

METHODS & TECHNIQUES

Refinement of a technique for collecting and evaluating the osmolality of haemolymph from *Drosophila* larvae

Misato Kurio¹, Yuma Tsukasa¹, Tadashi Uemura^{1,2} and Tadao Usui^{1,2,*}

ABSTRACT

Ex vivo physiological experiments using small insect models such as *Drosophila* larvae have become increasingly useful to address fundamental biological questions. To perform such experiments, various artificial saline solutions have been developed, but their osmolality varies significantly from one to the next. Such a variation of osmolality stems, in part, from the difficulty of determining the true value of haemolymph osmolality in *Drosophila* larvae. Thus, there is a pressing need to refine protocols for collecting and measuring the osmolality of the larval haemolymph. Two major obstacles are thought to impede the accurate analysis of haemolymph collected from small insects: melanin formation and gut-derived contamination. Here, we greatly refined existing haemolymph collection methods, evaluated the purity of the collected haemolymph under melanin-free conditions, and concluded that the true value of haemolymph osmolality is close to 306.0 mOsm kg⁻¹ in *Drosophila* larvae.

KEY WORDS: Insect model, Gut-derived materials, Osmometry

INTRODUCTION

In recent years, *ex vivo* physiological experiments using small insect models such as *Drosophila* larvae have become increasingly important (Handke et al., 2014; Hu et al., 2020; Kanaoka et al., 2023; Lemon et al., 2015; Li et al., 2023; Nakamizo-Dojo et al., 2023; Onodera et al., 2017; Redhai et al., 2020; Terada et al., 2016). To perform such experiments, various artificial saline solutions have been developed. Notably, the osmolality is significantly different between solutions: normal saline solution (also known as Standard saline), 300.0–315.5 mOsm kg⁻¹ (Jan and Jan, 1976); external saline solution, 305.0 mOsm kg⁻¹ (Xiang et al., 2010); Baines external solution, 310–320 mOsm kg⁻¹ (Jovanic et al., 2016); Haemolymph-Like No.3 (HL3), 343.0 mOsm kg⁻¹ (Stewart et al., 1994); HL3.1, 309.1 mOsm kg⁻¹ (Feng et al., 2004); HL4/HL5, 361.0 mOsm kg⁻¹ (Stewart et al., 1994); HL6, 341.0 mOsm kg⁻¹ (Macleod et al., 2002); and Schneider's *Drosophila* medium, 360–374 mOsm kg⁻¹ (Schneider, 1972). These variable settings of osmolality rely on the single reported value of osmolality of 390±5.5 mOsm kg⁻¹ (mean±s.d.) (Pierce et al., 1999). Therefore, once another reliable value of haemolymph osmolality is determined,

the saline solution can be further optimized accordingly. Towards this objective, it is critically important to refine protocols for collecting pure haemolymph and measuring its osmolality.

In relatively large insects, such as tobacco hornworm larvae and adult honeybees, a substantial volume of fluid (≥0.2 ml) can quickly be collected without contamination, as it leaks spontaneously from an incised site on a leg or antenna (Adams and Wilcox, 1973; Borsuk et al., 2017; Łoś and Strachecka, 2018). The osmolality of haemolymph can be assessed by analysing either freezing-point or vapour-pressure depression. In contrast, for small insects, the haemolymph volume in each animal is extremely limited, necessitating the simultaneous processing of dozens of animals to obtain enough for osmolality measurement (≥10 µl). Although a capillary-type osmometer requires only a tiny amount of liquid (≥2 nl) for measurement, it is still difficult to extract haemolymph from legless animals such as *Drosophila* or honeybee larvae without contamination. In addition, two major obstacles hinder the accurate measurement of the haemolymph collected from small insects: (1) various biochemical reactions, such as melanization, are initiated as it takes longer to collect haemolymph from a batch of animals; (2) gut-derived contents contaminate the collected haemolymph (Borsuk et al., 2017; Palomino-Schätzlein et al., 2022; Tabunoki et al., 2019). Specifically, the melanization reaction yields heterogeneous biopolymers, such as eumelanin and pheomelanin, by consuming organic solutes in haemolymph such as tyrosine and cysteine, thereby probably reducing its osmolality. Conversely, when the haemolymph is contaminated by gut-derived materials, the measured osmolality should spuriously increase because soluble components of intestinal contents are highly concentrated in the hindgut lumen (Harpur and Popkin, 1965). It should be noted that various experimental conditions such as sample temperature might influence osmolality values, as the vapour-pressure depression method depends on thermodynamic assumptions (Grattoni et al., 2008). To fully address this issue, we need to employ membrane osmometry as a direct method instead of the vapour-pressure depression method. Nonetheless, Grattoni et al. (2008) reported that, in the physiological range, the difference in osmolality values obtained using these two methods is not significant. Hence, the vapour-pressure depression method seems to be a reasonable alternative under our experimental conditions.

