggs (14% of fertile eggs at both early and late points) relative to COI eggs (4% early and 3% late); the SPIDES treatment most effectively rescued late mortality, decreasing it to 5%, whereas early mortality remained high at approximately 11% (Table 3). Early mortality was extremely high in both 6- and 12-h PI eggs, at 35 and 63%, respectively; in comparison, late mortality in both treatments almost returned to control levels, with 7% late mortality in 6-h eggs and 2% in 12-h eggs. It is possible the dramatic rise in early mortality in these groups and high late mortality in NSP eggs represents a developmental stage or stages at which long-term storage increases sensitivity to suboptimal incubation conditions. In this study, we did not alter incubation temperatures in response to egg surface temperatures to maintain a constant egg surface temperature of 37.8°C, as recommended by Lourens et al. (2005); further, CO_2 levels were not monitored or adjusted during incubation. These factors may have inadvertently introduced small stresses to which long-stored eggs or embryos at sensitized developmental stages were not able to adjust.

In the current study, early chick mortality within the first week posthatch was extremely low and did not differ significantly between treatments. This finding was in contrast to those of earlier large-scale studies (French et al., 2011; Nicholson et al., 2011), suggesting the small scale of this study and altered thermal profile experienced by developing embryos may have an effect on early mortality. The reduction in late mortality of 6- and 12-h treatments may be the result of significantly increased early mortality (Table 3); it is likely the majority of sensitive embryos died early during development, whereas most remaining embryos were robust and survived to hatch. Based on results achieved by Fasenko et al. (2001b), we expected the 6 h PI to improve hatchability. However, 2 main differences existed between the Fasenko et al. (2001b) study and this series of experiments. First, the PI delivered by Fasenko et al. (2001b) was administered at the onset of storage, rather than following 4 d of storage. Additionally, the duration of storage of this study exceeded that of Fasenko et al. (2001b) by 1 wk. It is possible an interaction between the length of the PI and the time at which it is administered during storage exists; certain combinations of PI length and time of delivery may result in different effects as embryos may be in a state of higher stress during delivery and progress to a more stable stage as a result of the PI. For example, in the case of Fasenko et al. (2001b), the single 6-h PI had a large positive effect if performed before any storage and may be able to compensate for storage-induced stress over a shorter duration of time. Commercial trials have shown that a single short PI treatment (eggs heated to 35–37°C and immediately cooled down) during storage was beneficial when imposed roughly halfway through the storage period (Nicholson et al., 2012). Thus, although we were unable to recapitulate the benefits of an initial single long PI when delivered within a prolonged storage period, it is probable modifications to the PI duration and time of delivery may protect against storage-induced losses.

In further contrast to previous studies by Fasenko et al. (2001b) and Reijrink et al. (2009), we found a positive effect on hatchability when advancing SPIDES embryos to early primitive streak formation (HH 3) over several short PI; advancement of embryos to hypoblast formation (EGK XIII) or primitive streak formation in one 6- or 12-h PI, respectively, showed a detrimental effect. Several possibilities exist to explain this difference. These earlier studies and our single PI treatment advanced embryos to these stage in one PI before prolonged uninterrupted storage, whereas SPIDES embryos did not reach hypoblast formation, the stage recommended by Fasenko et al. (2001b) and Reijrink et al. (2009), until after treatment with 2 PI, at d 8 or 9 of storage, and reached primitive streak formation after 4 PI, d 19 or 21 of storage. As a result, embryos were not stored at these advanced stages as long as in earlier studies (Fasenko et al., 2001b; Reijrink et al., 2009) or those featuring a single PI. We therefore speculate that although long storage at advanced developmental stages is detrimental to embryo survival, short bursts of incubation and development during short storage intervals may overcome this effect, perhaps by resetting the time in storage perceived by the embryo. Additionally, short frequent incubations during storage may also provide the opportunity for embryonic cells to engage in housekeeping activities otherwise suppressed by storage conditions; this may allow cells to repair cellular

