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A fluorescent biosensor enables high-sensitivity quantification of hemolymph trehalose in individual aphids

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Abstract

Objective Trehalose is a major hemolymph sugar in aphids; however, its accurate quantification at the individual level has been technically challenging because of the small body size of aphids. To quantify hemolymph trehalose at the single-insect level in aphids using a GFP-based fluorescent biosensor (Tre-C04), benchmark it against high-performance liquid chromatography (HPLC), and examine species- and wing-morph differences.

Results HPLC of pooled adult *Acyrthosiphon pisum* hemolymph measured 218.9 ± 34.4 mM trehalose, while Tre-C04 quantified 183.5–261.7 mM in individual adults (mean 227.3 ± 10.0 mM); the two methods did not differ significantly. Across nine species, single-insect measurements clustered near ~ 200 mM (191.7–249.2 mM), and we did not reproduce previously reported values > 600 mM in *Aphis gossypii* or *Myzus persicae*; pooling required by ^{1}H-NMR likely increased concentrations via tissue contamination. Winged *Sitobion ibarae* had higher trehalose than wingless conspecifics, whereas *Megoura crassicauda* showed no morph-dependent difference in trehalose levels under rainy or sunny conditions. These data validate Tre-C04 for rapid, sensitive single-insect sugar quantification, reveal a conserved trehalose set-point of approximately 200 mM with modest species-level variation, and indicate that morph effects are species-dependent.

Keywords Trehalose, Aphid, Hemolymph, Fluorescent biosensor, cpGFP, HPLC, Single-insect quantification, Wing morph, Carbohydrate homeostasis

Introduction

Trehalose, the principal sugar in insect hemolymph, consists of two glucose units and serves as an essential energy source for ATP production in insects [1, 2]. In most insects, hemolymph trehalose is homeostatically maintained at 0.1–100 mM to ensure a continuous carbohydrate supply to peripheral tissues [3, 4]. For example, *Tribolium castaneum* maintained hemolymph concentrations of 120 µM trehalose and 55 µM glucose [3], whereas fifth-instar *Bombyx mori* larvae exhibited ~ 13.7 mM trehalose [4]. By contrast, adult *Acyrthosiphon pisum* (pea aphid) maintain substantially higher trehalose levels (~ 270 mM) [5, 6]. Inter-specific variation

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was notable among aphids: species such as *Lachnus tropicalis*, *Megoura crassicauda*, *Megoura viciae*, and *A. pisum* cluster near ~ 200 mM, while *Aphis gossypii* and *Myzus persicae* have been reported at 600–900 mM [5]. These observations suggest a species-specific regulation of hemolymph sugar levels. The high levels of trehalose in aphid hemolymph compared to those in other insects suggest the potential for applying trehalose metabolism and transport as novel active ingredients. This allows for the control of aphids while minimizing the risk to non-target organisms [7]. However, the molecular basis for the exceptionally high trehalose levels in aphids remains unclear [8], in part because the small body size complicates accurate analysis. Many previous measurements pooled hemolymph from multiple individuals, a procedure that may introduce contamination from tissues and obscure inter-individual variations [9–11].

Historically, trehalose in *A. pisum* has been quantified using pooled samples. Radioisotope assays used approximately 1 µL of pooled hemolymph labeled with U-^{14}C sugars [9], and gas chromatography–mass spectrometry analyses required 275–600 nL per sample from approximately 15 adult females [10]. These approaches underscore the practical barriers to single-insect quantification. However, even with low genetic variation and standardized rearing, individual-level measurements improve resolution and are critical for understanding physiological and developmental responses. Thus, highly sensitive tools are needed to quantify trehalose at the single-insect level in small-bodied aphids, such as *A. gossypii* and *M. persicae*.

Tre-C04, a single fluorescent protein biosensor based on green fluorescent protein (GFP), has demonstrated high sensitivity in detecting trehalose in individual *Escherichia coli* cells [11]. This biosensor integrates a ligand-specific binding protein with circularly permuted GFP (cpGFP). In the absence of a ligand, cpGFP emits weak fluorescence; however, upon trehalose binding, it undergoes a conformational change that leads to a strong green fluorescence. The fluorescence intensity ratio correlates with trehalose concentration, enabling sensitive quantification. Given the need for precise, individual-level measurements [6, 12], we applied Tre-C04 to quantify hemolymph trehalose across multiple aphid species and compared wing morphs where relevant.

