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Numts: A Challenge for Avian Systematics and Population Biology

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The development of the polymerase chain reaction (PCR) and dramatic improvements in technologies for DNA sequencing over the last decade have provided new opportunities in avian systematics (Mindell 1997) and the study of population genetic structure (e.g. Quinn 1992). For reasons associated with its rapid rate of evolution, haploidy, and maternal inheritance (e.g. Moore 1995, Zhang and Hewitt 1996), recent work has focused on mitochondrial DNA (mtDNA). Direct sequencing of PCR products amplified from extracts of genomic DNA has circumvented the need for purified mtDNA. Almost any material is now a workable source of DNA for PCR, including blood, feathers, eggshells, feces, and other tissues from live birds; skin, feathers, cartilage, and

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bone from museum specimens; and even subfossil bones (e.g. Cooper et al. 1992, Cooper 1994).

PCR amplification of mtDNA from total genomic DNA, however, is associated with a significant problem: the potential for nuclear sequences to be amplified instead of or in addition to the targeted mtDNA sequence. The transposition of mtDNA sequences to the nucleus has been documented in a wide variety of taxa from fungi to insects to vertebrates, and mounting evidence suggests that it is a common if not ubiquitous phenomenon (see Zhang and Hewitt 1996). Lopez et al. (1994) suggested the term "Numt" to refer to nuclear sequences of mitochondrial origin, and we will use the term here.

Dating to transposition events from thousands to tens of millions of years ago (e.g. Collura and Stewart 1995, Sorenson and Fleischer 1996), Numts and mitochondrial sequences diverge following transposition, evolving at different rates and under different constraints (Arctander 1995, Zischler et al. 1995). If mistaken for mtDNA, Numts may introduce significant error in phylogenetic analyses (see below). If coamplified with mtDNA, Numts greatly complicate the accurate determination of mitochondrial sequences. Nonetheless, Numts present interesting opportunities both in the study of molecular evolution and in phylogenetic analysis (see Zhang and Hewitt 1996, Quinn 1997). Several recent studies using genomic DNA extracted from avian blood samples have encountered nuclear homologues of mitochondrial genes (Quinn 1992, Arctander 1995, Sorenson and Fleischer 1996). Because red blood cells in birds (and reptiles) are nucleated and relatively depauperate in mtDNA, nuclear "contamination" is particularly problematic with blood samples. We briefly review published examples of this phenomenon in birds, provide a new example showing that the problem is not limited to blood samples, and provide suggestions for avoiding, recognizing, and working with Numts.

Quinn and White (1987) provided the first example of a Numt in birds, demonstrating through DNA hybridization experiments that sequences homologous to mitochondrial genes occur in the nuclear genome of Snow Geese (*Anser caerulescens*). In addition, Quinn (1992) found that blood and tissue samples from Snow Geese yielded control-region sequences falling into two divergent groups. Because blood had been collected from one population and liver tissue from another, the mistaken conclusion that these populations were differentiated in their mtDNAs might have been made. Careful evaluation, however, revealed faint shadow bands (representing the mtDNA copy) in sequences from blood and led to the design of specific primers that allowed independent amplification of either the mitochondrial or Numt sequence.

Arctander (1995) and Sorenson and Fleischer (1996) also amplified nuclear sequences from avian blood samples, but in these cases mitochondrial and nuclear copies were approximately equally represented in the PCR product, resulting in sequences with ambiguities wherever the two copies differed. Interestingly, the pattern of phylogenetic relationships among Numts and mtDNAs differed greatly in these two studies. Arctander found that Numt cytochrome-*b* sequences from several tapaculos (*Scytalopus* spp. and *Myornis senilis*) were more closely related to each other than to any of the mtDNA sequences of these species, suggesting a single ancient transposition event in their common ancestor. In contrast, Sorenson and Fleischer found that each of six different Numts derived from the mtDNA control region in diving ducks (Aythyini) was a close relative of the mtDNA sequence of the species in which it was found, suggesting six recent, independent transposition events. The implication of these contrasting results is that the magnitude of the error introduced by an unrecognized Numt in a phylogenetic analysis is unpredictable.

