TECHNICAL COMMENT

Tissue Damage Precludes the Use of the Everted Sleeve Technique to Measure Nutrient Uptake in a Small Migratory Shorebird, the Western Sandpiper (*Calidris mauri*)

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Introduction

In vitro measurement of nutrient (e.g., glucose and proline) uptake rates across the brush border of the small intestine has played an integral role in the current understanding of the adaptive responses of the digestive system to artificial selection for rapid growth in domestic poultry (*Gallus gallus*; Obst and Diamond 1992; Jackson and Diamond 1996) and the energetic demands of provisioning young (Dykstra and Karasov 1992; Konarzewski and Diamond 1994; Hammond et al. 1996). In 1983, Karasov and Diamond introduced a simplified methodology for making in vitro measurements of nutrient uptake across the brush border of the small intestine, called the everted sleeve technique. Accurate assessment of modulation of mediated uptake with the everted sleeve technique requires measurement of (1) passive uptake and (2) the sum of passive and mediated (combined) uptake across intact mucosal epithelium with cytologically intact enterocytes (Starck et al. 2000). Recently, Starck et al. (2000) demonstrated that extensive tissue damage may occur due to eversion and, subsequently, to stirring during incubation with the everted sleeve technique. They recommended that histological evaluations of tissue damage be conducted to assess the reliability of uptake measurements when this technique is applied to a new species or life stage.

The majority of the studies that have examined changes in digestive performance, that is, modulation of mediated nutrient uptake rates, of small endotherms (mainly rodents and birds)

using the everted sleeve technique have done so with animals maintained in captivity on a relatively long-term basis. In captivity, environmental variability can be controlled and treatments can be applied uniformly to experimental groups; this circumstance offers an effective means of demonstrating causal relationships and the limits of digestive performance (Dykstra and Karasov 1992; Caveides-Vidal and Karasov 1996; Hammond et al. 1996; McWilliams et al. 1999; Kristan and Hammond 2000). However, for birds and mammals it may not be possible to extrapolate accurately from results obtained from captive individuals to those expected from free-living individuals (Starck et al. 2000). Captive conditions can result in changes in the absolute size of digestive organs, which may be the indirect result of a change in body composition, specifically lean mass, due to changes in diet composition and activity patterns associated with captivity (Brugger 1991).

This study was designed to examine the functional significance of an age-related difference in small intestine size in a long-distance migrant shorebird, the western sandpiper (*Calidris mauri*). Guglielmo and Williams (2003) reported that during the first southward migration juvenile western sandpipers have the heaviest small intestine, relative to body mass, of their entire life. This age-related difference in small intestine size reflects a difference in intestine length, which appears to be related to growth, at least initially (Stein 2002, p. 114). We attempted to validate the everted sleeve technique for use in western sandpipers, both recently captured juvenile migrants and long-term captive adults. In the fall of 1999, we conducted experiments to validate the use of the everted sleeve technique to measure nutrient uptake in vitro, as described in Karasov and Diamond (1983), for this migratory shorebird. In 2000, it came to our attention that a histological evaluation of tissue integrity was necessary to ensure reliability of measurements made with this technique (Starck et al. 2000). Therefore, in 2000 we conducted histological evaluations of tissue sections before, during, and after the use of this assay to measure nutrient uptake for migrants and captives. Here, we assess the reliability of mediated proline uptake measurements from recently captured migrants, describe changes in the morphometry of the small intestine associated with acclimation to captivity, and evaluate tissue damage associated with the everted sleeve

technique when it was applied to recently captured migrants and long-term captives.

Material and Methods

During the fall of 1999 and the spring and fall of 2000, sandpipers were collected while refueling on migration at Boundary Bay, British Columbia (49°10′N, 123°05′W), the first major stopover site south of the breeding grounds. To minimize the number of individuals collected, females were sampled exclusively. In 1999, seven adults (July 10–13) and 13 juveniles (August 8–27) were collected during fall migration for use in nutrient uptake experiments. In 2000, four adults (May 1–9) were brought into captivity during spring migration and four juveniles (August 9–27) were collected during fall migration to assess tissue damage associated with the everted sleeve technique. Sandpipers were captured with mist nets (1.25-inch mesh, Avinet, Dryden, N.Y.) and collected in accordance with permits from Environment Canada. Animal handling protocols were approved by the Simon Fraser University Animal Care Committee (permit 529B) and conformed to the Canadian Committee for Animal Care Guidelines.

Immediately after capture, each bird was weighed (capture mass, ± 0.001 g) and culmen length was measured to determine gender. By the time the juveniles arrive at Boundary Bay during southward migration, that is, the beginning of August, they have completed structural growth (Guglielmo and Williams 2003); therefore, birds with a culmen length greater than 24.8 mm were considered to be females (Page and Fearis 1974). Birds were transported to Simon Fraser University within 2 h of capture, where dissections were conducted in accordance with nutrient uptake experiments. During the spring of 2000, adult females were brought into captivity and maintained in a large outdoor aviary. These birds were exposed to the natural light cycle and were fed a diet of Clarke's dry trout chow, which was available ad lib. The birds were able to fly in the aviary and had constant access to fresh running water. Captives were maintained on this diet for 40 d before conducting experiments to assess tissue damage.