Materials & methods

Identification of aphid species

Single adult females were homogenized in 150 mM NaCl (1.5-mL tube) using sterile pestles. Genomic DNA was extracted using the HotSHOT method [13]. A partial fragment of mitochondrial cytochrome c oxidase subunit I (COI) was amplified with KOD One PCR polymerase (TOYOBO, Tokyo, Japan) using the following primers: forward 5′-GGTCAACAAATCATAAAGATATTGG-3′ and reverse 5′-TAAACTTCAGGGTGACCAAAAAATC A-3′. Cycling: 94 °C 10 s, 53 °C 5 s, 72 °C 10 s, 30 cycles. Amplicons were Sanger-sequenced (Eurofins Genomics, Tokyo, Japan) and identified by BLAST (NCBI). Species other than *Myzus persicae* were collected at the Ibaraki University Ami Campus farm (Ami, Ibaraki, Japan; Table S1). *M. persicae* was obtained from Sumika Techno Service (Takarazuka, Hyogo, Japan).

Purification of the recombinant trehalose biosensor

Trehalose was quantified using the GFP-based biosensor Tre-C04. The expression plasmid pTKEI-Tre-C04 (Addgene #79754; RRID:Addgene_79754) was a gift from David Savage [11]. The plasmid was transformed into *E. coli* BL21 (DE3) (Nippon Gene, Tokyo, Japan). Expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside at 15 °C for 24 h. Recombinant Tre-C04 was purified using Ni–NTA affinity chromatography (Profinity IMAC resin; Bio-Rad, Hercules, CA, USA). The columns were washed three times with 20 mM Tris–HCl (pH 8.0) and 10 mM imidazole, and the proteins were eluted with 20 mM Tris–HCl (pH 8.0) and 500 mM imidazole. The purified protein was diluted in distilled water for subsequent assays.

Quantification of hemolymph trehalose with the fluorescent biosensor

Hemolymph collection and trehalose quantification were performed as previously described [6, 12]. The collected hemolymph was diluted with distilled water to a concentration of less than 1,000 nM of detectable trehalose to quantify the trehalose concentration using Tre-C04. Aphids were collected on several independent days (Table S1). We returned the aphids to the laboratory using the leaves. Hemolymph from a single aphid was obtained within 60 min of collection and stored at – 30 °C until analysis. Fluorescence was measured using a Qubit 3 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA). The trehalose concentration was calculated from the fluorescence ratios using a calibration standard (50 nM trehalose). Statistical tests were Welch's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-hoc test (Prism 10; GraphPad Software, San Diego, CA, USA). All assays used independently prepared sensor batches ($n = 3$). The results were reproducible across batches, and representative datasets are presented.

HPLC quantification in *A. pisum*

For benchmarking, trehalose in *A. pisum* hemolymph was quantified by high-performance liquid chromatography (HPLC) using an Aminex HPX-87C column (Bio-Rad). Hemolymph (100 nL) was diluted in 80% (v/v) ethanol containing 1% (w/v) sorbitol as an internal

