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ACCURACY OF EGG FLOTATION THROUGHOUT INCUBATION TO DETERMINE EMBRYO AGE AND INCUBATION DAY IN WATERBIRD NESTS

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Abstract. Floating bird eggs to estimate their age is a widely used technique, but few studies have examined its accuracy throughout incubation. We assessed egg flotation for estimating hatch date, day of incubation, and the embryo's developmental age in eggs of the American Avocet (*Recurvirostra americana*), Black-necked Stilt (*Himantopus mexicanus*), and Forster's Tern (*Sterna forsteri*). Predicted hatch dates based on egg flotation during our first visit to a nest were highly correlated with actual hatch dates ($r = 0.99$) and accurate within 2.3 ± 1.7 (SD) days. Age estimates based on flotation were correlated with both day of incubation ($r = 0.96$) and the embryo's developmental age ($r = 0.86$) and accurate within 1.3 ± 1.6 days and 1.9 ± 1.6 days, respectively. However, the technique's accuracy varied substantially throughout incubation. Flotation overestimated the embryo's developmental age between 3 and 9 days, underestimated age between 12 and 21 days, and was most accurate between 0 and 3 days and 9 and 12 days. Age estimates based on egg flotation were generally accurate within 3 days until day 15 but later in incubation were biased progressively lower. Egg flotation was inaccurate and overestimated embryo age in abandoned nests (mean error: 7.5 ± 6.0 days). The embryo's developmental age and day of incubation were highly correlated ($r = 0.94$), differed by 2.1 ± 1.6 days, and resulted in similar assessments of the egg-flotation technique. Floating every egg in the clutch and refloating eggs at subsequent visits to a nest can refine age estimates.

Key words: *age-determination techniques, egg flotation, embryo age, hatching date, incubation day, nest age.*

Exactitud de la Flotación de los Huevos a lo Largo de la Incubación para Determinar la Edad del Embrión y el Día de Incubación en Nidos de Aves Playeras y Gaviotines

Resumen. Hacer flotar a los huevos de las aves para estimar su edad es una técnica empleada ampliamente, pero pocos estudios han examinado su exactitud a lo largo de la incubación. Evaluamos la flotación para estimar la fecha de eclosión, el día de incubación y el estadío de desarrollo del embrión en huevos de *Recurvirostra americana*, *Himantopus mexicanus* y *Sterna forsteri*. Las fechas de eclosión predichas con base en la flotación durante nuestra primera visita a un nido estuvieron altamente correlacionadas con las fechas reales ($r = 0.99$) y presentaron una exactitud con diferencias de 2.3 ± 1.7 (DE) días en comparación con las fechas reales. Los estimados de la edad basados en la flotación de los huevos se correlacionaron tanto con el día de incubación ($r = 0.96$) como con la edad de desarrollo del embrión ($r = 0.86$) y fueron exactos, con variaciones de 1.3 ± 1.6 días y 1.9 ± 1.6 días, respectivamente, con respecto a los valores reales. Sin embargo, la exactitud de la técnica varió significativamente a lo largo de la incubación. La flotación de los huevos sobrestimó la edad de desarrollo del embrión entre los días 3 y 9, la subestimó entre los días 12 y 21 y presentó una exactitud máxima en los días 0 a 3 y 9 a 12. En general, los estimados de la edad basados en la flotación fueron exactos (con variaciones de hasta 3 días con respecto a la edad real) hasta el día 15, pero estuvieron sesgados progresivamente hacia valores menores que los reales más adelante en la incubación. La flotación de los huevos fue inexacta y sobrestimó la edad del embrión en nidos abandonados (error medio: 7.5 ± 6.0 días). La edad de desarrollo del embrión y el día de incubación se correlacionaron fuertemente ($r = 0.94$), difirieron en 2.1 ± 1.6 días y resultaron en evaluaciones similares de la técnica de flotación de los huevos. Hacer flotar a todos los huevos de la nidada y volverlos a hacer flotar en visitas posteriores a un nido puede refinar los estimados de la edad.

INTRODUCTION

Most studies monitoring bird nests require that the age of eggs be determined. Accurately assessing an egg's age is not only necessary for estimating nest-survival rates (Dinsmore et al. 2002, Shaffer 2004), it also is useful for planning work in the field, such as banding or radio-marking precocial chicks before they leave the nest (e.g., Ackerman et al. 2008a). The three most common ways to age eggs nondestructively in the field are egg candling, density, and flotation. Candling eggs involves using the translucency of eggshells to assess an embryo's age visually, but this method does not work well for thick-shelled, dark, or mottled eggs (Westerskov 1950, Hanson 1954, Weller 1956, Lokemoen and Koford 1996). Using an egg's

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density to assess the embryo's age involves precisely measuring each egg's size and weight to determine its relative weight loss since it was laid, but this technique can be slow and cumbersome in the field and inaccurate at later stages of incubation (Westerskov 1950, O'Malley and Evans 1980).

Another technique to age embryos that relies on the specific gravity of eggs is the egg-flotation method. As the embryo develops, an egg loses mass at a relatively constant rate via moisture loss and respiration, and the air cell grows (Westerskov 1950, Ar and Rahn 1980). This process causes an egg's specific gravity to change from a value greater than that of fresh water (1.0 g ml^{-1}), to a value less than that of fresh water. Therefore, a freshly laid egg with an undeveloped embryo sinks and lies along its long axis when immersed in fresh water, then gradually tips upward and floats toward the surface as the embryo develops and the air cell grows. Several studies have quantified this embryonic development for use in the field (Westerskov 1950, Hays and LeCroy 1971), and egg flotation has become the technique most widely used to age embryos in bird eggs (Alberico 1995, Walter and Rusch 1997, Mabee et al. 2006, Liebezeit et al. 2007).

