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Embryo Injections for CRISPR-Mediated Mutagenesis in the Ant Harpegnathos saltator
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1 **TITLE:**

2 Embryo Injections for CRISPR-Mediated Mutagenesis in the Ant *Harpegnathos saltator*

3

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21 **KEYWORDS:**

22 social insect, ant, CRISPR, mutagenesis, microinjection

23

24 **SUMMARY:**

25 Many characteristics of insect eusociality rely on within-colony communication and division of

26 labor. Genetic manipulation of key regulatory genes in ant embryos via microinjection and

27 CRISPR-mediated mutagenesis provides insights into the nature of altruistic behavior in eusocial

28 insects.

29

30 **ABSTRACT:**

31 The unique traits of eusocial insects, such as social behavior and reproductive division of labor,

32 are controlled by their genetic system. To address how genes regulate social traits, we have

33 developed mutant ants via delivery of CRISPR complex into young embryos during their syncytial

34 stage. Here, we provide a protocol of CRISPR-mediated mutagenesis in *Harpegnathos saltator*, a

35 ponerine ant species that displays striking phenotypic plasticity. *H. saltator* ants are readily

36 reared in a laboratory setting. Embryos are collected for microinjection with Cas9 proteins and *in*

37 *vitro* synthesized small guide RNAs (sgRNAs) using home-made quartz needles. Post-injection

38 embryos are reared outside the colony. Following emergence of the first larva, all embryos and

39 larvae are transported to a nest box with a few nursing workers for further development. This

40 protocol is suitable for inducing mutagenesis for analysis of caste-specific physiology and social

41 behavior in ants, but may also be applied to a broader spectrum of hymenopterans and other

42 insects.

43

44 **INTRODUCTION:**

45   The evolution of eusociality in insects, namely those of the orders Hymenoptera and Blattodea
46   (formerly Isoptera), has resulted in unique and often sophisticated behavioral traits that manifest
47   on both the individual and the colony levels. Reproductive division of labor, a trait characterizing
48   the most advanced groups of social insects, often involves caste systems composed of several
49   behaviorally and often morphologically distinctive groups. Such behavioral and morphological
50   diversity between castes is controlled not only by their genetic system, but also often by the
51   environment[1-4], making eusocial insects attractive subjects for genetic and epigenetic research.
52
53   The ability to manipulate the genetic system of eusocial insects has proven to be challenging as
54   many species do not mate and reproduce in laboratory settings. Most eusocial insects also have
55   very few reproductive individuals in a colony, limiting the number of offspring that can be
56   produced and consequently, limiting the sample size for genetic manipulation[5]. Additionally,
57   many eusocial insects have long generation times compared to insects commonly used for
58   genetic studies (such as *Drosophila*), adding to the difficulty of establishing genetic lines[5]. Some
59   eusocial species, however, can generate a large proportion of reproductively active individuals in
60   a colony, which alleviates the challenges and provides opportunities to establish mutant or
61   transgenic lines.
62
63   In the case of the ponerine ant species, *Harpegnathos saltator*, all female workers can become
64   reproductively active upon the death of a queen or social isolation. These workers are referred
65   to as "gamergates" and can be used to generate new colonies[6]. Furthermore, there may be more
66   than one gamergate present in a colony, thus increasing offspring production[5,7,8]. Thus far,
67   mutant and/or transgenic lines have been developed in the European honeybee, *Apis mellifera,*
68   and in the ant species, *H. saltator*, *Ooceraea biroi,* and *Solenopsis invicta*[9-15]. Genetic analyses in
69   social bees and ants have paved the way toward a better understanding of eusociality, providing
70   an array of opportunities to study genes and their impacts on eusocial insect behavior and caste-
71   specific physiology.
72
73   Here, we provide a protocol for genetic modification via the CRISPR/Cas9 system in *H. saltator*.
74   Specifically, this technique was used to generate a germline mutation in *orco*, the gene encoding
75   the obligate co-receptor of all odorant receptors (ORs)[10]. *OR* genes have been remarkably
76   expanded in hymenopteran eusocial insects[16], and *orco* plays an essential role in insect olfaction;
77   in its absence, ORs do not assemble or function normally. Mutations of the *orco* gene therefore
78   disrupt olfactory sensation, neural development, and associated social behaviors[9,10].
79
80   In this protocol, Cas9 proteins and small guide RNAs (sgRNAs) are introduced into ant embryos
81   using microinjection for the purpose of inducing mutagenesis of a target gene. Here, we will
82   describe the microinjection procedure in detail along with directions regarding the care of
83   colonies and injected embryos. These methods are appropriate for inducing mutagenesis in a
84   variety of different genes in *H. saltator* ants and may be applied to a broader spectrum of
85   hymenopteran insects.
86
87   **PROTOCOL:**
88

1. Regular maintenance of *Harpegnathos saltator* colonies

1.1.   Maintain wild-type colonies of *H. saltator* in transparent plastic boxes in an ant rearing room at 22–25 °C and a photoperiod of 12 h light: 12 h dark (12L:12D) lighting schedule.

1.1.1.   Use small boxes (9.5 x 9.5 cm^2) to rear individual workers or small colonies. Use medium boxes (19 x 13.5 cm^2) or large boxes (27 x 19 cm^2) to rear larger colonies (**Figure 1**).

1.1.2.   To create nest boxes, use plaster to make floors for the boxes. As the wet plaster is drying in the medium and the large boxes, press a foam block into the plaster a few centimeters deep and a few centimeters from the back of the box to designate a lower nest region. Once the plaster has dried, cover the designated nest region with a square piece of glass.

NOTE: In small boxes, there is no need to designate a lower nest region. If ants are neglecting to use the designated lower nest region, it may help to cover the glass with a square piece of red cellophane. This gives the impression of a dark underground space resembling nests that *H. saltator* use in the wild and may encourage them to move their brood to the designated region.

