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RESEARCH ARTICLE

Invertebrate Biology

Snail sex and the city: Divergence in genital anatomy in an urban land snail

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Abstract

Urbanization causes changes in the function and structure of ecosystems. This leads to adaptive and non-adaptive evolution of organisms living in cities. Habitat fragmentation can play an important role, especially for less mobile species like land snails. One evolutionary impact of urban fragmentation that has been understudied is changes in morphological traits related to sexual selection. The present study aims to investigate the effect of increased urbanization on co-evolution of the male and female reproductive organs in the grove snail (*Cepaea nemoralis*), a simultaneous hermaphrodite. Grove snails were collected in three different cities in the Netherlands along urban-rural transects. Male (sperm donor) and female (sperm recipient) reproductive traits were measured for 271 individuals. The expectation was that, due to a decrease in population density in urban areas, both the male and female reproductive organs would show changes that can be explained as resulting from reduced mating competition. Our results confirmed that there is an effect on the male spermatophore-producing parts and the female spermatophore-receiving parts. In addition, there was a positive correlation between the size of the male spermatophore-producing and female spermatophore-receiving organs. These findings invite for further research into the biotic and abiotic factors that influence sexually-selected morphological traits in urbanized areas.

KEYWORDS
hermaphrodite, reproduction, mollusc, urban environment

1 | INTRODUCTION

Urbanization continues unabated; in 2018, approximately 55% of the world's population lived in urban areas, compared to only 30% in 1950 (United Nations, 2019). This percentage is expected to increase to 68% by 2050 (United Nations, 2019). Increasing urbanization is usually associated with large changes in biotic and abiotic factors (Grimm et al., 2008; Haddad et al., 2015), which leads to adaptive and non-adaptive evolution (Santangelo et al., 2018). A few well-studied examples are morphological changes in the Great tit (*Parus major*), Puerto Rican crested anole (*Anolis cristatellus*), (Caizergues et al., 2018; Winchell et al., 2018), and changes in flowering and pollination traits in Yellow jasmine (*Gelsemium sempervirens*) and Common ragweed (*Ambrosia artemisiifolia*) (Gorton et al., 2018; Irwin et al., 2018). Additionally, increased tolerance to abiotic factors has been observed in urban Acorn ants (*Temnothorax curvispinosus*) (Diamond et al., 2018).

While the afore-mentioned studies compared urban with non-urban populations, research done along rural-urban gradients has given a more detailed picture of the effect of urbanization on adaptive traits. For example, research on white clover

(*Trifolium repens*) has shown that along rural-urban gradients, adaptation to anti-herbivore defense changes, with concentrations of hydrogen cyanide (HCN) produced changing with urbanization (Santangelo et al., 2022). While most urban-evolutionary research has focused on natural selection and neutral evolution, it has also been hypothesized that sexual selection can play a major role in the divergence of urban and non-urban populations (Cronin et al., 2022). For example, it was found that male Túngara frogs (*Physalaemus pustulosus*) change their mating calls in urban environments, which consequently attracts more females than their forest counterparts (Halfwerk et al., 2019). Examples are also found in invertebrates: in damselflies, increased flight endurance was detected, a trait that is reinforced by sexual selection (Tüzün et al., 2017).

One of the major changes in urban environments is the increase in habitat fragmentation (Dobbs et al., 2017). This leads to reduced gene-flow and genetic drift, which can lead to non-adaptive evolution (Miles et al., 2019), especially in less mobile species (Balbi et al., 2018; Denny, 1980). Research on the population structure of land snails has shown that these organisms are highly affected by urbanization (Hodges & McKinney, 2018). In addition, it is hypothesized that, because of land snails' limited dispersal and high dispersal costs (Denny, 1980), urbanization can result in a decrease in population density and an increase in population fragmentation.

In hermaphroditic land snails, male reproductive organs are under evolutionary pressure caused by population density, with a higher population density correlating with a longer love-dart and larger mucus-producing glands (Abdelli et al., 2018; Lodi et al., 2017). Furthermore, it is also known that the male and female reproductive system of land snail co-evolve over time (Koene & Schulenburg, 2005; Sauer & Hausdorf, 2009). Therefore, urbanization could indirectly play a role in sexual selection in land snails, as an increase in urbanization is expected to lead to a decrease in population density, which in turn could lead to a decrease in the size of both the male and female reproductive system, when compared to their non-urban counterparts.