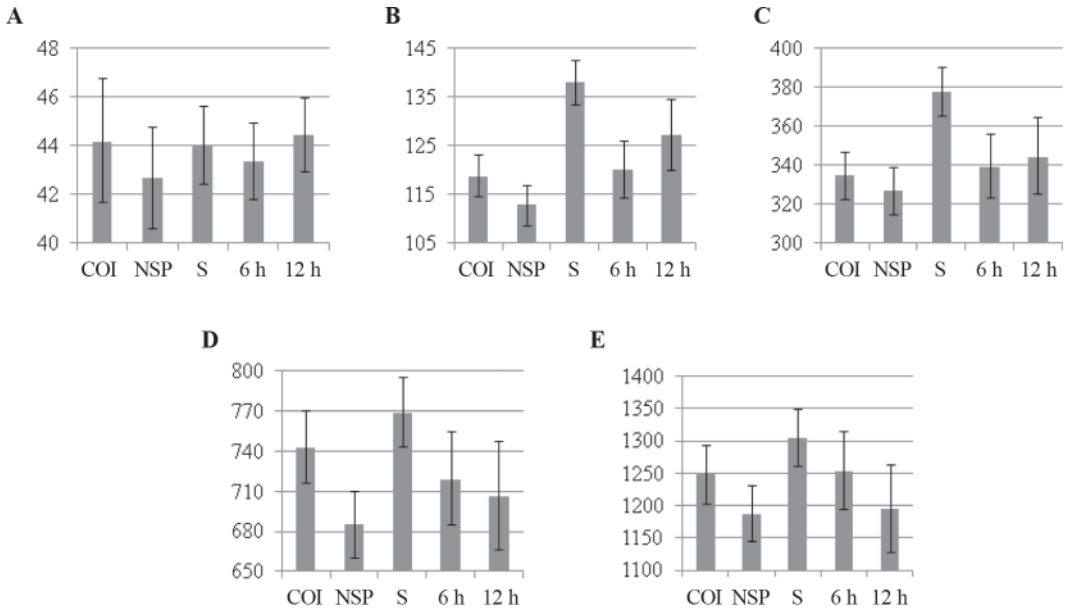

Figure 3. Chicks treated with short periods of incubation during egg storage (SPIDES) exhibit higher BW in wk 1 to 4 posthatch. The x-axis shows treatment, and the y-axis shows BW (g). A) BW at hatch, B) BW 1 wk posthatch, C) BW 2 wk posthatch, D) BW 3 wk posthatch, and E) BW 4 wk posthatch. Bars represent 95% CI. COI: control incubation; NSP: non-SPIDES; S: SPIDES; 6 h: single 6 h PI; 12 h: single 12 h PI.

damage induced by storage (Ar and Meir, 2002). It remains a formal possibility that such cellular repair is more important than embryonic development and stage progression. Additional study of the cellular stress response and repair mechanisms in SPIDES versus long-stored embryos is warranted.

A strong overall treatment effect was apparent in the length of incubation required to achieve a 50% hatch rate ($P = 0.03$). As previously reported, prolonged storage resulted in an increase of the incubation time required to achieve a 50% hatch rate of NSP eggs relative to COI (Mather and Laughlin, 1976; Kirk et al., 1980; Meir and Ar, 1998; Christensen et al., 2002; Reijrink et al., 2010), with 21-d-stored NSP eggs requiring approximately 8 h more time in the incubator than the nonstored COI (Table 3). Interestingly, the SPIDES treatment surpassed even COI in incubation time, requiring 7 h less to reach the 50% hatch mark. Although

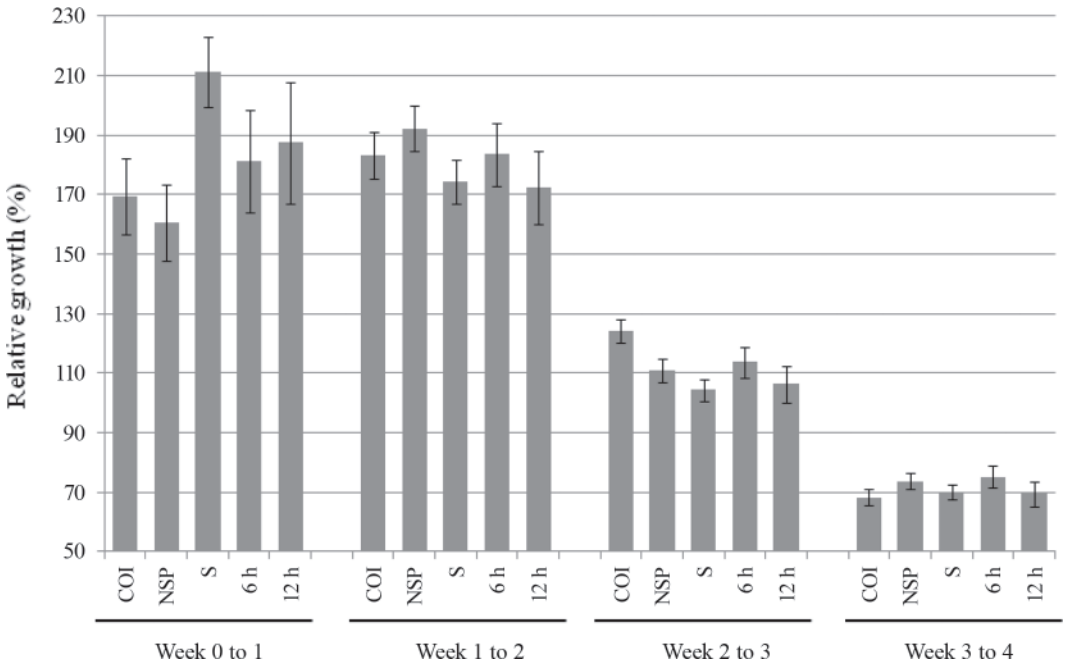

Figure 4. Chicks treated with short periods of incubation during egg storage (SPIDES) exhibit high relative growth in wk 1 posthatch. Relative growth is defined as the difference in bird mass between 2 wk, expressed as a percentage of the total bird mass during the prior week. Bars represent 95% CI. COI: control incubation; NSP: nontreated with short periods of incubation during egg storage (SPIDES); S: SPIDES; 6 h: single 6 h PI; 12 h: single 12 h PI.

