Effects of the Space Environment on Silkworm Development Time

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Abstract As aviation technology has developed, there has been more emphasis on exploitation and utilization of the space frontier. The lepidopteran insect *Bombyx mori* has advantages, including small body size, light weight, short life cycle, and well-characterized genetics, when used as a model for biological investigations in space compared with other animals. In preparation for experiments in space, we carried out a simulation experiment, and the number of embryos and the culture temperature and humidity were optimized. The silkworm incubator was launched with China's SJ-10 recoverable microgravity experimental satellite and was in orbit for 12 days and 15 h in 2016. The embryos were cultured in space. Images of the silkworm embryos were obtained during flight. The embryos cultured in space hatched properly after returning to the ground, but silkworm larva obtained from cultures grown on the SJ-10 satellite grew more rapidly than the ground control group. Analyses of subsequent generations and genome, transcriptome, and proteome analyses are ongoing.

Abbreviations

BmNPV	*B. mori* nuclear polyhedrosis virus
cDNA	Coding DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
ISS	International Space Station
mRNA	Messenger RNA
RNAi	RNA interference
TALENs	Transcription activator-like effector nucleases

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ZFNs Zinc finger nucleases
ZFP Zinc finger proteins

1 Silkworms Are Ideal Subjects for Life-Science Research in Space

1.1 Silkworms Are of Economic Importance

The silkworm, *Bombyx mori*, is an economically important insect due to its ability to produce silk. These insects originated in China and have been raised in the region for thousands of years. Each silkworm larva eats 20–25 g of fresh mulberry leaves during its life and produces approximately 0.2–1 g of silk with a length of 700–1500 m. Thus, the conversion efficiency of leaves to silk is approximately 5%. The silk gland, which is the tissue that biosynthesizes and secretes the silk, is formed at the embryonic stage but grows very slowly during the first four larval stages. The gland has three parts: the anterior silk gland, the middle silk gland, and the posterior silk gland. In the fifth (and final) instar, the silk gland grows rapidly, and the worms begin to spin and form a cocoon at the end of the larval stage. During the fifth larval instar, high levels of polyploidization result in an increase in the production of silk thousands of times higher relative to levels in the fourth instar. Due to the long history of sericulture in China, silk is widely used in the textile industry, in medicine, and in military applications.

1.1.1 The Silkworm as the Lepidopteran Model Insect

The silkworm is also an important model lepidopteran insect. The genome of the silkworm was first sequenced in 2004, and sequences of 40 different silkworm strains were reported in 2006 (Xia et al. 2004, 2009). This genomic information enabled many post-genomic studies. In 2000, Tamura and his colleagues used the *piggyBac* transposon to insert one reporter gene into the silkworm genome, successfully establishing the transgene system in the silkworm (Tamura et al. 2000). Genome editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems provide us with more choices for loss-of-function analysis in the silkworm (Wang et al. 2013a, b, 2014b). The fact that these tools have been successfully used in silkworm genome editing means that the silkworm is considered the model insect for Lepidoptera.

1.2 The Lifespan of the Silkworm

The silkworm life cycle has four stages: embryo, larva, pupa, and adult. In each stage, the morphology of the silkworm is different. The embryo stage lasts approximately 10 days before hatching into the larval stage. Larvae eat fresh mulberry leaves continuously. The larval stage lasts approximately 18 days. During this stage, the insect normally moults four times; at each moulting, the body becomes slightly yellower and the skin becomes tighter. The larva then stops eating, and when the gut is empty, it enters the wandering stage, indicating that the insect is preparing to enter the pupal phase of the lifecycle. The silkworm encloses itself in a cocoon made of raw silk produced by the salivary glands; building the cocoon takes approximately 2 days. The final moult from larva to pupa takes place within the cocoon, which provides a vital layer of protection during the vulnerable, almost motionless pupal state. The silkworm pupal stage lasts 10 days. At the end of the pupal stage, the insect releases proteolytic enzymes that make a hole in the cocoon so that it can emerge as an adult moth. The female moth produces approximately 300–500 eggs after mating. The entire lifespan of the silkworm is approximately 40–50 days and depends on temperature.

1.3 Suitability of the Silkworm for Space Flight

A large number of scientists are now focused on space-based life science research. In comparison to other model animals, the silkworm has certain advantages for space flight-based research. First, the volume of the silkworm embryo is small, and embryos do not need to be fed during short space flights. Second, the silkworm embryo stage is short, lasting approximately 10 days. Third, differentiation, diapause, metamorphosis, and genetic characteristics of silkworms are well understood (Kotani et al. 2002). The embryos can be maintained in the diapause state for at least two years by controlling the storage temperature (Furusawa et al. 1982). Fourth, radiation of silkworm eggs is an efficient strategy for generating mutations, so the effects of a range of doses of radiation on these insects are understood (Furusawa et al. 2001; Henneberry and Sullivan 1963; Kotani et al. 2002). Most importantly, space research using the silkworm has and will provide insights expected to improve silk production and sericulture.