Nutrient Uptake Measurements

Immediately before dissection, culmen and tarsus were measured with digital calipers (± 0.01 mm), and each bird was weighed (dissection mass, ± 0.001 g). Birds were killed with an intramuscular injection (4.0 mL 25 g^{-1}) of a 1 : 1 mixture of ketamine hydrochloride (100 mg mL^{-1}) and xylazine (20 mg mL^{-1}), exsanguinated, and dissected immediately thereafter. The small intestine was separated from the gizzard at the pylorus and from the large intestine immediately proximal to the cecae. After removal, the small intestine was rinsed in ice-cold avian Ringer's (composition in mM: 50 mannitol, 136 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 20 $NaHCO_3$

oxygenated with 95% O_2 and 5% CO_2; osmolality was 350 mOsm kg^{-1} H_2O). The proximal end of the small intestine was sutured to a gavauge needle, and its contents were gently purged with ice-cold avian Ringer's. Adherent fat and mesenteries were removed, and the length of the small intestine was measured using a modified version of the Brambell method (Freehling and Moore 1987). The small intestine was suspended vertically beside a ruler (± 0.5 mm) with the gavauge needle attached to the proximal end. The weight of the gavauge needle (2.2 g) straightened but did not stretch the small intestine. Subsequently, the small intestine was blotted dry and weighed (± 0.001 g). At the end of each dissection the gender was verified, the keel was measured with digital calipers (± 0.01 mm), and the carcass was stored at $-20°C$.

We measured passive and combined (i.e., mediated plus passive) uptake of L-[^{14}C(U)] proline (Amersham) across the brush border membrane of everted sections of small intestine in vitro, as described by Karasov and Diamond (1983). The small intestine was everted so that the mucosa faced outward. A mucous-like material was extruded from the small intestine during this procedure. Next, 1-cm sleeves of everted small intestine were preincubated at 40°C in avian Ringer's for 5 min. Incubation proceeded for 2 min in a proline solution that was stirring at 1,200 rpm. To allow for correction of unabsorbed L-proline in adherent fluid, the incubation solution also contained tracer concentrations of a membrane impermeable marker, inulin ([^{3}H] inulin, Amersham). In other avian species, 2 min has been sufficient to allow adherent preincubation solution to equilibrate with the labeled marker in the incubation solution. In addition, uptake rates have remained linear during this time period, ensuring measurement of unidirectional flux of proline (Karasov and Levey 1990; Levey and Karasov 1992). After incubation, tissues were blotted to remove adherent incubation solution, weighed, dissolved with tissue solubilizer (TS-2, Research Products International), and counted in scintillation cocktail (Hionicflour, Packard) in a Beckman LS 6500 liquid scintillation spectrometer to obtain disintegrations per minute. Counts for each isotope were corrected for quench and for counts due to the other isotope appearing in each counting channel.

To determine the optimal concentration of proline for incubation, it is necessary to determine the concentration of proline that saturates the amino acid transporters (Karasov and Diamond 1983). To determine the proline concentration that saturates the amino acid transporters for proline, intestinal sleeves from 20 western sandpipers were incubated in solutions (350 mOsm) containing 1, 5, 10, 25, and 50 mM L-proline and a trace amount of L-[^{14}C(U)] proline. Mediated L-proline uptake is Na^+ dependent. Therefore, to measure passive uptake of L-proline, Na^+ must be excluded from the incubation solution; this was accomplished by isosmotic replacement of Na^+ with choline. Sleeves were prepared from the entire length of the small intestine, except for the first and last 2 cm. After

correcting for unabsorbed L-proline, passive and combined uptake values were normalized to intestinal sleeve mass (pmol mg^{-1} min^{-1}) and were expressed relative to absolute combined uptake at 50 mM in the same intestinal region of the same individual. Using relative uptake minimizes the effect of positional and interindividual variation (Karasov and Diamond 1983). The apparent passive permeability coefficient of L-proline was determined by simple linear regression of relative passive uptake on L-proline concentration:

$$\text{Relative passive uptake} = (A \times [P]).$$

The apparent passive permeability coefficient of proline is estimated by A, while $[P]$ is the proline concentration (mmol) of the incubation solution. The analysis of relative combined uptake provides a second estimate of the apparent passive permeability coefficient of L-proline. The apparent carrier-mediated components of relative L-proline uptake were determined by nonlinear regression of relative combined uptake on L-proline concentration. The model for relative combined uptake incorporates passive and mediated (one carrier) uptake:

$$\text{Relative combined uptake} =$$
$$(A \times [P]) + [(J_{max} \times [P]) \times (K_m \times [P])^{-1}].$$

The apparent maximal mediated uptake rate was estimated by J_{max}. The apparent Michaelis constant, that is, the proline concentration at which uptake is $0.5 \times$ apparent J_{max}, was estimated by K_m, which is uncorrected for the effects of unstirred layers.