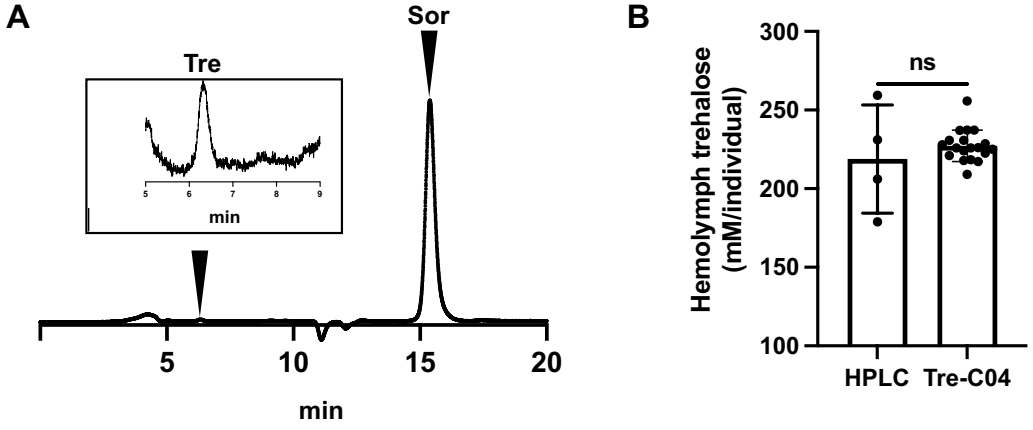

Fig. 1 Detection and quantification of hemolymph sugars in *Acyrthosiphon pisum*. **A** HPLC chromatogram of sugars in the hemolymph of wingless adult *A. pisum*. Tre, trehalose; Sor, sorbitol (internal standard). **B** Comparison of trehalose concentrations obtained by HPLC and the Tre-C04 biosensor. Data are mean ± SD (HPLC, n = 4; biosensor, n = 19). Each dot represents an individual. Welch's *t*-test, $p = 0.66$

standard and completely dried at 70 °C. The pellet was dissolved in distilled water. The detailed procedure was described in a previous study [14].

Results and discussion

HPLC validation and biosensor benchmarking in *A. pisum*

HPLC using a sugar separation column detected a dominant peak corresponding to trehalose in the hemolymph of wingless adult *A. pisum*, confirming trehalose as the principal hemolymph sugar (Fig. 1A), which is in agreement with a previous study [5]. The trehalose concentration measured by HPLC in pooled hemolymph from mature *A. pisum* was 218.9 ± 34.4 mM (mean ± SD; Fig. 1B). Because HPLC analysis was below the detection limit for individual samples, ≥ 10 females were pooled per measurement. In contrast, the Tre-C04 fluorescent biosensor quantified trehalose in individual adult *A. pisum*, yielding 183.5–261.7 mM (mean 227.3 ± 10.0 mM; Fig. 1B). No significant difference was observed between the two methods (Welch's t-test, p = 0.66). Thus, Tre-C04 provided rapid, sensitive, single-insect measurements that were concordant with HPLC. Single-insect resolution reduces potential artifacts from pooling (e.g., tissue contamination) and preserves inter-individual variability, which is critical for linking physiology to morph or environmental context. Practically, Tre-C04 overcomes the sample-volume constraints inherent to chromatography and enables hypothesis testing at the individual level while remaining anchored to an HPLC benchmark.

Species-specific hemolymph trehalose across nine aphids

The aphid species tested were identified using COI sequences (Table S1). Next, we quantified trehalose within 1 h of collection across nine species using Tre-C04. Mean (± SD) concentrations were: *Megoura crassicauda* 220.6 ± 12.5 mM, *Aphis gossypii* 204.7 ± 11.9 mM, *Sitobion ibarae* 191.7 ± 16.3 mM, *Uroleucon sonchi* 217.2 ± 9.0 mM,

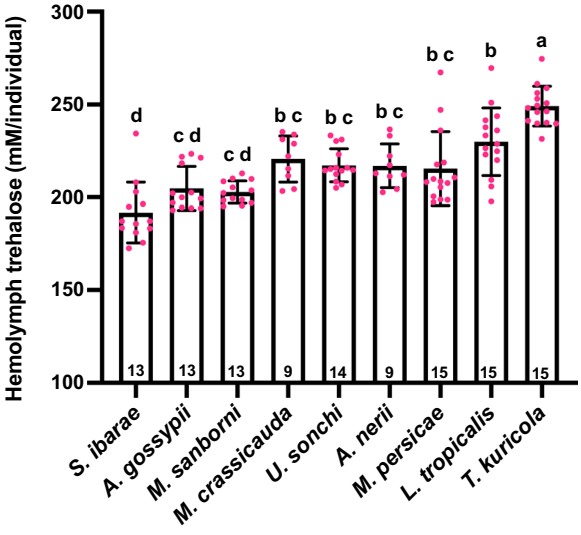

Fig. 2 Species-specific hemolymph trehalose levels in aphids. Trehalose concentrations in wingless adult females across nine species were measured using Tre-C04. Data are mean ± SD (n ≥ 9 per species). Each dot represents an individual. One-way ANOVA followed by Tukey's multiple-comparison test

