JOURNAL OF
EMERGING INVESTIGATORS

Article

Male Feminization of the Common Pillbug *Armadillidium vulgare* by *Wolbachia* bacteria

Lakshana Ramanan[1], Jay Lothe[1], Saanvi Prasad[1], Robert Kuhn[1]
[1] Innovation Academy, Alpharetta, Georgia

SUMMARY

***Wolbachia pipientis (Wolbachia)* is a maternally inherited endosymbiotic bacterium that infects over 50% of arthropods, including pillbugs, and acts as a reproductive parasite in the host. In the common terrestrial pillbug *Armadillidium vulgare (A. vulgare)*, *Wolbachia* alters the sex ratio of offspring through a phenomenon called feminization, where genetic males develop into reproductive females. Previous studies have focused on the presence or absence of *Wolbachia* as a sex ratio distorter in laboratory cultured and natural populations mainly from sites in Europe and Japan. Our three-year study is the first to evaluate the effects of the *Wolbachia* sex ratio distorter in cultured *A. vulgare* offspring in North America. We asked whether *Wolbachia* bacteria feminize *A. vulgare* isopod male offspring from infected mothers and if this effect can be detected in F1 offspring by comparing the male/female offspring ratios. If so, the F1 offspring ratio should show a higher number of females than males compared to the offspring of uninfected mothers. Over three years, pillbug offspring were cultured from pregnant *A. vulgare* females and developed into adults. We determined the *Wolbachia* status of mothers and counted the ratios of male and female F1 progeny to determine feminization effects. In each year sampled, significantly more female offspring were born to *Wolbachia*-infected mothers than those from uninfected mothers. These ratio differences suggest that the *Wolbachia* infection status of mothers directly impacts the *A. vulgare* population through the production of reproductive feminized males, which in turn provides an advantage for further *Wolbachia* transmission.**

INTRODUCTION

Wolbachia is an intracellular bacterium found in 50% of all arthropods, including insects and crustaceans. *Wolbachia* are maternally inherited, obligate endosymbionts (1). Since males cannot transmit *Wolbachia* to offspring, *Wolbachia* bacteria often act selfishly with host-specific effects on reproduction. These effects include male killing, cytoplasmic incompatibility, and feminization (2). Male killing has been documented in ladybirds and some species of butterfly, in which *Wolbachia* infection leads to the embryonic death of males due to DNA damage and repression of specific genes that control cell division and development (3). Cytoplasmic incompatibility is the most common type of reproductive disruption by *Wolbachia*. When *Wolbachia*-infected males mate with uninfected females, gene expression in male testes causes improper segregation of chromosomes, resulting in a failure to form embryos (4).

Feminization by *Wolbachia* is seen in isopods, including the common terrestrial pillbug *Armadillidium vulgare (A. vulgare)*. Feminization results in chromosomal males developing into reproductive females. In uninfected *A. vulgare* populations, a balanced ratio of males and females is expected. However, in populations where *Wolbachia* infection occurs, female bias is common (5). Feminization from cytoplasmic *Wolbachia* infection results in a reproductive benefit for the bacteria because *Wolbachia* are maternally transmitted to future offspring. Normally, sex determination is controlled by genes found in nuclear DNA. Imbalances of male/female ratios could lead to increased fitness for *Wolbachia* at the cost of decreased fitness for infected pillbugs (6).

Genetic processes like feminization, that skew expected male to female ratio in populations, are referred to as sex-ratio distorters (7). *A. vulgare* has a sex determination system with two chromosomes, Z and W, in which ZW organisms are female, and ZZ organisms are male. At birth, there is no differentiation between sexes, but shortly after birth, males differentiate through the secretion of an androgenic hormone (8). Feminization of isopod males is due to the *Wolbachia* interfering with male hormone-producing androgenic glands (9). In these cases, feminized ZZ males increase in the population, and there is a shift from chromosomal sex determination to *Wolbachia*-induced cytoplasmic sex determination (10). Over time, cytoplasmic feminization replaces the natural occurrence of the W chromosome, which is eventually lost in the population (11).

