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Source: The American Midland Naturalist, 171(1):139-146. 2014.

Published By: University of Notre Dame

DOI: <http://dx.doi.org/10.1674/0003-0031-171.1.139>

URL: <http://www.bioone.org/doi/full/10.1674/0003-0031-171.1.139>

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# Field Identification of Sympatric *Peromyscus leucopus noveboracensis* and *P. maniculatus gracilis* in Wisconsin from External Measurements

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**ABSTRACT.**—In Wisconsin white-footed mice (*Peromyscus leucopus noveboracensis*) and woodland deer mice (*P. maniculatus gracilis*) are difficult to distinguish. Recent climatic trends have facilitated encroachment of *P. leucopus* north into the range of *P. maniculatus*, necessitating unambiguous species identification as researchers begin to untangle the ecological implications of such community changes. Cranial and external measurements have been used by previous investigators to differentiate these species in other regions. However, because large geographic morphological variation occurs and most previous studies used measurements from dead specimens, definitive morphological characteristics need to be identified that can quickly and effectively classify live Wisconsin *Peromyscus* in the field. During the summer of 2010, we collected tissue samples and measured ear length, tail length, hindfoot length, and body weight of 84 *P. maniculatus* and 293 *P. leucopus* live-trapped in six Wisconsin counties. We used mDNA analysis to identify species. We developed discriminate function analysis (DFA) equations to identify characteristics that best distinguished species. Ear length correctly classified 97.9% of the samples with all but one *P. leucopus* <17 mm and all but seven *P. maniculatus* ≥17 mm. By adding body weight to the function, we were able to achieve 99.2% classification accuracy and with the addition of tail length were able to achieve 99.5% accuracy.

## INTRODUCTION

The woodland form of the deer mouse (*Peromyscus maniculatus gracilis*) and white-footed mouse (*P. leucopus noveboracensis*) are sympatric in the Great Lakes and Appalachian regions of the United States. Ecologically, these species can occupy similar habitats, have similar niche requirements, and closely resemble each other morphologically (Wolff, 1985; Long, 1996). A number of external characteristics such as pelage color, degree of caudal hair pencilation, and bicoloration of the tail can assist identification of *P. maniculatus* and *P. leucopus* (Choate, 1973; Long and Long, 1993; Finnell, 2000). However, subjectivity and intraspecific variation make these characteristics unreliable for definitive identification in many geographic regions (Feldhamer *et al.*, 1983; Rich *et al.*, 1996). To circumvent these shortcomings, previous studies have attempted to identify morphological characteristics to differentiate between *P. maniculatus* and *P. leucopus* (Stromberg, 1979; Feldhamer *et al.*, 1983; Kamler *et al.*, 1998; Reed *et al.*, 2004). These studies often use external measurements taken from dead animals (Feldhamer *et al.*, 1983; Sternburg and Feldhamer, 1997; Reed

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*et al.*, 2004) and, therefore, may not reflect accurately measurements from live animals (Bruseo *et al.*, 1999). Cranial characteristics alone, or in conjunction with external measurements, have also been used (Long and Long, 1993; Rich *et al.*, 1996; Sternburg and Feldhamer, 1997; Reed *et al.*, 2004) but these also require sacrificing animals (Lindquist *et al.*, 2003). Additionally, given geographic morphological variation of *P. maniculatus* and *P. leucopus* (Tolliver *et al.*, 1987; Palas *et al.*, 1992; Sternburg and Feldhamer, 1997), studies using morphological characteristics often only apply to small geographical regions or are only applicable to one subspecies (Bruseo *et al.*, 1999).

Electrophoresis of salivary amylase has proved effective for unequivocal distinction of *P. maniculatus* from *P. leucopus* (Aquadro and Patton, 1980; Bruseo *et al.*, 1999); however, insufficient salivary sequestration or degradation can severely reduce its utility (Sternburg and Feldhamer, 1997; Lindquist *et al.*, 2003). Differentiation using molecular markers also provides unambiguous distinction among *Peromyscus* species. Nevertheless, both these procedures require specialized equipment and must be performed in a lab, which can be expensive for studies with a large volume of samples (Sternburg and Feldhamer, 1997; Reed *et al.*, 2004). Furthermore, these procedures are time consuming and are not practical for field studies that may require rapid species determination, *e.g.*, in removal studies in which one species is removed and the other retained (Bruseo *et al.*, 1999).