Despite the widespread use of the egg-flotation technique, few studies have assessed its accuracy in determining the age of embryos by stage of incubation (but see Liebezeit et al. 2007, Reiter and Andersen 2008). Most studies assessing the accuracy of flotation have compared the embryo's age estimated from flotation to the age in days estimated from the clutch's completion date and the assumption that incubation begins on the day the last egg is laid (Nol and Blokpoel 1983, Sandercock 1998, Mabee et al. 2006, Liebezeit et al. 2007, Rizzolo and Schmutz 2007) or by back-calculation from observed dates of hatching and the assumption of an average incubation period (Carrol 1988, Sandercock 1998, Walter and Rusch 1997, Mabee et al. 2006, Liebezeit et al. 2007, Reiter and Anderson 2008). However, the egg-flotation technique actually measures an embryo's developmental age rather than its calendar age (Westerskov 1950, Ar and Rahn 1980). Estimating embryo age from clutch-completion or hatch dates also can introduce variation into assessments of the accuracy of the egg-flotation technique because birds often begin incubating before the last egg is laid (Loos and Rohwer 2004) and incubation periods can vary, often with nest-initiation date and clutch size (Feldheim 1997, Wells-Berlin et al. 2005). Assessing the developmental age of an embryo directly by sacrificing and opening the egg is a more accurate method (Fant 1957, Hays and LeCroy 1971, Liebezeit et al. 2007), but is generally not desirable.

As part of a larger study of contaminant concentrations in waterbird eggs (Ackerman and Eagles-Smith 2009), we had the opportunity to assess the accuracy of the egg-flotation method by directly comparing the estimate from egg flotation to both the embryo's developmental age and the number of days the clutch had been incubated. We evaluated the egg-flotation technique in the American Avocet (*Recurvirostra americana*), Black-necked Stilt (*Himantopus mexicanus*), and Forster's Tern (*Sterna forsteri*). These species build relatively uninsulated nests, have biparental incubation, and have an average incubation period of 24 days (Robinson et al. 1997, 1999, McNicholl et al. 2001). In addition, they lay eggs with opaque shells that preclude age determination via candling in the field. We compared age estimates from egg flotation to embryo ages determined by dissecting eggs and identifying the developmental age of embryos in both active nests, which were still being tended by their parents, and abandoned nests. For nests found during egg laying, we also compared age estimates by egg flotation to the number of incubation days from the time of clutch completion. Additionally, for nests not found during egg laying, we compared the hatch date predicted on the basis of egg flotation at our first visit to the nest to the actual hatch date observed during our subsequent nest visits.

METHODS

NEST MONITORING AND EGG COLLECTION

We monitored avocet, stilt, and tern nests and collected eggs during the nesting season (April–August) as part of a larger study from 2005 to 2007 assessing bioaccumulation of contaminants and its effects on avian reproduction in San Francisco Bay, California (Ackerman et al. 2008a,b, Ackerman and Eagles-Smith 2009). We monitored nests in several colonies within former salt ponds and marshes throughout the South San Francisco Bay within the Don Edwards San Francisco Bay National Wildlife Refuge and Eden Landing Ecological Reserve. We entered colonies every 6–8 days, marked each new nest with a uniquely numbered anodized aluminum tag at the nest and a 40-cm colored pin flag placed 2 m north of each nest, and numbered each egg in a clutch with an indelible marker. At every visit to a nest, we floated each egg in the clutch individually in a clear, plastic, wide-mouthed beaker filled with fresh water and, following the criteria of Westerskov (1950), Hays and LeCroy (1971), and Alberico (1995), estimated the egg's age from its buoyancy, the height at which the egg floated within the water column, the egg's flotation angle, and, if the egg protruded out of the water, its height above the water's surface. Fresh water in the beakers was changed regularly to ensure that the water's density remained consistent and visibility remained high. To prevent the potential for bias, observers floated eggs before they looked at the data previously recorded for cues as to the age of eggs during the last visit to the nest.

We calculated the number of calendar days from the date the clutch was completed to when the eggs were collected (hereafter, incubation day) in a subset of nests that were found during egg laying. To calculate incubation day, we assumed that birds laid one egg per day and began incubation the day when the clutch was completed (Robinson et al. 1997, 1999, McNicholl et al. 2001); thus we calculated incubation day at collection as the difference between the collection date and the date we estimated the full clutch to have been laid.

During our routine nest monitoring, we collected eggs randomly at various stages in incubation as well as eggs that were abandoned. If we collected more than one egg per clutch (as with abandoned clutches), we randomly picked only one egg to include in our analyses. We classified collected eggs as either from active clutches that were being tended by their parents at the time of collection or from clutches that had been abandoned. We considered nests active if parents were observed incubating the eggs or if eggs were evenly warm, eggs had advanced in incubation from the last visit, and nests were maintained. We considered them abandoned if eggs were cold, unevenly warm (if heated by the sun), dirty, and had not advanced in incubation the expected amount since the last nest visit, as estimated via floating. To ensure that we did not erroneously sample active nests thought to be abandoned, we waited 2 weeks from the date we suspected a nest to be abandoned before collecting the eggs. We floated abandoned eggs at the time we collected them.

