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Non-lethal genotyping in *Drosophila suzukii*, *Zeugodacus cucurbitae*, *Bactrocera dorsalis*, and *Aedes aegypti* for functional genomics and genetic control

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With 4 figures

Abstract: Non-lethal genotyping has advanced ecological and genetic research by enabling molecular analyses without sacrificing specimen viability. Concurrently, genome-editing tools such as the CRISPR/Cas9 system have spurred innovations in pest genetic control. A rapid, efficient, non-invasive genotyping method is therefore essential for functional genomics and genetic control studies. We evaluated tissue-specific genotyping in three economically important fruit flies – *Drosophila suzukii, Zeugodacus cucurbitae*, and *Bactrocera dorsalis* – and the public-health pest *Aedes aegypti*. PCR amplicon yield and quality were compared across up to three tissue types; genotyping accuracy was confirmed by PCR and Sanger sequencing; and effects on survival and reproduction were recorded. Pupal shells provided sufficient DNA for PCR and sequencing without any impact on insect survival or fecundity, whereas other tissues showed trade-offs between genotyping reliability and fitness. Based on these results, we propose optimized non-lethal genotyping protocols that minimize fitness costs, facilitating strain development for pest management and CRISPR-based research.

Keywords: genetic screening; CRISPR/Cas9; genetic control; insect pests; vector surveillance; tissue sampling; fecundity; ecological monitoring; functional genomics

1 Introduction

Non-lethal DNA sampling is fundamental to modern biology, underpinning species identification, population genetics, and functional genomics (Hebert et al. 2003). In entomology, extracting sufficient DNA without sacrificing individuals is increasingly critical, particularly in studies requiring live insects for breeding, behavioral assays, or longitudinal monitoring (Ali et al. 2019). Traditional protocols for small Diptera often entail whole-body extractions, precluding further use of valuable specimens. Non-lethal approaches that preserve survival and fecundity are therefore preferred (Carvalho et al. 2009).

Genome-editing breakthroughs, most notably CRISPR/Cas9, have transformed insect functional genomics by enabling precise, targeted modifications across both model and non-model taxa (Jinek et al. 2012). In *Drosophila melanogaster*, versatile tools such as Flp/*FRT* and Cre/*LoxP* recombinases, zinc-finger nucleases, transposable elements, and CRISPR/Cas9 systems facilitate complex spatiotemporal control of gene expression (Gratz et al. 2013). A persistent bottleneck is rapid, accurate genotyping of edited or transgenic individuals, especially at early developmental stages, where molecular-marker-based screening accelerates workflows and reduces dependence on labor-intensive phenotypic selection or multi-generational crosses (Kane et al. 2021).

Non-lethal genotyping also underpins applied genetic-control strategies against invasive and disease-vectoring insects (Ali et al. 2019; Yan & Schetelig 2024). Approaches

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such as Release of Insects carrying a Dominant Lethal gene (RIDL), precision-guided sterile insect technique (pgSIT), and CRISPR-based gene drives rely on releasing engineered cohorts into wild populations to suppress or modify target species (Yan et al. 2025). Following microinjection of genetic constructs into wild-type strains, G_0 adults are backcrossed to wild-type; non-invasive genotyping of G_1 progeny is then essential to identify those carrying desired alleles for further breeding. Although some insects possess phenotypic markers (e.g., eye color or fluorescent reporters) (Shen et al. 2015), many pest species lack robust, optimized screening systems. CRISPR-engineered genetic sexing strains, involving heat-inducible, temperature-sensitive mutations, similarly demand non-lethal genotyping to select viable, positively engineered individuals (Yan et al. 2021).

Vertebrate studies exploit feces, hair, saliva, and other shed materials for non-invasive sampling (Wheat et al. 2016; Bach et al. 2022), but insects' small size, rigid cuticle, and rapid metabolic turnover impede adoption of standard micro sampling methods. Recent proofs of concept include tarsal clipping in honeybees (Bubnič et al. 2020), frass-based DNA recovery in *Drosophila* (Ali et al. 2019), butterfly wing-tip sampling (Lushai et al. 2000), pupal cases from *Culex Pipiens* and *B. dorsalis* (Ashok et al. 2023; Jones et al. 2024). However, these methods lack broad, high-throughput validation, and further refinement is needed to maximize DNA yield and integrate rapid, field-deployable workflows.

Here, we systematically evaluate non-lethal genotyping protocols in four key pest species – *D. suzukii, Z. cucurbitae, B. dorsalis*, and *Ae. aegypti*. We compare DNA yield and PCR/sequencing success from up to seven tissue types, and assess impacts on survival and fecundity. By defining tissue-specific trade-offs between genotyping efficiency and organismal fitness, we present optimized protocols to support genetic screening and control programmes in these economically and medically important insects.

2 Material and methods

2.1 Insect rearing and maintenance

Zeugodacus cucurbitae adults (collected in Haikou City, Hainan Province, China, 2016) were reared at 27 ± 0.5 °C, 70 ± 5% RH, 14:10 h L:D. Larvae were reared on a semi-artificial pumpkin–cornmeal diet, and adults were provided with a 3:1 mixture of sugar to hydrolyzed yeast. Rearing procedures followed the protocol of Wei et al. (2020).

Bactrocera dorsalis populations (field-collected in Haikou, Hainan Province, China, 2008) were reared at 27± 0.5 °C, 70 ± 5% RH, 14:10 h L:D. Larvae were reared on a cornmeal-based medium, while adults were provided with an artificial diet consisting of yeast powder, honey, sugar, vitamin C and water. Rearing procedures followed the protocol of Shen et al. (2013).