Feminization in *A. vulgare* has been well studied but limited to Europe and Japan and in these studies, molecular methods were used to identify proportions of multiple sex ratio distorters in non-generational laboratory populations (5,7,9,12,13). One additional study looked at the prevalence of sex ratio distorters by culturing offspring and assessing the *Wolbachia* status of the mother (12). In this study, the main goal was to explore the relationship between mitochondrial DNA (mtDNA) variation, the prevalence of feminization, and their impact on population structure.

Here, we studied two populations of *A. vulgare* in the southeastern United States, an unstudied region. First, we documented the occurrence of *Wolbachia* infection in pillbug mothers. We further determined the prevalence of *Wolbachia* as a feminizing sex ratio distorter by comparing infection status of mothers and the sex ratios of offspring. Finally, we investigated whether the feminization effects in these populations are being maintained over time.

We hypothesized that if feminizing *Wolbachia* bacteria

https://doi.org/10.59720/23-299

were present in pillbug mothers, the ratio of the resulting offspring, the F1 generation, would show a higher proportion of females to males compared to the offspring of uninfected mothers. By counting the F1 offspring of infected and uninfected mothers, we found that the proportion of female offspring was significantly higher in infected mothers than in uninfected mothers. Our findings provide insights into the potential impact of *Wolbachia* on pillbug reproduction and contribute to our understanding of the interplay between parasitic microorganisms and the evolution of their host species. The feminization of pillbug offspring due to *Wolbachia* infection is an important area of research and has significant implications for the evolutionary population dynamics of these isopods in their widespread natural habitat. The geographical spread of *Wolbachia* as a sex ratio distorter of *A. vulgare* is largely unknown in the United States and our study serves as a starting point for future investigations.

RESULTS

Each year a new population of pregnant female *A. vulgare* pillbugs was sampled at the same locations. After birth of pillbug young, we used the molecular methods of DNA extraction, PCR, and gel electrophoresis, to assess *Wolbachia* infection status in the mothers. In each year of our three-year study (2019-2021), the proportion of females was higher in offspring of *Wolbachia*-infected mothers than in those from uninfected mothers**.** In 2019 and 2020, 91% of 370 individuals and 93% of 380 individuals were female, respectively. In 2021, 78% of 274 individuals were female **(Figure 1)**. In 2019 and 2020, the mean ratios of female progeny from uninfected mothers were similar, 52% and 56%, respectively, but in 2021, the mean female ratio, 28%, was much lower **(Figure 1)**. This discrepancy may be partly explained by the fact that two cultures from 2021 had no female progeny (**Table 1**). We cannot definitively explain why no females were counted for these cultures but is possibly an artifact of offspring survival in culture. While we set a lower limit of at least 10 offspring per soil culture for culture inclusion in the study, we did not set an inclusion limit for the male/female percentage mainly because of the male reduction potential due to feminization.

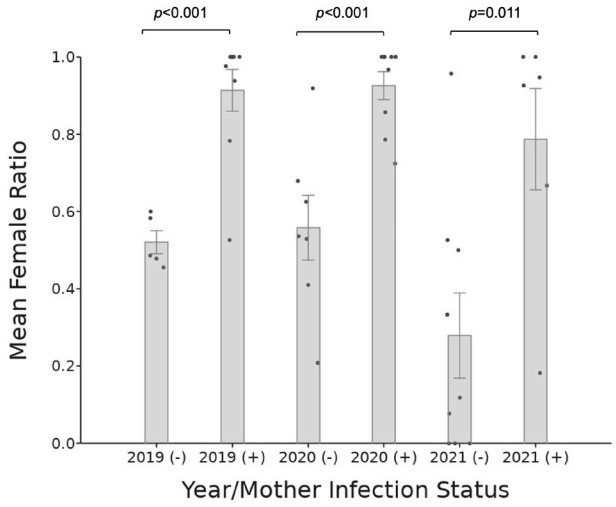

Figure 1: Mean female ratio in pillbug offspring from 2019 to 2021. Mean female ratio comparison of offspring produced from *Wolbachia* uninfected (-) and infected (+) pillbug mothers. Each data point represents the offspring ratio of females to males from a single mother. Error bars are ± 1 SEM. Mean female offspring ratio was significantly higher for mothers who were infected with *Wolbachia* (2019 *p*-value < 0.001, n = 370; 2020 *p*-value < 0.001, n = 380; 2021 *p*-value = 0.01, n= 274).

