



# Proximate mechanisms of drought resistance in *Phytoseiulus persimilis* eggs

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## Abstract

Under drought stress, *Phytoseiulus persimilis* females are able to lay drought-resistant eggs through an adaptive maternal effect. The mechanisms making these eggs drought resistant still remain to be investigated. For this purpose, we studied the physiological differences between drought-resistant and drought-sensitive eggs. We compared the volume and the surface-area-to-volume ratio (SA:V) of the eggs, their sex ratio, their chemical composition (by gas chromatography-mass spectrometry), their internal and external structure [by scanning electron microscope (SEM) and transmission electron microscope (TEM) images], and their developmental time. Our results show that drought-resistant and drought-sensitive eggs have a different chemical composition: drought-resistant eggs contain more compatible solutes (free amino acids and sugar alcohols) and saturated hydrocarbons than drought-sensitive eggs. This difference may contribute to reducing water loss in drought-resistant eggs. Moreover, drought-resistant eggs are on average 8.4% larger in volume, and have a 2.4% smaller SA:V than drought-sensitive eggs. This larger volume and smaller SA:V, probably the result of a higher water content, may make drought-resistant eggs less vulnerable to water loss. We did not find any difference in sex ratio, internal or external structure nor developmental time between drought-resistant and drought-sensitive eggs. These results mark the first step in the understanding of the strategies and the energetic costs involved in the production of drought-resistant eggs in *P. persimilis* females.

**Keywords** Egg · Desiccation · Maternal effect · Phytoseiidae · Metabolomic profiling

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## Introduction

Parental care is a behavioral strategy through which parents increase the survival and growth of their offspring, often at a cost to their own survival and reproduction (Royle et al. 2012). Some insect and arachnid species demonstrate elaborate forms of parental care, and a broad diversity in extent and forms of care (Costa 2006; Wong et al. 2013; Gilbert and Manica 2015). For example, the burying beetle *Nicrophorus vespilloides* Herbst provides food to its larvae via regurgitation, and acacia thrips in Australia (*Dunatothrips aneurae* Mound) build small niches, called ‘domiciles’, to protect their larvae from desiccation (Wong et al. 2013; Gilbert 2014). In some spider and mite species, eggs are covered with silk layers or debris, to protect them against predators or stressful abiotic conditions (Marquardt et al. 2016; Santos et al. 2017). Besides these parental traits enhancing offspring fitness, parental care can also benefit offspring through adaptive maternal effects (Benowitz et al. 2015). Adaptive maternal effects are defined as mechanisms of transgenerational phenotypic plasticity whereby, in response to a predictive environmental cue, a mother can change the phenotype of her offspring, and prepare it for the environmental conditions predicted by the cue (Mousseau and Fox 1998; Fox et al. 1999). The key characteristic of adaptive maternal effect phenotypes is their plasticity, providing organisms with the ability to respond to environmental variations. Whereas the consequences of adaptive maternal effects are often clear, i.e., an increase in offspring fitness in a specific environment, the physiological adaptations responsible for this higher fitness are more complicated to discern. Understanding the physiological adaptations involved in adaptive maternal effects is key to better estimate their consequences for offspring performance, as well as the costs of such maternal effects for the mothers, and finally the constraints to their evolution.

Here, we focus on an adaptive maternal effect triggered by low relative humidity (RH) conditions in the predatory mite *Phytoseiulus persimilis* Athias-Henriot. In a previous study, we demonstrated that *P. persimilis* females start laying drought-resistant eggs when they are exposed to constant low RH (65% RH) (Le Hesran et al. 2019b). The physiological adaptations making these eggs drought resistant remain to be investigated.

Protection against desiccation in insects and mites includes various non-mutually exclusive strategies. One strategy consists in increasing the initial body water content, mainly stored in the hemolymph and the tissues (Gibbs et al. 1997; Gray and Bradley 2005; Gefen et al. 2006). For example, body water content increased by 30% after selection for drought resistance in populations of *Drosophila melanogaster* Meigen (Gibbs et al. 1997). Insect and mite eggs have a large surface-area-to-volume ratio (SA:V): they contain a small volume of water, that can be lost through a relatively large surface. Although the relationship between egg composition and egg volume is not clear, we can expect that an increase in initial water content would result in an increase in egg volume and a decrease in SA:V. In the butterfly *Bicyclus anynana* (Butler), for example, two lines of females were artificially selected: a line producing large eggs (L) and a line producing smaller eggs (S). Eggs from the L line contained more water and lipids, and had a higher hatching success when reared at low RH (Karl et al. 2007). Another strategy for drought resistance consists in reducing water loss rate (Gibbs et al. 1997; Gray and Bradley 2005; Gefen et al. 2006). Water loss occurs mainly via evaporation through the cuticle, and to a lesser extent via respiration and excretion (Dittrich and Streibert 1969; Kühsel et al. 2017). A reduction in water loss rate can be achieved via changes in the chemical composition of the cuticle. For example, lipid analysis after drought acclimation in the springtail *Folsomia candida* Willem revealed changes in membrane fatty acids (Bayley et al. 2001). Furthermore, microbial symbionts

confer desiccation resistance to the saw-toothed grain beetle *Oryzaephilus surinamensis* (L.) by changing the cuticle thickness, melanization and hydrocarbon profile (Engl et al. 2018). Instead of a cuticle, insect and mite eggs are surrounded by a chorion (or eggshell), which should reconcile, even under drought stress, the opposing demands of water retention and respiration (Lees 1961). To make their eggs more waterproof and reduce water loss rate, some terrestrial arthropods change the physical structure of the chorion. In the mite *Petrobia latens* (Müller), females add an external coating of hard wax to their eggs to protect them from desiccation during diapause (Beament 1959; Lees 1961). Alternatively, in other mite species, the egg is covered with a protective exochorion layer, formed before or during oviposition (Marquardt et al. 2016). In *Tyrophagus perniciosus* Zakhvatkin, for example, the exochorion takes the form of locular chambers on the surface of the egg, and may contribute to reduction of water loss (Witalinski 1993). A third strategy to protect eggs against desiccation might be egg retention, leading to a shorter egg developmental time. In the phytoseiid mite *Phytoseius hawaiiensis* Prasad, females retain their eggs until just before the larvae hatch, when the substrate is unsuitable for oviposition. Extreme variations in egg hatching time were observed in this species, from 10 min to over 4 days (Sanderson and McMurtry 1984). Similarly, females of *Neoseiulus cucumeris* (Oudemans) reduce oviposition when exposed to a predator by retaining the egg inside their body, gaining some time to search for less risky oviposition sites, and leading to a shorter egg developmental time (Montserrat et al. 2007).

