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Size and sex in early developmental stages in a frog-biting mosquito

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Abstract

Sexual size variation in adult holometabolous insects may arise from selective pressures impacting ontogenetic stages associated with diverse habitats and resource use. In addition, scaling relations of these sexually dimorphic traits play an important role in morphological diversification. In mosquitoes, given the sexual differences in feeding strategies, investigations of the ontogeny of sexually dimorphic traits are of particular interest to understanding their reproductive biology and implementing early sex-separating technologies for vector control. However, our current knowledge of the morphological scaling of body parts over development across sexes is centered around a few well-known species of anthropophilic mosquitoes. In general, there is a noticeable gap in our understanding of the developmental biology of mosquitoes with limited medical consequences. One such mosquito is *Uranotaenia lowii* (Diptera: Culicidae), a species of growing interest due to its unique host use of feeding exclusively on frogs by eavesdropping on their mating calls. This study takes a step forward toward filling this gap by investigating sexual size dimorphism during the ontogeny of *Ur. lowii*. We examined larval and pupal stages to focus on traits that allow sex identification to evaluate various sex-sorting techniques that provide a foundation for experimental manipulation. We found that sex identification in *Ur. lowii* is possible during both larval and pupal stages. In the fourth larval instar, thorax length, abdomen length, and total body length differ significantly between the sexes, showing allometric scaling. In the pupal stage, the allometry of the head and thorax to body size remains consistent, as these parts fuse into the cephalothorax. Successful sorting based on cephalothorax length enables highly accurate pupal sex identification. This research sheds light on the biology of *Ur. lowii,* an understudied mosquito species, and lays the foundation for future studies on the developmental and reproductive biology of frog-biting mosquitoes.

KEYWORDS
body size, holometabolous insect, ontogeny, sexual size dimorphism, *Uranotaenia lowii*

INTRODUCTION

Like in most insects (Stillwell et al., 2010), in adult mosquitoes there is female-biased sexual size dimorphism (SSD) as a result of fecundity selection imposed on females favoring large size associated with high egg production and nutrient storage (Wormington & Juliano, 2014). Such SSD underscores the specialized ecological roles and different life-history strategies of male and female mosquitoes. Adult size differences between the sexes also prompt the question of when this divergence occurs during development. Given that selective forces operating at the juvenile stage often differ radically from those acting on sexually mature individuals, this disparity in selective pressures

Kanishka Singh and Krisha Shah contributed equally to this study.

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may constrain the expression of sexual size differences in adults (Badyaev, 2002; Reeve & Fairbairn, 2001). Despite potentially conflicting selective pressures at different developmental stages, sexual differences in size are often not restricted to adulthood.

In insects, sexual differences in body size during development can arise through three distinct but not mutually exclusive mechanisms. Individuals of a particular sex can be larger due to a faster growth rate at a certain ontogenetic stage, an overall longer growth period, or a larger size at the time of hatching (Blanckenhorn et al., 2007). Sexual differences in egg or hatchling size are uncommon in insects (Ernsting & Isaaks, 2002; Tammaru et al., 2010). In contrast, differences in developmental time (Jarošik & Honek, 2007; Stillwell & Davidowitz, 2010), growth rate (Blanckenhorn et al., 2007), or both of these factors (Ernsting & Isaaks, 2002) are widespread. There is growing evidence suggesting that the larvae of the larger sex have longer developmental periods than the smaller sex (Stillwell et al., 2010; Tammaru et al., 2010; Teder, 2014; Wormington & Juliano, 2014).

Given that the final instar larval stage is crucial for developing adult organs from imaginal disks, growth differences between sexes are expected during this time (Yasuda & Dixon, 2002). For instance, in scarab beetles, SSD arises from the longer rapid growth period of males during the final larval stage, despite the sexes showing similar third instar growth rates (Vendl et al., 2016, 2018). Such variation in growth trajectories between sexes across ontogenetic stages reflects the complexity in the ontogeny of SSD (Tammaru et al., 2010; Vendl et al., 2016, 2018). The degree of sexual dimorphism, however, can also be affected by environmental quality during development. Environmental stress, like extreme temperature, limited resources, or high larval density, often reduces SSD by impacting the larger sex more (Alcalay et al., 2018; Cordeschi et al., 2024; Teder & Kaasik, 2023). Therefore, to understand SSD development, detailed studies tracking growth in both sexes across ontogeny in optimal conditions are essential.

In mosquitoes, understanding the pattern of ontogenetic SSD is crucial for developing effective vector control strategies. Early identification of females, for instance, has been widely used for sex separation for vector control (Papathanos et al., 2009, 2014), which results in considerable savings in time, labor, and money (Lutrat et al., 2019). Sexing mosquitoes early in development also offers additional benefits, such as the ability to examine the role of sex in behavioral and physiological mechanisms and conduct experimental manipulations at an earlier stage (Lounibos & Escher, 2008; Yamada et al., 2019). Sexual size dimorphism can provide an opportunity for cost- and time-effective sorting of males and females. Size-based separation has traditionally been used to distinguish culicine male and female pupae (Bellini et al., 2018), but anophelines show greater size overlap between males and females, making sexing less accurate (Papathanos et al., 2009). It is unclear, however, how widespread pupae size sexual dimorphism is across mosquitoes.

