CRISPR-Cas9 Gene Editing in Lizards
Through Microinjection of Unfertilized Oocytes

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1   **Abstract**

2   CRISPR-Cas9 mediated gene editing has enabled the direct manipulation of gene

3   function in many species. However, the reproductive biology of reptiles presents unique

4   barriers for the use of this technology, and there are currently no reptiles with effective

5   methods for targeted mutagenesis. Here we present a new approach that enables the

6   efficient production of CRISPR-Cas9 induced mutations in *Anolis* lizards, an important

7   model for studies of reptile evolution and development.

8

9   **Main**

10   Squamates (lizards and snakes) comprise a diverse group of reptiles represented by

11   over 10,000 recognized species[1]. However, mechanistic studies of gene function in

12   squamates and other reptiles lag behind other major vertebrate groups. While the

13   adoption of CRISPR-Cas9 mediated gene editing has enabled direct manipulation of

14   gene function in many fish[2,3], amphibian[4,5], avian[6], and mammalian species[7,8], there

15   remain no reptilian model systems with established methods for the production of

16   targeted sequence alterations. To date, attempts to manipulate gene function in reptiles

17   have been limited to a small number of studies employing whole embryo culture

18   coupled to viral- or electroporation-based methods to alter gene expression[9,10]. These

19   methods produce transient, localized, and highly mosaic patterns of transgenesis.

20   Moreover, these techniques have not been used to engineer targeted gene

21   modifications in any reptile species.

22

23   Among squamates, *Anolis* lizards are compelling candidates for the establishment of

24   gene editing methods. Over the past 50 years, anoles have become one of the central

25   model systems for studies of reptile evolution, physiology, and development[11]. This

26   group has experienced an extensive adaptive radiation in the Caribbean with hundreds

27   of described species that display a wide range of morphological, behavioral, and

28   physiological differences. Studies of the convergent evolution of similar sets of *Anolis*

29   "ecomorphs", or habitat specialists, on different Caribbean Islands has produced a rich-

30   literature on the biology of *Anolis* lizards. Although many *Anolis* species can be

31   successfully raised in the lab, we chose to develop genome editing in the brown anole

32   lizard, *Anolis sagrei*. This invasive lizard is now found far beyond its native Caribbean

33   range and is ideal for genetic studies due to its small size, ease of husbandry, long

34   breeding season, and relatively short generation time.

35

36   CRISPR-Cas9 mediated genome editing is an effective method for producing

37   genetically modified vertebrates[12]. Typically, CRISPR-Cas9 components are

38   microinjected into vertebrate embryos at the one-cell stage to generate individuals

39   potentially harboring alterations at the locus of interest. However, there are significant

40   challenges associated with microinjection of *Anolis* zygotes. These challenges include

41   internal fertilization and the long-term storage of sperm within the oviducts of adult

42   females, which makes timing the microinjection of single cell embryos extremely

43   difficult. At the time of ovulation *Anolis* eggs are also quite large (~8 mm in length) and

44   are filled with substantial amounts of yolk; these oocytes are fragile and are difficult to

45   manipulate without rupturing. Furthermore, after fertilization the egg shell must be

46   deposited around the egg and embryonic development is initiated before the egg is laid.

47   Finally, unlike the hard shells of birds, the egg shells that enclose *Anolis* embryos are

48   pliable and no air space is present within the egg, presenting obstacles for embryo

49   manipulation within the egg shell. Most of these reproductive challenges for

50   microinjection are not unique to anoles, but are features that are typical of many

51   reptiles. Here we demonstrate the use of CRISPR-Cas9 to mutate the *tyrosinase* gene

52   in *Anolis sagrei* using an approach that is generalizable to other loci. The procedure we

53   have developed involves microinjection of CRISPR-Cas9 components into immature,

54   unfertilized *Anolis* oocytes within the ovaries of female lizards.

