CHAPTER TEN

Cricket: The third domesticated insect

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Abstract

Many researchers are using crickets to conduct research on various topics related to development and regeneration in addition to brain function, behavior, and biological clocks, using advanced functional and perturbational technologies such as genome editing. Recently, crickets have also been attracting attention as a food source for the next generation of humans. In addition, crickets are increasingly being used as disease models and biological factories for pharmaceuticals. Cricket research has thus evolved over the last century from use primarily in highly important basic research, to use in a variety of applications and practical uses. These insects are now a state-of-the-art model animal that can be obtained and maintained in large quantities at low

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cost. We therefore suggest that crickets are useful as a third domesticated insect for scientific research, after honeybees and silkworms, contributing to the achievement of global sustainable development goals.

1. Introduction

Crickets belong to the family Gryllidae (Insecta, Orthoptera). There are more than 2400 described species of crickets belonging to this family. The main cricket species used for research in the United States and Japan is *Gryllus bimaculatus*. The most commonly used wild type laboratory strain of this cricket originated from a population on Ishigaki Island in southern Japan, and was brought to Hiroshima City around 1970 by Midori Nishioka (Amphibian Research Center, Hiroshima University) as frog food. Today, these crickets are commonly sold for use as live food for amphibian and reptilian pets, and together with European crickets, are widely cultivated around the world.

In order to achieve Zero Hunger, the second of the 17 goals of the global sustainable development goals declared in 2015 (Atukunda, Eide, Kardel, Iversen, & Westerberg, 2021), namely efforts to improve the efficiency of food production, is gaining attention. Crickets, in particular, hold new potential for an edible insect as an alternative protein source to compensate for protein deficiencies in human food and livestock feed. In Japan, locusts are a representative of an edible insect. In Southeast Asia, including Thailand and Cambodia, crickets and other insects are a part of many people's daily diet. Recently, in the United States and Europe, a variety of foods using crickets have been developed. In recent years, products using crickets, such as "cricket rice crackers," have also been released in Japan.

It is noteworthy that many of these crickets, including *G. bimaculatus*, can lay eggs and breed all year round at a rearing temperature of about 28 °C. Their reproductive cycle is about 40–50 days at 28 °C, and each female produces more than 1000 eggs in her lifetime. The dry weight of one cricket is about 0.2 g, and current commercial cricket farming industries can harvest over 50 million tons per week. Because of their rapid growth and environmental friendliness, these crickets are very useful as a new source of protein in food and food products.

In addition to this important applied use of crickets, important findings have also been obtained in basic research. In particular, from the perspective of entomology, an outstanding problem is to understand the mechanism of

morphogenesis of hemimetabolous insects, to elucidate the process by which insects evolved indirect development (holometabolous development), and crickets have been used as model insects for this purpose. RNA interference is an effective research tool for cricket gene function analysis, and has the advantage of being very simple and efficient. Genome editing technology can also be used, which makes cricket species very useful as research organisms.

In the future, it should be possible to use these genome editing technologies to develop a variety of health-enhancing properties in crickets, and to make crickets themselves into health foods and medicines that can be eaten and used in various types of production industries. Thus, we suggest crickets will become the third domesticated insect, following successful domestication of honeybees (*Apis mellifera*) and silkworms (*Bombyx mori*), thereby contributing to the fulfillment of global sustainable development goals for the betterment of humanity.

2. Advantages as a model system

The insect that has been used the most in genetic laboratory research is the fly *Drosophila melanogaster*. *Drosophila*-based work has been associated with five Nobel Prizes in Physiology or Medicine. This illustrates the utility of basic research on insects in elucidating basic biological principles of many organisms, including humans. Some major advantages of using crickets as insect model organisms, and examples of new biological and evolutionary insights contributed by research using crickets, are described below.