Recently, we found an apparent Numt homologous to the mitochondrial cytochrome oxidase subunit I gene (COI) in the Wandering Whistling-Duck (*Dendrocygna arcuata*). In the process of sequencing the mitochondrial genome of this species (Mindell et al. unpubl. data), we used primers L6615 (5′-CCTCTGTAAAAAGGACTACAGCC-3′ [Miranda et al. 1997]; L and H numbers designate the location of the 3′ base in the light or heavy strand, respectively, of the published chicken mtDNA sequence [Desjardins and Morais 1990]) and H7032 (5′-TTGCCAGCTAGTGGGGGGTA-3′ [Miranda et al. 1997]) to amplify a 416-bp fragment including parts of COI (387 bp) and the flanking transfer RNA (29 bp). We obtained a single unambiguous sequence from the PCR product but it did not perfectly match overlapping sequences amplified from the same sample with other primer pairs. In addition, the inferred sequence of amino acids included substitutions at seven sites otherwise conserved in vertebrates (Table 1). Amplification of a larger fragment with primers L6615 and H7539 (5′-GATGTAAAGTAGGCTCGGGTGTCTAC-3′ [Miranda et al. 1997]) yielded a different unambiguous sequence that matched overlapping sequences for *D. arcuata* and that did not include the unusual amino-acid substitutions.

To explore this problem further, we sequenced six other whistling-ducks and the White-backed Duck (*Thalassornis leuconotus*; sequences deposited in Genbank, accession numbers U97731 to U97739), using primers L6615 and H7032 or H7338 (5′-CCGAAGAATCAGAAKARRTGTTG-3′, degenerate sites as follows: K = G or T, R = A or G). None of these sequences had unusual amino-acid substitutions. The sequence we initially obtained for *D. arcuata* is not the result of contamination from, for example, a bacterial or invertebrate source. Phylogenetically, it is a whistling-duck COI sequence, closer to several pri-

TABLE 1. Inferred amino-acid sequence for the putative *D. arcuata* Numt compared with that of seven *Dendrocygna* (all seven species identical; see Fig. 1 for species names) and *Thalassornis leuconotus* mtDNAs and comparison of H7032 primer sequence with *D. arcuata* mtDNA. Amino-acid substitutions at sites conserved across vertebrates[a] are marked with an asterisk.

Taxon	
	COI amino-acid sequence
	* *
Dendrocygna spp.	VTFINRWLFSTNHKDIGTLYLIFGAWAGMIGTALSLLIRAELGQPGTLLG
D. arcuata Numt	.....W.....S.....H.....
Thalassornis	.....
	* ***
Dendrocygna spp.	DDQIYNVIVTAHAFVMIFFMVMPIMIGGFGNWLVPLMIGAPDMAFPRMNN
D. arcuata Numt	.....L.....LLW...
Thalassornis	.....I.M.....
	*
Dendrocygna spp.	MSFWLLPPSFLLLLASSTVEAGAGTGWTV
D. arcuata Numt	.....D...S.
Thalassornis	.....
	Primer sequence
H7032 Primer	TTGCCAGCTAGTGGGGGGTA
D. arcuata mtDNA (H-strand)	..T..T....AA..T..A..

[a] Including two fish (*Cyprinus carpio*, GenBank accession number X61010; *Crossostoma lacustre*, M91245), an amphibian (*Xenopus laevis*, M10217), four mammals (*Homo sapiens*, J01415; *Mus musculus*, J01420; *Ornitorhynchus anatinus*, X83427; *Didelphis virginiana*, Z29573), a turtle (*Chrysemys picta*), *Alligator mississippiensis*, and several birds (Mindell et al. unpubl. data).

marily Australasian taxa than to *D. autumnalis*, *D. arborea*, or *Thalassornis* (Table 2, Fig. 1). That the sequence codes for nine amino-acid substitutions relative to other *Dendrocygna*, strongly suggests that it is no longer evolving under the same selective constraints and supports the conclusion that it is a Numt. Although we have not determined the flanking sequence of this presumed Numt, the H7032 primer matches *D. arcuata* mtDNA very poorly (Table 1), suggesting that this primer preferentially amplified a nuclear sequence that it matched somewhat better. It is possible that this apparent Numt actually represents an intra-mitochondrial duplication of a part of the COI gene, but this has not been detected in sequencing over half of the mitochondrial genome of this species.

TABLE 2. Corrected percent genetic distance (Kimura 1980) between *D. arcuata* Numt and mtDNA COI sequences and other Dendrocygnini mtDNA COI sequences.