Collected eggs were placed in labeled Whirl-paks (Nasco, Modesto, CA) and stored unsealed in fiber egg cartons. Egg cartons were carefully placed on ice packs in small, padded, portable coolers in the field and stored in a refrigerator at 4 °C for 14 ± 1 days (mean ± SE) before the eggs were dissected. We opened each egg with scissors, removed the contents into a glass petri dish, and examined the physical characteristics of each embryo to determine its developmental age. We defined an embryo's developmental age as the number of days from the time the egg was laid if the egg had been incubated under ideal and constant conditions. Therefore, our assessment of an embryo's developmental age was based on the physical characteristics of embryos incubated under optimal conditions. To determine the embryo's developmental age in our sample of collected eggs, we used criteria reported by Hamburger and Hamilton (1951), Fant (1957), Hays and LeCroy (1971), and Caldwell and Snart (1974). For avocets and stilts, we followed the standardized procedures for determining the age of embryos of the Recurvirostridae developed by J. Skorupa, U.S. Fish and Wildlife Service (unpubl. data), for studies of egg contaminants and embryo deformities. For the standardized procedure, the embryos' ages had been determined by artificially incubating fresh avocet and stilt eggs at 37.5 °C and 56% humidity and sacrificing eggs every 2–3 days for description and photography of embryo development (P. Martin, Department of Avian Sciences, University of California, Davis, unpubl. data). In addition, we compared the photographic guides for determining the ages of chicken (Hamburger and Hamilton 1951) and Mallard (*Anas platyrhynchos*; Caldwell and Snart 1974) embryos to the avocet and stilt guide and adjusted to appropriate equivalents for avocet and stilt embryos for further information. For Forster's Tern, we followed the criteria for the related Common Tern (*Sterna hirundo*; Hays and LeCroy 1971). We assumed that we could determine the embryo's developmental age with reasonable accuracy by opening eggs and comparing the embryo's development to the photographs and descriptions in these guides, but we acknowledge that identification error may have reduced our accuracy. We excluded infertile eggs from analyses.

For nests from which no eggs were collected, we also compared estimated hatch dates determined via egg flotation when we first visited the nest to actual hatch dates. We considered the actual hatch date to be the day we observed a recently hatched chick still in the nest bowl (≥1 chick), the day after we observed a clutch in which all eggs were pipping and had at least four star fractures in the shell, or 2 days after we observed a clutch in which eggs had two or three star fractures in the shell (Robinson et al. 1997, 1999, McNicholl et al. 2001). We projected hatch dates by averaging the age estimated by flotation of all eggs in the clutch during the first nest visit, subtracting this quantity from an average incubation period of 24 days (Robinson et al. 1997, 1999, McNicholl et al. 2001), and adding the remaining number of days in incubation to the date when eggs were floated. We did not include nests that were found during egg laying in this analysis since those nests provided additional cues that could be used to estimate egg age by flotation more precisely.

STATISTICAL ANALYSES

We began our analyses following the traditional approach used by most studies assessing the accuracy of the egg-flotation technique (e.g., Carroll 1988, Walter and Rusch 1997, Rizzolo and Schmutz 2007), comparing actual hatch dates to hatch dates predicted from egg flotation. We used a mixed-effects ANCOVA and the Satterthwaite method to estimate the degrees of freedom (PROC Mixed, SAS v9.2; SAS Institute 2005). The dependent variable was the estimated hatch date via egg flotation and the independent variables were the actual hatch date as a fixed effect, species as a random effect, and the interaction species × actual hatch date as a random effect. Actual and predicted hatch dates were converted into days of the year.

In the next stage of our analysis, we assessed the accuracy of the egg-flotation technique for estimating both the incubation day (on the basis of clutch-completion date) and the embryo's developmental age (on the basis of eggs being collected, dissected, and aged by examination of the embryo) throughout the incubation period. First, we tested whether the age estimated via egg flotation was positively related with either incubation day or the embryo's developmental age. We used mixed-effects ANCOVAs with the age estimate from egg flotation as the dependent variable, and the independent variables were either incubation day or the embryo's developmental age as a fixed effect, species as a random effect, and the interaction species × incubation day or embryo developmental age as a random effect. Next, we categorized both incubation day and the embryo's developmental age into eight 3-day age classes (0–3, >3–6, >6–9, >9–12, >12–15, >15–18, >18–21, and >21–24 days of age) to evaluate whether the bias and error of the egg-flotation technique varied as incubation progressed. We excluded the 21–24-day age class from tests assessing the effectiveness of the egg-flotation technique because most eggs >21 days of age were at the pipping stage, which would have influenced observers' assessment of the age estimate via egg flotation. Using mixed-effects ANOVAs, we examined whether the difference (bias) or absolute difference (error) between the age estimated from egg flotation and either the incubation day or the embryo's developmental age differed by age class. The dependent variable was the difference or absolute difference between the age estimated from egg flotation and either the incubation day or the embryo's developmental age, and the independent variables were the incubation day or the embryo's developmental age class as a fixed effect and the combination of species × incubation day or embryo developmental age class as a random effect. For these tests, a positive difference reflected a positive bias and a negative difference reflected a negative bias, whereas the absolute difference was inversely related to accuracy. We then used Student's *t*-tests, with a sequential Bonferroni-corrected α level (Rice 1989), to test, for each age class, whether the least-squares mean estimate of the difference or absolute difference between the age estimated from egg flotation and either incubation day or embryo developmental age was significantly different from a value of zero. We also used Tukey–Kramer pairwise comparisons to test which of the age classes differed from one another. We used least-squares means, accounting for any effects of species × incubation day or embryo developmental age class, to graph the bias and error of the egg-flotation technique relative to either the incubation day or the embryo's developmental age class. The species × incubation day or embryo developmental age class effects were not significant for any model ($P > 0.05$), indicating that the global tests with all three species pooled were appropriate.