This study, therefore, focused on the combination of the above-mentioned factors, namely: population fragmentation and rapid evolution by sexual selection along urbanization gradients. Specifically, how does urbanization affect the co-evolution of the male spermatophore-producing and the female spermatophore-receiving organs in the grove snail, *Cepaea nemoralis*? Previous research has shown that an increase of urbanization correlates with the decrease of the population density of land snails (Hodges & McKinney, 2018). Combined with the hypothesis that a higher population density results in larger male reproductive organs and the co-evolution of both the male and female reproductive organs, our expectation was that this would result in smaller reproductive organs in more urban areas. This is due to the combination of a decreased population density in urban environments (Hodges & McKinney, 2018) and the correlation between reproductive organs and population density (Abdelli et al., 2018; Lodi et al., 2017). Therefore, due to decreased sexual competition for mates in urban environments we expected smaller reproductive organs.

2 | METHODS

2.1 | Collection

Adult *Cepaea nemoralis*, Linnaeus, 1758 were collected in three cities in the Netherlands: Amsterdam, Leiden, and Utrecht. Individuals in Amsterdam and Leiden were collected along previously determined urban-rural transects (Santangelo et al., 2022); the transect in Utrecht was newly created (for maps of the transects, see Supplementary Material S1). Each transect sampling point was classified into one of three categories: Urban, Suburban, and Rural. In each of the three cities, 30 fully developed individuals were collected for each category, totaling 90 per city; one additional individual was collected in suburban Utrecht, totaling 271 individuals. Only fully developed individuals were collected. Individuals were relaxed in water without air bubbles at room temperature for 24 hours. Then, they were labelled and put into 70% ethanol at room temperature. The ethanol was replaced once after 7 days and the specimens were stored until dissection.

Before dissection, the volume of each individual was estimated by taking the shell height (the length of the central axis of the shell from the apex to the basis of the aperture) and width (from the umbilicus to the right and left edge of the shell) with calipers, measured in millimeters to 2 decimal points (Figure 1). The volume was then calculated using the formula of Locher and Baur (2000): $\text{Volume} = 0.312 \times \left(\text{shell width}^2 \times \text{shell height}\right) - 0.038$. During dissection, the snail was carefully removed from the shell, after which the reproductive system was dissected with fine forceps and stored in labelled 1.5 mL tubes with 70% ethanol.

2.2 | Photographs and measurements

The genital system was extended and pinned on a soft dark background and photographed (Figure 2). The following characteristics of the reproductive system were measured with ImageJ software (Supplementary Material S2): total length of mucus glands, length of phallus, length of epiphallus, length of flagellum, length of bursa tract, and length of diverticulum. To ensure accurate measurements, we kept tension on the elastic parts to a minimum and they were never stretched in such a way that it influenced the thickness of a coiled part. Furthermore, the continuous length of the parts was measured in centimeter to 2 decimal points, therefore the length of stretched-out coiled parts was still accurately measured. Next, the dart-sac was removed and the volume was determined by using the

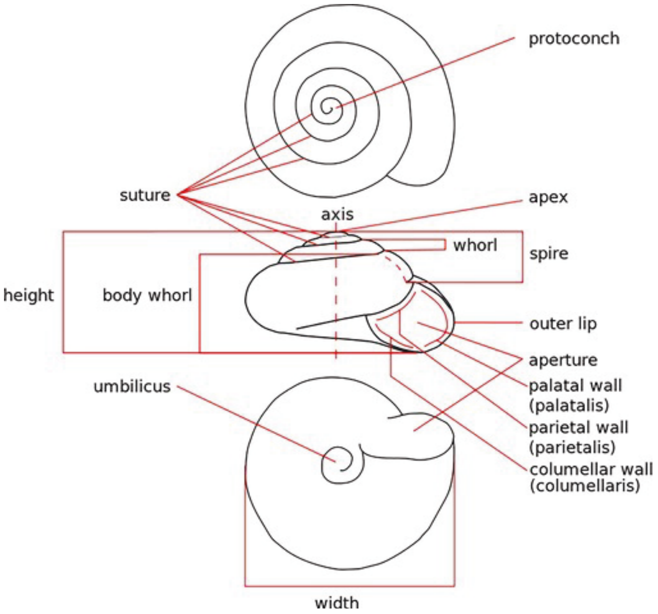

FIGURE 1 Diagram of a snail shell. (Maňas, 2009).