	D. arcuata mtDNA	*D. arcuata* Numt
D. arcuata mtDNA	—	11.6
D. javanica	5.8	13.1
D. guttata	6.9	12.8
D. bicolor	5.8	12.5
D. eytoni	8.0	13.1
D. autumnalis	11.6	14.7
D. arborea	12.3	14.7
Thalassornis leuconotus	16.1	17.0

We find this example significant because we initially obtained a single unambiguous sequence with DNA extracted from muscle tissue, presumably with a ratio of mitochondrial to nuclear genome copies of 1,000s to 1 (Robin and Wong 1988). Had our intent been to infer the phylogeny of *Dendrocygna* based on this single 416-bp fragment, the unusual amino-acid sequence would have been the only indication of a problem. Although quite divergent from *D. arcuata* mtDNA, this apparent Numt has sustained no mutations introducing stop codons or reading frame shifts. In phylogenetic analysis, substituting the *D. arcuata* Numt for its mtDNA sequence leads to a very different conclusion about the relationships of this species (Fig. 1).

Based on our experiences with Numts in birds and published observations, we make the following comments and practical suggestions for dealing with this phenomenon. Similar but less detailed recommendations were made by Zhang and Hewitt (1996) and Quinn (1997).

Avoiding Numts.—Because of their relatively high ratio of nuclear to mitochondrial genome copies, avian blood samples are particularly likely to yield Numts and should be avoided for mtDNA sequencing. Although more expensive and time consuming, preparations of purified mtDNA are the least likely to yield Numts, followed by extractions of total DNA from mtDNA-rich tissues. Neither, however, guar-

antees that Numts will not be amplified (e.g. Collura and Stewart 1995). Numts may have high copy number in the nucleus (e.g. Lopez et al. 1994), and PCR primers may favor a Numt over the mtDNA sequence (see Smith et al. 1992, Zhang and Hewitt 1996). In our experience, tissues that might seem relatively poor sources of DNA, such as hardened feather quills, work well for amplification of mtDNA and are less problematic than blood samples with respect to nuclear contamination.

Primer design is a critical issue in comparative sequencing studies. Because Numts may evolve more slowly than mtDNA following transposition (Arctander 1995, Collura and Stewart 1995, Zischler et al. 1995, Sorenson and Fleischer 1996), they diverge less from the ancestral sequence and may be more similar to the sequences of related taxa. As a result, primers based on sequences from species other than the study taxa and so-called "universal" primers may be particularly prone to amplification of Numts. In addition, primers designed to accommodate a number of taxa by using a consensus rule to determine the nucleotide at each position will tend to approximate ancestral sequences and as such may favor Numts.

One solution is to use primers targeted to highly conserved sequences that vary little (or not at all) among all birds, such as conserved blocks in the 12S and 16S rRNA genes and the anticodon stem and loop of some tRNA genes. For protein-coding genes, however, few 20- to 25-base stretches of highly conserved sequence exist, because most 3rd positions are free to vary. In contrast to Zhang and Hewitt (1996), we suggest that primers incorporating degenerate sites will more consistently amplify mtDNA from tissue samples. Primers with degenerate sites that accommodate alternative nucleotides at 3rd positions will be less likely to preferentially amplify one sequence over another, allowing the usually high ratio of mitochondrial to nuclear copies rather than asymmetry in primer matching to determine the PCR product. This assumes that variable sites in taxa already sequenced will predict the likely variation in additional species, an assumption that is probably reasonable for 3rd positions in a region coding a conserved sequence of amino acids, for example. See Kwok et al. (1994) for suggestions on the design of degenerate primers.

Ideally, the groundwork for a sequencing project should include a careful evaluation of primer sequences in relation to published sequences for a taxonomic range somewhat broader than that represented in the study and sequencing of flanking regions for a sample of the study taxa so that specific primers with appropriate degenerate sites can be designed for the study. If possible, this initial work should be done with purified mtDNA (see Dowling et al. 1996).

Another potential means of avoiding Numts is to amplify the entire mtDNA or large portions of it using protocols for extended or long-PCR (e.g. Cheng et al. 1994). Although large mtDNA fragments may be transposed to the nucleus (e.g. Lopez et al. 1994), most published examples involve sequences less than 4 kB in length (Zhang and Hewitt 1996), such that primers separated by 5 to 16 kB may amplify only mtDNA. Even if the entire mitochondrial genome is transferred to the nucleus, a pair of primers facing away from each other that amplify the circular mtDNA will not amplify a linear Numt, unless the Numt begins and ends precisely in the region not amplified by the primers, or unless the Numt is repeated tandemly in the nucleus (e.g. Lopez et al. 1994). Once obtained, long products can serve as templates for re-amplifications using internal primers for the gene of interest. This technique has been used to eliminate apparent Numts where amplification of smaller fragments yielded sequences with numerous ambiguities (Sorenson unpubl. data). This approach does not always work, and we speculate that heteroduplex formation between Numts and incomplete mtDNA extension products (as in "jumping PCR"; Pääbo et al. 1990) results in the incorporation of some Numt sequence in the final PCR product.