Histology and Morphometry

To obtain descriptive data on the internal morphology of the small intestine, histological sections were prepared from the proximal and distal duodenum, jejunum, and proximal and distal ileum of recently captured juvenile migrants ($n = 4$) and long-term captive adults ($n = 4$). To evaluate the effects of tissue handling during nutrient uptake experiments, histological sections were prepared from the jejunum (1) before eversion, (2) after eversion but before incubation, and (3) after eversion, preincubation for 5 min, and incubation for 2 min in a proline solution that was being stirred at 1,200 rpm. As before, a mucous-like material was extruded from the small intestine during eversion. Tissue sections were fixed in 10% formalin in 0.1 M phosphate-buffered saline, pH 7.4, for 48–72 h at room temperature. Fixed tissue sections were divided into subsections with a razor blade. Tissue subsections were dehydrated in 70% ethanol and 30% xylene, followed by 100% xylene, and then embedded in paraffin. Cross sections of the tissue subsections were cut at 5 μm on a rotary paraffin microtome, mounted onto microscope slides, and stained with hematoxylin and eosin.

Digital images of individual cross sections were obtained at four-power magnification with an Olympus Vanox microscope connected to a computer. Images were analyzed with a PC-based image analysis software (Empix Imaging 2001). Before analysis, digital images were converted to 8 bit gray scale, which allows contrast manipulation. For each section, total cross-sectional area, inner cross-sectional area (determined by the distinction between the mucosa and smooth muscle [*tunica muscularis*]), circumference, villus length (broken and intact villi were measured separately), and villi number were measured. These measurements were made on digitized images of eight to 10 cross sections from three to four tissue subsections for each tissue section. The thickness of the smooth muscle layer was not uniform. In order to determine the mean thickness of the muscle layer, the circular shape of the intestinal cross sections was used to calculate the lengths of the radii of circles with areas equal to the total cross sectional and inner cross-sectional areas. The difference between these calculated radii was reported as the mean muscle thickness. Lumen area of intact cross sections and mucosal area of everted sections were obtained by adjusting the contrast of the digital images and then employing threshold analysis. Threshold analysis calculates the area within a defined polygon and partitions this area based on pixel contrast. Subsequently, the percentages of smooth muscle, mucosa, and lumen were calculated based on cross-sectional area. To avoid pseudoreplication, statistical analyses were performed on the mean values for a single tissue section.

Data Analysis

As a measure of structural body size, we used the first component (PC1) from a single principal components analysis (PCA) of culmen, tarsus, and keel length (Rising and Somers 1989) performed on all of the birds included in this study. PC1 explained 57% of the variation in the univariate measures of structural body size, which all had large positive loadings (culmen $= 0.60$, tarsus $= 0.64$, and keel $= 0.49$) on PC1 (eigenvalue $= 1.71$). Within each age class, the two sample t-test was used to detect annual differences in body size (univariate: culmen, tarsus, and keel; multivariate: PC1) and body mass (capture and dissection). ANCOVA was used to examine the influence of year and acclimation to captivity on small intestine length and mass. The time that elapsed between capture and dissection was used as a covariate in the analysis of intestine length, because intestine length is known to increase after feeding (Robel et al. 1990); it is expected to contract while fasting. Small intestine length was used as a covariate in analysis of small intestine mass; this resulted in an analysis of length-corrected mass. Relationships between variables were examined using linear and nonlinear regression. Repeated-measures ANOVA was used to examine changes in body mass of captive sandpipers, variation in morphometry along the length of the
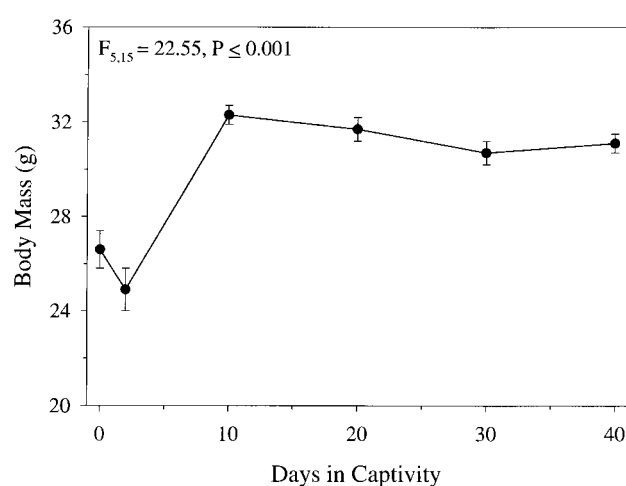
Figure 1. Changes in the body mass of female western sandpipers during acclimation to captivity ($n = 4$). Values are means $\pm$ SEM.

intestine, and damage due to tissue eversion and subsequent incubation. Where possible, the significance of interactions was examined. In univariate analyses, each dependent variable was considered separately, and the overall experiment-wise error rate was controlled by Bonferroni correction ($\alpha = 0.05$). Statistical analyses were conducted in SAS (SAS Institute 1990). The nonlinear regression component of Sigma Plot was used to fit the kinetics model to the combined uptake data (SPSS Science 1999).