Research on sexual dimorphic traits throughout ontogeny has focused on the well-known human disease-transmitting mosquitoes, such as *Aedes* and *Anopheles* species, resulting in a notable gap in our understanding of sexual differences during early developmental stages in most species in this family (Culicidae). Mosquitoes that feed on frogs and toads have received little attention, but recent findings suggest all mosquitoes likely evolved from an amphibian-feeding ancestor (Soghigian et al., 2023). This supports earlier hypotheses that mosquitoes initially exploited amphibian blood 217 million years ago when their ancestral habitat provided abundant amphibian hosts (Pyron, 2014). In addition, frog-biting flies are an emergent model system to understand the behavioral ecology of eavesdropping in animal communication systems (Ambrozio-Assis et al., 2019; Bernal et al., 2006; Campos et al., In review; Leavell et al., 2022; Legett et al., 2021; Pantoja-Sánchez et al., 2023; Singh et al., 2024; Toma et al., 2019). Here, we examine an eavesdropping frog-biting mosquito, *Uranotaenia lowii* Theobald, 1901, to investigate early SSD.

Uranotaenia lowii, also known as the pale-footed *Uranotaenia*, is a small mosquito (2.5 mm) with stripes and patches of iridescent blue scales on the head, thorax, abdomen, and wings (Burkett-Cadena, 2013). This species occurs in North America, mostly in the southeastern states along the coast, and in Central and South America (Global Biodiversity Information Facility; www.gbif.org/occurrence/search?taxon_key=1654276). While males feed on nectar, females exclusively feed on anuran hosts (Reeves et al., 2018) by using auditory cues to locate calling male frogs (Borkent & Belton, 2006; Pantoja-Sánchez et al., 2023). This species undergoes complete metamorphosis with immature aquatic stages: egg, larva, pupa, and adult (Figure 1). Females produce egg rafts, breeding in small ponds and grassy lake edges, similar to other Culicinae mosquitoes (Gillett, 1972). A low number of eggs, compared with other raft-laying species, are produced by *Ur. lowii* (up to 74 eggs per raft, Singh et al., 2024 versus 400 eggs in *Culex pipiens* and 150–200 eggs in *Culex fatigans*, Christophers, 1945), but a similar number of eggs relative to *Uranotaenia sapphirina* (45–50 eggs; Dyar, 1901). The eggs of *Ur. lowii* (0.7 mm) are smaller than those of *Ur. sapphirina* (2 mm) (Dyar, 1901), but larger than the eggs produced by *Aedes aegypti* (0.58 mm; Mundim-Pombo et al., 2021), *Anopheles stephensi* (0.59 mm; Malhotra et al., 2000), and *Culex saltanensis* (0.5 mm; Santos-Mallet et al., 2021). Larval development (10 days, Singh et al., 2024) is comparable to other species (*Anopheles gambiae*: 9.9–11 days, Bayoh & Lindsay, 2004; *Ae. aegypti*: 10 days, Tun-Lin et al., 2000). Overall, the unique combination of developmental features of *Ur. lowii* highlights the value of this species to broaden our understanding of ontogenetic patterns in Culicidae.

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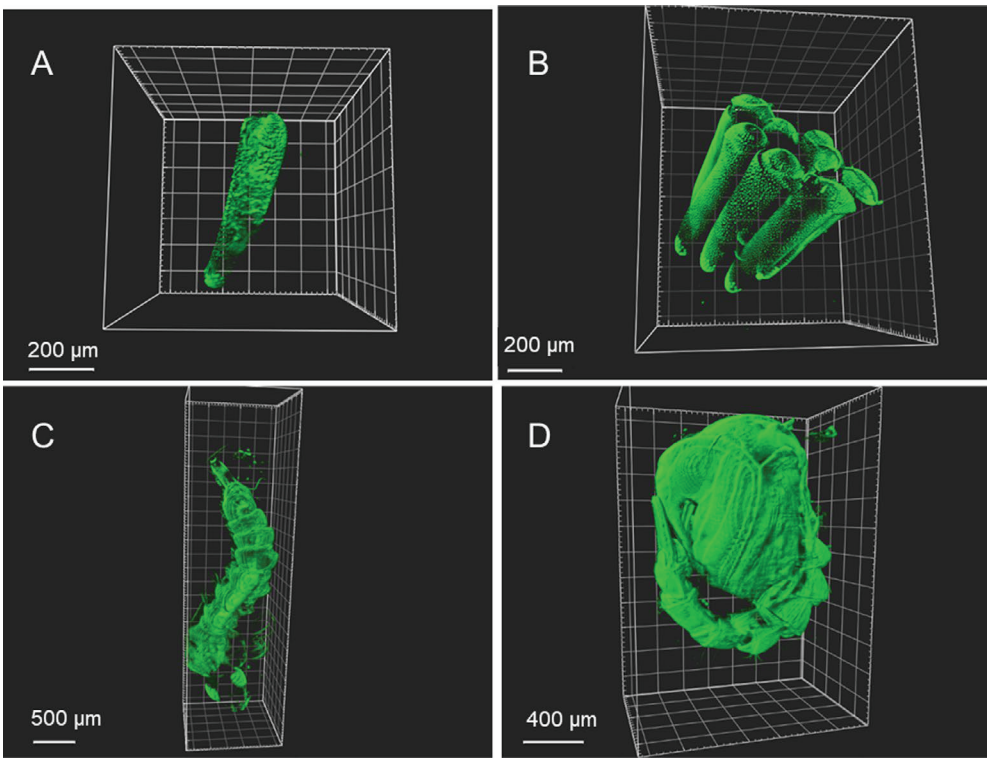