Recognizing Numts.—In our experience, Numts often coamplify with the mtDNA copy, producing ambiguous sequences in affected species. A careful examination of sequencing gels and electropherograms for positions with two bands or two peaks should be routine. Particularly when found at corresponding positions of complimentary strands, the temptation to simply "call" the stronger base and disregard the weaker as an artifact should be resisted. A comparison of sequences from tissue sources that vary in the ratio of mitochondrial to nuclear genome copies (e.g. avian blood versus muscle tissue) may provide a strong inference that a Numt is responsible for such ambiguities (e.g. Quinn 1992, Sorenson and Fleischer 1996).

Other lines of evidence, such as unusual amino-acid substitutions, stop codons, and length mutations in protein-coding regions may lead to the conclusion that a "clean" sequence is a Numt. Sequences of rRNA and tRNA genes should be reconciled with appropriate secondary structure models and checked for changes incompatible with that structure. Mismatches in overlapping sequences from a given individual should also raise suspicions. Indeed, sequencing broadly overlapping PCR products is a good precaution against Numts because a low copy number Numt is unlikely to be preferentially amplified by two different primer pairs. Finally, significant disagreement for a particular taxon in genetic distances or phylogenetic relationships derived from different gene fragments may indicate that one is a Numt. Numts also may yield significantly shorter branches in phylogenetic analyses as a result of their slower rate of evolution (Sorenson and Fleischer

