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Warmer incubation temperatures and later lay-orders lead to shorter telomere lengths in wood duck (*Aix sponsa*) ducklings

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Abstract

The environment that animals experience during development shapes phenotypic expression. In birds, two important aspects of the early-developmental environment are lay-order sequence and incubation. Later-laid eggs tend to produce weaker offspring, sometimes with compensatory mechanisms to accelerate their growth rate to catch-up to their siblings. Further, small decreases in incubation temperature slow down embryonic growth rates and lead to wide-ranging negative effects on many posthatch traits. Recently, telomeres, noncoding DNA sequences at the end of chromosomes, have been recognized as a potential proxy for fitness because longer telomeres are positively related to lifespan and individual quality in many animals, including birds. Although telomeres appear to be mechanistically linked to growth rate, little is known about how incubation temperature and lay-order may influence telomere length. We incubated wood duck (*Aix sponsa*) eggs at two ecologically-relevant temperatures (34.9°C and 36.2°C) and measured telomere length at hatch and 1 week after. We found that ducklings incubated at the lower temperature had longer telomeres than those incubated at the higher temperature both at hatch and 1 week later. Further, we found that later-laid eggs produced ducklings with shorter telomeres than those laid early in the lay-sequence, although lay-order was not related to embryonic developmental rate. This study contributes to our broader understanding of how parental effects can affect telomere length early in life. More work is needed to determine if these effects on telomere length persist until adulthood, and if they are associated with effects on fitness in this precocial species.

KEYWORDS
early developmental environment, incubation temperature, lay-order sequence, parental effect, precocial bird, telomere

1 | INTRODUCTION

Across taxa, parents can have profound effects on the development of their offspring. For example, nest site selection, diet during pregnancy/egg-laying, and postnatal provisioning of food, warmth, and protection can all differ among individuals of the same species, and small changes in these parental effects can have large consequences for offspring morphology, physiology, and survival (Bernardo, 1996; Dixon et al., 2016; Lindström, 1999; Monaghan, 2008; Mousseau & Fox, 1998).

In birds, two important parental effects are lay-order and incubation. The order in which eggs are laid within a nest is related to differences in egg size, egg hormone/nutrient composition, and hatching asynchrony (Groothuis et al., 2005; Magrath, 1990;

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Williams, 1994). Egg and offspring quality generally decrease with lay-order, although some species appear to have mechanisms to counteract the disadvantages of later-laid eggs. For example, in some species egg size or hormones that favor fast growth increase with lay-order (Griffith & Gilby, 2013; Groothuis et al., 2005), which may lead to rapid development that allows later-laid offspring to "catch-up" to their earlier-laid siblings (Hepp & Kennamer, 2018; Węgrzyn, 2012). Just after (or during) egg-laying, avian parents must then incubate their eggs, which is an energetically costly and time-consuming task (Nord & Williams, 2015; Tinbergen & Williams, 2002), especially for uniparental incubators. Parental incubation behavior can vary substantially among individuals due to differences in parental experience, clutch size, and environmental factors (Aldrich & Raveling, 1983; Coe et al., 2015; Conway & Martin, 2000; Haftorn & Reinertsen, 1985; Hope et al., 2020), which can then lead to differences in egg incubation temperatures both among and within nests (Boulton & Cassey, 2012; Coe et al., 2015; Hope et al., 2021). This is important because small changes in average incubation temperature (<1°C) have been shown to have large effects on avian offspring phenotype. For example, low incubation temperatures generally lead to avian offspring with smaller body masses, slower growth rates, weaker immune function, weaker thermoregulatory abilities, weaker locomotor performance, and even shorter long-term survival (Belnap et al., 2019; Berntsen & Bech, 2016; DuRant et al., 2010, 2012; Hepp & Kennamer, 2012; Hopkins et al., 2011; Nord & Nilsson, 2011, 2021; Ospina et al., 2018; Wada et al., 2015). On top of this, one study in wood ducks (*Aix sponsa*) showed that both lay-order and incubation temperature affect development, where embryonic developmental rate increased with incubation temperature, and increased with lay-order at an intermediate incubation temperature (Hepp & Kennamer, 2018). Understanding how lay-order and incubation temperature are individually and interactively related to offspring phenotype is important to understand how prenatal parental effects may affect the variation of traits within a population.