Last, for nests that were both found during egg laying and from which we collected, dissected, and aged an egg within the clutch by examining the embryo, we compared the embryo's developmental age to the number of days it had been incubated based on the clutch completion date. For these analyses, we included the 21–24-day age class since pipping eggs would not have influenced either the assessment of incubation day or the embryo's developmental age. These mixed-effects ANOVAs were similar to those described above, where the difference or absolute difference between the embryo's developmental age and incubation day was the dependent variable and the independent variables were incubation-day age class as a fixed effect, species as a random effect, and the interaction species × incubation-day age class as a random effect. Conversely, we also conducted a mixed-effects ANOVA in which the difference or absolute difference between incubation day and the embryo's developmental age was the dependent variable and the independent variables were embryo developmental age class as a fixed effect, species as a random effect, and the interaction species × embryo developmental age class as a random effect. We then used Student's *t*-tests, with a sequential Bonferroni-corrected α level (Rice 1989), and Tukey–Kramer pairwise comparisons as described above.

RESULTS

Using only one randomly selected egg per clutch, we estimated the age by egg flotation and determined the developmental age of embryos for 583 eggs from active clutches (avocet: 281; stilt: 86; tern: 216) and 124 eggs from abandoned clutches (avocet: 72; stilt: 12; tern: 40). For the 232 eggs collected from active nests found during egg laying, we also calculated incubation day from clutch-completion dates (avocet: 106; stilt: 36; tern: 90). For an additional 149 nests found after the clutch was completed and from which no egg was collected, we also predicted hatch dates based on egg flotation during our first visit to the nest and compared them to actual hatch dates (avocet: 80; stilt: 9; tern: 60) on which we observed either pipping eggs or newly hatched chicks in the nest.

EGG-FLOTATION TECHNIQUE FOR PREDICTING HATCH DATES

Predicted hatch dates based on egg flotation were highly correlated ($r = 0.99$) with actual hatch dates (Fig. 1; mixed-effects ANCOVA: $n = 149$, $F_{1,12.8} = 11285.2$, $P < 0.0001$). On average,

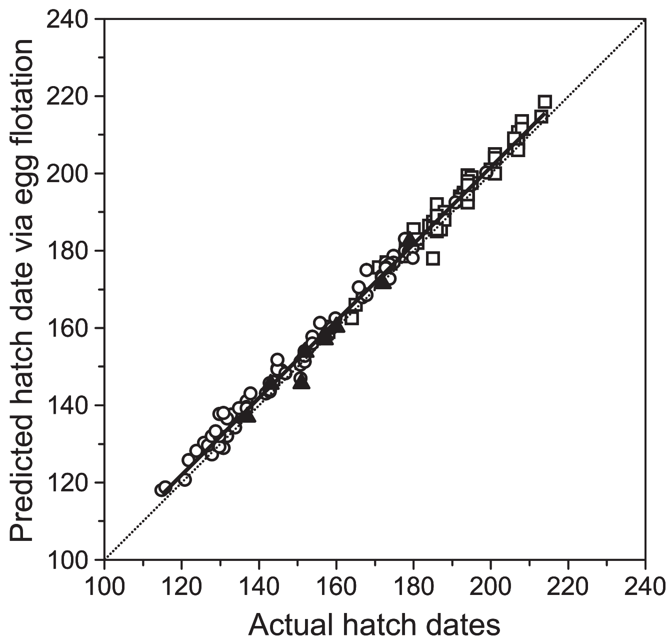

FIGURE 1. Hatch dates estimated from the egg-flotation technique in relation to actual, observed hatch dates for the American Avocet (open circles), Black-necked Stilt (closed triangles), and Forster's Tern (open squares) in San Francisco Bay, California ($n = 149$ nests). We predicted hatch dates from the first nest visit when eggs were floated by averaging the estimated age of all embryos in the clutch, subtracting this quantity from an average incubation period of 24 days, and adding the remaining number of days in incubation to the calendar date when eggs were floated. We did not include nests found during egg laying because these offered additional cues from which the age of embryos in those nests could be estimated more precisely. The stippled line indicates a relationship of one to one.

