

**Comparison of DIPA-CRISPR and SYNCAS gene editing methods in
the predatory bug *Orius strigicollis* (Hemiptera: Anthocoridae)**

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Abstract

CRISPR/Cas9-mediated gene editing in insects conventionally relies on embryonic microinjection, which is challenging for non-model species. Therefore, adult injection-based methods have been developed to overcome this obstacle. One such simple and accessible method—direct parental CRISPR (DIPA-CRISPR)—enables gene editing by injecting the Cas9 ribonucleoprotein (RNP) into adult females. Subsequently, a modified approach called SYNCAS was developed by supplementing Cas9 RNPs with branched amphipathic peptide capsules and saponins as delivery enhancers. SYNCAS has been reported to yield higher gene editing efficiency (GEF) than DIPA-CRISPR in the spider mite. However, a direct experimental comparison of these methods under consistent conditions has not been conducted in insects. In the present study, we compared DIPA-CRISPR and SYNCAS methods in the predatory flower bug *Orius strigicollis* (Poppius) (Hemiptera: Anthocoridae), an important biocontrol agent. While peak GEF values were comparably high for both methods, SYNCAS-treated females continued to lay eggs with higher editing efficiency over a longer period than DIPA-CRISPR-treated females. It resulted in up to 4.8-fold higher yield of gene-edited progeny in the former group than in the latter group. These findings suggested that SYNCAS offers greater flexibility in terms of overall experimental design and gene editing outcomes than DIPA-CRISPR.

Keywords: biocontrol agent, *cinnabar*, CRISPR/Cas9, flower bug, integrated pest management

Introduction

Recent advances in CRISPR/Cas9 technology have enabled highly efficient and broadly applicable targeted gene editing in insects and other arthropods (Doudna and Charpentier 2014; Gantz and Akbari 2018; Terradas et al. 2023). Conventional gene editing methods typically involve Cas9 protein and single guide RNA (sgRNA) injections into early embryos. However, embryo injection is challenging in certain insect species due to several limitations, such as injection lethality, hard eggshells or oothecae, or viviparity. To circumvent these obstacles, adult injection-based gene editing methods have been developed.

The first such method, termed Receptor-Mediated Ovary Transduction of Cargo (ReMOT), enhances Cas9 ribonucleoprotein (RNP) transduction in oocytes by fusing Cas9 with a yolk protein-derived peptide ligand. This approach enables gene editing in various insects and ticks (Chaverra-Rodriguez et al. 2018; Chaverra-Rodriguez et al. 2020; Sharma et al. 2022; Shirai and Daimon 2020). However, ReMOT is a time-consuming method as it involves recombinant Cas9 protein production and ligand designing for target species.

In contrast, the direct parental CRISPR (DIPA-CRISPR) method employs commercially available Cas9 protein and sgRNA, offering a simpler and more accessible approach. This method has been successfully used for gene editing in various insect orders, including Blattodea (Shirai et al. 2022), Coleoptera (Shirai et al. 2022), Diptera (Shirai et al. 2023), Hemiptera (Matsuda et al. 2024; Zhang et al. 2024), and Hymenoptera (Zhang et al. 2024).

Building on DIPA-CRISPR, De Rouck et al. (2024) recently developed an enhanced gene editing method termed SYNCAS. This method incorporates two

additional reagents in the Cas9 RNP solution—branched amphipathic peptide capsules (BAPCs) as transfection reagents and saponins as endosomal escape reagents. In the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), SYNCAS yielded a 64-fold increase in gene editing frequency compared to DIPA-CRISPR (De Rouck et al. 2024). Notably, this increase in efficiency exceeded the individual enhancement exhibited by BAPC (12-fold) and saponins (3.2-fold), suggesting a synergistic action of the two reagents in SYNCAS. SYNCAS-mediated gene editing has since been employed in several other insects, including the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (De Rouck et al. 2024), the silverleaf whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Mocchetti et al. 2025), the southern green stinkbug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae) (Mocchetti et al. 2025), and the jewel wasp, *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (Guerra et al. 2025).

However, these studies lacked direct comparisons of gene editing efficiency between DIPA-CRISPR and SYNCAS under consistent conditions, making it difficult to directly compare the performance of the two methods. For instance, both methods were employed in *N. vitripennis*; however, they were evaluated in separate studies using different reagent concentrations (Guerra et al. 2025; Zhang et al. 2024). Comparative studies conducted under consistent experimental conditions are warranted to assess the broader applicability of SYNCAS.

