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Aggregated oviposition in *Rhodnius prolixus*, sensory cues and physiological consequences



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ABSTRACT

Females of the haematophagous bug *Rhodnius prolixus* attach their eggs in clusters on substrates related to their hosts, such as nests or avian feathers. Because the hosts are an enormous food resource as well as potential predators, the choice of the site and pattern of oviposition could have an important adaptive value. Here we investigated proximate and a potential ultimate cause of this aggregated pattern of laid eggs. First, we studied proximate causes by analyzing the use of chemical or physical cues associated with aggregated oviposition in *R. prolixus*. For all terrestrial organisms there is a trade-off between exchange of respiratory gases and water loss. Particularly, insect eggs are highly susceptible to this trade-off because they do not obtain water from the environment, hence our second objective is to study the possible mechanisms involved in dehydration resistance in this species. Therefore we examined the dynamics of change in CO₂ release rate ($\dot{M}CO_2$), and water loss rate ($\dot{M}H_2O$) in relation to embryo development as energetic demands increase, and tested the energetic or hygric efficiency hypothesis as a potential ultimate cause of aggregated oviposition. This hypothesis states that grouped eggs consume less energy or lose less water than equal numbers of isolated eggs, the latter being more susceptible to dehydration. Results indicated the use of physical external cues such as dummy eggs or edges of the oviposition substrates, but we did not find any chemical cues associated with the aggregated pattern of oviposition. There are no energetic or hygric benefits associated with egg's aggregated pattern. However, when we analyzed the $\dot{M}CO_2$ and $\dot{M}H_2O$ change in relation to embryo development, we found a fairly constant and low $\dot{M}H_2O$ albeit a clear increase in $\dot{M}CO_2$, suggesting a tightly control of egg's desiccation tolerance. This high resistance to desiccation coupled with a temporal strategy of hatching allows *R. prolixus* embryos to successfully develop and hatch under harsh environmental conditions.

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1. Introduction

Aggregation of organisms within species is a widespread biological phenomenon. There are multiple possible reasons for the occurrence of this phenomenon including a reduction in predation risk (e.g., Ruxton and Sherratt, 2006), increased mating success (e.g., Sullivan, 1981) and growth rates (e.g., Knapp and Casey, 1986), improved thermo-regulation (Klok and Chown, 1999) and

energetic or hygric efficiency (e.g., Benoit et al., 2007; Killen et al., 2012; Yoder et al., 1992).

In particular, whether or not individuals of an insect species aggregate is probably determined by selection pressures. Availability of food in the surroundings could represent a major pressure on larval development. Unlike the nymphal and adult stages, eggs have a limited amount of energy and water reserves available for development until hatching and since they are immobile they will be exposed to changes in environmental abiotic factors. Therefore, the choice of the oviposition site by females will greatly influence the survival of their progeny, and consequently implies a very important process in insect's life-history (Resatarits, 1996). There are many hypotheses about the ultimate causes of aggregated oviposition, including maximizing the survival of embryos and the proximity of a favorable habitat for the progeny (for a review of the subject see Refsnider and Janzen, 2010). These hypotheses

Abbreviations: WL, water loss; DCM, dichloromethane; $\dot{M}CO_2$, rate of CO₂ expressed in $\mu\text{mol h}^{-1}$; $\dot{M}H_2O$, rate of H₂O expressed in $\mu\text{mol h}^{-1}$.

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have been tested with phytophagous insects in the context of insect–plant interaction (Gripenberg et al., 2010), as well as in many species of mosquitoes (Bentley and Day, 1989).

In all terrestrial organisms including insects, gas exchange presents the problem of imposing a needless water loss (WL). This trade-off has been extensively analyzed in the context of respiratory physiology of insects, particularly the different patterns of gas exchange (e.g., Chown et al., 2013; Moerbitz and Hetz, 2010; Rolandi et al., 2014; Schilman, 2016; Schilman et al., 2005; Terblanche et al., 2010; among others). In the case of insect's eggs, although they are small (therefore they could continuously exchange gases and lose water by simple diffusion), their surfaces are complex and efficient in providing mechanical defense and protection against dehydration (Hinton, 1981). Woods and colleagues have extensively studied this trade-off in eggs of the moth *Manduca sexta* (Woods, 2010; Woods and Hill, 2004; Zrubek and Woods, 2006). They found that embryonic metabolic rates increase with development, and water loss rates follow a very similar trajectory (Woods et al., 2005). Moreover, by varying oxygen levels they found that eggs under hypoxia (15% O₂) had significantly higher WL than under normoxic conditions (21%) suggesting that the change in egg's conductance might be associated with variations of energetic requirements (Zrubek and Woods, 2006).