Results

Variation in Body Size, Body Mass, and Small Intestine Size

Annual variation in body size, body mass, and small intestine size was examined separately for the two age classes. For juveniles, culmen, tarsus, and keel lengths were independent of year ($P > 0.05$ in all cases), as was structural size (PC1, $t_{15} =$

1.33, $P > 0.2$). At capture, juveniles caught in 1999 were 12% heavier than those caught in 2000 ($t_{15} = 2.45$, $P \leq 0.05$). At dissection, however, body mass was independent of year for juveniles ($t_{15} = 1.44$, $P > 0.17$). Small intestine length was independent of year for juveniles ($F_{1,14} = 0.79$, $P > 0.4$), as was the length-corrected mass of the small intestine ($F_{1,14} = 0.47$, $P > 0.5$). Similarly, for the adults, culmen, tarsus, and keel lengths were independent of year ($P > 0.05$ in all cases), as was structural size (PC1, $t_9 = 0.36$, $P > 0.7$). At capture, body mass was independent of year for adults ($t_9 = 0.61$, $P > 0.6$). At dissection, however, long-term captive adults were 27% heavier than recently captured adult migrants ($t_9 = 4.61$, $P \leq 0.001$). After adult migrants were brought into captivity in the spring of 2000, their body mass increased 12%, reaching a plateau by day 10 ($F_{5,15} = 22.55$, $P \leq 0.001$; Fig. 1). Small intestine length did not vary between adult migrants and captives ($F_{1,8} = 0.23$, $P > 0.7$; Table 1); however, the length-corrected mass of the small intestine mass was 22% lower in captive adults ($F_{1,8} = 9.34$, $P \leq 0.025$).

Uptake Measurements

To determine the proline concentration that saturates the amino acid transporters, which actively transport proline across the brush border membrane, nutrient uptake experiments were conducted on 20 recently captured refueling female western sandpipers ($n = 13$ juveniles and $n = 7$ adults). If measurements are made on intact tissue, then the proline concentration that saturates the available transporters should be apparent graphically as a plateau in the expected hyperbolic relationship between relative L-proline uptake and L-proline concentration. However, we observed a near linear fit with the kinetics model for combined uptake, and the relationship between combined uptake and proline concentration closely approximated the linear relationship between passive uptake and proline concentration (Fig. 2). The relative passive uptake model yielded an apparent passive per-

Table 1: Body mass and small intestine size of recently captured migrant and long-term captive female western sandpipers ($n = 28$) used in uptake experiments and histological studies

Parameter	Juveniles		Adults	
	1999 Migrants' Uptake	2000 Migrants' Histology	1999 Migrants' Uptake	2000 Captives' Histology
Capture mass (g)	29.1 $\pm$ .8[A]	25.4 $\pm$ .3[B]	25.8 $\pm$ 1.0[A]	26.6 $\pm$ .8[A]
Dissection mass (g)	26.2 $\pm$ .7[A]	24.4 $\pm$ .5[A]	23.4 $\pm$ 1.0[A]	29.8 $\pm$ .5[B]
Small intestine (cm)[a]	22.1 $\pm$ .4[A]	23.1 $\pm$ .6[A]	20.7 $\pm$ .5[A]	20.3 $\pm$ .7[A]
Small intestine (g)[b]	1.28 $\pm$ .06[A]	1.18 $\pm$ .12[A]	.97 $\pm$ .04[A]	.76 $\pm$ .06[B]
n	13	4	7	4

Note. Comparisons were made within age classes, and values are means $\pm$ SEM. Different superscripts or subscripts indicate statistically significant differences, $P < 0.05$.

[a] Least squares means, controlling for time to dissection.

[b] Least squares means, controlling for small intestine length, that is, length-corrected mass.
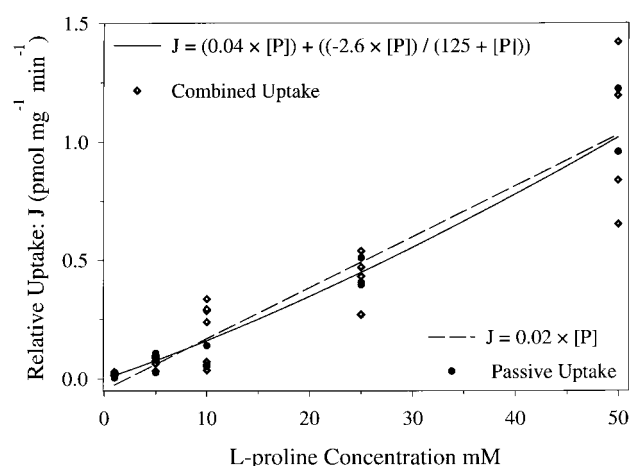
Figure 2. Relative combined (mediated plus passive) and passive proline uptake in the small intestine of recently captured migrant western sandpipers. Measurements were made in vitro, using the everted sleeve technique. [P] is proline concentration.

meability coefficient of 0.02 pmol proline × mg tissue^{-1} × proline concentration^{-1} ($F_{1,16} = 17.66$, $P < 0.001$, $r^2 = 0.95$), while the relative combined uptake model yielded an apparent passive permeability coefficient of 0.04 pmol proline × mg tissue^{-1} × proline concentration^{-1}. The relative combined uptake model also yielded estimates of the apparent $J_{max} = -2.6$ pmoles min^{-1} mg tissue^{-1} and apparent $K_m = 125$ mM proline ($F_{2,24} = 66.60$, $P < 0.001$, $r^2 = 0.85$). Due to the unexpected linear relationship between relative combined uptake and proline concentration, a negative J_{max} parameter estimate, and a very large K_m parameter estimate, we pooled the relative passive and relative combined uptake data and fit the pooled data with the passive uptake model, including an interaction term. In this analysis, the interaction between relative uptake (passive vs. combined) and proline concentration was not significant ($F_{1,41} = 0.41$, $P > 0.5$).