In the present study, we used the predatory flower bug *Orius strigicollis* (Poppius) (Hemiptera: Anthocoridae), a biocontrol agent, as the insect model to evaluate the frequency of SYNCAS-mediated gene editing. As conventional embryo injection is not feasible in this insect species due to its oviposition behavior, we previously

established a DIPA-CRISPR protocol as a practical alternative (Matsuda et al. 2024). However, DIPA-CRISPR requires precise staging of adult females and frequent handling of plants to recover newly laid eggs, limiting its overall feasibility. To explore whether SYNCAS can improve on these aspects, we directly compared gene editing efficiency and edited progeny yield of DIPA-CRISPR and SYNCAS methods under consistent experimental conditions. Although both methods achieved comparable peak gene editing efficiency, SYNCAS prolonged the effective oviposition window, resulting in a substantially higher number of edited offspring than that obtained from DIPA-CRISPR. These findings demonstrated that SYNCAS improves the efficiency and feasibility of gene editing in *O. strigicollis*, thus providing a foundation for advanced genome editing in this species.

Materials and methods

Insects

A colony of *O. strigicollis* was purchased from Agrisect (Ibaraki, Japan) and reared in a plastic mass-rearing container (150 × 103 mm, 47 mm deep) under a 14:10 h light:dark cycle at 24°C ± 1°C. Pieces of a Post-it note (Cat#658R, 3M, Saint Paul, USA) were used to carry the eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), purchased from Agrisect, that served as the food source for *O. strigicollis*. The twigs of the Mexican stonecrop, *Sedum mexicanum* Britton (Rosales: Crassulaceae) served as an oviposition substrate and a moisture/water source for the predatory bug. Newly emerged adults were collected daily and transferred to new containers for staging.

Cas9-sgRNA RNP formulation

We targeted the *cinnabar* gene, which encodes kynurenine 3-monooxygenase, which is involved in ommochrome biosynthesis (Lorenzen et al. 2002; Quan et al. 2002). The two sgRNAs (sgRNA #2 and #3) targeting *O. strigicollis cinnabar* used in this study were designed previously (Matsuda et al. 2024) and synthesized by Integrated DNA Technologies (IDT, Coralville, USA). The target sequences of sgRNAs were 5'-CGAATGATACACCCGGTATA+CGG-3' for sgRNA #2 and 5'-GACGAGCAAATAAAAAACCA+CGG-3' for sgRNA #3. Alt-R Cas9 Nuclease V3 (Cat#1081058, IDT) and the two sgRNAs were mixed at an approximate molar ratio of 1:2 and incubated for 10 min at room temperature to facilitate Cas9 RNP formation. After incubation, BAPC (BAPtofect-25, Phoreus Biotech, Olathe, USA) and saponins (Cat#558255, Merck Millipore, Tullagreen, Ireland) were added to the mixture (Fig. 1A), and the injection mix was incubated for 30 min on ice (De Rouck et al. 2024). To further evaluate the effects of BAPC and saponins and the synergism between them, we also mixed the Cas9-RNP mix with either BAPC or saponins, obtaining “DIPA-CRISPR + BAPC” or “DIPA-CRISPR + saponins” mixture, respectively, for injection experiments. The final concentrations of the compounds in the injection mix are shown in Fig. 1A. The concentration ratios of BAPC/Cas9 (= 5/75) and saponins/Cas9 (= 7.6/75) were adopted from a previous study employing SYNCAS in other arthropod species (De Rouck et al. 2024).

Adult injections

Adult females were carefully staged based on days after emergence (AE), with day 0 AE defined as the day of adult emergence. The females that were subjected to DIPA-

CRISPR and SYNCAS experiments were administered with the injection mix on days 1, 2, or 8 AE. However, those being subjected to DIPA-CRISPR + BAPC and DIPA-CRISPR + saponins experiments were administered only on day 2 AE, because gene editing efficiencies were most remarkably different between DIPA-CRISPR and SYNCAS on this injection day. At each injection session, approximately 300 nL of the respective injection mix was administered to 20 or more females (Fig. 1B, C, refer to Table S1 for detailed numbers of injected females), as described previously (Matsuda et al. 2024). The injected females were pooled and transferred to the mass-rearing container along with their sibling males. Stonecrop twigs, used as oviposition substrates, were collected and replaced daily until 4 days post-injection to investigate the relationship between oviposition timing and gene editing efficiency. Twigs with laid eggs were individually stored in plastic vials for fruit flies (25 mm in diameter and 90 mm deep; Cat#AS519, Fisher Scientific, Waltham, USA) containing a Post-it note (Cat#715RP-K, 3M) carrying *E. kuehniella* eggs. Two or three independent experiments were conducted for each injection time point. Some DIPA-CRISPR experimental data were adopted from a previous study conducted under the same experimental conditions (Table S1) (Matsuda et al. 2024).