55

56   Reproductively active *A. sagrei* females lay approximately 1 egg every week, similar

57   to *Anolis carolinensis*[13]. Each ovary contains a series of approximately 10 maturing

58   ovarian follicles arranged by size, with the smallest follicle closest to the germinal bed

59   and the largest vitellogenic follicle positioned distally (Fig. S1). With the exception of the

60   largest follicle, these follicles are previtellogenic or in the early stages of vitellogenesis

61   and are largely similar in size between the left and right ovaries. In our approach,

62   female lizards are anesthetized and are placed on a surgical platform underneath a

63   standard dissecting scope. Left and right ovaries are separately accessed via vertical

64   incisions positioned along the left or right flank, respectively (Fig. 1). During surgery, the

65   ovary can be gently moved to allow easy observation and injection of the oocytes under

66   a dissecting microscope. Oocytes are microinjected with Cas9 ribonucleoprotein

67   complex (Cas9 RNP) while remaining associated with the ovary (Fig. 1, Fig. S1, and

68   Video S1). In our hands, oocytes that are 0.75 to 5 mm in diameter yield the highest

69   frequency of mutant animals (Fig. S2). A typical anole ovary has 4 to 6 oocytes in this

70   range. Therefore, approximately 10 oocytes in this size range can be injected per

71   animal. Oocytes equal to or larger than 6 mm in diameter are not injected due to the

72   increased risk of rupturing these large, yolk-filled oocytes. After microinjection of

73   oocytes is completed on one side, the incision is sealed with veterinary glue. The

74   procedure is then repeated on the opposite side. We routinely inject 5 females per day

75   for a total of about 50 oocytes per injection session.

76

77   Following recovery, the microinjected oocytes continue to mature within the female

78   and are eventually ovulated and fertilized through natural mating with an introduced

79   male or via stored sperm from previous matings. The ovaries have an ovulatory cycle

80   that ranges from two to four weeks, but the left and right ovaries are out of phase

81   relative to each other, which results in eggs laid in alternating order from follicles on the

82   left and right ovaries[14]. Because of the alternating follicle contributions from the left and

83   right ovaries, a putative "follicle train" can be established by ranking follicles based on

84   size and by designating ovarian turns. By ordering follicles in this manner, one can infer

85   the range of follicle sizes which yielded crispant lizards. For instance, follicles in the 3 to

86   5 mm range develop into eggs that are laid within three to four weeks post injection (Fig.

87   S2).

88

89   To assess the effectiveness of our approach, we targeted the second exon of the

90   *tyrosinase* (*tyr*) gene. *Tyrosinase* was chosen for this study because loss-of-function

91   mutations are viable in a wide-range of vertebrates, the resulting pigmentation

92   phenotypes are readily detected, and this allowed us to develop a new *Anolis* model to

93   investigate human albinism. Cas9 protein coupled to a mixture of three different

94   synthetic *tyr* guide RNAs was injected into immature oocytes. The decision to

95   simultaneously inject three guide RNAs was motivated by the presence of a number of

96   single-nucleotide polymorphisms (SNPs) in *tyr* exon 2 within the population of lizards

97   used for these experiments. For these experiments, a total of 146 oocytes from 21

98   reproductively active females were microinjected over the course of eight surgical

99   sessions.

100

101   We obtained nine F0 animals harboring mutations in *tyr* exon 2. Four of these

102   animals were phenotypically albino and harbored loss-of-function mutations at both *tyr*

103   alleles. Five animals carried heterozygous loss-of-function mutations and exhibited

104   normal pigmentation. Mutant alleles could be visualized by PAGE after PCR

105   amplification across *tyr* exon 2 using genomic DNA prepared from F0 embryos or

106   hatchlings as a template. The changes associated with each of the CRISPR-Cas9

107   induced mutations was determined by sequencing the amplicons from each animal and

108   comparing the sequence to that of wild-type lizards in the colony. The overall mutation

109   frequency in terms of mutant lizards per follicle injected was 6.2%. A mutation frequency

110   of 9.7% was obtained from microinjected follicles that were 1.5 to 2.5 mm in diameter,

111   while follicles 0.75 to 1.0 mm and 3 to 5 mm in diameter yielding frequencies of 9.3%

112   and 5.6%, respectively. No mutations were obtained from microinjection into follicles

113   smaller than 0.5 mm in diameter. Consistent with results in other vertebrates, CRISPR-

114   Cas9 genome editing in lizards resulted in indels that typically ranged in size from 3 to

115   17 base pairs.

116

117   Our discovery that microinjection of genome editing reagents into oocytes generates

118   F0 lizards carrying bi-allelic mutations in the targeted gene, demonstrates that our

119   approach offers an efficient path for directly testing gene requirement in anoles. For

120   genes where loss of function mutations are homozygous lethal, or studies requiring

121   heterozygous animals, we expect that naturally occurring SNPs can be used to facilitate

122   targeting of one allele only.