The wild-type (WT) USA strain and transgenic *D. suzukii* were reared at 25 ± 0.5 °C, 55 ± 5% RH, 14:10 h L:D. Larvae were housed individually in 9 cm × 2.5 cm vials with food consisting of a cornmeal–yeast–agar diet composed of cornmeal, yeast, agar, sugar, methylparaben and propionic acid (Yan & Schetelig 2024).

Aedes aegypti Higgs White Eye (HWE) strain, a spontaneous white-eye mutant of the Rexville D strain from Puerto Rico, was reared at 27 °C, 70% RH, 12:12 h L:D. Larvae were reared in deionized water and fed with Tetra TabiMin (Tetra) fish food pellets ad libitum; water was exchanged as needed; adults received 10% (w/v) sucrose via cotton pads; females were blood-fed weekly on fresh porcine blood. Eggs were collected 3 days post blood feeding by putting a filter paper-lined cup with about 1 cm deionized water into the cages over night to allow oviposition. Eggs were then stored for another 48 h under humid conditions to allow completion of embryogenesis, before drying for 40 min at 27 °C and 70% RH on a paper towel and storing in a zipper bag at 27 °C until further use. For hatching, eggs were submerged in a jar with deionized water over night with some crumbs of fish food. Hatched larvae were transferred to rearing trays and fed ad libitum.

2.2 Tissue sampling for *D. suzukii*, *Z. cucurbitae*, *B. dorsalis*, and *Ae. aegypti*

For tissue removal of adult *D. suzukii, Z. cucurbitae*, and *B. dorsalis*, individuals (2–3 days post-eclosion) were briefly CO_2-anesthetized in 53 mm × 100 mm Drosophila vials sealed with a Ceaprene stopper, then transferred to a CO_2 stage. For leg sampling, ethanol-sterilized micro-scissors clipped the selected leg pair at the femur–tibia joint to minimize cuticular damage. For wing sampling, ethanol-sterilized fine forceps grasped the distal margin of the wing and removed the blade at its basal joint (Fig. S1). Pupal shells were collected within 12 h of eclosion and transferred individually to PCR tubes. Scissors and forceps were thoroughly rinsed with water after each individual.

For leg removal of adult *Ae. aegypti*, individual mosquitoes were briefly CO_2-anesthetized in 53 mm × 100 mm Drosophila vials sealed with a Ceaprene stopper, then placed on a CO_2 stage. For mating, fecundity and fertility assessment, both mid legs or both hind legs were clipped at mid-femur with micro-scissors, and adults were immediately transferred to the mating cages. For NLG, one mid leg was cut and the adults returned to the Drosophila vials supplied with 10% sucrose solution until the end of the genotyping. Cut legs were transferred individually to PCR tubes and submerged in 20 µl extraction buffer (20 µl lysis buffer, 0.6 µl Proteinase K (Platinum™ Direct PCR Universal Mater Mix kit, Invitrogen)). Scissors and forceps were extensively cleaned after each individual with water. *Ae. aegypti* exuviae were between 0 and 30 min old at the time of collection and were either immediately transferred to a PCR tube and stored at −20 °C (0 h), or were transferred to

1.5 ml Eppendorf tube with 1 ml distilled water and incubated for 1, 6, 12 or 24 h at 27 °C before transfer to a PCR tube and storage at −20 °C until further use.

2.3 Fecundity and fertility assays after non-lethal tissue sampling

To assess fecundity of dissected *D. suzukii, Z. cucurbitae*, and *B. dorsalis*, sampled males were each mated with three untreated females to assess male fertility, whereas sampled females were each mated with three untreated males to assess female fecundity. All mating partners remained unmanipulated. Female egg production was recorded over the experimental period to quantify reproductive output. For *D. suzukii*, adult flies were allowed to mate and oviposit in standard Drosophila vials (2.4 × 9.4 cm) containing grape juice agar as the oviposition substrate. For *B. dorsalis*, flies were placed in 9 × 6 cm cages for mating, and oviposition was stimulated using a 3 cm Petri dish containing orange juice. The dish surface was covered with plastic wrap perforated with small holes to allow oviposition. For *Z. cucurbitae*, flies were also placed in 9 × 6 cm cages for mating, with a fresh zucchini slice (approximately 2 × 1 cm) provided as the oviposition substrate. All oviposition substrates were replaced daily, and the number of eggs per female was counted under a stereomicroscope. Five biological replicates (n = 5 cohorts) were conducted for each treatment, with at least three successful experimental measurements obtained for each treatment.

Mosquito mating assays were carried out in cages (20 × 20 × 20 cm) containing five males and five females, with each cross performed in triplicate. Five pairings were set up: (1) females with both hind legs removed plus untreated males; (2) females with both mid legs removed plus untreated males; (3) untreated females plus males with both hind legs removed; (4) untreated females plus males with both mid legs removed; (5) untreated control. Females were blood-fed at 3- and 9-days post-mating; eggs were collected from both blood feeds and dried as described above. 1–2 weeks after collection eggs were counted to determine female fecundity. Larvae were hatched and counted three days after hatching to determine fertility.

2.5 Survival rate after non-lethal tissue sampling

For survival analysis, each treatment group consisted of 90 adults (45 males and 45 females) sampled as described above. After tissue sampling, flies were maintained under the previously described rearing conditions and provided with the same food. Survival was recorded at 24, 48, and 72 hours post-sampling, and subsequently monitored daily until day 7.