Therefore, the interaction term was removed from the model, and this resulted in relative uptake = 0.02 × [P] ($F_{1,43} = 17.88$, $P < 0.001$, $r^2 = 0.88$). This result suggests either that passive proline uptake was predominant or that there was extensive tissue damage.

Small Intestine Morphometry

Variation in morphometry was examined along the length of the small intestine in recently captured juvenile migrants and long-term captive adult western sandpipers. Circumference decreased distally by 20% in migrants ($F_{4,12} = 9.36$, $P \leq 0.001$; Table 2) and by 26% in captives ($F_{4,12} = 20.71$, $P \leq 0.001$; Table 3). Smooth muscle thickness increased distally by 130% in migrants ($F_{4,12} = 15.76$, $P \leq 0.001$) and by 55% in captives ($F_{4,12} = 6.50$, $P \leq 0.01$). Villus length decreased distally by 37% in migrants ($F_{4,12} = 17.72$, $P \leq 0.001$) and by 36% in captives ($F_{4,12} = 16.98$, $P \leq 0.001$). Villi number was independent of intestinal subregion for migrants ($F_{4,12} = 1.52$, $P > 0.15$) and captives ($F_{4,12} = 2.08$, $P > 0.15$). The percentage of the cross-sectional area occupied by smooth muscle increased distally by 23% in migrants ($F_{4,12} = 33.10$, $P \leq 0.001$) and by 16% in captives ($F_{4,12} = 11.64$, $P \leq 0.001$). The percentage of the cross-sectional area occupied by mucosa decreased distally by 27% in migrants ($F_{4,12} = 21.60$, $P \leq 0.001$) and by 18% in captives ($F_{4,12} = 18.96$, $P \leq 0.001$). The percentage of the cross-sectional area occupied by lumen was independent of intestinal subregion for migrants ($F_{4,12} = 4.35$, $P > 0.02$) and captives ($F_{4,12} = 0.38$, $P > 0.8$).

Histological Evaluation of Tissue Damage

The histology of noneverted sections of jejunum was similar for recently captured migrants and long-term captives (Fig. 3A and 3B, respectively). The inner mucosal membrane was

Table 2: Small intestine morphometry of recently captured juvenile female western sandpipers ($n = 4$)

Morphometric Parameter	Proximal Duodenum	Distal Duodenum	Jejunum	Proximal Ileum	Distal Ileum
Linear measures:					
Circumference (mm)	7.3 ± .4[A]	7.0 ± .2[A]	6.9 ± .2[A]	5.7 ± .3[B]	5.8 ± .4[B]
Muscle width (μm)	80 ± 7[A]	105 ± 7[AB]	99 ± 10[A]	138 ± 14[B]	184 ± 19[B]
Villus length (μm)[a]	830 ± 60[A]	815 ± 42[A]	640 ± 46[AB]	543 ± 30[B]	519 ± 28[B]
Villi number	29 ± .4[A]	29 ± .4[A]	27 ± 1.2[A]	27 ± .6[A]	27 ± 1.0[A]
Area percentages:					
Muscle	13 ± 1[A]	18 ± 1[A]	17 ± 2[A]	28 ± 2[B]	36 ± 3[B]
Mucosa	77 ± 1[A]	74 ± 2[AB]	63 ± 4[B]	58 ± 5[BC]	50 ± 2[C]
Lumen	10 ± 1[A]	8 ± 1[A]	20 ± 3[B]	14 ± 4[AB]	14 ± 3[AB]
n	4	4	4	4	4

Note. Values are means ± SEM. Different superscripts indicate statistically significant differences, $P < 0.05$.

[a] Intact villus length.

Table 3: Small intestine morphometry of long-term captive adult female western sandpipers ($n = 4$)

Morphometric Parameter	Proximal Duodenum	Distal Duodenum	Jejunum	Proximal Ileum	Distal Ileum
Linear measures:					
Circumference (mm)	6.0 ± .2[A]	5.9 ± .2[A]	5.0 ± .1[B]	4.6 ± .2[BC]	4.4 ± .0[C]
Muscle width (μm)	80 ± 2[A]	81 ± 3[A]	90 ± 4[AB]	112 ± 10[B]	124 ± 16[AB]
Villus length (μm)[a]	692 ± 51[A]	656 ± 51[A]	521 ± 59[AB]	436 ± 25[B]	441 ± 15[B]
Villi number	30 ± .9[A]	30 ± 1.0[A]	29 ± .2[A]	28 ± .7[A]	31 ± .5[A]
Area percentages:					
Muscle	16 ± 1[A]	17 ± 1[A]	22 ± 2[B]	28 ± 2[C]	32 ± 4[C]
Mucosa	70 ± 1[A]	66 ± 2[A]	62 ± 3[B]	56 ± 3[B]	52 ± 3[B]
Lumen	14 ± 1[A]	17 ± 1[A]	16 ± 2[A]	16 ± 2[A]	16 ± 3[AB]
n	4	4	4	4	4

Note. Values are means ± SEM. Different superscripts indicate statistically significant differences, $P < 0.05$.