123

124   The establishment of CRISPR-Cas9 editing in this inexpensive reptilian system will

125   finally permit mechanistic studies of gene function to be performed in reptiles. We

126   anticipate the gene-editing strategy we have developed in anoles can also be

127   successfully applied to many other squamate species.

128

129

Acknowledgments

137

Author contributions

139   A.M.R., J.D.L., and D.B.M conceived and designed the experiments. A.M.R., performed

140   the majority of the experiments with support from S.P., A.J.A. and R.E.B. Data

141   interpretation was carried by A.M.R., S.P., R.E.B., J.D.L., and D.B.M. The manuscript

142   was written by A.M.R., D.B.M., and J.D.L. with help from all other authors.

143

Competing interests

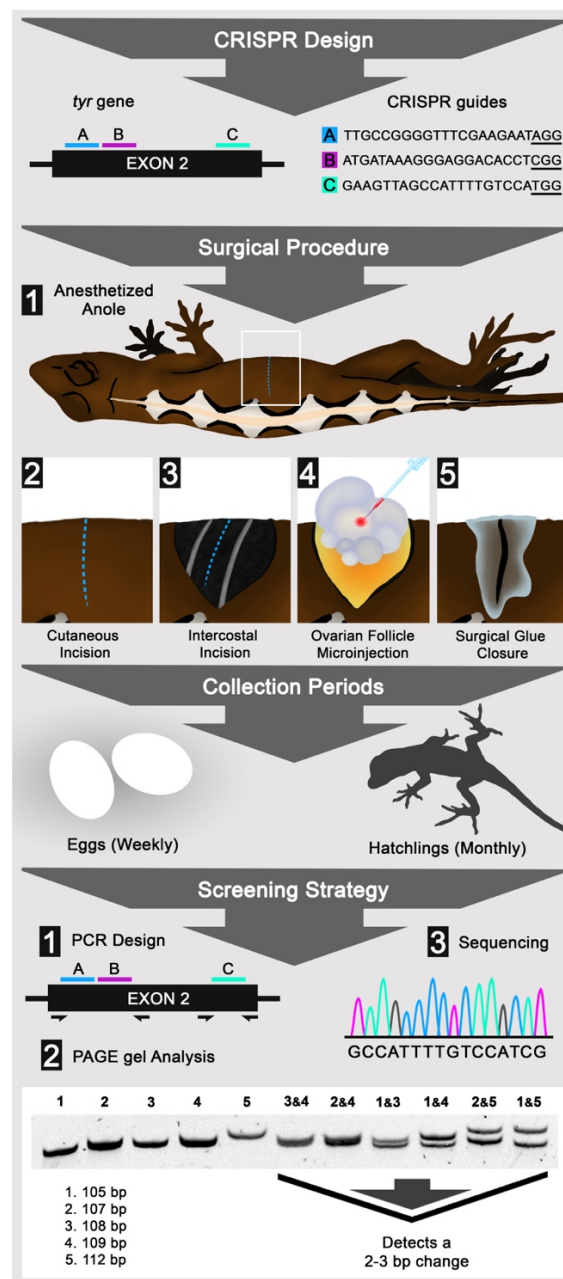145   The authors declare no competing interests.