Two tailed t-tests were used to directly compare mean female ratios from *Wolbachia*-infected and uninfected mothers from each year sampled. A *p*-value of < 0.05 was used to determine significance level. In all three years, the difference in mean female ratios was found to be statistically significant with *p*-value < 0.001 for 2019 and 2020 and *p*-value = 0.01 for 2021.

A two-way ANOVA test with a significance level of *p*-value < 0.05 was used to evaluate whether a high mean female ratio in offspring was being maintained over the three-year study. The test resulted in a *p*-value of 0.38, indicating that there is no statistical difference in mean female ratio of offspring born to *Wolbachia*-infected mothers. This supports the hypothesis that bias towards females is being maintained in these populations in each year of the study **(Figure 1)**.

DISCUSSION

Our results show a difference in sex ratio between progeny born to infected females versus those born to uninfected females. In each year sampled, progeny ratios were skewed towards females when born from a *Wolbachia*-infected mother. In general, the persistence of high mean female ratios in each culture born to infected females allowed us to conclude that feminization is both strongly present in these populations and that it is being maintained. This finding could have both evolutionary and ecological significance to these populations over time.

Wolbachia transmission rates in populations of *A. vulgare* have been previously estimated to be around 70-80% (14). In one previous study, progenies born to infected females were shown to reach 60-80% (15). However, sex ratios in wild pillbug populations can be variable with expected ratios to be between a balanced 1:1 ratio of males and females and an undetermined upper limit of female bias (16).

Evolutionarily, the terrestrial pillbug *A. vulgare* has become a model organism for sex ratio distorter-induced feminization. In isopods infected with *Wolbachia*, there is a dynamic struggle between genetic sex determination (isopod chromosomes) and cytoplasmic sex determination (*Wolbachia* infection) (17). *A. vulgare* populations not under the influence of sex ratio distorters like *Wolbachia* should be reproductively balanced between genetic males (ZW) and females (ZZ) (5). In these populations, evolutionary fitness is assumed to be determined by the genetic composition of the population over time, under the influence of random and non-random evolutionary forces. Selfish sex-ratio distorters like *Wolbachia* spread at the expense of their host. Since *Wolbachia* is maternally inherited, feminization of males into reproductively capable females gives *Wolbachia* a fitness advantage.

Within the *A. vulgare* population, however, feminization has the potential to influence population survival and mate choice through a decrease in males. As directional selection of females through feminization occurs, intraspecies consequences can arise. One such example is a decrease in fertility caused by sperm depletion as females outnumber males in a population (18). Theoretical models have predicted

https://doi.org/10.59720/23-299

2019				2020				2021			
Mother Wolbachia Status	Progeny % Female	Progeny % Male	N	Mother Wolbachia Status	Progeny % Female	Progeny % Male	N	Mother Wolbachia Status	Progeny % Female	Progeny % Male	N
Negative	48	52	23	Negative	41	59	39	Negative	12	88	17
Negative	45	55	22	Negative	53	47	17	Negative	33	67	39
Negative	49	51	72	Negative	63	37	16	Negative	96	4	23
Negative	58	42	12	Negative	68	32	28	Negative	53	47	19
Positive	100	0	47	Negative	92	8	37	Negative	0	100	20
Positive	53	47	19	Negative	21	79	24	Negative	0	100	11
Positive	94	6	16	Negative	54	46	28	Negative	8	92	13
Positive	100	0	19	Positive	100	0	37	Negative	100	0	24
Positive	78	22	23	Positive	79	21	14	Negative	18	82	11
Positive	100	0	18	Positive	100	0	14	Positive	67	33	21
Positive	98	2	41	Positive	100	0	22	Positive	93	7	27
Positive	100	0	13	Positive	100	0	20	Positive	95	5	19
Positive	100	0	20	Positive	97	3	41	Positive	100	0	30
Positive	100	0	11	Positive	86	14	14				
Positive	100	0	14	Positive	72	20	29				

