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Article

# Harvesting Silk Fibers from *Plodia interpunctella*: Role of Environmental Rearing Conditions in Fiber Production and Properties

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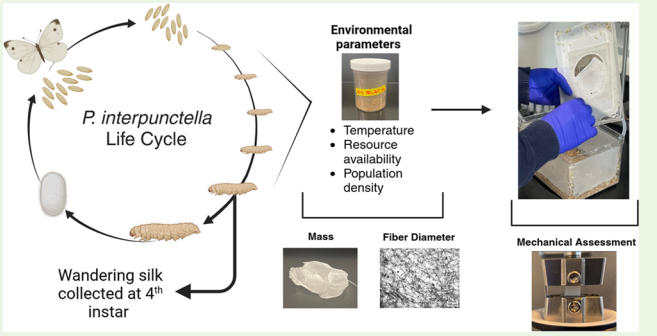
**ABSTRACT:** Silk fibers are produced by a wide variety of insects. The silkworm *Bombyx mori* (*Bombyx*) was domesticated because the physical properties of its silk fibers were amenable to the production of fine textiles. Subsequently, engineers have regenerated silk fibroin to form biomaterials. The monocular focus on *Bombyx* silk has underutilized the expanse of diverse silk proteins produced by more than 100,000 other arthropods. This vast array of silk fibers could be utilized for biomedical engineering challenges if sufficient rearing and purification processes are developed. Herein, we show that the moth, *Plodia interpunctella* (*Plodia*), represents an alternative silk source that is easily reared in highly regulated culture environments allowing for greater consistency in the silk produced. We controlled the temperature, resource availability (larvae/gram diet), and population density (larvae/mL) with the goal of increasing silk fiber production and improving homogeneity in *Plodia* silk proteins. We determined that higher temperatures accelerated insect growth and reduced life cycle length. Furthermore, we established initial protocols for the production of *Plodia* silk with optimal silk production occurring at 24 °C, with a resource availability of 10 larvae/gram and a population density of 0.72 larvae/mL. Population density was shown to be the most prominent driving force of *Plodia* silk mat formation among the three parameters assessed. Future work will need to link gene expression, protein production and purification, and resulting mechanical properties as a function of environmental cues to further transition *Plodia* silk into regenerated silk fibroin biomaterials.




**KEYWORDS:** *silk fibroin, biomaterials, insect biopolymers, entomology, natural biopolymers*

## 1. INTRODUCTION

*Plodia interpunctella* (hereafter referred to as *Plodia*), commonly known as the pantry moth or Indianmeal moth, is a Lepidopteran insect that has been extensively explored as an agricultural pest,[3−7] but *Plodia* also has potential utility in the biomaterial community as a nonmulberry silk-producing insect. While traditional mulberry silkworms (*Bombyx mori*, hereafter referred to as *Bombyx*) are the most studied silk-producing insects for biopolymer-based biomaterial design and applications,[8−10] *Plodia* has several potential advantages[11,12] that make it an attractive alternative to current methods. While *Bombyx* has been used for a variety of technologies,[8,13,14] continued translation and use in an industrial environment will require sourcing that maintains tight control over insect strain and culture conditions during silk fiber production. Current industrial-scale sericulture protocols, the methods for raising larvae for silk production, have been driven by the textile industry, which requires substantially less stringent batch-to-batch regulation. Companies, such as Mulsun Biotech, are making strides toward controlled *Bombyx* sericulture, but production has not yet matched outdoor sericulture practices. *Bombyx* primarily feeds on fresh mulberry leaves, where the nutritional composition can impact various physiological processes including silk production.[15] Shifts in the climate and environment can impact food sourcing, growth, silk fibers, and life cycle length of many insects,[16−23] including *Bombyx* and other silkworms. One of the primary drivers of how diet can influence silk fiber properties is through the alteration of the secondary structure self-assembly mechanism of silk fibroins.[24−26] As silk proteins traverse through the silk gland, a transition occurs from the random coil and/or $\alpha$-helix

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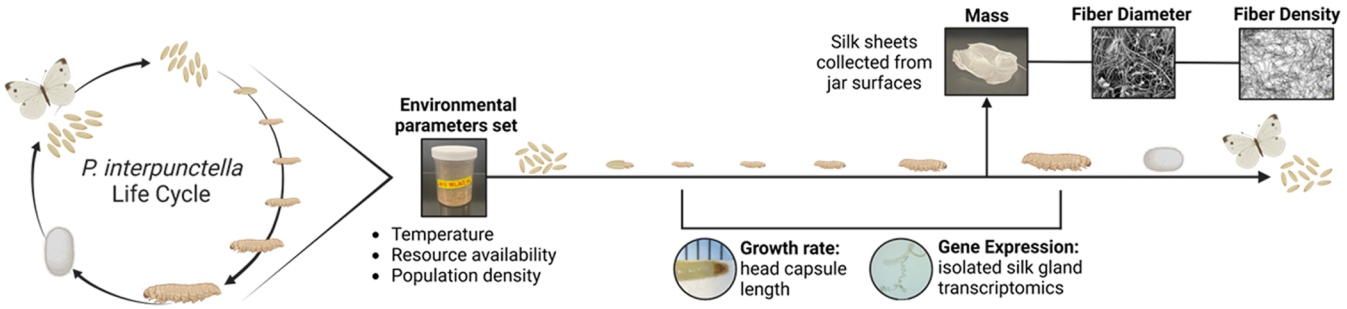


**Figure 1.** Life cycle of *Plodia* in a laboratory setting. Assessments of silk production, fiber diameters, and fiber density are evaluated prior to the emergence of the moths from cocoons. Alterations to this process, including changes in temperature, resource availability, and population density per container, are quantified in this article.

conformation to $\beta$-sheets when they undergo pH change, ion gradient shifts, and shear or flow. The $\beta$-sheet content in silk fibroins corresponds to better mechanical performance, and Cheng et al.[24] demonstrated a mechanism underlying how a diet with increased levels of amino acids prevalent in fibroin proteins increased the silk gland potassium content, inducing higher crystallinity. However, currently, the addition of dietary additives has not been incorporated into large sericulture farms. Furthermore, Ito et al.[27] indicated that the sericin-to-fibroin ratio is altered when *Bombyx* is reared on variable diets, indicating that not only are ion gradients shifting as a function of diet, but they are also impacting the silk fiber composition. Thus, industrial outdoor sericulture and small farmers[21] may struggle to ensure consistent raw materials suitable for biomedical product development at industrial scales over time, especially given the impacts of climate change.[17] Therefore, two of the main advantages for utilizing *Plodia* is that (1) this species has well-established indoor laboratory rearing protocols and (2) a standardized diet has been established,[12] eliminating both outdoor rearing and the use of fresh mulberry tree leaves. When examining the current paradigm in sourcing silk fibers from *Bombyx* for use in biomaterials, there is a distinct lack of standardization from a variety of standpoints including the country of origin and the quality of mulberry leaves provided based on annual rainfall and temperatures. For example, a *Bombyx* sericulture farm in India will experience different climates and rainfall totals each year (yearly variation), but these conditions are also distinct from those of Japan or South Korea (latitude variation). However, all three countries are major producers of quality silk fibers for the textile industry and rely on local mulberry trees for the *Bombyx* diet.