2.1 Availability and ease of husbandry

Crickets of multiple species are generally available in large quantities at low cost for use in research and teaching. In addition, for applications where uniformity of the genetic background is important, multiple laboratories maintain inbred strains of the cricket *G. bimaculatus*, using the white-eyed trait as a genetic marker.

2.2 Genome sequence and genetic manipulation

The number of chromosomes 2n in *G. bimaculatus* is 28 + XX (female)/XO (male). The only sex chromosome is the X chromosome (Yoshimura, Nakata, Mito, & Noji, 2006). The 1.66 billion base pair genome of the

cricket has been sequenced, assembled and annotated (Ylla et al., 2021). For context, it is useful to consider the history of insect genome sequencing.

The *D. melanogaster* genome (Adams et al., 2000) was the first publicly available insect genome, and the second animal genome to be fully sequenced, preceded only by the genome of the nematode *Caenorhabditis elegans* (C. elegans Sequencing Consortium, 1998). After the *Drosophila* genome, the insect genomes that followed to be sequenced were those with human health or economic interests. These included the malaria-carrying mosquito *Anopheles gambiae* (Holt et al., 2002), the silkworm *B. mori* (Mita et al., 2004), the honeybee *A. mellifera* (Weinstock et al., 2006), and the pest beetle *Tribolium castaneum* (Richards et al., 2008). All of these insect species belong to the monophyletic Holometabola (also called Endopterygota), which undergo complete metamorphosis.

In 2010, nearly 10 years after the publication of the *D. melanogaster* genome, the genome of the pea aphid *Acyrthosiphon pisum* was published, representing the first fully sequenced genome of a hemimetabolous insect (International Aphid Genomics Consortium, 2010). This was closely followed by the genome of the hemimetabolous human parasite, the louse *Pediculus humanus corporis* (Kirkness et al., 2010). The pea aphid belongs to the order Hemiptera, and the louse belongs to the order Phthiraptera. These two orders are typically grouped under the superorder Paraneoptera, although it is not clear whether Paraneoptera is monophyletic or paraphyletic (Belles, 2020; Misof et al., 2014; Wang et al., 2016; Wipfler et al., 2019). In either case, paraneopteran species, despite displaying the ancestral state of hemimetabolous metamorphosis (direct development), are more closely related to the Holometabola than to the other 14 hemimetabolous orders, 10 of which belong to the superorder Polyneoptera.

In 2014 the first polyneopteran genome assembly was published. This was the genome of the dessert locust *Locusta migratoria*, which, at 6.5 Gb, represented the largest animal genome sequenced to date at the time of its release (Wang et al., 2014). The genomes of more Polyneoptera, including the termites *Zootermopsis nevadensis* and *Macrotermes natalensis* (Poulsen et al., 2014; Terrapon et al., 2014), quickly followed. The first cricket genome assembly was that of the Hawaiian cricket *Laupala kohalensis*, published in 2018 (Blankers, Oh, Bombarely, & Shaw, 2018), followed by the genome assemblies of the Australian field cricket *Teleogryllus oceanicus* (Pascoal et al., 2020), the Asian cricket *Teleogryllus occipitalis* (Kataoka et al., 2020), the house cricket *Acheta domesticus* (Gupta et al., 2020), and the Mediterranean field cricket *G. bimaculatus* (Ylla et al., 2021).

21 years after the publication of the first insect genome, at the time of writing NCBI contains the genome assemblies of 705 insect species. However, most of them belong to only a few specific groups of insects (Fig. 1A). 617 of them (87.52%) belong to holometabolous species, while there are only 87 (12.34%) genomes of hemimetabolous species and 1 (0.14%) is from an ametabolous species. Of the 87 hemimetabolous genomes, 54 belong to the Paraneoptera and only 27 belong to the Polyneoptera (Fig. 1A). Thus, despite the fact that Polyneoptera are one of the largest winged insect lineages with

