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Subtle pedipalp dimorphism: a reliable method for sexing juvenile spiders

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Abstract. Quantifying primary sex ratios is necessary for studies in a wide range of areas including adaptive sex ratio modification, population demography, and sex-biased developmental mortality. Adult and penultimate male spiders are easy to sex, due to the great thickening of the male pedipalps, which are used for delivering sperm to the female reproductive tract. However, in many spider species, males and females are apparently monomorphic at hatching, are difficult to rear, and cannibalize their siblings, making assessment of primary sex ratios problematic. One technique for sexing spiders is karyotyping, but this can be challenging and time-consuming, particularly for species with high fecundity, and often requires destructive sampling. Here we report that, although apparently monomorphic, early-instar juveniles of two species of black widow spiders (*Latrodectus hasselti* Thorell 1870 and *Latrodectus hesperus* Chamberlin & Ivie 1935) can be sexed reliably. Palp width measurements are significantly different for males and females at the 3rd instar, with the palpi of juvenile females thinner than those of males. Moreover, sex identification with 89–100% accuracy can be achieved by an experienced observer visually inspecting the palpi of 3rd instar spiderlings under a dissecting microscope. Our results suggest that minimal investment in a pilot study can yield an accurate method for sexing juvenile spiders in the laboratory or field. The suitability of this method should be examined in other species with apparently monomorphic spiderlings, particularly those in which adult males have significantly enlarged palpi.

Keywords: *Latrodectus*, black widow spiders, monomorphic spiders, sexual dimorphism, sex identification

Assessing primary sex ratios, or sex ratios of juveniles observed in the field, is desirable for study in a variety of areas (e.g., adaptive sex ratio modification, population phenology, population dynamics and development of sex-specific growth patterns or behavior). For example, adaptive sex ratio modification has been demonstrated in a variety of species (Austad & Sunquist 1986; Emlen 1997; Nager et al. 1999; Cameron et al. 1999; West & Sheldon 2002; West et al. 2002), including social spiders (e.g., Aviles & Maddison 1991). However in non-social species, there is continued speculation about the possible importance of sex ratio modification since this has only been demonstrated in one non-social species, *Pityohyphantes phrygianus* (C.L. Koch 1836) (Gunnarsson & Andersson 1992; Uhl & Gunnarsson 2001).

Primary sex ratios (ratio of male:female at hatching) are difficult to determine in many spiders in which males and females are apparently monomorphic at hatching (Foelix 1996). Karyotyping has been used to determine sex ratios of fertilized eggs (Aviles & Maddison 1991), and this eliminates the need to rear spiders to later instars. However, this method does not work for all species (Watt, Hasenkampf & Andrade, unpublished), may not be practical for field studies, requires destructive sampling of eggs, and may be prohibitively time-consuming for species with high fecundity. Rearing spiderlings to advanced instars or adulthood to assess sex is problematic because there may be variable success in rearing juveniles due to challenging diet requirements and sibling cannibalism. Even in species that show pronounced sexual dimorphism as adults, juveniles may be apparently monomorphic to the naked eye until later instars when male and female body shape or size diverge significantly and the male's palpi thicken dramatically in the penultimate instar (Kaston 1970; Mahmoudi, Jovovic, Andrade & Brandt, unpublished). Nonetheless, a number of authors have noted that the pedipalps of males thicken gradually over several instars prior to the penultimate instar (e.g., *Latrodectus* - Bhatnagar & Rempel 1962; *Cyrtophora* – Berry 1987).

Our study describes a method of assessing spiderling sex using the relative size of the palpi in early-instar spiders of two species of black widows (*Latrodectus hasselti* Thorell 1870 and *Latrodectus hesperus* Chamberlin & Ivie 1935). In this genus, males and females appear to be monomorphic at hatching, although they show extreme reversed sexual size dimorphism at adulthood (Kaston 1970). Initial studies of development in *L. hesperus* by one of us (Y. Brandt) suggested there might be differences in the dimensions of pedipalps of males and females very early in development. The objective of this study was to determine whether early sex differences in palp dimensions could provide a simple, non-destructive, morphology-based method for sexing spiderlings. We examined early-instar spiderlings of both species and predicted their sex based on visual inspection and measurement of pedipalp width. We then reared spiderlings to adulthood and report this method yields 94% accuracy or better in determining the sex of 3rd instar spiderlings.