There are many other silk-producing species, such as the golden orb-weaver spider (*Trichonephila clavipes*) and the Chinese oak silkworm (*Antheraea pernyi*), yet many of these species pose substantial obstacles for laboratory rearing or rearing in closed, controlled environments. Other species that are routinely reared in the laboratory, such as the fall armyworm (*Spodoptera frugiperda*), do not produce silk fibroin proteins at quantities required for collection and advanced manufacturing, highlighting the challenge in developing applications for these natively formed biomaterials. As researchers obtain and study silk fibroins from other species, they have identified useful traits, such as the presence of mammalian cell integrin binding sequences (e.g., Arg-Gly-Asp (RGD)). RGD is present in the protein sequence of silk fibroins produced by *Antheraea mylitta*;[28] however, *Antheraea mylitta* is difficult to rear in a laboratory setting with detrimental impacts on silk production with current indoor rearing protocols.[29] *Antheraea mylitta* has seasonally dependent life cycles that are around 2 months long, where production is impacted by external factors (e.g., climate change[30]). Current collection methods for *Antheraea mylitta* include rearing in a forest with human-based collection from the forest,[30,31] meaning that production is outdoors, eliminating a researcher's or company's control over rearing conditions prior to use in manufacturing. This is a common challenge for translating alternative silk-producing species to biomedical applications, and while *Plodia*'s fibroin proteins do not contain RGD, its standardized indoor rearing parameters warrant further investigation on how its unique sequence in the silk biomaterial space can be leveraged. An additional advantage is that *Plodia* can produce collectable silk fibers in a shorter life cycle compared to many other silkworms, such as *Bombyx* or *Antheraea* species.[32,33] The life cycle length for *Bombyx* ranges between 45 and 55 days,[34] while the *Plodia* life cycle length ranges from 20 to 35 days (temperature-dependent). *Antheraea mylitta* is also longer, ranging from 35 to 45 days.[35] Traditional silkworm sericulture is exceptionally labor-intensive;[36] thus, decreased life cycles can lead to improved manufacturing potential as less time is spent on maintenance and growth of the insect.

Another difference between *Bombyx* and *Plodia* is the source of the silk fibers used in biomedical applications. Despite having a smaller and less dense cocoon, *Plodia* produces significant amounts of silk fibers while wandering around their environment under certain conditions. This has been observed by the agricultural community when these pests cover food sources and environments, like grain elevators or silos, in large silk mats.[11] We take advantage of this natural response and wandering behavior to generate silk mats from larvae (third−fifth instar) in the laboratory, which we call "wandering silk fibers." Therefore, not only can *Plodia* be reared indoors with a standardized diet but also silk fibers can be collected as mats of silk fibers, generating a relative abundance of silk material compared to what would be collected from the cocoon of this insect. This allows for continued maintenance of the silkworm population, as we do not need to sacrifice the silkworm to collect the silk fibers as is required for cocoon-based silk production (*Bombyx* sericulture protocols).

Overall, the investigation of *Plodia* silk for biomedical applications or the production of other silk-based technologies is driven by these four main advantages: (1) this species has well-established indoor laboratory rearing protocols, (2) a standardized diet has been established,[12] (3) short life cycle of 20−35 days, and (4) substantial silk fiber secretion during the


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Table 1. Rearing Parameters of *Plodia* under Evaluation[a]||

| Parameters | | | | | Abbreviation | Symbol |
|---|---|---|---|---|---|---|
| Temperature [C] | Diet (g) | Larvae | Resource Availability (# larvae/ g diet) | Population Density (# larvae/ 250 ml container) | | |
| 24 | 18 | 90 | 5 | 0.36 | 5 (0.36) | △ |
| 24 | 9 | 90 | 10 | 0.36 | 10 (0.36) | □ |
| 24 | 18 | 180 | 10 | 0.72 | 10 (0.72) | ▲ |
| 24 | 9 | 180 | 20 | 0.72 | 20 (0.72) | ■ |
| 26 | 18 | 90 | 5 | 0.36 | 5 (0.36) | △ |
| 26 | 9 | 90 | 10 | 0.36 | 10 (0.36) | □ |
| 26 | 18 | 180 | 10 | 0.72 | 10 (0.72) | ▲ |
| 26 | 9 | 180 | 20 | 0.72 | 20 (0.72 | ■ |
| 30 | 18 | 90 | 5 | 0.36 | 5 (0.36) | △ |
| 30 | 9 | 90 | 10 | 0.36 | 10 (0.36) | □ |
| 30 | 18 | 180 | 10 | 0.72 | 10 (0.72) | ▲ |
| 30 | 9 | 180 | 20 | 0.72 | 20 (0.72) | ■ |

[a]Rearing parameters in this article are expressed as an abbreviation of the conditions (temperature/resource availability/population density) and represented by symbols outlined in the far-right column. Resource availability and population density calculations are detailed in Supplemental eqs 1 and 2.

wandering stage, yielding large silk mats within the rearing vessel. To leverage these advantages, we seek to develop rearing protocols that include silk fiber mat collection, optimizing conditions for silk production.

When it comes to rearing *Plodia* or understanding its wild-type behaviors, it has been observed that diet and food scarcity can have a significant impact on its life cycle and reproduction.[37] While *Plodia* can feed on a wide variety of stored products, the quality and composition of its diet have an impact on silk production. When food is scarce, the population of *Plodia* may decrease and larvae may only utilize their available nutrients for basic life processes, which can reduce the number of individuals available for silk production and reduce fiber quality. Additionally, *Plodia* may be forced to feed on lower quality food sources, which can negatively impact the quality and quantity of silk produced. Studies have reported that the nutritional status of species can affect the composition and properties of the silk,[15,38] and thus, we aimed to explore laboratory rearing conditions for *Plodia* to understand the impact on advanced manufacturing and overall silk production. Our goal was to determine a set of environmental rearing conditions that led to consistent and reliable production of silk fibers while maximizing total wandering silk fiber production.

In this work, we confirm that temperature can affect the life cycle of *Plodia*, but we also explore other rearing conditions, such as resource availability and population density, as shown in Figure 1 and detailed in Table 1. We show that higher population densities can lead to production of silk fiber mats during the wandering stage of the larval life cycle, demonstrating alternative ways to collect and produce silk materials without collecting individual cocoons. This work represents the first step in addressing the challenges posed by current *Bombyx* sericulture and reducing the unknowns for using *Plodia* as an alternative source of silk biopolymers. Protocols developed herein set the stage for future investigation of *Plodia* silk biopolymers for applications in biotechnology and advanced manufacturing.

## 2. MATERIALS AND METHODS

**2.1. Insect Strain.** The *Plodia* (Hübner) (Lepidoptera: Pyralidae) wild-type strain was collected from a wild population in 2014 and maintained at the United States Department of Agriculture Center for Medical, Agricultural, and Veterinary Entomology (USDA-CMAVE) before being donated by Dr. Paul Shirk and established within our laboratory. They were reared on a standardized wheat bran diet similar to Silhacek et al.[33] in a 16 h light:8 h dark cycle at the various conditions described in Table 1 and a relative humidity of 65 ± 3.2% as measured using a Govee hygrometer/thermometer. The artificial diet ingredient list and composition can be found in Table S1.