Macrosiphoniella sanborni 202.8 ± 6.0 mM, *Lachnus tropicalis* 229.9 ± 18.3 mM, *Tuberculatus kuricola* 249.2 ± 10.7 mM, *Aphis nerii* 216.9 ± 11.8 mM, and *Myzus persicae* 215.5 ± 19.9 mM (Fig. 2). Overall, hemolymph trehalose levels clustered near ~ 200 mM across diverse aphids. Prior work reported 925.5 mM in *A. gossypii* and 678.9 ± 170.3 mM in *M. persicae* [5]. Our single-insect measurements did not reproduce such high values. Differences may arise from methodological factors: the previous study used ^{1}H-NMR requiring > 1 µL hemolymph, which, for these small species, necessitates pooling 20–400 individuals [6]. During pooling, contamination from tissues (e.g., fat bodies) could inflate the apparent trehalose concentrations (Fig. S1). Biosensor

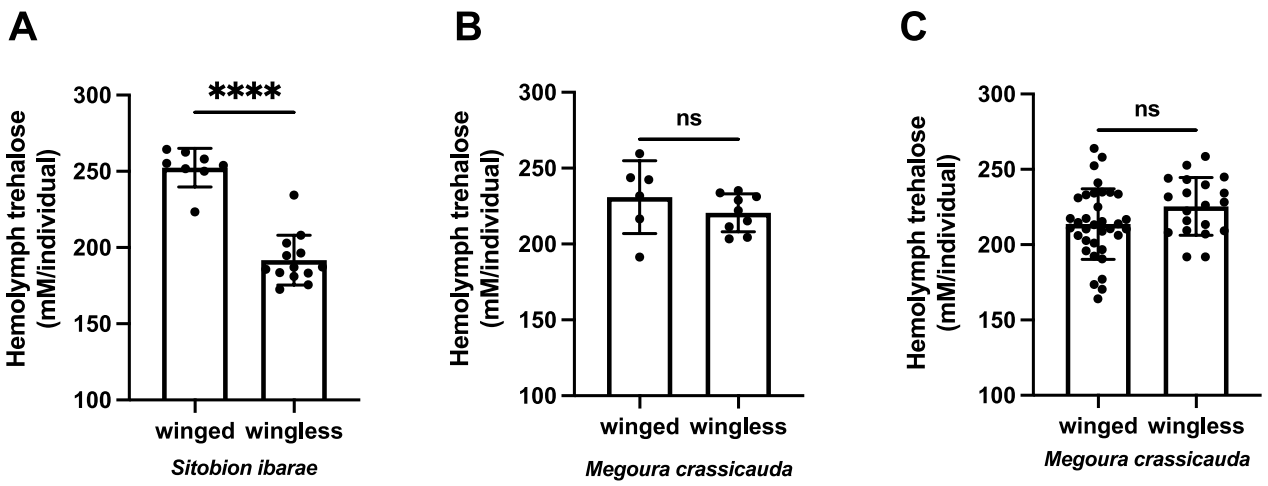

Fig. 3 Comparison of trehalose levels in winged and wingless morphs. Hemolymph trehalose in *Sitobion ibarae* and *Megoura crassicauda* was quantified using Tre-C04. Data are mean ± SD (n ≥ 6 per morph). Each dot represents an individual. Welch's *t*-test. **A** *S. ibarae*. **B** *M. crassicauda* collected on rainy days. **C** *M. crassicauda* collected on sunny days. ****$p < 0.0001$ where indicated

measurements from single aphids minimize such contamination and are likely to better reflect physiological levels. The convergence around ~ 200 mM suggests a conserved homeostatic set-point balancing osmotic pressure with the energetic needs of peripheral tissues and bacteriocytes [4, 14]. Species-level deviations within this narrow band may reflect differences in trehalose transport (e.g., TreT/TRET1 activity) or endocrine control of carbohydrate mobilization (e.g., adipokinetic hormone signaling) in aphids [4, 10, 14]. Importantly, our data suggest that extremely high values (> 600 mM) are not typical for single individuals under the conditions tested and are more parsimoniously explained by pooling-related artifacts.