Archimedes principle (Hughes, 2005). Then, the dart-sac was left to dry and the dry mass was measured with a Sartorius BP 2215 precision scale, weight in milligrams to 1 decimal point. The love-dart was isolated by dissolving the dart-sac in 2M NaOH for 2 days at room temperature, and left to dry for another 2 days, before being hand scanned with a NeoScan N80 micro-CT-scanner to determine the cross-sectional area.

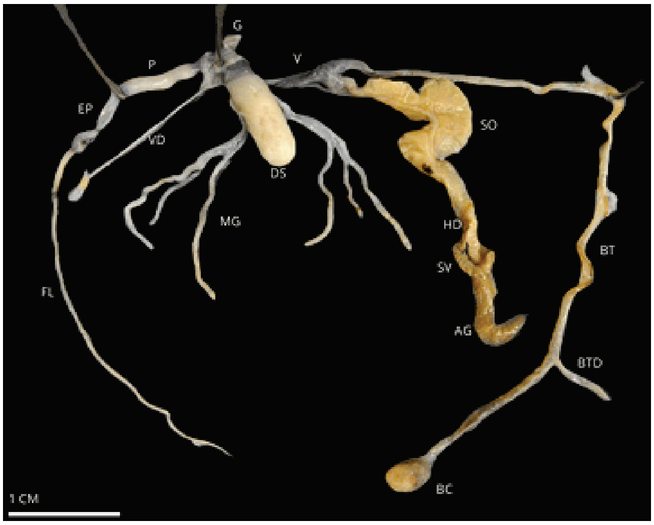

FIGURE 2 Photograph of the reproductive system of *C. nemoralis*. Abbreviations: AG - albumen gland, BC - bursa copulatrix, BT - bursa tract, BTD - bursa tract diverticulum, DS - dart sac, EP – epiphallus, FL – flagellum, G - genital pore, HD - hermaphroditic duct, MG - mucus glands, SV – seminal vesicels, P – phallus, SO – spermoviduct, V - vaginal duct, VD - vas deferens.

2.3 | Statistical analysis

The full list of specimens, their collection details and locations along the transects, and the morphological measurements are given in Supplementary Material S3. Statistical models were made for every part of the reproductive system. Individuals were considered as individual replicates. Linear Mixed Effect models were created with habitat and shell volume as explanatory variables and city where the snails were collected included as a random effect. All models were simplified and the Akaike information criterion was used to determine the best fitting model. After the best model was selected, assumptions like normal distribution and homoscedasticity were tested. If the best model did not meet the assumptions, the model was transformed using Tukey's Ladder of Powers. All final models, including the factors and transformations, can be found in Supplementary Material S4. For ANCOVA analyses, all models met the assumptions of linear relationships, correlation, variance, and normal distribution. P-values were later corrected for false discovery rate using the method described by Benjamini and Hochberg (1995). All analyses were conducted in R (R Core Team, 2014). Outliers were carefully considered; ultimately there was no valid reason to exclude the outliers, therefore all values were included in the analysis.

3 | RESULTS

When comparing the morphological measurements across the three different habitat types, there were significant differences in length of the flagellum, length of the epiphallus and flagellum combined, and length of the bursa tract.

For length of the flagellum, a significant difference was found when comparing the three habitats overall (ANOVA, P-adj. = 0.0006, *F* = 7.397, df = 2, 268, Figure 3, Supplementary Material 5). Post-hoc analysis also showed a significant difference between the length of the flagellum in individuals from rural habitats (mean ± SD: 4.31 ± 0.71 cm, sample size = 90), when compared to urban and suburban habitats (respectively, 3.89 ± 0.72, sample size = 90 and 4.08 ± 0.75 cm, sample size = 91), with the flagellum being significantly longer in rural habitats compared to urban habitats (Post-hoc Tukey, P-adj. = 0.0002) and suburban habitat (post-hoc Tukey, *p* = 0.027).