Currently, *P. leucopus* is expanding its range northward in Wisconsin and Michigan, ostensibly due in part to recent climatic changes (Long, 1996; Myers *et al.*, 2009). Moreover, *P. leucopus* may be replacing *P. maniculatus* within habitats formerly occupied only by *P. maniculatus* (Long, 1996). Range extensions of *P. leucopus* and concomitant declines of *P. maniculatus* have been noted in Michigan (Myers *et al.*, 2009) and anecdotally in Wisconsin (Long, 1996). Considering *P. maniculatus* serve many ecological functions and often dominate northern small mammal communities, replacement by its congener, *P. leucopus*, could precipitate considerable community changes with unknown consequences (Myers *et al.*, 2009). Additionally, *P. leucopus* are the primary reservoir for Lyme disease in the Midwest and Eastern United States giving rise to public health concerns (Meyers *et al.*, 2009).

Where the species are sympatric, the need to understand this trend and the potential ecological impacts is crucial; and much information is lost when researchers forgo species identification during ecological studies because of uncertain identification (Evrard, 1998; Chapman and Ribic, 2002; Kaminski *et al.*, 2007). To our knowledge, there has only been one study conducted on morphological characteristics of *P. maniculatus gracilis* and *P. leucopus noveboracensis* in Wisconsin (Long and Long, 1993). The results are difficult to use in the field because external measurements were taken from dead animals and cranial characteristics were extensively used (Long and Long, 1993). Additionally, relatively few animals were used and genetic testing was not used to confirm species identification, creating a situation where metrics used to identify species *a priori* were also used in the analysis. The objective of our study was to determine external measurements that could be taken in the field to distinguish live *P. maniculatus gracilis* and *P. leucopus noveboracensis* in Wisconsin, regardless of their sex or age.

## METHODS

### FIELD METHODS

As part of a larger study on ecological communities of small mammals and trap efficacy, *P. maniculatus gracilis* and *P. leucopus noveboracensis* were live trapped in natural plant communities in northern and central Wisconsin from Jul. 21 through Aug. 22, 2010 (for full study design see Stephens, 2012). For the purpose of this research, we sampled

*Peromyscus* from five study areas in northern Wisconsin: Burnett (45.896857, -92.617313), Douglas (46.263973, -92.038955), Oconto (45.30081, -88.409459), Portage\Waupaca (44.616497, -89.172604), and Taylor (45.246532, -90.622131) Counties. We established eight transects of 190 m at each study area in habitats ranging from open wetland to upland forest. Within study areas, transects were located an average of 8.7 km (range 0.09–30.51 km) apart and placed  $\geq 10$  m from the edge of a habitat to minimize edge effects. Each transect had 30 traps; Sherman live traps (7.6  $\times$  8.9  $\times$  23.9 cm; H.B. Sherman Inc., Tallahassee, Florida) were placed every 10 m ( $n = 20$ ) and pitfall traps (38 cm deep, 20 cm diameter, 10 L, floral cooler buckets, Syndicate Sales, Inc., Kokomo, Indiana) every 20 m ( $n = 10$ ). All traps were baited with peanut butter spread 4 mm thick between sheets of paper towel and cut into 2.5  $\times$  2.5 cm squares. Traps were checked twice daily for 4 d consecutively, resulting in three diurnal and four nocturnal survey periods. Captured animals were marked with a slight ear notch to identify recaptures.

We took standard museum measurements on initial captures of all *Peromyscus*. The first author performed all measurements of tail length (sacrum to caudal tip, excluding hairs), right hindfoot length (calcaneus to longest claw), and right ear length (basal notch to tip, excluding hairs) to the nearest mm using a flexible clear plastic ruler. Weight to the nearest gram was taken using a pull scale. Total length (tip of nose to caudal tip) was not taken because of the variability of this measurement on live animals (Bruseo *et al.*, 1999). We also collected ear tissue samples during the marking process. Tissues were placed in individually marked vials filled with 70% ethanol. The trapping and tissue sampling protocol was approved by the University of Wisconsin- Stevens Point Institutional Animal Care and Use Committee (protocol #201004.14) and followed guidelines outlined by the American Society of Mammalogists (Sikes *et al.*, 2011).

