Chapter 8
The Cricket *Gryllus bimaculatus*: Techniques for Quantitative and Functional Genetic Analyses of Cricket Biology

Arpita Kulkarni and Cassandra G. Extavour

Abstract All extant species are an outcome of nature's "experiments" during evolution, and hence multiple species need to be studied and compared to gain a thorough understanding of evolutionary processes. The field of evolutionary developmental biology (evo-devo) aspires to expand the number of species studied, because most functional genetic studies in animals have been limited to a small number of "traditional" model organisms, many of which belong to the same phylum (Chordata). The phylum Arthropoda, and particularly its component class Insecta, possesses many important characteristics that are considered favorable and attractive for evo-devo research, including an astonishing diversity of extant species and a wide disparity in body plans. The development of the most thoroughly investigated insect genetic model system to date, the fruit fly *Drosophila melanogaster* (a holometabolous insect), appears highly derived with respect to other insects and indeed with respect to most arthropods. In comparison, crickets (a basally branching hemimetabolous insect lineage compared to the Holometabola) are thought to embody many developmental features that make them more representative of insects. Here we focus on crickets as emerging models to study problems in a wide range of biological areas and summarize the currently available molecular, genomic, forward and reverse genetic, imaging and computational tool kit that has been established or adapted for cricket research. With an emphasis on the cricket species *Gryllus bimaculatus*, we highlight recent efforts made by the scientific community in establishing this species as a laboratory model for cellular biology and developmental genetics. This broad toolkit has the potential to accelerate many traditional areas of cricket research, including studies of adaptation, evolution,

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neuroethology, physiology, endocrinology, regeneration, and reproductive behavior. It may also help to establish newer areas, for example, the use of crickets as animal infection model systems and human food sources.

8.1 Introduction

All cellular life forms share a last common ancestor. Every extant species is a current outcome of an evolutionary process that has taken place over hundreds of millions of years. Thus, each species that exists today can be used as a data point toward increasing our understanding of the living world. Comparative study of the developmental biology of multicellular organisms motivates the field of evolutionary developmental biology, or "evo-devo." However, to date only a relatively small number of species has been used to study the functions of genes that regulate animal developmental processes, which limits our understanding of how developmental genetic changes underpin evolution. If the idealized goal of evo-devo research is to do a species comparison that includes representatives of all major evolutionary transitions, then this calls for the establishment of many more animals as laboratory model organisms than is currently the case. As a step in this direction, developmental biologists are increasingly choosing new animal models that are suitable to address thus far neglected areas of research, while simultaneously selecting these candidates for their ability to fulfill other criteria relevant for evo-devo work. Some criteria that could maximize the scientific gains achieved by establishing new models include choosing organisms that belong to clades that are representative of a wide range of ecological niches, and those belonging to phyla that are species-rich, display high diversity in form and function, have interdisciplinary scientific appeal, are economical to maintain in the laboratory, and could inform issues that directly affect humans (e.g., disease or agriculture). Strategically choosing and studying examples satisfying some or all of these criteria may therefore be impactful, evolutionarily informative, and a good use of limited resources.

The phylum Arthropoda contains multiple species that satisfy many of the above criteria and has thus played a prominent role in modern evo-devo research. Importantly, good fossil records exist for this phylum, providing researchers with snapshots into the evolutionary past and aiding in comparative work. For example, the EDNA fossil insect database lists over 23,000 species (Giribet and Edgecombe 2013; Mitchell 2013), with the earliest records of arthropod fossils dating back to nearly 555 million years ago (Mya) during the Cambrian era (Harvey et al. 2012; Zhang et al. 2010; Vaccari et al. 2004). Undoubtedly, access to such fossil records is essential to understanding the key phenotypic innovations that have made Arthropoda species-rich and evolutionarily successful (Mayhew 2007).

Extensive studies on insects, which account for the majority of all species described on earth (Wheeler 1990; Grimaldi and Engel 2005) and for ~85% of all arthropod diversity (Giribet and Edgecombe 2013), have pioneered and shaped the evo-devo field. This work has informed us of the genetic and evolutionary basis of

pivotal developmental mechanisms. For example, the description of the first homeotic mutant (Bridges and Morgan 1923), and the realization of the significance of conserved developmental genes in body patterning and in the evolution of different body plans across animals, come from studies in the insect *Drosophila melanogaster* (Lewis 1978; Nüsslein-Volhard and Wieschaus 1980; Duboule and Dolle 1989; Graham et al. 1989; Panganiban et al. 1997; Heffer et al. 2013). Other insect-based research that has broadened our understanding of evolutionary processes includes work on key evolutionary innovations such as the insect body plan (Grimaldi and Engel 2005), metamorphosis (Truman and Riddiford 1999), development of wings (Nicholson et al. 2014; Alexander 2018; Bruce and Patel 2018; Linz and Tomoyasu 2018), morphological novelties (Kijimoto et al. 2013), and insect eusociality (Toth and Rehan 2017). The widespread scope of such research is a result of the practical advantages that come with working on insects: insect phylogeny is well established (Giribet and Edgecombe 2013), many species are easy to maintain and culture in the laboratory, are often amenable to functional genetic analysis, in many instances produce easily accessible large broods suitable for external manipulation, and often have life cycles sufficiently short to allow laboratory rearing and multigenerational analysis.

To date, popular insect models to study the genetic basis of development have included the fruit fly *Drosophila melanogaster* (Diptera) (Demerec 1950), the flour beetle *Tribolium castaneum* (Coleoptera) (Sokoloff 1966, 1972, 1974, 1977; Denell 2008), the honeybee *Apis mellifera* (Hymenoptera) (Gould and Grould 1995; Oldroyd and Thompson 2006), the wasp *Nasonia vitripennis* (Hymenoptera) (Werren and Loehlin 2009), and the silk moth *Bombyx mori* (Lepidoptera) (Xia et al. 2004; Goldsmith et al. 2005; Meng et al. 2017). All of these insects, however, share a commonality: they belong to the same insect superorder of Holometabola, or insects that undergo complete metamorphosis during development. Complete metamorphosis is characterized by a pupal stage in the transition from larvae to adults, with neither the larval nor the pupal stages resembling the final adult form (Truman and Riddiford 1999). Such insects display a number of evolutionarily derived developmental characters that are not generally representative of all insects, let alone all arthropods (Mito and Noji 2008). To correct the overrepresentation of holometabolous insects in modern comparative developmental literature, insects branching basally to the Holometabola should be studied, as they, based on parsimony, appear to display characters that are likely ancestral to insects and in some cases also to arthropods. These insects are the Hemimetabola, insects displaying incomplete metamorphosis and lacking pupal stages during development. The embryo in such insects develops into a miniature adult (referred to as a nymph or a juvenile) which then undergoes several successive molts before reaching adulthood and sexual maturity. Orthopterans (crickets, grasshoppers, and locusts), one of the most abundant and dominant terrestrial insect groups, are in this category (Grimaldi and Engel 2005). Orthopterans display extraordinary diversity in developmental processes and are also economically important herbivores, which has resulted in them becoming popular for functional genetic research in recent years.

For the rest of this chapter, we focus on crickets as promising evo-devo models, deserving of serious regard. We discuss *Gryllus bimaculatus*, a field cricket species, introduce this model system, and discuss recent advances in establishing this animal for cell, developmental, and genetic research.

8.2 A Hemimetabolous Insect Model: The Cricket *Gryllus bimaculatus* De Geer

G. bimaculatus is a cosmopolitan orthopteran belonging to the family Gryllidae and is, to our knowledge, the most widespread of all *Gryllus* species for laboratory studies (Otte and Cade 1984). Although its use for developmental genetics is relatively recent, this species is by no means new to biological research: *G. bimaculatus* has been extensively used to inform areas such as neurobiology, insect physiology, reproduction, and behavior since the 1960s (Huber et al. 1989; Engel and Hoy 1999; Paydar et al. 1999; Wenzel and Hedwig 1999; Hedwig and Poulet 2004; Nakamura et al. 2008a, b; Horch et al. 2017b). The discovery of RNA interference (RNAi) as a mechanism for abrogating gene function (reviewed by Sen and Blau 2006) has greatly accelerated *G. bimaculatus* research (Mito et al. 2011), yielding important information about the developmental biology of this organism and unveiling its potential as an upcoming functional genetics laboratory model.

