Laboratory breeding of Phortica spp. (Diptera: Drosophilidae), vectors of the zoonotic eyeworm Thelazia callipaeda

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Research Article

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Abstract

Background - The establishment of arthropod colonies is an important tool to manipulate disease vectors, investigate their life history traits, and their vector competence, prevention of vector borne pathogen transmission, or to assess insecticidal/repellent activities. Some species of drosophilid flies belonging to the genus *Phortica* feed on ocular secretions of mammals, acting as biological vectors of the zoonotic eyeworm *Thelazia callipaeda*. This study describes an effective breeding protocol of *Phortica variegata* and *Phortica oldenbergi* in insectary conditions.

Materials and methods - Gravid flies of *P. oldenbergi*, *P. variegata* and *Phortica semivirgo* were field collected in wooded areas of Lazio region (Italy) and allowed to oviposit singularly to obtain isofamilies. Flies were maintained in ovipots (200ml) with a plaster-covered bottom to maintain high humidity level inside. Adult feeding was guaranteed by freshly apple and a liquid dietary supplement containing NaCl and mucin proteins, while larval development was obtained by *Drosophila*-like agar feeding medium. The breeding performances of two media were compared: a standard one based on cornmeal flour and an enriched medium based on chestnut flour. All conditions were kept in a climatic chamber with a photoperiod of 14:10h light:dark, 26±2°C and 80±10% RH.

Results - From a total of 130 field collected *Phortica spp*. three generations (i.e., 783 F1, 109 F2, 6 F3) were obtained. *Phortica oldenbergi* was the species with higher breeding performance, being the only species reaching F3. Chestnut-based feeding medium allowed higher adult production and survival probability in both *P. oldenbergi* and *P. variegata*. Adult production/female was promising in both species (*P. oldenbergi*: 13.5 F1/f; *P. variegata*: 4.5 F1/f), indicating the possibility to obtain stable colonies.

Conclusions - This standardized breeding protocol, based on controlled climatic parameters and fly densities, together with the introduction of an enriched feeding medium, allowed to investigate aspects of life history traits of *Phortica spp*. involved in the transmission of *T. callipaeda*. Obtaining F3 generation of these species for the first time paved the road for the establishment of stable colonies, an essential requirement for future studies on these vectors in controlled conditions.

Background

From the early 1900s to now, breeding of several invertebrate taxa (e.g., moths, mosquitoes, beetles, marine copepods, and fruit flies) has been pivotal to study subjects in many fields of science, including evolution, ecology and physiology [1]. In medical and veterinary context, the establishment of arthropod colonies is pivotal to investigate their life cycles, genetics, behaviour, interaction with vector-borne parasites, as well as their vector competence or susceptibility to insecticides [2]. Several rearing protocols have been developed for studying vectors of medical and veterinary concern such as mosquitoes, sand flies, tsetse flies [3–5] as well as flies of the *Phortica* genus [6]. The latter group of drosophilids includes around 130 species worldwide distributed, especially in mountain areas of the Oriental Region, where many species occur [7]. Most of the species of the subgenus *Phortica* have adapted to feed on ocular secretions of mammals [8–11]. Thanks to this lachryphagous behaviour, *Phortica variegata* and *Phortica okadai* have been identified as intermediate hosts and vectors of the zoonotic eyeworm *Thelazia callipaeda* (Railliet & Henry; Spirurida: Thelaziidae) [9]. This eyeworm infests the ocular apparatus of dogs, cats, wild carnivores (e.g., foxes, wolves, bears), lagomorphs, and humans in Europe and Asia [12, 13]. Currently, several cases of human thelaziosis by *T. callipaeda* have been reported in Asia (i.e., China, Korea, India, Thailand and Japan) and Europe, with an increasing trend in recent decades [14, 15, 12]. Recently, a third species, *Phortica oldenbergi*, has been experimentally demonstrated as intermediate host of *T. callipaeda* (Bezerra Santos *et al*., submitted). To date, five *Phortica spp*. (i.e., *Phortica erinacea*, *Phortica goetzi*, *P. oldenbergi*, *Phortica semivirgo*, and *P. variegata*) have been identified in Europe [16], with *P. variegata* being the most prevalent species in many regions [17]. Conversely, for the other species no information is available about their natural life history, pre- and post-mating behaviour and ecology [7].

This study aims to describe a novel, standardized rearing method of *P. variegata* and *P. oldenbergi* flies, based on an artificial diet and characterized parameters of density and climatic conditions suitable to create a potentially stable colony. Results will represent an important starting point for controlled studies on *Phortica spp*. life cycle and vector role, toward in vitro testing of new insecticidal drugs and reducing the vector capacity of these drosophilids.