Recently, telomeres have been recognized as an important trait. Telomeres are noncoding sections of DNA that form protective caps on the ends of chromosomes and are evolutionarily conserved across taxa (Blackburn, 1991). Each time a cell divides, a small portion of the telomere is lost due to the "end replication problem" (Aubert & Lansdorp, 2008; Xin et al., 2008). There is evidence that this telomere shortening leads to cellular senescence (Campisi et al., 2001), and thus may be a molecular mechanism underlying aging. Indeed, telomere length and shortening are related to longevity and aging in many animals (Epel et al., 2004; Heidinger et al., 2012; Monaghan, 2010; Wilbourn et al., 2018). Further, telomere attrition can occur through oxidative stress, which can be caused by environmental stressors (Reichert & Stier, 2017). Thus, telomeres may also be responsive to environmental stressors that an organism has experienced (Angelier et al., 2018; Chatelain et al., 2020; Monaghan, 2014; Salmón & Burraco, 2022), or provide an indication of longevity and fitness (Angelier et al., 2019; Bichet et al., 2020; Heidinger et al., 2021). For example, studies find that stressors such as anthropogenic noise, pollutants, and increased predation risk lead to shorter telomeres (Angelier et al., 2018; Chatelain et al., 2020; Kärkkäinen et al., 2019). Moreover, telomeres also appear to be important for growth, where telomere shortening is accelerated during phases of growth due to increased cell divisions and energy expenditure (Monaghan & Ozanne, 2018). In some cases, telomeres can also be repaired and lengthened by the enzyme telomerase, although this comes with trade-offs such as increased cancer risk (Monaghan & Ozanne, 2018). Interestingly, studies find that telomere lengths differ among individuals of the same species even at the very start of life and, importantly, telomere length and shortening in the first few days of life predicts lifespan, and even lifetime reproductive success, better than adult telomere length in some species (Boonekamp et al., 2014; Eastwood et al., 2019; Heidinger et al., 2012, 2021). Thus, determining how environmental conditions and parental care decisions while an embryo is developing may ultimately influence the individual's telomere dynamics early in life will shed light on how the changing environment and parental care can influence aging and lifespan.

In birds, telomeres could differ due to heritability (Asghar et al., 2014; Bauch et al., 2021), parental effects (e.g., parental age; Heidinger et al., 2016; Sparks et al., 2021), the prenatal environment (e.g., hormone deposition to egg; Haussmann et al., 2012; Parolini et al., 2019), and the early postnatal environment (e.g., sibling competition; Mizutani et al., 2016). However, still little is known about how lay-order and incubation temperature may affect telomeres, especially in wild bird species. To date, only one study has investigated lay-order, where it was found that telomere length decreased with lay-order in captive zebra finches *Taeniopygia guttata* (Noguera et al., 2016). Two studies have investigated incubation temperature. Vedder et. al. (2018) found that a 1°C increase in the incubation temperature of wild common terns (*Sterna hirundo*) led to shorter telomere lengths at hatch and, similarly, Stier et al. (2020) found that telomere lengths were shorter in captive Japanese quail (*Coturnix japonica*) hatchlings that were incubated at high or unstable incubation temperatures compared to low or medium temperatures.

In this study, we investigated whether incubation temperature and lay-order influence telomere length at hatch or telomere shortening in the first week of life in a wild bird. To do this, we collected wood duck (*A. sponsa*) eggs with known lay-orders (i.e., order in which eggs are laid within a specific nest) from the field, experimentally incubated them at two different temperatures (34.9 or 36.2°C) which are ecologically-relevant and known to produce phenotypic differences in this species, and collected blood samples from ducklings at hatch and again after 1 week to measure telomere length. We chose to study wood ducks because they are one of the most well-studied species for examining the effects of incubation temperature on phenotype, and differences in egg temperature are known to lead to changes in embryonic developmental rate, posthatch growth rate, glucocorticoid levels, and behavior (DuRant et al., 2013).

We tested three hypotheses related to the impacts of incubation temperature and lay order on telomere length. The first hypothesis is

that telomere length is an indicator of the environmental stressors that an individual has experienced, their individual quality, and potential lifespan (Angelier et al., 2018; Monaghan, 2014). If this is the case, we would predict that, because a low incubation temperature leads to lower wood duck survival and phenotypic changes that are negatively related to fitness (reviewed in DuRant et al., 2013; and Hope et al., 2021), low incubation temperatures would produce ducklings with short telomeres.

The second hypothesis is that telomeres are related to the pace of development, where faster embryonic/posthatch growth rates lead to more cell divisions, greater energy expenditure, and greater oxidative stress, leading to shorter telomeres (Monaghan & Ozanne, 2018; Vedder et al., 2018). If this is the case, because warm incubation temperatures lead to faster embryonic development and faster growth rates during the first week of life (DuRant et al., 2010; Hepp et al., 2006), we would predict that warm incubation temperatures would produce ducklings with short telomeres at both hatch and after 1 week.