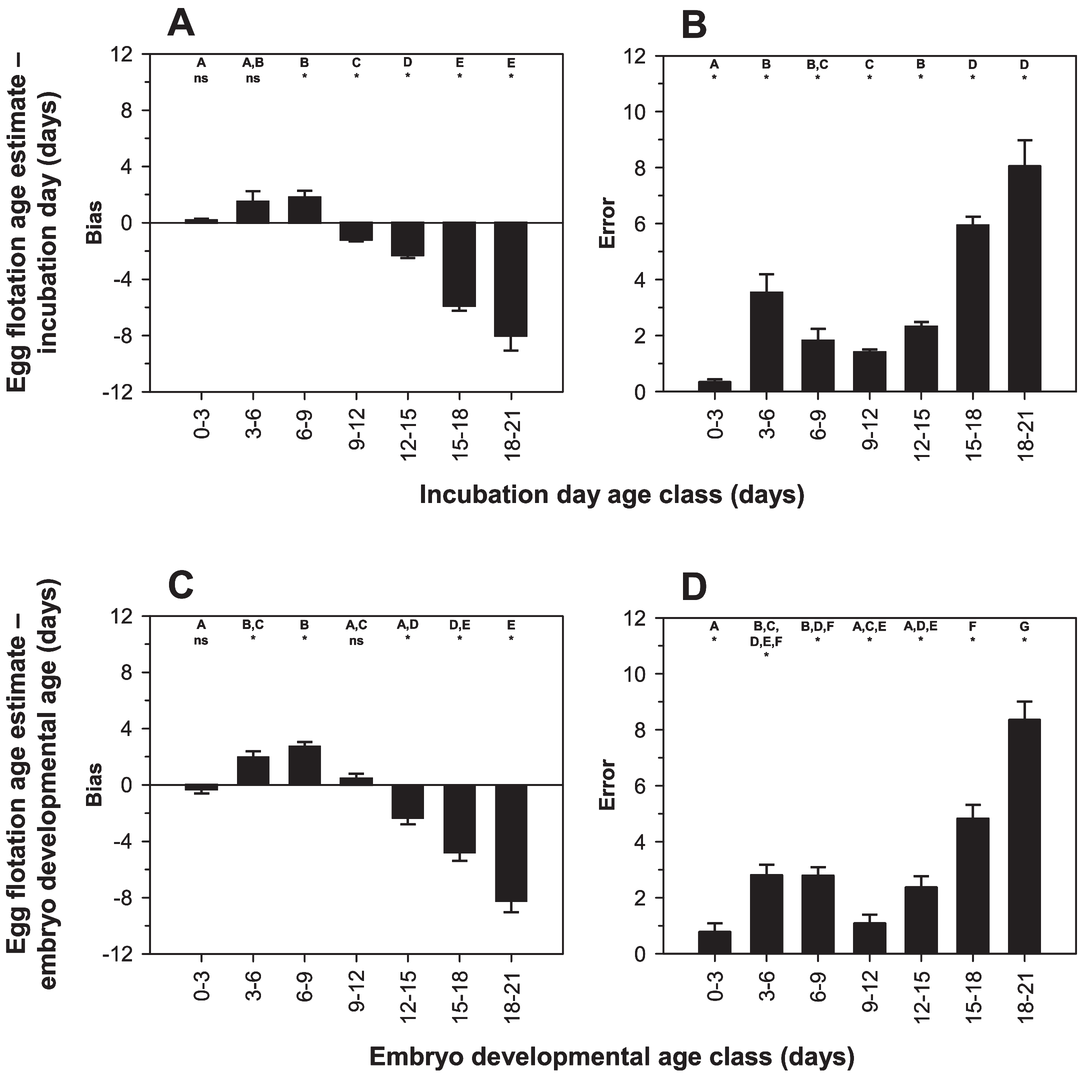

FIGURE 2. The difference (bias: left panels) and absolute difference (error: right panels) between the age estimate from egg flotation and either the (A, B) incubation day ($n = 213$) or (C, D) embryo developmental age ($n = 583$) throughout the incubation period (about 24 days) for active American Avocet, Black-necked Stilt, and Forster's Tern nests in San Francisco Bay, California. The incubation day for clutches was determined from nests found during egg laying, under the assumption that one egg was laid per day and incubation began the day the clutch was completed. The embryos' developmental age was determined by dissection of the egg and examination of the embryo's development. Eggs >21 days of age were excluded from the assessment of the egg-flotation technique's accuracy because these eggs were typically pipping and provided additional cues to help observers estimate age. Different letters above bars indicate statistical differences between age classes (Tukey pairwise comparisons, $P < 0.05$), whereas the asterisk (significant) or ns (nonsignificant) below the letters denotes whether the estimate was significantly different from a value of zero (sequential Bonferroni correction was applied).

predicted hatch dates were within 2.3 ± 1.7 (SD) days of actual hatch dates, and 73% of predicted hatch dates were within 3 days of actual hatch dates.

EGG-FLOTATION TECHNIQUE FOR ESTIMATING INCUBATION DAY IN ACTIVE NESTS

For nests found during egg laying, the egg's age according to flotation was correlated ($r = 0.96$) with the number of days from the time of clutch completion (mixed-effects ANCOVA: $n = 213$, $F_{1,76.1} = 2408.61$, $P < 0.0001$). However, the bias and accuracy of the egg-flotation technique varied substantially throughout the incubation period. The difference between the age estimate from egg flotation and incubation day differed among age classes based on clutch-completion dates (Fig. 2A; mixed-effects ANOVA: $n = 213$, $F_{6,206} = 71.81$, $P < 0.0001$). Egg flotation overestimated incubation day for age class 6–9 days, underestimated it for age classes 9–12, 12–15, 15–18, and 18–21 days, and was not biased for age classes 0–3 and

3–6 days (Fig. 2A). The absolute difference between the age estimate from egg flotation and incubation day also differed among age classes (mixed-effects ANOVA: $n = 213$, $F_{6,206} = 71.34$, $P < 0.0001$) but was always significantly greater than zero for each age class (Fig. 2B). The age estimated via egg flotation was generally within 4 days of the incubation day up until day 15, after which the egg-flotation technique's age estimate was severely biased low by as many as 8.0 days when eggs had been incubated for 18–21 days (Fig. 2A, B). On average, egg flotation estimated incubation day to within 1.3 ± 1.6 (SD) days.