Insects of the subfamily Triatominae (Hemiptera, Reduviidae) are obligate haematophagous bugs with more than 100 species distributed in the Americas (from the USA to Argentina and Chile) and several of them being vectors of Chagas disease (Schofield and Dujardin, 1999). Some triatomines lay their eggs free, e.g., in crevices while others attach them to a substrate, for example to feathers of their host, as is the case of females of *Rhodnius prolixus* (Lent and Wygodzinsky, 1979; Schofield and Dujardin, 1999). The genus *Rhodnius* has radiated from an arboreal common ancestor in the Orinoco–Amazon region giving three main lines, one of them including *R. prolixus* (distributed in the northern part of South America) with sylvatic populations associated with palm trees and birds and domestic populations associated to man (Schofield and Dujardin, 1999). It has been shown that *R. prolixus* lay their eggs on the substrate in clusters (Schilman, 1998); the type of substrate (Schilman et al., 1996) and the presence of olfactory cues from hosts (Guidobaldi and Guerenstein, 2015) modulate the fecundity of females. In consequence, the choice of oviposition site would not be important merely for successful hatching, but also plays an important role in modulating the number of eggs laid. In addition, there is a temporal strategy displayed: most of the eggs hatch synchronously towards the end of the night, when temperature is low and relative humidity (RH) is high (Schilman et al., 2009). The same temporal strategy was observed in the related species *Triatoma infestans* (Lazzari, 1991). All studies evaluating RH and its influence on hatching success of triatomine eggs (Clark, 1935; Guarneri et al., 2002; Schilman et al., 2009), with the exception of *T. infestans* (Roca and Lazzari, 1994) showed a deleterious effect of low RH. These results support the hypothesis of a temporary timing of hatching to avoid high temperature and low RH or high water vapor pressure saturation deficit during hatching. In addition, aggregated oviposition would improve hygric efficiency by depressing rates of water loss per egg due to the boundary layer effect. Water loss from individual eggs raises the local water vapor pressures (RH) around the other nearby eggs, so that the gradient in vapor pressures is reduced causing eggs to lose collectively less water.

The surface of *R. prolixus* eggs is not homogeneous (Fig. 1). It is composed of several layers that form the chorion (Beament, 1946a); the wax layer, which is secreted by the oocyte during development in the ovary, waterproofs the eggs (Beament, 1946b). The edge between the cap and the neck is the region with the highest permeability. This region has a series of

pseudo-micropyles through which occurs the highest proportion of gas exchange (Tuft, 1950). Although egg's structure in this species is known in detail, there is no information about changes in gas exchange and water loss rate during embryo development or in response to increases in metabolic rate. In addition, there is no information whether the aggregated oviposition pattern has some energetic or hygric advantage for this insect species. Thus, the aim of this study was to analyze proximal causes of the aggregation pattern of egg laying in the haematophagous insect *R. prolixus*, as well as to find a potential ultimate cause of this pattern such as a potential hygric or energetic advantage. To this end, we considered the question at two different levels: 1) at a behavioral level by analyzing the possible existence of chemical or physical cues associated with the choice of oviposition substrate; 2) at a physiological level by examining the effects of embryo development on CO₂ production (as a proxy for metabolic rate) and water loss rates, as well as the effects of egg group size and aggregation on energetic or hygric efficiency.

2. Materials and methods

2.1. Insects

Rhodnius prolixus adult females and eggs were used through the study. The insects were reared in the laboratory at 28 °C and 12:12 light–dark (L/D) cycle (light on 08:00 am) and they were fed weekly on live hens.

2.2. Scanning electron microscopy (SEM)

The external structures of *R. prolixus* eggs were scanned by means of SEM. The eggs were mounted with double-sided tape on a standard aluminum stub. All samples were coated successively during 180 s with gold/palladium (40/60%) before examination under a Philips XL 30 scanning electron microscope.

2.3. Behavioral assays

In order to investigate the existence of chemical or physical cues associated with the spatial pattern of egg-laying, we used an experimental cylindrical acrylic container (Fig. 2A). Both bases were made of filter paper as oviposition substrate (Fig. 2B). Experimental groups of 1 or 3 *R. prolixus* females (ca. two months post-ecdysis) were used. Since in *R. prolixus* blood feeding is necessary for egg production (Buxton, 1930) females were fed on live hens in a common jar before each test. Females were individualized by painting a color code on their legs with acrylic paint and individually weighed to the nearest 0.1 mg using an analytical balance (Mettler AJ100, OH, USA) before and 3 h after feeding. Thereafter, 1 or 3 fed females were placed inside the container for 13 days. After this time, the number and distribution of laid eggs was analyzed. Each paper wall was divided into eight identical sectors (Fig. 2B) to analyze the spatial arrangement of eggs, and taking in consideration the ones laid on the edges from the rest of the substrate.

We conducted two different treatments and their respective controls to test the existence of physical or chemical cues used by females of *R. prolixus* to select their oviposition substrate.

In each experimental container 1/8 of each substrate was treated with:

- a) Chemical cue: 50 µL of extracts of eggs by dichloromethane (DCM; a solvent of broad spectrum with an intermediate polarity between hexane and water). 200 eggs were washed with 200 µL of DCM in a 2 mL vial for 3 min and the liquid phase of the extract collected with a micropipette.