2 Space Flight History of the Silkworm

2.1 Silkworm Studies in Space Conducted by Researchers Worldwide

In 1997, researchers from the Department of Applied Biology at Kyoto Institute of Technology loaded silkworm eggs onto to the US space shuttle Atlantis (STS-84) with the goal of investigating the effects of cosmic radiation and microgravity on embryogenesis and post-embryonic development in *Bombyx* eggs. Insects in two different developmental stages were studied during the 9-day flight: eggs from the early stage after oviposition and diapause-terminated eggs (Furusawa et al. 2001). Approximately 85% of the eggs in space and in the ground-based control experiments matured; approximately 56% of eggs in the flight group and 43% of eggs in the ground control group hatched. Researchers also found that silkworms in the flight group had morphological disorders, such as segmental fusion of the 4th and 5th segments, and the percent of individuals with abnormal crescent marking was significantly higher than in the control group. It was assumed that the higher rate of abnormalities in the space-based samples than in the ground-based controls was caused by microgravity rather than the influence of cosmic rays, as there was no significant difference in the absorbed dose of radiation between the normal and abnormal larvae that were hatched from the eggs that had been in space (Shimada et al. 1986). These results show that the silkworm could be a useful model to investigate the biological effects of microgravity and cosmic rays on insect development (Kotani et al. 2002).

A series of experiments on silkworms were conducted aboard the Columbia space shuttle on the STS-107 mission. For STS-107, a series of miniature habitats were developed by BioServe Space Technologies, and the BioServe's Commercial Generic Bioprocessing Apparatus was loaded with three developmental silkworm larvae in the growth phase, two larvae in the wandering stage, and three pupae that were expected to eclose during flight. The instruments enabled strict control of temperature and humidity (it was necessary to keep the food moist). In the flight group, the three larvae grew normally despite the fact that the food was not fixed because of the weightlessness in the space environment. One of the wandering-stage larval insects successfully spun a cocoon in the flight vehicle, and the other died before cocooning. One of the pupae eclosed on the first day during the flight, whereas the second pupa eclosed on the seventh day of the mission, and the third pupa had not eclosed by the end of the mission. In the ground control group, one of the wandering larva cocooned on the eighth day of the mission, and the other wandering larva had begun cocooning but did not complete it before the end of the mission. One of the pupae started eclosion on the first day, and the other two moths had emerged by the end of the mission. The wide range of developmental timelines observed between the flight and ground units was attributed to the difficulty in determining the ages of the larvae and pupae at loading (Carla and Goulart 2004).

Scientists in the Japanese 'Kibo' facility in the International Space Station (ISS) (Furusawa et al. 2001) and the European Space Agency (Ohnishi 2016) performed

experiments on the silkworm. The Japan Aerospace Exploration Agency took *B. mori* eggs to the International Space Station(ISS) and studied the insects for 3 months with the aim of examining the biological effects of cosmic rays. A black-striped strain (P^S/P^S) and a normally marked strain ($+^p/+^p$) were crossed, and heterozygous silkworm eggs ($P^S/+^p$) were obtained. When heterozygous silkworm eggs ($P^S/+^p$) were exposed to heavy ion particles of carbon, neon, or iron in ground-based experiments, white spots were present on the backs at the fifth instar (Toshiharu Furusawa et al. 2009). In the larvae of the heterozygous silkworm eggs maintained in the ISS that hatched when returned to the ground, no mutants were detected in the first generation. Surprisingly, larvae from the second and third generations had white spots. These results suggest that space radiation affects primordial germ cells during embryonic development (Ohnishi 2016).

2.2 *Space Flight Studies of Silkworm Performed by Chinese Researchers*

To explore the impact of the space environment on the development of the silkworm, China used several return satellites to carry 12 batches of silkworm eggs into space. The first time silkworm eggs were sent to space in a Chinese recoverable satellite was in 1988, but the silkworm eggs were dehydrated upon return to the ground because the satellite lacked a biological protection cabinet (Gui et al. 2001). Two batches of silkworm experiments resulted in no data due to the failure of the satellite launches, and another six batches of silkworm eggs died after returning to the ground due to high capsule temperature.

In October 1990, silkworm eggs spent 8 days in space on a recoverable satellite. The goal of this space experiment was to study the effect of microgravity on the development and heredity of silkworm eggs. Another objective was to determine whether mutation induced in space might improve silkworm cocoon yield or silk quality (Shi et al. 1994). After being returned to the ground, the embryonic development of the silkworms was analysed and compared to eggs maintained on the ground. A number of interesting observations were made. First, the embryonic development of the silkworms was completed normally in the flight environment, but the mean lifespan of silkworms that had been in space was shorter than that of the ground control group. Second, the hatching rate of stagnant hybrid eggs was unaffected by the time in space, but that of the purebred eggs decreased. Third, the development of the silkworm eggs that had been in space was more rapid than the control group: the larval stage was shortened by a mean of 7 days, and eggs hatched a mean of 1 day sooner than ground controls. Fourth, the mean body weight of silkworm larvae was not significantly altered by flight, but the rate of digestion and absorption, the transformation rate of silk, the r-glutathione activity of the midgut, and the activity of GTPase in the silk gland were increased. Finally, the quality of the silk produced by the silkworms was also improved by space flight. None of these effects were observed

when silkworms were sent into space at the diapause stage. These results showed that the silkworm could complete normal embryonic development in microgravity. Moreover, this experiment suggested that new varieties of silkworm that produce better quality or more silk can be cultivated in a special space environment (Shi et al. 1994).