2.6 DNA extraction and PCR amplification

Drosophila suzukii, Zeugodacus cucurbitae, and Bactrocera dorsalis: Samples were placed into 0.2 mL PCR tubes (Sorenson Bioscience, USA) using sterilized forceps and incubated in 10 µL lysis buffer (Buffer A: 10 mM Tris-Cl, pH 8.2; 1 mM EDTA; 25 mM NaCl) supplemented with 400 µg/mL Proteinase K (MP Biomedicals, USA). Tubes were incubated at 37 °C for 60 min, then heated to 95 °C for 2 min to inactivate Proteinase K. Homogenization was unnecessary given the small tissue volume and enzyme efficiency. Extracted DNA was stored at −20 °C until PCR.

PCR was performed in 20 µL reactions using Platinum™ Direct PCR Universal Master Mix (Thermo Fisher Scientific): 10 µL 2× master mix, 4 µL 5× GC Enhancer, 0.2 µL each primer (10 µM), 1 µL DNA template and 4.6 µL nuclease-free water. Cycling conditions were: 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 60 °C for 15 s and 68 °C for 20 s; then 72 °C for 5 min. Products were resolved on 1% agarose gels stained with GelRed™ (Vazyme). DNA fragments were purified from agarose gels using the FastPure Gel DNA Extraction Mini Kit (Vazyme). DNA extracted from different tissues was subjected to PCR, and each tissue type was tested in ten independent reactions.

Aedes aegypti experiments tissue samples were transferred to 0.2 mL PCR tubes and incubated for at least 30 min in 20 µl extraction buffer (see tissue sampling) at room temperature. The protease was then heat inactivated at 98 °C for 1 min. Tissue extraction solutions were either stored at 4 °C until further use (up to four weeks possible) or directly subjected to PCR analysis. PCR reactions contained 5µl 2X Platinum™ Direct PCR Universal Master mix, 0.2 µl 10 µM forward and reverse primer, 1 µl of the tissue extraction solution, and 3.6 µl nuclease-free water. Cycling conditions were as specified in the manufacturer's protocol, using 39 cycles. In case of subsequent restriction digest of the PCR product, 5 µl of the restriction master mix (3 µl ddH_2O, 1.5 µl CutSmart buffer (New England Biolabs), and 20 U restriction enzyme (NEB)) were added to the PCR reaction and incubated for 2 hours at the enzyme-specific temperature. For sequencing, PCR bands were cut from the agarose gel and DNA extracted with the Zymoclean Gel DNA Recovery Kit (Zymo Research Corp.) following the manufacturer's instructions.

2.7 Sequencing

PCR products were Sanger sequenced with Macrogen Europe (*Ae. aegypti*) and BGI Genomics Co., Ltd (*D. suzukii, Z. cucurbitae*, and *B. dorsalis*).

2.8 Statistical analysis

Statistical analyses were performed using SPSS Statistics 22.0 (IBM, Chicago, IL, USA). Fecundity data were analyzed by one-way analysis of variance (ANOVA), and when a significant overall effect was detected, pairwise comparisons were performed using Tukey's honestly significant difference test. ANOVA results are reported in the text as F (df_between, df_within) = F-value, p = p-value. Survival data were analyzed using the Kaplan–Meier method, and differences among treatments were assessed by the log-rank test. When

the overall log-rank test indicated a significant difference among groups, pairwise comparisons were conducted. Log-rank results are reported as χ^2= χ^2-value, p = p-value.

3 Results

3.1 Development and evaluation of non-lethal genotyping protocols in *D. suzukii*

We compared DNA yield and PCR success for the endogenous *Obp19c* gene (stable, abundant, reproductive-related expression) from single pro-, meso-, and metafemurs, single wings, both wings, half wings, and pupal shells in *D. suzukii* (Fig. 1A). Leg excision was universally lethal, causing 100% mortality by 48 h (Fig. 1B). Survival dropped sharply after wing removal (one wing $\chi^2 = 57.39$, $P < 0.001$; half wing $\chi^2 = 40.75$, $P < 0.001$; both wings $\chi^2 = 163.60$, $P < 0.001$) (Fig. 1B). Female fecundity was significantly reduced by wing removal (one-wing: F (4, 14) = 16.98, $P < 0.001$; both wings: F (4, 14) = 16.98, $P < 0.001$; one-half wing: F (4, 14) = 16.98, $P < 0.001$; both half-wings: F (4, 14) = 16.98, $P < 0.001$) (Fig. 1C), whereas male fertility remained unchanged (Fig. 1D). Pupal shells, collected within 12 h of eclosion, provided robust PCR templates with zero mortality or reproductive cost and are therefore the optimal non-lethal source.