This species was first described by Baron Charles de Geer in 1773 (Geer 1773) and named *Gryllus* (meaning "cricket" in Latin) *bimaculatus* (from the Latin "macula" for "spot"). Indeed, this species is commonly referred to as the "two spotted field cricket," for the white spot that this species displays on the dorsal surface of the forewings next to the pronotal margin (Fig. 8.1a) (Otte and Cade 1984). Some of the morphological keys used to distinguish adult *G. bimaculatus* from other similar looking field cricket species, both within and outside the genus *Gryllus* (Otte and Cade 1984), include the white spots, a black colored adult body size of ~30 mm lacking any bands, forewings nearly covering and large hindwings extending well beyond the abdomen, and an ovipositor (in females) slightly longer than the hind femora (Fig. 8.1a). As an additional tool in the field, entomologists have documented that *G. bimaculatus* does not undergo an obligatory or facultative winter diapause (i.e., developmental arrest in response to adverse environmental conditions such as temperature and/or photoperiod), at any stage of its life cycle (Bigelow 1962). Such a lifestyle is called a homodynamic life cycle, and is in contrast to the heterodynamic lifestyle observed in many other *Gryllus* species entering diapause. Examples of overwintering species in this genus include *G. pennsylvanicus*, *G. campestris*, *G. fultoni*, *G. veletis*, *G. vernalis*, and *G. firmus* (Bigelow 1962). This means that it is possible to collect *G. bimaculatus* in the field across seasons and makes it easy to culture and breed this species in the laboratory all year-round. Additionally, in the field, *G. bimaculatus* is often found close to human settlements, on the ground surface or in soil cracks (Otte and Cade 1984).

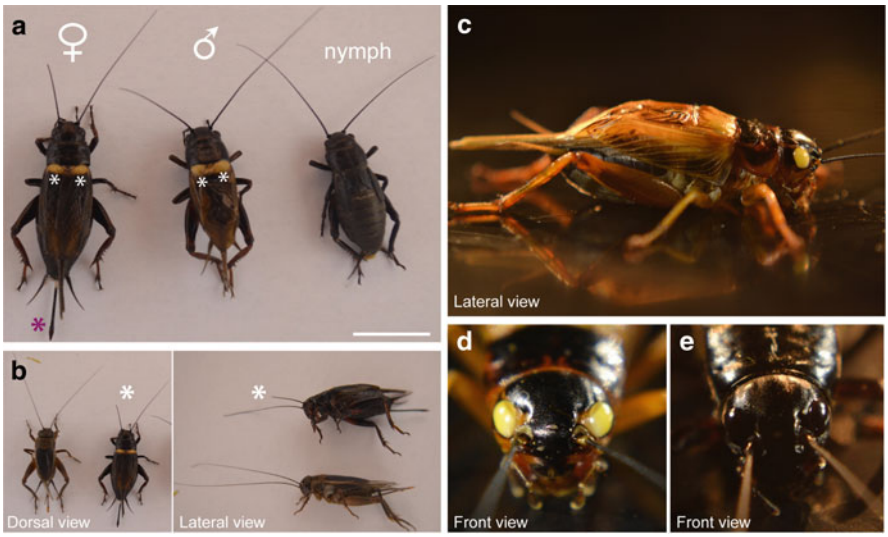

Fig. 8.1 *G. bimaculatus* morphology. (**a**) *G. bimaculatus* adult female (left), adult male (center), and male nymph (right) displaying some morphological keys used to identify this species. The characteristic white spots (marked with white asterisks on the adult female and male) on the forewings next to the pronotal margin are shown. Animals are black in color and are therefore vernacularly also known as the "black cricket" in some parts of the world. The body (in both sexes) lacks any bands of contrasting pigmentation; forewings nearly cover the abdomen, and hindwings extend well beyond the abdomen; females possess an ovipositor (marked with pink asterisk), used to deposit eggs. The presence or absence of an ovipositor can be used to sex animals in nymphal stages (developmental stages prior to becoming an adult). Note: The adult female and male photographed in this picture have broken left antennae; normally both antennae are of similar lengths. Scale bar is 1.5 cm. (**b**) Comparison (dorsal and lateral view) between an adult *G. assimilis* (unmarked) and *G. bimaculatus* female (marked with white asterisk). Note the brown body color, leaner and less bulky adult body, and the absence of the white spots on the forewings in *G. assimilis*. (**c**) A lateral view of an adult *G. bimaculatus* male white-eye mutant and (**d**) higher magnification images of the cricket head (front view) showing the white eye color in these mutants (left) compared to wild-type pigmented eyes shown in (**e**) (right)

While one could collect this species from the wild, the easiest way to establish a laboratory culture of *G. bimaculatus* is from animal pet suppliers. Multiple online pet suppliers based in various countries (e.g., Pets at Home, UK; Bugs International, Germany) culture and distribute this species as live crickets for captivity feeding. It is important to note, however, that at the time of writing, the United States is an exception: the United States Department of Agriculture (USDA) does not permit commercial distribution of *G. bimaculatus* in the United States, and the cricket species commonly commercially available for purchase are *Gryllodes sigillatus* and *Acheta domesticus* (examples of online retailers selling cricket species in the United States at the time of writing include Ghann's Crickets, Top Hat Cricket Farm, Fluker's Cricket Farm, and Premium Crickets). Being bulkier and meatier than other cricket species (Fig. 8.1b dorsal and lateral view), *G. bimaculatus* is

preferentially exploited in multiple countries as an inexpensive food source for humans (described below, Sect. 8.8) and for insectivorous animals housed in captivity (Mito and Noji 2008). This has significantly raised their potential economic importance in recent years. Because *G. bimaculatus* is quite heat tolerant and one of the very few insects that can be reared at 37 °C (human body temperature), this species is also promising as a simple animal infection model system and has found application in studying human pathogenic bacteria (e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*) (Kochi et al. 2016) and fungi including various *Candida* species (Kochi et al. 2017). Epizootic viral diseases are devastating in crickets (and for cricket-rearing facilities), wiping out entire colonies and becoming difficult to eradicate. Researchers who wish to culture *G. bimaculatus*, therefore, should be aware that this species is susceptible to the *G. bimaculatus* nudivirus (GbNV), known to infect nymphs and adults (Wang and Jehle 2009) and the cricket iridovirus (CrIV) (Kleespies et al. 1999), but is reportedly resistant to the cricket paralysis virus (CrPV) and the potent *A. domesticus* densovirus (AdDNV) (Szelei et al. 2011).

As a hemimetabolous insect, *G. bimaculatus* displays a short germ band during embryonic development and thus differs substantially from the well-studied long germ band characteristic of *Drosophila*. Short germ band development refers to a form of insect body patterning that is thought to be ancestral to arthropods (reviewed by Davis and Patel 2002) and present in many extant insects including crickets. In this form of development, only the anterior body segments (head only or the head and thorax) are specified in the early embryonic rudiment before gastrulation, whereas posterior segments (the thorax or the thorax and abdomen) are formed sequentially later in development during a secondary growth phase (reviewed by Krause 1939; Davis and Patel 2002; Liu and Kaufman 2005). In contrast, insects such as *Drosophila* follow the presumed derived long germ band type of development, whereby all segments are specified near simultaneously during the early blastoderm stage (Krause 1939; Campos-Ortega and Hartenstein 1985; Lohs-Schardin et al. 1979; Liu and Kaufman 2005). Another way in which crickets may display putative ancestral insect characteristics is in the structure of its ovaries. The *G. bimaculatus* ovary is panoistic, meaning that there are no germ-line-derived nurse cells that provide cytoplasmic content to growing oocytes (Büning 1994). Instead, every germ-line cell (i.e., every cystoblast) in the adult female ovary is thought to give rise to an oocyte (Büning 1994). In contrast, in the meriostic type of ovaries, as seen in *Drosophila* and nearly all other holometabolous insects (for details see Bilinski et al. 2017), the oocytes are connected to groups of germ-line cells called nurse cells. Panoistic ovary type and short germ development are, based on parsimony, thought to be features ancestral to insects and possibly to Pancrustacea. Consequently, it has been proposed that this species has the potential to serve as a representative study model for basally branching, hemimetabolous insect and arthropod lineages (Sander 1997; Mito and Noji 2008).

Sex determination in *G. bimaculatus* is thought to follow the XX/X0 system, with females being the homogametic sex and having a chromosome complement of $2n = 28 + XX$ (Yoshimura 2005) and having a predicted genome size of a

few gigabases (Mito and Noji 2008). *G. bimaculatus* is polyandrous—females are known to mate with several males and exert postcopulatory mate choice (Tregenza and Wedell 1998). This polyandry is associated with increased egg-hatching rates and is hypothesized to prevent effects of inbreeding in wild populations (Simmons 1986, 1987; Tregenza and Wedell 1998, 2002; Bretman and Tregenza 2005). Indeed, *G. bimaculatus* females can lay many hundreds or thousands of eggs over their lifetime in the lab and, in our hands, have been maintained successfully as an inbred line (originally founded from a few dozen individuals) for over a decade, without any noticeable decline in health that might be attributed to inbreeding depression (Extavour lab, unpublished observations).

8.3 Cricket Sources, Animal Husbandry, Life Cycle, and Available Strains

At the time of writing and to the best of our knowledge (as described above), most current working laboratory cultures of *G. bimaculatus* were either established from adults purchased from commercial vendors (e.g., Tsukiyono Farm, Gunma, Japan; Scope Reptile Pet Store, Okayama, Japan; Livefood UK Ltd., UK; Kreca Ento-Feed BV, the Netherlands) or caught in the wild. However, this genus contains many species that are morphologically very similar, many species are known to overlap with *G. bimaculatus* in their local distribution, and adequate species-level, prominent morphological keys are lacking within this genus. Although some keys have been described (e.g., Nickle and Walker 1974), including the morphological characters described above, to an inexperienced eye, many of these features are often distinguishable only in comparison with another species present, or are easier to observe in preserved specimens than in live animals. We thus recommend performing molecular barcoding (e.g., using 16s ribosomal DNA or the *cytochrome b* mitochondrial DNA sequence) of the founding adults of a new colony, whether purchased commercially or captured in the wild, to ensure that all founding adults are indeed *G. bimaculatus* (Ferreira and Ferguson 2010).