After these experiments, Chinese scientists used a Russian biosatellite to explore the biological effects of space microgravity and cosmic radiation on silkworm eggs. The biosatellite was launched into orbit on 29 December 1992 and returned to the ground on 10 January 1993 (Shi et al. 1995; Zhuang et al. 1995); the total flight time was 12 days. The temperature in the satellite capsule was between 20 and 26 °C. The dose of flight radiation was 1.849 mGy, and the daily dose was 0.154 mGy. The silkworm eggs were divided into two groups: a space flight group and a ground control group. The volume of the container carried by the satellite was $45 \times 75 \times 150\ mm^3$, and it was divided into four small cabins to carry larval stage insects, cocoons, pupae, and eggs. There were four (2♂, 2♀ line: H1 × jia90) wandering stage larvae for investigation of the behaviour of silk production, cocooning, and pupation. The goal of sending the four cocoons (2♂, 2♀ line: 54A) was to study pupation. There were eight pupae (4♂, 4♀ line: 54A) to investigate mating, oviposition, fertilization, and other adult behaviours. Finally, there were six kinds of diapaused silkworm eggs of different strains to allow investigation of the influence of genetic variation during flight. The results showed that the silkworm could complete silk spinning, cocooning, pupation, moth, mating, oviposition, fertilization, embryo formation, embryo development, and larval hatching stages during the space flight. It was of utmost importance that the variation of the pupae and the trimester were found in the recovered samples. Thus, the mutations induced in space were inherited upon subculture. Moreover, these characteristics were not observed in the ground control group (Shi et al. 1998).

A Chinese satellite that launched on 27 September 2005 and returned on 15 October of the same year carried another experiment into space. In this experiment, the total time in space was 18 days (Wu et al. 2005). This experiment showed that space travel had a negative impact on embryos. Variations in cocoon and egg colour were observed in the next generation of those embryos that survived. This confirmed that time in space could cause genetic variation in offspring.

To explore gene expression in silkworm embryos under space conditions, we sent embryos into space on the SJ-10 satellite in 2016. The results of this experiment will be discussed in detail in Sect. 4.

In 2016, China sent the Tiangong-2 space station into space and conducted many scientific experiments, including a silkworm culture experiment. This experiment was designed to observe (1) the cocoon stage, (2) the development of silkworm chrysalis, (3) the pupae, (4) the moth's movement in space and the process of mating, and (5) the oviposition status of female moths after mating. Five larvae are expected to spin cocoons, which is important progress in space experiments. When the spacecraft returns to Earth, the research team will observe whether the "space silkworm" silk spinning behaviour is different from the behaviour of ground silkworms and whether these changes can improve silkworm breeding technology.

3 Silkworm Research Platforms

3.1 Silkworm Genomic Sequence

The draft genome sequence of *B. mori* was simultaneously released by scientists from China and Japan in 2004 (Mita et al. 2004; Xia et al. 2004, 2014). The estimated silkworm genome size is 428.7 Mb; 18,510 genes were identified. The silkworm genome databases—SilkDB (China) and KAIKObase (Japan)—were established and are updated independently. These are important bioinformatic resources for the scientific community (Duan et al. 2010; Shimomura et al. 2009; Wang et al. 2005). In 2007, with the combination of nine-fold-coverage whole-genome sequencing data, fosmid and BAC sequencing data, and full-length coding DNA (cDNA) sequencing data, a 432-Mb more complete genome sequence was achieved (International Silkworm Genome 2008). Furthermore, to reveal the secrets of domestication of *B. mori* from the wild silkworm, *B. mandarina*, a single-base-pair-resolution silkworm genetic variation map was constructed from 29 phenotypically and geographically diverse domesticated strains and 11 wild varieties (Xia et al. 2009).

In addition to the general silkworm genome sequence, scientists have also explored other detailed genomic information, including microsatellite DNA (Prasad et al. 2005), microRNA expression (Liu et al. 2010), segmental duplication (Zhao et al. 2013), Z chromosome genes (Arunkumar et al. 2009), and expressed genes in wild silkmoths (Arunkumar et al. 2008). Illumina high-throughput bisulfite sequencing revealed that 0.11% of all genomic cytosines are methylcytosines that mainly occur in CG dinucleotides (Xiang et al. 2010); the level of methylation is much lower than in plants and mammals. Methylation in silkworms is positively correlated with the expression of corresponding genes (Xiang et al. 2010). In 2013, researchers created 21 full-length cDNA libraries derived from 14 tissues and performed full sequencing by primer walking to obtain a full-length sequence for 11,104 cDNAs, which enabled researchers to annotate the silkworm genome more accurately (Suetsugu et al. 2013). Despite this progress, compared with organisms such as *Drosophila*, information is lacking for the silkworm. In particular, information on functional small RNAs, including piRNAs and lncRNAs, is needed, and additional sequencing should be performed to make the genomic blueprint of silkworms more comprehensive and complete.