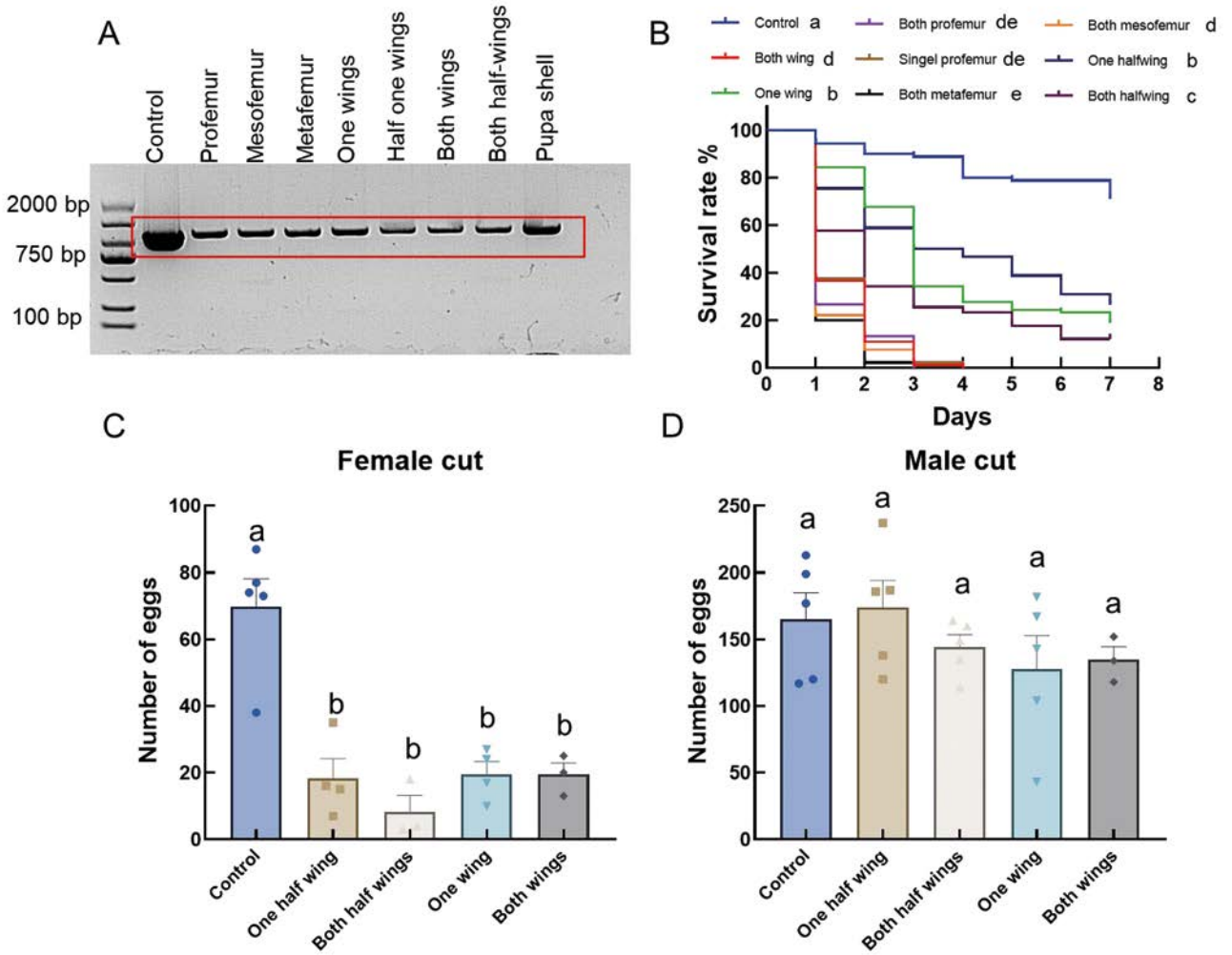

Fig. 1. Validation of tissue sampling methods for genomic PCR and fitness in *D. suzukii*. (A) Agarose gel of PCR products targeting the *Obp19c* gene from various tissues. Lane 1: Biomed 2000 DNA marker; lane 2: whole body; lane 3: pro-femur; lane 4: meso-femur; lane 5: metafemur; lane 6: single wing; lane 7: half wing; lane 8: both wings; lane 9: both half-wings; lane 10: pupal shell. The expected amplicon is boxed in red. **(B)** Kaplan–Meier survival curves of *D. suzukii* following each sampling method (n = 90 flies per treatment: 45 females, 45 males). Statistical differences versus control by log-rank test. Different letters indicate significant difference ($p < 0.05$, pairwise log-rank test). Female fecundity **(C)** was measured as the number of eggs laid per day by sampled females mated with three untreated males. Male fertility **(D)** was assessed as the number of eggs laid by three untreated females after mating with sampled males. Data are presented as means (± SE). Different letters indicate significant difference (ANOVA followed by Tukey's post hoc test, $p < 0.05$).

3.2 Development and evaluation of non-lethal genotyping protocols in *Z. cucurbitae*

Single-leg (mesofemur), wing and pupal-shell samples from *Z. cucurbitae* all yielded DNA suitable for PCR of the *clock* gene (stable, abundant expression; Valerio et al. 2022) (Fig. 2A; Fig. S4A–B). Wing clipping significantly reduced 7-day survival (one wing: $\chi^2 = 11.20$, $p < 0.001$; both wings: $\chi^2 = 36.87$, $p < 0.001$), whereas single mesofemur removal had no significant effect ($\chi^2 = 1.91$, $p = 0.167$), and removal of both mesofemur moderately reduced survival ($\chi^2 = 5.57$, $p = 0.018$) (Fig. 2B). Female fecundity showed a decreasing trend following one- or both-wings removal, but the changes were not statistically significant (both wings: $F(4,17) = 2.07$, $p = 0.199$; both mesofemur: $F(4,17) = 2.07$, $p = 0.231$) (Fig. 2C). Male fertility also tended to decrease after both-wing removal or removal of both mesofemour, but these effects were not significant (both wings: $F(4,18) = 1.10$, $p = 0.861$; both mesofemur: $F(4,18) = 1.10$, $p = 0.359$) (Fig. 2D). Thus, pupal shell is optimal for *Z. cucurbitae* non-lethal genotyping; single-mesofemur sampling is an acceptable alternative as it preserves survival and reproduction.