1.2.   Feed colonies with live crickets twice per week.

NOTE: Colonies should be fed enough that they consume all crickets prior to their next feeding. Feed any isolated ants and mutant ant colonies with crickets pre-stung by workers of a regular colony 2–3 times every week.

1.3.   Apply water regularly to the plaster nest box flooring using a wash bottle.

NOTE: The plaster should be moist enough that it does not feel dusty to the touch, but it should be dry enough that all added water is absorbed by the plaster. It is important that the nests are not watered excessively. On average, nest boxes will need a small amount of water added once per week.

1.4.   Whenever feeding occurs, remove trash and dead individuals. Freeze all waste and dead ants overnight at -30 °C before disposing of these materials as regular garbage.

1.5.   Add a pinch of dried sawdust to colonies periodically; this helps larvae as they undergo pupation and helps workers keep the nest box clean.

2. Preparation of quartz glass microinjection needles

2.1.   Use a micropipette puller to pull glass microinjection needles.

2.2.   Select the glass to be pulled. Ensure that the glass being used has been stored in a dust-free and clean environment.

133 NOTE: Here, thin-walled filamentous quartz glass with an outer diameter of 1.0 mm, inner
134 diameter of 0.5 mm, and length of 7.5 cm has been used to produce microinjection needles. If
135 injecting a soft-bodied embryo, borosilicate needles may also be applicable, but borosilicate
136 needles are not able to penetrate hard chorion.
137
138 2.3.   Set the parameter settings of the puller. Use a two-step process to pull microinjection
139 needles for *H. saltator* embryos: parameters for the first step include heat of 575, velocity of 35,
140 delay of 145, and a pull of 75; parameters of the second step include heat of 425, velocity of 15,
141 delay of 128, and a pull of 200. Following the second step, ensure that the resulting needle has a
142 2 mm taper and a tip of 0.5 µm (**Figure 2**).
143
144 NOTE: This set of parameters, along with parameters for other needle types, can be found in the
145 Operation Manual[17]. Manuals for pipette pullers often provide recommended parameters for a
146 variety of techniques. Some trial and error may be required to determine which parameters
147 generate the best needles for specific needs. A short taper is ideal for H. saltator injections, as it
148 can penetrate the hard chorion of H. saltator embryos. If injecting a soft embryo, such as one
149 that has been dechorionated (e.g., Drosophila), a longer taper around 10 mm may yield better
150 results. Operation manuals for pipette pullers usually provide specific parameters for pulling
151 needles suitable for different needs. It is important that gloves are worn while handling glass
152 filaments and pulling needles. Oils from bare hands may transfer to the glass if gloves are not
153 worn.
154
155 2.4.   Once parameters are set, use a micropipette puller to pull needles for microinjection.
156 Ensure that needles are kept in a dust-free and clean environment until used.
157
158 NOTE: It is recommended to use freshly pulled microinjection needles. If pre-pulled needles are
159 used, they should be stored properly in a box to prevent damage of needle tips and potential
160 contamination. Needles that have undergone long-term storage are not recommended for
161 embryo injections.
162
163 **3.   Preparation of microinjector**
164
165 3.1.   Use a microinjector to inject desired materials into ant embryos.
166
167 3.2.   Prepare the microinjection mixture of Cas9 proteins and *in vitro* synthesized small guide
168 RNAs (sgRNAs)[10]. Keep the mixture on ice until it is time to load a microinjection needle. When
169 not in use, store the microinjection mixture at -80 °C.
170
171 NOTE: The concentrations vary in different species. High concentration may cause high mortality,
172 whereas low concentration may reduce efficiency. We use 0.2 µg/µL Cas9 proteins and 0.2 µg/µL
173 sgRNAs for *H. saltator* embryo injection. Design of our sgRNAs followed a previously established
174 protocol[18]. Gene sequences were obtained from DNA databases. The genome sequence of *H.
175 saltator* was also previously reported[19,20].
176

177   3.3.   Adjust the injection parameters for *H. saltator* embryos: an injection pressure of 140
178   hectopascal (hPa), a constant pressure of 70 hPa, and a time of 0.4 seconds. Adjust constant
179   pressure such that material only flows in one direction. Adjust the injection pressure and time
180   only if no material is flowing from the needle into the embryo.
181
182   NOTE: If injecting a different type of embryo, the primary parameter that may change is constant
183   pressure, which is responsible for ensuring that fluid from the embryo does not flow back into
184   the needle. Volume is not controlled for in this protocol. Setting the parameters of the
185   microinjector is sufficient for obtaining consistent injections.
186
187   3.4.   Load a microinjection needle with 2 µL of the mixture using microloader pipette tips. Do
188   this slowly to ensure that no bubbles are formed in the mixture.
189
190   NOTE: If bubbles form, it may be difficult to maintain consistent microinjections.
191
192   3.5.   Break just the tip of the needle by breaking along the edge of the tape such that a narrow
193   taper is still maintained. Ensure that the needle is broken just enough that the tip is opened, but
194   not so much that the taper is broken off.
195
196   NOTE: Importantly, if the opening of the needle is too wide after breaking, the user will see liquid
197   run out of the needle when mounted to the pressurized microinjector prior to application of
198   injection pressure. Some microinjection protocols advise breaking needles by cutting the tip with
199   scissors. This method is not advised, as scissors may cause the tip of the needle to shatter.
200   Injecting embryos with a shattered needle will be highly damaging.
201
202   3.6.   Mount the needle to the micromanipulator
203
204   **4.   Injection of embryos**
205
206   4.1.   Select embryos for microinjection from the syncytial stage: the time during development
207   in which nuclei divide without cytokinesis.
208
209   NOTE: This is the ideal time during development for genome editing by microinjection, as
210   discovered previously in *Drosophila*[21]. *H. saltator* embryos pass the syncytial stage and reach
211   cellularization around 36 h after egg deposition[10]. Higher efficiency is achieved if younger
212   embryos are used for injections.
213
214   4.2.   Line embryos on a piece of double-sided tape stuck to a glass microscope slide. Ensure
215   that embryos are secured well to the tape to prevent movement during injection. Lay the
216   embryos in a vertical orientation, such that the lateral side of the embryo is at the edge of the
217   tape (**Figure 3**). Place the slide and lined embryos onto the stage of the microscope at a
218   designated microinjection workstation.
219
220   NOTE: It is recommended that the embryos are arranged such that successive injections can be