METHODS

Spiders for this study were acquired from outbred populations of redback spiders (*Latrodectus hasselti*) and western black widow spiders (*L. hesperus*), that originated from field-mated, adult females collected in New South Wales, Australia (2002) and San Diego, California (2007), respectively. Voucher specimens have been deposited in the Entomology collection of the Royal Ontario Museum (*L. hasselti*: ROMEnt 112065 through 112067; *L. hesperus:* ROMEnt 112068 through 112070). Egg sacs (*L. hasselti*, $n = 4$ sacs; *L.*

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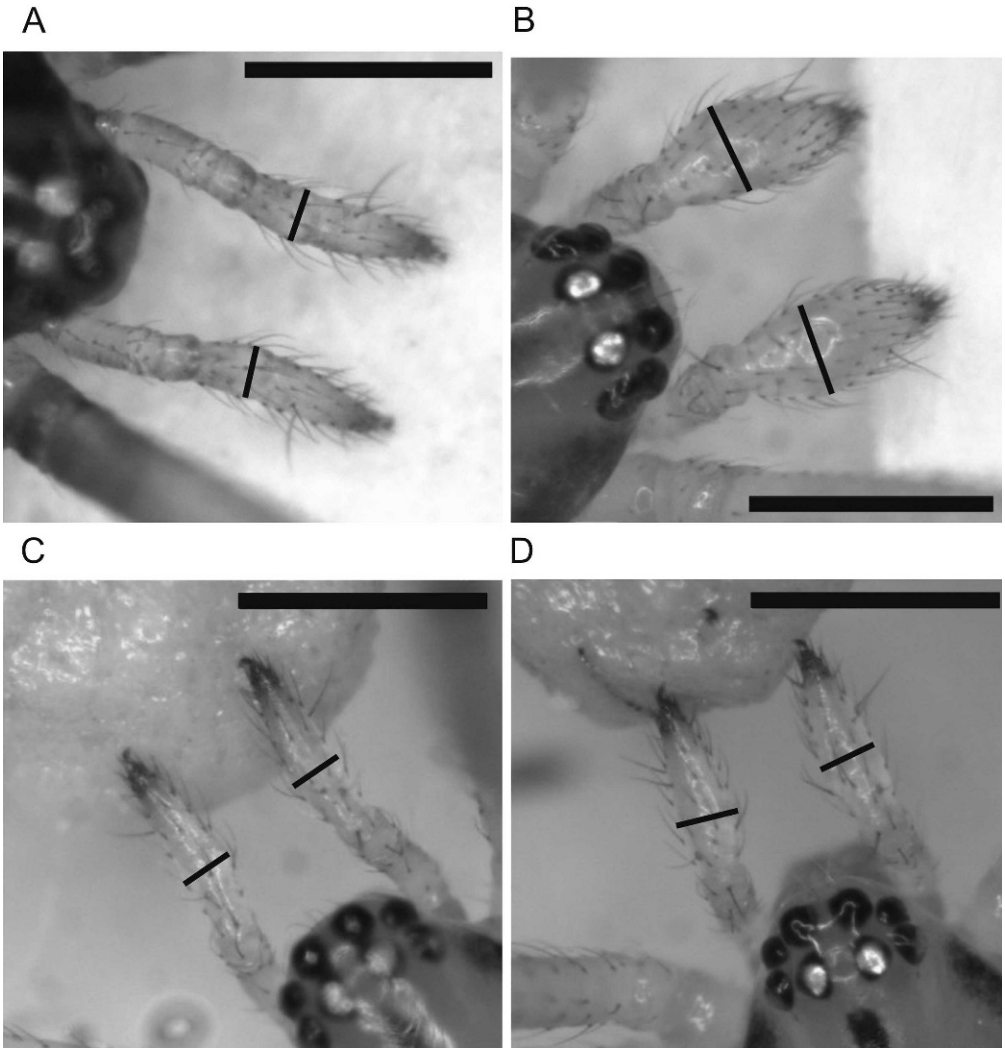

Figure 1.—Digital photographs of dorsal view of portion of the cephalothorax and pedipalps of spiderlings of *L. hasselti* (4th instar female [A] and male [B]) and *L. hesperus* (3rd instar female [C] and male [D]) showing location of measurement of palpal tibia width (black line). Scale bars are 0.50 mm.

hesperus, $n = 2$ sacs) were removed from females' cages shortly after production. Each egg sac was cut open, 40–75 unhatched eggs were randomly chosen and each egg was transferred into an individual clear plastic cage measuring 2.3 × 3.0 × 2.3 cm (Amac Plastic Products Ltd). Eggs and spiders were kept in a controlled-environment room at 25 ± 5° C, 12 hr light : 12 hr dark cycle throughout the study.