EGG-FLOTATION TECHNIQUE FOR ESTIMATING THE AGE OF EMBRYOS IN ACTIVE NESTS

Egg age according to flotation was correlated ($r = 0.86$) with the developmental age of embryos (mixed-effects ANCOVA: $n = 583$, $F_{1,1.87} = 267.92$, $P = 0.01$). The difference between the age estimate from flotation and the embryo's developmental age differed among age classes based on the embryo's developmental age (Fig. 2C; mixed-effects ANOVA: $n = 583$, $F_{6,6.04} = 54.74$, $P < 0.0001$). Egg flotation overestimated the embryo's developmental age for age classes 3–6 and 6–9 days, underestimated it for age classes 12–15, 15–18, and 18–21 days, and was not biased for age classes 0–3 and 9–12 days (Fig. 2C). The absolute difference between the age estimated from flotation and the embryo's developmental age also differed among age classes (mixed-effects ANOVA: $n = 583$, $F_{6,8.63} = 26.17$, $P < 0.0001$) but for each age class was always significantly greater than zero (Fig. 2D). The age estimated via egg flotation was generally within 3 days of the embryo's actual age up until day 15, after which the egg-flotation technique's age estimate was severely biased low by as many as 8.3 days when embryos were 18–21 days of age (Fig. 2C, D). On average, egg flotation estimated an embryo's developmental age to within 1.9 ± 1.6 (SD) days.

EGG-FLOTATION TECHNIQUE FOR ESTIMATING THE AGE OF EMBRYOS IN ABANDONED NESTS

The egg-flotation technique was not useful for determining the age of embryos in abandoned nests. The age estimated via egg flotation was only weakly correlated ($r = 0.26$) with the embryo's developmental age (mixed-effects ANCOVA: $n = 124$, $F_{1,121} = 5.75$, $P = 0.02$). The difference between the age estimated from flotation and the embryo's developmental age was as high as 9.2 days for embryos 0–3 days of age, and this error did not vary significantly among age classes (mixed-effects ANOVA: $n = 124$, $F_{5,13.4} = 2.77$, $P = 0.06$). Neither did the absolute difference between the age estimated from flotation and the embryo's developmental age (mixed-effects ANOVA: $n = 124$, $F_{5,13.7} = 0.83$, $P = 0.55$). Overall, 97% of abandoned eggs floated at a level implying a more advanced stage than the embryo's actual developmental age, and estimates from flotation of eggs from abandoned nests were 7.5 ± 6.0 (SD) days different from the embryo's developmental age.

COMPARING THE EMBRYO'S DEVELOPMENTAL AGE WITH INCUBATION DAY IN ACTIVE NESTS

The developmental age of an embryo was correlated ($r = 0.94$, slope: 0.95 ± 0.05, intercept: –0.37 ± 0.71) with the number of incubation days from the time of clutch completion (mixed-effects ANCOVA: $n = 232$, $F_{1,2.19} = 442.17$, $P = 0.001$), indicating that characteristics of the embryo could be used to estimate its developmental age with good certainty. On average, the developmental age of embryos was within 2.1 ± 1.6 (SD) days of the incubation day. The difference between the developmental age of the embryo and incubation day differed among age classes based on clutch-completion dates (Fig. 3A; mixed-effects ANOVA: $n = 232$, $F_{7,5.81} = 10.36$, $P = 0.01$). The developmental age of embryos was less than their corresponding incubation day for age class 12–15 days but was not significantly biased for any other age class (Fig. 3A). The absolute difference between the developmental age of the embryo and incubation day also differed among age classes (mixed-effects ANOVA: $n = 232$, $F_{7,11.6} = 15.66$, $P < 0.0001$) and was significantly greater than zero for each age class except 0–3, 3–6, and 21–24 days (Fig. 3B). The difference between incubation day and the developmental age of the embryo also differed among age classes based on the embryo's developmental age (Fig. 3C; mixed-effects ANOVA: $n = 232$, $F_{7,5.64} = 48.46$, $P < 0.0001$). Incubation day overestimated the embryo's developmental age for age classes 3–6, 6–9, and 9–12 days, underestimated it for age class 21–24 days, and was not significantly biased for age classes 0–3, 12–15, 15–18, and 18–21 days (Fig. 3C). The absolute difference between incubation day and the embryo's developmental age differed among age classes (mixed-effects ANOVA: $n = 232$, $F_{7,5.93} = 18.16$, $P = 0.001$) and for each age class was always significantly greater than zero (Fig. 3D).

DISCUSSION

As have most studies assessing the accuracy of the egg-flotation method (Carroll 1988, Walter and Rusch 1997, Rizzolo and Schmutz 2007), we found that hatch dates predicted from egg flotation during our first visit to a nest were highly correlated with actual hatch dates, and the relationship in the three species we studied was similar (Fig. 1). In parallel, Liebezeit et al. (2007) modeled the relationship between egg flotation and embryo age determined from either known clutch-completion dates or known hatching dates for 24 species of shorebirds and found little effect of species on the relationship between predicted and actual hatch dates. Therefore, they developed a standardized equation for all species to predict an egg's hatch date from its flotation angle and height (Liebezeit et al. 2007). We found that predicted hatch dates were generally within 3 days of the actual hatch dates, as have other studies (typically 1–3 days; van Paassen et al. 1984, Walter and Rusch 1997, Sandercock 1998, Liebezeit et al. 2007). Like many investigators who have monitored nests weekly, we found most nests early in incubation (all within 14 days of completion of the