3.2 Silkworm Transcriptomic Research

3.2.1 Transcriptome Analysis in Insects

With the advent of the post-genome era, technologies to study transcriptomes, proteomes, and metabolomes have been developed and have been widely used in biological studies (Lockhart and Winzeler 2000). The genetic central dogma demonstrated

that genetic information is precisely transferred from the DNA to protein by the messenger RNA (mRNA). Therefore, mRNA is considered to be a "bridge" for biological information transfer between DNA and proteins, and the identification of all expressed genes and their transcriptional levels is collectively referred to as transcriptomic analysis (Costa et al. 2010). A transcriptome is the sum of all RNA transcribed in a specific tissue or cell at a developmental stage or functional state, including mRNA and non-coding RNA (Costa et al. 2010; Wang et al. 2009).

In the mid-1990s, two transcriptomics research methods emerged almost simultaneously: DNA microarray technology (Lockhart et al. 1996) and serial analysis of gene expression (Velculescu et al. 1995), which are based on northern hybridization and expressed sequence tag analysis, respectively. Next-generation DNA sequencing technology, also known as high-throughput sequencing, has also been applied in transcriptomic analysis (Ansorge 2009; Rusk and Kiermer 2008; Schuster 2008).

3.2.2 Transcriptomic Analysis in the Silkworm

The silkworm is a heteromorphic insect, and different subsets of genes are expressed in different feeding stages and in the moulting period. High-throughput sequencing has been used to analyse differential gene expression in different stages, and these data will help us to understand the gene transcription profile throughout the developmental process.

Using high-throughput paired-end RNA sequencing, Li et al. explored the genes specifically expressed in different developmental stages (Li et al. 2012). Around the same time, Shao et al. identified 320 novel genes and identified thousands of alternative splicing and 58 trans-splicing events at different developmental stages and different tissues using Illumina sequencing technology (Shao et al. 2012).

In 2014, Kiuchi et al. performed deep sequencing (RNA-seq) of silkworm embryos and identified 157 transcripts that are expressed in significantly different amounts in female and male embryos (Kiuchi et al. 2014). Among these differentially expressed transcripts, they found one transcript that is highly expressed in females at all stages of embryogenesis but not in male embryos. This transcript is expressed from the W chromosome and is a precursor of a female-specific piRNA. On the basis of this work, they performed clustering analysis of sexually biased transcripts and divided them into 10 groups according to their expression patterns (Kawamoto et al. 2015). Parthenogenetic reproduction can be either obligate or facultative. There are complex variations between species of silkworm (Kellner et al. 2013), and parthenogenesis occasionally occurs in the domesticated silkworm. Liu et al. investigated the gene expression profile in silkworms undergoing thermal parthenogenesis and found that differentially expressed genes are mainly involved in reproduction, chorion formation, female gamete generation, and cell development pathways (Liu et al. 2015).

How silkworms respond to the environment is of great interest. Ogata et al. analysed the expression of drug resistance-related genes in silkworm fat body cells cultured in medium and harvested from silkworms grown under natural conditions. The comparison of transcriptomes in natural conditions and in cultured tissues revealed

that fewer genes represent a larger portion of the transcriptome in the natural fat body than the cultured fat body (Ogata et al. 2012). In 2014, researchers constructed four digital gene expression libraries from the silkworm fat body of females and males to analyse the effects of temperature. After constant high-temperature treatment, there were significant changes in gene expression in the fat body, especially in binding, catalytic, cellular, and metabolic processes (Wang et al. 2014a). Nie et al. analysed gene expression in neonatal larvae after hyperthermia-induced seizures in the contractile silkworm and found that the most common differentially expressed genes were up-regulated and that these genes encoded heat shock proteins (Nie et al. 2014).

Host-pathogen interactions are complex processes, and understanding these interactions is critical to the silk industry. Fungal infections induce a variety of responses in silkworms. To obtain an overview of the interaction between silkworm and an entomopathogenic fungus, Hou et al. identified a subset of genes in silkworm larvae that exhibit altered expression in response to *Beauveria bassiana* infection. These genes are involved in many biological processes, such as defence and response to pathogens, signal transduction, phagocytosis, regulation of gene expression, RNA splicing, biosynthesis and metabolism, and protein transport (Hou et al. 2014). In 2015, Wang et al. performed transcriptomic profiling of the brains of healthy silkworm larvae and larvae infected with *B. mori* nuclear polyhedrosis virus (BmNPV). The transcriptional level changes observed in the BmNPV-infected brain samples provided new clues regarding the molecular mechanisms that underlie BmNPV infection (Wang et al. 2015).

