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EGG MORPHOLOGY IS AN UNRELIABLE INDICATOR OF INTRASPECIFIC NEST PARASITISM IN WOOD DUCKS

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Abstract. We examined the utility of the maximum Euclidean distance (MED) method, which was developed for the Common Goldeneye (*Bucephala clangula*) and is based on egg morphology, to detect parasitism in the Wood Duck (*Aix sponsa*). We used five polymorphic microsatellite loci to identify parasitized clutches and contributions by multiple females. Three-egg clutches randomly assembled from eggs of one female were less variable than those of three females, indicating that variation within females was less than variation among females. Although variation in egg morphology in parasitized clutches was greater than among eggs laid by one female, extensive overlap in MED values precluded the identification of a clear boundary to distinguish between parasitized and unparasitized clutches. The best MED criterion misclassified 28% of nests. Our results suggest that egg morphology should not be used to detect parasitism in Wood Ducks.

Key words: *Aix sponsa*, egg morphology, egg size, intraspecific nest parasitism, Wood Duck.

La Morfología de los Huevos No es un Indicador Confiable del Parasitismo de Cría Intraespecífico en *Aix sponsa*

Resumen. Examinamos la utilidad del método de la distancia euclidiana máxima (DEM), que fue desarrollado para *Bucephala clangula* y está basado en la morfología de los huevos, para detectar el parasitismo en *Aix sponsa*. Empleamos cinco loci microsatélites polimórficos para identificar las nidadas parasitadas y las contribuciones de varias hembras. Las nidadas de tres huevos ensambladas de forma aleatoria usando huevos de una hembra fueron menos variables que las formadas por huevos de tres hembras, lo que indica que la variación entre los huevos de una misma hembra fue menor que la variación entre huevos de distintas hembras. Aunque la variación en la morfología de los huevos en las nidadas parasitadas fue mayor que la existente entre los huevos puestos por una hembra, la amplia superposición en los valores de DEM impidió identificar un límite claro para distinguir entre nidadas parasitadas y no parasitadas. El mejor criterio basado en la DEM clasificó de forma incorrecta el 28% de los nidos. Nuestros resultados sugieren que la morfología de los huevos no debería emplearse para detectar el parasitismo en *A. sponsa*.

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Intraspecific nest parasitism is relatively common among birds, particularly among waterfowl (Eadie et al. 1998). Recently, this behavior has received considerable attention in waterfowl (Anderson and Åhlund 2000, Semel and Sherman 2001, Roy Nielsen et al. 2006a, 2006b, 2006c, 2008, Pöysä and Pesonen 2007). Studies of this behavior rely on numerous methods to detect parasitism, some of which can be fairly labor intensive and expensive. Some of the more laborious methods include observing individual nests daily (Semel and Sherman 2001) and calculating the rate of egg deposition on the basis of the egg-laying rates of individual females (Jones and Leopold 1967, Clawson et al. 1979, Semel and Sherman 1992). Microsatellite and fingerprinting techniques are accurate (MacWhirter 1989, Andersson and Åhlund 2000, Roy Nielsen et al. 2006a) but are also labor intensive and come with the additional drawback of being relatively expensive compared to other methods. A less expensive and minimally laborious method is the use of large clutch size as an indicator of parasitism, but this method suffers from poor accuracy and consistently underestimates parasitism (Semel et al. 1988, Semel and Sherman 1992). Another comparatively easy method, the use of nonterm embryos to indicate parasitism, is also common but misses parasite eggs that hatch with the rest of the clutch (Jones and Leopold 1967, Clawson et al. 1979). Therefore, an inexpensive technique with high accuracy would greatly simplify the study of nest parasitism. Egg morphology has been used successfully in studies of the Common Goldeneye (*Bucephala clangula*; Pöysä et al. 2001), but the utility of this method for other species of waterfowl remains unexplored.