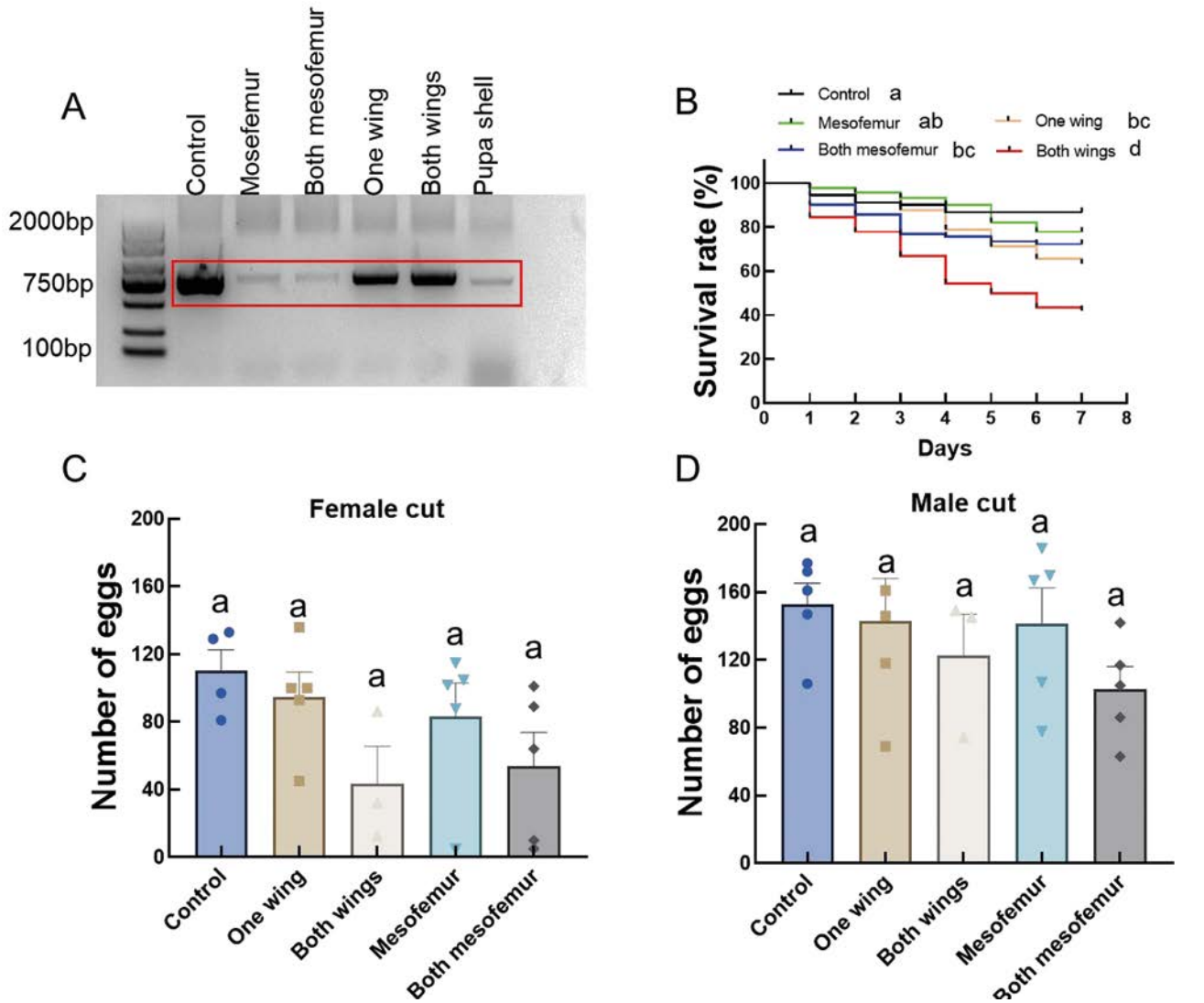

Fig. 2. Validation of tissue sampling methods for genomic PCR and fitness in *Z. cucurbitae*. **(A)** Agarose gel of PCR products targeting the *clock* gene from various tissues. Lane 1: Biomed 2000 DNA marker; lane 2: whole body; lane 3: single mesofemur; lane 4: both mesofemurs; lane 5: single wing; lane 6: both wings; lane 7: pupal shell. The expected amplicon is boxed in red. **(B)** Kaplan–Meier survival curves of *Z. cucurbitae* following each sampling method (n = 90 adults per treatment: 45 females, 45 males). Different letters indicate significant difference ($p < 0.05$, pairwise log-rank test). Female fecundity **(C)** was measured as the number of eggs laid per day by sampled females mated with three untreated males. Male fertility **(D)** was assessed as the number of eggs laid by three untreated females after mating with sampled males. Data are presented as means (± SE). Different letters indicate significant difference groups (ANOVA followed by Tukey's post hoc test, $p < 0.05$).

3.3 Development and evaluation of non-lethal genotyping protocols in *B. dorsalis*

Wing and leg tissues from *B. dorsalis* yielded reliable PCR amplification of the *clock* gene (Fig. 3A; Fig. S5A–B). One wing removal significantly reduced 7-day survival (χ^2 = 11.35, p < 0.001), and both wings removal caused a stronger reduction (χ^2 = 22.77, p < 0.001), compared with controls. Single mesofemur excision had no significant effect (χ^2 = 2.32, p = 0.128), whereas both mesofemur removal moderately reduced survival (χ^2 = 6.38, p = 0.012) (Fig. 3B). Female fecundity was significantly reduced by wing removal (one wing: F (4, 20) = 3.27, p = 0.030; both wings: F (4, 20) = 3.27, p = 0.049), but mesofemur removal had no significant effect (Fig. 3C). Male reproductive output also declined significantly following wing removal (one wing: F (4, 19) = 18.50, p < 0.001; both wings: F (4, 19) = 18.50, p < 0.001). Removal of mesofemur further reduced offspring per mating (one mesofemur: F (4, 19) = 18.50, p < 0.001; both mesofemur: F (4, 19) = 18.50, p < 0.001) (Fig. 3D). Pupal shells thus represent the only non-invasive source that does not compromise survival or fecundity in *B. dorsalis*.