3.2.3 Problems and Future Development of Transcriptomic Research

Compared with traditional sequencing, next-generation sequencing technology has several advantages, including high throughput, high sequencing speed, and low cost. There are shortcomings, however. First, the sequence length obtained using next-generation sequencing is usually short, which makes efficient assembly challenging. Second, although useful for studying tissue-specific gene expression, the technique is not suitable for single-cell gene expression studies (Wolf 2013). Third, transcripts of overlapping genes encoded on different chains are not effectively distinguished, which makes it difficult to annotate genes because next-generation sequencing analyses single-stranded mRNAs. Fourth, the mRNA is only an intermediate in the gene expression process, and mRNA level changes are not necessarily reflective of protein level changes. Finally, next-generation sequencing technology is essentially PCR-based sequencing, and some mismatch incorporation occurs during the PCR process, which adversely impacts the accuracy of next-generation sequencing (Wolf 2013).

3.3 Development of Transgenic Silkworms and Applications

Germline transformation in silkworms was first achieved using a *piggyBac* transposon-derived vector (Tamura et al. 2000). The *piggyBac* transposase randomly recognizes the sequence TTAA in the genome and integrates the foreign expression cassette that this site (Uchino et al. 2008). Other transgenic elements, including the Minos transposase (Uchino et al. 2007), ψ31 integrase (Long et al. 2013), and FLP recombinase (Long et al. 2012), have also been demonstrated to mediate genetic transformation in silkworms.

With the ability to generate transgenic silkworms, researchers have developed many genetic tools for silkworm reverse genetics research. The binary GAL4-UAS gene expression system was established to explore gene functions (Kimoto et al. 2014; Kobayashi et al. 2011; Sakai et al. 2016; Tan et al. 2005; Tsubota et al. 2014) and has been used to reveal the roles of small RNAs (Ling et al. 2015). Techniques for transient expression in specific tissues (i.e., the epidermis) have also been applied to explore certain gene functions (Yoda et al. 2014). Many tissue, sex, and stage-specific promoters have been identified by the transgenic expression of marker genes (Deng et al. 2013; Xu et al. 2015), and these will greatly benefit future research. As the silk gland from silkworms are a highly effective protein production biofactory, specific promoters have been used to engineer the expression of foreign proteins in the silk glands of transgenic silkworms (Tatematsu et al. 2010; Wang et al. 2013a).

As an insect with economic importance, efficient systems to separate the male individuals from females, which will enable higher silk yields, are desired. Transgenic platforms have been used to allow genetic sexing using a W chromosome-linked transgene and a transgene-based female-specific lethality system (Tan et al. 2013; Ma et al. 2013). Also of economic importance is the development of methods to engineer resistance to viral infection, particularly BmNPV. With the combination of transgenics and the RNA interference (RNAi) system, researchers have generated transgenic silkworms that are more resistant to different viruses than wild-type insects (Ito et al. 2008; Subbaiah et al. 2013; Zhang et al. 2014; Zhou et al. 2014).

After almost two decades of development, the transgenic platform in silkworms has proven its power in both fundamental and applied research. Work in silkworms has also been horizontally transferred to pest species in the lepidopteran order. Some improvements could be made, such as the establishment of a genome-scale gene RNAi (shRNA) library and the application of an enhancer trap system to identify more expression regulatory elements for accurate and specific gene expression control.

3.4 Genome Editing Techniques for Silkworms

During the first decade of the 21st century, genome editing tools began to be established in various organisms, including the silkworm. The first generation genome editing methods are ZFNs and TALENs, which are based on the combination of

domains that bind specifically to a particular DNA sequence (the zinc finger proteins and TALEs, respectively) and a non-specific DNA-cleaving nuclease. The first successful targeted mutagenesis in the silkworm was of the epidermal colour marker gene *BmBLOS2* using ZFNs (Takasu et al. 2010; Daimon et al. 2014). Subsequently, the application of TALEN-mediated gene disruption was also achieved in the *BmBLOS2* gene (Ma et al. 2012; Sajwan et al. 2013; Takasu et al. 2013, 2016b); this gene has become the ideal target for testing new genome engineering techniques. In addition to its use in gene depletion for functional research (Daimon et al. 2015), the TALEN system has been applied to mediate precise genetic transformation (Nakade et al. 2014; Takasu et al. 2016a; Wang et al. 2014a, b), to transform the silkworm silk gland into a highly efficient bioreactor (Ma et al. 2014b), and for genetic sexing (Xu et al. 2014).

More recently, the newly emerged tour de force genome editing tool, the CRISPR/Cas9 system, was established in silkworms (Wang et al. 2013a, b). The CRISPR/Cas9 system enables multiplex targeted mutation, large genomic fragment deletion, and heritable mutagenesis in silkworm cells and in vivo (Li et al. 2015; Liu et al. 2014; Ma et al. 2014a; Wei et al. 2014; Xu et al. 2016; Zhang et al. 2015). Researchers have also tried to enhance gene targeting by knocking out factors in the non-homologous end joining pathway; this will facilitate the application of homologous recombination-mediated gene insertion in silkworm individuals (Zhu et al. 2015). A highly efficient virus-inducible CRISPR/Cas9 system was also established in silkworm cells, which suggested the possibility of a CRISPR/Cas9-based anti-virus strategy (Dong et al. 2016). Finally, U6 promoter-mediated N20NGG-type sgRNA expression for gene disruption in vitro and in vivo, which is different from that in mammals, has expanded the targetable engineering sites in the silkworm genome (Zeng et al. 2016).