Standard rearing conditions prior to this set of experimentation was 26 °C and 70% relative humidity in plastic display boxes (tristate plastics, 079-C, 7 7/16 × 5 5/16 × 3 3/4 in.) modified for insect rearing. A circular hole (diameter: 76 mm) was cut in the top of the lid, where a fine wire mesh was superglued to cover the hole. In brief, *Plodia* was reared within these boxes by first adding 130 g of standard wheat bran diet and then placing 50 mg of *Plodia* eggs on top of the diet. The boxes were stored in the incubator under conditions as described above. After the adults emerge, they are placed within a state of quiescence using carbon dioxide and transferred to a mason jar fitted with a fine wire mesh at the top, which allows for the eggs to pass through but not the adults. Once the eggs are collected, the adults are frozen for at least 24 h for euthanasia and new boxes are created. See Figure S1 for the schematic representing this process.

**2.2. Establishing Conditions for *Plodia* Rearing in Table 1.** In these experiments, the *Plodia* colony was maintained in custom cylindrical plastic containers (height: 9 cm, diameter: 6 cm) with a circular hole (diameter: 2.5 cm) cut in the bottom of the lid where the fine wire mesh was superglued to cover the hole, Figure 1. The amount of diet in each container was massed using a balance (Mettler Toledo XPE205). To set up each jar, eggs were collected and put in the respective incubator for their condition. Once hatched, the newly emerged larvae were counted and added to containers using a paint brush (Artistrove 4/0) and immediately placed within their respective incubator (ThermoFisher Model 3900 Series). Each condition contained 3+ sets of replicate containers.

**2.3. Silk Mass Assessment.** The *Plodia* larvae were allowed to progress through their natural life cycles. Once they began pupation, the silk around the entire jar, both the lid and side walls, was collected, except for a 2 mm margin around the base of the container to avoid additional mass caused by diet contamination. Additionally, once the silk was collected, any visible debris remaining on the mats (frass, head capsules, etc.) was meticulously removed using a fine paint brush to eliminate as much contamination as possible. Cleaned mats were then placed between aluminum foils and stored within a fume hood. The silk mass was measured on a balance. The full set of samples analyzed is given in Table S2.



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**2.4. Silk Mat Analysis.** To analyze the silk fiber mats, 8 mm biopsy punches were taken at different heights of the container to determine whether variability existed within one silk mat. Images were taken on a Keyence microscope (BZ-X800 Analyzer) at 10× magnification. Subsequently, DiameterJ[39] was used to calculate the average silk fiber diameters for all conditions by selecting four random images from each condition and analyzing three random fibers from the image. Representative scanning electron microscopy (SEM) images were also taken (Phenom Pure) for each condition.

**2.5. Scanning Electron Microscopy (SEM).** Sections of silk mats were excised using an 8 mm biopsy punch and added to an adhesive sticker (Catalog No. JN81435, Ted Pella, Inc., Redding, Ca) placed on top of a ZEISS/LEO SEM Pin Stub Mount, Ø 12.7 mm × 9 mm pin height. Samples were dried in a fume hood for 48 h and mounted on a metallurgic charge reduction sample holder before being imaged at 5 kV on a Phenom Pure benchtop SEM.

**2.6. Developmental Time Analysis.** To analyze the progression of developmental time, the head capsule size was documented. Within each instar, the head capsule size remains constant. As the larvae develop to the next instar, they will shed their old head capsule and grow a new, larger one.[40] Initial images were taken when the larvae were large enough to grab with feather light forceps. Images were taken by placing larvae next to a ruler and using a microscope (Fisher Science Education Stereomicroscope Intermediate+ Series). Measurements were taken every 3 days, using 4 larvae from each jar. ImageJ software[41] was used to measure the head capsule sizes.

**2.7. Fiber Density.** To analyze the fiber density, 8 mm biopsy punches were taken at different heights of the container. Different heights account for variability within the silk mat. Images of the biopsy punches were taken using 10× magnification on a Keyence microscope (BZ-X800 Analyzer). Fiber density was analyzed on three clear images at each condition using the Histogram feature of software ImageJ.[41] The histogram feature counts the pixels under each color condition. For a black and white image, the colored pixels are considered fibers, with white pixels being open space. Therefore, the fiber density was calculated by comparing the number of colored pixels to the total pixels in the image. Each fiber density was for the same size image (966 $\mu$m × 725 $\mu$m).

**2.8. Fibroin-Heavy and Fibroin-Light Transcript Analysis.** RT-qPCR primers were designed using MacVector V17.0.5 with genomic assemblies for *Plodia* described in Shirk et al.,[42] Childers et al.,[43] and Kawahara et al.[44] Total RNA was extracted from 12 silk glands from wandering-phase *Plodia* larvae (four from each of the three replicate containers for each condition) using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA). The silk glands were stored in RNAlater stabilization solution (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocols after isolation until RNA extraction. cDNA was created from 1 $\mu$g of total RNA extract for each group using an AffinityScript QPCR cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). RT-qPCR was performed on 1 $\mu$L of cDNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) with fibroin-heavy and fibroin-light primers (Integrated DNA Technologies, Coralville, IA) as described in Table S3. As reference genes, the transcript levels of $\beta$-actin (Pi_Bac3F and Pi_Bac3R) and ribosomal protein 7S (Pi_RPS7AF and Pi_RPS7AR) were quantified. All biological samples were run in triplicate. The RT-qPCR reactions were conducted in a C1000 touch thermal cycler operated by the CFX96 real-time system (Bio-Rad, Hercules, CA) programmed for 35 cycles of 95 °C, 10 s; 54 °C, 30 s; and 95 °C, 10 s. To confirm that there was minimal genomic DNA contamination coextracted during total RNA isolation, no reverse transcriptase control (NRT) reactions were made using the whole RNA extract from each sample. The relative gene expression was determined following the $2^{-\Delta\Delta Ct}$ method as a standardized ratio to the geometric mean of the reference gene transcripts.[45,46] To calculate the ΔΔCt, all of the conditions were normalized to the 30/20/0.72 fibroin-heavy expression value as represented by its relative expression being a value of 1 (Table S4).

**2.9. Dynamic Mechanical Analysis of Silk Mats.** Mechanical assessment of unprocessed *Plodia* silk was conducted on 10 mm × 10 mm sections excised from silk mats that were collected from the lids of our standard rearing boxes (tristate plastics, 079-C, 7 7/16 × 5 5/16 × 3/4 in.) (Figure S1) that were reared in the recommended conditions for silk production described in this article (24 °C, resource availability of 10 larvae/g diet, and population density of 0.72 larvae/mL). The cross-section of each silk mat was determined by SEM (Figure S2). Five (5) samples were collected from two different boxes and pooled for analysis. Dynamic mechanical analysis (DMA) was performed on an Anton Paar MCR 702e rheometer (Anton Paar, Graz, Austria). Each sample was loaded into tensile clamps (upper clamp: U-SRF5; lower clamp: L-SRF5/LD) with Emery Cloth (3M) folded within each clamp and tightened to 10 centinewton/meter with a torsion screwdriver. Anton Paar RheoCompass software was used to perform static tensile testing where the sample was loaded and prestretched to a force of 0.01 N to remove any sag. The gap width was then recorded as the height of the sample between the clamps. Static testing was performed at room temperature and used to extend the samples from 0 to 50% of the original length at 1% per minute. The average Young's modulus was calculated from the stress−strain curves at a strain range of 0.01−0.08. Ultimate tensile strength was calculated as the maximum point on the stress−strain curve.