Figure 1. Gene editing in lizards through microinjection of ovarian follicles. Flow diagram detailing CRISPR design, surgical procedure, collection periods, and screening strategy. CRISPR design shows the placement and sequence of CRISPR guides A (blue), B (pink), and C (cyan) within exon 2 of the *tyr* gene; PAM sites are underlined. The surgical procedure panel depicts lizard anesthesia and surgical steps to access and microinject ovary follicles. The collection periods panel highlights the time between gathering eggs and raising hatchlings. The screening strategy panel illustrates the steps used to detect *tyr*-crispants including: 1) PCR primer design, 2) PAGE analysis, which can reliably detect 2-3 bp changes, and 3) Sanger sequencing.
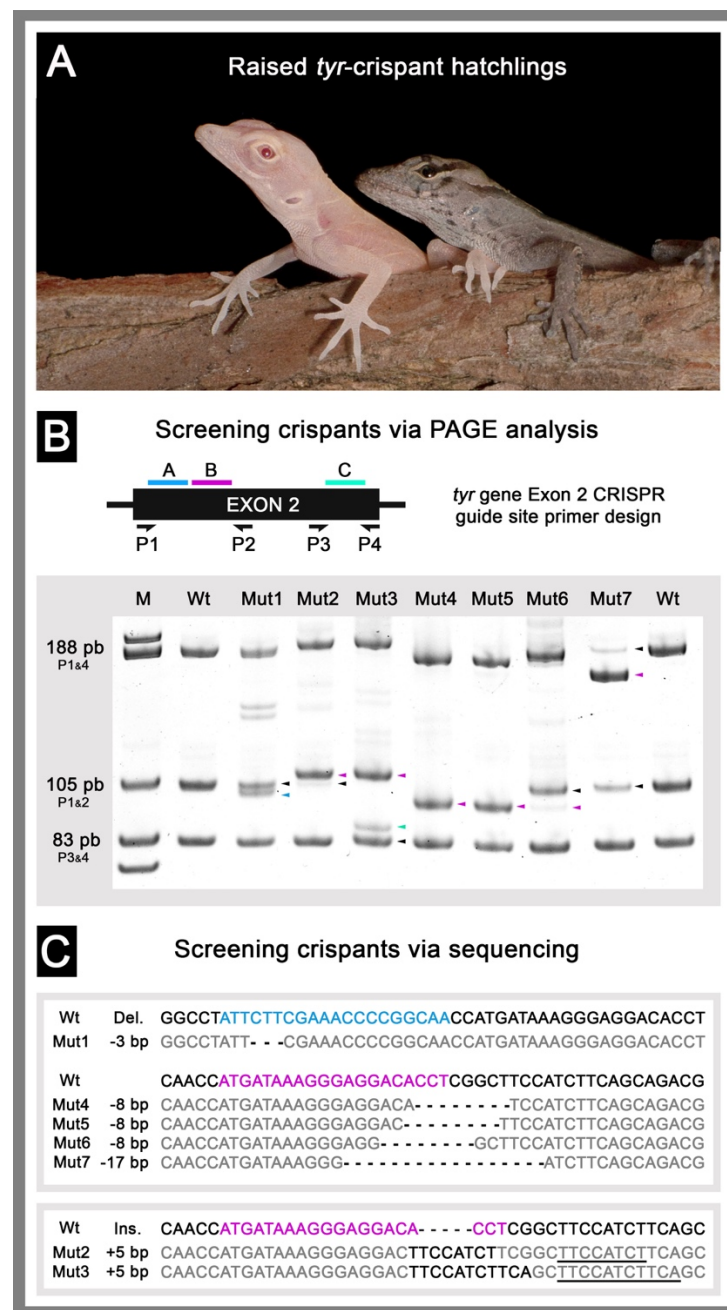
Figure 2. Detection and sequencing of *tyr*-crispants. (A) albino *tyr*-crispant (left) and wildtype (right) aged-matched hatchlings. (B) PCR primer placement (P1-P4) relative to CRISPR target sites A (blue), B (pink), and C (cyan). Representative PAGE results are shown for seven of the mutant lizards. Colored arrows denote bands with altered mobility