Third, since lay-order is related to multiple traits in wood ducks (Hepp & Kennamer, 2018; Kennamer & Colwell, 1997), we hypothesized that it would also be related to telomere length. Because egg quality (e.g., egg size, hatch success, posthatch survival) tends to decrease with lay-order (Magrath, 1990; Nager et al., 2000), and because later-laid eggs can have faster embryonic and posthatch growth rates to "catch-up" to their earlier-laid siblings (Hepp & Kennamer, 2018; Węgrzyn, 2012), we predicted that telomere length would be negatively related to lay-order.

2 | METHODS

2.1 | Study species

Wood ducks (*A. sponsa*) are common, cavity-nesting waterfowl that are widely distributed throughout North America (Hepp & Bellrose, 2013). Wood ducks nest in tree cavities and nest boxes in or near wooded bodies of water, and breed from February to July (Hepp & Bellrose, 2013). The average clutch size is 12, but clutch sizes can reach >20 eggs in some populations due to conspecific brood parasitism (Roy Nielsen et al., 2006; Semel & Sherman, 1986; Semel et al., 1988). Females solely incubate the clutch and spend most of their time incubating, but usually take 1–2 off-bouts per day to forage (Hepp & Bellrose, 2013), although incubation behavior varies among individuals (Manlove & Hepp, 2000). For example, the daily amount of time spent on the nest can differ by >5 h among individuals (Hope et al., 2020). Ambient temperature (Hope et al., 2018), clutch size (Hope et al., 2018), and female incubation behavior (Hope et al., 2020) all influence incubation temperature of wood duck eggs and, as a result, incubation temperatures can vary substantially (i.e., >3°C) both among and within nests (Hope et al., 2018, 2021). Ducklings are precocial, and thus incubation is one of the most important aspects of parental care in this species. Importantly, small changes (<1°C) in average incubation temperature have been shown to determine a wide array of fitness-related traits in offspring of this species (reviewed in DuRant et al., 2013; and Hope et al., 2021).

2.2 | Egg collection and incubation

We monitored nest boxes in ephemeral wetlands on the Department of Energy's Savannah River Site in SC, USA (33.1°N, 81.3°W; elevation: 157 m) from February 20 to 26, 2019. Because wood ducks in our study system lay eggs from January to June, effects of lay-date (i.e., the calendar day on which an egg is laid) on physiological and developmental traits are common (e.g., Hope et al., 2019; Sedinger et al., 2018). Thus, to focus specifically on the effects on incubation temperature and lay-order, our study controlled for lay-date by constraining sampling to a narrow time window (i.e., only including lay-dates between February 20 and 26). We checked boxes daily, marked new eggs with lay-date and lay-order (i.e., the order that an egg is laid within the laying sequence of each nest), collected up to 6 eggs (mean ± SD: 3.9 ± 1.8; range 1–6 eggs) from each nest before the hen began to incubate, and replaced eggs with wooden eggs to prevent the hen from abandoning the nest (Hepp et al., 1987). If a nest was discovered with eggs already in it, we randomly assigned lay-order values to all eggs and then began collecting fresh eggs the next day. We only collected new eggs with known lay-dates. Because we aimed to represent a large range of lay-orders in our study, as well as complete egg collection during a 1-week period (described above), we opportunistically collected all eggs with known lay-dates, without discriminating among lay-order values. Conspecific brood parasitism is common in wood ducks and, thus, it is not unusual to find multiple new eggs in a nest with the same lay date. In these cases, eggs were assigned the same lay-order value, which is the common practice for determining lay-order in this species (Hepp & Kennamer, 2018). In total, we collected 99 eggs from 25 nests, with lay-orders ranging from 1 to 25. We transported eggs to Virginia Tech at room temperature, weighed all eggs, and rotated unincubated eggs twice daily before beginning incubation. To stagger hatching, we held eggs for 5 days after their lay-date (all eggs were held for the same number of days) before beginning incubation, which does not affect hatchability (Hope et al., 2018, 2020; Walls et al., 2011).