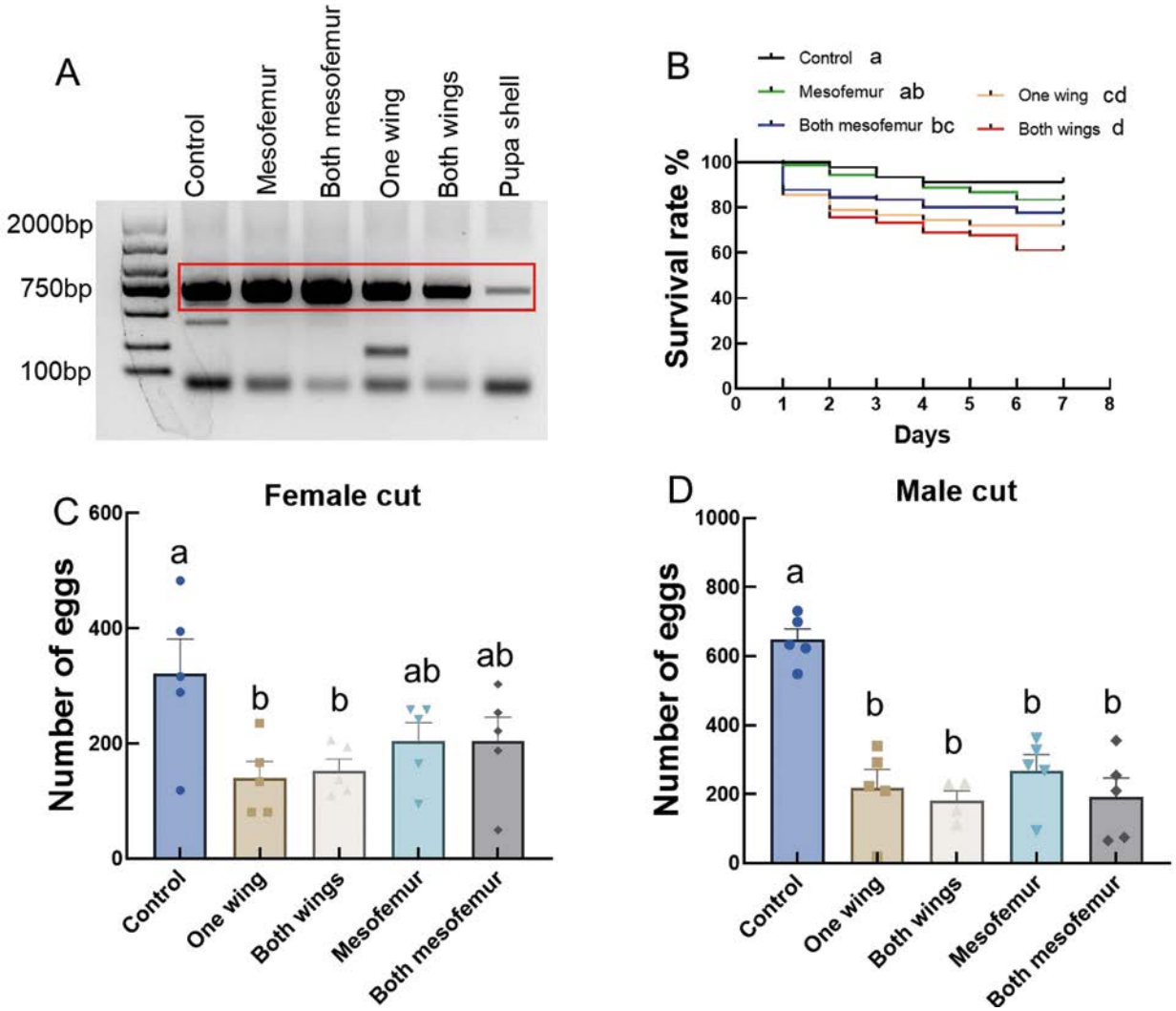

Fig. 3. Validation of tissue sampling methods for genomic PCR and fitness in *B. dorsalis*. **(A)** Agarose gel of PCR products targeting the *clock* gene from various tissues. Lane 1: Biomed 2000 DNA marker; lane 2: whole body; lane 3: single mesofemur; lane 4: both mesofemurs; lane 5: single wing; lane 6: both wings; lane 7: pupal shell. The expected amplicon is boxed in red. **(B)** Kaplan–Meier survival curves of *B. dorsalis* following each sampling method (n = 90 adults per treatment: 45 females, 45 males). Statistical differences versus control by log-rank test. Different letters indicate significant difference (p < 0.05, pairwise log-rank test). Female fecundity **(C)** was measured as the number of eggs laid per day by sampled females mated with three untreated males. Male fertility **(D)** was assessed as the number of eggs laid by three untreated females after mating with sampled males. Data are presented as means (± SE). Different letters indicate significant difference (ANOVA followed by Tukey's post hoc test, p < 0.05).

3.4 Development and evaluation of non-lethal genotyping protocols in *Ae. aegypti*

Aedes aegypti depend on flight ability to form mating couples, grabbing each other by their legs. Therefore, the effect of removing both mid or hind legs from male or female *Ae. aegypti* for NLG was particularly interesting. Another option for NLG in mosquitoes is exuviae, the pupal skin that remains in the water once the adult mosquito emerged.

DNA extracted from single legs reliably yielded PCR amplicons for hundreds of samples, which were also readily digestible (exemplary gel in Fig. 4A). Sequencing gel-extracted PCR products consistently resulted in high quality sequencing results for legs (Fig. S6A, showing 12 out of 12 successful sequencing events). For exuviae, a PCR band was obtained only in 14 out of 36 sampled exuviae. The failure to amplify DNA was not clearly associated with the age of the exuviae, as at least 50% of the DNA extractions yielded a product for the 0 h and 1 h old samples, but also for the 18 h samples (Fig. 4B). Moreover, the band intensity was overall markedly weaker than that obtained for the leg samples at the same cycling conditions. Sequencing of the PCR bands returned 11 out of 13 samples with high quality reads (Fig. S6B). Noticeably, however, the quality at the start of the sequencing reads was lower than from the leg samples (Fig. S7).