relative to wild-type (WT). (C) Sequences of CRISPR-Cas9 induced indels from representative *tyr*-crispants. (Top) Mut1 and Mut4-7 sequences with deletions. (Below) Mut2 and 3 sequences with insertions. Targeted guide sites A (blue), B (pink), and C (cyan) are highlighted in wildtype reference sequences. *tyr*-crispants deletions/insertions are indicated in black and sequences matching wildtype in grey.
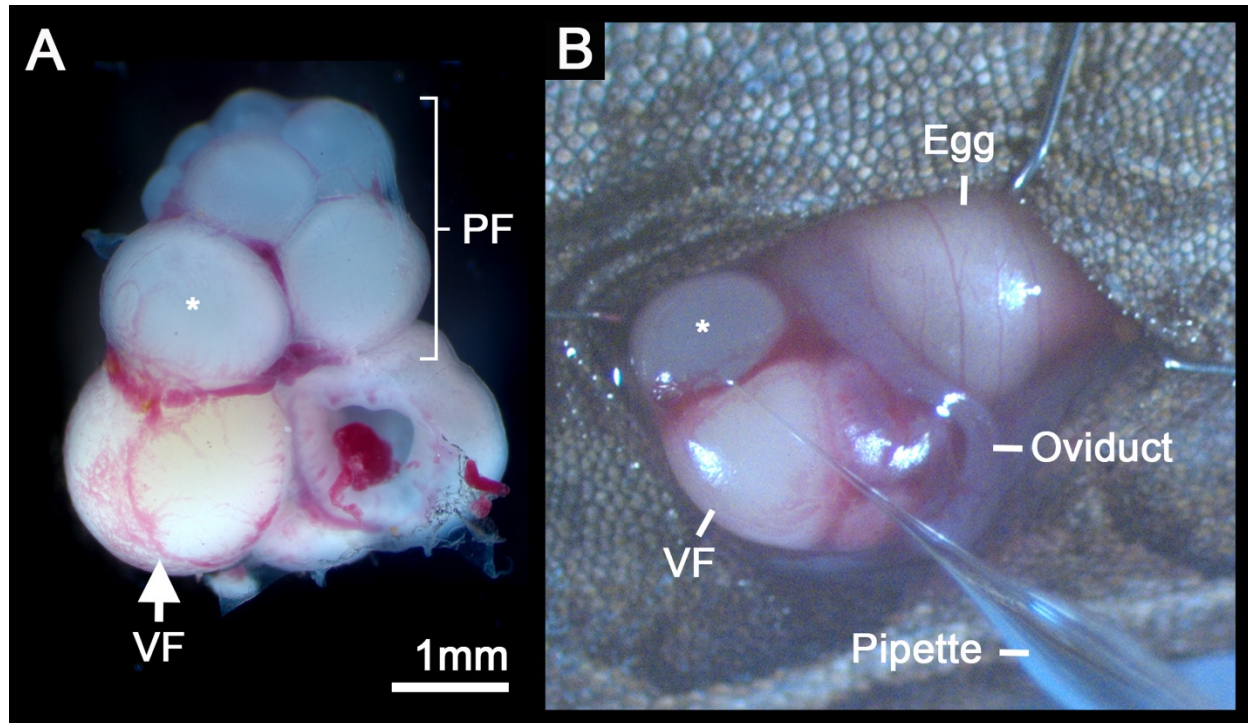
Figure S1. Anole lizard ovary. (A) Dissected ovary showing previtellogenic (PF) and vitellogenic (VF) follicles. (B) Same ovary prior to dissection showing microinjection of a 1.5 mm diameter follicle (asterisk in panels A and B).
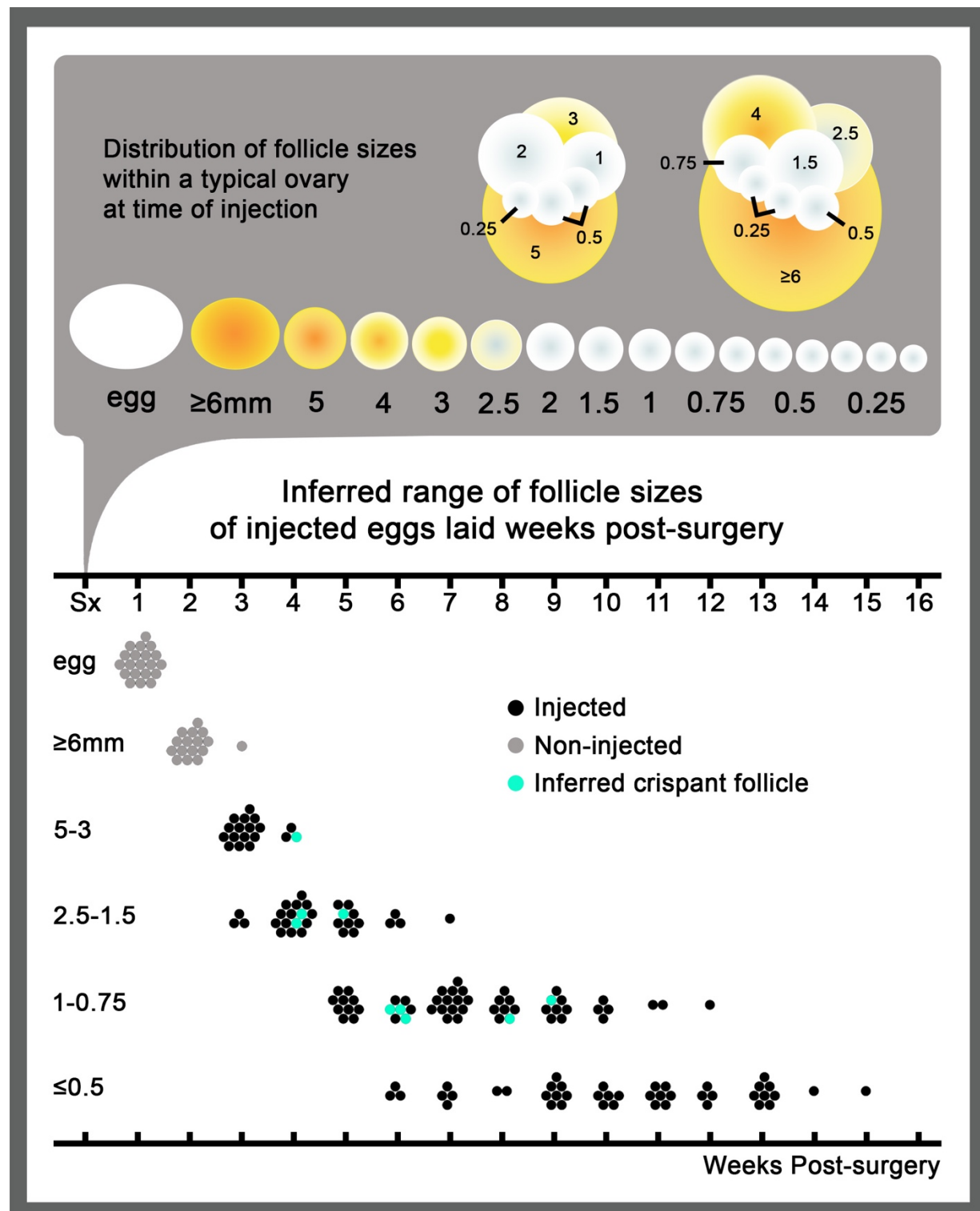
Figure S2. Inferred relationship between follicle size and crispants. (Top) the distribution of follicles sizes within ovaries at time of injection depicted as a "follicle train." (Below) a distribution graph showing inferred follicle sizes over weeks post-surgery. Non-injected eggs and large yolky follicles are shown in grey, while injected follicles are in black and follicles that likely produced crispant lizards are in cyan.