221 performed by moving the slide on the stage instead of adjusting the needle position with every
222 injection. This allows for successive injections to be performed more efficiently.
223
224 4.3.   Align the needle with the first embryo to be injected using the micromanipulator (**Figure**
225 **4a**).
226
227 4.4.   Laterally puncture the needle into the first embryo along its dorsal/ventral axis under a
228 microscope.
229
230 4.5.   Inject the microinjection mixture. Look for slight movement of the embryo, indicating an
231 increase in internal pressure due to the injected liquid. Additionally, watch for the formation of
232 a small droplet containing visible trace of tissue and/or lipid on the outer membrane of the
233 embryo (**Figure 4b**).
234
235 NOTE: Presence of trace of tissue and/or lipid in the droplet indicates that the needle has
236 successfully punctured both chorion and vitelline membrane of the embryo. If traces of these
237 materials are not present, the injection was not performed successfully and should be repeated.
238 After a few seconds, the droplet will be reabsorbed by the embryo and will no longer be visible.
239
240 4.6.   Gently remove the needle from the embryo immediately, and proceed to the next by
241 adjusting the position of the microscope slide. Repeat until all the embryos have been injected.
242
243 4.7.   Once all embryos on the slide have been successfully injected, transfer the slide to a
244 humid box for 1 h to give the embryos time to recover from the injection process prior to being
245 removed from the slide.
246
247 5.   **Rearing of injected embryos**
248
249 5.1.   After 1 h of incubation in a humid box, gently remove the injected embryos from the tape
250 using featherweight forceps, and transfer them to a tube filled with a small amount of 70%
251 ethanol. Invert the tube several times to transfer the embryos to the bottom of the tube. Repeat
252 the ethanol wash once, followed by three washes with autoclaved water.
253
254 5.2.   Using a small and soft paintbrush, transfer all injected embryos to 1% agar plates with 2%
255 Antibiotic-Antimycotic. Apply the Antibiotic-Antimycotic after poured agar plates have cooled by
256 spreading over the surface of the plate using a cell spreader. Seal the agar plate with parafilm to
257 prevent agar desiccation.
258
259 NOTE: Do not attempt to return injected embryos to a colony after injections as workers may
260 destroy most injected embryos. Survival is therefore optimized by allowing embryos to develop
261 on agar plates outside of a normal colony environment.
262
263 5.3.   Incubate the agar plates at 25 °C for approximately 4 weeks. Check regularly for hatching.
264

265 5.4.   Once the first embryo has hatched into a larva, return all embryos and larvae to a nest
266 box with a few young nurse workers to care for the hatchlings. Feed using crickets pre-stung by
267 a larger wild-type colony, remove waste products, and add water following the same protocol
268 discussed in Section 1.
269

270 NOTE: Small boxes (9.5 x 9.5 cm^2) are ideal for such colonies. *H. saltator* reproduces well in
271 captivity. Therefore, mutant embryos can be reared to adulthood. Isolation of mutant adult(s)
272 induces transition to the reproductive gamergate stage. Controlled crosses are used to establish
273 mutant colonies with heterozygous or homozygous individuals (**Figure 5**).
274

275 **REPRESENTATIVE RESULTS:**
276 Using the protocol provided here, genome editing in *Harpegnathos saltator* embryos was
277 performed successfully. These results were validated via polymerase chain reaction and pGEM
278 cloning of DNA extracted from injected embryos followed by DNA sequencing. Efficiency of
279 somatic mutagenesis using this protocol reached approximately 40%. F1 mutant males were
280 mated to wild type females to produce heterozygous F2 females which, if not mated, produced
281 F3 males. Mutant F3 males were mated to heterozygous females to produce F4 homozygous
282 mutant females. Absence of the target peptide was further confirmed via mass spectrometry of
283 these F4 homozygous female individuals. Wings were clipped from males using microdissection
284 scissors and used for genotyping purposes. As workers do not have wings, they are normally
285 sacrificed and genotyped after experiments. As a result of successful mutagenesis, unusual
286 behaviors were observed, which correlated with loss of the target gene. The loss of *orco* resulted
287 in abnormal behaviors related to loss of pheromone sensing, inability to detect prey, impaired
288 fecundity, and wandering from the colony. Furthermore, *orco* mutant ants exhibited a decreased
289 number of odorant receptor neurons and antennal lobe glomeruli, suggesting that
290 neuroanatomy in ants is dependent on odorant receptor functionality[10].
291

292 **FIGURE AND TABLE LEGENDS:**
293 **Figure 1: *Harpegnathos saltator* nests.** (**A**) External features of a 19 x 13.5 cm^2 nest box
294 containing an *H. saltator* colony. (**B**) Internal features of a 19 x 13.5 cm^2 nest box containing an
295 *H. saltator* colony. Note the presence of a lower nest region under a square piece of glass. (**C**)
296 External features of a 9.5 x 9.5 cm^2 nest box containing a small *H. saltator* colony. Such a nest box
297 is also suitable for isolated workers and mutant colonies. (**D**) Internal features of a 9.5 x 9.5 cm^2
298 nest box containing a small *H. saltator* colony.
299

300 **Figure 2: Needle for *Harpegnathos saltator* embryo microinjection.** Note the thin taper of the
301 needle (marked with an arrow). The needle is opened by breaking the tip slightly.
302