With the aim to understand the physiological adaptations providing drought resistance to *P. persimilis* eggs, we investigated *P. persimilis* eggs laid by females exposed to constant low (drought-resistant eggs) and to constant high humidity (drought-sensitive eggs). We focused on the three strategies described previously: increase in initial egg water content, reduction of water loss rate through the chorion, and egg retention. We first measured the volume and the SA:V of the two groups of eggs. In *P. persimilis*, a change in egg volume could reflect a change in water content, a thicker chorion, but also a change in offspring sex ratio, since female eggs are larger than male eggs (Toyoshima and Amano 1998; Walzer and Schausberger 2015). In phytoseiid mites, short-term variations in sex ratio are related to temperature, humidity, wind speed, mating duration, and prey density (Amano and Chant 1977; Dyer and Swift 1979; Toyoshima and Amano 1998; Lü et al. 2019). Therefore, in parallel with egg volume, we assessed the sex ratio of the offspring coming from drought-resistant and drought-sensitive eggs. Secondly, we analyzed the chemical composition of the two groups of eggs, by Gas Chromatography-Mass Spectrometry (GC-MS), and we studied the internal and external structure of the eggs, by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Finally, we compared the developmental time of drought-resistant and drought-sensitive eggs.

## Materials and methods

### Predatory mites

The *P. persimilis* strain used in this study was obtained from a mass rearing (Koppert Biological Systems), and reared in closed Petri dishes containing around 100 individuals each. Each Petri dish (7.5 cm diameter, 3 cm high) contained an agar layer (1/100 diluted agar powder; VWR Chemicals, Amsterdam, The Netherlands) on which a disk (7 cm diameter) of a cucumber leaf (*Cucumis sativus* ‘Pyralis’) infested with a mixture of all stages of

spider mites (*Tetranychus urticae* Koch) was placed, with the adaxial side facing the agar plate. Predatory mites were transferred to new Petri dishes once a week, and maintained in a climate chamber at  $18 \pm 1$  °C and  $65 \pm 2\%$  RH, with L16:D8 photoperiod (see Le Hesran et al. 2019a for details).

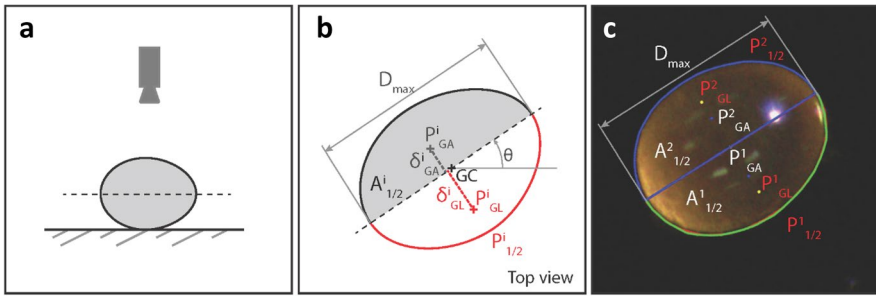
To control for the influence of *P. persimilis* female age, we used even-aged cohorts of young adult females (7 days since the egg stage). These females were collected as eggs and kept for 7 days in a closed Petri dish in a climate chamber at  $70 \pm 2\%$  RH and  $25 \pm 1$  °C. Inside the Petri dish, a cucumber leaf disk infested with spider mites was placed upside down on an agar layer, to provide optimal conditions for the development of these eggs to adults.

## Humidity treatments

Consecutive groups (blocks) of 15–20 *P. persimilis* females were exposed to either of two humidity treatments: ‘constant low’ (65% RH) or ‘constant high’ (95% RH). For this, females were placed in individual plastic cups (3.5 cm diameter, 2.8 cm deep), covered by a lid with a fine gauze (90 µm mesh). To provide them with an oviposition substrate, a thin layer of cardboard with hairy surface facing up was fixed to the bottom of the cup with double-sided tape (Tesa®). For humidity treatment ‘constant low’, a block consisted of 15–20 cups, placed upside down on top of a wire platform (2.5×2.5 cm spacing) inside a closed plastic box (L×W×H=40×25.5×16.5 cm). The relative humidity inside the box was regulated with a humidifier (Cigar Oasis Excel, Farmingdale, NY, USA), to achieve constant humidity of 65% RH (average  $64.7 \pm 1.6$ ). For humidity treatment ‘constant high’, a block consisted of 15–20 cups placed upside down on an agar layer at the bottom of a closed plastic box. The relative humidity inside the box was maintained at 95% RH (average  $96.2 \pm 1.7$ ). The two boxes containing the cups were placed in a climate chamber at  $70 \pm 2\%$  RH and  $25 \pm 1$  °C (L16:D8 photoperiod). The females were provided with fresh *T. urticae* (larvae, nymphs and adults) ad libitum every day. A data logger (LogTag TRIX 8) was placed in each box containing the cups, to measure relative humidity and temperature. For logistic reasons, in the five experiments described hereafter, *P. persimilis* females were exposed to the two humidity treatments for different time periods (from 48 to 120 h). After 48 h exposure to constant low RH, around 60% of *P. persimilis* females lay drought-resistant eggs, which is high enough to detect a difference between eggs from both treatments (Le Hesran et al. 2019b). Moreover, the percentage of females laying drought-resistant eggs increases with time exposure to low RH. It is, therefore, unlikely that this difference in exposure time prevented us from detecting differences between drought-resistant and drought-sensitive eggs.

## Egg volume and surface-area-to-volume ratio

*Phytoseiulus persimilis* females were exposed to either of both humidity treatments for 72 to 102 h, and 0- to 6-h-old eggs laid by these females were collected. For this, we removed all eggs from each cup after 72 h of exposure, and collected the freshly laid eggs 6 h later, i.e., after 78 h of exposure. We repeated the same procedure between 96 and 102 h of exposure. The collected eggs were placed on a smooth black PVC plate (L×W×H=8×4×0.5 cm) in such a way that the longest semi-major axis was facing up (Fig. 1a). A picture of each egg was taken, using a Dino-Lite Digital Microscope installed on a stereo microscope (Stemi 508) with KL 300 LED light source at level 3 (Zeiss



**Fig. 1** Scheme of the automated egg geometry estimation. **a** Egg placed on the observation surface. **b** Derived characteristics of the top-view egg section; maximum diameter ( $D_{max}$ ), geometrical center of the area ( $P^i_{GA}$ ) and of the outer perimeter ( $P^i_{GL}$ ) at each symmetrical lobe, and distance from the geometrical centers and the main symmetry axis ( $\delta^i_{GA}$  and  $\delta^i_{GL}$ ). **c** Example of a post-processed egg image

Nederland, Breda, The Netherlands). The camera was re-calibrated for each block. A computer vision algorithm (OpenCV, Python) was developed to automate the estimation of geometrical characteristics from egg images. This consisted of a color-based segmentation algorithm to derive the egg contour. Then, the maximum egg diameter was estimated ( $D_{max}$ ). The egg top-view projection was divided into two symmetrical lobes (Fig. 1b), for which the cross-section area ( $A^i_{1/2}$ ) and outer perimeter ( $P^i_{1/2}$ ) were computed. The three-dimensional geometry of the egg was derived from a single image under the assumption that the egg is approximated by a solid of revolution and that the computed  $D_{max}$  represents the symmetry axis. The first and second Pappus’s centroid theorems were used to estimate the volume ( $V^i_{egg}$ ) and surface area ( $S^i_{egg}$ ) of each egg as:

$$\begin{aligned} V^i_{egg} &= 2\pi \cdot \delta^i_{GA} \cdot A^i_{1/2} \\ S^i_{egg} &= 2\pi \cdot \delta^i_{GP} \cdot P^i_{1/2} \end{aligned} \tag{1}$$

where  $\delta^i_{GA}$  represents the distance between the axis of symmetry and the centroid of  $A^i_{1/2}$ , and  $\delta^i_{GP}$  is the distance between the axis of symmetry and the centroid of the egg semi-perimeter (Fig. 1b). Egg volume and surface area were derived as the mean from those computed from both symmetrical lobes (Fig. 1c).