146 **Methods**

147 *Animals*

148 Animals used in this study were wild-caught *Anolis sagrei* from Orlando, FL. Lizards

149 were housed at University of Georgia following published guidelines[15]. Breeding cages

150 housed up to 4 females and 1 male together. Twenty-one adult females from cages that

151 consistently produced eggs were selected for this study. All experiments followed the

152 National Research Council's Guide for the Care and Use of Laboratory Animals and

153 were performed with the approval and oversight of the University of Georgia Institutional

154 Animal Care and Use Committee (A2016 09-008-Y2-A3).

155

156 *Selection of crRNA guide sequences and preparation of Cas9 RNP*

157 The CRISPOR target selection tool (version 4.4) was used to select target regions with

158 efficiency scores of 50% or greater within the second exon of the *A. sagrei tyr* gene[16].

159 The *tyr* gene reference sequence was obtained from a draft genome assembly of *Anolis*

160 *sagrei*. Alt-R CRISPR-Cas9 crRNAs, tracrRNA, and Cas9 V3 were purchased from

161 Integrated DNA Technologies, Inc. The crRNA target sequences were as follows:

162

163   *AsagTyrEx2A*: 5′-TTGCCGGGGTTTCGAAGAAT-3′

164   *AsagTyrEx2B*: 5′-ATGATAAAGGGAGGACACCT-3′

165   *AsagTyrEx2C*: 5′-GAAGTTAGCCATTTTGTCCA-3′.

166

167 Cas9 RNP was prepared by following manufacturer recommendations. A 5 µM injection

168 solution was made using standard microinjection solution (10 mM Tris-HCl, pH 7.4)

169 containing phenol red to help verify that injected solutions entered the oocytes.

170

171 *Anesthesia and analgesia*

172 Lizards were anesthetized by administering 30 mg/kg of Alfaxalone (Alfaxan, 10 mg/mL,

173 Jurox) in combination with 0.1 mg/kg of dexmedetomidine (Dexdomitor 5 mg/10 mL,

174 Zoetis/Orion). To ensure accurate dosing, these drugs were administered by

175 subcutaneous injection in the cervical area as an Alfaxalone/Dexmedetomidine (A/D)

176 mixture. Preoperative analgesia was obtained by subcutaneous injection of meloxicam

177   (0.3 mg/kg, Loxiject, 5 mg/mL, Henry Schein) in the dorsal epaxial area just above the

178   shoulder and topical application of lidocaine (2.0 mg/kg, Lidocaine HCL, 2%, Hospira)

179   over the surgical site. See video for injection sites. The anesthetic combination A/D

180   provided approximately 30 min of surgical anesthesia time. Lizards typically recovered

181   about 40-45 min following A/D administration. If a longer anesthesia time was required,

182   a second dose of 30 mg/kg of alfaxalone alone was administered 25-30 min post

183   induction dose, providing an additional 30 min of surgical anesthesia.