FIGURE 1 Development stages of *Uranotaenia lowii* photographed using lightsheet microscopy. (A) single egg, (B) egg raft, (C) fourth instar larva, and (D) pupa.

By focusing on *Ur. lowii*, we characterize the development stages and investigate ontogenetic sexual dimorphic traits and their allometric scaling in a frog-biting mosquito. In particular, we focus on identifying early, effective sexing techniques by examining sexual dimorphism during the larval and pupal stages. In doing so, we shed light on the development of this species and provide insights into key life-history traits of a mosquito species from an understudied group.

MATERIALS AND METHODS

Following an established rearing protocol (Singh et al., 2024), *Ur. lowii* mosquitoes (strain MFRU-FL; NCBI BioSample: SAMN33601576) were maintained at the Department of Biological Sciences, Purdue University (West Lafayette, IN, USA). At the colony, adult mosquitoes were fed a variety of anuran hosts, including cane toads (*Rhinella marina*) and Cuban treefrogs (*Osteopilus septentrionalis*) while the larvae were fed using a 3:2 ratio of bovine liver powder and brewer's yeast; detailed feeding protocols can be found in Singh et al. (2024). To document the life stages of *Ur. lowii*, after females were blood-fed with anuran hosts to support egg production, we monitored their development from the day of egg appearance to the pupal stage. Photographs of the mosquitoes at each developmental stage were collected using a Celestron Digital eyepiece (5MP CMOS microscope imager) connected to a Stereozoom Motic microscope (SMZ-160-BLED; 4.5 to 2× magnification). Sexing of adults was also performed using this setup. Images from all stages were analyzed using ImageJ software (Version 1.53, National Institute of Health, USA).

Larval stages

A total of 53 larvae were monitored individually by observing their development from the first instar until they reached the pupal stage. The first instar larvae were placed in individually labeled Petri dishes (60 mm diameter x 15 mm) and their morphometric characteristics (head length, thorax length, thorax width, abdomen length, and total larval length) were examined and measured following Timmermann and Briegel (1999) and Bar and Andrew (2013). To determine early sexual dimorphism, each final instar larva was observed till pupation, after which the sex was confirmed upon emerging into adulthood. The larvae were fed on alternative days, and the food amount was standardized among individuals by providing 1/64th tablespoon of a bovine liver powder and brewer's yeast diluted in 10 mL of deionized water for each larva. Images of the larvae were taken daily to document morphometric changes throughout the instars.

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Pupal stage

To investigate potential sexing methodologies to discriminate between male and female pupae, we used three approaches that vary in the traits observed and the time required for sorting individuals. To examine a quick and often-used approach, we visually inspected pupae ($n = 68$) to sex them based on their body size. Following previous work (Bellini et al., 2018; Koenraadt, 2014) and mirroring differences in adult body size in *Ur. lowii*, we assumed small individuals were males and large individuals were females. When the individuals matured into adults, we examined their genitalia under the microscope to assess sorting accuracy. To examine sexual dimorphic morphometry, we measured cephalothorax length following previous work that identified this trait as sexually dimorphic in other mosquito species (Koenraadt, 2014). We performed morphometric analyses for 84 randomly selected pupae. Each pupa was placed in a mesh-covered 50 mL vial to track them individually until adult emergence. The pupae were separated based on the size of the cephalothorax, with females assigned to individuals with a length greater than 1.5 mm and males shorter than 1.5 mm. This value was selected after conducting preliminary measurements for both sexes, suggesting this threshold resulted in a conservative approach. The sex assigned at the pupal stage was checked upon maturation. Finally, we examined sexual dimorphism based on shape, focusing on the apex of the abdomen to assign individuals as male (tapered) or female (rounded). A total of 28 pupae were individually photographed from a ventral view. Based on the photographs, their sex was predicted based on abdomen shape, and the pupae were placed back into individual vials to check their sex upon the emergence of the adults.