We incubated eggs in Grumbach incubators (Model BSS 420) at two different average temperatures within the natural range for wood ducks: 34.9°C and 36.2°C. We chose these temperatures because they lead to a wide range of different phenotypes in wood duck ducklings, as shown in previous studies (DuRant et al., 2013). Incubation temperature treatments were assigned randomly to eggs (34.9°C: 54 eggs; 36.2°C: 45 eggs), although we allocated more eggs to the low incubation temperature in the anticipation of lower hatching success (Hepp et al., 2006; Hope et al., 2018). We also ensured that eggs from the same nest and with the same lay-date were distributed equally between treatments. Further, there were no differences in egg mass ($F_{1,97} = 0.70$; $p = 0.40$) or lay-order ($F_{1,97} = 0.22$; $p = 0.64$) between treatments, and egg mass and

lay-order were not related ($F_{1,97} = 0.28$; $p = 0.60$). Incubators were programed with two daily cool-down periods to mimic the two daily recesses that female wood ducks take to forage (Manlove & Hepp, 2000). This is a standard protocol for egg incubation in this species and makes our experiment more applicable to incubation conditions experienced by eggs under natural field conditions (Hope et al., 2021). During the cool-down periods, incubators turned off and temperatures passively dropped, leading to different minimum temperatures for each incubator (mean ± SD minimum temperature: 34.9°C treatment: 32.1 ± 0.72; 36.2°C treatment: 32.7 ± 0.74). However, we ensured that each incubator maintained the above-mentioned overall average temperatures for their respective treatment group using iButtons© throughout incubation. The average humidity for each incubator was maintained between 60% and 65%.

We candled eggs every 7–11 days and removed eggs that were infertile ($N = 12$ eggs). Once eggs pipped externally, we moved them to a hatcher with a constant temperature (36.3°C) and humidity (73.5%). We checked incubators for pipping at least two times per day and checked the hatcher for hatching at least every 3 h between 09:00 and 17:00 daily. Out of 99 eggs, 58 ducklings hatched (29 ducklings from each temperature treatment), and hatch dates ranged from April 1 to 12, 2019.

2.3 | General husbandry

Once hatched (Day 0), we collected a blood sample from each duckling (see below). We then color-banded ducklings and housed them in same-treatment groups of 2–3 in cages (46 × 32 × 24.5 cm) in a 3 × 4 rack system. Each cage had a 50 W heat lamp hanging 32.5 cm above the cage floor, which created a thermal gradient (28–35°C). We cleaned cages daily and provided ducklings with ad lib food (DuMOR Chick Starter/Grower) and water. We measured body mass (g), tarsus length (mm), and culmen (mm; bill) length on Days 0 and 7, and collected another blood sample on Day 7. After this blood sample, ducklings were humanely euthanized using carbon dioxide followed by cervical dislocation. Then, we determined sex by examining external and internal genitalia. From those that hatched, 12 died before reaching Day 7 (10 from 34.9°C; 2 from 36.2°C). All procedures were approved by Virginia Tech Institutional Animal Care and Use Committee.

2.4 | Blood collection

On Days 0 and 7, we collected a blood sample from the femoral vein of each duckling (sample sizes: 34.9°C Day 0: $N = 29$ from 22 nests; 34.9°C Day 7: $N = 19$ from 18 nests; 36.2°C Day 0: $N = 29$ from 18 nests; 36.2°C Day 7: $N = 27$ from 17 nests). We collected a blood volume of <0.25 ml within 16.5 min (7.0 ± 3.3; range: 2.0–16.5 min). We then stored blood samples on ice until centrifugation (within 2 h) at 3.5 *g* for 5 min, separated plasma and red blood cells, and stored red blood cells at −80°C until analysis.

2.5 | Telomere analyses

We conducted telomere analyses following standard methods (Criscuolo et al., 2009; Meillère et al., 2015). We extracted DNA from red blood cells using the Qiagen DNeasy Blood & Tissue Kit. We quantified telomere length using real-time quantitative PCR and the BioRad SYBR Green Supermix. Telomeric DNA (TEL) and a reference gene (RAG1) were amplified by running three plates (McLennan et al., 2019; Molbert et al., 2021; Sebastiano et al., 2020). The RAG1 primers (forward and reverse) of this study were specifically designed for wood ducks. qPCR runs were conducted using 5 ng of DNA per reaction and the telomere primers and RAG1 primers were used at a concentration of 800 nM. The efficiency of the telomere and RAG1 assays were 94.93 (±1.88) and 102.23 (±4.58). The standard curves for controlling the amplifying efficiency of the reactions were performed by using a serial dilution of DNA from a pooled sampled of wood ducks. All samples were randomly distributed across the PCR plates. A reference sample was run in triplicate on all plates. Interplate variation for TS ratio was 7.60% (CV).