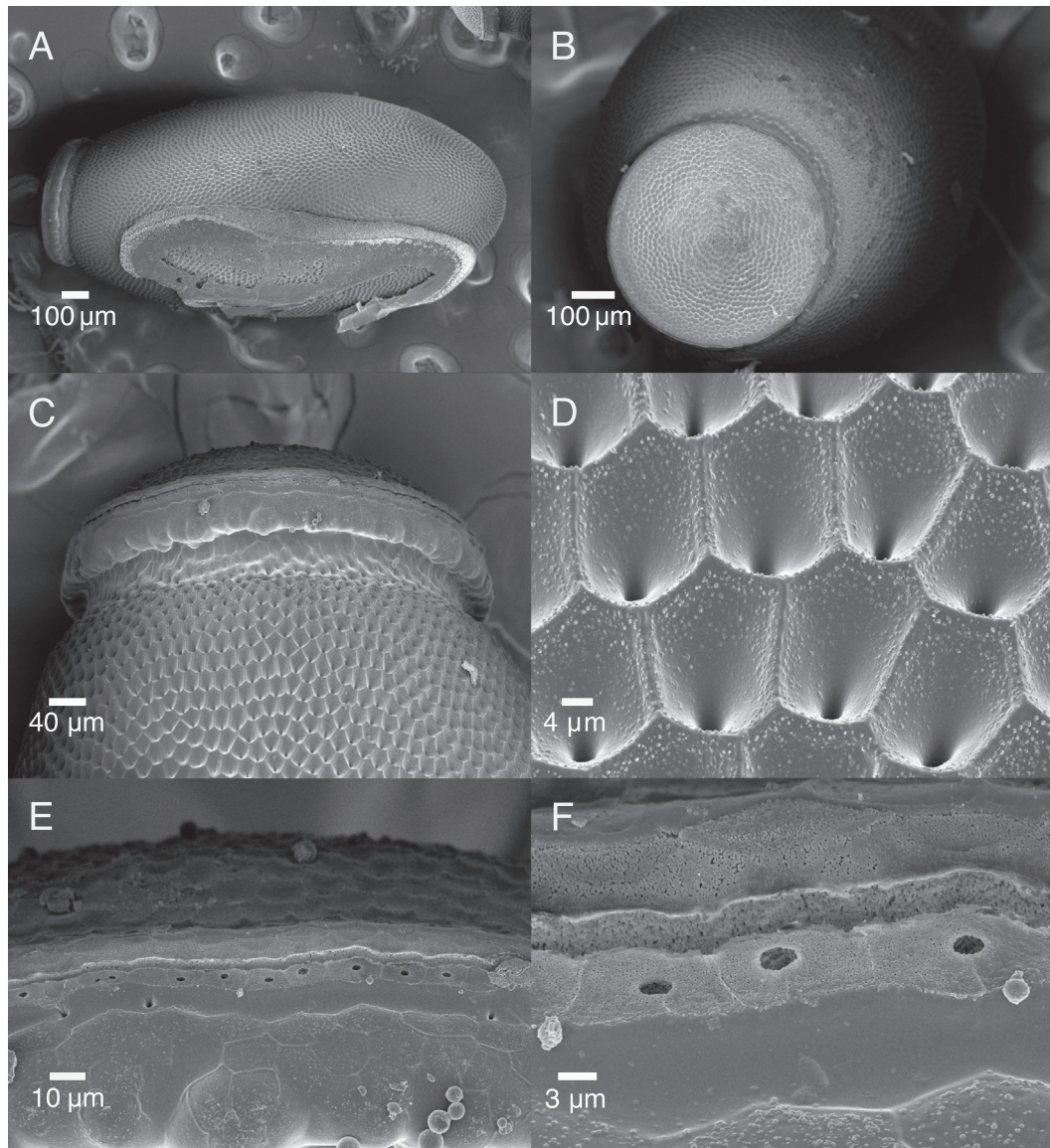


Fig. 1. SEM photos of *Rhodnius prolixus* eggs. A) Lateral view of whole egg. Note the cement from adhesion to the substratum in the lower part of the image. B–C) Top and lateral view of the operculum of the egg. D) Follicular polygons, the depression in the centre of each polygon corresponds to the follicular clefts. E–F) Detail of the operculum's edge. Note the pores distributed uniformly along the edge where gas exchange occurs.

b) Control: 50 µL DCM.

c) Physical cue: a cluster of 7 glass beads of 2 mm in diameter (egg size *ca.* 1 × 2 mm), simulating a group of eggs, glued to the inner face of the filter paper with double-sided tape.

d) Control: double-sided tape glued to the outer side of filter paper.

For each treatment group the number of eggs laid on the marked sector of the oviposition substrate was compared with the unmarked sectors using a Wilcoxon signed-ranks test for paired samples. The number of eggs in each sector was normalized by the number of segments: 2 for the marked and 14 for unmarked sectors.

2.4. Respirometry

We used a Sable Systems International (SSI) flow-through respirometry system (Las Vegas, NV, USA) with a Li-Cor 6251

carbon dioxide (CO₂) analyzer (Lincoln, NE, USA) attached to a SSI RH-300 water vapor analyzer to measure real time CO₂ production and water vapor emission of *R. prolixus* eggs. Briefly, room air was scrubbed of water vapor and CO₂ using a drierite – ascarite II – drierite column followed by a magnesium perchlorate column in order to obtain a better water vapor baseline trace. Air free of CO₂ and H₂O was drawn at a flow rate of *ca.* 40 ml min⁻¹ by a SS4 sub-sampler (SSI) through low-permeability, Bev-A-Line tubing (to minimize errors associated with CO₂ and water vapor absorbance), and a RC-M precision miniature sealed glass/metal respirometer chamber (length: 5 cm, radio: 0.9 cm; volume *ca.* 13 ml; SSI). The velocity of the air or lineal flow rate inside the chamber calculated from formula (1) was *ca.* of 15.75 cm min⁻¹ or 0.26 cm s⁻¹.

$$\text{Linear flow rate} \left(\frac{\text{cm}}{\text{min}} \right) = \frac{\text{Volumetric flow rate} \left(\frac{\text{cm}^3}{\text{min}} \right)}{\text{cross section area} (\text{cm}^2)} \quad (1)$$