Rearing *G. bimaculatus* is straightforward, and detailed cricket husbandry protocols are well described for this species (Mito and Noji 2008; Kainz et al. 2011; Kochi et al. 2016). Crickets (nymphs and adults) can be kept as inbred lines at 26–30 °C in well-ventilated plastic cages with egg cartons (Fig. 8.2a) or crumpled paper for shelter, and can be maintained on either a 12 h light/12 h dark (Kainz et al. 2011) or a 10 h day/14 h dark photoperiod (Mito and Noji 2008). They can be fed on general insect food or artificial insect diets (e.g., Oriental Yeast Co., Ltd., Tokyo, Japan), artificial fish food, finely ground dry cat food (e.g., Purina Kitten Chow), a mixture of oils and whole grain cereals (Kainz et al. 2011), or a combination of these food sources (Fig. 8.2b). Cricket Quencher water gel (Fluker Farms) can be used as a water source. Alternatively, a 50 mL falcon tube filled with water and stopped with cotton, or wet tissue or cotton in petri dishes, can also serve as water sources

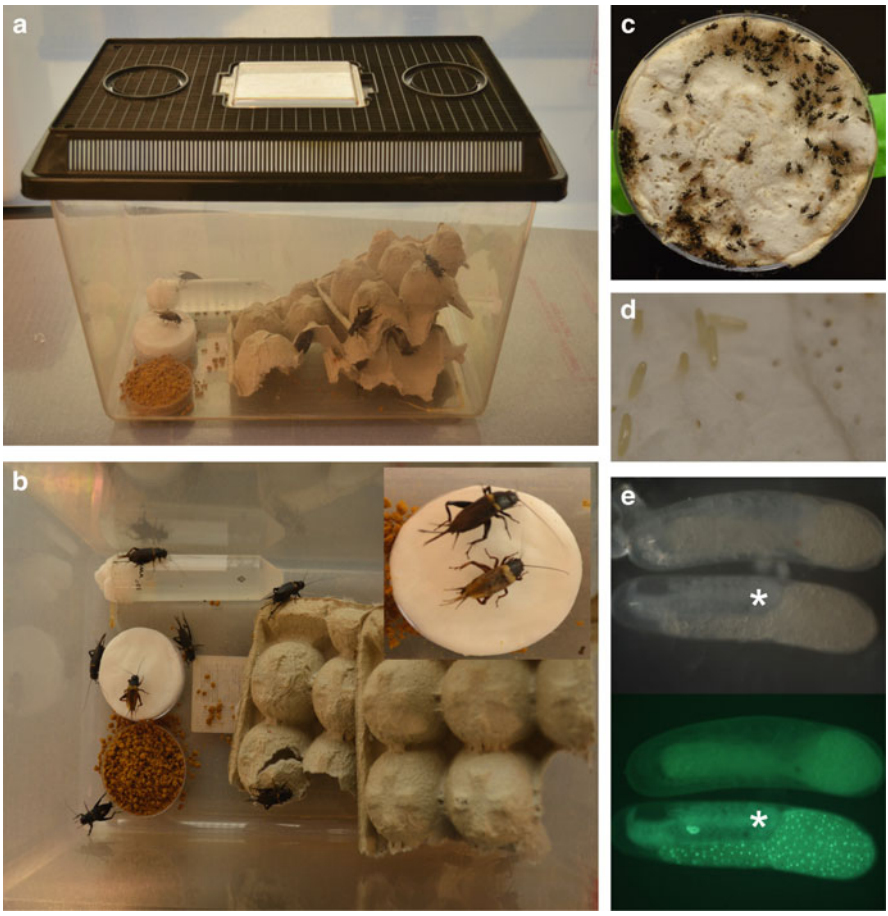

Fig. 8.2 *G. bimaculatus* husbandry. (**a**) (side view) and (**b**) (top view) showing a well-ventilated plastic container used for housing a cricket colony. Note the use of egg cartons for providing shelter, ground cat food, and a 50 ml Falcon filled with water and stopped with cotton as a food and water source, respectively. A wet cotton plate (seen in **a**, **b**, and **b** inset) is placed in the adult cages for females to oviposit fertilized eggs. Oviposited eggs (higher magnification shown in (**d**)) need to be kept moist and clean until the eggs hatch. (**c**) A close-up of a cotton plate showing newly emerged cricket hatchlings, which can then be transferred into new plastic cages with food, water, and shelter until they reach adulthood. (**e**) Two *G. bimaculatus* eggs (6 days after egg laying) imaged under bright field white light (top) and green florescent light (bottom). Both eggs are progeny obtained from a cross between the histone2B-GFP (H2B-GFP) transgenic and wild-type *G. bimaculatus* line. Embryos carrying the H2B-GFP transgene (bottom egg marked with white asterisk) can be distinguished from non-transgenic embryos (top egg) based on the presence of bright florescent nuclei, which is detectable from day 5 until day 10 after egg laying

(Fig. 8.2b). Crickets will oviposit fertilized eggs into damp sand (e.g., Sandtastik Sparkling White Play Sand, Product Code PLA0050), wet paper towels, Whatman paper, or wet cotton placed in petri plates in cricket cages (Fig. 8.2b inset, d). These eggs will develop successfully and hatch in 12–14 days (Fig. 8.2c) under the following conditions: incubation at ~28–29 °C with 70% humidity, dead or moldy

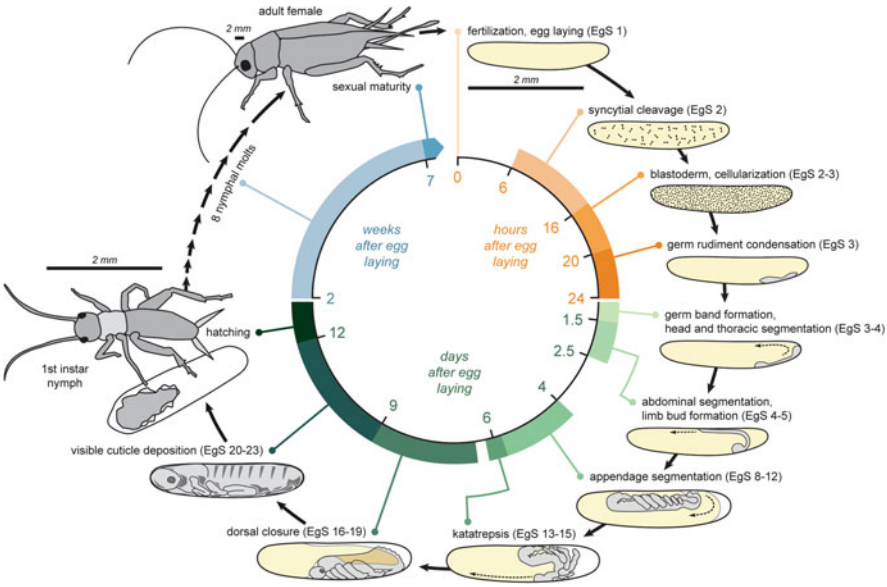

Fig. 8.3 A schematic showing an overview of the *G. bimaculatus* life cycle. Selected embryonic and nymphal developmental stages are shown, alongside the duration of each developmental stage depicted in hours (orange arc and lines), days (green arc and lines), or weeks (blue arc and lines) after egg laying. The colored arcs indicate the entire duration of time occupied by the indicated developmental stage, whereas the lines show a cartoon schematic of the selected stages within this time window. Each displayed embryonic stage during embryogenesis shows the position of the embryo (in gray) relative to the yolk (yellow) within the egg and has a small description of features that are characteristic of that developmental stage (depicted as egg stage "EgS"). Dotted lines with arrowheads indicate the different movements that the embryo makes during the course of development in this species. Upon hatching, nymphs undergo eight nymphal molts (indicated by solid black arrows) to reach adulthood. Newly emerged adult animals are sexually mature at molting and begin mating soon after. This figure is modified from Donoughe and Extavour (2016)

embryo removal on a regular basis, and maintenance of a moist and clean substrate (Mito and Noji 2008; Donoughe and Extavour 2016). Embryonic development for this species has been divided into 16 stages based on morphological features of eggs, developing embryos and their appendages (Niwa et al. 1997; Donoughe and Extavour 2016). After hatching, nymphs undergo eight nymphal molts to finally become adults over the next 5 weeks. The generation time (total time to adulthood and sexual maturity) of *G. bimaculatus* is thus approximately 7 weeks at 29 °C (Fig. 8.3). Adults are thought to reach maximum fecundity 1 week after the final molt (Mito and Noji 2008). Sexing and identification of virgin males and females are straightforward: late-stage male and female nymphs can be separated based on the presence or absence of an ovipositor and then isolated until they undergo the final molt to sexual maturity (Fig. 8.1a). This also helps in setting up single mating crosses, for example, to establish genetically modified lines. Similarly, precisely timed egg collections are possible by placing egg collection petri dishes in the cages and removing them at desired intervals (described in Donoughe and Extavour 2016).