Winged versus wingless morphs

In *A. pisum*, winged morphs displayed higher trehalose than wingless morphs, consistent with the energetic demands of flight [6]. We tested whether this pattern generalizes to other species by comparing field-collected *S. ibarae* and *M. crassicauda* (Fig. 3). In *S. ibarae*, winged adults had significantly higher trehalose levels than wingless conspecifics (Fig. 3A). In *M. crassicauda*, trehalose did not differ between morphs when collected on rainy days (Fig. 3B). Notably, the environmental conditions during collection differed: *M. crassicauda* individuals were collected on a rainy day, whereas *S. ibarae* were sampled under controlled greenhouse conditions. Because flight activity may be suppressed in rainy weather, the lack of elevated trehalose levels in winged *M. crassicauda* may reflect a reduced metabolic demand. To test this possibility, we reanalyzed the trehalose levels in *M. crassicauda* collected on sunny days. Trehalose concentrations remained unchanged between the winged and wingless morphs, suggesting that factors other than immediate flight activity may regulate the hemolymph sugar levels in this species. Therefore, we analyzed trehalose levels in the hemolymph obtained from the winged and wingless morphs of *M. crassicauda* on sunny days. Unexpectedly, trehalose levels were not significantly different in *M. crassicauda* (Fig. 3C). These results indicate that the winged morphs of *M. crassicauda* did not have elevated trehalose levels compared to the wingless morphs. Elevated trehalose levels in winged *S. ibarae* are consistent with morph-specific carbohydrate mobilization supporting flight readiness, as reported for *A. pisum* [6, 10]. In contrast, the absence of a morph effect in *M. crassicauda*, even under fair weather, suggests species-specific metabolic strategies, such as greater reliance on alternative fuels or tighter trehalose homeostasis, rather than a simple suppression by weather at the time of collection. Discriminating among these possibilities will require coupling single-insect trehalose with complementary endpoints (e.g., glycogen or lipid content, expression of trehalose transport and synthesis genes [4, 14]) under controlled conditions.

Concluding remarks

Tre-C04 provides sensitive, HPLC-benchmarked trehalose quantification from single aphids, enabling analyses that were previously limited by sample volume. The narrow distribution around ~ 200 mM across species is consistent with tight osmotic and metabolic homeostasis. The mixed morph effects point to species-specific strategies—potentially involving trehalose transport and hormonal mobilization—rather than weather-driven, immediate flight demand alone. This platform opens the door to mechanistic tests integrating trehalose dynamics with glycogen/lipid reserves and gene expression under defined conditions.

Limitations

Aphis gossypii exhibits seasonal color morphs (e.g., yellow and dark green) that depend on host plant conditions [15, 16]. We attempted to compare hemolymph trehalose levels among these morphs, but we could not obtain individual hemolymph samples from the yellow morph because of its markedly smaller body size [17, 18], which made single-insect sampling infeasible. Future studies should evaluate morph-specific physiology, including the relationship between trehalose and color phenotypes, using approaches compatible with very small sample volumes.

Abbreviations

ANOVA	Analysis of variance
cpGFP	Circularly permuted green fluorescent protein
COI	Cytochrome c oxidase subunit I
GFP	Green fluorescent protein
HPLC	High-performance liquid chromatography

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-025-07543-2.

Additional file 1.

Additional file 2.

Additional file 3.

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Author contributions

Ryusei Tomari: Conceptualization (equal), data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), and visualization (equal). Writing—original draft (equal). Sakura Yamaguchi: Conceptualization (equal), data curation (equal), formal analysis (equal), investigation (lead), methodology (equal). Writing—original draft (equal); writing—review and editing (equal). Naomi Soma: Data curation (equal), formal analysis (equal), investigation (equal), writing—original draft (equal). Takahiro Kikawada: Data curation (equal); formal analysis (equal); investigation (equal) Shingo Kikuta: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (equal); supervision (equal); validation (equal); visualization (lead); writing—original draft (lead); writing—review and editing (lead).

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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