Statistical analyses

Statistical analyses were performed in Rstudio (v. 2023.12.0 + 369, PBC, Boston, MA) and GraphPad Prism (v. 10.0.0, Boston, MA, USA). All the variables were tested for normality using Kolmogorov–Smirnov tests. The duration of different larval instars was examined using ANOVA followed by Tukey's multiple comparison analysis. Independent Kruskal–Wallis tests followed by the Mann–Whitney *U*-tests were performed to analyze morphometric measurements of various parameters for larval size. To examine the allometric relationship between larval length and other larval parameters as well as for male and female pupal body size and cephalothorax length, we performed ANCOVA with sex as a fixed factor, larval length/pupal length as a covariate, and multiple comparisons were adjusted by Bonferroni correction. A *t*-test was performed to compare morphometric parameters in male and female larvae. Chi-squared tests were performed to assess whether sexual dimorphism in the pupae based on visual size separation, cephalothorax length, and abdomen shape were correctly assigned or not. A Mann–Whitney U-test was used to compare the sexual dimorphism in pupae based on cephalothorax length. To examine sexual differences and allometric relationships over development, we performed independent linear regression. We calculated the SSD index for body size following Lovich and Gibbons (1992) as females are the larger sex:

$$\text{SSD} = \frac{\text{Mean size of female}}{\text{Mean size of male}} - 1$$

RESULTS

General life history

Like other mosquitoes, the larvae of *Ur. lowii* go through four instar stages (see Figure S1A). The time to reach the third and fourth instars is more variable compared with the time spent at the first and second instars (ANOVA, $F_{3,208} = 79.30$, $p < 0.01$, Figure S1B), suggesting that the most prominent growth occurs between the third and fourth instar and from the fourth instar to the pupal stage (Figure S1B; see Table S1). On progressing through successive stages, larvae increase in size across all parameters measured: head length (Kruskal–Wallis test: $H_{3,496} = 355.38$, $p = 0.001$); thorax length (Kruskal–Wallis test: $H_{3,496} = 440.56$ $p < 0.001$); thorax width (Kruskal–Wallis test: $H_{3,495} = 441.42$, $p < 0.001$); abdomen length (Kruskal–Wallis test: $H_{3,496} = 441.51$, $p < 0.001$); total body length (Kruskal–Wallis test: $H_{3,496} = 441.41$, $p < 0.001$; Table S1; Figure S2).

Sexual dimorphism in ontogenetic stages

Uranotaenia lowii exhibits female-biased SSD, with noticeable differences in development time between sexes beginning at the fourth larval instar and continuing through the pupal stage (Figure 2A). This indicates that females tend to grow larger than males, and this size difference becomes more apparent as the larvae progress through later developmental stages. The SSD index, which measures the degree of size difference between sexes, gradually increased from each larval stage to the pupal stage, but the most pronounced changes were observed in the fourth larval instar and pupal stages (Figure 2B). This suggests that while both sexes develop at relatively similar rates in the earlier larval stages, a divergence occurs later, with females taking longer to develop compared with males.

The development of SSD significantly influences the size of various body parts. At the fourth instar stage, head length between males and females does not differ significantly ($t = -0.81$, $df = 27$, $p > 0.05$), indicating similar head development in both sexes at this stage. However, other body measurements, such as thorax and abdomen dimensions, show clear differences. Thorax length ($t = -4.09$, $df = 27$, $p < 0.01$), thorax width ($t = -3.35$, $df = 27$, $p < 0.01$),