Genotyping

Genomic DNA was extracted individually as described previously (Matsuda et al. 2024). Genomic PCR was performed using KOD FX Neo (Cat#KFX-201, TOYOBO, Osaka, Japan). The mutations were screened by analyzing the PCR products through a heteroduplex mobility assay (HMA) using the MultiNA Microchip Electrophoresis System (MCE-202, Shimadzu, Kyoto, Japan). In the HMA, heteroduplexes were

identified as bands with reduced mobility (Delwart et al. 1993). The Primers for the HMA of *O. strigicollis cinnabar* were designed previously to amplify the 156 bp fragment containing sgRNA target sites 5'- TCCATAAATCTCGCGCTCTC-3' and 5'-TTTGTAACGGGATCGTATGG-3') (Matsuda et al. 2024). Only bands within the 156–242 bp range were considered to be heteroduplexes owing to the presence of nonspecific PCR products in some individuals. The efficiency of G₀ gene editing was calculated using two measures: (i) gene editing efficiency (GEF; the proportion of gene-edited G₀ individuals out of the total G₀ individuals), and (ii) effort efficiency (EEF; the proportion of gene-edited G₀ individuals out of the total number of injected females).

Results

Comparison of GEF

We had previously demonstrated that GEF of the DIPA-CRISPR method depends on the timing of adult injection and the injection dose of Cas9 RNPs (Matsuda et al. 2024; Shirai et al. 2022). In the present study, to compare the GEFs of DIPA-CRISPR and SYNCAS methods in *O. strigicollis*, we injected adult females on days 1, 2, or 8 AE with either Cas9 RNPs alone or Cas9 RNPs in combination with BAPC and saponins. From the 311 injected females, we obtained 3,954 G₀ progeny, all of which were individually genotyped using HMA (Fig. 2A, refer to Table S1 for detailed results).

To evaluate the performance of the two methods, we calculated the GEF for each day of injection and oviposition as a measure of efficiency (Fig. 2B). The day on which GEF peaked varied depending on the injection timing. The GEF peaked on the first day of oviposition in females injected on day 1 AE and then declined, whereas it peaked on the second or third day of oviposition in females injected on day 2 or 8 AE.

On each injection day, the peak GEF value was higher for SYNCAS than for DIPA-CRISPR (52.8% vs. 36.8% on day 1 AE; 45.1% vs. 29.5% on day 2 AE; 36.3% vs. 23.3% on day 8 AE), although the difference was statistically insignificant (t -test, $P > 0.05$). Notably, SYNCAS maintained relatively high GEF values even after the peak period, particularly in females injected on day 2 AE. These results indicated that SYNCAS not only increases the peak GEF values but also extends the time window to collect gene-edited G_0 progeny following injection.

De Rouck et al. (2024) demonstrated that BAPC and saponins synergistically increase the GEF in the spider mite subjected to SYNCAS. To evaluate the effects of BAPC and saponins and the potential synergism between them in *O. strigicollis*, we additionally prepared DIPA-CRISPR + BAPC and DIPA-CRISPR + saponins injections and administered them on day 2 AE (Fig. 3, refer to Table S1 for detailed results). From the 116 injected females, we obtained 1,384 G_0 progeny (Table S1), all of which were individually genotyped using HMA. In both experimental groups, the GEF peaked on the second day of oviposition. The highest GEFs observed in DIPA-CRISPR + BAPC and DIPA-CRISPR + saponins experiments exceeded those of DIPA-CRISPR experiments, indicating that BAPC and saponins increased the GEF when added individually to Cas9 RNP. Notably, the peak GEF observed in DIPA-CRISPR + saponins (40.7–52.8%) was higher than that of SYNCAS experiments (29.1–45.1%), although the difference was statistically insignificant (t -test, $P = 0.44$). This finding indicated an absence of synergistic effects between BAPC and saponins in terms of peak GEF. However, the synergistic effects between BAPC and saponins was observed on day 6 AE: the GEFs in DIPA-CRISPR + saponins experiments (1.3–6.6%) were comparable to those in DIPA-CRISPR experiments (1.7–3.5%) and much lower than

those in SYNCAS experiments (12.3–17.9%). These results indicated that SYNCAS provides the widest time window for collecting gene-edited G₀ progeny.