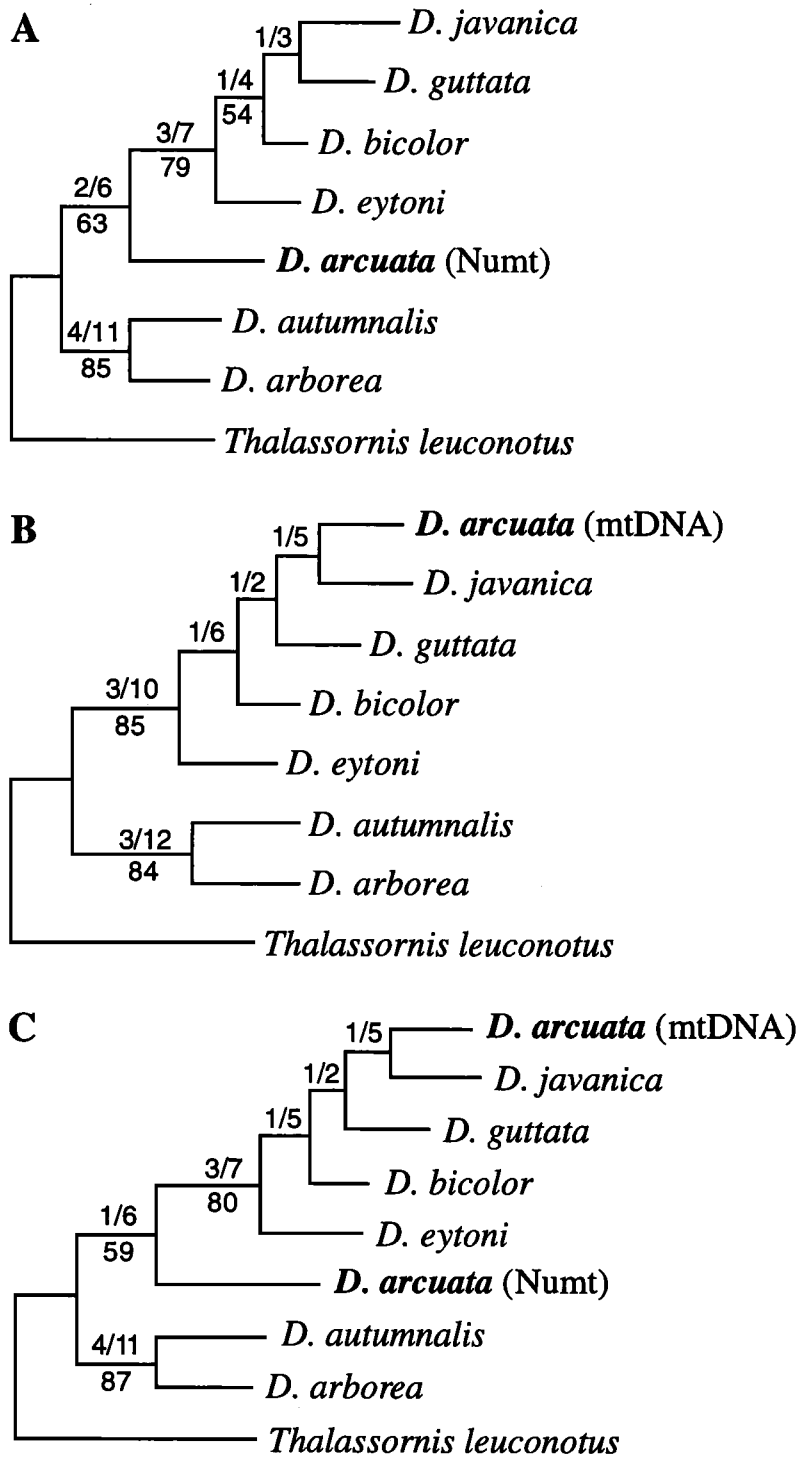

FIG. 1. Alternative phylogenetic hypotheses for *Dendrocygna* using (A) the putative *D. arcuata* Numt, (B) the *D. arcuata* mtDNA sequence, and (C) both. A single most parsimonious tree was found in each analysis using equal weights for all characters in PAUP 3.1 (Swofford 1993). Branches are drawn proportional to the

1996). Note, however, that in the absence of selective constraints, Numts eventually will accumulate substitutions at sites conserved in mtDNAs (Arctander 1995; Fig. 1), resulting in a greater perceived rate of change for older Numts. Such changes in addition to length mutations and recombination eventually will make older Numts unavailable to PCR primers.

Of course, phenomena such as mitochondrial heteroplasmy (Mundy et al. 1996), duplication events within the mitochondrial genome (Moritz and Brown 1986), and cross-contamination of samples or reagents may account for PCR products including more than one sequence. Numts, however, have been the most common of these phenomena.

Working with Numts.—When Numts and mtDNA are co-amplified, a number of techniques may be used to separate the two copies. First, the PCR product can be cloned and several clones sequenced to determine the different sequences included in the PCR product (e.g. Arctander 1995). Second, primers internal to the original PCR primers can be designed to discriminate between the two sequences (e.g. Quinn 1992, Sorenson and Fleischer 1996). Considerations of primer design are exactly opposite to those discussed above in that a primer that maximally discriminates between the two sequences is needed. Ideally, the 3′ base of the new primer(s) should be located where Numt and mtDNA differ by a transversion (see Kwok et al. 1990) and near additional differences. This approach will be more cost effective than cloning for studies of intraspecific variation. Third, the two copies can be separated by digesting either the genomic DNA prior to PCR or PCR product with a restriction enzyme that cuts only one of the copies between the PCR primers. Choice of an enzyme is based on ambiguous positions in initial sequencing. The digested PCR product is run out on an agarose gel to separate the fragments, which can then be excised, purified, and sequenced (e.g. Sorenson and Fleischer 1996). Although very effective, this technique is limited by the locations of differences between the two copies. Once two clean sequences are obtained, identifying them, respectively, as Numt and mtDNA may be based on a number of potential criteria discussed above. Alternatively, the expressed mtDNA copy can be obtained using reverse-transcription of mRNA followed by PCR amplification of the resulting cDNA (Collura et al. 1996). This approach is powerful in that it automatically identifies the functional mitochondrial copy, but it is limited to primer pairs within a single gene. The relative instability of mRNAs also may limit the samples to which this technique is applied.

To the extent that Numts provide a historical record of ancestral mtDNA sequences, they also provide opportunities for phylogenetic analyses. Numts may be ideal outgroups for extant mtDNA haplotypes (Quinn 1992, Zischler et al. 1995), their presence or absence among species can provide evidence of relationships (e.g. Sorenson and Fleischer 1996), and they provide a means to compare mechanisms and rates of molecular evolution between the mitochondrial and nuclear genomes (Sorenson and Fleischer 1996, Zhang and Hewitt 1996). For these reasons, researchers may want to discover Numt sequences intentionally.