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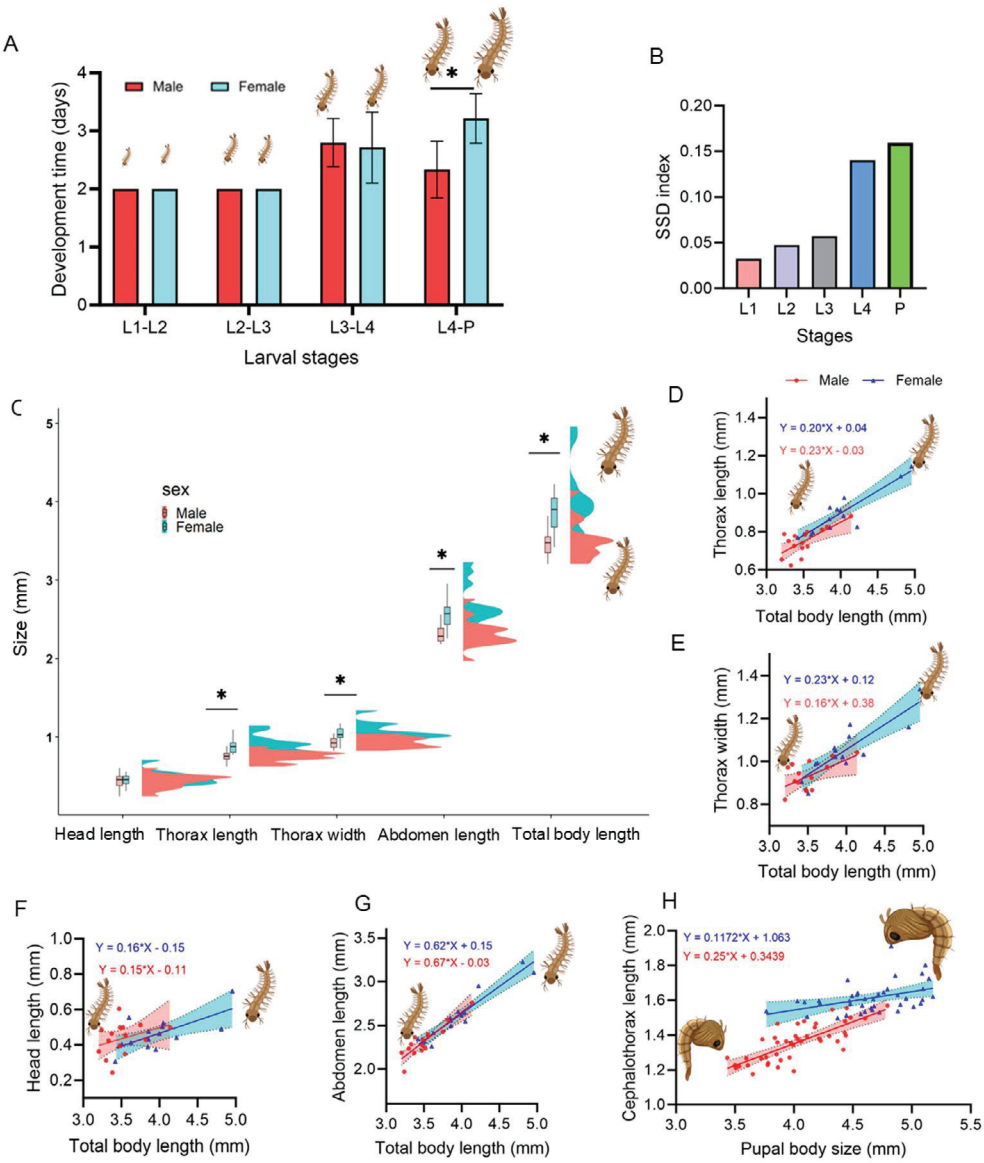

FIGURE 2 Sexual dimorphism in larval and pupal stages of *Ur. lowii*. (A) Total development time for each sex across larval stages. (B) Sexual size dimorphism (SSD) index across larval and pupal stages. (C) Sexual dimorphism across all morphological traits measured in fourth instar larvae. Boxplots and their corresponding histograms are shown for four body parts and body length for females (blue) and males (red). (D–G) Allometric relation of different morphometric parameters with total larval length in the fourth instar stage for both sexes. (H) Allometric relation of cephalothorax length with pupal body size in male and female pupae.

abdomen length ($t = -3.40$, df = 27, $p < 0.01$), and total body length ($t = -3.59$, df = 27, $p < 0.01$) are all significantly larger in one sex compared with the other. These differences indicate that while head size remains consistent, SSD becomes evident in other body regions, such as the thorax and abdomen, contributing to the overall body size variation observed between sexes at this developmental stage (Figure 2C).

We examined the effect of sex on thorax length, thorax width, head length, and abdomen length, controlling for larval length, by performing an ANCOVA using Bonferroni correction to account for multiple comparisons. Our results showed that larval length had a significant effect on all measured traits: head length ($F_{1,28} = 10.41$, $p < 0.01$), thorax length ($F_{1,28} = 68.89$, $p < 0.01$), thorax width ($F_{1,28} = 43.17$, $p < 0.01$), and abdomen length ($F_{1,28} = 187.73$, $p < 0.01$), suggesting that larval length plays a crucial role in shaping the overall body proportions. After controlling for larval length, sex was found to have a significant main effect on thorax length ($F_{1,28} = 2.50$, $p < 0.01$), thorax width ($F_{1,28} = 0.45$, $p < 0.01$), and abdomen length ($F_{1,28} = 0.01$, $p < 0.01$), all corrected with the Bonferroni adjustment. However, no significant effect of sex was observed for head length ($F_{1,28} = 1.14$, $p > 0.05$), indicating that while sex significantly influences thorax and abdomen size, head length remains unaffected after accounting for larval length. Additionally, our findings highlight that abdomen length exhibited the strongest allometric scaling effect and head length showed the

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weakest allometric response (Figure 2D–G), suggesting that abdomen length is more sensitive to changes in overall body size compared with other traits like thorax length and width, with head length showing the least variability in response to growth.