Comparison of EEF

As an additional measure of the gene editing outcomes, we calculated the EEF values for each injection day (Fig. 4). Injections on day 1 AE in SYNCAS experiments resulted in EEF values of up to 363%, indicating that 363 gene-edited progeny could be expected per 100 injected females. Similarly, injections on days 2 and 8 AE yielded EEF values of up to 551.5% and 203.7%, respectively. These values represent 4.8-, 4.2-, and 1.3-fold increases, respectively, compared to the corresponding values in DIPA-CRISPR experiments. These findings suggested that SYNCAS is superior to DIPA-CRISPR in terms of EEF, enabling the recovery of more gene-edited G₀ progeny from fewer injected adults.

DIPA-CRISPR + BAPC and DIPA-CRISPR + saponins injections on day 2 AE did not markedly increase the EEF values, leading to only 1.5- and 2.2-fold increases, respectively, compared to DIPA-CRISPR injections (Fig. 4). These EEF increases were substantially smaller than those achieved in the SYNCAS experiments on the same injection day. These results indicated that while BAPC or saponins alone can improve the EEF, their combination leads to even greater improvements.

Collectively, our findings suggested that SYNCAS provides greater flexibility in the experimental design and superior gene editing performance in *O. strigicollis*.

Discussion

In this study, we established and evaluated a SYNCAS-based gene editing method in the

predatory bug *O. strigicollis*, a key biocontrol agent. While SYNCAS was originally developed in the spider mite *T. urticae* (De Rouck et al. 2024), it has since been applied to other arthropods, including thrips, whiteflies, stinkbugs, and parasitic wasps (De Rouck et al. 2024; Guerra et al. 2025; Mocchetti et al. 2025). In the present study, we quantitatively assessed the individual and combined contributions of BAPC and saponins within the same experimental framework, providing important insights into the further refinement of adult injection-based gene editing in insects.

The central concept of SYNCAS lies in the synergistic action of BAPC and saponins. BAPC is considered to enhance the cellular uptake of Cas9 RNPs via endocytosis, whereas saponins promote their endosomal escape (De Rouck et al. 2024). In the current study, SYNCAS injection led to a higher peak GEF and a longer peak GEF duration compared to DIPA-CRISPR. While our findings demonstrated a synergistic improvement in the EEF, the precise molecular mechanisms underlying this synergy between BAPC and saponins remain unclear.

The SYNCAS method improved the GEF by approximately twofold compared to the DIPA-CRISPR method in *O. strigicollis*; however, this enhancement was relatively milder compared to the dramatic increases reported in other species. For instance, in *T. urticae*, SYNCAS led to a 64-fold higher GEF (3.24% vs. 0.05%) (De Rouck et al. 2024). In *N. vitripennis*, a 34-fold improvement (2.73% vs. 0.08%) was observed using the SYNCAS method (Guerra et al. 2025; Zhang et al. 2024). These interspecies differences might partially be attributed to baseline DIPA-CRISPR efficiencies. *O. strigicollis* already exhibits a very high GEF (29.5%) with the DIPA-CRISPR method without the additional reagents (Matsuda et al. 2024), potentially limiting the room for further enhancement. This finding suggested that SYNCAS might

be particularly valuable in species where DIPA-CRISPR is ineffective or poorly optimized, such as *T. urticae*, *N. vitripennis*, or *Drosophila melanogaster* (Diptera: Drosophilidae) (Shirai et al. 2022). Investigating such challenging models might help elucidate the factors that limit the effectiveness of DIPA-CRISPR and SYNCAS.