As nuclease-based genome-editing techniques have demonstrated great potential in silkworm research, further studies should be performed to enhance and improve these methods. There are still some issues to address, such as finding ways to reduce off-target effects, expanding the target range and realizing CRISPR-mediated gene insertion, and achieving specific mutations and single-nucleotide correction. Genome editing techniques optimized and validated in the silkworm will also find application in genetic control of lepidopteran pests. The CRISPR-based platform has already been established in mosquitoes (Gantz et al. 2015; Hammond et al. 2016).

4 SJ-10 Satellite Experiment

4.1 The Silkworm Incubator, SJY102-10/Z01-1, Used Aboard the SJ-10 Satellite

Based on our research goals and comprehensive consideration of the conditions aboard the SJ-10 satellite platform, we developed an incubator to study the influ-

Fig. 1 The silkworm incubator used aboard the SJ-10 satellite

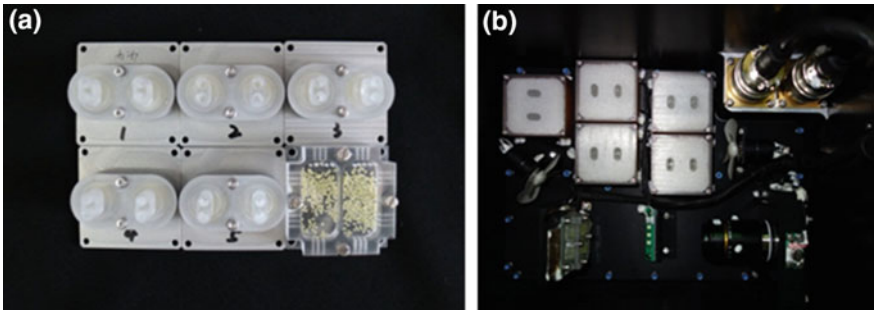

Fig. 2 **a** Silkworm embryos in the incubator. **b** The culture units installed in the silkworm incubator

ence of the space environment on silkworm embryonic development (Fig. 1). This system allowed the culture of silkworm eggs in space for 12 days and 15 h of flight, providing full control of temperature and enabling continuous monitoring of environmental conditions (Fig. 2). Biological samples were collected and stored during the cultivation process within the incubator.

Considering the effects of the space environment, we carried out a simulation experiment on the ground before launching. The number of embryos and the culture temperature and humidity were optimized in the ground simulation (Fig. 3). Loading of the silkworm incubator with embryos was completed on April 4, 2016, and installation and tower trans-shipment work was completed on April 5, 2016. The satellite was launched on April 6, and the incubator was retrieved on April 18, 2016.

The silkworm incubator was used for the in-orbit experiment, and there were five units in the incubator. The culture temperature was 21 °C, but the temperature was changed to 4 °C every two days in one unit during the entire culture process. Digital

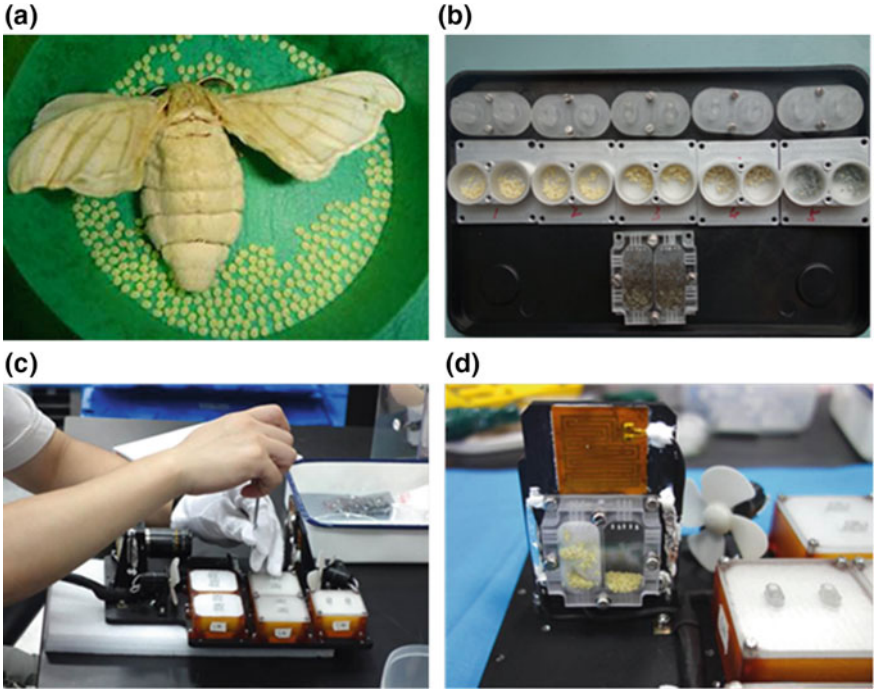

Fig. 3 Silkworm embryo collection and sample loading. **a** Collection of the embryos. **b** Image of the incubator after the simulation experiment on the ground. **c** The cryogenic fixation of samples. **d** The transparent unit enables digital images to be taken during culture