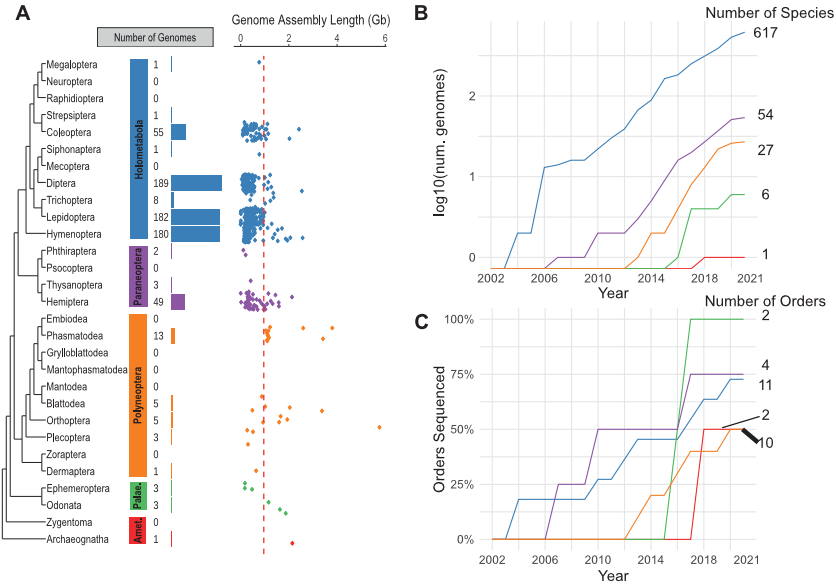

Fig. 1 (A) Phylogenetic tree of the main orders of insects based on Misof et al. (2014), modified to merge the former order Isoptera into Blattodea (Inward, Beccaloni, & Eggleton, 2007), maintaining monophyletic Paraneoptera (Wang et al., 2016) , and splitting Psocodea into Psocoptera and Phthiraptera, keeping NCBI-taxonomy ranks, showing the number of species with a publicly available NCBI genome assembly at the time of writing (bar plot with number shown at the base). Colored dots represent the reported lengths of each genome assembly in gigabases (Gb). The red-dashed vertical line indicates a genome length of 1 Gb. Colors indicate Holometabola (blue), Ametabola (red), Paraneoptera (purple), Polyneoptera (orange), and Palaeoptera (green). (B) Number of species with a publicly available NCBI genome assembly per year since 2002 in log10 scale for each of the five insect lineages shown in colors in (A). The number on the right indicates the number of insect species with a publicly available NCBI genome assembly available at the time of writing. (C) Proportion of insect orders with at least one publicly available NCBI genome assembly per year since 2002. The number on the right indicates the total number of insect orders in the given lineage; colors as in (A).

circa 40,000 described species (Belles, 2020; Wipfler et al., 2019), and their study being essential to fully understand insect evolution and biodiversity, fewer than 1% of species of this clade have genomes available.

Using the submission dates of the genome assemblies deposited in NCBI as a proxy for studying the progress on insect genome sequencing, we observe an exponential growth in the number of genomes per year for the major insect lineages (Fig. 1B). Although this exponential growth in the number of insect genomes is very exciting for the scientific community, the number of newly sequenced orders grows at a much slower pace (Fig. 1C). As result, we have a representative genome from only 72.71% of the Holometabola orders (8 out of 11), 50% of the Polyneoptera (5 out of 10), and 75% of the Paraneoptera (3 out of 4) (Fig. 1C).