In the present study, we aimed to establish a more reliable and reproducible osmolality measurement protocol for small insects. To this end, we re-examined each step of the preceding haemolymph collection methods (Palomino-Schätzlein et al., 2022; Pierce et al., 1999) and evaluated the purity of the collected haemolymph by employing a dye-tracing technique to detect food-derived materials (Sano et al., 2015), while haemolymph melanization was sufficiently suppressed by introducing a specific genetic mutation (Nam et al., 2012).

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MATERIALS AND METHODS

Fly husbandry

The flies were raised on a standard cornmeal–yeast–agar food at 25°C in constant-dark conditions. The following *Drosophila* stocks were used: $y^1 w^{67e23}; +; P\{CaryP\}attP2$ (BDSC 8622) and *Hayan*¹ (a gift from Won-Jae Lee, Seoul National University, Republic of Korea; Nam et al., 2012).

Collection of haemolymph using centrifugation and measurement of its osmolality

For tracing food-derived materials, 10 g l⁻¹ Brilliant Blue FCF (027-12842, Wako Pure Chemical Industry) was added to the food. For analysis, we crossed 20 females and 10 males in a food vial without blue dye for 3 days, and then transferred these flies to a new food vial with blue dye. In this vial, the flies laid eggs, and then the hatched larvae fed on the blue dye-labelled food.

Before starting each experiment, the osmometer was calibrated carefully. Sixty late third-instar larvae that were vigorously crawling on the inner wall of the vial were recovered with a paint brush. Larvae that had stopped moving were not collected. Hereafter, these crawling larvae will be referred to simply as ‘wandering larvae’. Male and female larvae were randomly selected. Subsequent operations up to centrifugation were performed in temperature-controlled rooms, at 4 or 25°C [cold temperature (CT) and room temperature (RT), respectively], and all instruments and reagents were precooled to the respective temperature before starting the procedures. Larvae were quickly washed twice in a 35 mm plastic dish (1000-035, AGC TECHNO GLASS Co., Ltd, Shizuoka, Japan) filled with pure water (06442-95, nacalai tesque, Inc., Kyoto, Japan) and transferred onto a sheet of absorbent paper (KimWipes®, S200 62011J-240, Nippon Paper Crexia Co., Ltd, Tokyo, Japan), and excess water was removed with a dry brush (Fig. 1). Larvae were processed in groups of 30 individuals at a time. Under a binocular dissecting microscope, the dorsal epidermis of the second thorax (T2) was carefully torn with tweezers (0208-5-PS, Dumont, Montignez, Switzerland) to make a small slit, and 30 larvae were then carefully transferred to two separate 0.2 ml PCR tubes (NN-2719S, TERUMO Corp., Tokyo, Japan) punctured slightly off-centre at the bottom so that the hole faced vertically downward when the tube was placed in a fixed angle rotor. The 0.2 ml PCR tube was then placed in a 1.5 ml tube

(131-615c, Fukae-kasei Co., Ltd, Tokyo, Japan) and centrifuged at 500 *g* for 1 min at 4 or 25°C using a refrigerated microcentrifuge (MX-300, TOMY). The first PCR tube was then discarded, and a second batch of 30 larvae, processed similarly and placed in a punctured 0.2 ml PCR tube, was placed in the same 1.5 ml tube and centrifuged, thus combining the haemolymph from all 60 larvae. After centrifugation, the second PCR tube was discarded. Importantly, for experiments under the CT condition, the second PCR tube was chilled at 4°C until just before centrifugation. Notably, the duration of manipulating larvae and haemolymph under ice-cold conditions should be more than 10 min and less than 20 min, as longer cooling of larvae may trigger their cold-tolerance responses such as osmolality changes in haemolymph (Zachariassen, 1985; Olsson et al., 2016). After confirming that the osmometer was calibrated correctly using the 290 mOsm kg⁻¹ standard solution (this procedure usually takes 2–3 min), an aliquot of 10 µl supernatant from the collected haemolymph in the 1.5 ml tube was transferred to the osmometer (VAPRO 5520, Wescor). It should be noted that osmolality is expressed in mmol kg⁻¹ when using this osmometer, which is the same as the value expressed in mOsm kg⁻¹.