Removing both mid or hind legs from male or female *Ae. aegypti* did not affect egg production or egg hatch rate across treatment cages. Specifically, fecundity was not significantly affected by tissue sampling (BF1: $F(4,5) = 0.78$, $p = 0.583$; BF2: $F(4,10) = 0.38$, $p = 0.820$), and egg hatchability likewise showed no significant differences among treat-

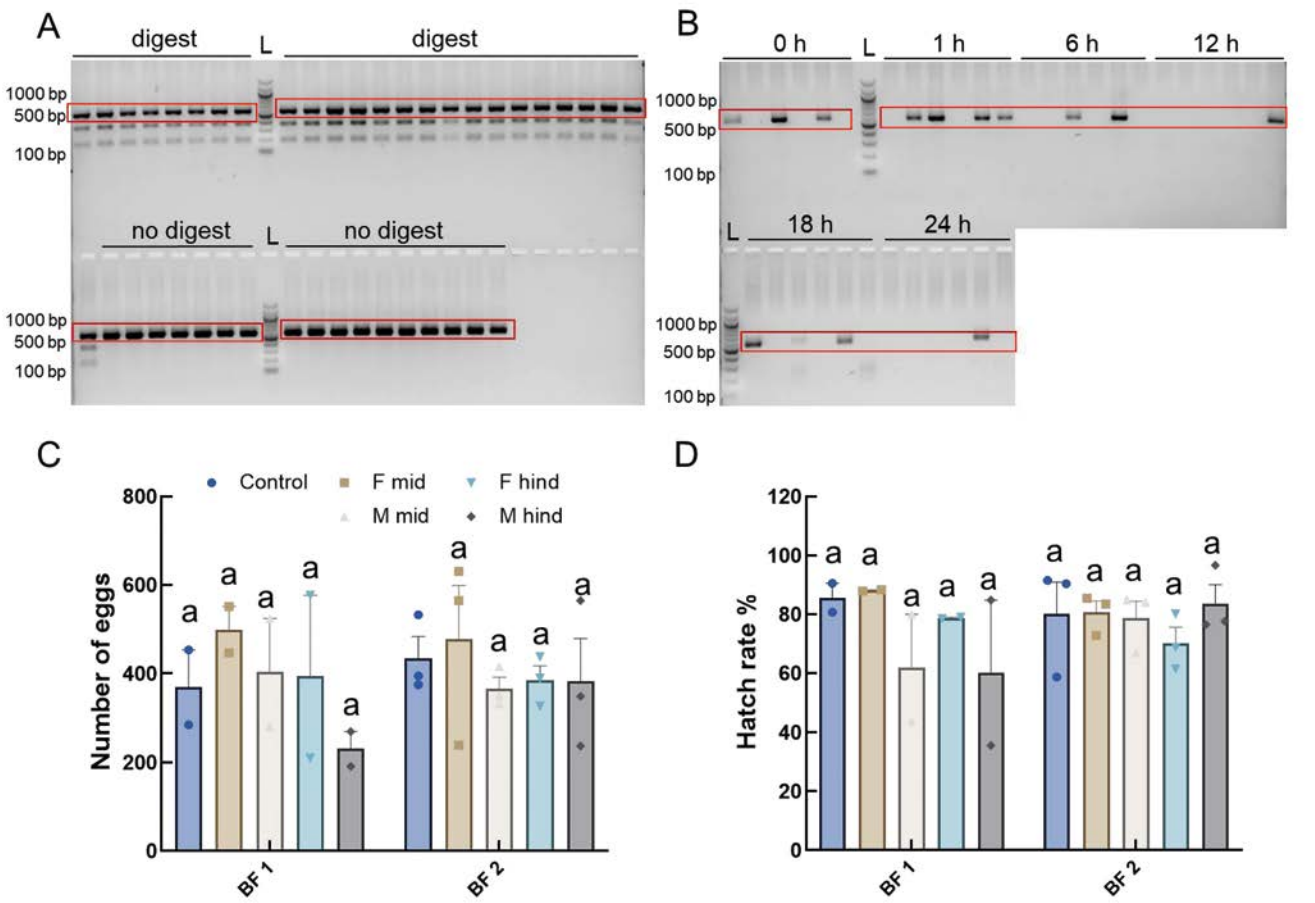

Fig. 4. Validation of *Ae. aegypti* leg and exuviae sampling for genomic PCR and reproductive output. (A) Agarose gel of PCR products from genomic DNA of single-leg samples digested: PCR product was restriction enzyme digested; no digest: PCR product without digest. Each lane corresponds to an independent replicate; **(B)** Agarose gel of PCR products from single exuviae, incubated between 0 and 24 h before DNA extraction; Each lane corresponds to an independent replicate (**A, B**) L = ladder: NEB 100 bp ladder; the expected amplicon is boxed in red. **(C)** Mean number of eggs collected per cage following the first blood feed (BF 1; 3 days post–leg removal) and second BF (BF 2; 9 days post–leg removal). Each BF 1 datum represents two replicates of five treated or control adults; BF 2 represents three replicates. Error bars show standard deviation. Treatments: females with mid legs removed (F mid), males with mid legs removed (M mid), females with hind legs removed (F hind), males with hind legs removed (M hind), and untreated control (Ctrl). (D) Mean hatch rate of eggs from BF 1 and BF 2 (same replicates as in C). Data are presented as means (± SE). Different letters indicate significant difference (ANOVA followed by Tukey's post hoc test, $p < 0.05$).

ments (BF1: $F(4,5) = 0.90$, $p = 0.527$; BF2: $F(4,10) = 0.54$, $p = 0.708$) (Fig. 4C, D). The only effect observed was a reduced egg production in the 1st blood feed when cutting the males' hind legs, but this effect was not significant. This was not visible after the 2nd blood feed. Additional replicates would be required to confirm this effect.