This sampling bias might lead to inaccurate or erroneous conclusions regarding the evolution of various aspects of insect biology, given that holometabolous genomes appear to be quite different from those of polyneopteran species in many respects. For example, while most Holometabola do not perform genome-wide DNA methylation (the few known exceptions being primarily confined to the Hymenoptera), most polyneopteran genomes display signs of widespread DNA methylation (Bewick, Vogel, Moore, & Schmitz, 2017). Similarly, the genome sizes of the Holometabola and Paraneoptera are very different from those of Polyneoptera (Fig. 1A). Most of the genome assemblies in NCBI of Holometabola (589 out of 617) and Paraneoptera (45 out of 54) are under 1 Gb, and only 5 holometabolous species and 1 paraneopteran species have a genome larger than 2 Gb, the largest one being 2.6 Gb. By contrast, most of the polyneopteran genome assemblies (20 out of 27) exceed 1 Gb, and 6 of them exceed 2 Gb. The largest insect genome assembly available in NCBI to date belongs to the orthopteran *L. migratoria*, with an assembly length of 5.76 Gb (reported as 6.5 Gb in the original publication (Wang et al., 2014)). The genome sizes of some other Orthoptera, although not yet available in NCBI, are even larger, such as the recently assembled 8.55 Gb genome of the locust *Schistocerca gregaria* (Verlinden et al., 2020), and the estimated 16–18 Gb genome of the grasshopper *Podisma pedestris* (Bensasson, Petrov, Zhang, Hartl, & Hewitt, 2001; Camacho et al., 2015; Westerman, Barton, & Hewitt, 1987). This makes the Orthoptera one of the animal lineages with the widest range of genome sizes, and therefore one of the most interesting lineages in which to study genome size evolution and the mechanisms of genome size expansion and constraint.

At the time of writing, there are five cricket genome assemblies available, four of which have been annotated: *T. oceanicus*, *T. occipitalis*, *L. kohalensis*,

and *G. bimaculatus* (Kataoka et al., 2020; Pascoal et al., 2020; Ylla et al., 2021). These four crickets have fairly similar genome sizes (ranging from 1.6 to 2 Gb), numbers of annotated protein-coding genes (ranging from 12,000 to 19,000), and proportions of reported repetitive content (ranging between 34% and 45%). These annotated cricket genomes have also provided new insights into cricket genomics, and allowed us to gain an understanding of the evolution of polyneopteran genomes. For example, comparing the genome annotations for two cricket species, *G. bimaculatus* and *L. kohalensis*, revealed that nearly 45% of annotated protein-coding genes display fingerprints of DNA methylation. Furthermore, these genes show significantly stronger signatures of purifying selection, and higher conservation across distant species, than genes predicted to be unmethylated, suggesting a putative biologically relevant role for DNA methylation in Polyneoptera that may have been lost in many species of Holometabola.

Genetic manipulation techniques achieved by injection of fertilized cricket eggs, including genome editing using CRISPR/Cas9, have been established (Watanabe, Noji, & Mito, 2017). In addition, the ReMOT control genome editing method, in which a complex of Cas9 and guide RNA injected into the abdominal hemolymph of an adult female insect is transferred to the developing eggs, wherein genome editing occurs, has been developed in mosquitoes (Chaverra-Rodriguez et al., 2018; Macias et al., 2020) and applied successfully in other arthropods (Chaverra-Rodriguez et al., 2020; Heu, McCullough, Luan, & Rasgon, 2020; Sharma et al., 2022). With this method, microinjection into fertilized eggs, which can be initially challenging for inexperienced researchers, becomes unnecessary for genome editing. This method should be applicable to crickets as well.

2.3 Germ cell formation and evolution

In animals that reproduce sexually, germ cells are the only cell lineage that transmits genetic information to the next generation. Elucidating the characteristics of germ cells and the mechanisms that control their formation is one of the most important issues in life science research. Differentiated cells specialized for reproductive ability are an evolutionary novelty typically acquired concomitantly with the evolution of multicellularity (Veit, 2019). Histological, embryological and molecular studies have shown that germ cell formation in animals can be broadly classified into two modes: (a) somatic induction by signals from somatic cells, and (b) cytoplasmic inheritance by localization of maternal effectors (germ plasm) during egg formation (Extavour, 2007; Extavour & Akam, 2003). It is hypothesized that

the inheritance mode evolved from the somatic induction mode (Extavour, 2007; Extavour & Akam, 2003). However, the molecular mechanisms involved in the formation of primordial germ cells have only been elucidated in a few model organisms.