Studies on numerous avian taxa have come to disparate conclusions about the accuracy of using egg morphology to detect intraspecific nest parasitism. In addition to the Common Goldeneye, studies of the Ring-billed Gull (*Larus delawarensis*; Fetterolf and Blokpoel 1984), Herring Gull (*Larus argentatus*; Baerends and Hogan-Warburg 1982), Northern Masked Weaver (*Ploceus taeniopterus*; Jackson 1992), and Emperor Goose (*Chen canagicus*; Petersen 1992) have found egg morphology to be effective at discriminating between parasitized and unparasitized nests. Studies of the Barn (*Hirundo rustica*) and Cliff Swallow (*Petrochelidon pyrrhonota*; Brown and Sherman 1989), Black-legged Kittiwake (*Rissa tridactyla*; Coulson 1963), Common Moorhen (*Gallinula chloropus*; McRae 1997), and Guira Cuckoo (*Guira guira*; Cariello et al. 2004), however, have found the opposite, that egg morphology is unreliable for the detection of nest parasitism. Therefore, evaluation of morphology-based methods appears to be necessary on a case-by-case basis for each species.

Morphology-based techniques depend on greater variation in egg size among females than among eggs laid by a single female. When this condition is true, a parasitized clutch should have greater variation in egg size than a clutch laid by a single female. Pöysä et al. (2001), with techniques described by Eadie (1989), used Euclidean distances between *z*-score standardized measurements of egg length, breadth and the mass of the two most different eggs within a clutch to identify a threshold criterion that distinguished parasitized clutches from those laid by a single female. This maximum Euclidean distance (MED) could then be used as a criterion to correctly classify parasitized clutches.

Our objective was to evaluate the efficacy of the MED method for the Wood Duck (*Aix sponsa*), a species well known for egg-dumping or parasitic behavior. Previous comparisons of methods to detect parasitism (Semel and Sherman 1992, Roy Nielsen et al. 2006a) clearly demonstrated that clutch size is an inaccurate method for assessing intraspecific parasitism in the Wood Duck, but comparably easy methods are unavailable. We used genetic methods to verify parasitism in Wood Duck clutches and compared these results to those based on the MED method of Eadie (1989) and Pöysä et al. (2001).

METHODS

FIELD

We studied a population of the Wood Duck nesting in natural cavities at Union County Conservation Area and LaRue Pine Hills Natural Resource Area in southern Illinois from 2000 to 2003. We located nests by climbing 63 trees reported to have Wood Duck nests from 1993 to 1998 (Ryan et al. 1998, Zwicker 1999). We also used swim-in traps to capture hens and placed radio transmitters (Montgomery 1985) on 43 hens in 2001–02 according to animal-care protocols of the University of Missouri–St. Louis (W01-3) and Ohio State University (00A0001). We collected blood samples (<200 μ l) from the captured ducks' medial metatarsal vein with a 25-gauge, 15.9-mm needle and stored the blood in lysis buffer (Longmire et al. 1988). We followed radio-tagged birds back to nest trees and discovered three additional nest trees through chance observations of Wood Ducks flying into or near cavities or feathers on a cavity entrance.

When a nest was located, we collected a sample of nest feathers to obtain DNA from females from which we did not have a blood sample. We collected unhatched eggs and eggs from nests that had been abandoned because of flooding or other reasons. Eggs were not removed before nest termination because of the difficulty of returning all eggs safely to cavities \leq 160 cm deep. We collected tissue from embryos for DNA analysis. In all cases, DNA from nest feathers matched DNA from some eggs within the cavity. We measured the length and width of collected eggs to the nearest 0.01 mm with calipers and weighed eggs to the nearest 0.5 g with a Pesola scale. We also calculated mass by the equation used by Rohwer (1988) for more direct comparisons with the method of Pöysä et al. (2001).