Table 1: The *Wolbachia* status of pregnant pillbug mothers and the ratio of progeny over the 3-year study period, where N designates the total number of progeny for each culture.

the rates at which feminization might spread within a population (19). While natural populations can experience 80-90% female bias, some models predict that all females should be infected with *Wolbachia*, given enough time (20). Some hypotheses have been proposed as to why large populations never reach 100% feminization. These hypotheses include the risk of extinction due to the reduction in males, sexual selection against *Wolbachia*-infected males and genes found in the nucleus of the male pillbug that may protect against infected females (21). While we do report some progeny from *Wolbachia*-infected females with a 100% female bias, we believe this result is an artifact of culture population size.

Our study had some limitations, primarily with molecular screening of feminized males and sample size. We assumed that a greater mean female ratio in offspring of *Wolbachia*-infected female mothers indicates feminization of males. Ideally, testing females for the presence of male ZZ sex chromosomes would be ideal. Unfortunately, the ability to molecularly test females for ZZ male chromosomes does not exist currently. Once molecular tests are developed, we will incorporate them into future projects.

In addition, strains of *Wolbachia* causing feminization in *A. vulgare* isopods have not been studied in North America like they have in Europe. So far, our samples were limited to a small geographic location. Even though collection times were a year apart, there is a risk of sampling genetically similar populations in sampled locations. We have already begun studying sex ratio distorters in *A. vulgare* from multiple states in the US. We hope to gain greater insight to the spread and impact of Wolbachia with larger sample sizes and greater geographic area. Locally, sampling in the spring and summer of 2021 was limited by the prevalence of SARS-CoV-2. This contributed to fewer cultures being made and less overall pillbugs analyzed. This study provided us a starting point for expanding our study of feminizing sex ratio distorters to populations across the United States.

MATERIALS AND METHODS

Live Culture

Pregnant *A. vulgare* females were collected from two sources: Big Trees Forest Preserve, a protected forest fragment in Sandy Springs, Georgia, United States, and a backyard compost bin in Dunwoody, Georgia, United States. The pillbugs were sexed on site, and pregnant females were identified in the field by their ventral marsupium containing offspring (23). Mothers were placed in a soil litter culture until the offspring were born. Soil cultures contained moist compost and were kept under a 16-hour light period at room temperature (22ºC). Cultures were kept humid by spraying Publix supermarket brand spring water onto the soil monthly. Each week, pillbugs were fed pieces of carrot and dried Linden leaves. Upon giving birth, each mother was euthanized by freezing at -20ºC, placing into 75% ethanol, and storing at -20ºC. The pillbug offspring were allowed to develop in the soil cultures and sexed at three months. The sex of the pillbug was determined by examining the presence or absence of the copulatory first pleopod organ, which is only present in males (24).

Cultures that maintained brood sizes greater than 10 were considered for the study each year. In 2019 and 2020, 15 cultures were used, and individual culture brood sizes ranged from 11 to 72 (**Table 1**). Of these, 11 mothers in 2019 and 8 in 2020 were determined to be *Wolbachia*-positive. In 2021, 13 cultures were used, and individual brood size ranged from 11 to 39. Of the 13 mothers collected, 4 were *Wolbachia*-positive (**Table 1**).

DNA Extraction

In 2019, the New England Biolabs (NEB) Monarch Genomic DNA Purification Kit (T3010S) was used to extract DNA. In 2020 and 2021, Zymo Quick Insect/Tissue DNA kits (D3024) were provided at our new lab and therefore used to extract DNA. Pillbug heads were removed under the microscope using a sterile blade and placed in a 1.5 mL microcentrifuge tube with lysis buffer (NEB) or bead bashing buffer (Zymo). Tissues were lysed either by using a sterile plastic micro pestle (NEB) or bead bashing (Zymo). Spin column purification was used in both kits according to each manufacturer's directions. Both processes yielded 50 µL of eluted DNA, which was then used for polymerase chain reaction (PCR).