For each egg, the maximum diameter, volume, surface area and SA:V were estimated. We carried out seven blocks for humidity treatment ‘constant low’ and seven blocks for humidity treatment ‘constant high’, with 6–46 eggs per block. In total, 238 eggs were examined (127 eggs for treatment ‘constant high’, 111 eggs for treatment ‘constant low’). An error analysis was conducted to assess the measurement repeatability and the influence of relative positioning misalignments (see Electronic Supplementary Material, ESM1 and ESM2). Successive observations of samples produced standard deviations of  $\sigma_{Dmax}=0.0044$  mm;  $\sigma_V=0.00024$  mm<sup>3</sup> and  $\sigma_{SA:V}=0.55$  mm<sup>-1</sup>, and coefficients of variation of 0.92, 2.62 and 0.95%, respectively.

**Offspring sex ratio**

*Phytoseiulus persimilis* females were exposed to either of both humidity treatments for 78–102 h. During this time interval, we collected 0- to 18-h-old eggs from each treatment,

and placed them in two Petri dishes containing an agar layer, a cucumber leaf disk and enough spider mites to guarantee their development to adults. One week after egg collection, we counted the number of males and females present in each Petri dish. In case of any doubt about an individual, we made a glass slide preparation and placed the mite in a drop of lactic acid. The glass slide was then covered with a cover slip and placed on a heated plate (33 °C) for 72 h. After 72 h, males and females could be differentiated by the external morphology of the anal region: *P. persimilis* females have the anus inside a circular shield, whereas males do not have such a shield structure (see ESM3). We carried out seven blocks for treatment ‘constant high’, and nine blocks for treatment ‘constant low’, with 15–20 females and 15–56 offspring individuals per block.

### Chemical composition of eggs

Adult females were exposed to either of both humidity treatments for 72–102 h. During this time interval, we collected 0- to 18-h-old eggs. For each block, 100 eggs from each humidity treatment were placed in two Eppendorf tubes (one tube per treatment) and stored at –18 °C. We carried out 10 blocks per treatment.

### Sample preparation

Each sample (Eppendorf tube) was extracted in 1 mL methanol under ultra-sonication for 20 min. Subsequently, the samples were centrifuged at 13,000 rpm for 10 min. The supernatant (500 µL) was collected for each sample, transferred to a new micro-tube and dried completely in a centrivap concentrator (Labconco). The dried extracts were then re-dissolved in 100 µL pyridine under ultra-sonication for 5 min. Then, 100 µL of *N,O*-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+TMCS (99:1); Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) were added to the samples and they were incubated at 80 °C for 50 min. The samples were cooled down to room temperature and methyl palmitate was added as internal standard with a final concentration of 20 ng/µL. Finally, the samples were transferred to micro-inserts for GC–MS analysis.

### GC–MS analysis and data extraction

The samples were analyzed with an Agilent 7890A gas chromatograph equipped with a 7693 automatic sampler and coupled to a 5975C mass single-quadrupole detector (Agilent, Folsom, CA, USA). The samples were separated using a DB-5 GC column (30 m×0.25 mm, 0.25 µm film; J&W Science, Folsom, CA, USA) and He (99.9% purity) as a carrier gas at a flow rate of 1.2 mL/min. The injection port in splitless mode was set at 270 °C and the sample injection volume was 1 µL. The oven temperature was programmed to start at 60 °C, kept for 1 min, then increased to 202 °C at 7 °C/min and kept again for 1 min. The temperature then increased to 212 °C at 4 °C/min, was held for 1 min, and then finally increased to 290 °C at 7 °C/min and was held for 3 min. The interface temperature was 280 °C, and the ion source and quadrupole temperature of the mass detector were 230 and 150 °C, respectively. The ionization energy in EI mode was 70 eV and peaks were identified by comparison of their ion spectra with the NIST library (version 2008), or by comparison of their retention time with standard compounds.

For GC–MS data acquisition, all of the total ion chromatograms were automatically integrated and peaks were identified using Mass Hunter Qualitative Analysis software

v.B.07.00 (Agilent). The area of the identified compounds was normalized to the area of the internal standard (20 ng/ $\mu$ L of methyl palmitate).

### Internal and external structure of eggs

*Phytoseiulus persimilis* females were exposed to either of both humidity treatments for 120 h. After 120 h, 0- to 24-h-old eggs were collected from each treatment. To analyze the internal and external structure of the eggs, we used SEM and TEM.

### Scanning electron microscopy (SEM)

Non-dehydrated samples and dehydrated samples were used for the SEM analysis. For the non-dehydrated samples, we used two specimen preparation protocols. First, eggs were either high-pressure frozen (HPF) using Leica EM PACT or frozen by plunging in liquid nitrogen slush, using Cryo ALTO 2500 (Gatan). Just before the HPF, we placed three *P. persimilis* eggs from each humidity treatment on two copper specimen carriers (one carrier per humidity treatment, 1.5 mm diameter, Leica), that were then filled in with 20% Bovine Serum Albumin (BSA). For the plunging method, we placed the eggs onto a SEM specimen holder (aluminium) covered with Tissue-Tek<sup>®</sup> (EMS). After freezing, samples were transferred into a liquid nitrogen bath where they were longitudinally fractured with a scalpel under optical microscope control. Some eggs from the plunging method were kept intact, to analyze their external structure. The eggs were then transferred under vacuum to a Cryo ALTO 2500 chamber of a JEOL 7401F SEM, cooled at  $-140$  °C. Inside the chamber, sublimation was conducted at  $-95$  °C for 5 min. Temperature was then decreased to  $-140$  °C and the samples were sputter coated with platinum-palladium for 100 s. Finally, samples were examined by JEOL 7401F SEM at 1–2 kV using the Everhart–Thornley detector of secondary electrons.

For the dehydrated samples, eggs were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) overnight, at 4 °C, then washed in 0.1 M PB with 4% glucose 3 $\times$  for 15 min and post-fixed in 2% OsO<sub>4</sub> for 2 h. The samples were washed and dehydrated in a graded acetone series (30, 50, 70, 80, 90, 95 and 100% acetone in water), for 15 min in each solution. The samples were dried using Critical Point Dryer (CPD2, Pelco), mounted using a carbon tape on aluminium stub and sputter coated with gold (Bal-Tec). Specimens were observed using a JEOL 7401F and SEM Apreo (Thermo Fisher Scientific) at 4 kV.