LABORATORY

We used a standard phenol:chloroform extraction protocol and subsequent ethanol precipitation (Maniatis et al. 1982) to extract DNA from blood samples and embryos. For feathers, we added 30 μ l of 100 mg ml⁻¹ dithiothreitol to each sample to dissolve 4 to 10 feather tips and used a DNeasy tissue kit from Qiagen to extract the DNA. We used a Biometra Trio Thermoblock 48 to amplify five polymorphic microsatellite loci: Sfi μ 4, Sfi μ 5 (Fields and Scribner

1997), Bca μ 11 (Buchholz et al. 1998), cmaat28, and cmaat35 (Stai and Hughes 2003). The number of alleles at each locus was 6, 9, 6, 25, and 20, respectively, and for each locus observed heterozygosities were high (0.75, 0.90, 0.93, 0.74, 0.66). We visualized amplicons on a Kodak Digital Science ID 3.0.2 Imaging System, after running 7.5% polyacrylamide gels and staining with SYBR green. Homozygous individuals were rerun to minimize errors caused by allelic dropout. Size standards of known genotypes were run on every gel to enable comparisons from gel to gel.

We used permutation tests in FSTAT to examine deviations from Hardy–Weinberg equilibrium and linkage equilibria (version 2.9.3, Goudet 2001). We determined exclusion probabilities by using the method of Gerber et al. (2000) for single-parent exclusion. We adjusted for the possibility of host–parasite relatedness according to equations in Neff et al. (2000). Parasitic young were identified through exclusion analysis. We used the program Kinship (version 1.2; Goodnight and Queller 1999) to determine the relatedness, *r*, of clutch mates according to equations described by Queller and Goodnight (1989).

STATISTICAL ANALYSES

We standardized egg weight, breadth, and length by *z*-score. We determined the Euclidean distance for each pair of eggs in the clutch. The MED was determined for the two most dissimilar eggs in each clutch. We also constructed three-egg clutches (*n* = 36) by using eggs from one or three females to examine variation within and between females and tested for differences in MED with a one-tailed independent-samples *t*-test. We expected relatedness of clutch mates to be lower in parasitized nests than in nests to which only a single female had contributed. Therefore, we expected relatedness to be negatively related to clutch size and MED, but because clutch size is known to be correlated with MED (Pöysä et al. 2001), we did partial correlations to test for the strength of the relationship between relatedness and MED after controlling for clutch size. We also compared the average measurements of excluded (parasite) eggs and nonexcluded (host) eggs of each clutch with paired *t*-tests, to determine whether eggs laid parasitically differ from eggs laid by females that incubate. Statistical analyses were performed in SAS version 9.1.3 (SAS Institute, Cary, NC). We used α = 0.05 in all tests. All results are reported as means \pm SE.

RESULTS

The assumptions of Hardy–Weinberg equilibrium and linkage equilibrium were met at all loci (P = 0.05–0.88 and P = 0.10–0.89, respectively). Exclusion probability for nonparents was 96.4% if the host was not a relative of the parasite and 72.0% if host and parasite were first-order relatives.

Mean weight of 216 eggs from 16 clutches was 39.0 \pm 0.4 g, but mass calculated from length and breadth was 44.10 \pm 0.85 g. Mean egg length was 51.29 \pm 0.14 mm, mean egg breadth was 38.70 \pm 0.10 mm, and mean volume was 39.05 \pm 0.27 cm³. The mean size of excluded eggs within a clutch (parasite eggs) was similar to the average size of non-excluded eggs (host eggs) within a clutch for all dimensions (P > 0.30 for all comparisons, Table 1).

The MED of one-female clutches of three eggs (1.65 \pm 0.18) was less than the MED of three-female clutches of three eggs (3.73 \pm 0.56, $t_{19,2}$ = 3.50, P = 0.002, Fig. 1). The MED of parasitized nests (3.82 \pm 0.37) was greater than the MED of nests containing only host eggs (3.28 \pm 0.29, t_{25} = 2.59, P = 0.03, Fig. 2). MED increased with clutch size (R^2 = 0.50, P = 0.007), and clutch

TABLE 1. Mean egg dimensions and statistical comparison (by *t*-test) of 83 host and 80 parasite eggs in 13 Wood Duck clutches in southern Illinois, 2000–2003.