**2.10. Atomic Force Microscopy (AFM).** Imaging of single *Plodia* silk fibers was conducted on a Bruker NanoWizard 4 XP AFM in contact imaging mode with a NT_B30_v0030 cantilever (Nano-AndMore, resonance frequency: 13 kHz, force constant: 0.2 N $m^{-1}$). Fibers were isolated by placing larvae on glass microscope slides and allowing them to wander until silk fibers were visibly accumulated on the slide. Single fibers were carefully stretched and secured over a glass microscope slide for imaging in air.

**2.11. Degumming Silk Fibers.** Bombyx silk cocoons were degummed according to the established Rockwood et al.[2] protocol. *Plodia* silk mats were cleaned to remove frass, head capsules, and other waste on its surface. Cleaned mats were degummed by boiling in deionized water for 10 min. The silk mats were then dried for 2 days in air before the final weighing or assessment via SEM.

**2.12. Statistics.** All statistics were performed in GraphPad Prism version 9.4.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. All statistics were performed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test with an $\alpha$ value of 0.05, except for mass of silk produced, which was analyzed as a one-way ANOVA. All graphs are shown with an average of three or more biological replicates ($n \geq 3$) plus or minus a standard deviation (±SD).

## 3. RESULTS AND DISCUSSION

To investigate the potential of *Plodia* silk fibroin protein for applications in biomaterials, we must first understand the relationship between the insect's rearing conditions and its life cycle as well as the production of silk fibers. Second, we must develop consistent rearing strategies to reduce variability in fiber properties and allow one to maximize silk production relative to culture inputs, with the goal of achieving consistent silk fibers from batch to batch (Figure 1). Previous work in the sericulture literature has shown that temperature (e.g., climate change) and resource availability can influence developmental timelines and silk fiber production.[11,47,48] Thus, we are investigating a range of relevant conditions, as outlined in Table 1 with the goal of determining a set of rearing conditions for advanced manufacturing of *Plodia* silk fibers for future uses in biomedical applications.

**3.1. Temperature Alters the Rate of Growth of *Plodia* Larvae.** As demonstrated in the previous literature, insects from the Lepidopteran order are impacted heavily by even small shifts in temperature.[49] As temperatures increase above their developmental threshold, life cycles shorten as captured by a concept called accumulated degree days.[50] Additionally,


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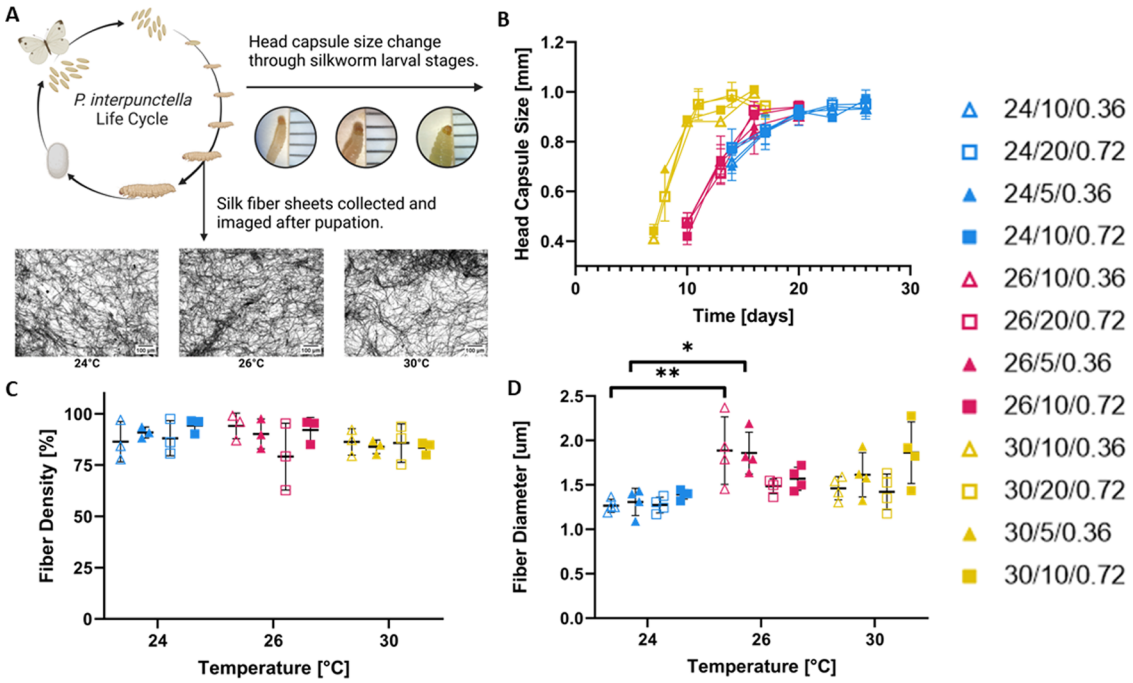


**Figure 2.** Role of temperature on *Plodia* silk production. (A) Head capsule size is measured, and silk mats are collected during larval stages of the life cycle. (B) Head capsule measurements characterize how rearing parameters affect the larvae growth cycle ($n$ = 4). Rearing parameter impacts on (C) fiber density (number of colored pixels, silk, to total area in a representative image) and (D) fiber diameter. For a given resource availability and population density, each temperature was statistically compared for fiber density and diameter. *$p$ = 0.025, **$p$ = 0.01, $n$ = 3.

when temperatures drop below a threshold, production of nonessential proteins decreases in favor of essential pathways.[11] To leverage *Plodia* for future applications, it is critical to understand which laboratory rearing conditions yield consistent silk fibers while maintaining the population viability. In Figure 2, the relationships between growth rates and fiber properties are compared between *Plodia* populations with respect to feasible parameters for rearing in the laboratory, including temperatures (24−30 °C), resource availability (5, 10, 20 larvae/g diet), and population density (0.36 vs 0.72 larvae/mL) by evaluating growth via head capsule size and overall silk production and fiber properties, as depicted in Figure 2A. First, we evaluated the impact of rearing temperature on the growth rates of *Plodia* larvae, determining that the life cycle decreases from an average of 35 ± 2 to 24 ± 2 days when the temperature increases from 24 to 30 °C (Figure 2B). Furthermore, our data suggest that the supplied diet (5, 10, and 20 larvae/g diet) did not have an influence on the developmental rate. This suggests that we did not reach a resource limiting threshold that affected the life cycle of surviving individuals (Figure 2B). This led us to hypothesize that temperature, under the conditions we provided, would have the greatest potential to impact physiological processes, including silk production.