[a] Intact villus length.

formed by long, delicate villi. The connective tissue core (*lamina propria mucosae*) of the villi was characterized by the presence of smooth muscle cells and small blood vessels. The mucosal epithelium (*lamina epethelialis mucosae*) was composed primarily of enterocytes with few goblet cells (characterized by large vesicles). Crypts of Lieberkhun opened at the base of the villi. The muscular wall (*tunica muscularis*) was quite thick; however, the *lamina muscularis mucosae* was not discernible with light microscopy.

We measured mucosal area, mean villus length (weighted average of intact and broken villi), and percentage of unbroken villi in sequential tissue sections from the jejunum before eversion, after eversion, and after eversion and incubation in a proline solution that was being stirred at 1,200 rpm. The normal morphometery of the small intestine of recently captured juvenile migrant and long-term captive adult western sandpipers predisposed them to tissue damage from the everted sleeve technique. The distance between opposing villi, that is, the lumen, in the jejunum was 25% and 20% of its diameter in migrants and captives, respectively. This allowed little room through which to evert the small intestine. Therefore, eversion of the small intestine resulted in pronounced effects on the
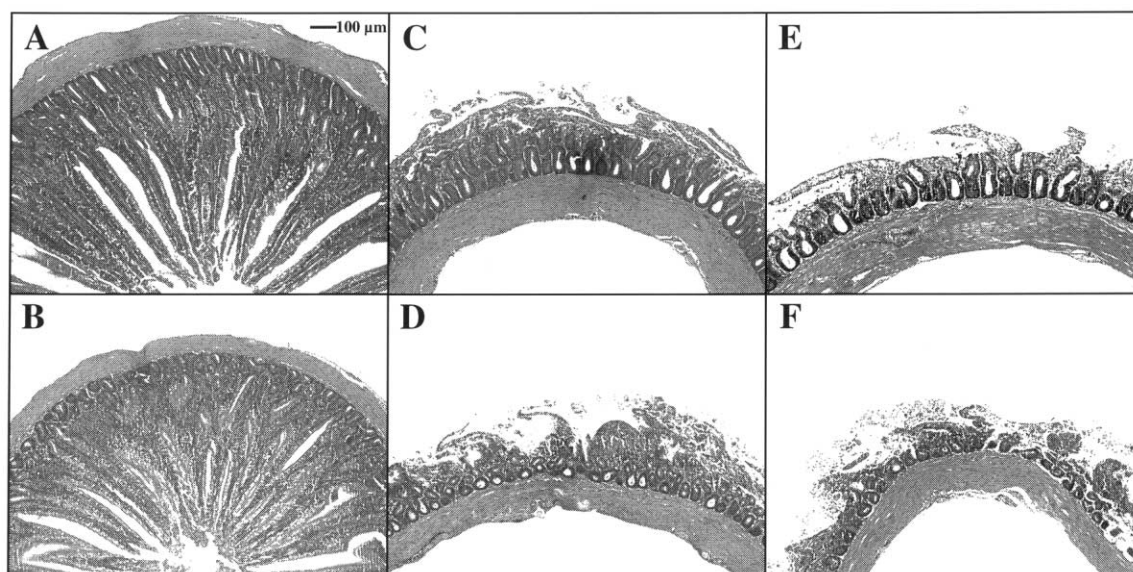

Figure 3. Histological cross sections of the jejunum of western sandpipers demonstrating damage due to eversion and subsequent incubation with the everted sleeve technique. *A, C,* and *E* are from recently captured juvenile migrants, while *B, D,* and *F* are from long-term captive adults. *A* and *B* depict intact tissue sections; *C* and *D* depict everted tissue sections; and *E* and *F* depict everted and subsequently incubated tissue sections.

histology of jejunal sections of migrants and captives (Fig. 3C and 3D, respectively). Many villi were missing or broken, and remaining villi often appeared as distorted structural artifacts. Following eversion, mucosal area decreased 67% in migrants ($F_{2,3} = 200.32$, $P \leq 0.001$; Table 4) and 62% in captives ($F_{2,3} = 93.47$, $P \leq 0.01$). Mean villus length decreased 44% in migrants ($F_{2,3} = 23.11$, $P \leq 0.01$) and 51% in captives ($F_{2,3} = 80.94$, $P \leq 0.01$). The percentage of broken villi increased three-fold in migrants ($F_{2,3} = 34.31$, $P < 0.01$) and in captives ($F_{2,3} = 939$, $P < 0.001$). After eversion, the mucosal epithelium was no longer intact, as the majority of the villi had sustained extensive damage. Incubation of everted tissue sections in a nutrient solution stirring at 1,200 rpm caused little additional damage (Fig. 3E and 3F, respectively), because the damage due to eversion was so extensive (Table 4).