#### LAB METHODS

DNA was extracted from ear tissue samples using QIAamp<sup>®</sup> DNA Mini Kit Tissue Protocol (QIAGEN Inc., Valencia, CA) at Marshfield Clinic, Marshfield, Wisconsin. Manufacturer's recommendations were followed except that samples were incubated overnight at 56 C in buffered ATL and proteinase K. A polymerase chain reaction (PCR) was used to amplify a 358 base pair product from mitochondrial *cytochrome b* with primers BMI and BM2 (Boakye *et al.*, 1999; Lee *et al.*, 2002; Meece *et al.*, 2005) using the HotStarTaq Master mix kit (QIAGEN) according to manufacturer recommendations. The PCR product then underwent restriction fragment length polymorphism (RFLP) analysis using restriction enzyme *Hae*III. *Hae*III cut amplified PCR products from *P. leucopus* into fragments but did not cut *P. maniculatus* products. Digested PCR products were sized by gel electrophoresis on 2.0% LE agarose gel stained with ethidium bromide and identified to species.

#### DATA ANALYSIS

We used a two-tailed student's *t*-test to determine if measurements differed between males and females for both *P. maniculatus* and *P. leucopus*. There was no indication of sexual dimorphism for either species, as noted by other authors (Long and Long, 1993; Kamler *et al.*, 1998), and subsequently sexes were grouped for analyses. Additionally, we compared measurements among sites using a one-way analysis of variance and found little variation in measurements for either species. Because we did not differentiate among juveniles, subadults, and adults in the field, we choose to pool them for analysis to broaden the applicability of the identified metrics to all age cohorts of a population. We visually inspected univariate measurements for normality using histograms and used a two-tailed student's *t*-test to compare univariate measurements between species. Pearson's correlation

TABLE 1.—Mean, standard deviation, range, and results of a *t*-test comparing univariate external measurements of live *Peromyscus maniculatus* and *P. leucopus* from central and northern Wisconsin, 2010

	<i>P. maniculatus</i> (n = 84)*			<i>P. leucopus</i> (n = 293)*			<i>t</i>	<i>df</i>	P-value
	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range			
Tail	87.1	6.8	66–103	74.8	5.8	56–92	-16.35	373	<0.001
Hindfoot	20.6	0.7	18–22	20.3	0.8	18–22	-2.74	375	0.006
Ear	17.9	1.0	16–20	14.4	0.8	12–17	-32.47	375	<0.001
Weight	17.4	3.9	7–27	19.8	4.7	9–33	4.70	375	<0.001

\* *Peromyscus maniculatus* and *P. leucopus* had sample sizes 292 and 83, respectively, for the tail measurement because of exclusion of tail length for two individuals with docked tails

coefficient was used to test for collinearity of variables. Discriminant function analysis (DFA) was used to develop equations based on measurements that could be used in distinguishing between the two species. We created four *a priori* functions that included the following measurements: (1) ear length, (2) ear and tail length, (3) ear length and weight, (4) ear length, tail length, and weight. We did not use hindfoot length in any function given its high rate of intraspecific variation and interspecific overlap (see Results). We tested for homogeneity of variance-covariance using Box's M statistic and used Wilk's  $\lambda$  to test if group means differed between species with  $\alpha = 0.05$  (McGarigal *et al.*, 2000). To validate the accuracy of functions we used leave-one-out classification, in which each case was independently left out and was classified by the functions derived from all other cases. All statistical analyses were performed in PSAW Statistics version 18 (IBM SPSS Statistics, Chicago, Illinois).