### Transmission electron microscopy (TEM)

For TEM, the samples were high-pressure frozen (see details above) and transferred to the freeze substitution media containing 2% OsO<sub>4</sub> in 100% acetone cooled at  $-90$  °C (Leica AFS). After 96 h, the temperature was increased (5 °C/h) to  $-20$  °C and, after the next 24 h, to 4 °C. At room temperature, the samples were rinsed 3 $\times$  in 100% acetone (15 min each) and infiltrated in 25, 50 and 75% Embed 812 resin/acetone solutions, for 1 h in each solution. Finally, samples were incubated in 100% resin overnight in a vacuum desiccator. Samples were polymerized at 60 °C. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined using a JEOL 1010 TEM.

## TEM images analysis

The ImageJ program (WS Rasband, ImageJ, US National Institutes of Health, Bethesda, MD, USA; <https://imagej.nih.gov/ij/>, 1997–2018) was used to measure thickness of the exochorion on two TEM images. On each image, the exochorion thickness was measured 10×, at 10 random locations, and the average and standard deviation of these 10 measurements were calculated.

## Egg developmental time

*Phytoseiulus persimilis* females were exposed to either of both humidity treatments for 48–54 h. After 48 h of exposure, all eggs were removed from each cup, and the freshly laid eggs (0–6 h old) were collected 6 h later. Each egg was placed with a brush in a hole (0.7 cm diameter, 0.4 cm deep) in a platform made of polymethyl methacrylate (PMMA) ( $L \times W \times H = 17.5 \times 15 \times 4.5$  cm) containing 30 holes (one egg per hole). Each hole had a fine gauze at the bottom (90  $\mu\text{m}$  mesh), to ensure contact with ambient air. The hole was then covered with a coverslip to prevent the larvae from escaping after hatching. The PMMA platforms (one platform per humidity treatment, per block) were placed in a climate chamber at  $85 \pm 2\%$  RH,  $25 \pm 1$  °C and L16:D8 photoperiod. The number of hatched larvae was recorded after 24, 41, 43, 45, 47, and 49 h. For each humidity treatment, we carried out eight blocks, with 9–22 eggs per block. In total, we assessed the developmental time of 257 eggs.

## Statistical analysis

The statistical analysis was performed in R (v.3.5.1) and SIMCA P (v.14.1, Umeå, Sweden).

## Egg volume and surface-area-to-volume ratio

To compare egg volume and SA:V between humidity treatments ‘constant high’ and ‘constant low’, we used a linear mixed effect model with Gaussian error distribution for both analyses. The variable humidity treatment was expressed as a fixed effect in the model. The blocks which had been performed at the same date were assigned to the same block number, and the block factor was expressed as a random effect in both models (by-block random intercept). We used the model-fitting method of the maximum likelihood, and used likelihood-ratio tests to select the most parsimonious models.

## Offspring sex ratio

To compare the proportion of males in the offspring of females exposed to the humidity treatments ‘constant high’ and ‘constant low’, we used a generalized linear mixed model with a binomial error distribution and logit link function. Humidity treatment was expressed as a fixed effect in the model, and block was expressed as a random effect



(by-block random intercept). We used the model-fitting method of the maximum likelihood, and used likelihood-ratio tests to select the most parsimonious model.

### Egg chemical composition

Multivariate data analysis (MVDA) was performed using SIMCA P. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed for GC–MS data with two classes (constant low and constant high humidity). For this model, a unit-variance (UV) scaling method was used to suppress the effects of other random factors and identify differences caused by humidity. All normalized data was scaled by UV method for PCA and PLS-DA models. An area value corresponding to half of the lowest area detected in the whole analysis was assigned to the compounds that were not detected in some samples. The correlated compounds to each treatment were selected by interpretation of the PLS-DA loading plot and variable importance for the projection (VIP) plot.

### Egg developmental time

For the analysis of egg developmental time, we studied the influence of the factor humidity treatment on hatching probability ('time to hatching', observed right-censored data) of *P. persimilis* eggs, during 49 h. For each humidity treatment, eggs that were observed during the same 49 h were grouped under the same block number. We used a mixed-effects Cox model, with humidity treatment as a fixed effect, and block as a random effect. We used likelihood-ratio tests to select the most parsimonious model, and plotted the estimated survival curves for each RH treatment using the Kaplan–Meier method (packages survival and survminer in R).

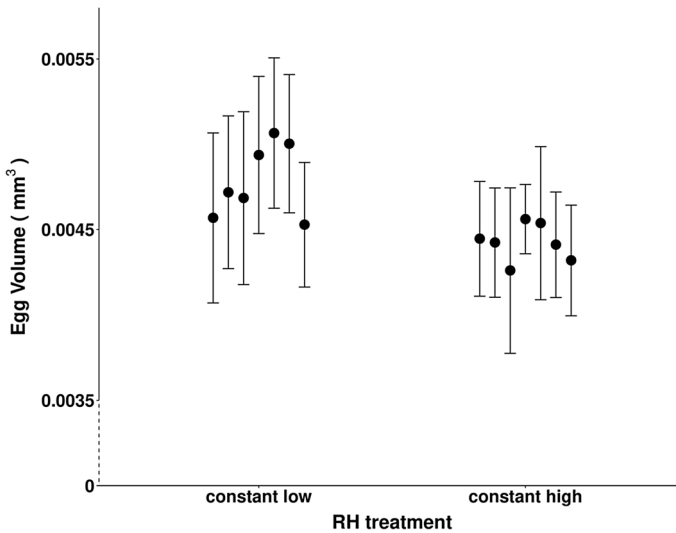
## Results

### Egg volume and surface-area-to-volume ratio

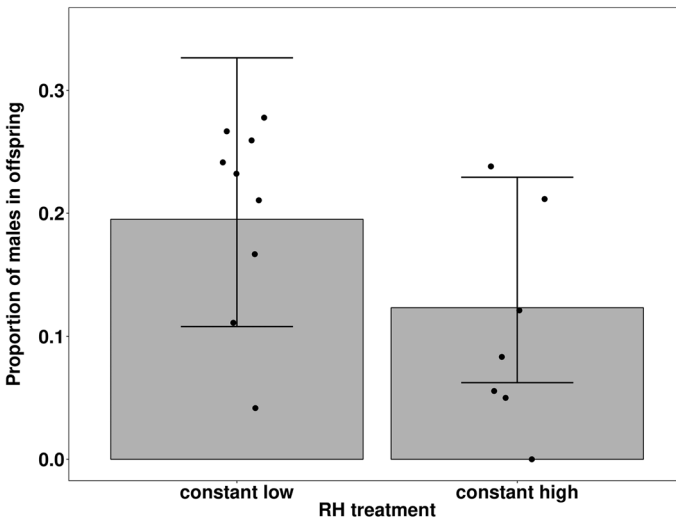
Humidity treatment had a significant effect on egg volume ( $\chi^2=31.6$ ,  $df=1$ ,  $P=2 \times 10^{-8}$ ; Fig. 2). On average, eggs laid by females exposed to constant low humidity had a bigger volume ( $4.8 \times 10^{-3} \pm 3 \times 10^{-4} \text{ mm}^3$ ) than eggs laid by females exposed to constant high humidity ( $4.4 \times 10^{-3} \pm 3 \times 10^{-4} \text{ mm}^3$ ) (estimated values + 95% CI). Humidity treatment also had a significant effect on egg SA:V ( $\chi^2=24.9$ ,  $df=1$ ,  $P=6 \times 10^{-7}$ ; see ESM4). On average, the eggs laid by females exposed to constant low humidity had a smaller SA:V ( $29.1 \pm 0.51 \text{ mm}^{-1}$ ) than those laid by females exposed to constant high humidity ( $29.8 \pm 0.51 \text{ mm}^{-1}$ ) (estimated values + 95% CI).