Fig. 4 Images of the silkworm embryonic development in space. **a** Image captured aboard the SJ-10 satellite on day 1 after launch. **b** Image captured after 6 days in orbit. **c** Image captured in the SJ-10 satellite on the final day in orbit

images of the silkworm embryos were obtained every day during flight (Fig. 4). After flight, the silkworm embryos were retrieved. All remained in good condition.

4.2 Studies of Silkworm Development in Space

4.2.1 Goals of the Silkworm Study Performed Onboard the SJ-10 Satellite

The space environment is significantly different from that on the earth with lower temperatures, higher radiation levels, vacuum characteristics, long-term microgravity, and weaker magnetic fields than on earth. We expected to find developmental differences between embryos that had been in space compared to ground-based controls that might be observed in subsequent generations. A goal is to identify genes that play dominant roles in the regulation of these processes. These experiments are continuing.

4.2.2 Study of Silkworm Phenotypes

The study of growth, development, ageing, and death of the silkworm may result in lessons that can be extrapolated to other organisms and even human beings. The silkworm is an animal model for radiation dose biometrics and basal metabolic resistance to external environment disturbances. In silkworms that had been aboard the satellite and in ground controls, a number of physiological indexes were measured, including embryonic development rate, hatching rate, larval digestion and absorption, larval weight, larval developmental period, total lipid droplets in larval haemolymph, protein concentration in larval haemolymph, larval mid-gut esterase activity, larval fat mass, soluble protein and total lipid droplet concentrations, juvenile hormone and ecdysone levels, silkworm cocoon yield, silk quality, silk gland GPTase activity, glutathione S-transferase activity, mating behaviour and oviposition.

4.2.3 Analysis of the Genome, Genes, and Non-coding RNA Expression

The rapid development of sequencing technology and silkworm genomics has made possible the study of differences in the genomes and transcriptomes of flight group s and ground control groups. We are performing whole genome re-sequencing, transcriptome and proteome analysis and using other methods to carry out correlation analyses of the flight group and the ground control group. We are also working to identify the differentially expressed genes and non-coding RNAs. We are eager to identify genetic variations and explore the candidate genes responsible for certain traits. We also want to perform long-chain non-coding RNA sequencing, circular RNA sequencing, and small RNA sequencing and plan to use whole-genome bisulfite sequencing techniques to study DNA methylation and mRNA and miRNA methylation, as well as transcription factor binding site methylation.

4.2.4 Functional Validation of Certain Space-Responsible Candidate Genes

Based on the cultivation of silkworm embryos in the SJ-10 satellite, a single environment of microgravity, heavy ions, protons, and neutrons was established to simulate the space environment. This will allow further study of the effects of the space environment on the gene expression and protein expression characteristics of the silkworm. Using the latest genome-editing technologies, such as CRISPR/Cas9 and TALENs, we are also trying to explore the functional and molecular mechanisms of space environment-sensitive genes.

4.3 Preliminary Research Results

After incubation in the space environment, the hatching rate of the silkworm embryos was not significantly different from that of the ground control group. The larvae in both the ground control group and the SJ-10 satellite group were reared on fresh mulberry leaves at 25 °C. In the larval stage, the development time of SJ-10 satellite group silkworms after 15 days was significantly shorter than that of the ground animals after 18 days. Furthermore, the flight group showed precocious wandering behaviour and earlier larval-pupal transition by 3 days relative to the ground-based controls (Figs. 5 and 6). No significant changes in larval or pupal body sizes or pupal development time were observed between the SJ-10 satellite group and the ground group. In the adult stage, the SJ-10 satellite group silkworms mated normally, and the oviposition rate did not differ significantly from that of the ground control. The next generation has begun breeding. Other data still need further analysis.

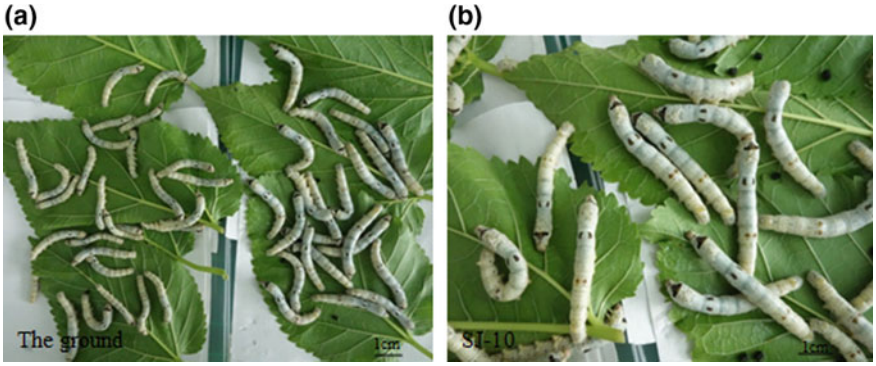

Fig. 5 The flight group made an earlier larval to pupal transition than the ground group. Photographs of **a** the ground group and **b** the SJ-10 satellite group captured on the same day. The ground group consisted of day 2 larvae at the fifth instar, and the SJ-10 satellite group consisted of day 5 larvae at the fifth instar