A *G. bimaculatus* spontaneous mutant strain with white eyes (Fig. 8.1c–e) was isolated by Isao Nakatani and colleagues at the University of Yamagata, Japan, in 1989 (referenced in Mito and Noji 2008). This is, to our knowledge, currently the only available mutant, and its phenotype is caused by an autosomal recessive mutation, referred to as *gwhite* (Niwa et al. 1997; Mito and Noji 2008). This mutant is sometimes preferred for whole-mount gene expression analysis at late stages of embryogenesis, owing to the fact that at this stage, the tissues are more transparent compared to wild type (Niwa et al. 1997; Mito and Noji 2008).

8.4 Techniques for Quantitative and Functional Genetic Analyses in *G. bimaculatus*

Here we discuss protocols and methodologies that have been established and are currently available in the cricket *G. bimaculatus*, with the aim of making new users aware of the plethora of techniques at their disposal. Detailed descriptions of these published techniques and step-by-step protocols are thus avoided in this chapter (we refer the reader to Horch et al. 2017a, b for detailed protocols). While these protocols are now well established in crickets, these tools are not limited to them and could in principle be modified or adapted for use in other hemimetabolous insects to further species-specific research.

8.4.1 Precise Embryonic Staging System

To make meaningful observations of deviations from normal embryonic development, one first needs a wild-type reference for any given species. Donoughe and Extavour (2016) have reported a detailed embryonic staging system for *G. bimaculatus* (Figs. 8.4 and 8.5). This system is based on externally observable characters of the developing cricket embryo that are visible through the eggshell, thereby circumventing the need for embryonic dissections to ascertain embryonic developmental stage. This is especially informative for studying early embryos of insects such as crickets, which are embedded within a large amount of opaque yolk, making direct observations through the eggshell difficult if not impossible. *G. bimaculatus* development, based on this staging, is presented as 24 "egg stages" and encompasses the entire development of the animal from fertilization to hatching, based solely on external observable egg characters (called stage identifiers). Each of the 24 "egg stages" described here corresponds to one or more of 16 "embryonic stages." For each stage, the authors provide a list of embryonic developmental features defining that stage, including features of body segmentation, mesoderm, and appendage formation. Determining the stage of embryogenesis through the eggshell is a useful complement to earlier described staging schemes that require dissection of the embryo (Mito and Noji 2008; Kainz 2009).

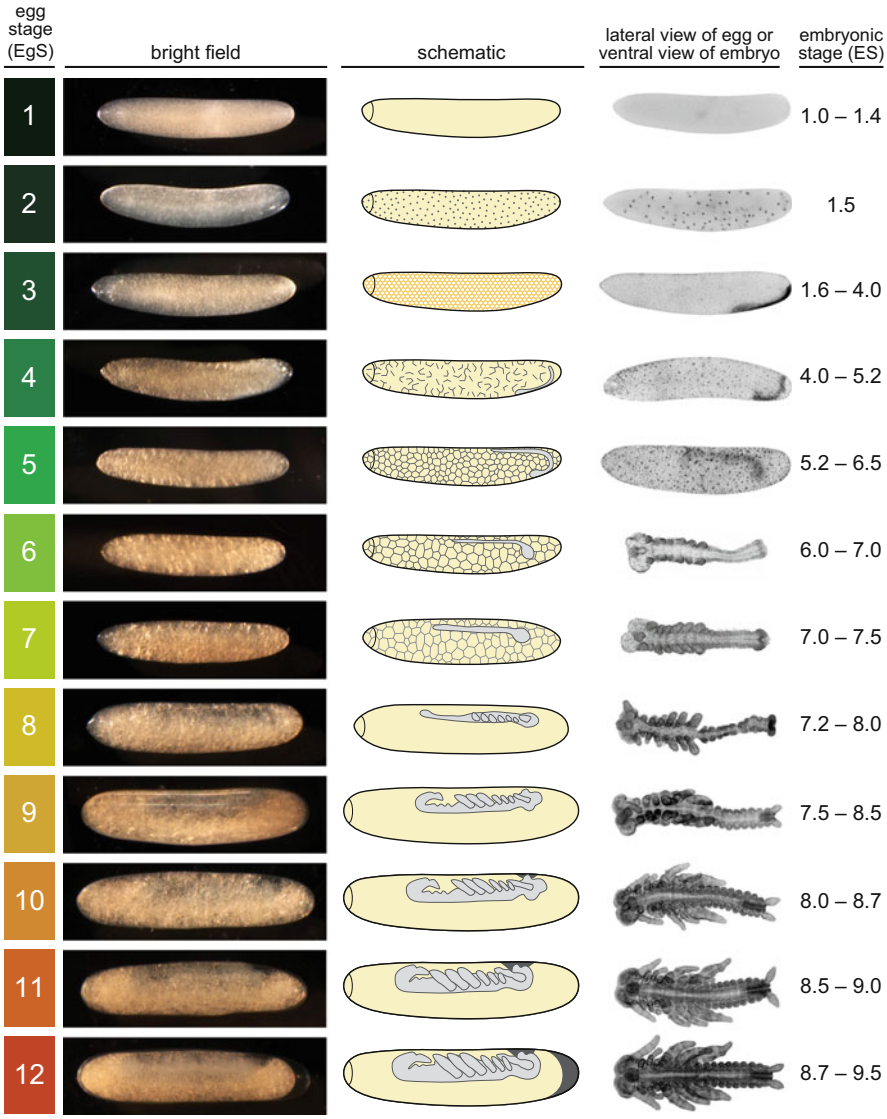

Fig. 8.4 A detailed description of the egg stages (EgS 1–12) in *G. bimaculatus.* Micrographs in second column from left help display the morphological features of the egg that can be used to assign embryos to an egg stage (EgS) and also describe the corresponding morphological features and embryonic stage (ES) of the embryo within the egg. In the schematic, the embryo is depicted in gray and yolk in yellow. Micrographs in the right second column from the right are not to scale, and are taken using lateral views of either a H2B-GFP transgenic live embryo (EgS 1–5) or ventral views of dissected and fixed, Hoechst 33342-stained embryos (EgS 6-182). Micrographs are not to scale. This figure is modified from Donoughe and Extavour (2016)

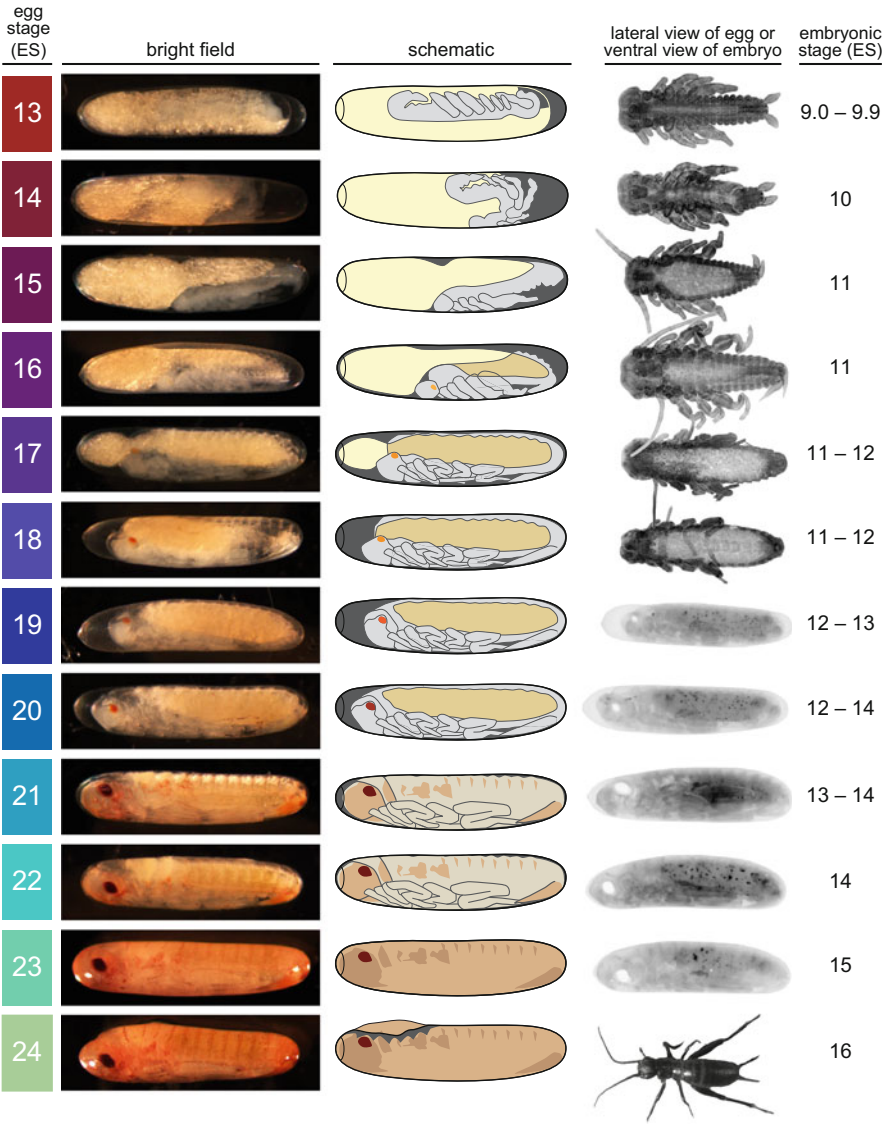

Fig. 8.5 A detailed description of the egg stages (EgS 12–24) in *G. bimaculatus*. Staging system for egg stages (EgS) 12–24 continued from Fig. 8.4. This staging system ends at hatching and does not include postembryonic development of nymphs to adulthood. This figure is modified from Donoughe and Extavour (2016)