303 **Figure 3: Alignment of embryos on double-sided tape.** Embryos should be aligned so that their
304 length is parallel with the long edge of the tape. This enables successive injections to be
305 performed with ease by moving the slide on the stage.
306

307 **Figure 4: Embryo and needle as seen during microinjection.** (**A**) Proper alignment of the needle
308 with the embryo prior to injection. The needle rests perpendicular to the mid-point of the

309   embryo's side, the typical site of injection. (**B**) The embryo and needle following a successful
310   injection. A small droplet protrudes from the embryo's side (marked with an arrow).
311
312   **Figure 5: Diagram of basic mutant crosses.** CRISPR may induce mutations on the target gene in
313   F0 females, which are subsequently isolated upon adulthood to induce the gamergate transition.
314   If mutations occur in germline cells, unmated gamergates may lay mutant male eggs. The F1
315   mutant adult males may be mated with wild-type females to generate heterozygous offspring,
316   or they may be mated with heterozygous females to generate homozygous or heterozygous
317   offspring.
318
319   **DISCUSSION:**
320   The evolution of eusociality amongst insects, including ants, bees, wasps, and termites, has
321   resulted in the appearance of novel behavioral and morphological traits, many of which are
322   understood to be influenced by a combination of environmental and genetic factors[1-4].
323   Unfortunately, the attractiveness and usefulness of eusocial insects as research models in the
324   field of genetics has been hindered by the difficulties associated with mutagenesis in this group.
325   This hindrance occurs due to reproductive division of labor, a key trait of eusocial insects in which
326   only a few members of a colony may reproduce. This trait places limitations on the number of
327   mutant offspring and makes the development of genetic lines challenging[5]. The ponerine ant
328   species, *Harpegnathos saltator,* provides a solution to this dilemma, as all females are capable of
329   becoming reproductive gamergates when isolated[5]. Here, we provide methods for mutagenesis
330   in *H. saltator* using the CRISPR/Cas9 system, delivered via embryo microinjection.
331
332   Proper colony maintenance and selection of embryos at the appropriate developmental stage
333   for microinjection are critical. Previous work established that the syncytial stage in insect
334   development is the ideal stage for genome editing[21], but the timing of this stage in *H. saltator*
335   embryonic development was previously unknown. Via nuclear staining of early embryo sections,
336   we were able to determine that the syncytial stage in *H. saltator* embryos lasts until 36 h after
337   egg deposition, allowing us to determine when to select new embryos for microinjection[10].
338
339   Selecting parameters for needle pulling and for microinjection can be challenging. Factors to
340   consider when selecting parameters for needle pulling include (1) the type of glass used, (2) the
341   desired purpose of the needle, (3) the desired tip size, (4) the amount of resistance the needle
342   will experience during injection, (5) the desired taper length, and (6) the type of cell or organism
343   that will be injected. Suggestions and guidelines for pulling various needle types can be found in
344   various operation manuals[17]. Similarly, there are factors to consider when selecting parameters
345   for microinjection including (1) size of the needle being used and (2) the embryos to be injected[22].
346   While this protocol focuses on microinjection in *H. saltator* embryos, the techniques may vary in
347   other insects with modification to these parameters. In particular, the parameters for needle
348   pulling and injection will differ if the embryo in question is soft or dechorionated. *H. saltator*
349   embryos have a tough chorion, and injection results are best when a needle with a short taper is
350   used (approximately 2 mm). Embryos that have less firm chorion may be injected with needles
351   with longer tapers (approximately 10 mm).
352