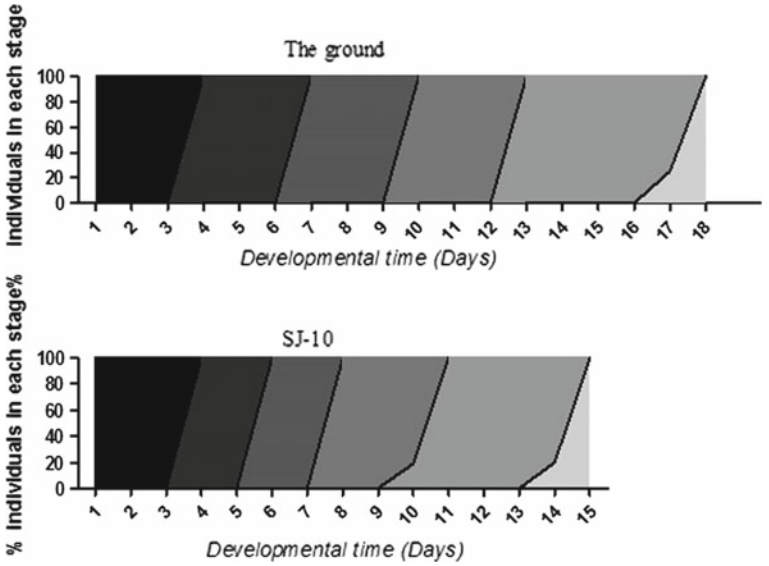

Fig. 6 The percentage of individuals in each stage of larval development in the ground group (**top panel**) and the SJ-10 satellite group (**bottom panel**)

5 Discussion and Prospective

Over the more than a half century that humans have been in space, valuable and meaningful data have been obtained on effects of space on various organisms, and these studies have laid the foundation for the future exploration and development of space. However, many problems and challenges remain. Firstly, satellites are typically built one at a time and at huge expense, making it expensive to launch and maintain satellite-based experiments. The time frame of an experiment is limited, and there is very little opportunity for reproducibility, so results obtained may not be reliable. Secondly, due to hardware limitations and the fact that satellites are not manned means that experiments cannot be performed on large organisms and complex life science experiments are not possible. Finally, the time in space experiment is limited. Therefore, a large number of ground-based simulations of space conditions are needed.

In the space environment, there are many factors, including weightlessness, strong ionizing radiation, and sub-magnetic field, that could alter physiological and biochemical functions during silkworm development. Our study of the influence of space on the development and genetic variation of the silkworm provided important data for the development of silkworm breeding, and it also provided a theoretical basis for exploring new breeding methods.

China is a major location for sericulture and is the homeland of silk; silkworms have been bred in captivity for thousands of years in China. The silkworm is an

important economic insect, and the silk industry is an ecologically sound component of China's economic strategy. At the present, there are more than 10 million farmers in China involved in the silkworm industry, and the sericulture areas are spread over 26 provinces, with a total of more than 800,000 ha of mulberry gardens. In 2011, cocoon production was approximately 800,000 tons, with Chinese silk production accounting for more than 75% of total world production. China is the center of the world silk industry, and the Chinese silk accounts for 50% of the world export trade. The output value of the silk industry is more than 160 billion yuan. The silkworm also contains valuable proteins and is a good germplasm resource.

In recent years, Chinese scientists have used space technology in an effort to enhance crop mutagenesis and to select new traits. The application of space mutagenesis technology to silkworms will lead to characterization of new silkworm mutant lines that will be beneficial to silkworm researchers and in industrial applications. The silk industry has played an important role in solving the problem of rural surplus labor, maintaining social stability. Using the space environment for systematic research on the silkworm thus has not only important theoretical significance but also economic value.

Interestingly, studies suggest that silkworms may be an ideal high-nutrition food for astronauts that can be grown efficiently in a controlled system like a space station. Studies of the nutritional composition, maturity time, and processing of these insects demonstrate that the silkworm can be used as a source of protein for astronaut recipes. Life-support technology is important for long-term viability of a manned space program, such as a lunar base, a space laboratory, or a space station. Five or six silkworm pupae are equivalent to an egg in terms of nutrition value. Based on weight, the content of protein in a silkworm chrysalis is much higher than that of eggs, and the amino acid content is also several times higher than pork, lamb, eggs, or milk by weight. The proteins produced by silkworm can be harvested in a short time, and silkworms do not need water and so do not produce waste water. In brief, the silkworm is an ideal protein source for astronauts in the space.

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