Evaluation of food-derived materials in haemolymph

The collected haemolymph was incubated overnight to complete the melanization reaction at RT. A 2 µl aliquot of haemolymph was then used for an optical absorbance spectrum from 190 to 840 nm with a Nanodrop 2000c spectrometer (ND-2000c, Thermo Fisher Scientific Inc.). By monitoring the 629 nm absorbance of blue dye, food-derived materials in the haemolymph were traced (Fig. 2). We employed pure water as a reference for absorbance measurements, as the 629 nm absorbance of haemolymph collected from *Hayan*¹ mutant larvae fed the normal food without blue dye was almost zero (0.0097±0.0015; Fig. S1A).

Collection of haemolymph without centrifugation and evaluation of its food-derived materials

Ten incised larvae were prepared as described above, and were then piled up on a strip of Parafilm® (P7543-1EA, Sigma-Aldrich). The leaking haemolymph was transferred into a 1.5 ml tube using a

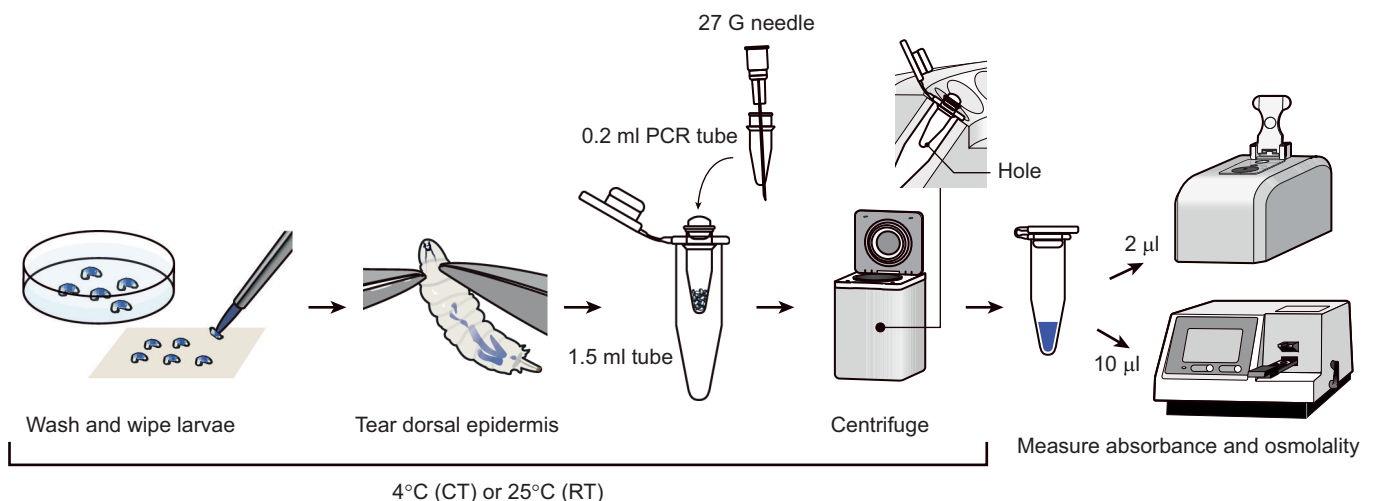


Fig. 1. Overview of the refined method for collecting haemolymph from *Drosophila* larvae by centrifugation. The procedure was carried out at 25°C (room temperature, RT) or 4°C (cold temperature, CT).