the SPIDES-COI relationship was not significant in terms of hours of incubation required to reach 50%, the SPIDES-NSP relationship was significant (Table 3).

Chick Performance

Prolonged egg storage has been shown to decrease chick quality and subsequent chick performance (Becker, 1960; Byng and Nash, 1962; Mather and Laughlin, 1979; Tona et al., 2003, 2004). To further examine the effect of prolonged storage on chick performance and to determine if the SPIDES treatment could rescue a storage phenotype, we chose to examine chick BW from hatch through 4 wk posthatch. Each bird was weighed on a weekly basis beginning with the day of hatch, and BW was compared. At wk 0 (hatch), the BW of all stored treatments (NSP, SPIDES, 6 h, and 12 h) did not differ significantly from that of unstored COI chicks (Figure 3A). By 7 d posthatch, chicks hatched from the SPIDES-treated eggs were heavier than all the other treatment groups, a weight advantage that persisted until the trial ended when the chicks were 4 wk old (Figure 3). This suggests the SPIDES treatment can improve chick performance. Weekly relative growth (**RG**) comparing COI and the different egg storage treatments (NSP, SPIDES, 6 h, and 12 h PI) was examined and an overall treatment effect was identified ($P = 0.002$). Although we anticipated the SPIDES chicks may have a higher RG through 4 wk of age, this was not the case; initially SPIDES chicks did exhibit a high RG (211.3% of the previous week's mass, significantly greater than 169.4% in COI chicks); however, by wk 1 posthatch the growth rate of SPIDES chicks decreased to a level not significantly different from nonstored controls (174.3 and 183.2%, respectively); RG of SPIDES chicks fell below that of nonstored controls during wk 2, then rebounded to control levels by wk 4 (Figure 4). Relative growth of NSP chicks also exhibited a slightly oscillatory behavior: at wk 1 and 2, RG of NSP chicks was comparable with that of COI chicks; at wk 3, NSP chicks showed a significant decreased RG relative to COI chicks (110.9 and 124.0%, respectively); however, at wk 4, RG of NSP chicks significantly exceeded that of COI chicks (73.8 and 68.3%, respectively). Similarly, the RG of 6 and 12 h chicks also fluctuated relative to COI chicks (Figure 4). Based on these results and