353　In *H. saltator*, injected embryos cannot be returned immediately to a colony, as doing so will risk
354　their destruction by nursing workers. If applying this protocol to other social insect species, this
355　may not be the case. Determining the best post-microinjection rearing methods in other species
356　may require some trial and error. In the case of *H. saltator*, embryos need to be reared on agar
357　plates until hatching. Once hatched, they can be returned safely to a small colony with a few
358　workers to care for the injected brood[10]. A similar method of embryo care is utilized in fire ants
359　(*Solenopsis invicta*) and clonal raider ants (*Ooceraea biroi*) to increase the survival rate of injected
360　embryos[9,15]. Upon adult eclosion, mature mutant females may be kept individually or in small
361　colonies to begin their reproductive cycle. Directions for the care of *H. saltator* mutants are
362　provided in this protocol, but if using a different species, directions for care of injected embryos
363　and mutant adults should be modified to fit the needs of the species.
364
365　The protocol provided here is optimized for mutagenesis, in which specifically non-essential
366　genes are targeted. In this case, the *orco* gene was targeted, and mutations in *orco* did not affect
367　ant survival to adulthood. Similarly, this protocol may be used to target other non-essential
368　genes, including the ones associated with other sensory receptors. If the target gene is essential,
369　transgenic ants will have to be generated instead, either via CRISPR or transposon. Transposon-
370　mediated transgenesis has been used in honeybees[13] and may apply to ants. If the desired
371　outcome is transgenic organisms, the injected materials will have to be different. However, post-
372　injection processes will be similar, and therefore some aspects of this protocol will be beneficial
373　despite the difference in the desired outcome.
374
375　Overall, *H. saltator* is ideal for use as a model organism and for performance of genetic crosses
376　due to its plastic reproductive system in which workers begin reproducing after isolation[10]. This
377　differentiates this ant species from other ants in which CRISPR/Cas9 technology has been
378　established, for example, *O. biroi*, a species in which all females reproduce clonally. *H. saltator*
379　presents unique research opportunities amongst organisms of its kind as genetic lineages can be
380　established and mutations can be maintained across generations in this species. The novelty of
381　this system allows researchers not only to generate mutant ants, but also to develop transgenic