For the combined length of epiphallus and flagellum, a significant effect was also observed when comparing the three habitats overall (ANOVA, P = 0.0007, *F* = 7.569, df = 2, 268, Figure 4, Supplementary Material S5). Post-hoc analysis again also showed a significant difference between the length of epiphallus+flagellum in individuals from rural habitats (mean ± SD: 4.99 ± 0.78, sample size = 90), when compared to urban and suburban habitats (respectively, 4.53 ± 0.80, sample size = 90 and 4.73 ± 0.83, sample size = 91), with the combined epiphallus+flagellum length greater in rural compared to urban habitats (Post-hoc Tukey, P = 0.0001) and suburban habitats (Post-hoc Tukey, P = 0.021). There was no significant difference between urban and suburban habitats, and there was also no effect

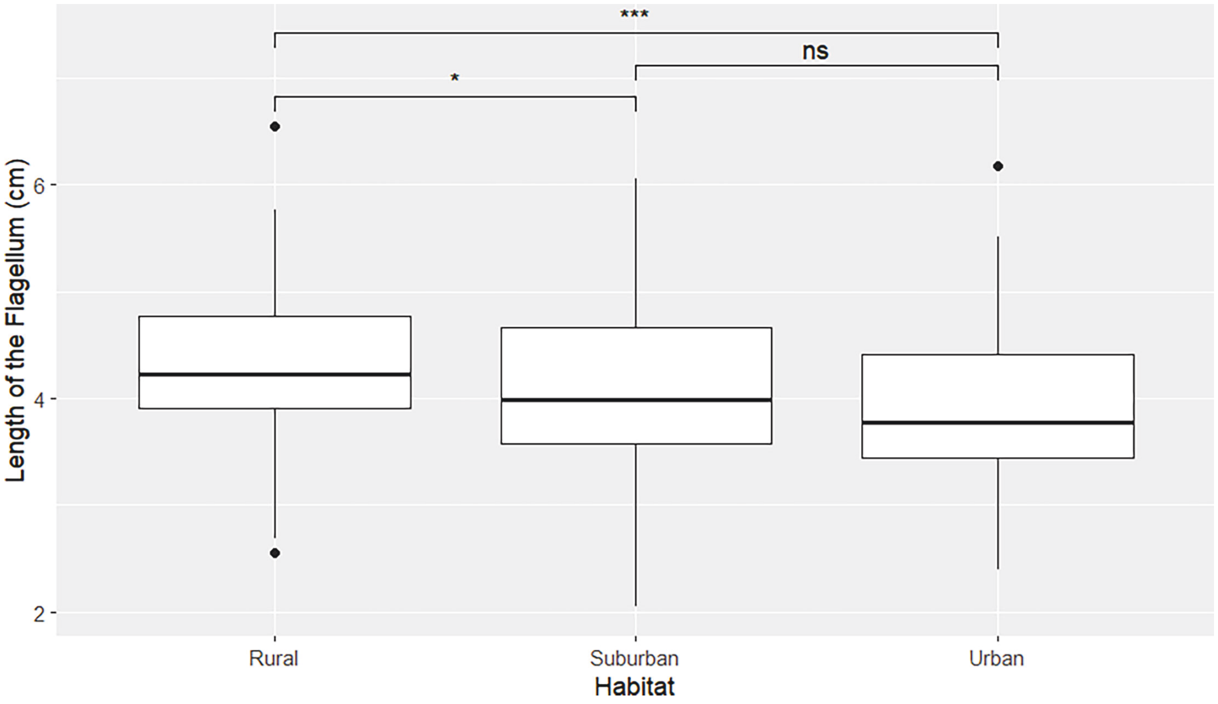

FIGURE 3 ANOVA; Boxplot of flagellum length for the different habitat types. The boxplot shows the means, 1st and 3rd quartiles, ranges, outliers, and significance levels. NS is P-value > 0.05, * is P-value < 0.05, *** is P-value < 0.001. • represents outliers.

of shell volume of the combined length of the epiphallus and flagellum.