184   The method and location where injections were made were specifically chosen to

185   avoid some of the challenges with administering drugs to reptiles. One such issue to be

186   aware of is the hepatic-first pass effect which is a phenomenon found in many reptiles

187   where, drugs, if administered in hindlimb or caudal regions, are rapidly cleared by the

188   ventral abdominal and hepatic portal veins and metabolized by the liver, inhibiting wide

189   systemic circulation. We have found that administering A/D subcutaneously in the

190   dorsal epaxial area just above the shoulder region resulted in only moderate to light

191   levels of anesthesia. Contrary to this, lizards administered subcutaneously in cervical

192   area are rapidly induced and reach a surgical plane of anesthesia within 1 min. Because

193   injection volumes can be large, a subcutaneous method was also preferred over an

194   intramuscular approach. For these reasons, the cervical area and a subcutaneous route

195   were used in this study.

196   Another important factor that can influence drug metabolism in reptiles is body

197   temperature. Lizards are ectothermic and are dependent upon the environmental temps

198   to regulate their body warmth which in turn impacts their metabolic rate. A decrease in

199   body temperature will lead to a decrease in drug metabolism potentially resulting in a

200   persistence of circulating drugs. In such an instance, this can result in an animal

201   responding poorly, prolongment of anesthesia recovery time, or in some cases lead to

202   death. The converse is also true. Animals maintained at too high a temperature may

203   metabolize and clear drugs quickly resulting in insufficient anesthesia time. To avoid

204   this, anesthetized anoles should be maintained on a heating source at around 32˚C until

205   fully recovered.

206

207

208   *Surgery and microinjection*

209   After successful anesthesia induction, the lizard becomes non-responsive to any

210   noxious stimuli (i.e., an absence of response to a cloacal/tail clamp that normally

211   induces severe discomfort). The anesthetized lizard was placed into right lateral

212   recumbency and the left flank was aseptically prepared by alternating disinfection with

213   70% ethanol and 7.5% povidone-iodine (Surgical Scrub Solution, 16 fl. oz. 473 mL,

214   Dynarex) wipes for 5 minutes.

215   Following standard surgical practices, sterile iris scissors (FST, item 15023-10)

216   were used to make an 8-10 mm vertical cutaneous incision on the left side, in the mid-

217   coelom region. A second incision between the ribs was made through the musculature

218   (i.e., internal/external intercostal and pigmented coelom muscle layers) to enter the

219   coelom. The ovary can be found dorsally in the mid-coelom region and was easily

220   accessible by shifting intestines gently aside using blunt forceps (FST: 45˚ angled

221   forceps, item 00649-11; FST: strait forceps, item 00632-11). Once located, the ovary

222   was carefully rotated and repositioned to expose immature follicles ranging anywhere

223   from 0.25 mm to 5 mm in size.

224   Using the blunt forceps to clasp and hold the ovary in place, a microinjection

225   needle was visually guided into the follicle center at an angle between 35-45˚ degrees

226   relative to horizontal. 5 µM Cas9 RNP solution was then injected into follicles at differing

227   volumes ranging from as little as 15 nL to as much as 575 nL which was dependent

228   upon needle and follicle size. Retrospectively, ideal injection volumes were determined

229   (3≤5 mm, 300-500 nL; 2≤2.5 mm, 200-250 nL; 1≤1.5 mm, 100-150 nL; and 0.75 mm, 25

230   nL) based on surgical sessions that produced mutants. Large yolky follicles greater than

231   5 mm in diameter, and eggs already present in the oviduct, were not injected. Sterile

232   drops of P-Lytes solution (Veterinary Plasma-Lyte A Injection pH 7.4) were applied

233   directly on the ovary or in coeliotomy opening throughout the procedure to prevent

234   tissue dehydration.

235   After injection, the ovary was gently returned into the coelom and overlying

236   musculature and skin was lightly pulled together to close the cavity. Tissue adhesive

237   (3M Vetbond, #1469SB) was carefully applied only to only the external surface of the

238   skin, avoiding the underlying musculature. Once the tissue adhesive was dry, the lizard

239 was re-positioned into left lateral recumbency and the procedure was repeated for a
240 right coeliotomy.