Discussion

The results of this study are unambiguous. For western sandpipers, acclimation to captivity resulted in an increase in body mass and a reduction in the length-corrected mass of the small intestine. This decrease in small intestine mass was associated with a reduction in circumference and villus length; however, it was not due to a decrease in intestine length. There was extensive damage to the mucosa of jejunal sections of recently captured migrants and long-term captives due to everting the small intestine and subsequently incubating sections of it in a nutrient solution that was being stirred at 1,200 rpm, as required by the everted sleeve assay. In migrants, mucosal area decreased by 67%, mean villus length by 44%, and the percentage of unbroken villi increased threefold. Similarly, in captives, mucosal area decreased by 62%, mean villus length by 51%, and the percentage of broken villi also increased threefold. The majority of the damage occurred as a result of everting

the small intestine. Tissue damage resulted in unreliable measurements of combined and passive L-proline uptake in recently captured migrants. Consequently, we were not successful in validating the everted sleeve technique for use in the western sandpiper.

Captivity Effects

Captive conditions may result in a decrease in the size of the digestive organs due to concentrated diets and/or decreased activity (Brugger 1991). Adult western sandpipers became obese after 10 days in captivity on an energy-rich diet fed ad lib. When these birds were dissected after 40 d in captivity, there was no difference in the length of the small intestine relative to recently captured adult migrants; however, the length-corrected mass of the small intestine was 22% lower in the captive adults. This decrease in the length-corrected mass of the small intestine was associated with a reduction in circumference and also in villus length. Brugger (1991) demonstrated that captive red-winged blackbirds (*Agelaius phoeniceus*) fed energy-rich diets showed reductions in small intestine length, circumference, and villus length. Similarly, Piersma et al. (1993) observed reductions in gizzard size in captive red knots (*Calidris canutus*) that consumed soft energy-rich food. Therefore, acclimation to captivity can result in substantial changes in the absolute size of the digestive organs. This is an important consideration when making inferences from results obtained on captives to those expected from free-living individuals.

Tissue Damage and Uptake Measurements

Mediated uptake measurements produced by the everted sleeve method have been reproduced reliably in some species. We

Table 4: Assessment of tissue damage to jejunal sections of the small intestine of recently captured juvenile migrant ($n = 4$) and long-term captive adult ($n = 4$) female western sandpipers due to the everted sleeve technique

	Jejunal Sections		
	Control	Everted	Everted and Incubated
Recently captured juveniles:			
Mucosal area (10^6 μm^2)	2.4 ± .2[A]	1.0 ± .2[B]	.8 ± .1[B]
Villus length (μm)[a]	569 ± 60[A]	367 ± 11[B]	318 ± 11[B]
Unbroken villi (%)	74 ± 10[A]	18 ± 6[B]	8 ± 3[B]
n	4	4	4
Long-term captive adults:			
Mucosal area (10^6 μm^2)	1.3 ± .1[A]	.7 ± .1[B]	.5 ± .1[B]
Villus length (μm)[a]	452 ± 60[A]	288 ± 8[B]	223 ± 17[B]
Unbroken villi (%)	71 ± 7[A]	12 ± 3[B]	2 ± 1[B]
n	4	4	4

Note. Values are means ± SEM. Different superscripts indicate statistically significant differences, $P < 0.05$.
[a] Weighted mean of broken and unbroken villi.

attempted to validate the everted sleeve technique for use on a small shorebird that was actively refueling during migration and were unsuccessful. One essential condition for successful application of the everted sleeve technique in either circumstance is that there must be little or, ideally, no tissue damage due to eversion and incubation. However, we observed extensive damage to the villi of the jejunum in histological sections, and this damage resulted in unreliable mediated proline uptake measurements for this species. Starck et al. (2000) observed a similar extent of tissue damage in the sunbird (*Nectarinia osea*), which also resulted in unreliable uptake measurements. However, they demonstrated an absence of tissue damage due to everting the small intestines of domestic chickens and mice (*Mus domesticus*); therefore, they were successful in validating the everted sleeve technique for use in those two species. The villi of domestic chickens and mice appear to be relatively short and stout compared to those of sunbirds and sandpipers; this may be part of the reason that no tissue damage occurred in the two domestic species.