For the bursa tract length, there was a significant effect of both habitat and volume, as well as a significant interaction between them (ANCOVA, P-adj. < 0.001, $F = 6.029$, df = 5, 265, Figure 5, Supplementary data S5). The analysis showed that when comparing the intercept of the different habitats, there is a significant difference of the intercept of the urban habitat (mean ± SD: 4.87 ± 1.03) compared to the rural habitat (5.21 ± 1.15) (ANCOVA, P-adj. = 0.048, $t = 2.338$). There was no significant difference between the intercepts of the urban (4.87 ± 1.03) and suburban habitats (4.86 ± 0.96) and between rural and suburban habitats. The slopes of the different habitats show a significant difference between urban and rural (ANCOVA, p-adj. = 0.028, $t = 2.656$). There is no significant difference when comparing the slopes of rural with suburban habitats and urban with suburban habitats. However, the slope of the urban habitat does not significantly differ from zero, indicating that there is no significant effect of volume when comparing bursa tract length in urban habitats.

Besides the comparison between the three habitats, there were also parts of the reproductive system that showed significant correlation with the volume of the shell of the individuals. These are summarized in Table 1, which also includes correlations that were not significant. Futhermore, we tested the effect of shell volume on the length of the diverticulum and glands length, as well as the parts of the reproductive systems. These did not show significance when looking at the effect of urbanization (summarized in Table 1).

4 | DISCUSSION

Our results suggest that both urbanization and shell volume play important roles in the size of parts of the reproductive system of the simultaneously hermaphroditic grove snail. Significant effects were found in traits associated with both the male (spermatophore-producing) and female (spermatophore-receiving) parts of the reproductive system when comparing the different habitats. Furthermore, male and female traits of the reproductive system were also correlated with each other. It was found that the combined length of the epiphallus and flagellum, the place where the body and the tail of the spermatophore, respectively, are created, was correlated with both length of the bursa tract and the diverticulum. The latter are both part of the spermatophore receiving organ. These results show that the reproductive system of *Cepaea nemoralis* is highly complex and under influence of several factors, including urbanization, but also body size and co-evolutionary factors.

Shell volume was used as a standard proxy for body size, and we saw an effect of body size on both the male and female part of the reproductive system. For the male part, the total length of all the mucus glands, and for the female part, the length of the bursa