2.6 | Statistical analyses

We used R version 3.5.1 (R Core Team, 2018) for all analyses. All models met the assumptions of normal and homoscedastic residuals. We used the lme4 package (Bates et al., 2015) to build mixed effects models and used the analysis of variance function of the car package (Fox & Weisberg, 2011) with Type III sums of squares, which determines *p* values using Wald χ^2 tests for mixed effects models. We treated incubation temperature and duckling age as categorical variables. We first confirmed that our incubation temperature treatment produced similar effects on incubation period, body size, and survival as those reported in previous studies (reviewed in DuRant et al., 2013; and Hope et al., 2021), and we report those results in the Supporting Information.

To investigate whether incubation temperature or lay-order were related to telomere length and how telomeres changed as ducklings aged, we built a linear mixed effects model with telomere length (T/S ratio) as the response variable. Incubation temperature, age (Days 0 or 7), and lay-order, along with all interactions, were included as predictors. Further, sex and its interaction with incubation temperature, as well as egg mass, were included as covariates. Duckling ID (repeated measures) and nest ID were included as random effects. We used a model selection procedure based on the second-order Akaike Information Criterion using the dredge function of the MuMIn package (Bartón,2018) to determine the top model (Table 1). We then used the *model.avg* function to apply a conditional model averaging procedure using models within 2 AICc units of the top model. We report the conditional average using *z*-values (Table 1), as provided by the MuMIn package.

To investigate the potential relationships between telomere dynamics and duckling development and survival, we constructed

TABLE 1 Conditional average of models and top model, using AICc model selection, to investigate the effect of incubation temperature, lay order, and age on wood duck duckling telomere length

	Dependent variable: telomere length (T/S ratio) $N = 58$ ducklings; 104 observations; 23 nests Conditional model average			
Term	**Estimate**	**SE**	***z***	***p***
Incubation temperature	−0.10	0.05	2.04	**0.042**
Age	0.22	0.24	6.34	**<0.001**
Lay order	−0.01	0.005	2.94	**0.003**
Egg mass	−0.01	0.01	1.15	0.25
Sex	−0.05	0.05	1.08	0.28
	Top model $R^2m = 0.27$			$R^2c = 0.58$
	X^2			***p***
Incubation temperature	4.19			**0.041**
Age	40.9			**<0.001**
Lay order	8.61			**0.003**

Note: Bold values indicate statistical significance $p < 0.05$.

an additional nine models. To determine whether telomere length was related to embryonic development, we employed a model with telomere length at Day 0 as the dependent variable and incubation period (the number of days from the incubation start date until hatching) as the independent variable. To determine whether the change in telomere length was related to duckling growth rate, we used a model with body mass on Day 7 as the dependent variable, the absolute change in telomere length between Days 0 and 7 as the independent variable, and body mass on Day 0 as a covariate. Next, to determine whether telomere length was related to body size, we used one model with body mass at Day 0 as the dependent variable and telomere length at Day 0 as the independent variable, and another similar model but with data from Day 7. We also built two similar models to investigate tarsus length, and another two models to investigate culmen length. In all six of these models, egg mass was included as a covariate to correct for differences in egg size. Then, to determine whether telomere length was related to duckling survival, we used a general linear mixed model with a binomial error distribution, with survival until Day 7 (yes or no) as the dependent variable and telomere length at Day 0 as the independent variable. All nine models included nest ID as a random effect. We did not include incubation temperature as an independent variable in these models to reduce problems of multicollinearity with telomere length and body mass, and because we were interested in overall relationships among telomere dynamics and duckling development. We did not use model selection for these nine models, and thus report the results of the full models.

3 | RESULTS

3.1 | Incubation temperature, lay-order, and telomere length

We found that telomere length was affected by incubation temperature, changed as ducklings aged, and was related to lay-order. Ducklings incubated at the higher temperature had shorter telomeres than those incubated at the lower temperature (Table 1; Figure 1), and telomeres were longer at Day 7 than they were at Day 0 (Table 1; Figure 1). Additionally, lay-order was negatively related to telomere length (Table 1; Figure 2). Thus, eggs that were laid later in the laying sequence produced ducklings with shorter telomeres. When we reran the top model while excluding eggs potentially laid by brood parasites (i.e., eggs with the same lay-date within the same nest), we found similar results (lay-order: $p = 0.022$; incubation temperature: $p = 0.056$; age: $p < 0.0001$). Because there was no interactive effect of incubation temperature and age on telomere length (i.e., this interaction term was not retained in the top model or the conditional average), this suggests that incubation temperature did not affect the rate of telomere change as ducklings aged. Telomere length was not related to egg mass or sex (Table 1).