382　lines in the future. This provides opportunities for novel research to study genetic control of
383　advanced eusociality.
384
392
393　**DISCLOSURES:**
394　The authors have nothing to disclose.
395
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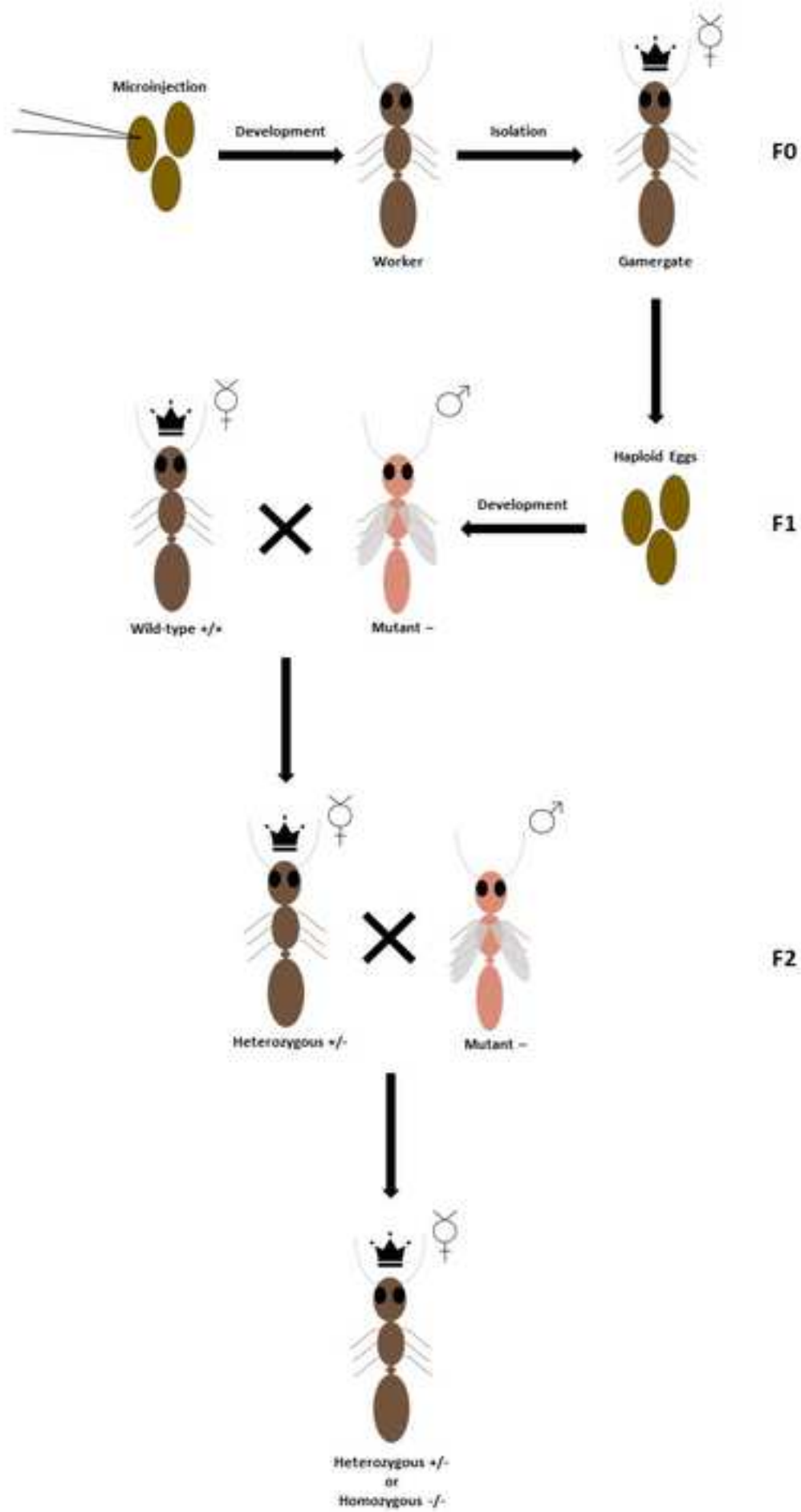447   Eppendorf Injectman® 4 and Femtojet® 4. (2019).
448
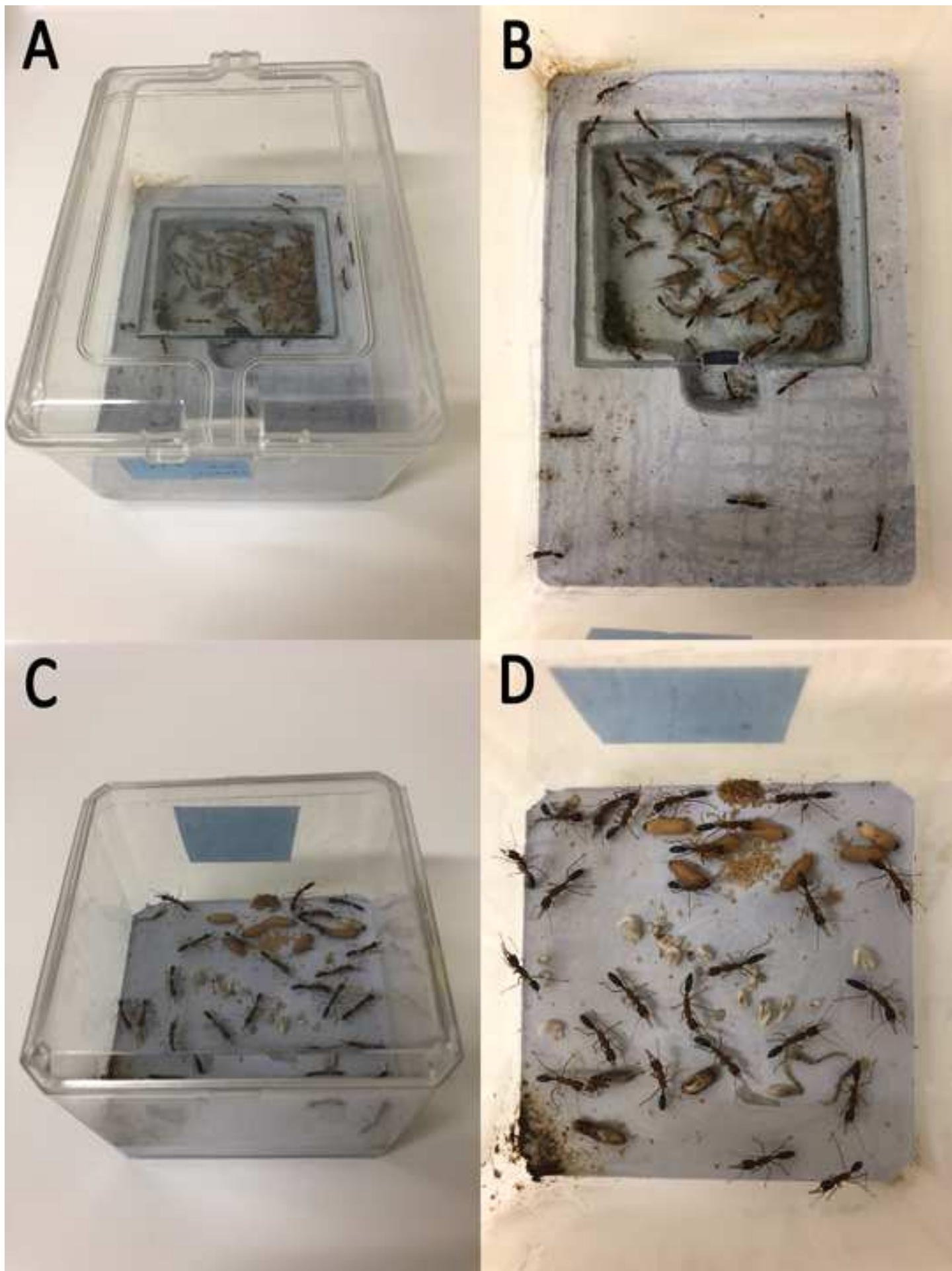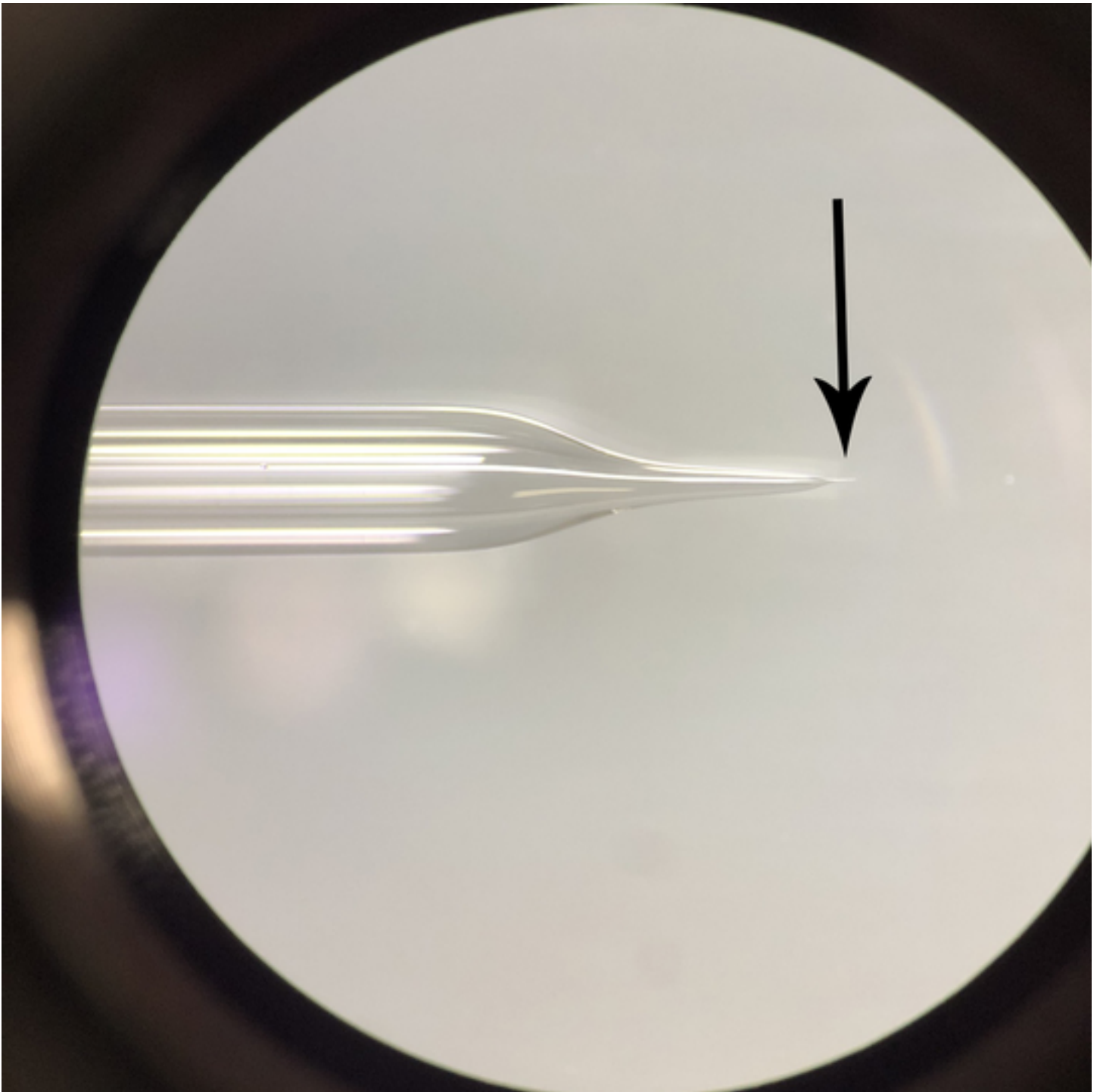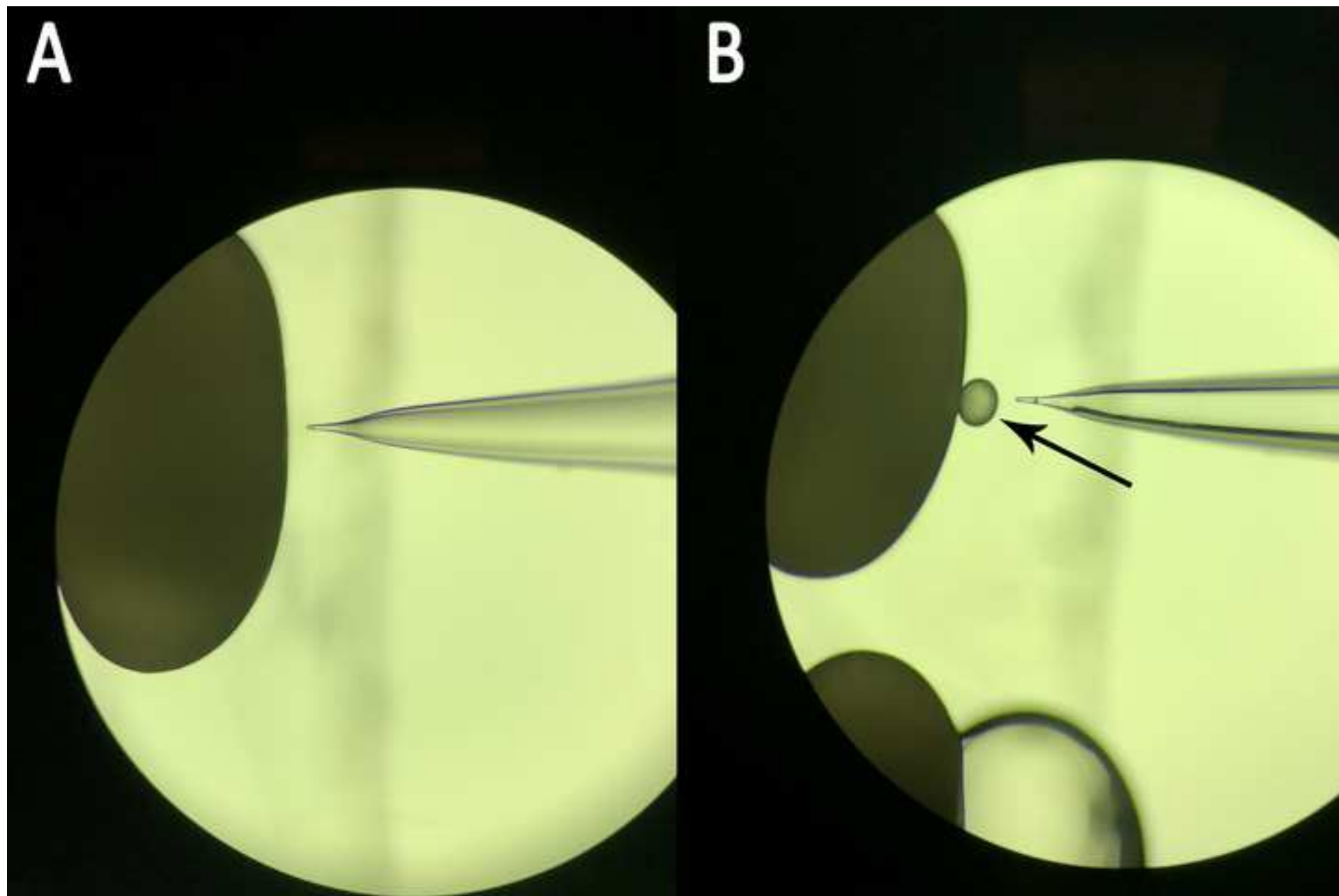
Name of Material/ Equipment	Company	Catalog Number
Antibiotic-Antimycotic (100X)	ThermoFisher	15240-062
Cas9 protein with NLS, high concentration	PNA Bio	CP02
Cellophane Roll 20 inch X 5 feet	Hypogloss Products	B00254CNJA
Eclipse Ci-S upright microscope	Nikon	Ci-S
Featherweight forceps, narrow tip	BioQuip	4748
FemtoJet ll microinjector	Eppendorf	920010504
Microloader pipette tips	Eppendorf	930001007
NCBI database	National Center for Biotechnology Information	Gene ID: 105183395
P-2000 Micropipette Puller	Sutter Instruments	P-2000/G
Plastic boxes (19 X 13.5 cm^2)	Pioneer Plastics	079C
Plastic boxes (27 X 19 cm^2)	Pioneer Plastics	195C
Plastic boxes (9.5 X 9.5 cm^2)	Pioneer Plastics	028C
Quartz glass without filament	Sutter Instruments	Q100-50-7.5
Vannas scissors, 8.5 cm	World Precision Instruments	500086
Winsor & Newton Cotman Water Colour Series 111 Short Handle Synthetic Brush - Round #000	Winsor and Newton	5301030