4 Discussion

Gene editing tools such as CRISPR/Cas9 have become indispensable in insect functional genomics, enabling targeted mutagenesis to probe gene roles in physiology, behavior, environmental response, and pesticide resistance (Li et al. 2021; Sun et al. 2017). Many mutations do not produce obvious phenotypes, so non-lethal genotyping is essential to identify edited individuals for downstream assays. Genetic control strategies such as CRISPR-engineered genetic sexing strains require stable lines for mass rearing and release; when engineered alleles lack visible markers, PCR-based selection of live insects is the only reliable method (Fig. S8). Transgene-based approaches, including RIDL, pgSIT, and gene drives (Yan et al. 2023; Champer et al. 2017) would also be impractical without efficient non-invasive genotyping (Yan et al. 2023; Ali et al. 2019).

Drosophila suzukii, Zeugodacus cucurbitae, and Bactrocera dorsalis inflict major crop losses worldwide (Dhillon et al. 2005; Clarke et al. 2005; Walsh et al. 2011) and *Ae. aegypti* is a rapidly expanding disease vector under climate change (Lahondère & Bonizzoni 2022). Proposed CRISPR-based controls for these species (Yan et al. 2023; Yan et al. 2025) depend on maintaining viable cohorts for genetic surveillance. Destructive sampling can reduce cohort size, skew sex ratios, impair mating, or alter behaviors critical to breeding. By comparing leg, wing, and pupal-shell sampling, we define methods that preserve viability while yielding reliable DNA.

Our results reveal clear trade-offs between genotyping efficiency and fitness for some of the tested insects. In *D. suzukii, Z. cucurbitae*, and *B. dorsalis*, wing removal reduced oviposition dramatically, reflecting loss of courtship cues such as wing-produced sounds and visual signals (Coen et al. 2014). In *D. suzukii*, leg or wing excision caused high mortality, with full-wing removal killing all adults within four days and partial excision reducing survival by more than 70%. In contrast, *Z. cucurbitae* and *B. dorsalis* tolerated mesofemur removal with survival above 70% and retained PCR success. We also observed sex-dependent effects of tissue damage. In *D. suzukii* and *Z. cucurbitae*, females lost more reproductive output after leg excision, possibly due to disrupted limb-borne sensory signals required for pheromone detection (Thistle et al. 2012). Male fertility remained largely unaffected after mesofemur sampling, suggesting that male-only sampling protocols could maximize throughput when targeting male-fertility genes – sampled males can be mated to wild-type virgin females to directly assay fertility (and to maintain/propagate lines), thereby reducing workload and avoiding female-specific confounding effects. For mosquitoes, wing removal wasn't tried as these insects mate only in flight. The males grab the females with their legs until they securely grasped their mid and hind legs (Aldersley & Cator 2019). While removal of either both mid or both hind legs didn't affect mating success significantly, we still observed a reduction in egg production in a single setup: when blood feeding three days after removal of the males' mid legs. This suggests that the mid legs might be more important than the hind legs, and that the males can cope with the situation and to learn to grab the females during flight without their mid legs. These species-specific differences highlight the need to tailor sampling to anatomical tolerance and resilience.

Pupal-shell sampling was uniformly non-invasive and caused zero mortality across all species. Although DNA yield is lower than from appendages, amplification of both endogenous and exogenous markers was consistently robust for all fly species. Pupal shells therefore offer the safest early-stage DNA source for founder screening in these species, especially when mutations may incur fitness costs. In contrast, amplification from *Ae. aegypti* exuviae yielded less than 50% success rate and also fainter bands than from leg samples. This was not surprising as insect exuviae consist of glycoproteins and don't contain DNA. That DNA can still be extracted from exuviae is probably due to epithelial cells from the foregut, hindgut and tracheae lining that are pulled out upon molting and cling to the molted skin. The number of cells remaining attached during the molting process and the time until analysis (Bertholf 1925) will have an effect on PCR amplification success, which has already been observed to be highly variable in the past when using endpoint PCR (Dhananjeyan et al. 2010), probably due to limited sensitivity. Here, real-time PCR might be more reliable (Jones et al. 2024) but would have to be tested for *Ae. aegypti*. If successful then genotyping from exuviae could be a promising method for field-collections.

Beyond initial screening for the development of genetic control strains, non-lethal genotyping also supports insect quality control and field monitoring in a release program. Strains can be repeatedly assayed during mass rearing to confirm genetic stability without loss of individuals. Field-collected pupal shells or fragments can provide DNA even when insect samples are damaged or degraded, enabling allele-frequency tracking in gene-drive or other release programs. Our study here provides a pipeline for refining genotyping approaches that minimize fitness costs. Future work could explore additional non-destructive matrices such as frass, cuticular hydrocarbons, or saliva that are already used in vertebrate ecology (Rizzo et al. 2021; Moore et al. 2021). Coupling these sources with portable assays such as loop-mediated isothermal amplification or mobile qPCR could bring real-time genotyping into the field. For both basic research and applied control, robust non-lethal genotyping is a strategic necessity for efficient and effective insect genetic screening and surveillance.

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