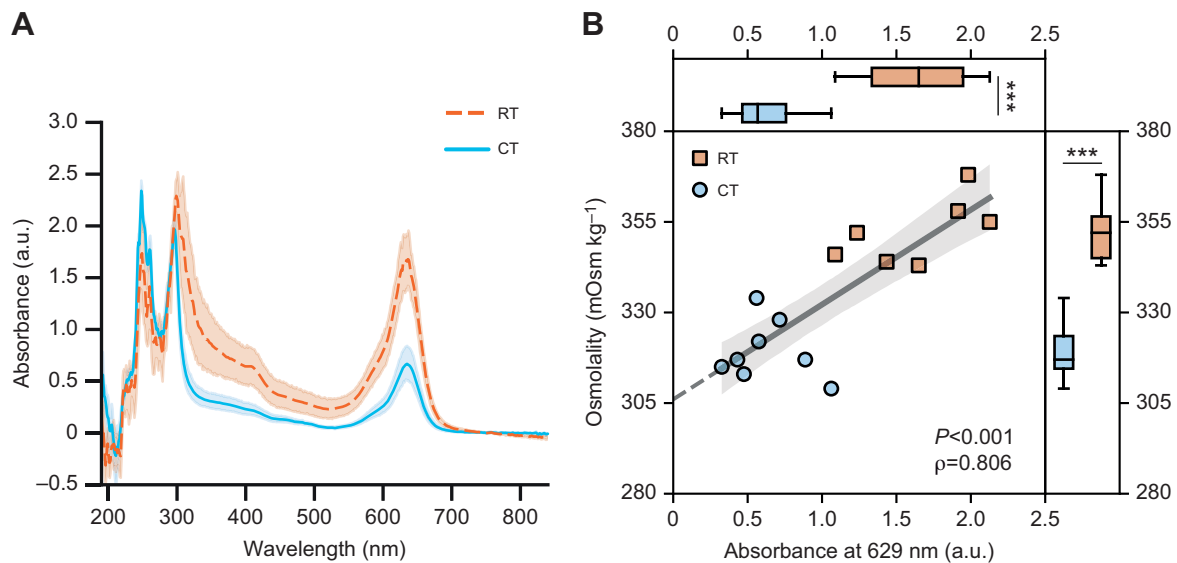


Fig. 2. The purity and osmolality of haemolymph collected from *Hayan*¹ mutant larvae by centrifugation depends on temperature conditions.

(A) Absorption spectra of haemolymph collected from *Hayan*¹ mutant larvae at RT or CT (means and 95% confidence intervals). (B) Absorbance at 629 nm and osmolality of haemolymph collected from *Hayan*¹ mutant larvae under RT or CT conditions, displayed as scatter plots (grey line and shading indicate linear regression and 95% confidence interval) and box plots. *** $P < 0.001$, Mann-Whitney *U*-test; $n = 7$ in RT and $n = 8$ in CT.

micropipette. The absorbance was measured immediately after collecting the haemolymph (Fig. 3A).

Data analyses

All data analyses were performed using Python 3 (version 3.7.6, Python Software Foundation) and R (version 3.6.1, R Development Core Team). Using Python packages, a linear model was applied to determine how the fluid osmolality correlated with contamination by gut-derived materials. To estimate the true value of haemolymph osmolality, we obtained the y -intercept of the linear equation using `LinearRegression()` in the Python package `sklearn.linear_model`. A Spearman's rank correlation test was performed to examine the correlation between absorbance at 629 nm and the osmolality of the haemolymph using `cor.test()`. The 95% confidence intervals were obtained using Python packages. A Mann-Whitney *U*-test was performed using `wilcox.exact()` in the R package `exactRanktests` (version 0.8.31).

RESULTS AND DISCUSSION

To minimize the melanization reaction that could potentially affect the osmolality of the collected haemolymph, we utilized a *Drosophila* strain with a loss-of-function *Hayan* mutation (*Hayan*¹; Nam et al., 2012); *Hayan* encodes a serine protease that cleaves pro-phenoloxidase to yield phenoloxidase, a key melanin-producing enzyme that is active during microbial infection or after epidermal injury (Dudzic et al., 2019; Nam et al., 2012). In addition, we labelled the larval food with blue dye (1 g l⁻¹ Brilliant Blue FCF, see details in Materials and Methods) to trace the intestinal contents of food origin during haemolymph collection.