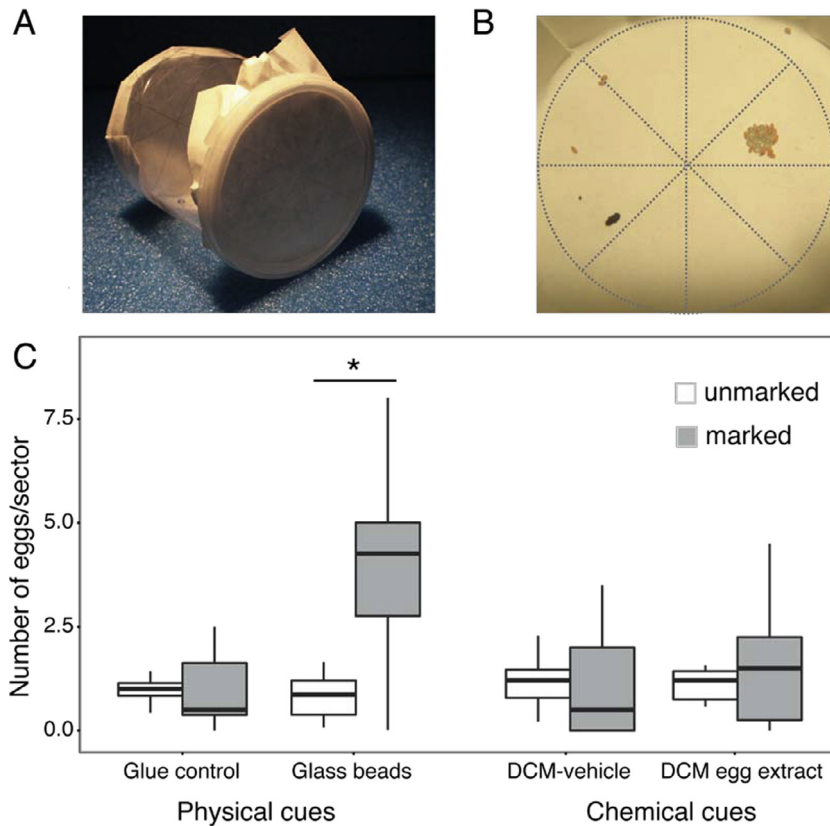


Fig. 2. Behavioral assays of egg-laying. A) Scheme of the experimental device. B) Each base made of filter paper acts as oviposition substrate and it is divided into eight equal parts (16 sectors per container). Note glass spheres (physical cue) and eggs attached to the substrate (small pink ovals). C) Number of laid eggs by a *R. prolixus* female. For each treatment to test the stimuli, marked sectors (2/16) vs. unmarked ones (14/16) of the oviposition substrate were compared using Wilcoxon signed-ranks test for paired samples. * $P < 0.001$.

The water vapor released by the eggs was measured by a SSI RH-300 (set to measure water vapor density in a range of $0\text{--}2\ \mu\text{g ml}^{-1}$ and $0.0001\ \mu\text{g ml}^{-1}$ of resolution), then passed through another magnesium perchlorate column to scrub the water vapor and entered the Li-Cor CO_2 analyzer (resolution $0.1\ \text{ppm CO}_2$) to measure CO_2 produced by the eggs. Measurements were performed at $30\ ^\circ\text{C}$, controlled by a SSI's Pelt-5 temperature controller and SSI's PTC-1 Peltier Effect cabinet. In addition, the temperature was measured by a thermocouple attached to a SSI TC-2000 thermocouple meter. The analog outputs from the analyzers measuring CO_2 , water vapor, temperature of the chamber and air flow rate were connected to an A/D converter (SSI UI-2) and stored in a computer by ExpeData data acquisition software (SSI) at a frequency of 1 Hz. Each recording lasted 40 min and it was corrected to a reading taken through the empty baseline chamber at the beginning and end of each recording to correct for instrument drift.

Previous to the measurements both, CO_2 and water vapor analyzers were calibrated (see Rolandi et al., 2014) for details of a similar setup and calibration).

2.4.1. Temporal dynamics of water loss and CO_2 production rate

We measured eggs of 0, 2, 4, 6, 8, 10 and 12 days after laid. The temporal dispersion between them did not exceed 24 h. In fact, this variation was most likely smaller because *R. prolixus* females lay most of their eggs with a daily rhythm at the beginning of the night (Ampleford and Davey, 1989). For each recording, we used a group of 20 eggs. All eggs hatched between 13 and 14 days after oviposition.

2.4.2. Water loss: Aggregate vs. individuals eggs

For all recordings, 1–4 days old eggs were used. Eggs were collected from rearing jars of adult *R. prolixus* where the filter paper, normally used as oviposition substrate, was replaced by aluminum foils. In this way, the substrate was avoided from absorbing moisture and interfering with the water signal during open flow respirometry measurements. Solitary eggs were fixed (2 mm apart) on a glass-cover slip with double-sided tape with the operculum looking upwards. To avoid differences in humidity between aggregated and solitary egg assays, aluminum foil and the cover slip with double-sided tape were used on both assays.

2.4.3. Analyses and statistics

Data were stored in a laptop computer and analyzed by ExpeData data acquisition and analysis software (SSI). The following corrections and conversions were made from the recordings: (1) CO_2 and H_2O baselines were subtracted, (2) CO_2 in ppm was converted to $\mu\text{mol h}^{-1}$ (for formulae see Lighton, 2008), (3) H_2O vapor density in $\mu\text{g ml}^{-1}$ was converted to WL rate in $\mu\text{mol h}^{-1}$ (see Lighton, 2008). After corrections and conversions were made, mean values of CO_2 production rate ($\dot{M}\text{CO}_2$) and water release rate ($\dot{M}\text{H}_2\text{O}$) from the last 7 min of the recording were extracted and saved in a spreadsheet for further data manipulations. The spreadsheet also included the water vapor saturation deficit from chamber temperature (see Lighton and Feener, 1989), the egg's mass and surface area (see formula 2) and, hence, we calculated the egg's surface permeability.

Shape of eggs was considered as a prolate spheroid; the length of the mayor and minor axes of 20 eggs were measured under

stereo microscope with a graduate ocular and the area calculated using the formula (2).

$$S_{\text{prolate}} = 2\pi \left(a^2 + \frac{a^*c^*}{\sin(\text{oe})} \right) \quad (2)$$

where $\text{oe} = \arccos\left(\frac{a}{c}\right)$, and a is the length of the minor and c of the mayor axes.

All statistical analysis were performed with R Core Team (2016).