Cages were examined every other day, and hatch dates and molt dates were noted. Normally, 1st instar spiderlings remain inside the egg sac and survive on yolk reserves until they molt, emerge from the sac, and begin to take prey (Foelix 1996). Beginning at the 2nd instar, the spiders were fed 1–2 small, wingless fruit flies (*Drosophila melanogaster*) three times each week. The number of fruit flies each spider received was gradually increased as the spider developed. After each molt, approximately 2 more fruit flies were added to each feeding. By the 4th instar, both males and females began receiving larger, heavy-bodied fruit flies (*D. hydei*) three times each week.

Preliminary work suggested that pedipalps of juvenile males might be wider than those of juvenile females, particularly at the palpal tibia (Jovovic, Brandt & Andrade, unpublished; e.g., Fig. 1A, B). To test whether the appearance of the palps reliably indicated sex differences, an experienced observer examined the pedipalps of each spiderling under a dissecting microsope or examined photographs of the palpi and predicted its sex based on overall pedipalp girth (*L. hasselti*: N. Mahmoudi; *L. hesperus*: M. Modanu). To determine the accuracy of these predictions, all spiders were reared until the 5th (*L. hasselti*) or 6th instar (*L. hesperus*), at which time males and females can be reliably distinguished by the development of notable swellings of the pedipalp in penultimate instar males (Bhatnagar & Rempel 1962; Kaston 1970).

For the visual examination predictions, intact spiderlings were placed under a dissecting microscope (Zeiss Stemi 2000C) and examined, or photographs were taken of their palps (see below). Palps were scored categorically as thick (male-like) or thin (female-like). For *L. hasselti*, sex was determined from 1 ($n = 30$) or 3 ($n = 58$) independent viewings of individuals at the 3rd instar. For *L. hesperus*, there were 3 independent viewings of individuals at the 2nd instar, then 3 independent

Table 1.—Accuracy of sex identification based on visual examination of palpal tibia width of spiderlings for two species of *Latrodectus* spiders. *Null expectation = 50% accuracy; §two-tailed test, *df* = 1 with Yates' correction.

Species	Instar	# Correct	# Incorrect	% accuracy	χ^{2*}	$P^{\S}$
L. hesperus	2nd	83	52	61.5%	3.143	0.0762
L. hesperus	3rd	94	6	94.0%	45.858	< 0.0001
L. hasselti	3rd	78	10	88.6%	29.093	< 0.0001

viewings of photographs of the spider's palps at the 3rd instar. Spiderlings were predicted to be males or females as a function of the majority of the independent assessments.

We determined whether palp width was sexually dimorphic in juveniles by comparing palpal tibia widths. Palps were measured in photographs taken at the 3rd instar (*L. hesperus*) or 4th instar (*L. hasselti*) using a high-resolution digital camera (Nikon DXM 1200) attached to a dissecting microscope. Each spider was anaesthetized by brief exposure to CO_2 and then laid flat on the abdomen. A small sheet of paper was placed under their pedipalps and this was briefly elevated to extend the pedipalps horizontally for photographing. The width of the tibia at the point of connection to the tarsus was measured for each palp using Image Tool (version 3.0, Fig. 1). In *L. hasselti*, maximum prosoma width of a subset of 4th instar spiderlings was also measured and relative palp width (palp width / prosoma width) calculated as this relative measure could allow more accurate predictions.

Analysis.—For each species and instar, we compared accuracy of the categorical assignment method to an expected null of 50% correct using χ^2 analysis (with Yates' correction). We examined whether or not absolute or relative palp width is dimorphic using *t*-tests (with unequal variances if Levene's test showed a significant difference in variance). We also determined the extent to which absolute or relative palp dimensions reliably predict sex using a logistic regression model with confirmed sex as the dependent variable and relative or absolute palp width as the predictor. *t*-tests and logistic analyses were completed in SPSS (version 13.0) and χ^2 in GraphPad (http://www.graphpad.com/quickcalcs/contingency1.cfm). Sample sizes vary because in some cases isolated eggs did not hatch, or photographs of spiderlings were of insufficient quality to accurately measure body dimensions.