8.4.2 Injection Methods for Eggs, Nymphs, and Adults

A basic requirement for many experimental procedures in modern developmental biology, including live imaging, RNA interference, and gene editing, is the delivery of synthetic or biological materials into the body of an animal, without disrupting its health or sacrificing its life. Direct manual injection is one such method and, in the case of crickets, has been well established and found effective in egg, nymphal, and adult stages. Two methodological variations are commonly in use for *G. bimaculatus* egg injections, differing essentially in the number and arrangement of embryos for injection, and are described in great detail in Horch et al. (2017a) and Barry et al. (2019). Both methods are thus described below in brief.

The first variant of the egg injection method, developed by the Noji lab (University of Tokushima), involves the construction of a mold to house eggs for injections (Horch et al. 2017a). Watson chambers (which resemble a rectangular mold) are glued onto microscopic glass slides using double-sided tape. Embryos are then lined up end to end along the length of the chamber, using a small stainless-steel spatula. The wall of the chamber and the adhesive of the double-sided tape (on the slide) help secure and hold the embryos in place during the injections. The second variant, optimized in the Extavour lab, uses rectangular troughs made using plastic molds set in low-melting agarose that hold eggs in place (Kainz et al. 2011; Barry et al. 2019). Using this setup, over 35 embryos per slide can be prepared for injection simultaneously, making it efficient in terms of preparation time and the number of embryos injected in one sitting.

Regardless of the egg injection method used, eggs are injected under a dissecting or compound microscope, using a needle held by a micromanipulator (Fig. 8.6a, b). The needle must be loaded with the desired injection material and connected to a pressure source, which may be manual (e.g., a syringe) or electronically controlled compressed gas (e.g., using a commercial micro-injector). The injected material may be mixed with a dye that is visible under white light (e.g., phenol red or fast green) or fluorescent light (e.g., fluorescein- or rhodamine-conjugated dextrans) depending on the user's preference. The choice of dye will determine the best microscopy and light regime to be used for injections. Following injections, embryos are allowed to develop normally in humid incubators at 28 °C on wet paper towels or are submerged in 1× phosphate buffered saline in closed petri dishes and monitored daily until embryos hatch. Eggs of developmental stages that are turgid and under high pressure, including very early stages in the first few hours following fertilization and middle stages following elongation of the germ band, are more difficult to inject than earlier stages.

For nymphal and adult injections, a manually held Hamilton syringe or any automated micromanipulator/microinjector system specifically designed for delicate microinjections and capable of injecting nanoliter volumes can be used effectively. Nymphs and adults are prepared for injections by first cooling them on ice to temporarily immobilize them, and then injected either between the abdominal segments (A2 and A3) or in the soft tissue between the T3 coxa and the thorax. For site- or tissue-specific injections (e.g., the leg or brain), the injection site should

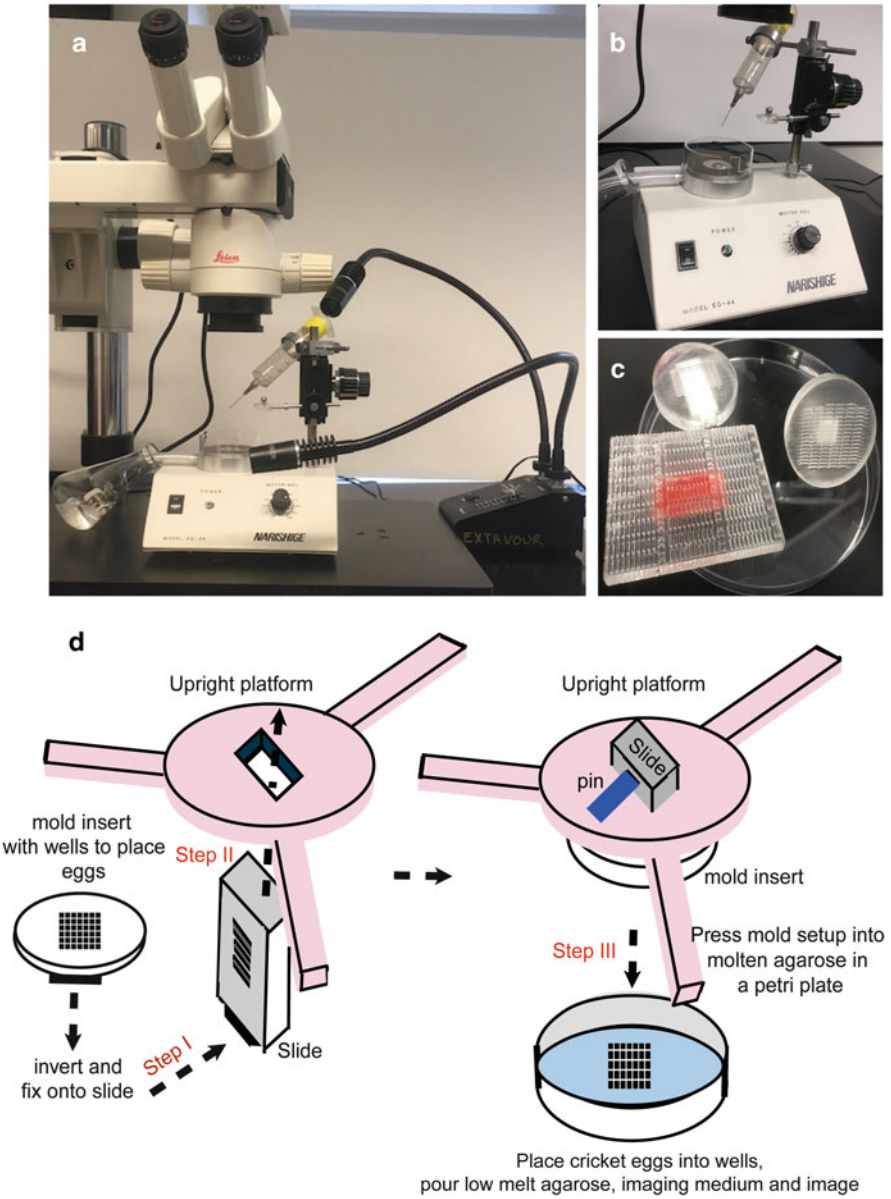

Fig. 8.6 Injection and OMMAwell setup. (**a**) The apparatus used for beveling glass needles for use in injecting crickets, consisting of a light source, a dissecting microscope, a micromanipulator, and a beveling stone (Narishige model EG-45 is shown here). (**b**) Higher magnification of the beveling setup shown in (**a**). (**c**) Different types of agarose mold inserts used for mounting cricket embryos for application in OMMAwell or, alternatively, for cricket egg injections. (**d**) OMMAwell (Donoughe et al. 2018) schematic for top-loaded microwells, used for injecting and imaging cricket embryos using a configuration for upright objectives. Different assembly components are shown: the mold insert (white) consists of wells that will house the cricket embryos and is inverted and attached to the base of the slide (gray). This is then placed into the upright platform (pink) and

be modified accordingly. However, locales of abundant fat tissue should be avoided as injection sites in crickets, to help facilitate dispersal of liquid into the body upon injection, prevent backflow of injected material or hemolymph into the needle, and prevent blockage of fine needle tips with insect tissue. Irrespective of the injection site, care should be taken while injecting the needle into the nymphal or adult body, so as to prevent injuring the internal organs, which could potentially kill the animal or disrupt recovery. Such injuries can be easily avoided by inserting the needle only deep enough into the animal body to prevent oozing of material at injection.

Other recommendations for successful injections of adult or juvenile *G. bimaculatus* include inserting the needle parallel to the body of the insect, rather than at a perpendicular angle, injecting larger volumes (relative to the insect size) as multiple pulses of smaller doses rather than all at once, injecting slowly to prevent leakage, minimizing handling stress for the animal, making sure the needle is not blocked prior to insertion, and maintaining basic cleanliness and sterility during the procedure. Following injection, animals should be allowed to recover in isolated cages with food and water at room temperature before proceeding with the desired study.