The cricket *G. bimaculatus* has played an important role in recent attempts to clarify the commonality and diversity of the molecular basis of the two modes of primordial germ cell formation from an evolutionary developmental perspective. The Extavour lab performed studies elucidating the molecular mechanisms of primordial germ cell formation in this cricket, which provided strong evidence for somatic cell-induced primordial germ cell formation by the hypothesized ancestral mode of induction, for the first time in an invertebrate animal. Interestingly, BMP signaling induces cricket primordial germ cells from somitic mesoderm by positively regulating the expression of the Blimp1 transcription factor, as is the case in mice (Donoughe et al., 2014; Nakamura & Extavour, 2016). In addition, the posterior Hox genes are required for correct cricket germ cell formation (Barnett, Nakamura, & Extavour, 2019).

2.4 Parental RNAi and the study of early development

Fertilized eggs of *G. bimaculatus* hatch in 10 days at 28 °C. 27 hours after fertilization, a large portion of the blastoderm cells assemble separately into dense condensations on the left and right sides of the ventral midline in the posterior third of the embryo. Later, they coalesce along the ventral midline to form a small embryonic rudiment in a posterior ventral position. At this stage both the anteroposterior and the dorsoventral axes of the embryo are determined, and are aligned with those of the egg. The rudiment tissues give rise to the head, thoracic, and telson primordia, whereas abdominal segments form at later stages, sequentially from anterior to posterior. Unlike the derivatives of the imaginal discs of Holometabola, limb buds in crickets are formed as lateral outgrowths from the thoracic segments, gradually elongate and eventually form legs, more similar to the developmental process that produces vertebrate limbs than to that involved in *Drosophila* leg formation.

Parental RNAi is efficient in male and female crickets. When double-stranded RNA is injected into the adult female abdominal cavity, typically more than 1000 eggs subsequently laid by the injected female gradually develop a strong phenotype (Ronco, Uda, Minelli, Noji, & Klingler, 2008). This is a very useful method for the analysis of genes involved in embryonic development.

2.5 Mechanisms of metamorphosis and its evolution

Insect development proceeds in one of two ways: full metamorphosis, seen in indirectly developing insects (e.g., flies, butterflies), or incomplete metamorphosis, seen in directly developing insects (e.g., dragonflies, crickets). Holometabolous insects undergo full metamorphosis, forming a pupa and then becoming an adult. As epitomized by the metamorphosis of butterflies, for example, the adult forms a completely different body plan from the larva. Holometaboly evolved from hemimetaboly, and hormones that control metamorphosis are present in both types of insects, but are deployed differently in the two groups. Studies in *G. bimaculatus* have been instructive in understanding how hormones regulating holometabolous metamorphosis operate in hemimetabolous insects, and thus in clarifying how insect metamorphosis evolved.

Juvenile hormone (JH) is a hormone that inhibits the metamorphosis of larvae into pupae in Holometabola, or of juveniles to adults in Hemimetabola. When JH activity is artificially and continuously increased by external treatment, larvae undergo additional growth and molt cycles, resulting in a dramatic transformation into giant-sized adults. On the other hand, when JH action is inhibited, the number of molts until metamorphosis is reduced, resulting in adults with smaller body size, referred to as precocious metamorphosis. In the final step of the juvenile hormone synthesis process, juvenile hormone acid methyltransferase (JHAMT) and epoxidase (Cytochrome P450: CYP15A1) are involved. JHAMT was first identified in the silkworm in 2003 by T. Shinoda and his colleagues (Shinoda & Itoyama, 2003). In the absence of this enzyme, JH is not activated. In early silkworm larvae, JHAMT is continuously expressed and produces juvenile hormones, but in final instar larvae, JH synthesis ceases due to a decrease in the amount of JHAMT, resulting in the induction of metamorphosis. Thus, the repression of JHAMT expression is an essential switch for insect metamorphosis. However, the mechanism of JHAMT repression has remained unclear. Elucidation of the molecular mechanism is very important for understanding the regulation of insect metamorphosis.