**3.2. Impact of Growth Rate and Temperature on Silk Fiber Properties.** When population density has reached an upper threshold at a given temperature, *Plodia* larvae will leave the food source and start to crawl or wander around their environment (Supporting Information Video S1). During this phase, they frequently spin silk fibers, leaving them in their path as they wander. This behavioral drive is not well understood;[11] however, our initial hypothesis is that this wandering silk activity is a function of resource protection and/or a stress-induced response. This phenomenon causes the exposed surfaces of the insect-rearing container to quickly become covered with silk fibers during the later half of the growth cycle to form silk fiber mats. We analyzed the influence of the rearing temperature on the total silk fiber density, Figure 2C, and silk fiber diameter, Figure 2D, while holding the total rearing vessel volume constant. Examples of silk fiber mats collected prior to moth emergence are shown in Figure 2A for all temperatures (Figure S2). Results show that the fiber density remains statistically similar across all temperatures, resource availability, and population densities when analyzed using ImageJ ($p \geq 0.4542$). The variability in the structure of the laid silk fiber mats was too high to draw conclusions related to how fiber density differed among conditions based on imaging of small mat sections. From SEM images (Figure S2), there are clear layers to each of the fiber mats, which hinders our ability to assess the true differences in fiber mat densities between the groups and does not align with the differences in the mass of silk fibers produced under these conditions (Figure 3). Future work evaluating the mechanical properties of the collected silk fiber mats and fiber microstructures may provide more insights into the influence of rearing conditions on the density of fibers within the mats, but these data are not necessary to determine ideal culture conditions for scale up of silk fiber production.

Previous research has shown that *Plodia* silk fibers have different diameters between wandering silk and pupal silk, leading to the conclusion that silk fiber production is a dynamic process and altered based on the insects' application for the fiber.[51] These are two distinct developmental time points that demand different outputs from the silk fibers, where pupal silk is needed to protect the organism for a prolonged period, during which the insect is in a vulnerable state and wandering silk is thought to be a response to environmental stress. Therefore, as we think about developing a set of


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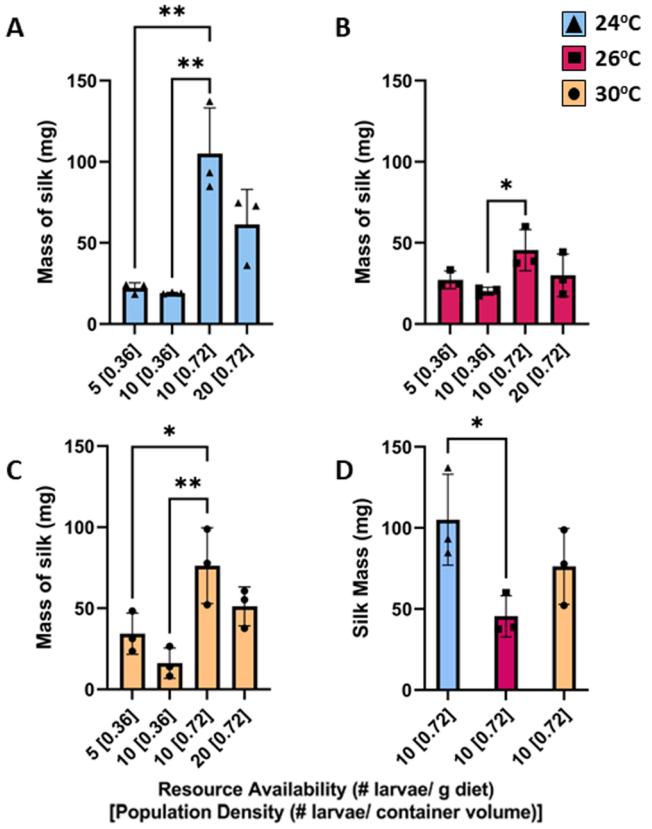


**Figure 3.** Mass of silk produced by *Plodia* under the rearing parameters. (A–C) Amount of silk produced across the various conditions outlined in Table 1, separated by temperature groups (24, 26, and 30 °C respectively). (A) $^{**}p = 0.002$, $^{**}p = 0.0016$, (B) $^{*}p = 0.048$, (C) $^{*}p = 0.04$, $^{**}p = 0.0059$, $n = 3$. (D) Highest silk production for each temperature (10 larvae/g diet and 0.72 larvae/mL) are further considered. $^{*}p = 0.0391$.

protocols for consistent production of *Plodia* silk fibers, there needs to be consideration for whether our range of rearing conditions, which provide population stress, cause the larvae to spin fibers with differing protein compositions. We analyzed the diameter of the silk fibers in each mat (three images per vessel) from three separate rearing vessels to obtain average silk fiber diameters (Figure 2D). Overall, trends show limited differences between observed fiber diameters, but some statistical differences were observed between the 24 and 26 °C groups, where 26 °C fibers were statistically larger in diameter. However, given that the 26 °C samples contained lower amounts of total silk fiber production, further analysis of time-dependent silk production and time-dependent shifts in silk fibroin transcript levels is required to understand if these changes in diameter are due to shifts in the protein composition or silk fiber structure.

There is not a distinct trend that can be concluded about what impacts the wandering-phase silk fiber diameter within the range of conditions suitable for advanced manufacturing and scale-up. Potentially, in more extreme conditions, there could be larger variability of fiber diameter or even shifts in total fiber production, as observed by Shim et al. for insects temporarily exposed to 4 °C.[11] However, within the scope of this publication, this result reinforces that wandering silk, while spun over a longer period than conventional cocoon silk used for biomedical applications, can be produced in a consistent manner with fiber diameters in the range of 1–2 $\mu$m. Within these rearing conditions, we can reliably expect silk fibers to be spun by larvae with a similar diameter for downstream applications. Still, future research needs to focus on the protein composition of the silk fibers within the *Plodia* silk mats, as it has been previously shown that diet and food scarcity[38] can influence the composition and output of the silk fibers in other silk-producing organisms.

**3.3. Greater Silk Fiber Production Achieved at 24 °C with Higher Population Density and Moderate Resource Availability.** Following assessments of temperature change, we further explored the impacts of larval population density and resource availability (eqs S1 and S2) on total silk fiber production (Figure 3). While all the conditions being analyzed will have some influence on total silk fiber production, we aim to elucidate which factor(s) is critical for maximizing the wandering silk production in the larvae. Results demonstrate that population density had a significant effect on *Plodia*'s production of silk fibers. This is shown in Figure 3A, where statistical differences in total silk fiber production within a given temperature (shown by color) were found when comparing production by larvae at 24 °C with a resource availability of 5, 10, or 20 larvae/g diet. When the resource availability is constant (10 larvae/g diet), but the population density changes (0.36 vs 0.72 larvae/mL), the total silk produced increases with increasing population density ($p < 0.0001$). This general trend was also present in the 26 and 30 °C conditions (Figure 3B,C); however, silk fiber production did not increase as dramatically within the 10 larvae/g diet [0.72 larvae/mL] conditions in the 26 °C group. Resource availability (larvae/g diet) also played a small role in the wandering silk behavior, where all temperature groups showed a general trend of increased silk fiber production as resource availability moved to the most restrictive state of 20 larvae/g diet (Figure 3A–C). Alternative assessments of these same data, where we compare individually across temperatures, are described in Table S2 and plotted in Figure S3.