By tearing the dorsal epidermis of a *Drosophila* third instar wandering larva at RT (25.0±2.0°C), we collected haemolymph leaking from the incised site (Fig. 1), and found some contaminating bluish material. To examine the fluid osmolality and evaluate the potential contaminants, we then collected more than 20 µl of haemolymph by centrifugating the incised larvae (60 larvae; Palomino-Schätzlein et al., 2022; see details in Materials and

Methods). We measured the osmolality and the absorbance spectrum (190–840 nm) of the collected fluid, in which the absorbance at 629 nm specifically indicates the presence of the blue dye (RT in Figs 2 and 3B,C). The optical absorbance at 629 nm was 1.6±0.3 (arbitrary units), and the measured osmolality was 352±6.6 mOsm kg⁻¹, indicative of contamination by food-derived materials ($n = 7$; Figs 1 and 2). Notably, the moderate magnitude of the absorbance in the range from 300 to 600 nm possibly originated from the intrinsic optical properties of the food-derived materials and/or melanin pigments produced by residual phenoloxidase activity in the *Hayan*¹ mutant (Dudzic et al., 2019). Importantly, the 629 nm absorbance of haemolymph collected from the mutant larvae fed the normal food containing no blue dye was negligible (0.0097±0.0015; Fig. S1B). Therefore, even if such activity remains in the mutant, it will hardly interfere with the assessment of absorbance. These results suggest that the measured osmolality could be markedly overestimated as a result of contamination by food-derived materials under RT conditions.

If the contaminated materials were excreted from the larval anus during haemolymph collection, suppression of sphincter muscle contraction by cooling should help reduce such contamination. To induce and maintain larvae in a state of cold anaesthesia, all the equipment and reagents used for measurement including surgical tools, sampling tubes and distilled water for testing were precooled, and all manipulations were performed at CT (4.0±2.0°C). We then examined the absorbance at 629 nm and measured the osmolality of the haemolymph collected under the CT condition. The optical absorbance at 629 nm was significantly reduced (CT in Fig. 2A; Fig. S1B and S2B; 0.6±0.2, $P = 3.11 \times 10^{-4}$, Mann-Whitney *U*-test, $n = 8$) and the measured osmolality was 319±5.7 mOsm kg⁻¹ (CT in Fig. 2B; $P = 3.11 \times 10^{-4}$, Mann-Whitney *U*-test, $n = 8$). These results imply that the gut-derived contamination through defecation was significantly inhibited during haemolymph collection under CT conditions; therefore, the osmolality of the haemolymph collected under CT conditions is considered closer