Materials And Methods

Sample collection - *Phortica* spp. gravid females were collected in Manziana (Lazio region, Italy, 42°07'09"N, 12°06'58"E; altitude 378 m a.s.l.) from May to October 2020. Wild females were collected with an entomological net around bait of decaying fruits (i.e., apples, bananas, peaches, pears) into a cloth tied with a string around the bark of Turkey oak trees (*Quercus cerris*). Specimens were subsequently identified as *P. oldenbergi, P. semivirgo* and *P. variegata* using identification keys [18]. Later on, the flies were individually transferred in plastic pots and immediately transported alive to the laboratory at the Department of Public Health and Infectious Diseases, Sapienza University of Rome (Italy). During the travel, pieces of fresh apple were added on the pots as food source and the containers were placed in a plastic box with a moist cloth pad to maintain high humidity and to protect flies from sunlight and excessive heat.

Rearing conditions - Specimens were reared in 200ml clear plastic containers (ovipots) with a hole at the bottom filled with a 2 cm layer of plaster to maintain high humidity without water condensation. The container was closed by a lid with a net to prevent escape of larvae and to allow adult feeding on a slice of apple placed on the top. During ovipot servicing, flies were temporarily put in an empty cage of 30cm^3. Checks were performed every two days, when the piece of apple was changed and eggs/larvae potentially present on it were gently transferred into a solid medium for hatching and larval development.

Two different solid media were used:

- - **Standard**: 86% water, 5.6% yeast, 3.9% sucrose, 0.5% agar, 3% cornmeal flour and propionic acid;
- - **Chestnut**: 84.8% water, 6.69% yeast, 4.46% sucrose, 0.66% agar, 2.67% chestnut flour, 0.66% banana, > 0.001% propionic acid.

Additionally, adult feeding was enriched by a liquid dietary supplement (i.e., 80% distilled water, 20% snail extract–based syrup and 0.009% sodium chloride) soaking a cotton wool inside the pot.

Ovipot moisture was regularly monitored wetting the plaster as needed, contemporarily avoiding excess of water and the consequent development of moulds. Sibling pupae were transferred and pooled in another plastic container in dry conditions during all pupation period in a plastic box and discarded if no adults emerged after 30 days. Adult progeny of a single wild female was kept in pool at the same parental conditions (maximum 10 flies/ovipot) maintaining both sexes in the same container in order to allow mating. All ovipots were stored in large plastic boxes (50x80x40 cm) placed in a climatic chamber with a photoperiod of 14:10h light:dark 26 ± 2°C and 80 ± 10% RH.

Statistical analysis - As only *P. oldenbergi* and *P. variegata* are known to be vectors of *Thelazia callipaeda*, a focus was made on these two species.

A negative binomial Generalized Linear Model was performed to test the differences in oviposition rates between field collected females of *P. oldenbergi* and *P. variegata*, as follows:

$$\log y_i = \beta_j X + \epsilon_i$$

Where y_i is the oviposition rate for the i^{th} pot and β is the effect of the j^{th} species, with *j* representing a factor with two levels (*P. oldenbergi* and *P. variegata*) [19].

Additionally, the same model structure using a Linear Model analysis was carried out to test pupae production of *P. oldenbergi* and *P. variegata* according to artificial diets. In this case y_i is the pupae rate (n. pupae/females/pot) for the i^{th} pot and β is the effect of the j^{th} artificial diet, with *j* representing a factor with two levels (Chestnut and Standard media).

Kaplan-Meier analysis was carried out to determine the survival probability of *Phortica* pupae and adults. The survival probability at time t_i, $S\ (t_i)$ is calculated as follows:

$S\ (t_i) = S\ (t_i - 1)$ x $(1 - d_i/n_i)$

Where S is survival, t_i is time, d_i is the number of events, and n_i is the number of flies alive just before t_i [20].

To test the robustness of the analysis, a log-rank test was performed approximately distributed as a chi-square function.

Results

Phortica species development and reproduction parameters - Field collected *Phortica spp.* females (N = 130) left to singularly oviposit in the plastic containers, lead to the production of three generations (F1 = 783; F2 = 109; F3 = 6) (Table 1). The oviposition rates for field collected females (based on mean number of eggs oviposited per female) were significantly higher for *P. oldenbergi* compared to *P. variegata* (negative binomial GLM; z-value: -2.637; p-value: 0.008; Table 1). Among F2 specimens, only those belonging to *P. oldenbergi* were able to produce F3 eggs. Looking at the mean number of pupae and adults per female in F1 and F2 generations, the highest value was reached by *P. oldenbergi* followed by *P. variegata*, while no production was obtained for *P. semivirgo* (Table 1). The mean number of development days from pupae to adults (pupation) varied from a minimum of 12 ± 1 (range 4–49 ± 1) in *P. oldenbergi* F1 to a maximum of 20 ± 1 (range 11–20 ± 1) in F3 of the same species. Sex ratio of F1 was slightly unbalanced in favour of females along the sampling season for both *P. variegata* and *P. oldenbergi* with an average value of female proportions of 57% and 58%, respectively (Table 2).