The highest production of wandering silk fibers was achieved at a resource availability of 10 larvae/g diet and at a population density of 0.72 larvae/mL across all temperatures (Figure 3). Keeping resource availability and population density constant, statistical differences in total silk fiber production were observed between 24 and 26 °C ($p = 0.0391$, Figure 3D), where the highest silk mass achieved across all parameters occurred at 24 °C. Furthermore, our findings established that 26 °C produces the least amount of wandering silk fibers, which is evident in the full comparisons provided in Figure S4. This finding supports our initial hypothesis that wandering silk production can be attributed to a stress response to *Plodia*'s environmental conditions. Silhacek et al.[33] established optimal *Plodia* rearing conditions at 28 °C, which, from our conclusion, would show that 26 °C is a temperature that is preferred by *Plodia* compared to 24 and 30 °C.

Through the evaluation of various environmental factors that could increase the behavioral drive to produce wandering silk, our data support the conclusion that on a per life cycle basis, 24/10/0.72 (°C, larvae/g diet, larvae/mL) is the optimal choice for laboratory rearing. However, when pairing this finding temporally (Figure 2B), it is clear that we need to consider which conditions will produce the maximum amount of *Plodia* silk fibers over the long term as a function of life cycle.

**3.4. Discussion of Total Silk Fiber Production per Life Cycle vs per Year.** When considering rearing conditions for


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Table 2. Dependence of Scale-Up Potential of Silk Production on Rearing Conditions[a]

| Parameter | Growth Cycle | Cycles in a year | Scaled up mass of silk per year |
|---|---|---|---|
| [°C/Resource availability/ Population density] | [days] | [-] | [Mass of silk*Life cycles/ year] |
| 24/10/0.36 | 35 | 10 | 199.32 |
| 24/20/0.72 | | | 638.92 |
| 24/5/0.36 | | | 231.55 |
| **24/10/0.72** | | | **1096.11** |
| 26/10/0.36 | 28 | 13 | 264.36 |
| 26/20/0.72 | | | 392.03 |
| 26/5/0.36 | | | 355.18 |
| **26/10/0.72** | | | **593.25** |
| 30/10/0.36 | 24 | 15 | 246.48 |
| 30/20/0.72 | | | 779.17 |
| 30/5/0.36 | | | 522.66 |
| **30/10/0.72** | | | **1160.65** |

[a]Silk mass produced was considered on a growth cycle per year basis to determine the theoretical mass of silk produced per year under each rearing conditions. The number of cycles per year is obtained by dividing the days in a year by the growth cycle.

advanced manufacturing and scale-up, it is important to consider not only the total mass of silk fibers produced in a life cycle but also the total amount produced over time. Similar to results reported in the literature, we observed that an increase in temperature decreases the total length of the *Plodia* life cycle[33] (Figure 2). Given that the life cycle length is substantially shorter at 30 °C, the total silk produced is the greatest for conditions at 30 °C, a resource availability of 10 larvae/g diet, and a population density of 0.72 larvae/mL. At 30 °C, we can achieve 15 total life cycles per year, while only 10 cycles are completed per year at 24 °C (Table 2). However, while the data support the use of higher temperatures for the maximum long-term production of wandering silk fibers, there are various considerations that make this temperature choice less than ideal. *Plodia* has been shown to have extensive mortality rates at 34 °C.[51] Rearing *Plodia* at higher temperatures over multiple generations can diminish the fitness and fecundity of the colonies[52] and therefore diminish the production over multiple generations. Furthermore, having an increased life cycle means that one will use 1.5 times more labor per year to change out and set up these insect-rearing boxes and pay additional costs to maintain a higher incubator temperature over room temperature. Therefore, our recommendation is to rear cultures at 24 °C, with a resource availability of 10 larvae/gram diet and population density of 0.72 larvae/mL to yield the greatest return on investment in terms of total silk fiber production over time.

**3.5. Silk Fibroin Gene Expression Impacted by Rearing Conditions.** While silk fiber mass is a good indicator of the total raw product produced, the fiber itself contains many different proteins. This is commonly assumed to be two main classes of proteins: fibroins and sericins. Fibroins are the main proteins used in silk biomaterial production, and silk fiber-processing protocols to create silk fibroin solutions focus on the removal of sericin proteins. Thus, these *Plodia* silk fiber mats are a combination of many proteins, however, mainly fibroins and sericins. The fibroins produced in the mats are predominantly responsible for mechanical properties such as elasticity and yield strength, with the majority of the mechanical properties being attributed to the fibroin-heavy protein. However, the silk fibroin regeneration process, specifically the degumming and dissolution steps, causes the silk fibroin protein to degrade and alters the mechanical properties from the native unprocessed fibers.[53−55] Therefore, it is important for us to understand the relative abundance of the fibroin proteins produced by the *Plodia* larvae during silk fiber mat production. We currently do not have methods to process the raw silk components into their respective aqueous fibroin and sericin states. To start to understand how gene expression and protein production are related as a function of environmental parameters, we analyzed silk fibroin-heavy and -light chain transcripts (primers given in Table S3), which are the two main fibroin proteins connected via disulfide bonds, in *Plodia* larvae at the early wandering stage. This stage correlates to a time point at roughly two-thirds of their total life cycle. Results in Figure 4 show that transcript levels tend to be the highest in larvae reared at temperatures outside of their optimal temperature range[33] and confirm the results we see which show that the greatest silk fiber mass is produced at 24 and 30 °C (Figure 3). The fibroin-heavy transcript was the highest at 30 °C and could be a result of *Plodia* being at a higher stressed state compared to the other groups. However, this heat-stressed response over a prolonged period could have negative consequences on the fecundity of the culture.[51,52] We also observe shifts in the ratio of silk fibroin-heavy chain to silk fibroin-light chain as a function of environmental conditions. While the *Bombyx* heavy and light chain molar ratio has been reported as 1,[56] this information is currently unknown for *Plodia* and the ratio is likely to be different due to more cysteine residues found in the *Plodia* heavy chain. Isolation of

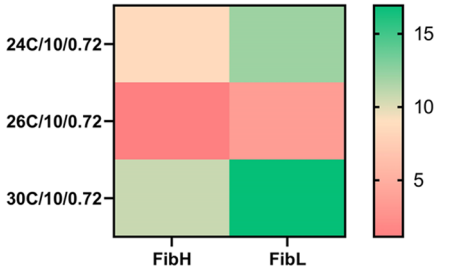


**Figure 4.** Rearing condition impact on fibroin-heavy and -light transcripts. Fibroin-heavy and -light chain expressions are compared for each temperature at a resource availability of 10 larvae/g diet and population density of 0.72. For a full summary of expression values for all conditions, see Table S4.