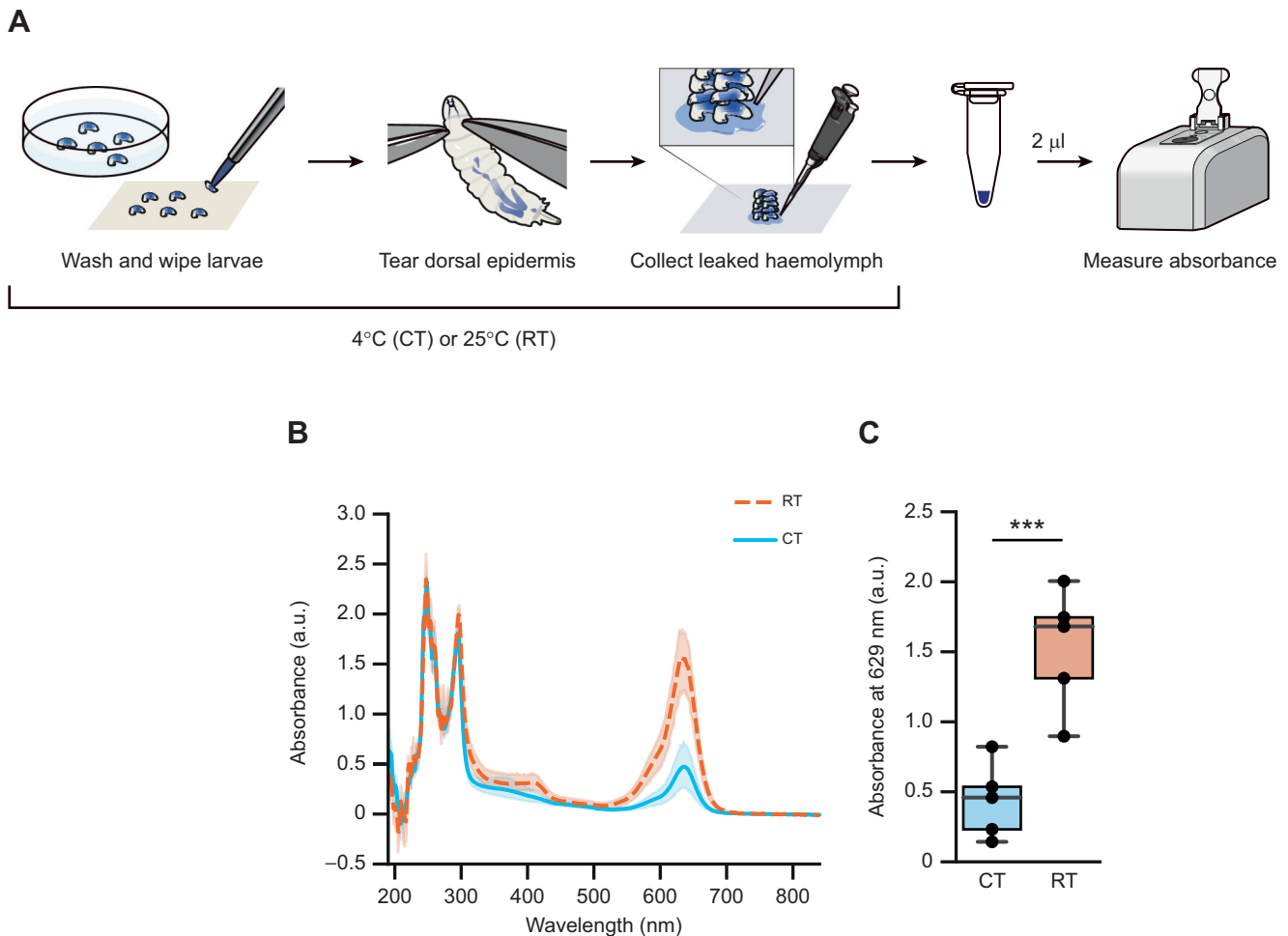


Fig. 3. Gut-derived materials contaminate samples before the centrifugation step under RT conditions. (A) Overview of a method for collecting haemolymph leaking spontaneously from *Drosophila* larvae without centrifugation at RT or CT. (B) Absorption spectra (a.u., arbitrary units) of haemolymph collected without centrifugation from *Hayan*¹ mutant larvae at RT or CT (means and 95% confidence intervals). (C) Absorbance at 629 nm of haemolymph collected from *Hayan*¹ mutant larvae under RT or CT conditions, displayed as box plots (median, upper and lower quartiles and 1.5 $\times$ interquartile range). *** P <0.001, Mann–Whitney U -test; n =5 in RT and n =5 in CT.

to the true value than that of the haemolymph collected at RT. Importantly, insufficient precooling of larvae resulted in varying levels of contamination (data not shown).

We then aimed to estimate the true value of haemolymph osmolality using these data. If the deviations of osmolality largely depend on the amount of contaminating gut-derived materials, the data for the fluid osmolality should be linearly correlated with the corresponding absorbance at 629 nm. In fact, a linear model provided a good fit to these data (Fig. 2B; $y=26.1x+306.0$, where x is absorbance at 629 nm and y is osmolality; Spearman's rank correlation: $\rho=0.806$, $P=2.85\times 10^{-4}$). Importantly, as noted above, we found that the 629 nm absorbance of haemolymph collected from *Hayan*¹ mutant larvae fed the normal food without blue dye was almost zero (0.0097 ± 0.0015 ; Fig. S1A). Based on this linear relationship, we propose that the true value of the haemolymph osmolality is close to 306.0 mOsm kg⁻¹, corresponding to the y -intercept of the linear equation.