## RESULTS

We collected live animal measurements and tissue from 381 individuals. Based on RFLP of *cytochrome b*, 84 individuals were identified as *P. maniculatus*, 293 as *P. leucopus*, and four as unknowns. *Peromyscus maniculatus* and *P. leucopus*, respectively, were captured in the following Wisconsin counties: Burnett (14, 68), Douglas (2, 2), Oconto (36, 120), Portage/Waupaca (0, 77), and Taylor (31, 22). Compared to open habitats, the majority of both *P. maniculatus* and *P. leucopus* were captured in forested habitats, 100% and 85% respectively. All external measurements were significantly different between *P. maniculatus gracilis* and *P. leucopus noveboracensis*, with *P. maniculatus* having a longer tail, ear, and hindfoot length but lower body weight than *P. leucopus* (Table 1). We noted a high degree of intraspecific variation for both species and no single univariate measurement unambiguously differentiated between the species (Table 1). Hindfoot length had the highest degree of overlap with both species, having a range from 18 to 22 mm. Ear length proved to be the most useful univariate measurement for differentiating the species, with all but one *P. leucopus* having ear lengths <17 mm and all but seven *P. maniculatus* having ear lengths  $\geq 17$  mm. Six of the seven *P. maniculatus* with ear lengths <17 mm had weights  $\leq 13$  g and one was only 7 g, suggesting that nearly all of these animals were juveniles (Dice and Bradley, 1942).

Based on histograms and residual assessment, all measurements were relatively normally distributed. None of the variables was highly correlated ( $r < 0.70$ ). The Box's M statistic indicated that homogeneity of variance-covariance matrices were not met for functions one, three, or four (Box's M = 4.442, P = 0.035; Box's M = 16.614, P = 0.001; Box's M = 28.019, P < 0.001, respectively) but was met for function two (Box's M = 7.474, P = 0.06). We

TABLE 2.—Standardized and unstandardized coefficients calculated from discriminant function analyses of external measurements from live *Peromyscus leucopus* and *P. maniculatus* in central and northern Wisconsin, 2010. Equations based on unstandardized coefficients;  $P. leucopus < 0 < P. maniculatus$  †

Variables	Function 1	Function 2	Function 3	Function 4
Unstandardized coefficients				
Ear	1.177	1.099	1.267	1.040
Tail		0.250		0.090
Weight			-0.529	-0.172
Constant	-20.008	-20.902		-22.333
Standardized coefficients				
Ear	1.000	0.933	1.076	0.883
Tail		0.151		0.543
Weight			-0.529	-0.775

\* Cutoffs for unstandardized coefficients of functions 1–4 were standardized to 0 by subtracting 2.110, 2.255, 2.553, and 2.819 respectively, from constants

† Equation for function 2 =  $-20.902 + (1.099)(\text{ear length}) + 0.250(\text{tail length})$

Equation for function 3 =  $-19.572 + (1.267)(\text{ear length}) - 0.117(\text{weight})$

Equation for function 4 =  $-22.333 + (1.040)(\text{ear length}) + (0.090)(\text{tail length}) - (0.172)(\text{weight})$

re-ran functions one, three, and four using separate covariance matrices and withheld 20% for cross-validation with nearly identical results. The Box's M test is extremely sensitive and based on our large sample size homogeneity of variance is likely not an issue (Tabachnick and Fidell, 2007).

Results of discriminant function analysis showed that all functions performed well and ear length was the best predictor of species identity as indicated by the standardized canonical discriminant function coefficients (Table 2). Function one, using only ear length, correctly classified 97.9% of individuals and had 97.7% accuracy based on cross-validation (eigenvalue of 2.81, canonical correlation of 0.86, Wilk's  $\lambda$  was 0.262, and  $P < 0.001$ ). This function misclassified the aforementioned one *P. leucopus* and seven *P. maniculatus*. Function two consisted of ear length and tail length and had an eigenvalue of 2.84, canonical correlation of 0.86, Wilk's  $\lambda$  of 0.261, and  $P < 0.001$ . However, although this function classified 97.9%, it achieved no additional classification accuracy above using ear length alone. Function three used ear length and weight and had an eigenvalue of 3.69, canonical correlation of 0.89, Wilk's  $\lambda$  of 0.213, and  $P < 0.001$ . This function correctly classified 99.2% of individuals and cross validation showed that two *P. leucopus* and one *P. maniculatus* were misclassified. Function four, comprised of ear length, tail length, and weight had an eigenvalue of 4.43, canonical correlation of 0.90, Wilk's  $\lambda$  of 0.184, and  $P < 0.001$ . This function achieved a classification accuracy of 99.7% and 99.5% cross validation accuracy. Cross validation showed that two *P. leucopus* were classified incorrectly and all *P. maniculatus* were correctly classified.