A more direct but technically demanding way to detect Numts is to use purified mitochondrial DNA as a probe against DNA isolated on a Southern blot. Total DNA extracted from blood or other tissues is restriction-endonuclease digested, size fractionated in an agarose gel, and transferred to a nitrocellulose or nylon membrane. In the absence of Numts, hybridization of a purified mitochondrial probe with the blot should reveal bands corresponding in position to those obtained from digesting purified mtDNA alone. If Numts are present, additional bands may be detected. Even if the Numt sequence retains all of the same restriction sites as the mtDNA, flanking regions should generate bands of unique size. Unique bands also may correspond to restriction fragments spanning the junction between tandemly repeated Numt copies (e.g. Lopez et al. 1994).

By including total DNA extracts from tissue types with different mitochondrial to nuclear DNA ratios, the nuclear origin of anomalous bands can be verified by tissue-specific variation in the relative intensity of the putative nuclear and mitochondrial bands. Lopez et al. (1994) and Quinn and White (1987) used information from Southern blots to complement anomalous PCR-based sequencing results. In each case, the Numt was present in multiple copies arranged in a tandem array. Note that such tandem repeats result in multiple nuclear targets for PCR primers, potentially offsetting the high ratio of mitochondrial to nuclear genome copies in most tissues. This approach provides a broader survey of the mitochondrial genome than the small regions usually studied with PCR techniques. One potential dif-

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number of steps using ACCTRAN character optimization. Support indices (the number of additional steps required in a tree without the node in question; Bremer 1988) and minimum branch length are displayed above each node. Bootstrap percentages are shown below nodes present in more than 50% of 1,000 replicates. For A: tree length = 190, CI = 0.69, RI = 0.49. For B: tree length = 172, CI = 0.70, RI = 0.50. For C: tree length = 201, CI = 0.68, RI = 0.47. A sister relationship between *D. arcuata* and *D. javanica* is consistent with analyses based on complete 12S rDNA sequences (Sorenson and Johnson unpubl. data).

ficulty is the high ratio of mitochondrial to nuclear genome copies in most tissue extracts, which might lead to large differences in the signal intensity between Numt versus mitochondrial targets. Avian blood is the least biased tissue in this respect and hence has the best chance of yielding Numts.

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Body Mass, Ambient Temperature, Time of Day, and Vigilance in Tufted Titmice

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Risk of starvation and risk of predation are two major evolutionary forces thought to shape an animal's foraging behavior (Lima 1986, McNamara and Houston 1990). If energetic demands are high, the risk of starvation could be high, and individuals should increase their energy reserves by foraging more intensively. If the risk of predation is high, individuals should become more vigilant to detect predators. However, when vigilant for predators, individuals could be forced to reduce other activities such as foraging (e.g. Caraco 1979, Elgar 1989).

McNamara and Houston (1986) modeled vigilance behavior in diurnal animals as a function of energy reserves and time of day. They predicted that vigilance rate should be low in the morning, a time when animals replenish fat stores lost during the nocturnal fasting period. Vigilance also was predicted to be low late in the day as animals accumulated energy reserves for the following night. In contrast, the reduced demand for energy gain around midday was predicted to allow a heightened vigilance rate. We are aware of no empirical tests of these predictions. In the present study, we examined the relationships among vigilance rate, energy reserves (body mass), and time of day in Tufted Titmice (*Baeolophus bicolor*). We also examined the effects of ambient temperature because vigilance has been shown to be positively correlated with temperature (Pravosudov and Grubb 1995).

Methods.—We studied vigilance in six male Tufted Titmice during the winter of 1994–95. The birds were caught at different locations in central Ohio and observed in isolation for eight days in an outdoor aviary 3 × 8 × 2 m high. During that period, food (unshelled sunflower seeds) and water were provided *ad libitum*. The aviary was open to the sky (except for wire netting) but was walled by translucent fiberglass panels that isolated the birds visually and maintained wind speeds within the aviary at or near zero. One end of the aviary was roofed by a translucent fiberglass panel 30 cm wide that protected a "recording" perch and feeder from rain and snow.

We observed the titmice from an observation chamber attached to one end of the aviary. Observations occurred under prevailing photoperiods, and ambient temperatures were recorded and stored every 30 min in a computer housed in an attached observation chamber (Weather Wizard III and Weatherlink software; Davis Instruments). An electronic balance inside the observation chamber was connected through a one-way glass wall to a perch used by the birds frequently during the day and for roosting at night. Readings from the balance were recorded to the nearest 0.01 g and stored in another computer, also housed in the observation chamber.

To evaluate a bird's vigilance, we divided each day into four 2-h blocks from 0900 to 1700 EST. During each block, we randomly selected one 30-min period during which we observed vigilance. We obtained a

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