The allometric relationship of the head and thorax relative to the body size in the fourth larval instar is maintained as individuals develop into the pupal stage in which those parts are fused in a cephalothorax. An ANCOVA was performed at the pupal stage to examine the effect of sex on cephalothorax length while controlling for pupal body size. The results showed that both sex and pupal body size had a significant effect on cephalothorax length ($F_{1,80} = 8.95$, $p < 0.01$); the length of the cephalothorax co-varies with female body size ($\beta = 0.106$; $R^2 = 0.18$; $p < 0.05$; Figure 2H) and male pupal body size ($\beta = 0.252$; $R^2 = 0.59$; $p < 0.0001$; Figure 2H) indicating a more pronounced allometric scaling effect in males compared with females.

Sex identification

Visual segregation of males and females was successful, and there were no significant differences in accuracy between the sexes (males = 84% correct, females 90% correct; $X^2 = 0.49$, df = 1, $p = 0.48$ $n = 68$, Figure 3A). Separation based on cephalothorax length was highly successful for identifying females (males = 94% correct, females 100% correct; $X^2 = 2.45$, df = 1, $p = 0.12$, $n = 84$). The cephalothorax of females is longer than that of males (Female: 1.613 ± 0.085 mm ($n = 40$); Male: 1.355 ± 0.10 mm ($n = 44$); Mann–Whitney $U = 31$, $p < 0.0001$, Figure 3B). Sexual dimorphism in the shape of the abdomen of pupae was also an effective sexing strategy (males = 88% correct, females 100% correct; $X^2 = 1.394$, df = 1, $p = 0.25$ $n = 28$, Figure 3C). The abdomen of males protrudes and has a less well-defined tip, whereas the female abdomen is pointier and less protruding. Using this trait, however, females are correctly assigned to their sex with a higher probability than males.

DISCUSSION

Knowing when the sexes in morphology diverge during ontogeny is important for earlier sex identification as well as to deepen our understanding of the development of sexual size differences. For example, if males and females diverge during the early larval stages, selection may act on growth parameters, such as growth rate, development time, or number of instars (Stillwell et al., 2010; Tammaru et al., 2010). Since body size is an important determinant of reproductive success in many systems, it becomes important to understand the sources of size variation. The present study confirms that SSD occurs early in development in *Ur. lowii* and both larval and pupal stages show female-biased sexual dimorphism. Our results thus add to the growing body of evidence showing that sexual size differences appear during an early larval stage in insect species with no sex-specific difference in the number of instars but development time (Jarošik & Honek, 2007; Stillwell & Davidowitz, 2010; Teder, 2014; Vendl et al., 2016, 2018; Wormington & Juliano, 2014).

In *Ur. lowii*, female final instar larvae are larger in size and take a longer time to develop compared with male final instar larvae. Female larvae likely require more energy reserves during development, as they need to accumulate resources for egg production in adulthood. Such differences in energy requirements may result in divergent foraging strategies or nutritional needs between male and female larvae. A lower nutritional threshold for continuing development in males, a phenomenon known for other mosquito species (Teder & Kaasik, 2023), may explain the shorter development time in males than in females. Shorter development time in males, however, may also result from protandry, a form of sexual selection whereby males sacrifice mass to develop faster to hatch early, gaining an advantage in competing for access to virgin females, who take longer to develop (Kleckner et al., 1995). The larger body size of fourth instar larvae allows greater energy storage for developing complex organ systems for adult life, and since female mosquito larvae typically have higher reserve requirements than males, differences in development time and size may be linked to sex-specific growth and differentiation of the larval midgut.

Our findings describe the development and SSD of *Ur. lowii* under colony conditions. These phenotypes are expressed under abiotic conditions typical of those experienced in nature (e.g., temperature, relative humidity, and dark:light cycle) and high abundance of resources. Numerous studies have demonstrated that the development of mosquito instar stages is influenced by factors, such as rearing temperature, quantity and quality of food, and larval density (e.g., *An. gambiae*, Agyekum et al., 2022; Lyimo et al., 1992; *Ae. aegypti*, Mohammed & Chadee, 2011; *Anopheles arabiensis*, Mamai et al., 2018; *Cx. pipiens*, *Culex quinquefasciatus*, and *Culex restuans*, Ciota et al., 2014). In insects, males and females often exhibit different plastic responses to environmental changes, such as food limitation, food quality, and larval density. While males and females differ significantly in their plastic response to diet, temperature-induced phenotypic plasticity is generally less pronounced (Teder et al., 2022). Typically, however, the larger sex, usually females, shows a more pronounced plastic response than the smaller sex (Rohner & Blanckenhorn, 2018). In *Ae. mariae*, for instance, increasing temperature results in reduced development time in both sexes, but females show a more accentuated reduction (Cordeschi et al., 2024). Similarly, in *Ochlerotatus taeniorhynchus*, salinity elicits similar responses in both sexes, though females consistently take longer to pupate than males (Clark et al., 2004). The specific factors that affect larval development, which sex is more susceptible, and the relative impact of those effects