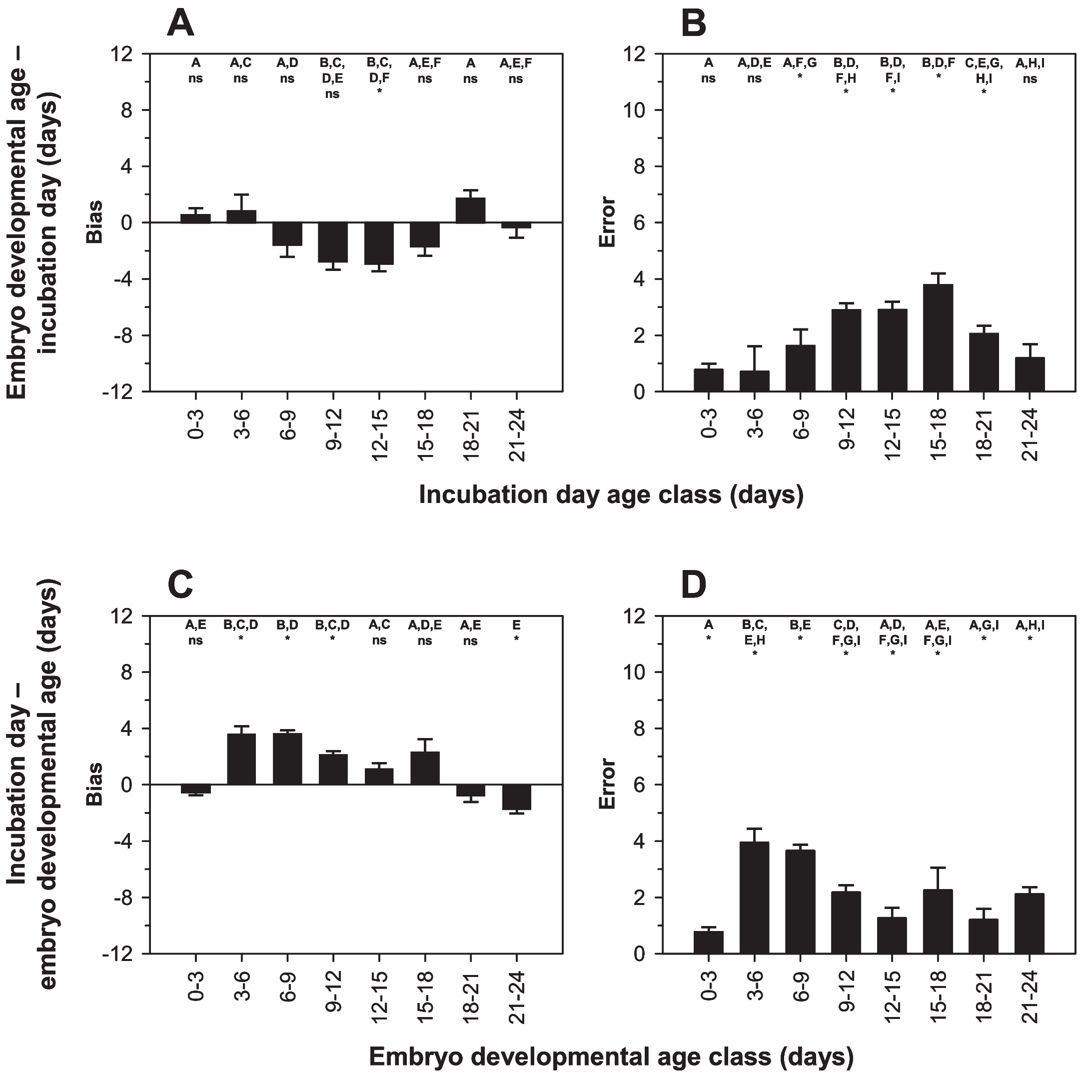

FIGURE 3. The difference (bias: left panels) and absolute difference (error: right panels) between (A, B) the embryo's developmental age and the egg's incubation day ($n = 232$) or (C, D) incubation day and the embryo's developmental age ($n = 232$) throughout the incubation period (about 24 days) for active nests of the American Avocet, Black-necked Stilt, and Forster's Tern in San Francisco Bay, California. The incubation day for clutches was determined from nests found during egg laying, under the assumption that one egg was laid per day and incubation began the day the clutch was completed. The embryo's developmental age was determined by dissection of the egg and examination of the embryo's development. Different letters above bars indicate statistical differences between age classes (Tukey pairwise comparisons, $P < 0.05$), whereas the asterisk (significant) or ns (nonsignificant) below the letters denotes whether the estimate was significantly different from a value of zero (sequential Bonferroni correction was applied).

clutch) when age estimates by egg flotation are most reliable. In addition, we used the average age according to flotation of all eggs in the clutch, further refining the accuracy of the predicted hatch date by pooling several age estimates. Therefore, most studies assessing the accuracy of the egg-flotation technique may underestimate the actual error associated with assessing an individual embryo's age.