Comments/Description

The product has many color variations. Purchase it in red for use in making ant nests.

This product is no longer sold or supported by Eppendorf. A comparable microinjector may be used instead.

Responses to reviewers' comments

We greatly appreciate all comments left by reviewers. Please see below our responses to comments.

Reviewer #1:

1. Lines 46-48. "… namely those of the order Hymenoptera …". This is incorrect. At the very least, termites should be included and they are not Hymenoptera.
Also in discussion (Line 291).
We have added further information to include termites in our description of the evolution of eusociality (lines 46-47 and 321).

2. Lines 66-70. CRISPR/Cas9 mediated mutagenesis of fire ants have also been demonstrated. Chiu et al 2020 Insectes Sociaux.
The paper by Chiu et al. 2020 has been added to our list of references, and the corresponding information about CRISPR/Cas9 mediated mutagenesis of fire ants has been added to the manuscript (line 68-69).

3. Line 129. "Select the glass to be pulled". I think the vendor(s) and catalogue number(s) should be provided (and for all reagents and equipment). The exact reagent or equipment is sometimes critical.
We have added this information to the revised manuscript (lines 130-132).
As per JoVE requirements, a Table of Materials is included with the manuscript submission as a separate Microsoft Excel document. Within this Table of Materials, one will find names, vendor(s), and respective catalogue number(s) for all reagents and equipment discussed in the manuscript.

4. Lines 134-141. In our experience when pulling needles, it is important to wear gloves to prevent transferring the oil from our hands onto the glass pipettes. Perhaps this could be included in this section.
We agree that wearing gloves is important to ensure cleanliness of the glass. This step has been added in as a Note after step 2.5 (lines 151-152).

4b. Additionally, I realize a lot of experimentation is "trial and error". Yet, I think it would benefit the readers if the authors could provide some insight into why the parameters were chosen or perhaps why the current shape of the needle works for them. This way, the readers may have a hint as to what to try first when adapting to other systems. Same for step 3.3.
And/or address in the discussion (associated with Lines 310-319).
We agree. Additional insight regarding proper needle shape has been added, particularly regarding shapes ideal for different levels of embryo firmness (lines 146-149 and lines 349-352). A short taper (approximately 2 mm) is ideal for *H. saltator* injections, as it can penetrate hard chorion of *H. saltator* embryos. If injecting a soft embryo, such as one that has been dechorionated, a longer taper around 10 mm may yield better results.
Regarding the parameters for the injection system, additional insight regarding injection parameters has also been added (lines 179-183): If injecting a different type of embryo, the primary parameter that may change is constant pressure, to ensure that fluid from the embryo does not flow back into

the needle. Injection pressure and time may be adjusted only if no material seems to be flowing from the needle into the embryo.

5. Lines 176-177. "Break just the tip of the needle…" I think more details or description for this step would be useful. "Break" can mean different things for different people.

Some additional details were added in an effort to provide further guidance (lines 193-196): The needle should be broken just enough that the tip is opened, but not so much that the taper is broken off. Importantly, if the opening of the needle is too wide after breaking, liquid will run out of the needle when mounted.

6. Regarding Steps 3.3 and 3.5. Do the authors control for volume? Is this important? Depending on the how big the hole is on the broken needle tip, the same pressure and time can produce different volumes.

Volume is not controlled for in this protocol. The amount of liquid that is injected is an estimate. Setting the parameters of the microinjector properly and breaking needle tips in a consistent manner is sufficient for controlling the volume of injected liquid. This information has been added as a Note following step 3.5 (lines 185-186).

7. Line 226. What type of tweezers (presumably the soft ones? Again brand and catalog number would be nice)?

The name of the tweezers, brand, and catalogue number have been added to the Table of Materials. We use featherweight forceps produced by BioQuip, which is added to line 252.

8. Lines 321-329. A similar situation is also the case for fire ants and Ooceraea biroi. Perhaps these points should also be noted here.

We appreciate your noting of this similarity in post-injection methods between species. This information has been added to the discussion (lines 359-361).

Minor Concerns:
1. Lines 40-41: "Following the emergence of the first embryo…, all embryos". Should both cases be "larva(e)"?
Revised, as suggested (lines 40-41).

2. Line 99. "square glass". Suggest "square piece of glass".
Revised (line 100).

3. Lines 107-111. Seems like steps 1.2 and 1.3 should be more like 1.2a and 1.2b.
These step values have been revised.

4. Line 183. "… genome editing by microinjection [in D. melanogaster]". The reference is for the fruit fly. Or the sentence needs to be restructured to be more precise.
Revised (line 208).

5. Line 212. "absorb" (or maybe "resorb") rather than "retreat"? Also, could it be evaporation?
Revised. We chose to replace the word with "reabsorbed" (line 238).

6. Line 257. "…obtained from out- and backcrossing haploid mutant males" Sentence unclear, especially in regarding the out and backcrossing part. Who is crossing to whom?
Revised (lines 284-286): F1 mutant males were mated to wild type females to produce heterozygous F2 females which, if not mated, produced F3 males. Mutant F3 males were mated to heterozygous females to produce F4 homozygous mutant females.

7. Line 277. Which edge?
Revised (line 306-307): Embryos should be aligned so that their length is parallel with the long edge of the tape.

Reviewer #2:
(1) I have no real comments on the study. The biggest issue, of course, is that there weren't any videos associated with the study. This makes it difficult to review for JoVE. But so be it
We appreciate comments by the reviewer.
Regarding videos, it is indicated by JoVE that filming would be accomplished following the peer-reviewing process. It is to our understanding that we will be receiving a notice to arrange a filming date with a videographer in the near future.

(2) The only other comment I might make is that the Discussion might include comments on how the current technique proposed by the authors differs from other studies that have done microinjection in social insects or other insects. Why is it better? How is it different? Is it innovative in any new ways?
We appreciate the suggestion to add comments regarding the difference between our technique and similar techniques in other social insects. Some additional text has been added, further explaining the benefits of the plastic reproductive system in *H. saltator*, and how it differs from the systems used by other ant species in which CRISPR/Cas9 has been established (lines 376-384). In brief, certain features in *H. saltator*, such as controlled crosses in the lab and transition of workers to reproductive gamergates, allow us not only to generate mutant ants, but also for future work in developing transgenic lines.