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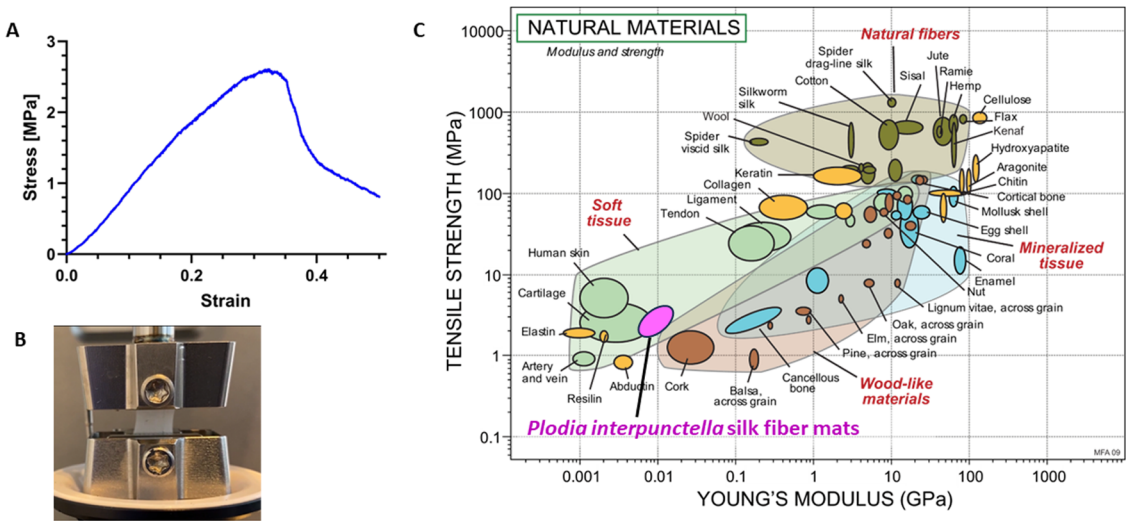


**Figure 5.** *Plodia* silk fibers occupy a new group of material properties compared to those of other commonly studied silk fibers. (A) Representative stress strain curve of a *Plodia* silk mat laid in our standard insect rearing chambers (Figure S1) at optimal conditions established in this article, obtained by measuring stress in response to strain at 1% strain per minute. (B) Representative image of the *Plodia* silk mat undergoing tensile testing. (C) *Plodia* silk fibers, labeled in pink, lie in the soft tissue region of natural materials and expand the region of properties that are found for natural fibers. (C) Reproduced from Gibson et al.[1] Copyright [2010, Cambridge University Press].

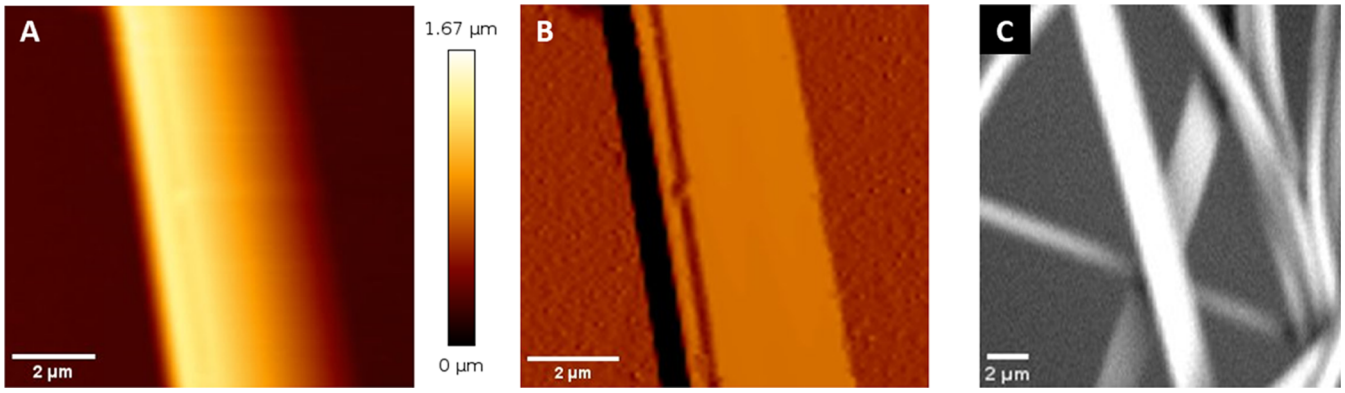


**Figure 6.** Imaging of single *Plodia* fibers produced under standard rearing conditions. (A) AFM surface image of the *Plodia* fiber. Relative surface height of the fiber is depicted by the color scale accompanying the image. (B) Corresponding AFM deflection error image of (A). (C) SEM micrograph of a silk mat.

the proteins from the silk gland or purification of the protein fiber is necessary to fully elucidate this information. However, our transcript data provide a snapshot of how silk fibroin gene regulation can be altered due to environmental impact, necessitating highly regulated rearing conditions.

Additionally, transcript data confirm trends previously reported in the literature for other silk-producing species where resource scarcity decreases silk fiber production (Table S4).[48,57] Our data suggest that resource scarcity has a fitness cost to silk fibroin production in *Plodia*. This reinforces the fact that we need stringent control over environmental conditions and resource availability, as inconsistencies in these parameters can lead to variation in silk fiber quality. When considering the production of *Plodia* silk for future applications as a biopolymer, choosing and maintaining rearing conditions that yield consistent silk fibroin gene expression ratios and subsequent protein production are important. Because the gene sequence of *Plodia*[44] is different from that of *Bombyx*, there is no reason to expect that the resulting proteins are similar. Thus, purification processes to isolate *Bombyx* fibroins[2] may not apply to *Plodia* or to the generation of a *Plodia* aqueous silk fibroin solution. Ongoing work outside the scope of this rearing study aims to address these gaps, allowing for future studies to address how environmental factors influence fibroin-to-sericin ratios in raw silk fibers.

**3.6. Future Perspectives and Requirements for Use of *Plodia* Silk Fibers in Advanced Manufacturing and Product Development.** *Plodia* offers the potential to be used as an alternative silk fiber source within the biomedical landscape, and methods for characterizing, purifying, and solubilizing the silk fibers to generate various material formats will be necessary. Processing of raw silk components into regenerated fibroin solutions modulates the crystallinity, viscosity, and molecular weight of the regenerated silk fibroin solutions and subsequent material properties[55,58] but is beyond the scope of this article. Initial mechanical and structural characterization of the unprocessed silk fiber mats in Figure 5 shows that these materials have an average Young's modulus and tensile strength of 10.6 ± 3.8 and 2.64 ± 0.40 MPa, respectively, when measured under extension at 1% strain per minute as shown via a representative stress strain curve in Figure 5A on an Anton Paar MCR 702 instrument equipped