3.2 | Telomere length and duckling development

We did not find any relationships between telomere dynamics and duckling development or survival. Telomere length at Day 0 was not related to the length of the incubation period ($\chi^2 = 2.52$; $p = 0.11$). Duckling growth rate was not related to the change in telomere

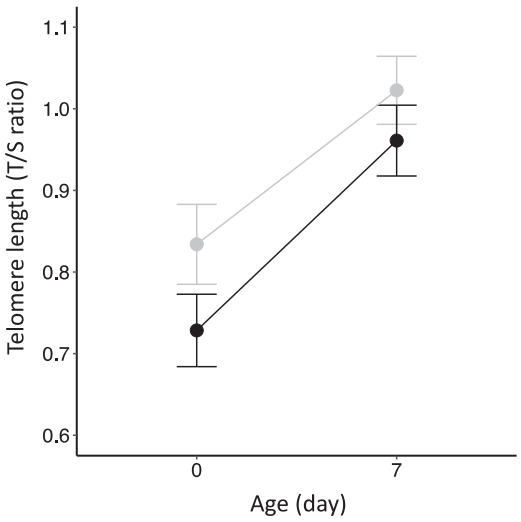

FIGURE 1 Telomere length of wood duck ducklings incubated at 34.9°C (gray) or 36.2°C (black) at hatch (Day 0) and Day 7. Telomere length is expressed as the ratio (T/S) of the telomere sequence copy number (T) divided by a reference single-copy gene number (S). All points represent mean ± SE.

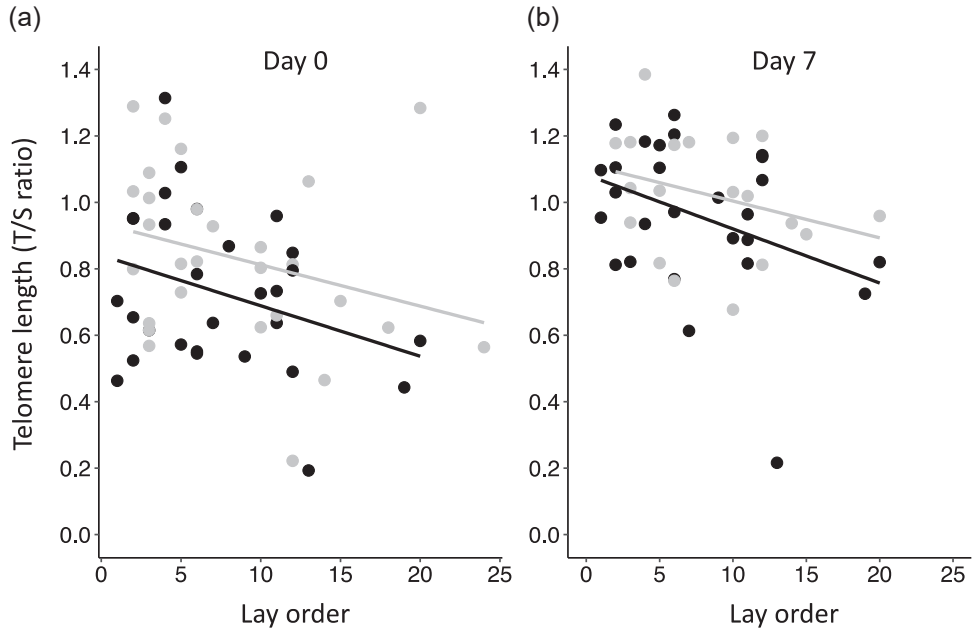

FIGURE 2 Telomere length of wood duck ducklings incubated at 34.9°C (gray) or 36.2°C (black) in relation to lay-order at (a) hatch (Day 0) and (b) Day 7. Telomere length is expressed as the ratio (T/S) of the telomere sequence copy number (T) divided by a reference single-copy gene number (S).

length between Days 0 and 7 (change in telomere length: $\chi^2 = 0.05$; $p = 0.83$; body mass on Day 0 [covariate]: $\chi^2 = 5.88$; $p = 0.02$). Further, neither body mass, tarsus length, nor culmen length were related to telomere length at Day 0 (mass: $\chi^2 = 0.23$; $p = 0.63$; tarsus: $\chi^2 = 0.28$; $p = 0.60$; culmen: $\chi^2 = 0.0008$; $p = 0.98$) or at Day 7 (mass: $\chi^2 = 0.02$; $p = 0.90$; tarsus: $\chi^2 = 0.003$; $p = 0.96$; culmen: $\chi^2 = 0.004$; $p = 0.95$). Lastly, there was no relationship between telomere length at Day 0 and duckling survival until Day 7 ($\chi^2 = 1.35$; $p = 0.25$). Those that died had a mean telomere length at Day 0 (mean T/S ratio ± SE) of 0.86 ± 0.07 and those that survived until Day 7 had a mean telomere length at Day 0 of 0.76 ± 0.04.