3. Results

3.1. Behavioral assays

Regarding physical cues females laid significantly more eggs on the sector of the substrate where the seven glass spheres were present compared with the others sectors (one female: $V = 163.5$, $N = 18$, $P < 0.001$, Fig. 2C; three females: $V = 231$, $N = 21$, $P < 0.0001$; Supplementary Fig. 1). However, there was no significant difference between the number of eggs laid on the sector of the substrate marked with 50 μL of extracts of eggs by DCM and the unmarked sectors (one female: $V = 49$, $N = 15$, $P = 0.53$; three females: $V = 58$, $N = 15$, $P = 0.91$). Both control groups, *i.e.*, double-sided tape attached to the outer side of filter paper and 50 μL of DCM, did not generate any effect on the oviposition pattern (Fig. 2C). In addition, we used these control groups to compare the number of eggs laid on the edges with the rest of the substrate; oviposition was significantly higher on edges (one female: $V = 342$, $N = 31$, $P < 0.01$; three females: $V = 307$, $N = 27$, $P = 0.005$).

3.2. Respirometry

Water loss rate and CO_2 release rate increased as a function of days after oviposition, *i.e.*, egg's development. However, the dynamics of change was different for each variable. Fig. 3A shows average values of $\dot{M}\text{H}_2\text{O}$ and its variation with development, which significantly fits a linear function ($F_{1,51} = 45.43$, $P < 0.0001$, $R^2 = 0.45$; Table 1). Average size ($\pm\text{SD}$) of 20 eggs was measured: length of mayor and minor axes were 1.982 ± 0.040 and 0.959 ± 0.023 mm respectively, and egg's surface area calculated was 21.045 mm^2 (see Eq. (1)). Based on this value and $\dot{M}\text{H}_2\text{O}$, a mean permeability of $0.36 \mu\text{g h}^{-1} \text{ cm}^{-2} \text{ Torr}^{-1}$ (where $1 \text{ Torr} \approx 133 \text{ Pa}$) was estimated.

In addition, the dynamics of $\dot{M}\text{CO}_2$ with development showed a slow increase until approximately day 6 (Fig. 3B). Data fits a quadratic equation ($F_{2,50} = 866.4$, $P < 0.0001$, $R^2 = 0.97$; Table 1).

As a measure of hygric efficiency, the ratio of $\dot{M}\text{CO}_2:\dot{M}\text{H}_2\text{O}$ was calculated (Fig. 3C). A local regression fit was conducted observing two distinct stages. During the first days there was a decrease in water cost, and then from the 4th day onwards, it remained relatively constant. Therefore, a linear model was fitted for each stage and found that although both regressions were significant, the model for the 1st stage had a better fit ($F_{1,22} = 95.87$, $P < 0.0001$, $R^2 = 0.80$) than the 2nd stage ($F_{1,27} = 7.15$, $P < 0.02$, $R^2 = 0.18$). Table 1 shows estimated values of the effect of days of embryo development; water cost decreased at a constant rate, and after the 4th day of development the decline rate decreased approximately one order of magnitude.

Fig. 4 shows (A) $\dot{M}\text{H}_2\text{O}$ and (B) $\dot{M}\text{CO}_2$ as function of the number of eggs. Aggregation did not have a significant effect on $\dot{M}\text{CO}_2$ ($P = 0.87$), nor $\dot{M}\text{H}_2\text{O}$ ($P = 0.91$). Both, $\dot{M}\text{H}_2\text{O}$ and $\dot{M}\text{CO}_2$, increased linearly with the number of eggs ($\dot{M}\text{CO}_2$:

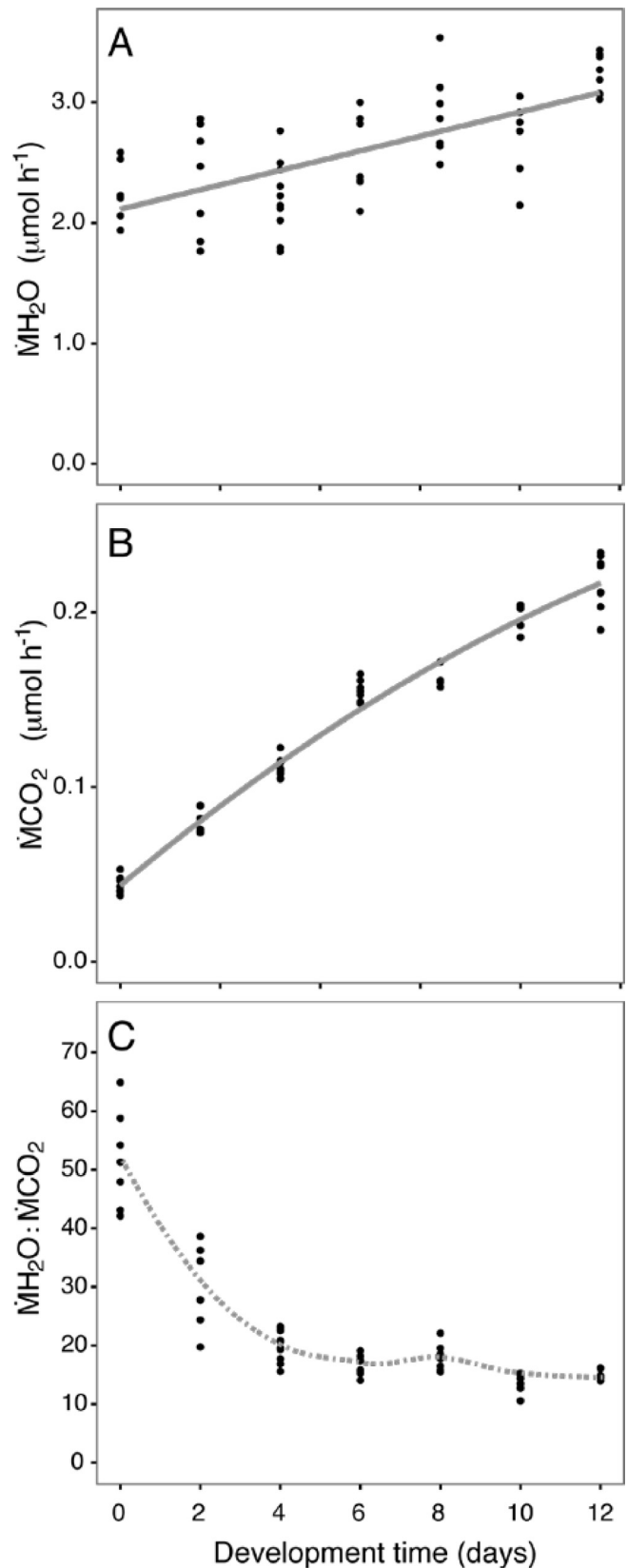


Fig. 3. Temporal dynamics of: (A) water loss rate ($\dot{M}\text{H}_2\text{O}$), (B) CO_2 release ($\dot{M}\text{CO}_2$) and (C) $\dot{M}\text{H}_2\text{O}:\dot{M}\text{CO}_2$ ratio. Each point represents a group of 20 eggs. Solid line corresponds to the setting of a linear function for (A) and quadratic function for (B). Dotted line is a local regression fit for (C).

Table 1Lineal models for $\dot{M}H_2O$, $\dot{M}CO_2$ and $\dot{M}H_2O : \dot{M}CO_2$ ratio. For the latter, data was divided in days 0–4 and 6–12.

Model	Factor	Estimated Value	Standard Error	t	P-value
$\dot{M}H_2O \sim \text{Days}$	Intercept	2.11	0.09	24.62	<0.0001
	Days	0.08	0.01	6.67	<0.0001
$\dot{M}CO_2 \sim \text{Days} + \text{Days}^2$	Intercept	4.36×10^{-2}	3.25×10^{-3}	13.41	<0.0001
	Days	1.92×10^{-2}	1.23×10^{-3}	15.57	<0.0001
	Days ²	-3.98×10^{-4}	9.68×10^{-5}	-4.11	<0.0002
[0 – 4] $\dot{M}H_2O : \dot{M}CO_2 \sim \text{Days}$	Intercept	49.66	2.23	22.28	<0.0001
	Days	-7.80	0.89	-9.79	<0.0001
[6 – 12] $\dot{M}H_2O : \dot{M}CO_2 \sim \text{Days}$	Intercept	19.82	1.53	12.93	<0.0001
	Days	-0.44	0.17	-2.67	<0.02

Slope = $0.0033 \pm 0.0003 \mu\text{mol h}^{-1} \text{egg}^{-1}$; $P < 0.0001$; $\dot{M}H_2O$: Slope = $0.13 \pm 0.01 \mu\text{mol h}^{-1} \text{egg}^{-1}$; $P < 0.0001$). In order to estimate the effect of group size on the hygric cost of gas exchange, the $\dot{M}H_2O : \dot{M}CO_2$ ratio was calculated and related with the group size or number of eggs. There was no significant linear relation between the $\dot{M}H_2O : \dot{M}CO_2$ ratio and the number of eggs ($F_{3,39} = 0.76$, $P = 0.52$, $R^2 = 0.02$), indicating that there was no effect of group size on the hygric cost of gas exchange.

4. Discussion

4.1. Chemical or physical cues associated with the aggregation pattern of laid eggs

It has been shown that *R. prolixus* females do not lay their eggs at random places of the oviposition substrate, but they do it in clusters (Schilman, 1998). Using a similar setup, Schilman (1998) compared the oviposition patterns produced by one or three females. As a measure of aggregation the “index of clumping” (*I*), was calculated for each container (Pielou, 1977). *I* values were statistically homogeneous between one and three females, indicating a similar degree of aggregation. In other words, the oviposition pattern is not only the result of a single female laying many eggs together (Schilman, 1998). In addition, *R. prolixus* lays most of its eggs at the beginning of the night (Ampleford and Davey, 1989), thus it is unlikely that they use visual external cues for guiding the oviposition behavior. Through a very simple experimental design we tested the hypothesis that *R. prolixus* females uses physical cues for laying their eggs in clusters. Using filter paper as oviposition substrate we glued a group of 7 glass spheres to a sector of the substrate. The glass beads were similar in size to eggs and contained no chemical cues as they were previously washed. Oviposition was higher in the sector containing glass spheres, particularly around them. In addition, for all experimental groups more eggs were laid on the edges of the substrate providing extra support for the use of physical cues guiding the pattern of oviposition. Two other species of bloodsucking Hemiptera, *Cimex lectularius* and *C. hemipterous*, also used physical cues to oviposit, choosing heterogeneous and rough surfaces to lay their eggs (Usinger, 1966). It has been suggested that these species use mechanoreceptors in the legs to detect the different textures of the substrates (Walpole, 1987), thus it would be interesting to find the mechanoreceptors involved in the choice of an oviposition substrate by *R. prolixus*.