From a practical perspective, SYNCAS substantially enhances the flexibility of gene editing workflows in *O. strigicollis*. Although DIPA-CRISPR has already been optimized for this insect species (Matsuda et al. 2024), it requires precise synchronization with adult emergence and egg laying. In contrast, SYNCAS allows a broader range of adult injection and sampling time points than DIPA-CRISPR. Notably, when SYNCAS injections were administered 1–2 days AE and eggs were collected 1–3 days post-injection, the mean GEF value remained at 23.4%. This GEF value was comparable to the optimal GEF value of 25.6% obtained under tightly regulated DIPA-CRISPR conditions (Matsuda et al. 2024). However, as high saponin concentrations have been shown to negatively affect the survival and oviposition in some insect species (Guerra et al. 2025; Heu et al. 2020), careful preliminary evaluation will likely be required when applying SYNCAS to new species. Additionally, the optimal formulation of SYNCAS reagents might vary depending on the insect species.

Besides, the robustness of SYNCAS might prove advantageous in gene editing applications beyond insects. A recent study has extended adult injection-based gene editing to tardigrades, non-arthropod ecdysozoans (Kondo et al. 2024). Given its high efficiency and experimental flexibility, SYNCAS exhibits strong potential to facilitate basic research, such as gene function analysis, and applied research, such as genetic improvement of agriculturally beneficial animals.

Supplementary Information

The online version contains supplementary material available.

Author contributions

NH and TD conceived the study. NM and TD designed experiments. NM performed experiments and analyzed the data. NM wrote the original draft of the manuscript, and TD commented on the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

Data are available upon request by contact with the corresponding author.

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Figures

A	DIPA-CRISPR	DIPA-CRISPR + BAPC	DIPA-CRISPR + saponins	SYNCAS
Cas9	3.3 µg/µL	3.3 µg/µL	3.3 µg/µL	3.3 µg/µL
sgRNA #2	0.63 µg/µL	0.63 µg/µL	0.63 µg/µL	0.63 µg/µL
sgRNA #3	0.63 µg/µL	0.63 µg/µL	0.63 µg/µL	0.63 µg/µL
BAPC	–	0.22 µg/µL	–	0.22 µg/µL
saponins	–	–	0.34 µg/µL	0.34 µg/µL

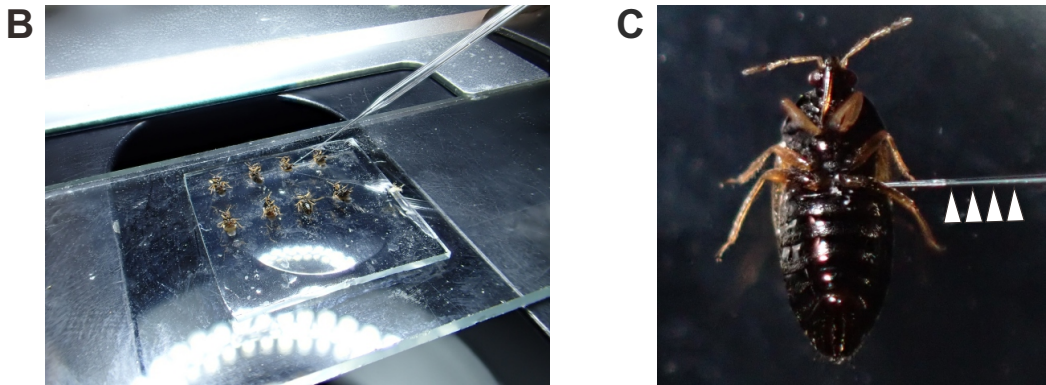


Fig. 1 Adult injection in *Orius strigicollis*. (A) Injection mix compositions. (B) Adult female *O. strigicollis* being injected. (C) Magnified image of the insect being injected. A glass capillary needle (white arrowheads) was inserted into the thoracic–abdominal intersegmental membrane.