RESULTS

Visual examination method.—Visual examination of palps yielded accurate sex identification in a high proportion of cases (Table 1), with no difference in accuracy between the two *Latrodectus* species ($\chi^2 = 1.109$, $df = 1$, $P = 0.2923$). When palps were visually examined at the 3rd instar, approximately 89% ($n = 88$) of the predictions made for *L. hasselti* and 94% ($n = 100$) of the predictions for *L. hesperus* were accurate (Table 1). Accuracy decreased significantly and prediction was no better than chance if spiderling sex was predicted based on direct examination of spiderlings at the 2nd instar (*L. hesperus*, 62% accuracy, Table 1).

Since the highest accuracy was achieved through visual inspection at the 3rd instar, we focused on understanding sources of error in these predictions. Prediction errors ($n = 6$) for *L. hesperus* spiders categorized at the 3rd instar were all cases of spiderlings initially predicted to be female that were in fact male. Most errors (5/6) occurred for spiderlings in which the 3 independent predictions were in disagreement. Although in 16 such equivocal cases, the majority categorization was correct, in 24% of these cases (5/21), 2/3 viewings suggested "female-like" palps, but spiders were in fact male. The one additional error occurred when 3/3 viewings suggested "female-like" palps (but the spider was male). We examined our error rate if we excluded from the data set all equivocal cases where the majority categorization was "female-like" ($n = 7$ cases, in two of which this was the correct categorization). In this reduced data set, accuracy increases to 99% (92/93) for sexing by visual examination in *L. hesperus* (although this is not a significant improvement in accuracy, $\chi^2 = 2.083$, $df = 1$, $P = 0.1490$).

Similarly, for 3rd instar *L. hasselti*, 90% (9/10) of prediction errors occurred when individuals considered to have "female-like" palps as spiderlings were actually male. For those *L. hasselti* spiderlings that were examined 3 times ($n = 58$), there were a total of 9 prediction errors (84% correct). In these data, there were 24 equivocal cases (1 of the 3 assessments inconsistent with the others), and in 8 (33%) of these cases, predictions were incorrect. We again examined our error rate if we excluded from the data set all equivocal cases where the majority categorization was "female-like" (n = 12 cases, in 5 of which this was the correct categorization). In this reduced data set for *L. hasselti*, accuracy of the visual examination method increases to 96% (44/46 correct, not a significant improvement in accuracy: $\chi^2 = 2.306$, $df = 1$, $P = 0.1289$).

Finally, for 3rd instar spiders, we examined the level of accuracy that could be achieved if only a single visual examination was used to identify sex, as this may be required in some field studies. For *L. hasselti* spiderlings examined independently 3 times (n = 58), accuracy was 76% at the first and second assessments, and increased to 86% in the third assessment. Accuracy was 97% for an additional 30 *L. hasselti* spiderlings that were assessed only once. For *L. hesperus* ($n = 100$), accuracy increased from 79% at the first examination, to 94% at the second and finally 98% at the third examination. In both species, accuracy of identification increased with each subsequent (blind) attempt at categorization. As was the case for categorizations based on the consensus of 3 rankings, most of the errors were spiders predicted to be "female-like" that were actually male (29/36 errors for *L. hasselti*, 27/29 errors for *L. hesperus*).

Pedipalp measurement method.—Absolute pedipalp width at the tibia-tarsal joint was sexually dimorphic in spiderlings of both species (Table 2, Fig. 2). Males had significantly wider pedipalps than females at the 3rd instar in *L. hesperus* ($t = -15.847$, $df = 82.504$, $P < 0.001$, Fig. 2A) and at the 4th instar in *L. hasselti* ($t = 14.00$, $df = 23$, $P < 0.001$, Fig. 2B). For *L. hesperus*, absolute pedipalp width accurately predicts the sex of 94.6% of 3rd instar spiderlings (logistic model $\chi^2 = 79.04$, $P < 0.001$). Similarly, absolute palp width accurately predicts

Table 2.—Mean absolute and relative palpal tibia dimensions ± S.D. for juvenile males and females of two species of *Latrodectus* spiders. *palpal tibia width/ prosoma width.