4 | DISCUSSION

This study contributes to our broader understanding of how parental effects can affect telomere length early in life. In this study, we found that a decrease of ~1°C in average incubation temperature resulted in ducklings with longer telomeres at hatch and after 1 week, compared to those incubated at a slightly warmer temperature. Further, lay-order had a negative relationship with duckling telomere length, which lasted from hatch until Day 7 and, to our knowledge, this is the first study that has documented this relationship in wild birds. However, we did not find any interactive effects of incubation temperature and lay-order on telomere length. Hatch success was not affected by incubation temperature (which is consistent with other studies; see Hope et al., 2021) or lay-order, and suggests that the results of the current study should not reflect a biased mortality rate of embryos with short telomeres at low incubation temperatures or earlier lay-orders. Altogether, our results suggest that incubation temperature and lay-order may help explain differences in telomere length among individuals within populations, and even within broods, early in life.

We found that a small increase in average incubation temperature produced ducklings with shorter telomeres at hatch and on Day 7. This agrees with the only two other studies in birds that investigated the effects of incubation temperature on telomere length (common terns: Vedder et al., 2018; Japanese quail: Stier et al., 2020). Our results, along with these two previous studies, support the hypothesis that telomere length early in life is negatively related to the pace of development (Monaghan & Ozanne, 2018). Indeed, compared to the low temperature, ducklings from the high temperature treatment developed faster as embryos (i.e., shorter incubation period) and had generally larger body sizes (see Supporting Information). However, we did not find support for the hypothesis that telomere length is positively related to offspring quality; although wood ducks incubated at warmer temperatures have greater short- and long-term survival compared to those incubated at lower temperatures (DuRant et al., 2010; Hepp & Kennamer, 2012), ducklings incubated at the higher temperature in this study had shorter telomeres compared to those incubated at the lower temperature.

Although incubation temperature affected telomere length, the mechanistic underpinnings of these differences remain elusive. We did not find correlations between incubation period and telomere length, growth rate and change in telomere length, or body size and telomere length. Thus, the mechanism underlying the effect of incubation temperature on telomere length must be more complex

than a simple difference in growth rate. One possibility is that high temperatures and/or fast growth rates lead to high levels of oxidative stress (Monaghan & Ozanne, 2018), which result in telomeres that are overall shorter in the high temperature treatment, but are not linearly related to small variations in embryonic/posthatch development within treatments. However, the one study that investigated oxidative stress and telomeres in the context of avian incubation temperature found no relationship between these factors (Stier et al., 2020). Glucocorticoids could be another potential mechanism underlying telomere differences. One study in chickens (*Gallus domesticus*) found that experimentally increased egg glucocorticoid levels led to telomere shortening early in life (Haussmann et al., 2012), while a study on yellow-legged gulls (*Larus michahellis*) found that experimentally increased egg glucocorticoid levels increased telomerase activity in embryos and led to longer telomeres at hatch (Noguera et al., 2020). In wood ducks, low incubation temperatures lead to high offspring glucocorticoid levels (DuRant et al., 2010) and, thus, if this leads to increased telomerase activity in wood duck ducklings, this could explain the long telomeres in low temperature-incubated ducklings that we observed in this study.

Contrary to what we expected, telomeres lengthened as ducklings aged. This is surprising and contrary to multiple studies that show that telomeres shorten over time (e.g., Boonekamp et al., 2014; Hall et al., 2004; Juola et al., 2006; Stier et al., 2020). However, telomere dynamics are not well studied in young individuals and several recent studies have also found that telomere length increases in early life. For example, telomere length increased between 0.5 and 3 months of age in house sparrows *Passer domesticus* (Bennett et al., 2021), between Days 15 and 30 in Magellanic penguins *Spheniscus magellanicus* (Cerchiara et al., 2017), and across larval ontology in the common frog *Rana temporaria* (Burraco et al., 2020). Further, telomeres lengthened in common gulls (*Larus canus*) during the first 11 days of life and, similar to our study, were not significantly correlated with growth rate during this time period (Sepp et al., 2020). This increase in telomere length could be linked to increased telomerase expression, which could be particularly important during early development, where rapid growth and many cell divisions occur. It is also important to note that the rate of telomere lengthening did not differ between incubation temperature treatments. This suggests that ducklings from the warm temperature treatment could not "catch-up" to the telomere lengths of their cold temperature counterparts, at least during the first week of life. Thus, the rate of posthatch telomere lengthening may be a relatively inflexible trait that is robust to changes in the developmental environment. However, more work is needed to determine whether the rate of posthatch telomere lengthening remains consistent across development and if ducklings from the warm temperature treatment do eventually "catch-up," or even surpass the telomere lengths of those incubated at the colder temperature.