### Offspring sex ratio

Relative humidity significantly affected the sex ratio of offspring ( $\chi^2=3.87$ ,  $df=1$ ,  $P=0.049$ ; Fig. 3). When females were exposed to constant low humidity, the proportion of males in their offspring was on average 0.19 (–0.08 to +0.13). For females exposed to constant high humidity, the proportion of males in their offspring was on average 0.12 (–0.06 to +0.11) (estimated values + asymmetric 95% CI).



**Fig. 2** Mean ( $\pm$ SD) observed egg volume ( $\text{mm}^3$ ), for eggs laid by *Phytoseiulus persimilis* females exposed to two humidity treatments (constant low RH: 111 eggs, and constant high RH: 127 eggs). Each symbol represents the average egg volume for one replicate (n=37, 15, 19, 9, 14, 11, 6 eggs for constant low RH and n=46, 15, 18, 14, 9, 13, 12 eggs for constant high RH)



**Fig. 3** Observed (symbols) and mean ( $\pm$ 95% CI) estimated (bars) sex ratio (proportion of males) in offspring from *Phytoseiulus persimilis* females exposed to two humidity treatments (constant high and constant low RH). Each symbol represents one replicate, with offspring from 15–20 females (n=15, 24, 29, 18, 18, 19, 18, 27, 56 for constant low RH and n=18, 24, 33, 20, 18, 21, 52 for constant high RH)

## Chemical composition of eggs

Through GC–MS analysis we detected primary metabolites such as amino acids, fatty acids, monoglycerides, sugars, alkanes, and phosphoric acid. There were qualitative and quantitative differences in chemical composition between eggs from constant low and constant high humidity. In the PCA model (see ESM5), the samples were principally separated by humidity treatment ( $R^2=0.47$ ). However, there was a certain degree of overlap between the two groups, which was eliminated when a third principal component was added to the model. Therefore, even if humidity treatment has an effect on the chemical composition of the egg extracts, there are other unknown factors influencing the plot separation.

To obtain a clearer separation by a supervised method, a PLS-DA model was applied to the same dataset and it showed a clear effect of humidity on the chemical composition of the samples ( $Q^2=0.46$ ,  $P=0.03$  by CV-ANOVA) (Fig. 4a). Metabolites more associated to extracts of eggs laid under constant low humidity were amino acids, sugars, one alkane, one fatty acid, and phosphoric acid. Metabolites more associated to extracts of eggs laid under constant high humidity were two fatty acids, one sugar and one monoglyceride (Fig. 4b and ESM6). Serine is the metabolite that accounts most for eggs from constant low humidity, and *trans*- $\beta$ -octadecenoic acid is the metabolite that accounts most for eggs from constant high humidity. Both groups of eggs contained serine, which could indicate quantitative differences in this metabolite. The *trans*- $\beta$ -octadecenoic acid, however, was detected only in eggs from constant high humidity.

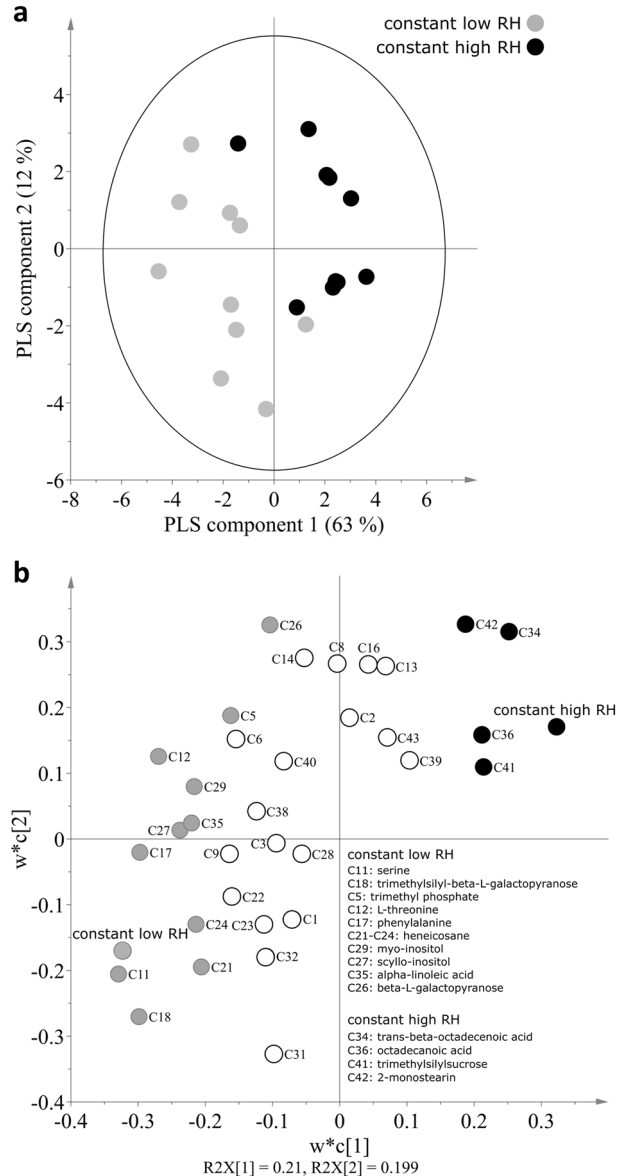
## Internal and external structure of eggs

We did not observe any clear structural difference between eggs laid under constant low and constant high humidity conditions. The external surface of all eggs was similar: smooth and without any external structure or pattern (Figs. 5a and 6a). Zooming in ( $\times 10,000$ ) on the external surface of the eggs did not reveal any visible respiratory pores. Regarding the internal structure, all eggs were covered with three layers: an exochorion, an endochorion, and a third fibrous layer, probably made of proteins (Figs. 5, 6 and 7). The most external layer, the exochorion, was thick and compact. Closely attached to the exochorion, was the endochorion, around  $10\times$  thinner than the exochorion. Between the endochorion and the embryo tissue was a fibrous layer, thicker than the endochorion, but thinner and less compact than the exochorion. This fibrous layer is the last layer torn by the hatching larva (Fig. 6b,c). On average (for 10 measurements on one egg), the exochorion was  $0.33 \pm 0.007 \mu\text{m}$  thick for an egg laid under constant low humidity, and  $0.43 \pm 0.009 \mu\text{m}$  thick for an egg laid under constant high humidity (Fig. 7b,d). Due to the limited number of TEM images, we were not able to measure more than one egg for each humidity treatment.