Polymerase Chain Reaction

https://doi.org/10.59720/23-299

PCR reactions were set up using a master mix of Promega GoTaq 2x polymerase (M3001), Promega nuclease-free water (MC1191), and forward/reverse oligonucleotide primers. To each reaction, 2 µL of eluted DNA was added. The *Wolbachia* Project at Vanderbilt University supplied *Wolbachia*-positive *Nasonia vitripennis* wasp DNA that was used in positive control reactions and the primer sets used for PCR. In negative control reactions, 2 µL nuclease-free water was used instead of DNA.

The two primer sets were used independently to confirm the presence of *Wolbachia* and to confirm pillbug species. The 16S rRNA portion of the prokaryotic 30S ribosomal subunit is commonly used to identify bacteria. The Wspec forward and reverse primers target the 16S region of *Wolbachia* and were used as a detection assay: F (5'−CATACCTATTCGAA GGGATAG−3') and R (5'−AGCTTCGAGTGAAACCAATTC−3') (25). The following PCR parameters were used: initial denaturation at 94ºC for 120 sec, followed by 34 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC for 45 sec, and extension at 72ºC for 60 sec, then a final extension at 72ºC for 600 sec (25). The cytochrome c oxidase (CO1) region of mitochondrial DNA (mtDNA) is commonly used for DNA barcoding (26). The CO1 region is a good target since eukaryotic cells have many mitochondria per cell and the amplified region of CO1 will be unique to each species (27). The forward and reverse primers used were: LCO11490F (5'-GT AAAACGACGGCCAGGGTCAACAAATCATAAAGATATTGG -3') and HCO2198R (5'-CAGGAAACAGCTATGACT AAACTTCAGGGTGACCAAAAAATCA-3') (27). The following PCR parameters were used: initial denaturation at 94ºC for 120 sec followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 50ºC for 45 sec, and extension at 72ºC for 45 sec, then a final extension at 72ºC for 600 sec (27).

The presence of *Wolbachia* 16S DNA and mitochondrial CO1 mtDNA was verified using gel electrophoresis. For each gel, 5 µL of each PCR product and 10 µL of NEB 100 bp DNA ladder (B7025) was used. *Wolbachia*-positive mothers were confirmed by amplicon bands at 438 bp and CO1 DNA at 710 bp (**Figure 2**). DNA barcoding was used to confirm visually identified pillbug species. Amplified CO1 DNA from PCR was used for Sanger sequencing and performed off-site by GeneWiz.

Counting and Data Analysis

For each culture of pillbug offspring, the number of female and male offspring were counted and recorded. These data were then inputted into an Excel spreadsheet differentiating the groups of mothers by *Wolbachia* infection status. DataClassroom was used for graphing and statistical analysis, including 2-way ANOVA and t-tests.

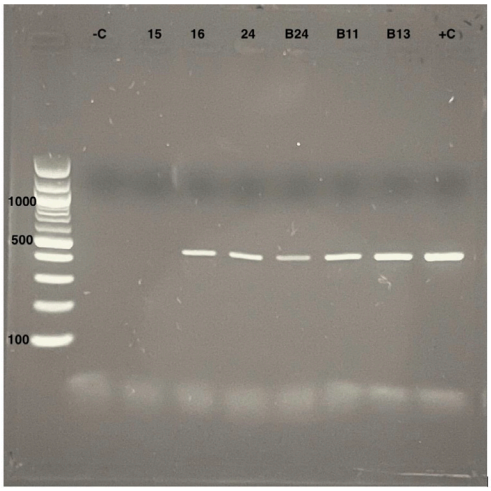

Figure 2: *Wolbachia* 16S DNA gel electrophoresis. 16S *Wolbachia* gel electrophoresis assay showing five pillbug mothers positive for *Wolbachia* infection (gel lanes 4-8) and one negative mother (gel lane 3). Lane 1 shows a 100 bp DNA ladder for size estimation. The negative control (-C, gel lane 2) substituted water for DNA in the PCR, while the positive control (+C, gel lane 8) used DNA extracted from *Wolbachia*-positive *Nasonia vitripennis* wasps. Positive bands on the gel correspond to 438 bp, which is the expected amplicon size for the Wspec 16S *Wolbachia* primers (25).