Few studies have had the opportunity to compare age estimates by egg flotation to the developmental age of embryos because examining the embryo results in the destruction of the egg (Hays and LeCroy 1971), but we had this opportunity as part of a larger study assessing concentrations of contaminants in eggs (Ackerman and Eagles-Smith 2009). We assumed that we determined the embryo's developmental age accurately by dissecting eggs and examining the embryo, and the correlation ($r = 0.94$) and the slope (0.95) between the embryo's developmental age (determined by dissecting the egg) and incubation day (based on calendar days since completion of the clutch) suggested that this assumption was valid. By randomly selecting one egg per clutch and dissecting the egg to assess embryo development, we were able to compare estimates from egg flotation to the developmental age of embryos throughout the incubation period. We found substantial variation throughout the incubation period in the accuracy of the egg-flotation technique for estimating the embryo's developmental age (Fig. 2C) and incubation day (Fig. 2A). Egg flotation tended to overestimate embryo age between 3 and 9 days of incubation and underestimate embryo age after 12 days of incubation. The difference between the egg-flotation estimate and the developmental age of embryos was generally within 3 days up until day 15, but, after that age, the egg-flotation estimate was inaccurate and biased progressively lower as incubation progressed. Similarly, flotation of Canada Goose (*Branta canadensis interior*) eggs overestimated the true age early in incubation (<13 days) and underestimated it later in incubation (Walter and Rusch 1997, Reiter and Andersen 2008).

The egg-flotation age estimate was not biased and was most accurate for embryo-age classes 0–3 and 9–12 days. Several other studies also have found that the egg-flotation technique is most accurate at earlier stages of incubation (van Paassen et al. 1984, Mabee et al. 2006, Liebezeit et al. 2007, Rizzolo and Schmutz 2007). Our results, however, were most accurate for embryos at 9–12 days of age. Early in incubation the angle and buoyancy of the egg changes relatively rapidly, allowing for more definitive age estimation (Mabee et al. 2006, Liebezeit et al. 2007). In particular, an egg's specific gravity changes from being greater than to less than that of fresh water at about 8 days of age, causing the egg to float vertically off the bottom of the container and eventually break the water's surface, with a portion of the egg 17–19 mm in diameter protruding out of the water near 12 days of age, as in the Ring-necked Pheasant (*Phasianus colchicus*; Westerskov 1950) and Common Tern (Hays and LeCroy 1971). It is possible that our increased accuracy of aging embryos at 9–12 days of age is due to the fact that eggs at this stage float relatively vertically and are just beginning to break the water's surface, which is easily identifiable in the field.

Testing the accuracy of the egg-flotation technique by either the embryo's developmental age or the incubation day produced similar results (Fig. 2). This outcome was expected because an embryo's developmental age is highly correlated with the number of days the egg has been incubated. Consequently, these two terms are often used interchangeably to refer to nest age (e.g., Liebezeit et al. 2007, Reiter and Andersen 2008). However, the egg-flotation technique actually measures an embryo's developmental age, not its calendar age (Westerskov 1950, Ar and Rahn 1980). An embryo's age derived from egg flotation often then is translated into incubation days for an estimate of potential hatch dates or combined with clutch size to estimate the number of days a nest has been exposed (i.e., nest age) to predation. The bias and accuracy of the age estimates from egg flotation were typically similar between embryo developmental age and incubation day, but we did find that egg flotation was more accurate for estimating an embryo's developmental age than for estimating incubation days at 9–12 days. On average, an embryo's developmental age was within 2 days of the incubation day and, in contrast to the age estimated via egg flotation, tended to converge toward the incubation day later in incubation. It is unclear why incubation days would slightly overestimate an embryo's age early in incubation from 3 to 12 days (Fig. 3C), but it could be that the assumptions underlying the estimation of incubation days were not valid. In order to calculate incubation days, we estimated dates of clutch completion for nests found during laying. We assumed that birds laid one egg per day and began incubation on the day the last egg was laid. However, avocets and stilts often take 4 to 5 days to lay a typical four-egg clutch (Robinson et al. 1997, 1999), and the interval between eggs laid by Forster's Terns can be 1 to 2 days for a typical three-egg clutch (McNicholl et al. 2001). Additionally, embryo development can be delayed in early incubation when eggs can withstand temporary neglect and parents refine their incubation bouts (Webb 1987, Gaston and Powell 1989, Astheimer 1991). Thus incubation days may slightly overestimate an embryo's true developmental age during early incubation.

The egg-flotation technique was not accurate for estimating embryo age in abandoned nests. This result was anticipated, though the magnitude of the error was larger than expected. Ninety-seven percent of abandoned eggs floated at stages later than the developmental age of their embryos, with an average error of 7.5 days. Avocet, stilt, and Forster's Tern nests afford little insulation (Robinson et al. 1997, 1999, McNicholl et al. 2001), and abandoned eggs were exposed and likely desiccated, mimicking the air cell expanding as a viable embryo develops. Although the buoyancy of abandoned eggs did not appear to progress as fast as that of viable, incubated eggs, the egg-flotation technique nonetheless produced estimates indicating that abandoned eggs had advanced approximately half as fast as eggs still being actively incubated.

Our results indicate that the egg-flotation technique can accurately predict hatch dates, incubation day, and the embryo's developmental age to within 3 days. Although the accuracy of the egg-flotation technique to determine incubation days or the developmental age of embryos varies substantially throughout the incubation period, much of this inaccuracy can be ameliorated by floating eggs early in incubation (i.e., <15 days of age). We do not recommend using egg flotation to estimate age if nests are found late in incubation, as these age estimates were biased significantly low. In addition to floating eggs early in incubation, we recommend floating every egg in the clutch to improve the estimate of incubation day. Monitoring nests regularly and floating eggs on subsequent visits to the nest can refine age estimates further.

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