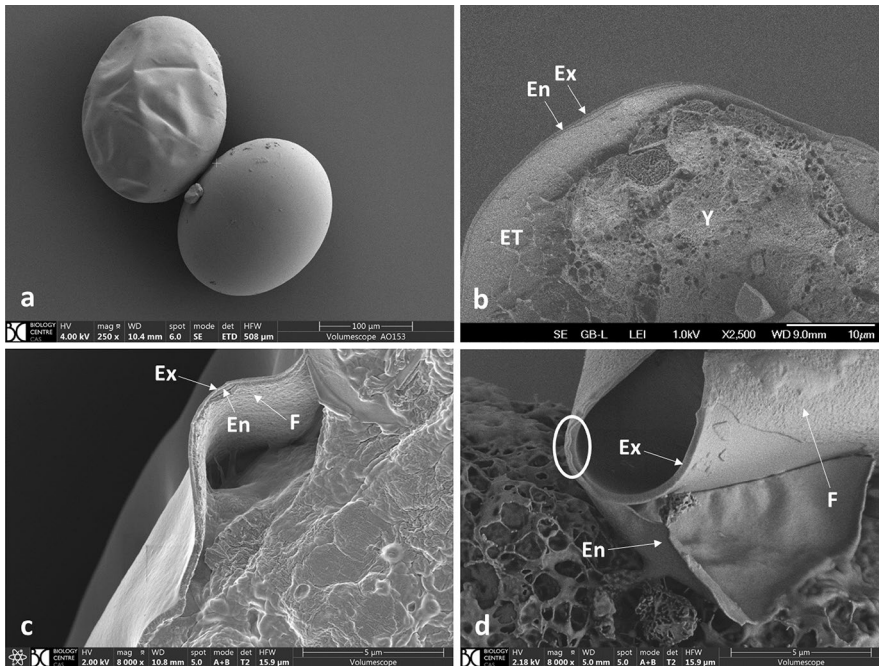
## Developmental time

Relative humidity did not influence egg developmental time ( $\chi^2=0.01$ ,  $df=1$ ,  $P=0.9$ ; Fig. 8). For both humidity treatments, more than 50% of the eggs hatched between 24 and 41 h after egg collection, and at least 98% of the eggs had hatched 49 h after egg collection. Since the eggs were between 0 and 6 h old when they were collected, the real developmental time of the

**Fig. 4 a** PLS-DA plot for chemical composition of eggs laid by *Phytoseiulus persimilis* females exposed to each of two humidity treatments (constant low and constant high RH) ( $Q^2=0.46$ ,  $P=0.03$ ). The ellipse represents the Hotelling's T2 (95%) border. Grey symbols represent 10 blocks for constant low humidity, black symbols represent 10 blocks for constant high humidity ( $n=100$  eggs per block). **b** Loading plot from PLS-DA analysis for *P. persimilis* eggs laid by females exposed to two humidity treatments (constant low and constant high RH). Eleven metabolites more correlated (loading value  $> 1$  on the VIP plot) to eggs laid under constant low humidity are represented by grey symbols. Four metabolites more correlated (loading value  $> 1$  on the VIP plot) to eggs laid under constant high humidity are represented by black symbols. White symbols represent metabolites that are not correlated to any humidity treatment. The full names of the chemical compounds corresponding to each labelled dot are listed in Electronic Supplementary Material ESM6



eggs could be up to 3 h longer than the developmental time presented in Fig. 8 (the 0–6 h difference was averaged to 3 h since multiple eggs were collected randomly between 0 and 6 h after laying).

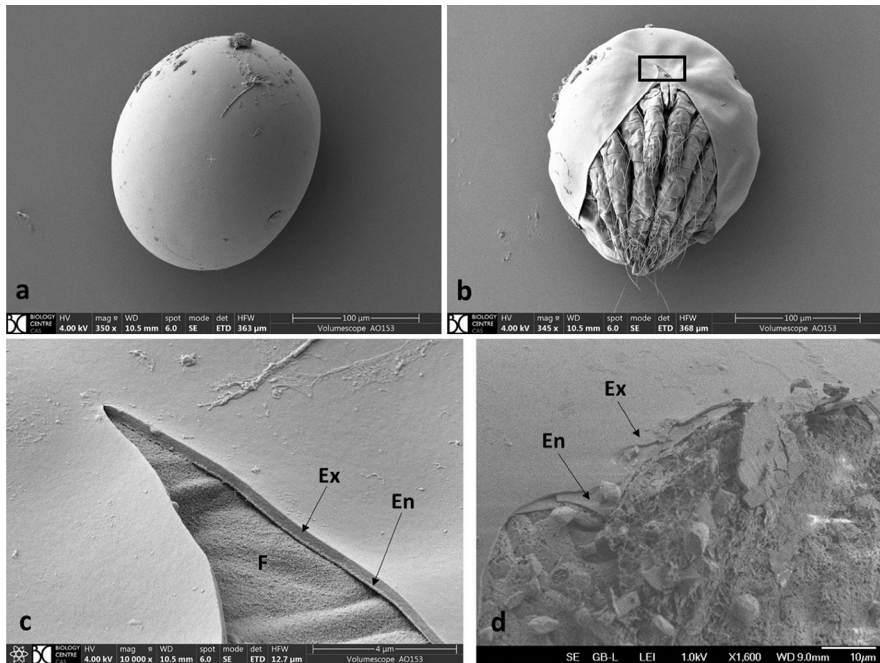


**Fig. 5** Scanning electron microscope (SEM) images of *Phytoseiulus persimilis* eggs laid by females exposed to constant low RH. **a** External view of a dehydrated egg (left), and a full egg (right). **b** Egg section, with exochorion, endochorion, embryo tissue and yolk. **c**, **d** Egg sections with external layers (exochorion, endochorion, fibrous layer) peeled off, due to dehydration. The circle in **d** highlights the three external layers. *Ex* exochorion, *En* endochorion, *ET* embryo tissue, *Y* yolk, *F* fibrous layer

## Discussion

The objective of our study was to better understand the physiological adaptations making *P. persimilis* eggs drought resistant. We show that humidity conditions experienced by adult *P. persimilis* females influence the chemical composition of their eggs. Drought-resistant eggs, laid under constant low humidity, contain higher levels of free amino acids, such as serine, phenylalanine, and threonine, higher levels of sugar alcohols, such as *myo*-inositol and *scyllo*-inositol, and higher levels of heneicosane, a saturated hydrocarbon.