We also found that lay-order was negatively related to telomere length, which is the first documentation of this relationship in wild birds. Our finding agrees with the one study on captive birds that investigated lay-order effect; the telomere lengths of domestic zebra finch embryos (72 h of incubation) and offspring at 20, 40, and 90 days after hatch were negatively related to lay-order (Noguera et al., 2016). There are at least three explanations for this result, although our data do not clearly support or eliminate any of them. First, it is possible that, similar to incubation temperature, the relationship between lay-order and telomere length is driven by the pace of development. For example, later-laid eggs could have exhibited compensatory growth either as embryos or hatchlings (Hepp & Kennamer, 2018; Węgrzyn, 2012), which would have increased growth rate and led to telomere shortening. However, we did not find any evidence for compensatory growth in later-laid ducklings. For example, lay-order was not related to body size measurements or incubation period (see Supporting Information), although it is possible that our measurement of incubation period was not precise enough (i.e., we did not continuously check the hatcher overnight and thus achieved an average resolution of 9.4 h for this variable) to reveal a relationship with lay-order. Second, telomere length could be related to offspring quality. In birds, later-laid offspring are usually weaker and have lower chances of survival than earlier laid offspring (Magrath, 1990; Nager et al., 2000), and thus, these weaker birds may have shorter telomeres. However, we did not find any evidence that later-laid ducklings were weaker or had lower chances of survival (see Supporting Information). Third, telomere length differences in relation to lay-order could be due to factors that occur before the embryo begins to develop, such as during ovulation, sperm storage, or nutrient deposition to the egg, which we did not investigate in this study. For example, it has been shown that the ovulation process can lead to oxidative stress (Miyamoto et al., 2010), and thus accumulated exposure to oxidative stress in later-ovulated eggs could reduce telomere length. Further, because female ducks can store sperm (Sasanami et al., 2013), sperm that is stored for a longer amount of time and used in later-laid eggs could incur greater oxidative damage (Noguera et al., 2016; Velando et al., 2008) and result in shorter telomeres. Additionally, lay-order is related to differences in egg composition (e.g., nutrients, hormones, antioxidants; Kennamer & Colwell, 1997; Royle et al., 2003; Schwabl, 1993), which can affect telomere dynamics (Parolini et al., 2019; Soler et al., 2018). Thus, more work is needed to determine the mechanism underlying the relationship between lay-order and telomere length.

It should be noted that, because wood ducks experience conspecific brood parasitism and we did not conduct genetic analyses on ducklings, we cannot be certain of whether all eggs from the same nest were laid by the same female. This adds some uncertainty to our data on lay-order because we are unaware of the precise number of eggs that any brood parasites potentially laid before we sampled the hosts' nests. However, we believe that our results are reliable for three reasons. First, when we excluded any eggs in which more than one new egg was found on the same day in a nest, our results remained the same. Second, even with the additional uncertainty in our data, we found the same result as the only other study that has investigated the relationship between lay-order and telomere length (Noguera et al., 2016). Third, brood parasites in non-waterfowl can generally match the timing of laying with their host nest (Pöysä et al., 2014), in which

case lay-orders of host and parasitic eggs would be consistent. Although little is known about this phenomenon in waterfowl, one study in black brant (*Branta bernicla nigricans*) showed that females that exhibit conspecific brood parasitism were able to closely match the egg size of their hosts (Lemons & Sedinger, 2011). Thus, it may be possible that waterfowl brood parasites could match their timing of laying to that of their hosts, which would greatly decrease any uncertainty in our data.

Our study shows that both lay-order and incubation temperature are related to telomere length at the beginning of life in a wild, precocial bird. Because telomere length early in life can be used to predict lifespan is some species (Boonekamp et al., 2014; Eastwood et al., 2019; Heidinger et al., 2012), this suggests that changes in telomere length could be used to evaluate the lasting consequences that parental effects can have for their offspring. However, long-term studies are needed to determine if the effects of incubation temperature and lay-order on telomere length persist later in life, or have long-term consequences for fitness.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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