	Parasite		Host		Statistics	
	Mean	SE	Mean	SE	<i>t</i>	<i>P</i>
Mass (g)	40.5	1.5	39.5	1.7	-1.00	0.34
Length (mm)	51.60	0.53	51.44	0.49	-0.32	0.75
Breadth (mm)	38.91	0.41	38.86	0.46	-0.26	0.80
Volume (cm ³)	39.77	1.25	39.53	1.23	-0.37	0.71

sizes were larger as a result of parasitism (Fig. 2). However, the best possible MED criterion, which resulted in the lowest number of misclassified nests, was 2.25 and misclassified 28% of nests. This value was determined by calculating the number of misclassified nests for each MED value and then minimizing that number.

DISCUSSION

The MED method of discriminating between parasitized and nonparasitized clutches was not effective for the Wood Duck. Although variation in egg morphology among females was greater than variation within females, the MED of clutches from single females overlapped with that of parasitized nests. Because MED is strongly influenced by clutch size, with larger clutches having a higher MED, small clutches with a few parasite eggs were misclassified as not parasitized and large clutches of single females were misclassified as parasitized (Fig. 2). Furthermore, variation in parasitized clutches was often similar to that of clutches laid by single females. Although some parasitized clutches had more variation than those of single females (Fig. 3a), this difference was not consistent (Fig. 3b,c). These results contrast with the success of the method for the Common Goldeneye, for which it had an accuracy of 96.7% (Pöysä et al. 2001).

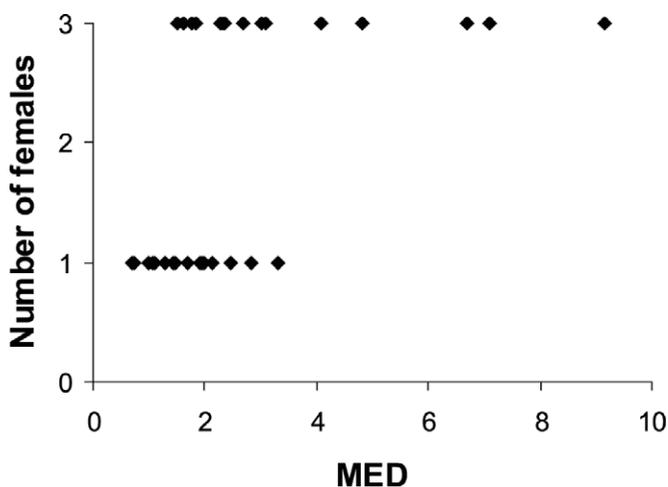


FIGURE 1. Comparison of maximum Euclidean distance (MED) values for three-egg clutches constructed from the eggs of one female and from the eggs of three females. This analysis controls for the effect of clutch size on MED.

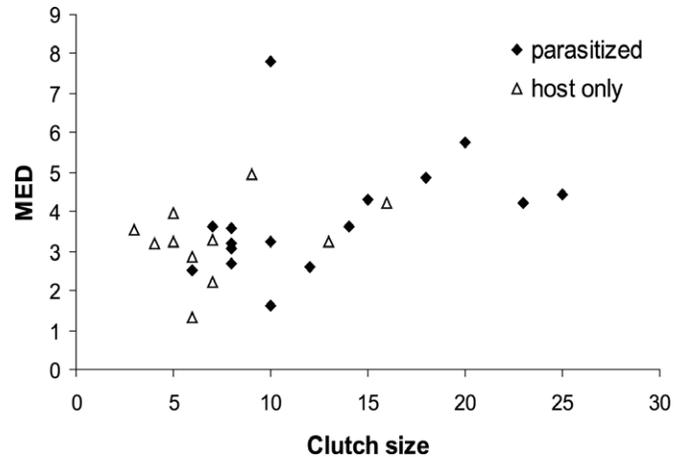


FIGURE 2. Overlap in maximum Euclidean distances (MED) from parasitized clutches and just the host's contributions to those clutches.