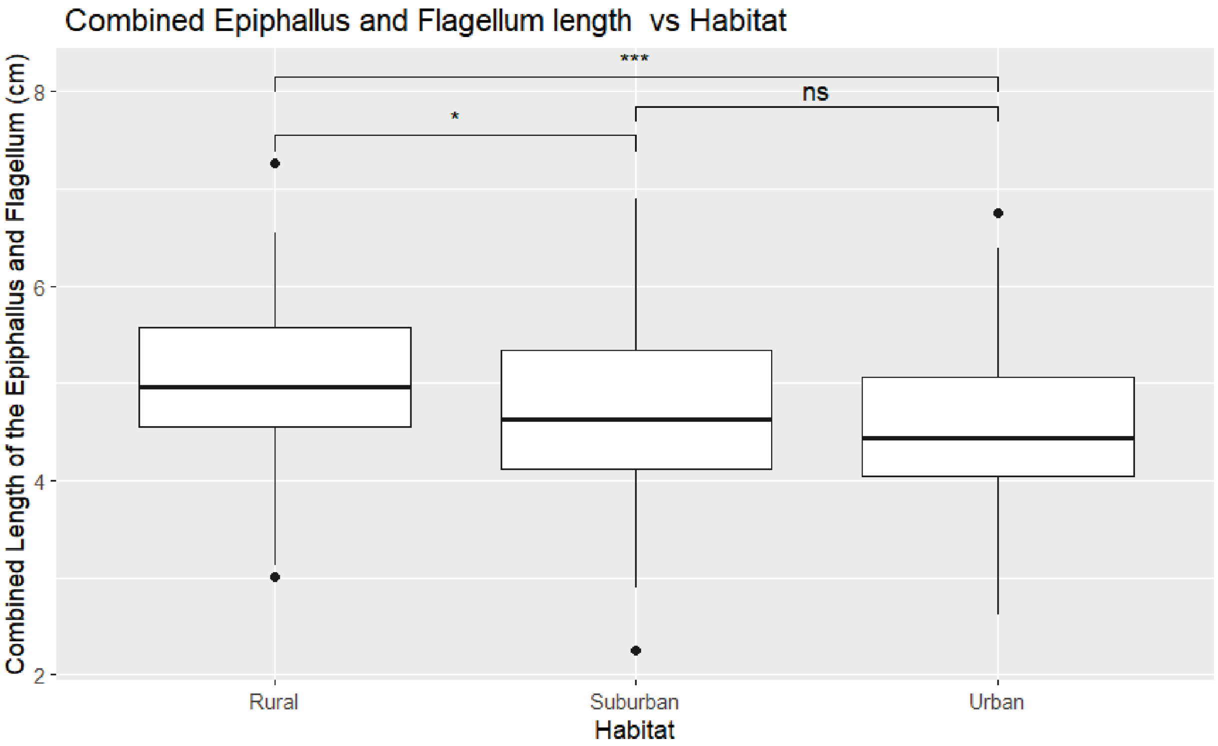

FIGURE 4 ANOVA; Boxplot of combined lengths of epiphallus and flagellum for the different habitat types. The boxplot shows the means, 1st and 3rd quartiles, ranges, outliers and significance levels. NS is P-value > 0.05, * is P-value < 0.05, *** is P-value < 0.001. • represents outliers.

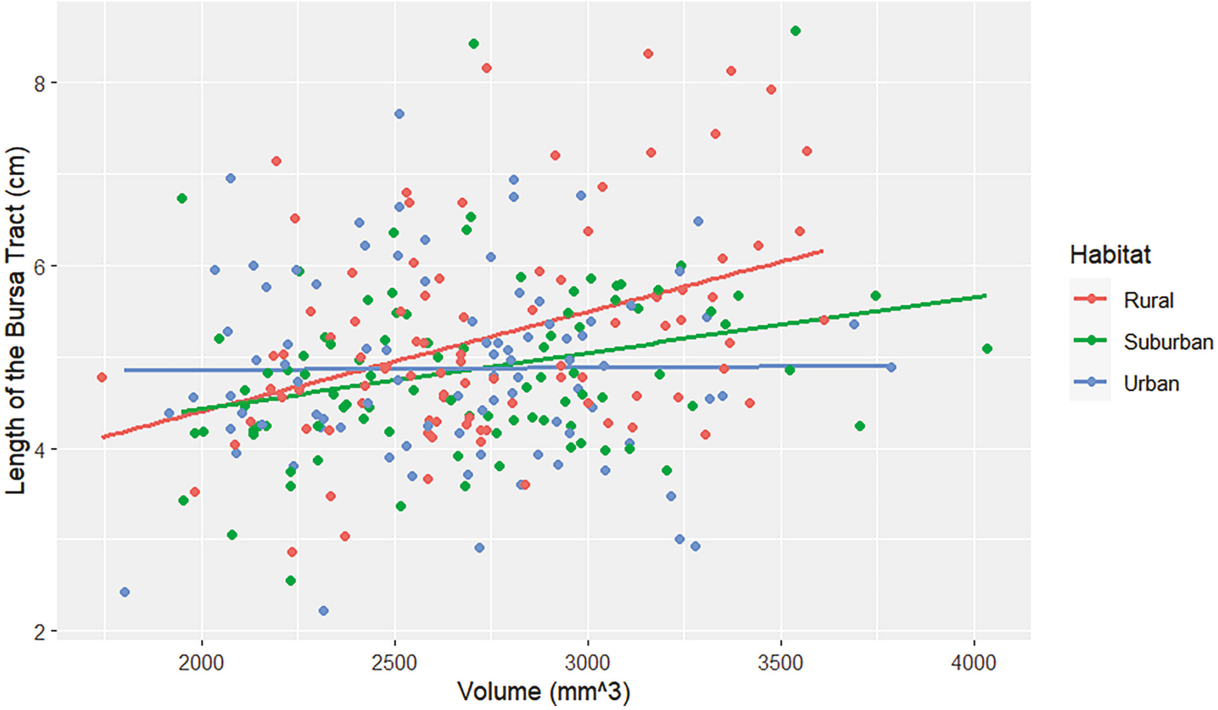

FIGURE 5 Plot of the length of the bursa tract against the volume of the shell, colored by Habitat type. Dots represent the individual measurements that are accompanied by their fitted slope line.