241   During recovery, triple antibiotic ointment (Bacitracin Zinc, Neomycin Sulfate,
242 Polymyxin B Sulfate) was applied topically to the surgical wounds. Lizards were
243 monitored daily for 1 week for any signs of infection, pain, or inflammation. After
244 recovery from anesthesia, females were housed together with their previous female
245 mates and allowed to recover for 7 days prior to reintroducing the male.

246   All surgeries were performed using sterilized equipment and tools (i.e. forceps
247 and iris scissors) under a dissecting scope (Zeiss Stemi SV11) with a top light
248 (AmScope 80-LED illuminator). Body temperature was maintained throughout the
249 procedure by placing lizards on a heating platform (Fisher Scientific: model 77, serial #
250 802N0041CAT 12-594) with surgical towels draped between the heat source and the
251 lizards to provide a barrier. The contact surface temperature was held at 32˚C and
252 readings continuously taken using thermometer strips. Each laparotomy was performed
253 within 10-12 minutes. Follicle injections were carried out using a standard
254 zebrafish/xenopus microinjection rig (Harvard Apparatus PLI-100 Pico-Injector) set at 20
255 PSI with an injection time of 50-60 msecs. Initially, a manual micromanipulator
256 (Märzhäuser Wetzlar; MMJ-rechts: 00-42-107-0000) was used to perform steady needle
257 injections. However, use of this limited the degrees of freedom to inject the ovary from
258 multiple directions and angles, therefore, a simple hand-guided technique using no
259 micromanipulator was ultimately preferred and proved to be more efficient. Injection
260 needles with a gradual taper typically used for zebrafish microinjection were made
261 following the Sutter Instrument Company Pipette Cookbook guidelines using a
262 Flaming/Brown Micropipette Puller (Model P-97) and cut to have a 20 to 40 μm diameter
263 opening.

264

265 *Mutation screening*

266   Cages were monitored for a specified number of weeks following surgery which
267 was based on the highest number of follicles injected in an ovary (e.g., if 8 and 5
268 follicles were injected in the right and left ovaries of one lizard, respectively, a cage
269 housing this lizard would be monitored for n=16 weeks). Because these females often

270   had 1 or 2 eggs in the oviduct as well as 2 large (>5mm) un-injected large follicles,

271   cages were monitored for an additional 3-4 weeks following surgery.

272   Embryos and hatchlings from surgery cages were screened via PCR PAGE

273   analysis under conditions that reliably detect a 2-3bp change[17]. DNA was extracted from

274   tail clips from hatchlings or from tissue collected from embryos following standard

275   protocols. PCR was performed using the following primers: P1, 5-

276   CAAGAACTTTGCAATGGAACAAATG-3'; P2, 5'-GAATTCAACGTCTGCTGAAGATG-

277   3'; P3, 5'-TGTTTAAGTCTGACTCAGTACGAAG-3'; P4, 5'-

278   GGATTACCTTCCAAAGTATTCCTG-3'. See Figure 2 for primer location relative to

279   targeting sites. Sanger sequencing was performed on PCR products to fully

280   characterize mutations.

281

282   *Follicle train assignment*

283   Follicles were ranked by size and by alternating left and right ovarian

284   contributions to infer a probable timeline of egg lay for a given follicle size at time of

285   injection. This method of follicle train assignment assumes 1) eggs present in the

286   oviduct will be laid within one week, 2) follicles greater than 8-10 mm in 2 weeks, and 3)

287   follicles less than or equal to 5mm in diameter will be laid no sooner than 3 weeks. Our

288   reason for including these 3 underlying assumptions derives from the observation that

289   females who had 1-2 eggs present in their oviduct, also had a follicle greater than 8-10

290   mm in diameter followed by a follicle between 3-5 mm in each ovary, suggesting at least

291   a week interval between these sizes. As each lizard possesses a "leading" ovary and

292   "lagging" ovary in follicle sizes, the leading ovary is given preferential ordering in train

293   position. It is important to note that this method of ordering does not account for any

294   potential loss of follicles accidently destroyed in the microinjection process and

295   assumes that if such an event occurred, the follicle developmental timeline of that ovary

296   is unaffected.

297

298   *Data availability*

299   The data that support the findings of this study are available from the corresponding

300   author upon request.

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