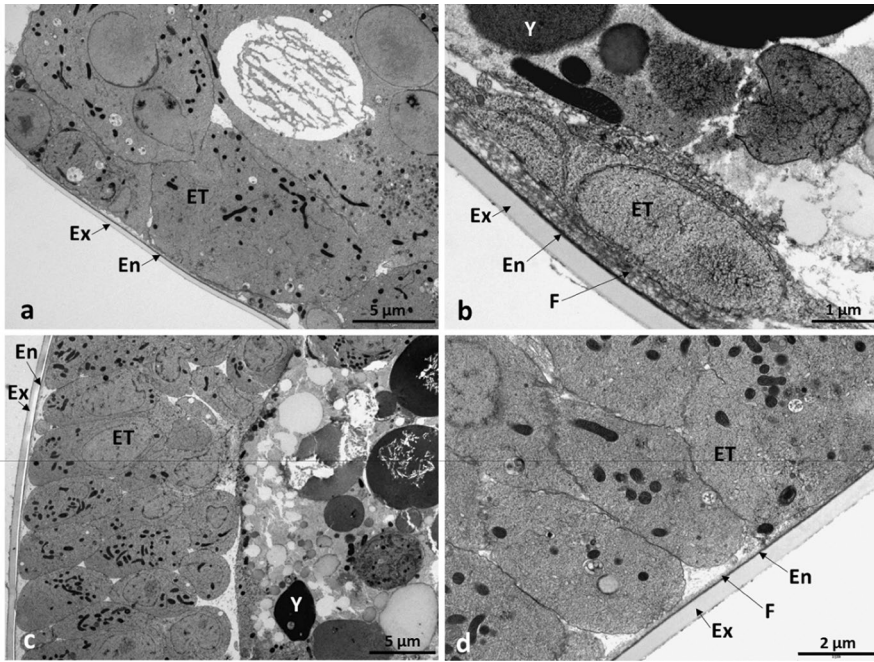
In water-stressed arthropods, the ability to regulate osmotic pressure of body fluids and to maintain cell volume is based on the accumulation of organic osmolytes, also called compatible solutes, such as sugar alcohols, free amino acids, and polyols (Miles 1966; Yancey et al. 1982; Holmstrup and Bayley 2013; Holmstrup et al. 2015; Holmstrup and Slotsbo 2018). Compatible solutes are small organic molecules with neutral charge and low toxicity at high concentrations, that act as osmolytes and help organisms survive extreme osmotic stress (Lang 2007). For example, when exposed to drought stress, the soil-dwelling springtail *F. candida* actively produces *myo*-inositol and glucose to increase its osmotic pressure and stop water efflux (Bayley and Holmstrup 1999). Furthermore, insects are able to adapt to climate variation by modifying the composition of their cuticle, which serves a variety of functions, including chemical communication and waterproofing (Nation et al. 1992; Woodrow et al. 2000). The cuticle of insects contains a wide range of organic



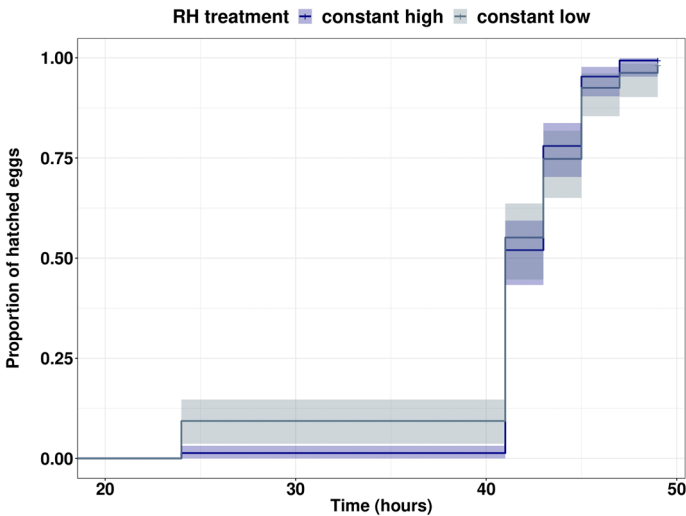
**Fig. 6** Scanning electron microscope (SEM) images of *Phytoseiulus persimilis* eggs laid by females exposed to constant high RH. **a** External view of a full egg. **b**, **c** External view of a larva hatching from an egg, **c** is an enlargement of a section (black rectangle) from image (**b**), where the eggshell splits, at the top of the egg. **d** Egg section, with exochorion and endochorion. *Ex* exochorion, *En* endochorion, *F* fibrous layer

compounds including fatty acids, alcohols, esters, ketones, glycerides, sterols, aldehydes and hydrocarbons. Hydrocarbons are the primary components of the lipid layer that are responsible for waterproofing the cuticle (Hadley 1978). In the mosquito *Aedes albopictus* (Skuse), for example, diapause eggs contain one-third more surface hydrocarbons and have a 50% lower water loss rate compared to non-diapause eggs (Urbanski et al. 2010). Our results suggest that the same mechanisms are involved in drought resistance of *P. persimilis* eggs: they contain higher levels of compatible solutes and hydrocarbons, likely reducing their water loss rate under dry conditions. The compatible solutes may be located inside the egg, in the tissues and the hemolymph, and the hydrocarbons may be located in the chorion. These metabolites are probably produced and allocated to the egg by the mother before laying, through a maternal effect triggered by constant low humidity. We previously showed that *P. persimilis* females start laying drought-resistant eggs after 6 to 24 h of exposure to low humidity (65% RH), and that the production of drought-resistant eggs can stop within 24 h if the females are transferred back to high humidity (Le Hesran et al. 2019b). These observations suggest that *P. persimilis* females start allocating more compatible solutes and hydrocarbons to their eggs shortly after the start of an exposure to constant low humidity.

Second, our data show that *P. persimilis* females exposed to constant low humidity lay larger eggs (on average 8.4% larger volume), with a 2.4% smaller SA:V than eggs laid by females exposed to constant high humidity. For an egg, a smaller SA:V results in a



**Fig. 7** Transmission electron microscope (TEM) images (cross sections) of *Phytoseiulus persimilis* eggs laid by females exposed to constant low (a, b) and constant high (c, d) RH. The embryo tissue and yolk are covered with three layers: exochorion, endochorion, and fibrous layer. In image (b), the exochorion is  $0.33 \pm 0.007 \mu\text{m}$  thick (10 measurements). In image d, the exochorion is  $0.43 \pm 0.009 \mu\text{m}$  thick (10 measurements). *Ex* exochorion, *En* endochorion, *ET* embryo tissue, *Y* yolk, *F* fibrous layer