This suggests that quasi-isotonic saline solutions, such as the external saline solution and HL3.1, would be more advantageous for preserving live specimens in a physiological state, particularly in terms of maintaining the appropriate osmolality (Feng et al., 2004; Li et al., 2023; Xiang et al., 2010). Notably, the proposed value

is comparable to the reported haemolymph osmolality in *Drosophila* adults (300–320 mOsm kg⁻¹; Jourjine et al., 2016; Senapati et al., 2019). Importantly, while various mechanisms maintain haemolymph osmolality in a certain physiological range, osmolyte concentrations in haemolymph can fluctuate as a result of various interferences such as environmental stress and metabolic changes. In addition, the water content of haemolymph shows not only developmental changes but also environmental variation depending on dietary composition (Olsson et al., 2016; Jourjine et al., 2016).

Next, we tried to determine the specific step at which gut-derived material contaminated the collected haemolymph. Our suspicion was that the strong centrifugal force during the centrifugation process could have extruded food-derived materials from the hindgut. To test this hypothesis, we tore 10 larvae on a piece of Parafilm and harvested the haemolymph leaking from the incised sites without centrifugation (Fig. 3A), comparing the procedure under RT and CT conditions. The absorbance at 629 nm of haemolymph collected under CT conditions was significantly smaller than that under the RT conditions (Fig. 3B,C; $P=0.00794$, Mann–Whitney U -test, $n=5$), indicating that gut-derived materials had mostly contaminated the sample even before the centrifugation step under RT conditions.

We then asked whether this refined osmolality measurement protocol (CT conditions, with centrifugation, as described in Materials and Methods) is also applicable for a strain that is commonly used as an experimental control ($y^1 w^{67c23}$). We measured the osmolality and absorbance at 629 nm of the collected haemolymph under RT and CT conditions and found that the optical absorbance values at 629 nm were significantly different (Fig. S2; RT 2.0 ± 0.2 , CT 1.5 ± 0.2 ; $P=0.0411$, Mann–Whitney U -test, $n=6$) and the osmolality values were also significantly different (Fig. S2; RT 334 ± 4.3 mOsm kg^{-1} , CT 310 ± 6.8 mOsm kg^{-1} ; $P=2.17 \times 10^{-4}$, Mann–Whitney U -test, $n=6$). Here, a linear model provided only a modest fit to the data (Fig. S2; $y=24.6x+279.6$, where x is absorbance at 629 nm and y is osmolality; Spearman's rank correlation: $\rho=0.587$, $P=0.049$). These findings suggest that our refined measurement protocol is also applicable for other strains with intact melanization ability. Nonetheless, the perturbations in osmolality measurement should be taken into account, and these may be attributed to the significant melanization. In other words, the 629 nm absorbance is composed of two factors: the blue dye and the melanization products. Therefore, the y -intercept does not simply correspond to the true haemolymph osmolality. Therefore, if we want to precisely calculate the true value of haemolymph of $y^1 w^{67c23}$, the amount of the 629 nm absorbance caused by melanization should be measured and the value adjusted appropriately. In addition, as Hayan^1 and $y^1 w^{67c23}$ may have different genetic backgrounds, this may lead to differences in the true value of haemolymph osmolality.

In this study, we refined a method to assess haemolymph osmolality in *Drosophila* larvae. Our new protocol allows us to reliably evaluate the osmolality of small insects, which has previously shown wide variability in reported values because of the technical difficulty in collecting pure haemolymph from small legless insects. Furthermore, our investigation illuminated the pivotal role of a careful precooling procedure in preventing contamination of the haemolymph by gut-derived materials. In addition, this protocol could be applied for robust measurement of haemolymph osmolality in other small insects such as mosquito larvae or *Leptopilina boulardi* larvae, a parasitoid wasp species that develops inside *Drosophila* larvae.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.K., Y.T., T. Usui; Methodology: M.K., Y.T.; Software: Y.T.; Validation: M.K., Y.T., T. Uemura, T. Usui; Formal analysis: M.K., Y.T.; Investigation: M.K., Y.T.; Resources: T. Usui; Data curation: M.K., Y.T.; Writing – original draft: M.K., T. Usui; Writing – review & editing: M.K., Y.T., T. Uemura, T. Usui; Visualization: M.K., Y.T.; Supervision: T. Usui; Project administration: T. Usui; Funding acquisition: Y.T., T. Uemura, T. Usui.

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Data availability

All relevant data can be found within the article and its supplementary information.

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