## DISCUSSION

Although all univariate measurements were statistically different between *P. maniculatus gracilis* and *P. leucopus noveboracensis*, none completely separated the species. Range of hindfoot length was identical in both species and tail length and weight overlapped substantially. Finnell (2000) noted significant differences in tail length, hindfoot length,

and ear length in *Peromyscus* from northern Wisconsin and Upper Michigan, but also with considerable overlap. Of these three measures, hindfoot length showed the greatest overlap (Finnell, 2000). Despite slight overlap of ear length measurements, we found that it could distinguish 97.9% of *Peromyscus* samples. Long and Long (1993) also found that ear length was the best distinguishing measurement. Ear length was better at distinguishing *P. leucopus*, but misclassified several young *P. maniculatus*. Nevertheless, it is interesting to note that ears from young *P. maniculatus*, even those of 7 g, were well developed, demonstrating that this measurement may be useful on animals shortly after weaning. Dice and Bradley (1942) noted that in *P. maniculatus* growth of the ear began to plateau even before weaning occurred. Thus, using ear length alone may be useful for separating most *P. leucopus* from *P. maniculatus*, particularly adults; animals with ears <17 mm are likely *P. leucopus* and animals with an ear  $\geq$ 17 mm are likely *P. maniculatus*. Although Long and Long (1993) suggested that the ear length measurement is often "slightly made in error," we feel trained persons can accurately measure ear length on live mice. Nonetheless, we caution against use of measurements from naïve field technicians, as previous research suggests that inexperience can lead to greater variation for this measurement than would normally be expected (Lindquist *et al.*, 2003).

Many DFA studies exclude juvenile animals from analysis (Feldhamer *et al.*, 1983; Long and Long, 1993; Kamler *et al.*, 1998); however, we concur with others (Rich *et al.*, 1996; Bruseo *et al.*, 1999; Lindquist *et al.*, 2003) that creating a function for all age and sex classes is much more practical for field applications. We also included weight in functions three and four. Moreover, some females were pregnant or lactating at the time measurements were taken, yet these individuals were still classified correctly. Thus, we feel our sample reflects actual demographics of *Peromyscus* populations encountered in northern Wisconsin during the summer.

Function two, using ear and tail length, gave no additional classification accuracy above that of using ear length alone. Mice are often captured with tails docked from the trap or other sources, further limiting use of this function for distinguishing individuals with damaged tails. Function three used ear length and body weight and differentiated 99.2% of individuals. Adding weight to the analysis helped separate young *P. maniculatus* with ear lengths of 16 mm. Function four used tail length, ear length, and weight and had the highest cross validation accuracy of 99.5%. Bruseo *et al.* (1999) identified the same measurements in their best DFA models for distinguishing between these species. Long and Long (1993) also found ear and tail to be the most useful external measurements, although weights were not available. Based on the slight increase in classification accuracy (0.3%) by adding tail length to the function, it is likely that ear length and weight produce an adequate degree of classification accuracy. Equations derived from the unstandardized coefficient from Table 2 can be used to distinguish animals captured and measured in the field.

With recent documentation that *P. leucopus noveboracensis* is advancing northward and *P. maniculatus gracilis* is declining in the Great Lakes region (Long, 1996; Myer *et al.*, 2009), the need to determine the current distribution of these species is becoming more important. Small mammal trapping, especially over extended temporal periods, is necessary to help elucidate the spatial extent of this phenomenon and its potential implications at a community scale. Thus, it is imperative that field researchers studying small mammals distinguish between *P. leucopus noveboracensis* and *P. maniculatus gracilis*. However, because research budgets are often small and genetic testing for large samples is costly, we believe that external morphological measurements from live animals offer a satisfactory alternative

for identifying live *Peromyscus* in the Great Lakes region. Additionally, even if electrophoresis of salivary amylase or genetic testing will be used to ultimately confirm species identification, the few minutes required to take external measurements will provide a substantial failsafe in the event that tissue samples are lost or degraded and fail to produce unequivocal identification.

*Acknowledgments.*—This study was funded by the Wisconsin Department of Natural Resources (Bureau of Endangered Resources), Prairie Biotic Research, Inc., and the University of Wisconsin – Stevens Point, Wisconsin. Particular thanks go to L. Ayers, who helped procure funding and supplied trapping and field equipment. This manuscript was improved by comments from three anonymous reviewers.

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SUBMITTED 13 NOVEMBER 2012

ACCEPTED 11 JULY 2013