ACKNOWLEDGMENTS

We would like to thank the following for their help and support to this research: Dr. Richard Cordaux, Diversity Institute Ecology and Evolution of Living Things, University of Paris Saclay for his expertise and mentorship throughout the project. Former student researchers who contributed significantly to the first two years of the project from Centennial High School, Roswell, GA include Allison Diaz-Fernandez, Hope Begashaw, Garrison Svay, and Zachary Svay (2019 -2020), and Carley Chinn, Ally Feinstein, and Sophia Laidhold (2020-2021). Zymo Research for their generous donation of DNA extraction kits. Aaron Reedy at dataclassroom.com for statistical and graphing support. The Bordenstein Lab and the *Wolbachia Project* at Penn State University for supplying positive controls, and primer reagents.

Received: November 09, 2023
Accepted: February 17, 2024
Published: June 30, 2024

REFERENCES

1. Weinart, et al. "The incidence of bacterial endosymbionts in terrestrial arthropods." *Proc.R.Soc.Lond.B Biol. Sci.*, vol. 282, May 2015. https://doi.org/10.1098/rspb.2015.0249.
2. Werren, J., et al." Wolbachia*:* master manipulators of invertebrate biology." *Nat Rev Microbiol,* vol. 6, pp.741–751, Oct. 2008. https://doi.org/10.1098/rspb.2015.024910.1038/nrmicro1969.
3. Hurst, GDD, et al. "Male killing Wolbachia in two species of insect." *Proc. R. Soc. B Biol. Sci.*, vol. 266, pp. 735-740, April 1999. https://doi.org/10.1098/rspb.1999.0698.
4. Kaur, Rupinder, et al. "Living in the Endosymbiotic World of *Wolbachia*: A Centennial Review." *Cell Host & Microbe*, vol. 29, no. 6, pp. 879–893, June 2021, https://doi.org/10.1016/j.chom.2021.03.006.
5. Cordaux, R.; Gilbert, C. "Evolutionary Significance of *Wolbachia*-to-Animal Horizontal Gene Transfer: Female Sex Determination and the *f* Element in the Isopod *Armadillidium vulgare*." *Genes*, vol. *8*, no.186, July 2017, https://doi.