Studies in *G. bimaculatus* by T. Mito, Y. Ishimaru and their colleagues revealed that in this cricket, JH is regulated by *dpp* and *myoglianin*, which are members of the TGFb family of ligands (Ishimaru et al., 2016). Dpp signaling regulates the biosynthesis of active JH through the induction of JHAMT expression and suppresses molting to adulthood at early stages. At later stages, when myoglianin signaling represses JHAMT expression, the final molt to adulthood is triggered. Thus, the timing of metamorphosis is strictly regulated by the effects of both signals on JHAMT expression.

In the molecular mechanism of juvenile hormone action in holometabolous insect metamorphosis, the mutually inhibitory functions of *Krüppel homolog* 1 (*Kr-h*1), *Broad-Complex* (*Br*), and *E93* at two stages ((terminal larva → pupa) and (pupa → adult)) are also observed in the final transition from juvenile to adult in hemimetabolous insects. Based on the timing of action of this evolutionarily conserved molecular mechanism, Ishimaru and colleagues proposed the hypothesis that in the cricket, the preterminal → larval stage of hemimetabolous development corresponds to the pupal stage of Holometabola (Ishimaru, Tomonari, Watanabe, Noji, & Mito, 2019). Thus, although the metamorphosis process differs greatly between holometabolous and hemimetabolous insects in terms of relative degree of change in body plan organization, many elements of the molecular mechanisms controlling the process appear conserved.

2.6 Surgical experiments and RNAi elucidate leg regeneration mechanisms

The legs of the cricket larvae are capable of complete regeneration following amputation. Pioneering studies on the regeneration of the legs of hemimetabolous insects were primarily conducted by European researchers using cockroaches, but these studies had nearly ceased by around 1980. Performing similar experiments with crickets, T. Bando and colleagues hypothesized that the protocadherins Fat/Dachsous (Ft/Ds) might determine positional information in cricket legs during regeneration, and tested this hypothesis using RNAi. A related hypothesis had previously been proposed by P. Lawrence and colleagues based on his work on *Drosophila* (Lawrence, Struhl, & Casal, 2008). They suggested that these molecules may be responsible for positional information, and proposed the Ft/Ds gradient model as a mechanism to determine the length of leg segments. Bando and colleagues found that when cricket legs regenerate, if the function of Ft is lost, the regenerated legs are thicker and shorter than controls. This phenotype is similar to that of loss of function mutations of the *fat* gene in *Drosophila*, which results in thicker legs (hence the name of the gene). Bando and colleagues found that signaling by the protocadherins Ft/Ds is involved in the regeneration process via the Hippo signaling pathway, and proposed a novel gradient model for the determination of leg size (Bando, Mito, Nakamura, Ohuchi, & Noji, 2011). Elucidation of the regeneration mechanism at the molecular level is ongoing (Bando et al., 2018).

Recently, it was reported that during recovery from damage to multiple organ systems in larval and adult zebrafish, the innate immune response is involved in their regeneration (Iribarne, 2021). To investigate whether

macrophages are similarly involved in the regeneration of cricket legs as an innate immune response, Bando and colleagues applied a pharmacological agent that kills macrophages to crickets subject to leg amputation, and observed no subsequent regeneration of the amputated leg (Bando et al., 2021). This suggests that the function of macrophages is necessary in early stages of regeneration, and that this function may be common to both insects and vertebrates. In addition, cricket regeneration requires a STAT protein that is also involved in the differentiation of human macrophages. A current model thus posits that when a cricket's leg is amputated, cells of the immune system, including macrophages, gather to seal the wound, and that substances secreted by these cells may determine the start of regeneration. Understanding how the innate immune response regulates regeneration in the cricket may provide important clues that could improve the therapeutic strategies for repairing injured mammalian tissues that do not have inherent regenerative capacity (Iribarne, 2021).