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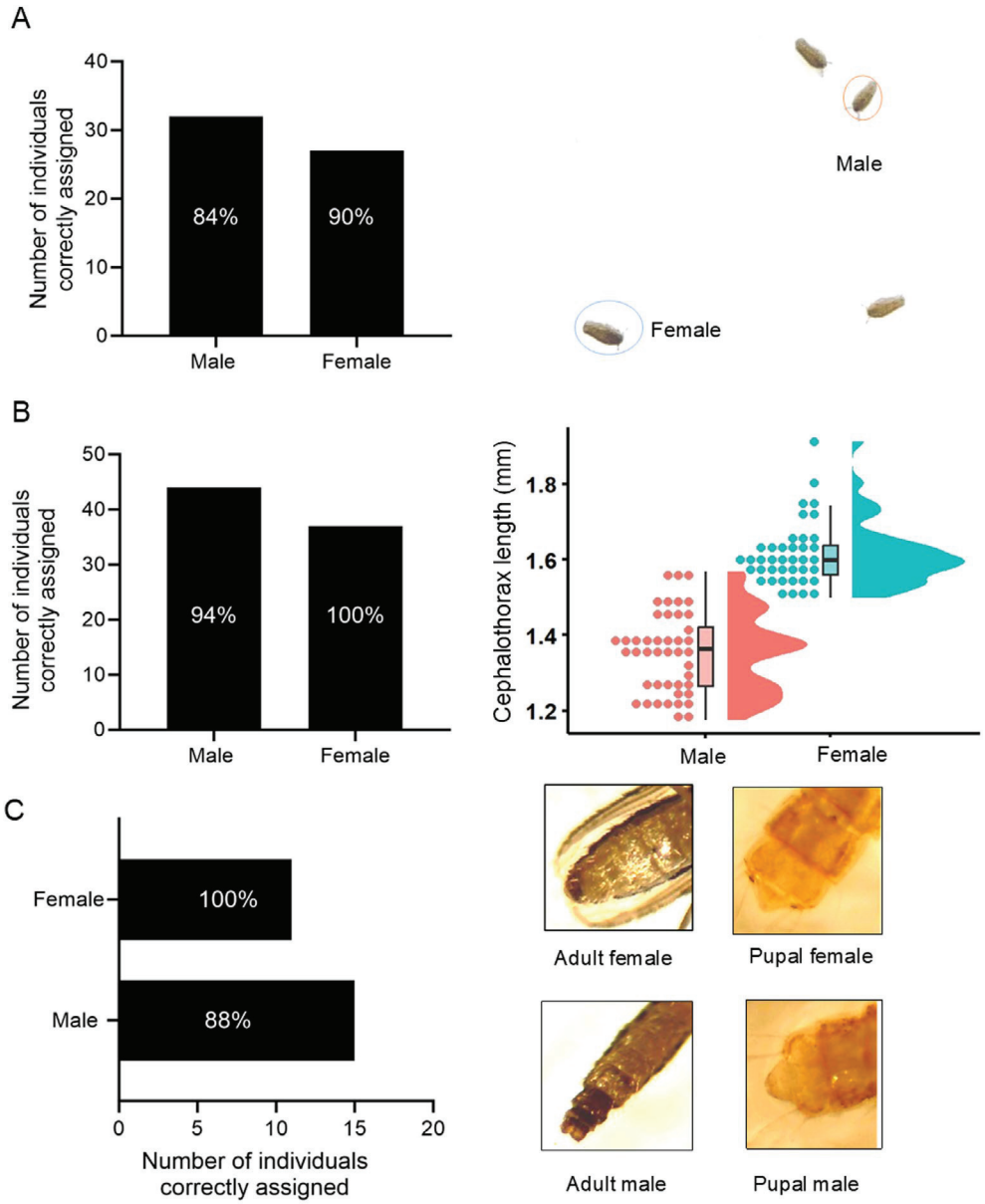

FIGURE 3 Effectiveness of three sexing methods for the early developmental detection of males and females in pupae of *Uranotaenia lowii*. (A) Visual-based sexual size differentiation; (B) Cephalothorax length-based sexual size separation; (C) Abdomen shape-based sexual separation. Black bars with percentages showing the number of individuals correctly assigned as male or female at the pupal stage.

can vary even within a species. In *Cx. pipiens*, for example, larval development time and subsequent body size are affected more strongly by increased larval density (especially in females) and temperature fluctuations (especially in males) than by increased solute concentration (Alcalay et al., 2018). It is expected that environmental conditions can also affect *Ur. lowii* larvae development, but further studies are necessary to understand how specific conditions may accentuate or tamper with the SSD of this species.