https://doi.org/10.59720/23-299

org/10.3390/genes8070186.
6. Correa, Claudia, et al. "Wolbachia associations with insects: winning or losing against a master manipulator." *Sec. Evolutionary and Genomic Microbiology*, vol. 3, Jan 2016. https://doi.org/10.3389/fevo.2015.00153
7. Rigaud T, Juchault P. "Conflict between feminizing sex ratio distorters and an autosomal masculinizing gene in the terrestrial isopod Armadillidium vulgare." *Latr. Genetics*, vol. 133, no.2, pp. 247-52, Feb. 1993, https://doi.org/10.1093/genetics/133.2.247.
8. Rigaud T., et al. "Feminizing endocytobiosis in the terrestrial crustacean Armadillidium vulgare Latr. (Isopoda): Recent acquisitions." Endosymbiosis and Cell Research, vol.7, pp. 259-273, Jan. 1991.
9. Becking, T., Giraud, I., Raimond, M. et al. "Diversity and evolution of sex determination systems in terrestrial isopods." *Sci Rep,* vol. 7, no. 1084, April 2017, https://doi.org/10.1038/s41598-017-01195-4.
10. Rigaud, Thierry. "Inherited microorganisms and sex determination of arthropod hosts." *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*, chapter 3, pp. 81-101, Oct. 1997. https://doi.org/10.1093/oso/9780198577867.003.0003
11. Leclercq, Sébastien, et al. "Birth of a W Sex Chromosome by Horizontal Transfer of *Wolbachia* Bacterial Symbiont Genome." *Proceedings of the National Academy of Sciences*, vol. 113, no. 52, pp. 15036–15041, Dec. 2016, https://doi.org/10.1073/pnas.1608979113.
12. Rigaud, Thierry. "Mitochondrial DNA polymorphism, sex ratio distorters and population genetics in the isopod *Armadillidium vulgare*." *Genetics Society of America*, vol. 152, pp. 1669-1677, August 1999. https://doi.org/ 10.1093/genetics/152.4.1669
13. Durand S. et al. "Heterogeneous distribution of sex ratio distorters in natural populations of the isopod *Armadillidium vulgare*." *Biol Lett*., Vol.19, no.1, Jan. 2023, https://doi.org/10.1098/rsbl.2022.0457.
14. Cordaux, R. et al. "Evidence for a new feminizing Wolbachia strain in the isopod Armadillidium vulgare" evolutionary implications." *Heredity*, vol.93, pp. 78-84, May 2004, https://doi.org/10.1038/sj.hdy.6800482.
15. Chevalier, F. et al. "Feminizing *Wolbachia*: a transcriptomics approach with insights on the immune response genes in *Armadillidium vulgare*." *BMC Microbiol* vol., vol12 (Suppl 1), S1, Jan. 2012, https://doi.org/10.1186/1471-2180-12-S1-S1.
16. Becking T. et al. "Diversity and evolution of sex determination systems in terrestrial isopods." *Sci Rep.,* vol. 7, no.1, April 2017, https://doi.org/10.1038/s41598-017-01195-4.
17. Rigaud, T. et al. "The evolution of sex determination in isopod crustaceans." *BioEssays*, no.19, vol.5, pp. 409-416, Feb. 1997, https://doi.org/10.1002/bies.950190508.
18. Rigaud T, Moreau J. "A cost of Wolbachia-induced sex reversal and female-biased sex ratios: decrease in female fertility after sperm depletion in a terrestrial isopod." *Proc Biol Sci.,* vol. 271 no. 1551, pp. 1941-6, Sept. 2004, https://doi.org/.1098/rspb.2004.2804.
19. Cordaux, R. et al. "*Wolbachia* infection in crustaceans: novel hosts and potential routes for horizontal transmission." *Journal of Evolutionary Biology,* vol.14, pp. 237–243, Dec. 2001, https://doi.org/10.1046/j.1420-9101.2001.00279.x.
20. Hatcher, M.J. "Persistence of selfish genetic elements: population structure and conflict." *Trends Ecol. Evol*, vol. 15, pp. 271–277, July 2000, https://doi.org/10.1016/S0169-5347(00)01875-9.
21. Verne, S. et al. "Effects of parasitic sex-ratio distorters on host genetic structure in the Armadillidium vulgare–Wolbachia association." *Journal of Evolutionary Biology,* December 2011, https://doi.org/10.1111/j.1420-9101.2011.02413.
22. Bouchon, D. et al. "The Terrestrial Isopod Microbiome: An All-in-One Toolbox for Animal-Microbe Interactions of Ecological Relevance. *Frontiers*, vol. 7, September 2016, https://doi.org/10.3389/fmicb.2016.01472.
23. Surbida, K.L. et al. "Embryo Tolerance and Maternal Control of the Marsupial Environment in *Armadillidium vulgare* Isopoda: Oniscidea)." *Physiological and Biochemical Zoology*, vol. 74, pp. 894-906, Nov. 2001, https://doi.org/10.1086/324474 .
24. "Featured Creatures: *Armadillidium vulgare*." *Entomology Department University of Florida*. https://entnemdept.ufl.edu/creatures/MISC/Armadillidium_vulgare.htm. Accessed 2019-2021.
25. Bordenstein, Seth R., et al. "Using the *Wolbachia* Bacterial Symbiont to Teach Inquiry-Based Science: A High School Laboratory Series." *The American Biology Teacher*, vol. 72, no. 8, pp. 478–83, Oct. 2010, https://doi.org/10.1525/abt.2010.72.8.3.
26. Folmer, O. "DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates." *Molecular Marine Biology and Biotechnology*, vol. 3, no. 5, Oct. 1994.
27. "DNA Barcoding 101: Using DNA barcodes to identify and classify living things." *Cold Springs Harbor Laboratory DNA Learning Center*. https://dnabarcoding101.org/files/using-dna-barcodes.pdf. Accessed 2019-2022.