Starck et al. (2000) demonstrated that the long and delicate villi of sunbirds could not sustain the mechanical forces of tissue eversion. Western sandpipers also have long and thin villi, and they exhibited a pattern of damage similar to that in the sunbirds. However, there may be other aspects of intestinal morphometry that make particular species prone to damage during eversion. For instance, lumen diameter, that is, the distance between opposing villi, represents the "working room" through which the small intestine must be everted. In the western sandpiper, the diameter of the jejunal lumen is small relative to its outside diameter. In recently captured juvenile migrants, the diameter of the jejunal lumen represents 33% of the outside diameter of the jejunum, and in long-term captive adults it represents only 23%. This amount of working room suggests that in some cases severe damage is inevitable. Muscle thickness is another aspect of intestinal morphometry that could make some species prone to damage during eversion. A thick muscle layer makes the initial stage of eversion difficult, and, when this occurs, the intestine must be held steady to enable eversion. This could cause damage to the distal end, where eversion begins. Therefore, it is good practice not to use sleeves from near the ends of the intestine. In addition, a thick muscle layer becomes stiff in ice-cold avian Ringer's; this makes the natural curvature of the small intestine firm, which may lead to additional damage along the entire length of the small intestine during eversion.

We reiterate the cautionary statements of Starck et al. (2000): it is essential to conduct a histological evaluation to assess reliability of the everted sleeve technique before using it on a species or in a circumstance where such an evaluation has not been conducted. Although acclimation to captivity can cause marked changes in the size of the digestive tract, we observed similar patterns of tissue damage in long-term captive adult and recently captured juvenile sandpipers. We concur with Starck et al. (2000) that long delicate villi predispose some species to extensive damage during eversion and suggest that there are other aspects of intestinal morphometry (lumen diameter and smooth muscle thickness) that make tissue damage more likely. These factors should be considered in future studies using the everted sleeve technique.

Acknowledgments

Literature Cited

Brugger K.E. 1991. Anatomical adaptation of the gut to diet in red-winged blackbirds (*Agelaius phoeniceus*). Auk 108: 562–567.

Caveides-Vidal E. and W.H. Karasov. 1996. Glucose and amino acid absorption in the house sparrow intestine and its dietary modulation. Am J Physiol 271:R561–R568.

Dykstra C.R. and W.H. Karasov. 1992. Changes in gut structure and function of house wrens (*Troglodytes aedon*) in response to increased energy demands. Physiol Zool 65:422–442.

Empix Imaging. 2001. Northern eclipse 6.0. Empix Imaging, Mississauga, Ontario.

Freehling M.D. and J. Moore. 1987. A comparison of two techniques for measuring intestinal length. J Wildl Manage 51: 108–111.

Guglielmo C.G. and T.D. Williams. 2003. Phenotypic flexibility of body composition in relation to migratory state, age, and sex in the western sandpiper (*Calidris mauri*). Physiol Biochem Zool 76:84–98.

Hammond K.A., K.C. Kent Lloyd, and J. Diamond. 1996. Is mammary output capacity limiting to lactational performance in mice? J Exp Biol 199:337–349.

Jackson S. and J. Diamond. 1996. Metabolic and digestive responses to artificial selection in chickens. Evolution 50:1638–1650.

Karasov W.H. and J. Diamond. 1983. A simple method for measuring intestinal solute uptake in vitro. J Comp Physiol 152:105–116.

Karasov W.H. and D.J. Levey. 1990. Digestive system trade-offs and adaptations of frugivorous passerine birds. Physiol Zool 63:1248–1270.

Konarzewski M. and J. Diamond. 1994. Peak sustained meta-

bolic rate and its individual variation in cold-stressed mice. Physiol Zool 67:1186–1212.

Kristan D.M. and K.A. Hammond. 2000. Combined effects of cold exposure and sub-lethal intestinal parasites on host morphology and physiology. J Exp Biol 203:3495–3504.

Levey D.J. and W.H. Karasov. 1992. Digestive modulation in a seasonal frugivore, the american robin (*Turdus migratorius*). Am J Physiol 262:G711–G718.

McWilliams S.R., E. Caviedes-Vidal, and W.H. Karasov, 1999. Digestive adjustments in cedar waxwings to high feeding rates. J Exp Zool 283:394–407.

Obst B.S. and J. Diamond. 1992. Ontogenesis of intestinal nutrient transport in domestic chickens (*Gallus gallus*) and its relation to growth. Auk 109:451–464.

Page G. and B. Fearis. 1974. Sexing western sandpipers by bill length. Bird-Banding 42:297–298.

Piersma T., A. Koolhaas, and A. Dekinga. 1993. Interactions between stomach structure and diet choice in shorebirds. Auk 110:552–564.

Rising J.D. and K.M. Somers. 1989. The measurement of overall body size in birds. Auk 106:666–674.

Robel R.R., M.E. Barnes, K.E. Kemp, and J.L. Zimmerman. 1990. Influences of time postmortem, feeding activity, and diet on digestive organ morphology of two emberezids. Auk 107:669–677.

SAS Institute. 1990. SAS/STAT User's Guide. Release 6.03. SAS Institute, Cary, N.C.

SPSS Science. 1999. SigmaPlot 5.05. SPSS Science, Chicago.

Starck J.M., W.H. Karasov, and D. Afik. 2000. Intestinal nutrient uptake measurements and tissue damage: validating the everted sleeves method. Physiol Biochem Zool 73:454–460.

Stein R.W. 2002. Busting a gut: age-related variation and seasonal modulation of digestive tract structure and function in the western sandpiper. MS thesis. Simon Fraser University, Burnaby, British Columbia.