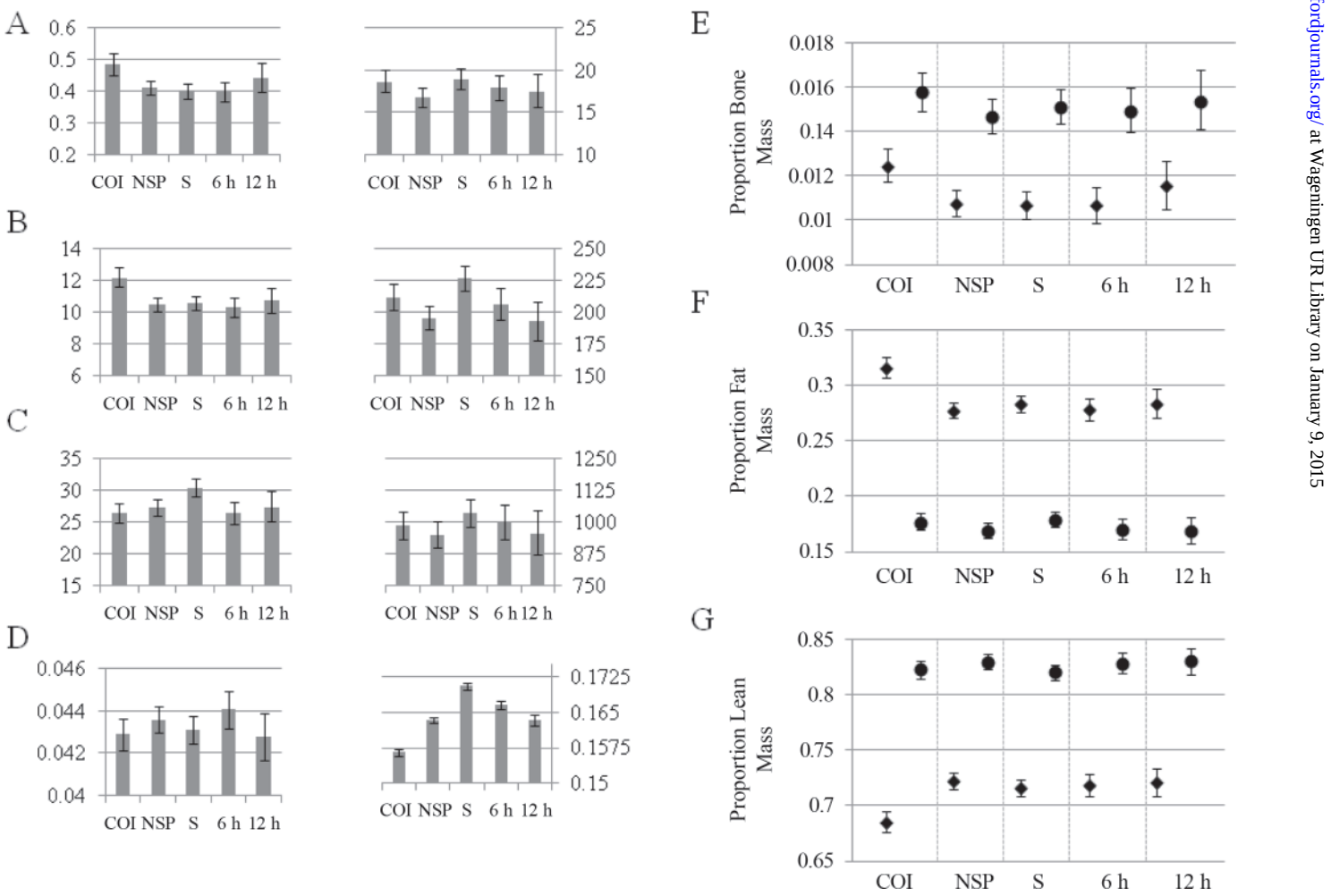

Figure 5. Body composition of control and treated chicks at hatch and wk 4 posthatch. A–D. Left panel, wk 0; right panel, wk 4; y-axes have the same units in both panels. Bars represent 95% CI. Diamonds: wk 0; circles: wk 4. COI: control incubation; NSP: nontreated with short periods of incubation during egg storage (SPIDES); S: SPIDES; 6 h: single 6 h PI; 12 h: single 12 h PI; BMD: bone mass density.

the continuing increased BW of SPIDES chicks relative to controls (Figure 3), we speculate that initial large changes in RG have a greater impact on BW than later smaller changes.

In an attempt to determine the physiological basis of the differing BW and RG of the stored egg treatments, chick body composition was examined by DEXA at wk 0 (hatch), and following 4 wk of growth (Mitchell et al., 1997). The DEXA-based body composition analysis measures fat, lean, and bone content, as well as bone density. At wk 0, no differences were apparent between any of the treatment and control classes for most of these metrics; however, SPIDES chicks did exhibit increased bone mass content relative to other treatments (Figure 5A–D). At wk 4, by which weight differences and developmental changes had begun to manifest, numerous differences in body composition were apparent (Figure 5A-D). As the class with the lightest BW (Figure 3), NSP chicks exhibited reduced bone, fat, and lean masses; conversely, the SPIDES chicks, having the highest BW, exhibited the highest bone, fat, and lean masses. Because the bone, fat, and lean masses are correlated with total BW, changes in body composition will only be apparent when proportions are examined. Interestingly, the proportion of bone mass was decreased in all stored egg treatments relative to COI at wk 0 ($P = 0.001$); this effect disappeared across all treatments by wk 4 ($P = 0.44$; Figure 5E). Given these results, it is surprising bone density is increased in all stored strains relative to COI by wk 4 (Figure 5D). The proportion of fat mass was higher in COI than in all stored embryos at hatch ($P < 0.0001$), similar to observed differences in bone density; by wk 4, the treatment effect on fat mass was diminished ($P = 0.22$). Predictably, as fat mass was lower in all stored egg treatments at hatch (Figure 5F), these strains also exhibited an increased proportion of lean mass relative to the COI treatment ($P < 0.0001$; Figure 5G). This effect is abrogated by wk 4 ($P = 0.24$; Figure 5G). Thus, the SPIDES treatment generates 4-wk-old chicks with increased BW, a marginally increased proportion of fat, and increased bone density relative to other stored and nonstored treatments. The biological basis of these phenomena is not yet understood and warrants further research.

We have shown SPIDES is an effective method of negating the detrimental effects of long-term storage. Further research into the mechanisms by which body composition parameters are altered in long-term storage and by the SPIDES treatment will provide insight both into the physiological stresses experienced by embryos in storage and mechanisms induced by SPIDES to overcome these impediments.

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