8.4.3 *High-Throughput Live Imaging of Embryos Using OMMAwell*

Open Modular Mold for Agarose Microwells (OMMAwell) is a simple, reusable, all-in-one device that allows users to easily mount and simultaneously image dozens of live *G. bimaculatus* embryos consistently and economically for 2D and/or 3D time-lapse analyses of early development (Donoughe et al. 2018). OMMAwell has the added advantage of being adaptable and customizable: it has been made to accommodate the imaging needs of researchers with different experimental designs, can be used on diverse species (OMMAwell has been successfully designed for and tested on nine animal species, including many traditional model organisms), and can be used for both inverted and upright objective microscopes (Fig. 8.6d). With this device, embryos can be efficiently and quickly lined up in arrays of agarose microwells, whose dimensions and spacing can also be customized as per individual user needs (Fig. 8.6c) (see Donoughe et al. 2018). In addition, OMMAwell has reservoirs to hold live imaging media and help maintain specimen-specific humidity, osmolarity, and oxygen levels during time-lapse live imaging, thereby enhancing embryonic survival and data quality. This device also allows positional tracking of

Fig. 8.6 (continued) secured at the desired height with the help of a pin (blue). The assembled components are then lowered into a petri plate containing molten low-melt agarose and allowed to set. Following the removal of the mold insert from the cooled and set agarose, specimens are added into the wells in the petri plate either individually or in bulk and covered with low-melt agarose (in microliter volumes of up to 100 μl) to hold them in place. Embryos can be oriented carefully using forceps prior to this step. Once the agarose sets, live-imaging media is poured into the dish

individual embryos and permits users to control sample orientation for imaging. The OMMAwell microwell array arrangement is also convenient to hold embryos in place during injections. OMMAwell has been used to image the development of as many as 102 *G. bimaculatus* live embryos simultaneously for 12 consecutive days (Donoughe et al. 2018). Hatching rates of these embryos were not significantly different from the hatching rates of controls, suggesting no lingering effects of phototoxicity, developmental delays, or defects on these embryos from the use of OMMAwell.

8.4.4 Gene Expression Analyses Using Embryonic or Whole Mount In Situ Hybridization and Immunohistochemistry

The ability to detect mRNAs [using in situ hybridization (ISH)] and proteins [immunohistochemistry using labeled antibodies (IAb)] of interest is central to whole mount gene expression analyses in any organism. Standard protocols to study gene expression in other vertebrate and invertebrate embryos have been applied successfully in *G. bimaculatus* (Niwa et al. 2000; Mito and Noji 2008). These include whole mount in situ hybridization using digoxigenin (DIG)-labeled antisense RNA probes (as per Wilkinson 1992), protein detection (Patel 1994), and double in situ hybridization using probes labeled with different haptens (e.g., Dietrich et al. 1997). Optimized ISH and IAb protocols have also been developed in this species for specific tissues including the brain, nymphal legs, and wings. Automated medium- or high-throughput gene expression assays on *G. bimaculatus* tissues using specialized robots (e.g., Intavis InsituPro VSi) are also possible (Extavour lab, unpublished).

8.4.5 RNA Interference

The Noji lab pioneered the establishment of RNA interference (RNAi) technology in the cricket *G. bimaculatus* (Miyawaki et al. 2004), and many researchers have since used this technique successfully to deplete mRNAs of multiple target genes in this species. Four main types of RNAi techniques have been developed for use in crickets: embryonic, nymphal, parental, and regenerative RNAi (Miyawaki et al. 2004; Mito et al. 2005; Nakamura et al. 2008a; Mito and Noji 2008; Ronco et al. 2008).

To perform RNAi, double-stranded RNA (dsRNA), preferably 300–500 nucleotides in length, complementary to a region in the *G. bimaculatus* gene of interest, is injected into the eggs (embryonic RNAi) or into the body cavity of nymphs (nymphal RNAi) or adults (parental RNAi). Successful concentrations of dsRNA have been reported to range from 2 to 6 μg/μl (e.g., Kainz et al. 2011). It is recommended that the dsRNAs designed should match a region close to or including the 3′ UTR of the target *G. bimaculatus* gene, which may minimize off-target effects. Typical specificity controls may include testing at least one other dsRNA designed

against a nonoverlapping fragment of this same gene. Injecting dsRNA against exogenous genes not encoded by the cricket genome (e.g., DsRed) and injecting the buffer alone can also serve as meaningful controls and are thus strongly recommended for every RNAi experiment. Together, these measures can help researchers distinguish between specific and nonspecific effects of RNAi, allowing meaningful interpretation of their results.

RNAi is systemic in *G. bimaculatus*, such that RNAi-induced phenotypes may be detected throughout the body of the embryo, nymph, or adult, regardless of the site of injection. Moreover, the injection of dsRNA into sexually mature adult females allows for observation of RNAi effects not only in the adult animal itself but also in its progeny (i.e., eggs) that the animal will lay over the weeks following a postinjection mating as long as the gene knockdown does not interfere with oogenesis, fertilization, or egg laying. Alternatively, nymphal RNAi can be conveniently used to determine gene functions in postembryonic stages. Regenerative RNAi was optimized in the Noji lab and has been performed as a specific application of nymphal RNAi in the cricket (Nakamura et al. 2008a). For this procedure, a leg of a third instar nymph is amputated following dsRNA injection, and the effects of RNAi are then assessed during the regeneration of the lost leg (which normally occurs over subsequent molts). Based on these observations, the RNAi response in crickets can be robust, stable, and even transmissible through subsequent molts (Nakamura et al. 2008a; Hamada et al. 2015). However, it is recommended that the robustness of RNAi response, its stability, and duration be determined on a case-to-case basis; in our hands, there have been instances where the RNAi response for some genes has lasted only a few days (see Kainz et al. 2011).

8.4.6 *Calcium Imaging to Study Neurobiology and Neuroethology*

The cricket has been an important model for neurobiology and neuroethological studies, and many physiological techniques are easily applicable to the cricket (Ogawa and Miller 2017). One such technique is that of calcium imaging, which uses florescent dyes and optical methods to monitor the changes in intracellular levels of calcium ions in live cells and tissues (Neubauer and MacLean 2010), including cricket neurons. Information on selection of calcium indicators, dye loading protocols, experimental designs, and calcium imaging techniques in the cricket are well described (Ogawa and Miller 2017). In 2013, Matsumoto and colleagues successfully expressed Yellow Chameleon (YC) 3.60, a genetically encoded calcium indicator (GECI) in the cricket brain via electroporation (Matsumoto et al. 2013), enabling prolonged deep imaging of the cricket brain for the first time. Together with high-resolution microscopy and gene editing techniques, calcium imaging is expected to facilitate major advances in our understanding of cricket neurobiology (Ogawa and Miller 2017). Calcium imaging is not limited to neurobiology, so its successful establishment in the study of cricket

nervous tissue suggests that this technique can now also be used to study physiology in other tissues and cell types in this animal.

8.4.7 *High-Sensitivity Trackball Recording Systems for Studying Phonotaxis and Auditory Neuronal Plasticity*

Acoustic communication is paramount in insects, both within and between species. Making precise recordings of insect locomotory behavior in response to auditory stimuli (phonotaxis), such as male calling songs, is often challenging under natural settings. Various laboratory assays for measuring cricket phonotactic behavior have been developed, making such studies possible. Examples of such assays include analyzing the number of crickets that reach an acoustic stimuli or sound target within a defined time period (Tschuch 1976; Stout et al. 1983), studying cricket behavior in mazes (Popov and Shuvalov 1977; Rheinlaender and Blätgen 1982), or steering responses of tethered flying female crickets (Pollack and Hoy 1979). The development of two different trackball recording systems in crickets has been paramount in enhancing our understanding of *G. bimaculatus* auditory steering behavior (Hedwig 2017) and provided detailed insights into insect locomotory behavior in general. All trackball recording systems measure the movements of the trackball, based on which insect velocity and direction of insect movement (walking) are inferred, without allowing the insect to reach the auditory target. In closed-loop trackball systems, the cricket is allowed to walk and turn freely on the trackball during recordings, with the trackball compensating for cricket movement by having the ability to counter-rotate. By contrast, in open-loop systems, the tethered cricket has the ability to walk but not change its orientation in an acoustic field. Due to their sophisticated design, these trackball recording systems can now easily be integrated into experimental setups using other forms of recording, including neuro- or electrophysiological and high-speed video recording experiments. Thus, combined with the GECI YC3.60 discussed above, and alongside other sophisticated imaging and video recording techniques (see below), trackball recording systems are expected to provide new insights not only into cricket biology but also into the study of insect phonotaxis in general.