In *Ur. lowii*, morphometric measurements of the head, thorax, abdomen, and larval body length show that their sizes increase exponentially with the instar stage, resulting in accelerated growth in the third and fourth larval stages. These findings are similar to those reported in other mosquito species. For instance, in *Cx. quinquefasciatus*, head capsule, thorax, and abdomen also increased exponentially with larval instar stages at ambient temperature (Ukubuiwe et al., 2019). In *Ae. aegypti, Aedes vexans, Cx. pipiens, An. gambiae, Anopheles abimanus*, and *Anopheles quadrimaculatus*, morphometric measurements of the thorax, head capsule, and body size also grow exponentially (Timmermann & Briegel, 1999). The allometric relationship of different larval body parts and body size in *Ur. lowii* varies across sexes in early development stages. While the thorax and abdomen show allometric relationships with larval length across sexes, the weaker allometric relationship for head length for

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males and females suggests that strong sclerotization of the head capsule may limit the growth of this body part (Dyar, 1890). Allometric relationships between body traits and body size can vary with environmental conditions during development in adult insects (Dillon & Frazier, 2013; Shingleton et al., 2007). For instance, in *Aedes albopictus*, temperature has a profound effect on allometry, with higher temperatures resulting in mosquitoes with shorter wings relative to their body size (Reiskind & Zarrabi, 2012). However, little is known about how the relationships between various larval body parts and body size change under different environmental conditions.

Sexual differences in scaling coefficients between body parts and body size or in absolute body size early in development provide an opportunity to detect males and females before they become adults. In the present study, of the three sex separation techniques examined, measuring cephalothorax length is the most accurate one. However, measuring cephalothorax length for each pupa is time-consuming and increases pupal mortality due to the additional handling required. Visual size-based segregation, while less precise, yields high accuracy in *Ur. lowii* and can be performed relatively quickly without compromising individual survival. While the effectiveness of this method varies across different mosquitoes (e.g., *Anophelini* Papathanos et al., 2009 versus Culicine Bellini et al., 2018), this study shows it is an appropriate and cost-effective approach to sort *Ur. lowii* males and females before metamorphosis is completed. It is unclear, however, whether visual size-based segregation is effective in other Uranotaeniini species, and further studies are necessary to examine whether early body size difference between the sexes is a trait widespread in this tribe. Finally, we observed differences in abdominal shape between male and female pupae in *Ur. lowii*, specifically in the ninth pupal abdominal segment (genital segment), a sexually dimorphic trait reported in other mosquito species (Vargas, 1968). Using this feature to sex pupae has been a standard approach for setting up crosses and collecting virgin females in *Ae. aegypti*. When implemented by experienced personnel, a large number of individuals can be sexed with minimal error (~500 pupae/hr. with a 0.05%–1% error rate, Papathanos et al., 2018). In this study, using abdominal shape proved to be a more cost-effective alternative to measuring cephalothorax length, segregating *Ur. lowii* by sex at the pupal stage.

We measured the efficacy of the sexing methodologies in mosquitoes from a laboratory-established colony of *Ur. lowii* (Singh et al., 2024). Environmental conditions, however, are expected to influence sex identification given that sexual dimorphism during development can vary with temperature, salinity, and nutrition due to differential phenotypic plasticity between the sexes (Alcalay et al., 2018; Cordeschi et al., 2024; Teder & Kaasik, 2023). In suboptimal conditions, the sexes can overlap in body size, reducing the efficiency of sexing methods based on pupal size dimorphism (Papathanos et al., 2009). In *Ae. albopictus*, size differentiation for sex separation efficacy is affected by larval density, water temperature, and diet composition (Balestrino et al., 2014). Some conditions, however, can improve sex separation accuracy. For instance, in *Ae. albopictus*, the addition of nutrients (e.g., brewer's yeast) improves sex separation accuracy by amplifying the size difference between male and female pupae (Puggioli et al., 2013). In general, since phenotypic plasticity in development time and body size is common in insects, optimizing the efficiency of SSD-based sorting methods can be achieved by minimizing competition among pupae to ultimately reduce within-sex individual variation in size. Further studies are necessary to determine the extent of context dependency of sex-sorting techniques in *Ur. lowii*, but the use of pupae abdominal shape provides a robust method for sex identification when environmental conditions temper SSD.

In conclusion, this study on life-history traits and sexual dimorphism provides a foundation for future research on *Ur. lowii* and other frog-biting mosquitoes. Our findings confirm general development patterns in this frog-biting species that are comparable to those of other mosquito species but also show species-specific differences likely to be associated with its unique natural history. Given the differences in sensory ecology between the sexes in *Ur. lowii*, effective early sex detection strategies like those characterized in this study provide the methodological foundation for advancing our knowledge of this understudied mosquito species.

AUTHOR CONTRIBUTIONS

Richa Singh: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; supervision; validation; visualization; writing – original draft; writing – review and editing. **Kanishka Singh:** Data curation; formal analysis; writing – review and editing. **Krisha Shah:** Data curation; formal analysis; writing – review and editing. **Ximena E. Bernal:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Purdue University Research Repository at https://purr.purdue.edu/publications/4486/1, reference number doi: 10.4231/2X06-RX08.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Development of different instar stages of *Uranotaenia lowii*. (A) Four instar stages in *Ur. lowii*, (B) Latency to reach the next larval stage.

Figure S2. Morphological traits across each larval instar stages.

Table S1. Quantitative measurements of morphometric larval traits in *Uranotaenia lowii*.

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