In addition, aggregation pheromones are present in eggs of several species of insects including haematophagous, mostly in Diptera (Seenivasagan and Vijayaraghavan, 2010). Hence, we also tested the hypothesis that *R. prolixus* females use chemical contact cues as guidance to oviposition sites. To do this, a region of the oviposition substrate was marked with extract of eggs washed with DCM. We did not find chemical cues associated with the aggregated pattern of eggs. However, it cannot be completely ruled

out their existence, as only one type of extraction was performed. DCM extracts non-polar compounds, but it has a wider spectrum than e.g., hexane. Nonetheless there could be more polar compounds acting as aggregation signals. Moreover, the process of extracting a compound did not take into account that synthesis and secretion of biologically active substances are dynamic processes (Haynes and Millar, 1998). Future research could be accomplished with extractions at different times of eggs development, with different solvents and with volatiles since it has been observed that olfactory cues from host (chicken feathers and live mice) modulate the spatial pattern of oviposition, and the number of eggs laid (Guidobaldi and Guerenstein, 2015).

4.2. Energetic and water loss modulation in relation to embryo development

The average rate of WL measured in *R. prolixus* eggs by open flow respirometry was $0.04 \mu\text{g min}^{-1} \text{egg}^{-1}$, which is equivalent to $0.01 \text{mg cm}^{-2} \text{h}^{-1}$ and different from the $0.09 \text{mg cm}^{-2} \text{h}^{-1}$ measured by Beament (1946a,b). Part of the difference could be the result of different experimental protocols: e.g., using laid eggs (this study) or eggs from the oviduct (Beament, 1946a,b). Moreover, we measured WL at 30 °C by open flow respirometry instead of 25 °C by gravimetric method (Beament, 1946a,b), however these differences should render opposite results (for comparison of both methods see: Schilman et al., 2007). Based on the WL rate measured, the egg surface area, and taking into account temperature and humidity at which they were measured, an egg permeability of $0.36 \mu\text{g h}^{-1} \text{cm}^{-2} \text{Torr}^{-1}$ was calculated. This is a very low value, similar to the cuticular permeability of xeric adapted arthropods (see Table 3.1 from Hadley, 1994), and even lower than values calculated for males of the same species (between 1.76 and $4.11 \mu\text{g h}^{-1} \text{cm}^{-2} \text{Torr}^{-1}$, depending on the temperature and days after feeding; Rolandi et al., 2014). Moreover, *R. prolixus* egg permeability is lower than the lowest value measured by open-flow respirometry, i.e., $0.90 \mu\text{g h}^{-1} \text{cm}^{-2} \text{Torr}^{-1}$ for the tenebrionid beetle *Eleodes obscura* (Schilman et al., 2008).

In relation to energy expenditure, our data were consistent with the work of Tuft (1950) in the same species and Woods et al. (2005) in eggs of *Manduca sexta*, where they showed an increase in consumption of O_2 (Tuft, 1950) or CO_2 production (Woods et al., 2005) from eggs just laid to nearly hatching. Our results showed that the metabolic rate approached a maximum value as the embryo was close to hatching, an increase of almost 5 times since the beginning of development to hatch; a similar increase was observed in eggs of *M. sexta* (Woods et al., 2005). The $\dot{M}CO_2$ of 12 days old embryos was $0.011 \pm 0.001 \mu\text{mol h}^{-1} \text{egg}^{-1}$ (mean \pm SD). It is interesting to note that the standard metabolic rate of unfed first instar nymph was ca. 1.5 times higher ($0.015 \pm 0.003 \mu\text{mol h}^{-1} \text{insect}^{-1}$; unpublished results).

Contrary to what occurs in *M. sexta*, where there is a change of the eggshell permeability according to O_2 requirements (Zrubek