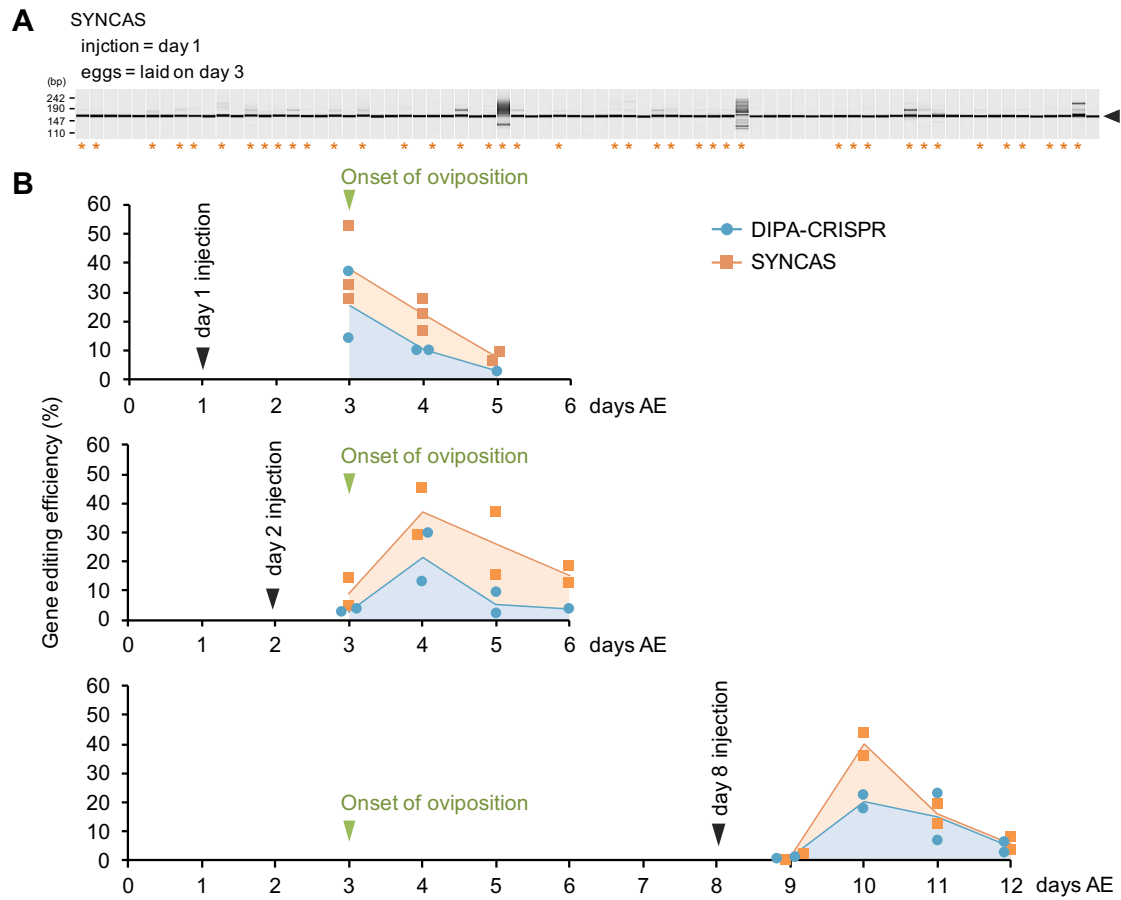


Fig. 2 Genotyping of G_0 individuals. (A) Representative results of the heteroduplex mobility assay. The arrowhead indicates the band from the wild-type allele, whereas the extra bands above it represent the heteroduplexes. Asterisks indicate G_0 individuals with edited alleles. (B) Relationship between gene editing efficiency and timings of injection and oviposition. Blue circles (DIPA-CRISPR) and orange squares (SYNCAS) indicate the results of independent experiments consisting of 19–180 G_0 hatchlings (refer to Table S1 for detailed results). Lines represent the means of pooled results. Note that *O. strigicollis* females began oviposition on day 3 after emergence (AE).