Species	Instar	Absolute width (mm)		Relative width*	
		Male (*n*)	Female (*n*)	Male (*n*)	Female (*n*)
L. hesperus	3rd	0.128 ± 0.013 (68)	0.097 ± 0.006 (24)	–	–
L. hasselti	4th	0.180 ± 0.006 (6)	0.111 ± 0.002 (19)	0.225 ± 0.014 (6)	0.119 ± 0.003 (15)

the sex of 100% of 4th instar *L. hasselti* ($n = 25$, logistic model $\chi^2 = 27.554$, $df = 1$, $P < 0.001$).

Relative palp width (palp width/prosoma width, $n = 25$ *L. hasselti* spiderlings) was also sexually dimorphic ($t = 7.438$, $df = 5.571$, $P < 0.001$, Table 2, Fig. 2C), and yielded 100% accuracy in predicting sex of 4th instar *L. hasselti* spiderlings ($n = 25$, model $\chi^2 = 27.554$, $df = 1$, $P < 0.001$).

DISCUSSION

We have shown that, in *Latrodectus hasselti* and *Latrodectus hesperus* juveniles, the palpal tibia is significantly wider in males than in females, and this allows accurate identification of sex using a simple visual examination of the spiderlings. In both species, accuracy of over 89% can be attained, and this may increase to 99% if the relatively rare individuals (7/100 for *L. hesperus,* 12/58 for *L. hasselti*) for which equivocal categorization predicts ''female-like'' palps are excluded (i.e., 3 independent predictions not in agreement, and the majority of predictions suggest ''female''). In *L. hesperus*, categorization of spiderlings based on visual examination of palps was incorrect in only 6/100 (6%) of cases. This relatively low error rate could be accommodated statistically in studies of population dynamics or sex ratio, particularly since the polarity of these errors was always males mis-categorized as females. There are a number of alternatives that could increase the accuracy of sexing. First, individuals with equivocal predictions may be reared through one or two additional molts until swelling of male palpi is more readily observable, thus providing highly accurate data with a much-reduced need for labor-intensive rearing than is possible without this technique. Second, repeated scoring of the same individuals may increase reliability. Third, the person scoring the spiders could examine a large number of spiders before attempting categorization. In our study, accuracy of the visual identification method increased with each subsequent attempt at categorization within each species, (i.e., from 76% to 97% in *L. hasselti* and from 79% to 98% in *L. hesperus*). Presumably, this was due to the increased experience of each investigator with each independent examination of the specimens (note that each species was scored by a different researcher, see methods). Fourth, since most of the ambiguous individuals are cryptic males, these individuals may be scored as males, regardless of whether they were scored more frequently as male-like or as female-like, with a relatively small increase in error rate.

Although the polarity of errors for *L. hasselti* was similarly consistent, the proportion of cases with equivocal categorization was far higher (41% of 58 cases). This would be more difficult to accommodate statistically, and would require labor-intensive rearing of almost half of the spiders examined for direct assessment. The higher proportion of equivocal cases for *L. hasselti* may arise because the use of direct observation of individuals is inferior to examination of photographs for assessment of sex. It is likely that the difference in the number of equivocal cases arises from observer error driven by the method of observation because, if anything, palpi are more dimorphic in *L. hasselti* than *L. hesperus* (Figs. 1, 2), but our predictions were more accurate for *L. hesperus* (Table 1). We note however, that the accuracy of categorization derived from a single examination can be very high after the observer has experience using this technique (i.e., for *L. hasselti*, accuracy = 97% for $n = 30$ spiderlings examined only once).

Our logistic analyses showed that a regression model using measurements of absolute or relative pedipalp width as a predictor could similarly yield up to 100% accuracy in assessing spiderling sex. While this method did not significantly improve accuracy over the visual categorization method it may widen the range of instars and situations in which this technique may be applied.

The practical application of this morphology-based technique to other species will depend on whether or not similar early sexual dimorphism in palp width exists. This may be particularly useful in species that are monomorphic to the naked eye at hatching but in which the male's palpi are notably enlarged at adulthood, as in the genus *Latrodectus*. If palp dimorphism generally develops gradually over several instars, then perhaps the best candidates for sexing spiders visually at an early instar are those species in which males attain sexual maturity in few instars. Applying this method to other species would critically require pilot studies in which individuals are reared until males and females are clearly distinguishable to confirm the applicability of this technique. Early indications that such studies may be fruitful would include significant bimodal clustering of absolute or relative pedipalp width measurements in early-instar spiderlings (e.g., Fig. 2). Such pilot studies should also identify the earliest instars at which this technique would work. In our data set for example, predictions were no better than chance for 2nd instar spiderlings, but highly accurate for spiderlings one instar later (Table 1).