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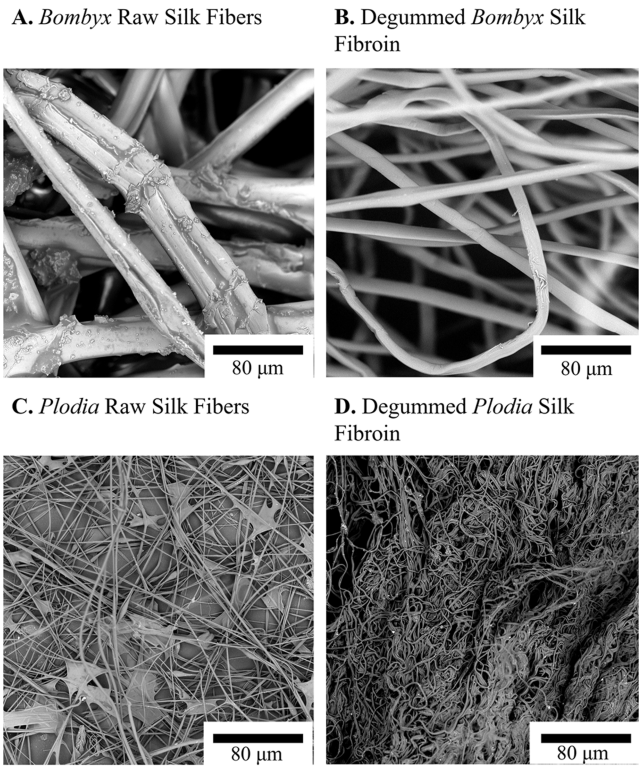
with a linear drive (Figure 5B). Individual replicates are listed in Figure S5. Compared to the previously studied silks which lie in the natural fiber range described in Gibson et al.,[1] shown in the upper right of Figure 5C, *Plodia* silk fiber mats reside in the soft tissue region where they display mechanical properties that more closely resemble that of cartilage, which are significantly lower than other common silk producers.[1,59] Furthermore, the *Plodia* silk mats had higher average strain at the maximum stress (28.5 ± 2.4%, Table S5) compared to the reported values for *Bombyx* (18 ± 2%[60]). However, these spun silk fiber mats are nonuniform and randomly oriented (Figures 2A and S2A), so we anticipate that substantial single fiber assessments and regenerated material assessments will be necessary to fully understand the mechanical and structural profiles of these materials, accounting for variability in measured ultimate strength, as noted by the movement of the peaks in the stress vs strain curve (Figure S5), which we attribute to this lack of fiber alignment and contributions of various fibers in the measurements. Further investigation of the contribution of individual fibers via single fiber analysis using atomic force microscopy provides greater information about the sources of variability within these samples (Figure 6). Analysis of single fibers by AFM shows variation in fiber diameters (Figure 6A,B), which can be visualized in the mat as raw *Plodia* silk fibers using SEM (Figure 6C).

The next step in analysis is the removal of sericin proteins from the silk fibers through a process known as degumming. In Figure 7, raw and degummed[2] Bombyx fibers (Figure 7A and Figure 7B, respectively) are compared to raw and degummed *Plodia* fibers (Figure 7C and Figure 7D, respectively). Estimations of silk fibroin recovery via degumming are given in Figure 7E. These results suggest that *Plodia* is a good candidate for further investigation as an alternative source for silk fibroin proteins, even though variations exist in the protein sequence and fiber structure. Ongoing work aims to regenerate *Plodia* fibers into silk solutions to produce a wide variety of silk materials, taking inspiration from previous work[2] in *Bombyx*. Therefore, with *Plodia* having a long-standing history of laboratory rearing and our demonstration of relatively robust and scalable silk production within a controlled environment, the advanced manufacturing potential for these materials is evident. Ongoing efforts outside the scope of this article to characterize the silk fibers and regenerate silk fibroin solutions will enable the use of *Plodia* silk fibroins in biomaterials or other biomedical applications.

## 4. CONCLUSIONS

Natural biopolymers and materials derived from them are poised to have a substantial impact on the market for new biomaterials in the coming decade. A major challenge with natural material sourcing is transitioning the production of the biopolymer into a controlled and reproducible process. To overcome these challenges, the use of microorganisms and bioreactor culture for recombinant protein production has become the gold standard for small protein and peptide production. Unfortunately, production of full-length, foldable, and manipulatable silk fibroin biopolymers of >300 kDa by recombinant production has been met with substantial challenges, despite intelligent and well-designed efforts.[61] Thus, for silk fibroin biopolymers and materials engineered from them, silk fibroin will continue to require sourcing from insect raw materials. Currently, the only commercially available silk fibers come from the cocoons of insects farmed fully




E. Comparison of Degummed Silk Fiber Recovery

| Silk Source | Avg. Weight of Initial Raw Silk | Avg. Weight of Degummed Silk | % Recovery |
|---|---|---|---|
| *Bombyx mori* | 5 g | 3.2-3.3 g | 64-66 % |
| *Plodia interpunctella* | 1 g | 0.45-0.55 g | 45-55 % |

**Figure 7.** Degummed silk fibers. Raw *Bombyx* fibers (A) and raw *Plodia* fibers (B) compared to degummed *Bombyx* fibers (30 min, C) vs degummed *Plodia* fibers (10 min, D) are shown via SEM images with a scale bar of 80 μm. (E) Estimation of silk fibroin recovery from degumming activities. *Bombyx* is degummed by standard protocols,[2] while *Plodia* is degummed in boiling DI water. Recovery estimations show that *Bombyx* is around 64−66% fibroins, while *Plodia* is around 45−55% fibroins following degumming.

outdoors or in large, open-air warehouses commonly in regions such as Asia and India. To meet the demands of the biomaterial field, new sources of silk fibers with well-controlled production criteria are needed. In this work, we present *Plodia interpunctella* (*Plodia*) as an alternative to the prevailing source for native silk fibers, *Bombyx mori*. Our efforts define a protocol for production of insect-produced silk fibers with tight control over the *Plodia* rearing conditions in an incubator. We explore the potential range of rearing conditions within this controlled environment to maximize native silk fiber production for future applications in advanced manufacturing and biomaterial-based products. Through evaluation of temperature, resource availability, and population density, we determined rearing and production within a 24 °C incubator at 65% humidity with a resource availability of 10 (larvae/gram diet) and a population density of 0.72 (larvae/mL container volume). These conditions led to the greatest amount of silk production per life cycle without causing generational decline, which has been previously observed at temperatures >30 °C.[51] Furthermore, we showed that the main driving force for silk production, aside from temperature, is the population density.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We hypothesize that these rearing conditions result in the greatest silk fiber production due to the potentially stressful conditions chosen. For example, in the wild, these insects will "silk over" grain in grain elevators and silos, presumably trying to protect themselves and their food from outside influence. We aim to leverage this natural response without causing substantial changes in the native gene expression to ensure that silk fiber properties remain consistent with time. Future work will need to link the gene expression and the resulting mechanical properties. To link the gene expression and mechanical properties, exploration of silk genes present within *Plodia* and subsequent protein expression profiles under various conditions is needed. This is a sizable challenge, but research groups such as Kawahara et al.[44] have established paths to improve sequencing techniques for highly repetitive regions and applied this to *Plodia*. We demonstrate initial expression changes within fibroin proteins for *Plodia* in various conditions and provide preliminary assessment of mechanical properties and methods to recover silk fibroin following degumming. Future work will need to expand upon this preliminary assessment, characterizing the currently unknown proteins that are present within the fiber and their impact on methods for silk fibroin solubilization, continuing investigations into recovery of purified silk fibroin and the impact on mechanical properties.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.3c01372.

Pictorial representation of the circular process of rearing Plodia for silk collection, *Plodia* wheat bran-based diet used for laboratory rearing, representative brightfield (top) and scanning electron microscopy (bottom) images of *Plodia* silk mats, rearing parameters of *Plodia* under evaluation, analysis of silk mass production for linear relationship, environmental impact on silk production, fibroin-heavy and -light chain relative gene expression for all conditions, and stress−strain analysis of *Plodia* silk mats (PDF)

Wandering *Plodia interpunctella* (MP4)

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### Notes

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