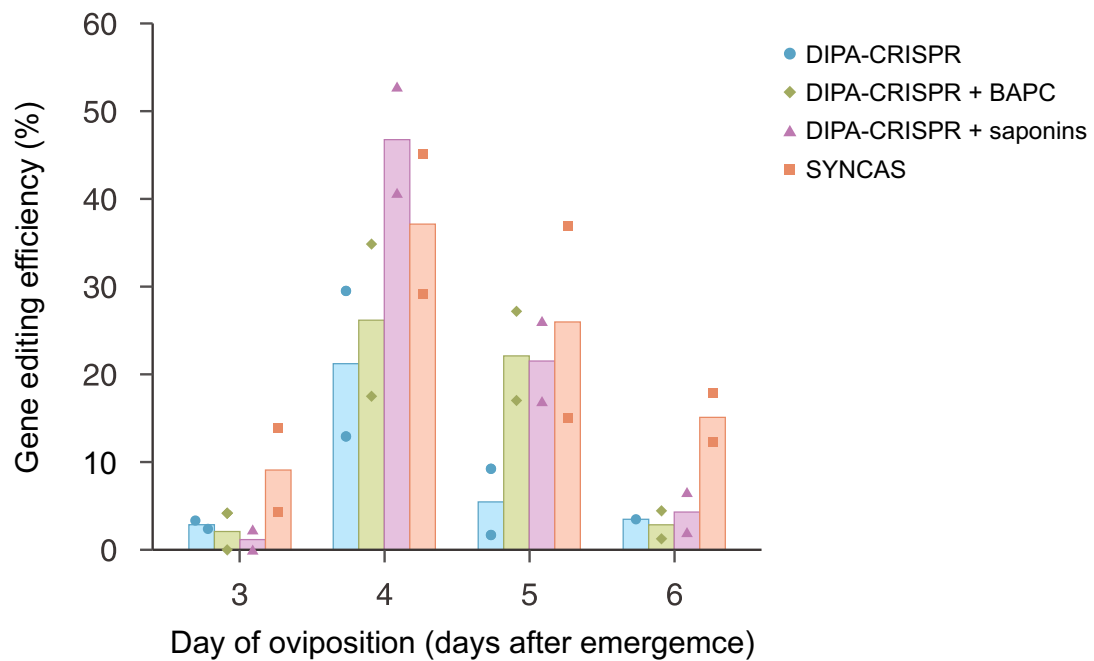


Fig. 3 Comparisons of gene editing efficiency in females injected on day 2 after emergence. Circles (DIPA-CRISPR), diamonds (DIPA-CRISPR + BAPC), triangles (DIPA-CRISPR + saponins), and squares (SYNCAS) indicate the results of independent experiments consisting of 24–180 G₀ hatchlings (refer to Table S1 for detailed results). Bars represent the means of two or three experiments.

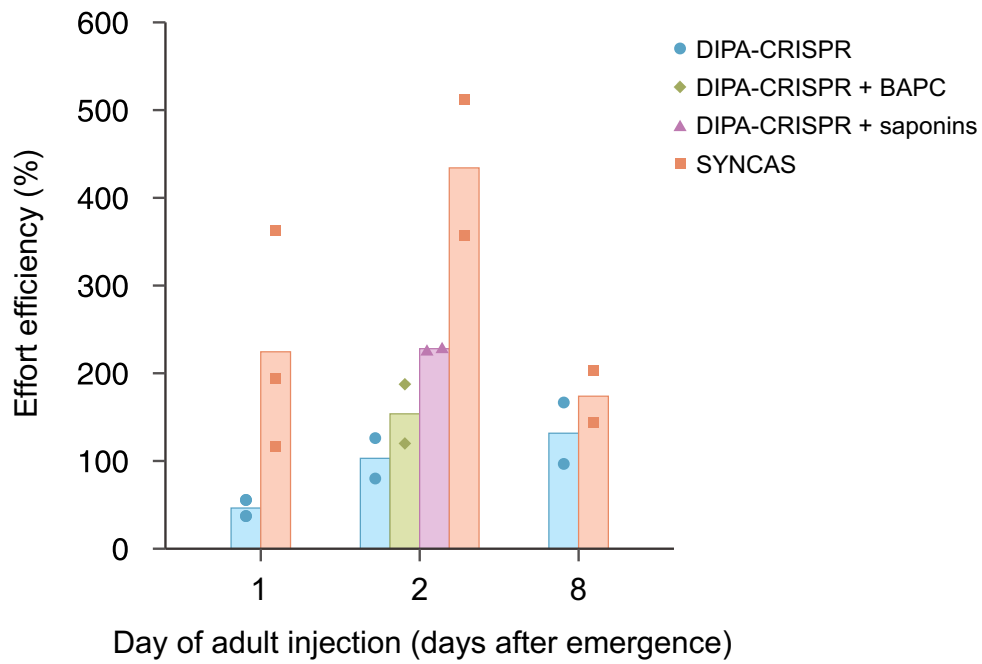


Fig. 4 Comparisons of effort efficiency. Circles (DIPA-CRISPR), diamonds (DIPA-CRISPR + BAPC), triangles (DIPA-CRISPR + saponins), and squares (SYNCAS) indicate the results of independent experiments consisting of 20–61 injected females (refer to Table S1 for detailed results). Bars represent the means of two or three experiments.