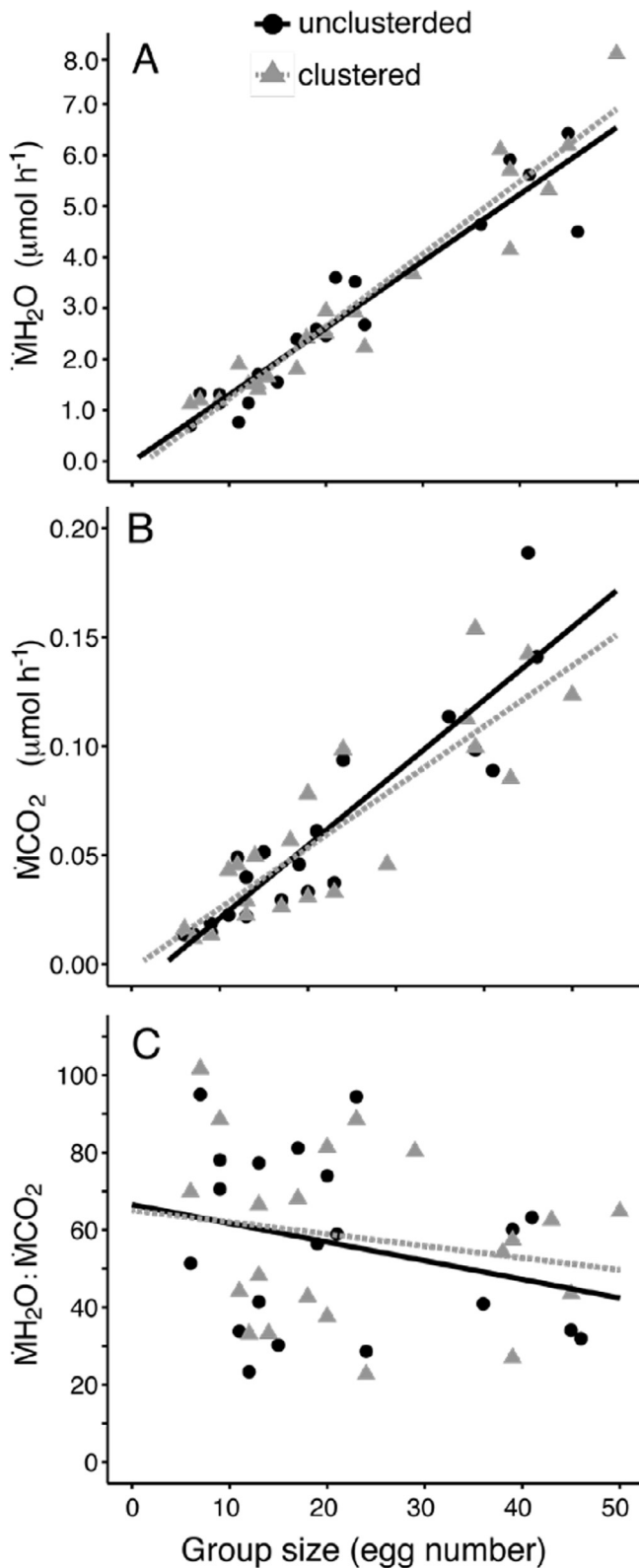


Fig. 4. Effect of aggregation on: (A) water loss rate ($\dot{M}H_2O$), (B) CO₂ release ($\dot{M}CO_2$) and (C) $\dot{M}H_2O : \dot{M}CO_2$ ratio. Each point represents groups of different size of clustered (triangles) and unclustered (circles) eggs. The effect of group size was tested by fitting a linear model for clustered (solid line) and unclustered (dotted line) eggs.

and Woods, 2006), in *R. prolixus*, WL remained relatively constant throughout development. Despite similarities in requirements for development, surface architecture, etc., across eggs of different

insect species (Hilker and Meiners, 2002), there are some differences. For example while in *M. sexta*'s eggs micropyles have an homogeneous distribution (Woods et al., 2005), suggesting uniform WL through the surface, *R. prolixus* eggshell was not homogeneous (Fig. 1). For *R. prolixus* eggs the proportion of gas exchange and probably WL was highest along the edge between the cap and the neck through the pseudo-micropyles (Tuft, 1950) with an air space between the outer shell and the wax layer improving gas exchange (Wigglesworth and Beament, 1950). These structural differences could explain the variation on temporal correlation between $\dot{M}CO_2$ and $\dot{M}H_2O$ found between these two species. Although speculative, the structure of regionalized micropyles and air space between the outer shell and the wax layer could create a low enough conductance that gas compositions inside the air-filled parts can be different from ambient (lower O₂ and higher RH), which resemble to the subelytral cavity and the pygidial cleft of tenebrionid beetles. In tenebrionid beetles, abdominal spiracles open into the subelytral cavity (an air space beneath the fused elytra), which is connected to the environment by the pygidial cleft. It is suggested that this arrangement helps reduce respiratory WL because the spiracles open into a chamber that is more humid than the drier atmosphere outside the subelytral cavity (Ahearn, 1970; Byrne and Duncan, 2003; Schilman et al., 2008).

Thus, the extremely low permeability probably due to morphological features of *R. prolixus* eggs could allow them to survive and successfully hatch under hostile environments.

4.3. Effects of eggs' aggregation on the energetic or hygric efficiency

Aggregation of organisms is a widespread biological phenomenon, and an ultimate cause associated with this phenomenon could be the improvement in energetic or hygric efficiency. In order to test this idea, we compared by open-flow respirometry, the rate of water loss and release of CO₂ (as proxy for metabolic rate) on isolated or aggregated groups of *R. prolixus* eggs of different sizes.

In other blood-sucking arthropod, the tick *Ixodes uriae* aggregation between individuals in each life stage is an effective strategy to reduce water loss (Benoit et al., 2007). Nonetheless our results did not validate the hypothesis that aggregation is a mechanism to reduce water loss. Similar results were found by Schoombie and collaborators (2013) recording groups of caterpillars. Water loss depends on the conductance of the eggshell as well as the conductance through the boundary layer surrounding the egg(s). This has been thoroughly studied on avian eggs showing that boundary layer conductance increases with decreasing egg's size (Paganelli, 1980). Given the very low permeability values calculated in this study together with the small size of insect's eggs, the boundary layers wouldn't play much of a role influencing the water loss of other nearby eggs, which could explain our findings. In addition, aggregated oviposition could render several other benefits to the bugs such as physical protection against mechanical aggressions (e.g., antiparasitic host behavior), reduced predation risk (Ruxton and Sherratt, 2006), or it may even result in synchronous hatch, facilitating surviving success. For example, synchronous hatching occurs when eggs of the locust, *Schistocerca gregaria*, are in contact with each other probably controlled by vibrational signals (Nishide and Tanaka, 2016). It has been shown that synchronous hatching may facilitate group emergence from the nest in a variety of taxa, which in turn, may limit predation by diluting an individual's risk of predation. However, there are costs associated to hatch synchronicity. These are, among others, neuromuscular developmental costs in individuals that hatch prematurely in order to synchronize hatching with more developed embryos (Colbert et al., 2010).

5. Conclusion

In this study we found that one of the proximate causes that guide the oviposition behavior of *R. prolixus* females, was the use of physical external cues such as previous laid eggs or borders of the oviposition substrates. Thus leading to an aggregated pattern of oviposition. In order to look for a possible ultimate cause for this pattern, it was tested the scaling of energetic or hygric efficiency with experimental manipulation of group size (i.e., number of eggs) and aggregation state. No benefits were found to be associated with egg aggregation. However the dynamics of $\dot{M}CO_2$ and $\dot{M}H_2O$ in relation to embryo development showed a fairly constant and low water loss rate and permeability albeit a marked increase of CO_2 release, suggesting a tight control of egg's water loss.

Author contributions

PES conceived the study; CR and PES design the experiments; CR gathered and analyzed the data and PES and CR wrote the paper.

Competing interests

No competing interests declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2016.12.001>.

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