**Fig. 8** Developmental time (h) of eggs laid by *Phytoseiulus persimilis* females exposed to two humidity treatments at 25 °C for 48–54 h (dark blue: constant high RH, light blue: constant low RH). Eight blocks per treatment, 9–22 eggs per block (Kaplan–Meier method, with 95% CI). (Color figure online)

relatively smaller area from which water is lost, allowing the egg to be less vulnerable to water loss and dehydration stress (Gibbs and Matzkin 2001; Kühsel et al. 2017). Drought-resistant *P. persimilis* eggs could have a larger volume for three main reasons: a higher initial water content, a thicker chorion, or a shift in offspring sex ratio. The production of larger eggs could be due to the fact that *P. persimilis* females produce more female offspring under drought stress (Toyoshima and Amano 1998; Walzer and Schausberger 2015). However, we show that females exposed to constant low humidity produce more males (male proportion: 0.19) than females exposed to constant high humidity (male proportion: 0.12). Therefore, the larger volume of eggs laid under dry conditions cannot be explained by a change in offspring sex ratio. Under optimal conditions, the percentage of females in *P. persimilis* offspring is 83.7% (Toyoshima and Amano 1998), and this percentage can decrease with maternal stress, such as food deprivation (Walzer and Schausberger 2015). In our study, the percentage of female offspring under maternal drought stress was 81%, lower than the 88% female offspring produced under high humidity conditions. This reduction in the percentage of female offspring under dry conditions could be due to the higher production costs of large female eggs than smaller male eggs. Regarding the chorion, we would have expected to see a thicker exochorion or an extra wax layer covering drought-resistant eggs, because many terrestrial arthropods rely on hard waxes for waterproofing (Beament 1959). However, we did not observe any structural difference between chorions of drought-resistant and drought-sensitive eggs. All eggs were covered with three layers: a thick exochorion, a thin endochorion, and a fibrous layer, probably attaching the embryo to the endochorion. We are not able to draw strong conclusions regarding exochorion thickness. However, on the basis of the two TEM pictures on which it was measured, the exochorion of the drought-resistant egg ( $0.33 \pm 0.007 \mu\text{m}$ ) was not thicker, but rather thinner than the exochorion of the drought-sensitive egg ( $0.43 \pm 0.009 \mu\text{m}$ ). More observations are needed to investigate potential differences in exochorion thickness between drought-resistant and drought-sensitive eggs. Ultimately, a higher initial water content is a plausible explanation for the larger volume of drought-resistant *P. persimilis* eggs. A higher initial water content, combined with decreased water loss rate due to different chemical composition and lower SA:V ratio, may explain how drought-resistant *P. persimilis* eggs survive under constant dry conditions. Previous studies on insects suggest that a decreased water loss rate plays a more important role in desiccation resistance than a higher water content. For example, desert *Drosophila* species have adapted to arid conditions primarily by reducing rates of water loss rather than by increasing the amount of water in their body or by tolerating the loss of a greater percentage of body water (Gibbs and Matzkin 2001). Similarly, in the moth *Manduca sexta* (L.), egg adaptation to drought stress was explained by the modification of eggshell conductance rather than by a greater amount of water in the eggs (Potter and Woods 2012). Which mechanism contributes most to drought resistance in *P. persimilis* eggs remains to be investigated.

Egg retention does not seem to be involved in the mechanisms allowing *P. persimilis* eggs to be drought-resistant. Drought-resistant and drought-sensitive eggs had the same developmental time under high humidity, confirming that *P. persimilis* females do not retain their eggs longer in their body under constant low humidity.

Regarding the costs of laying drought-resistant eggs, we showed in a previous study that *P. persimilis* females lay fewer eggs under constant low humidity (Le Hesran et al. 2019b). This lower oviposition rate is probably due to high energy costs of producing drought-resistant eggs, combined with a reallocation of the female's resources to somatic maintenance instead of reproduction. For *P. persimilis* females, drought-resistant eggs could be costly to produce because of their larger volume, or because of their different chemical



composition. Laying larger eggs likely costs more energy, and the selection for larger eggs in a population generally results in lower fecundity (Roff 1993; Fox 2000). For example, in the butterfly *B. anynana*, oviposition temperature induces a plastic response in egg size: at 20 °C, females lay significantly larger but fewer eggs than their sisters at 27 °C (Fischer et al. 2003). However, the assumption of a trade-off between egg size and egg number is challenged by the results of another study on *B. anynana*: despite an increase in egg size through artificial selection, there was no evidence for any correlated change in fecundity of *B. anynana* females. Due to adjustments in egg composition, females producing large eggs did not have to sacrifice fecundity (Karl et al. 2007). For water-stressed *P. persimilis* females, allocating more water to their eggs probably represents an important sacrifice, at the expense of water availability to themselves. The lower fecundity observed in *P. persimilis* females under low humidity is probably due to a lack of water to satisfy both the production of larger eggs and the physiological needs of the females.

Finally, during our research on the mechanisms of drought resistance in *P. persimilis* eggs, an important question emerged: is dry air fatal for *P. persimilis* eggs because the embryo dehydrates until it dies, or because the chorion becomes hard and impenetrable? When eggs of *Haematopinus asini* (L.) are exposed to dry air, the embryo dies before development is finished (Bacot and Linzell 1919). But in other cases, death is due to the inability of the larva to penetrate the chorion and hatch (Buxton 1932). The mechanical properties of the sternal cuticle of the locust were investigated under dry and wet conditions. The results show that water has a major impact on the mechanical properties of the cuticle (measured in GPa): after drying, the endocuticle became harder by a factor of up to 9 (Klocke and Schmitz 2011). Similarly, a decrease in water content of only a few percent can cause a 10-fold increase in stiffness in the cuticle of a maggot in *Calliphora erythrocephala* Meigen (Vincent 2004). During our experiments, several developed larvae were observed inside drought-sensitive eggs trying to hatch under dry conditions, without success. After approximately 15 min of struggling to break the chorion, the larvae would die inside the eggs (SLH personal observation). There may be two explanations for this: first, under constant low humidity, the chorion of *P. persimilis* eggs becomes too hard to break for the fully developed larva inside. Second, under constant low humidity, the embryo suffers from dehydration and at the end of its development, the larva inside the egg is too weak to break the chorion. In both cases, the larvae do not fail to hatch because the embryo inside is dead from dehydration, but rather because they cannot emerge. If this is the case, the higher water content in drought-resistant *P. persimilis* eggs could prevent the hardening of the chorion or the dehydration of the larva, by compensating the decrease in water content due to dry conditions. Alternatively, the exochorion of drought-resistant eggs may be easier to break because it is thinner, even after hardening due to desiccation.

In conclusion, two main mechanisms seem to be responsible for the drought resistance of *P. persimilis* eggs laid under low humidity: they are larger, probably because of a higher water content, and contain more compatible solutes (free amino acids and sugar alcohols) and hydrocarbons than drought-sensitive eggs. Why *P. persimilis* eggs do not hatch under dry conditions remains to be further investigated, but our observations suggest that the embryo fully develops in *P. persimilis* eggs under dry conditions, whereas the larva cannot break the eggshell, and dies inside the egg.

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**Author contributions** SLH, TG, MK and MD conceived and designed the experiments. SLH, JEN, and GB provided eggs for all experiments and carried out the sex ratio and developmental time experiments. LFSA, YHC and SLH conducted the chemical analysis. SLH and MV made the electron microscope images. SLH and AMMR measured the egg volume. SLH, JEN, LFSA, YHC, and AMMR analyzed the data. SLH prepared the manuscript with input from all co-authors. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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