8.4.8 *Automated and Customizable Video Tracking Systems, Artificial Crickets, and Cricket Robots for Synthetic Neuroethology and Social Behavior*

Crickets have been used over the past several decades as systems to study behaviors including mating, flight, aggression, wandering, obstacle avoidance, and importantly, to study the neurophysiology underlying these processes. When investigating, quantifying, and qualifying animal behavior, dependable and accurate measurement systems are needed to record animal responses to external stimuli, at both behavioral and

physiological levels. The advent of engineering approaches in crickets, especially robotics, is expected to greatly facilitate such research and is the genesis of the field of cricket synthetic neuroethology. Aonuma and colleagues have described a novel approach developed for crickets, where provoked animal behavior in response to computer-generated simulation and robots is captured to effectively bridge the gap between insect behavior and physiology (Aonuma 2017). Different commercially available automated video tracking systems designed to follow cricket movement have also been previously described (Noldus et al. 2001). Recently, another customizable tracking system based on a simple open-source solution called SwisTrack (Lochmatter et al. 2008) has been introduced for use in crickets. Using this system, multiple crickets can be video recorded and tracked simultaneously. Because the entire process is semiautomatic, data collection and its interpretation are more efficient than previous methods that were based exclusively on manual tracking. Using artificial crickets (e.g., Funato et al. 2011; Kawabata et al. 2012; Mizuno et al. 2012) or cricket robots (Funato et al. 2008, 2011) alongside computer modeling is another way of analyzing cricket behavior that has recently been reported. Further detailed information on artificial crickets, cricket robots, biomimetic robots (Ritzmann et al. 2000), and behavioral modeling in this species can be found in Aonuma (2017).

8.4.9 *Standardized Protocols for Assessing Learning and Memory*

G. bimaculatus has been reported to have a robust memory and thus has been exploited for studying the neural mechanisms underlying olfactory, auditory, and visual learning. Mizunami and colleagues have published detailed protocols for classical conditioning, operant testing, associative learning, memory retention, and subsequent data analyses in *G. bimaculatus* (Mizunami and Matsumoto 2017a). A "classical conditioning and operant testing" procedure has also been developed in crickets by these researchers. The establishment of such protocols has resulted in the elucidation of detailed cellular mechanisms and signaling cascades that are important for memory formation in crickets. These studies have additionally revealed that crickets display unexpected diversity in the mechanisms underlying these processes in comparison to other insects including *Drosophila* (Mizunami and Matsumoto 2017b). The use of such classical conditioning paradigms and their variants in crickets may provide novel breakthroughs in our understanding of learning, cognition, and memory across animals.

8.4.10 *Transgenic Lines*

Stable transgenic lines are an invaluable tool for developmental genetics and contribute to the successful establishment of a model animal system. Transgenesis using P elements, which are the transposon of choice for *Drosophila* transgenesis (Rubin

and Spradling 1982), have been found ineffective in crickets, such that other transposable elements need to be used to achieve transformation in this species. Zhang and colleagues (2002) showed that *Minos* transposons (Pavlopoulos et al. 2007) are active in *G. bimaculatus* embryos and highlighted the possibility of using these as gene vectors for germ line transformation in this species. However, to our knowledge, this transposon has not yet been used to establish stable transgenic cricket lines. Shinmyo et al. (2004) succeeded in somatic transformation of *G. bimaculatus* embryos, using the *piggyBac* transposon (Handler et al. 1998) to achieve somatic insertion of a construct containing an enhanced green florescent protein (eGFP) coding region driven by a *G. bimaculatus actin 3/4* promoter. Construction of plasmids and injection protocols for this line are as described in Shinmyo et al. (2004) and Zhang et al. (2002). Subsequently, this technique has been optimized to achieve germ line transmission of transgenes (Nakamura et al. 2010).

At the time of writing, a histone2B-GFP (H2B-GFP) transgenic line is stably maintained in multiple laboratories (Nakamura et al. 2010). In this line, the promoter of the *G. bimaculatus* actin orthologue (*Gb-Actin*) drives the expression of the Gb-histone2B protein tagged with eGFP. This transgene is ubiquitously and constitutively expressed and is maternally contributed to eggs. Based on viability ratios of the embryos laid by this line, it is likely that the transgene is sublethal in homozygosis (Extavour lab, unpublished observations). As there are no balancer chromosomes for *G. bimaculatus*, heterozygotes must be manually selected at every generation to maintain the transgene (Extavour lab, unpublished observations). Zygotic expression of this transgene begins at approximately the fourth day after egg laying (AEL) at 28 °C, and eggs expressing the transgene can then be easily identified and selected between 5 and 10 days AEL, based on the presence of brightly fluorescent nuclei under a fluorescent stereomicroscope (Fig. 8.2e top and bottom).

8.4.11 Genome Editing Using CRISPR/Cas9, TALEN, and Zinc-Finger Nucleases

Sophisticated functional genetics techniques commonly used to modify genomes in vivo at a specific site include *c*lustered *r*egularly *i*nter*s*paced *p*alindromic *r*epeats (CRISPR)/*C*RISPR-*a*ssociated nuclease 9 (Cas9), collectively known as the CRISPR/Cas9 system (Cong et al. 2013), *t*ranscription *a*ctivator-*l*ike (TAL) *e*ffector *n*ucleases (TALENs), and *z*inc-*f*inger *n*ucleases (ZFNs) (Porteus and Carroll 2005; Moscou and Bogdanove 2009; Remy et al. 2010; Miller et al. 2011; Jinek et al. 2012). All of these approaches work by generating double-stranded breaks in target DNA sequences, which in turn trigger the cell's DNA damage response (Remy et al. 2010), and this cellular response can then generate mutations (insertions or deletions) in the targeted gene. All of these techniques are now available and functional in crickets. The Mito lab established and reported the use of ZFNs and TALENs in

crickets in 2012, by successfully creating homozygous genetic knockouts (Watanabe et al. 2012). CRISPR/Cas9 has also now been effectively used for the generation of both knock-ins (Horch et al. 2017b) and knockouts (Awata et al. 2015) of cricket genes. Detailed protocols for knocking-in or knocking-out cricket genes using the CRISPR/Cas9 method are available in Horch et al. (2017b).

8.4.12 Genomics and Transcriptomics

While no genome sequence is currently publicly available for *G. bimaculatus*, a number of de novo transcriptomes have been published for this species, providing gene expression datasets for a number of different specific tissue types and developmental stages. To date, these include transcriptomes of the ovaries, embryos, the prothoracic ganglion, and regenerating legs (Zeng and Extavour 2012; Bando et al. 2013; Zeng et al. 2013; Fisher et al. 2018). Moreover, several transcriptomes reflecting gene expression at different life stages (Berdan et al. 2016), in the male accessory gland (Andres et al. 2013), fat body and flight muscles of different ecological morphs (Vellichirammal et al. 2014), and under cold-acclimation conditions (Des Marteaux et al. 2017; Toxopeus et al. 2019), are available for other species of the genus.

8.5 Novel Insights into Biological Processes Using Forward Genetics in a Hemimetabolous Insect

G. bimaculatus has been effectively used to study various disciplines of biological sciences over the past decades. These include early embryonic development and body patterning, tissue and organ system specification, regeneration, body size regulation, memory and learning, reproductive biology, ecology, physiology, and endocrinology (reviewed in Horch et al. 2017b). Here, we will therefore refrain from reiterating the contributions that these results have made to our understanding of biology. Instead, we will briefly discuss one example of a novel insight that the biological community has gained through the use of functional genetics in the cricket.

8.6 The Evolution of the *oskar* Gene and Its Implications for Germ-Line Research

Germ cells are the cells that give rise to eggs and sperm. They are therefore an important cell type in sexually reproducing organisms and are sometimes referred to as the ultimate totipotent stem cell (Cinalli et al. 2008), because they alone maintain a genetic link between generations. In a developing embryo, the first cells to give rise

to the germ cells by clonal mitotic divisions are known as the primordial germ cells (PGCs). Across metazoans, PGCs are specified using one of two mechanisms (Extavour and Akam 2003). In some animals, including *G. bimaculatus* and *Mus musculus* inductive cell–cell signaling among neighboring somatic cells instructs certain cells to adopt PGC fate; this method of PGC specification is known as "induction." In other animals, including the fruit fly *D. melanogaster*, the nematode worm *Caenorhabditis elegans*, the zebrafish *Danio rerio*, and the clawed frog *Xenopus laevis*, PGCs are instead specified by "inheritance." In this mechanism, PGCs are specified very early in development through the cytoplasmic inheritance of a maternally derived special cytoplasm called "germ plasm," which often contains determinants that confer germ-line fate.

oskar is an insect-specific gene critical for the establishment of germ plasm in *D. melanogaster* and is the only gene reported in the animal kingdom to be both necessary and sufficient for germ cell formation (Lehmann and Nüsslein-Volhard 1986; Ephrussi et al. 1991; Kim-Ha et al. 1991; Ephrussi and Lehmann 1992; Smith et al. 1992). Following its discovery in *D. melanogaster*, *oskar* orthologues were reported in the genomes of other holometabolous insects known to specify their germ cells using germ plasm (Goltsev et al. 2004; Juhn and James 2006; Juhn et al. 2008; Lynch et al. 2011). Interestingly, *oskar* appears to be absent from many insect genomes that are known to lack germ plasm, including the bee *A. mellifera*, the beetle *T. castaneum*, and the silk moth *B. mori* (summarized by Quan and Lynch 2016). Based on this observation and the fact that hemimetabolous insects reportedly lack germ plasm (see Ewen-Campen et al. 2013 and references therein), it was hypothesized that *oskar* was a novel gene that arose at the base of the Holometabola, concurrent with the advent of insect germ plasm (Lynch et al. 2011). However, Ewen-Campen and colleagues discovered an *oskar* orthologue in the cricket *G. bimaculatus* genome and demonstrated that in this species, *oskar* is neither expressed at high levels in PGCs nor required for PGC formation (Ewen-Campen et al. 2012). Instead, cricket *oskar* is expressed in the neuroblasts (stem cells that arise from the neural ectoderm and give rise to the nervous system in Pancrustacea) of the brain and central nervous system (CNS) of the developing embryo, and is required for proper embryonic CNS patterning (Ewen-Campen et al. 2012). This observation, taken together with the reports that *D. melanogaster oskar* also plays a neural role (Xu et al. 2013), suggests two novel hypotheses: (1) *oskar* arose at least 50 million years earlier in insect evolution than previously hypothesized, before the divergence of Hemimetabola and Holometabola, and (2) *oskar*'s ancestral role in insects may have been in the nervous system and not in the germ line. This implies that *oskar* may have been co-opted for its essential role in holometabolous germ plasm assembly rather than having originated concurrently with germ plasm as had been previously suggested. This significantly changes our understanding of the evolutionary origins and functional evolution not only of this gene but perhaps also of insect germ plasm. Moreover, it constitutes an important example of how novel genes may arise and become co-opted, across evolutionary time scales, to perform different biological roles in animals.