2.7 Internal clock and photoperiodism in crickets

Crickets, like most animals, have an internal circadian clock. Studies by Seymour Benzer and others of clock mutants in *Drosophila* (Konopka & Benzer, 1971; Price, 2005; Singh et al., 2019; Takahashi, 2004), and a detailed gene expression cascade (Claridge-Chang et al., 2001; Hardin, 2011) have laid important foundations for understanding the internal clock in this insect. While the regulation of insect physiological activities including reproduction and dormancy are regulated by photoperiodism (the response of an organism to the length of the light or dark period of the day), the mechanism of this regulation is still unclear. K. Tomioka and his group conducted experiments to address this problem using the cricket *Modicogryllus siamensis* (Miki, Shinohara, Chafino, Noji, & Tomioka, 2020). When the expression of myoglianin was suppressed by RNAi under long-day conditions, the number of molts increased, indicating short-day development. They also observed that the expression of myoglianin was regulated by day-length information through the brain. The question of what regulates the expression of myoglianin in the brain remains unclear, but its answer may be relevant to elucidation of the mechanism of hibernation in animals, given that the mammalian version of myoglianin is myostatin (GDF8), which may be involved in hibernation (Brooks, Myburgh, & Storey, 2011).

2.8 Brain and behavioral studies

The fact that crickets can learn and remember has been exploited by neurobiologists and neuroethologists to study the mechanisms of learning

and memory. Researchers use genetic manipulation, RNAi and surgical procedures such as the introduction of electrodes, to pursue research into the higher functions of the brain. For example, the mechanisms underlying the formation and retention of memory of crickets has been under investigation by the Mizunami group (Matsumoto, Matsumoto, & Mizunami, 2018). Recently, they found that the cricket has the ability to learn, not only by previously known olfactory stimuli (Matsumoto & Mizunami, 2002, 2004), but also by observation (Ebina & Mizunami, 2020). In humans, "observational learning" refers to the ability to learn by observing the behavior of other individuals without having any experience of their own. Evidence for this ability comes from experiments wherein a "demonstrator" cricket is allowed to freely visit a water reward associated with a particular scent, or salt water (punishment) associated with a second scent. An "observer" cricket is allowed to observe the behavior of the "demonstrator" through a transparent partition, and then tested to see which of the two waters they prefer (Ebina & Mizunami, 2020). The neural substrate of this learning model in crickets is the subject of ongoing work.

2.9 Crickets as food

Around 2016, the phrase "entomophagy" began to appear with increased frequency in multiple scientific journals, often in the context of global sustainable development goals. Crickets, in particular, appear increasingly frequently as an example of edible insects. Since the wealth of existing developmental research in crickets should be useful to enhance the cultivation and breeding of edible crickets, crickets as an edible resource may contribute effectively to solving the planets' global human food shortage. Multiple commercial and nonprofit organizations, including Aspire Food Group and Gryllus Inc., are working on the development of automatic cricket breeding equipment, a stable supply of cricket powder, and genetic enhancements of cricket nutritional value.

3. Conclusion

Studies of crickets have made critical contributions to multiple areas of basic and applied scientific knowledge. As the number of insect genomes continues to grow in the future, we suggest that increased focus on incorporating species from currently underrepresented lineages will yield a more complete picture of the diversity of insect genomes. As for cricket genomes, 2020 was a great year, with the release of four annotated genomes and an

unannotated fifth one. Hopefully, these genomes will help to establish crickets as even more widely used model organisms, promoting the development of enhanced research protocols, experimental techniques, and genome editing technologies.

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