TABLE 1 Table of additional measured effects, including correlation coefficients of significantly correlated reproductive organs. Analyses were perfomed on the total sample size, independant of habitat.

Trait	Adjusted P-Value	Correlation coëfficient
Effect of shell volume on the diverticulum	0.048	-
Effect of shell volume on the total gland length	0.048	-
Correlation between the combined length of the epiphallus and flagellum and the length of the bursa tract	7.8 x e^{-11}	0.163
Correlation between the combined length of the epiphallus and flagellum and the length of the SRO	6.79 x e^{-10}	0.145
Correlation between the length of the flagellum and SRO	1.17 x e^{-8}	0.127
Effect of urbanization on the length of the epiphallus	0.14	-
Effect of urbanization on the density of the dart-sac	0.993	-
Effect of urbanization on the cross-sectional area of the love dart	0.502	-

tract and the diverticulum were correlated with body size. Both of these findings are interesting, because in general for invertebrates, the size of genitalia does not generally, or at least not strongly, increase with body size (Eberhard et al., 1998; Eberhard, 2009). Another pattern we uncovered was that the male and female parts of the reproductive system (epiphallus+flagellum and bursa+diverticulum, respectively) showed a significant positive correlation with each other. In other words, individuals with a larger male reproductive system also had a larger female reproductive system, independent of body size. Previous research has already shown that the male and female reproductive systems are probably co-evolving (Koene & Schulenburg, 2005; Sauer & Hausdorf, 2009). Research had also shown that the length of the flagellum correlates with the spermatophore-receiving organ (Koene & Schulenburg, 2005). Therefore, our results support previous work.

For both male function (length of the flagellum and the combined length of the epiphallus and flagellum) and female function (length of the bursa tract), we found that lengths were significantly greater in rural habitats than urban and suburban habitats. This pattern supports our expectation that cities fragment and reduce populations (Hodges & McKinney, 2018), which leads to reduced sexual selection and consequently smaller sizes for organs involved in sperm competition (Lodi et al., 2017). Something that is also important to consider is that in different cities the urban, suburban, and rural habitats may differ from each other not just in population structure, but also in other biotic and abiotic factors. Such differences might play an important role in determining the effects of urbanization, as previous research has shown that micro-scale differences in environments can change how organisms within the same city adapt to urbanization (Santangelo et al., 2022). It is known that land snails are sensitive to micro-scale changes in environments (Nandy et al., 2022). A possible reflection of this can be seen in the results for the length of the phallus, where there is a significant effect of the city in which the individual was collected. Small-scale differences among cities in biotic and abiotic factors may be why no comprehensive effects of urbanization were found for the phallus and may consequently also affect other parts of the reproductive system. Therefore, future studies should pay further attention to such effects, to determine a more comprehensive understanding of how urbanization affects sexual selection in snails.

In conclusion, the effects observed in this study help us gain a better understanding of how urbanization can play a role in sexual selection. The results suggest that, under reduced sexual competition for mates, selection for large spermatophores with long tails may be relaxed, which, in a correlated evolutionary response, also reduces the size of the spermatophore receiving organ that digests it. For the female spermatophore receiving organs, overall body size also plays an important role. In addition, a correlation between the male spermatophore-producing and female spermatophore-receiving organs was found. Our study also triggers new questions on the effect of micro-climates in urbanized regions, how body size, which is known to be impacted by urban evolution (Merckx et al., 2018), influences sexual selection, and the mechanisms of interaction between the spermatophore-producing and spermatophore-receiving organs under changes in sexual competition for mates. Finally, although we assumed the effects of urbanization uncovered were evolutionary, a role for phenotypic plasticity cannot be excluded.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest

AUTHORS' CONTRIBUTIONS

The research was performed by the corresponding author and supervised by the co-authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in de summmplementary data or from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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

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