Once pilot studies are complete, the more simple visual examination method can potentially be used for a range of studies. In addition to non-destructive sampling, one advantage of this method is that an experienced observer should be able to classify the sex of juvenile spiders without the disturbance involved in collecting or measuring body parts. Clearly, this method should be very useful for laboratory studies. In addition, for larger species it may be possible to use macro-photography, a portable microscope, or visual inspection using a hand lens in the field to determine sex. One limitation of the visual examination method is that it requires

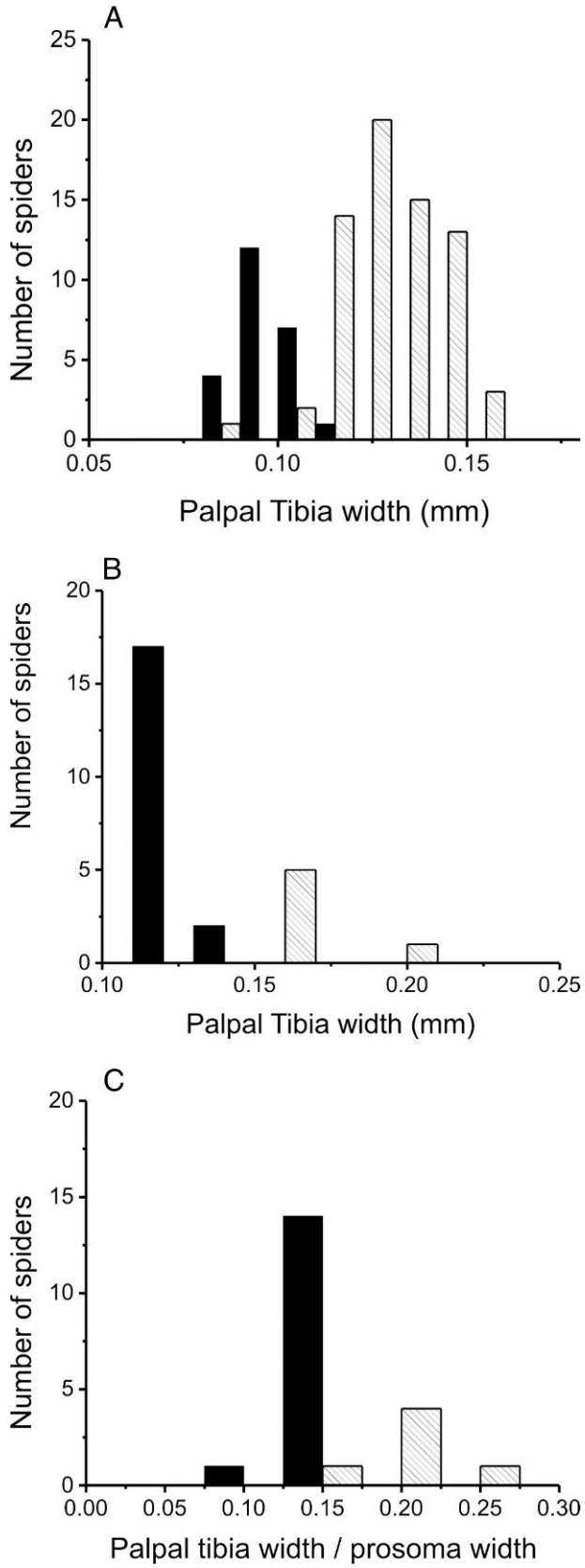

Figure 2.—Distribution of absolute dimensions of palpal tibia of male (cross-hatched bars) and female (black bars) spiderlings of (A) *L. hesperus* (3rd instar), (B) *L. hasselti* (4th instar) and (C) distribution of relative palp dimensions (palpal tibia width/ prosoma width) for 4th instar *L. hasselti.*

knowledge of spider instar, or at least that the spiders under consideration are in the same instar. In the field this may pose difficulties for multivoltine species unless populations are part of long-term monitoring studies. This problem will likely be greatly reduced if measures of relative pedipalp width are used, but confirming this requires additional study.

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