We explored possible reasons this method did not perform well for the Wood Duck, including reduced variation among eggs laid by different females and increased variation among eggs laid by the same female. We started by examining explanations for what would reduce variation among females. We explored the possibility that the MED method may be influenced by genetic similarities in characteristics of related hosts and parasites. In the Wood Duck, hosts and their primary parasites are more related than expected by chance (Roy Nielsen et al. 2006c), and egg size has a large heritable component (Ojanen et al. 1979, Van Noordwijk et al. 1980). Relatedness of host and parasites may make the MED method less effective by reducing variation in the characteristics of eggs in parasitized clutches so that clutches parasitized by relatives may have lower MED values than similarly sized clutches parasitized by nonrelatives. If the degree of relatedness among hosts and parasites varies from population to population, then the MED method may vary in its effectiveness from one population to another.

We did not have DNA samples from parasites in this population, but clutch mates should be more closely related when the parasite is a relative of the host than when the parasite is not a relative. We reasoned that MED would be higher for clutches with low relatedness of clutch mates than for clutches with high relatedness of clutch mates. However, we were concerned that the positive relationship between clutch size and MED ($R^2 = 0.50$, $n = 13$, $P = 0.007$) would result in a spurious relationship between MED and relatedness if unparasitized clutches had both higher relatedness of clutch mates and were smaller. We did find an inverse relationship between relatedness of clutch mates and clutch size ($R^2 = 0.53$, $n = 15$, $P = 0.002$). When we controlled for clutch size, however, relatedness of clutch mates was not correlated with MED ($R^2 = 0.26$, $P = 0.13$).

Thus, the poor performance of the MED method for the Wood Duck cannot be explained by relatedness of hosts and parasites reducing variation between their eggs. This is not surprising given that relatedness between hosts and parasites has also been reported for a population of the Common Goldeneye (Andersson and Åhlund 2000) and a similarly poor performance of MED would be expected for it. But, on the basis of egg-deposition rates and the appearance of new eggs during incubation, the method is