8.7 Genomic Resources in Other Orthopterans

For an organism to become widely used as a research model for comparative or evolutionary studies, an important contributing factor is whether resources and tools are also available to study its close relatives. With this in mind, we will discuss available resources in other orthopterans that may be of use in aiding comparative work with *G. bimaculatus*. To our knowledge, at the time of writing, large-scale genomic resources are available for only two other orthopterans, a locust and a Hawaiian cricket species.

8.7.1 Resources in Field Crickets

Several transcriptomes are available for tissues and stages of many species of the genus *Gryllus*, including (1) gene expression at different life stages in *G. rubens* (Berdan et al. 2016), (2) in the male accessory gland of *G. firmus* and *G. pennsylvanicus* (Braswell et al. 2006; Andres et al. 2013), (3) fat body and flight muscles of different ecological morphs of *G. firmus* (Vellichirammal et al. 2014), (4) under cold-acclimation in *G. veletis* (Des Marteaux et al. 2017; Toxopeus et al. 2019), or (5) adult femur-derived transcriptomes from *G. assimilis* (Palacios-Gimenez et al. 2018). Genomic resources are also available for an inbred line of *G. assimilis* (Palacios-Gimenez et al. 2018). Outside of the genus *Gryllus*, large-scale genomic resources are also available for the Hawaiian cricket *Laupala kohalensis* in the form of an EST resource from a nerve cord cDNA library (Danley et al. 2007) and a de novo draft genome (Blankers et al. 2018). In fact, the *L. kohalensis* genome is, to our knowledge, the only published cricket genome to date. Transcriptomic data are also available for *Allonemobius fasciatus* embryos (Reynolds and Hand 2009), male accessory glands of *Gryllodes sigillatus* (Pauchet et al. 2015), and the testis, accessory glands, and adult body of *Teleogryllus oceanicus* (Bailey et al. 2013).

8.7.2 Resources in Grasshoppers and Locusts

A de novo transcriptome spanning several stages is available for the grasshoppers *Chorthippus biguttulus* and *Oxya chinensis sinuosa* (Kim et al. 2016) and for nymphs, adult females and males of *Xenocatantops brachycerus* (Zhao et al. 2018). An organ-specific transcriptome is available for the gut of *Oedaleus asiaticus* (Huang et al. 2017), an EST database exists for transcripts from the central nervous system of *Schistocerca gregaria* (Badisco et al. 2011), and a de novo transcriptome for *Tetrix japonica* (Qiu et al. 2017) is also available. In addition, other tools including RNAi have been tested and reported to be successful in *S. americana*

(Dong and Friedrich 2005). *Locusta migratoria* is a well-studied locust species that has large-scale genomic resources available in the form of a de novo genome and transcriptome (Wang et al. 2014) and an EST database from whole body and dissected organs (Kang et al. 2004; Ma et al. 2006). In addition, RNAi has been established and reported to be successful in adults, nymphs, and embryos for this species (He et al. 2006).

8.8 Commercial Importance of Crickets as Edible Insects and "Food of the Future"

In this chapter we have primarily focused on crickets as emerging evo-devo models. In this final section, we would like to briefly highlight other reasons that crickets are gaining popularity as study systems. Given their cosmopolitan distribution (all areas of the world, except the arctic and subarctic regions) and over 2400 documented species, crickets represent the most diverse lineage of "jumping or leaping" insects (Horch et al. 2017b). While the chirping sounds made by males have historically given them acoustic appeal as pets and in research, many species are now becoming economically important as an alternative food source for humans (Huis et al. 2013; Horch et al. 2017b), as feedstock for poultry (Ravindran and Blair 1993), or as fish bait (Huis et al. 2013), all of which are multibillion dollar industries. With the human population predicted to reach nine billion by the year 2050 (Huis et al. 2013), meat production and consumption is soon expected to reach unsustainable levels (Boland et al. 2013). Insects, especially crickets, have therefore been proposed and marketed as a novel, alternative, environmentally efficient food source with high nutritional value (Oonincx and de Boer 2012; Huis et al. 2013; Deroy et al. 2015).

Crickets are reportedly common street snacks in some parts of the world and have been part of the traditional diet in Thailand, the Lao People's Democratic Republic, Vietnam, the Democratic Republic of Congo, and Nigeria for hundreds of years (Kuhnlein et al. 2009; Huis et al. 2013). As an example, over 20,000 farmers are reported to rear crickets in Thailand, resulting in an estimated production of over 7500 tons per year in this country alone (Hanboonsong et al. 2013). To increase their appeal in the West, crickets are now being advertised and marketed to be eaten whole, in granular or in paste form, and as ingredients in commercially available flours and protein bars (e.g., Aspire Food Group USA, Inc.). While many species of crickets are edible (e.g., *G. bimaculatus*, *Gryllodes sigillatus*, *A. domesticus*, *A. testacea*, *T. occipitalis*, *T. mitratus*, and *Brachytrupes portentosus*), to our knowledge currently only *G. bimaculatus*, *G. sigillatus*, and *A. domesticus* are farmed economically for human consumption (Huis et al. 2013).

The use of crickets has the potential to change the future of the food industry, because of how effective they are in maximizing nutrition for minimal resources. Cricket rearing is comparatively inexpensive, requires a fraction of input resources, and has fewer negative environmental impacts than rearing traditional vertebrate

protein sources (Halloran et al. 2017). Crickets produce only 1% of greenhouse gases compared to cattle and pigs, in addition to showing an approximate tenfold reduction in ammonia emission (Oonincx et al. 2010), all relevant factors when considering sustainable production in the age of climate change. They act as a complete protein source and consist of over 50% protein by volume (Wang et al. 2004). Other advantages of eating crickets include their high edible weight: Nakagaki and DeFoliart (1991) have estimated that over 80% of a cricket is edible and digestible compared to 55% for chicken and pigs and 40% for cattle. This translates into making crickets twice as efficient as chicken, at least 4 times as efficient as pigs, and 12 times more efficient as cattle in converting feed into meat (Huis et al. 2013). As a specific example, the food conversion efficiency of the house cricket *Acheta domesticus* has been reported to be five times higher than beef, and when their fecundity is considered, this has been shown to increase as much as 15- to 20-fold (Horch et al. 2017b; Nakagaki and Defoliart 1991). Farming crickets is projected to become a multimillion dollar industry, with the US market for edible insects alone expected to exceed $50 million by as early as 2023 (Ahuja and Deb 2018).

The development of a novel food source like crickets must include assessing the potential risks involved with consumption of such sources. Therefore, there is increased interest in understanding the biology of these insects. While studies addressing entomophagy-induced food allergies (especially ones arising from eating crickets alone) are few, there is some preliminary evidence to confirm that crustacean-allergic individuals (or people with seafood allergies) may also show cross-reactivity to edible insects in general (Srinroch et al. 2015; Pener 2016; Ribeiro et al. 2018). Another study has reported that some individuals can develop asthmatic symptoms upon ingesting insects belonging to Orthoptera (Auerswald and Lopata 2005). Overall, however, eating and/or exposure to insects is not expected to pose significant risks of allergenic reactions for most people, especially if the individual has no prior history of arthropod or insect allergen sensitivity (Huis et al. 2013). In summary, the disadvantages associated with eating insects like crickets currently seem few and the advantages many. Consuming reared insects is potentially more environmentally friendly, nutritious, cheap, and affordable for people in all parts of the world. Cricket rearing is one way to use land efficiently, reduce or lower pesticide use and greenhouse gas emissions, may boost human and/or animal immunity (Goodman 1989; Muzzarelli 2010; Taufek et al. 2016), and finally improve the livelihood of women and children in rural areas by supporting local economies (Huis et al. 2013).

8.9 Conclusion

The successful establishment of the many functional and genetic manipulation tools in crickets has contributed to a new era of non-drosophilid insect research, not limited to evo-devo research. We hope that scientists from various disciplines feel

encouraged to use the cricket as a system to address intriguing questions in their respective fields.

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