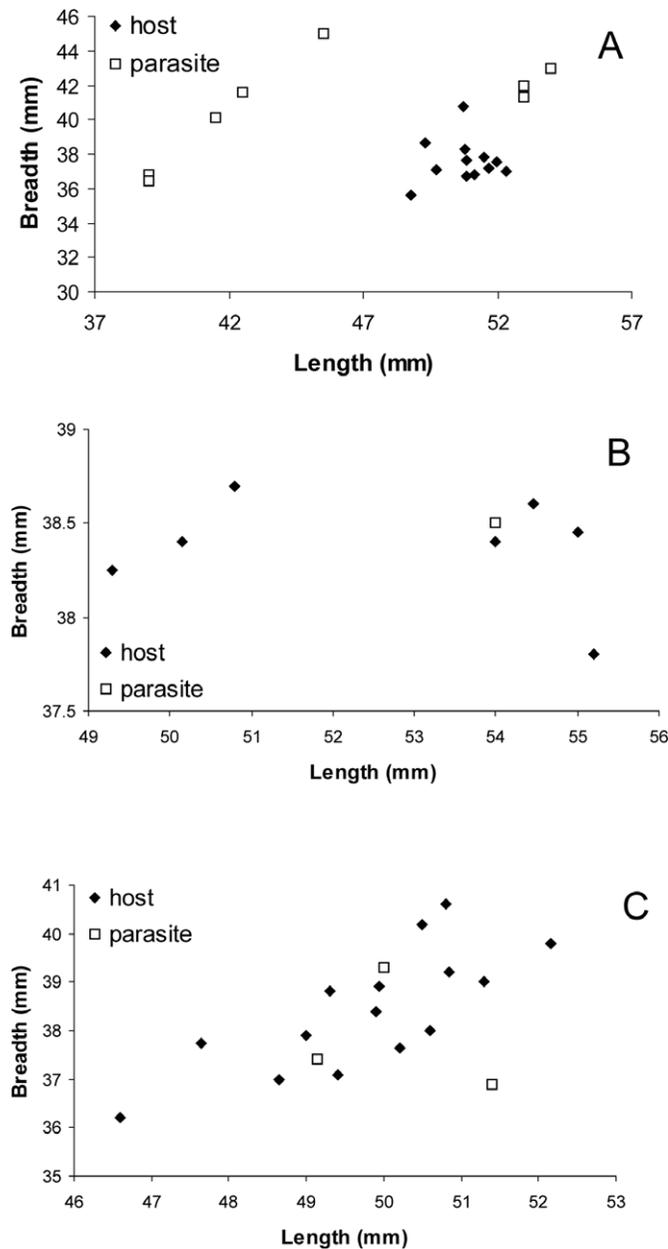


FIGURE 3. Measurements of eggs from three different clutches. Parasite eggs were sometimes noticeably different from host eggs and markedly increased variation within a clutch (A), but often the parasite's eggs were quite similar to those of the host (B, C).

reported to perform well for the Common Goldeneye (Pöysä et al. 2001). Genetic techniques, however, have not been applied in conjunction with the MED method for the Common Goldeneye.

We also considered the possibility that eggs laid by an individual Wood Duck may vary in size more than those laid by an individual Common Goldeneye. The Wood Duck lays smaller eggs at the beginning and end of the laying cycle, with larger eggs in the middle, so variation within a female can be quite substantial (Kennamer et al. 1997). Hepp et al. (1987) and Kennamer et al. (1997) reported considerable variation among eggs (CV 8.0%, 4.0%, 2.7% and 6.4%, 3.5%, 2.4% for mass, length, and

breadth, for each respective study). The Bufflehead (*Bucephala albeola*) and Barrow's Goldeneye (*B. islandica*) vary comparably in these same egg measurements with CVs of 5.89%, 3.33%, 2.13% and 5.04%, 3.06%, 2.10%, respectively (Lavers et al. 2006). Although we found slightly more variation, particularly in egg mass (CV 15.5%, 4.0%, 3.7% for mass, length, and breadth, respectively), variation in egg mass could easily be explained by differing stages of incubation. In some waterfowl egg weight decreases during development (Zicus et al. 2004). However, when we calculated mass from length and breadth (Rohwer 1988), as Pöysä et al. (2001) did for the Common Goldeneye, the CV was smaller and more comparable to that of other studies (10.2%). We used the calculated mass in the MED calculations, so greater variation in egg size within females cannot explain the inability of this method to perform well for the Wood Duck.

In this population rates of parasitism were very high (85%, Roy Nielsen et al. 2006a), which, if hosts and parasites invest in eggs differently, could explain higher variability within and among nests. However, we found no evidence of such a difference, as indicated by the size or mass of genetically determined host and parasite eggs. Therefore, we cannot explain the poor performance of this method by high variability in females' investments.

Thus it seems that the poor performance of the MED method for the Wood Duck is due to its inability to identify parasitism when parasites lay eggs similar in size to those of the host. We caution that this might also be true for other waterfowl. Furthermore, we suggest that researchers using the MED method test it with eggs of known maternity, so that parasite eggs that would be missed on the basis of egg-deposition rates can be detected reliably. Hosts may reduce their contribution to clutches in response to parasitism (Roy Nielsen et al. 2006b), and if this reduction coincides with parasites' laying, it may go undetected. In conclusion, we do not recommend this method for the Wood Duck and do not advise its use without appropriate investigation of its reliability. In the Wood Duck, the MED method was inferior to a blind assumption that all nests were parasitized.

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