Table 1
Average values of eggs, pupae and adult progeny obtained from field collected (WF), F1 and F2 females of *Phortica oldenbergi*, *Phortica semivirgo* and *Phortic* to feeding medium (Chestnut, Standard).

Species	Medium	Parental generation				F1 generation				F2 generation		
		Wild Females	Eggs/WF	Pupae/WF	Adults/WF	F1 females	Eggs/F1	Pupae/F1	Adults/F1	F2 females	Eggs/F2	Pup
Phortica oldenbergi	Chestnut	4	48.2 (min: 1, max: 41, SD: 8.7)	33.8 (min: 1, max: 19, SD: 4.9)	13.5 (min: 1, max: 79, SD: 13.2)	61	1.4 (min: 1, max: 30, SD: 3.2)	0.3 (min: 2, max: 6, SD: 0.7)	0.1 (min: 3, max: 13, SD: 2.4)	13	0	0
	Standard	67	33.0 (min: 1, max: 69, SD: 10.8)	8.2 (min: 1, max: 19, SD: 3.2)	2.9 (min: 1, max: 31, SD: 4.1)	189	5.9 (min: 1, max: 42, SD: 5.8)	0.7 (min: 1, max: 9, SD: 1.2)	0.3 (min: 1, max: 25, SD: 2.1)	43	3.2 (min: 1, max: 18, SD: 3.2)	0.2 1, n SD:
	overall		33.9 (min: 1, max: 66, SD: 10.6)	9.6 (min: 1, max: 24; SD: 3.5)	6.8 (min: 1, max: 79; SD: 5.9)		4.9 (min: 1, max: 42, SD: 5.6)	0.6 (min: 1, max: 9; SD: 1)	4.3 (min: 1, max: 25; SD: 2.2)		2.5 (min: 1, max: 18, SD: 3.1)	0.1 0, n SD:
Phortica semivirgo	Standard	3	63.7 (min: 1, max: 19, SD: 6.7)	27.3 (min: 1, max: 12, SD: 2.9)	7.0 (min: 4, max: 23, SD: 4.6)	21	10.8 (min: 1, max: 33, SD: 4.9)	0	0	0	0	0
Phortica variegata	Chestnut	6	11.9 (min: 1, max: 12, SD: 3.2)	9.2 (min: 1, max: 5, SD: 2.1)	4.5 (min: 1, max: 17, SD: 4.3)	19	1.8 (min: 1, max: 15, SD: 2.2)	0	0	0	0	0
	Standard	50	23.8 (min: 1, max: 44, SD: 5.6)	6.8 (min: 1, max: 11, SD: 1.8)	1.8 (min: 1, max: 29, SD: 2.6)	85	1.3 (min: 1, max: 10, SD: 1.3)	0.01 (min- max: 1, SD: 0.06)	0.01 (min- max: 1, SD: 0.05)	1	0	0
	overall		22.6 (min: 1, max: 33, SD: 4.8)	7.1 (min: 1, max: 12; SD: 1.8)	4.6 (min: 1, max: 29; SD: 2.7)		1.4 (min: 1, max: 15, SD: 1.6)	0.008 (min: 1, max: 1; SD: 0.06)	0.008 (min: 1, max: 1; SD: 0.05)		0	0

Table 2
Proportion of F1 female progeny obtained from field collected females of *Phortica variegata* and *Phortica oldenbergi* according to the sampling day.

Collection date	*Phortica variegata*	*Phortica oldenbergi*
28/05/2020	60%	71%
04/06/2020	50%	
12/06/2020	52%	72%
19/06/2020	65%	49%
02/07/2020	44%	57%
09/07/2020	41%	53%
13/08/2020	67%	
27/08/2020	67%	46%
03/09/2020	44%	88%
10/09/2020	74%	41%
15/09/2020		46%
19/09/2020	56%	
01/10/2020	65%	
Mean proportion	57%	58%

Influence of artificial diets on Phortica species breeding - The Chestnut rearing medium led to a higher production of *P. oldenbergi* F1 adults as compared to Standard medium (4.6:1; negative binomial GLM: z-value: -2.940; p-value:0.003). Conversely, no significant difference for *P. variegata* F1 adults was obtained between the two rearing media (2.5:1; negative binomial GLM: z-value: -1.19; p-value: 0.23; Table 1). Comparing the two feeding media, the average development time of *P. oldenbergi* progeny was lower for larvae fed with the Standard medium (F1: 12 ± 1 days; F2: 15 ± 1 days) as compared with Chestnut

medium (F1: 14 ± 1 days; F2: 23 ± 1 days). Conversely, F1 development of *P. variegata* showed no difference between the two media (13 ± 1 days with both feeding conditions; Table 3). The survival probability of Chestnut-reared pupae of *P. oldenbergi* was higher as compared to the Standard-reared ones (log-rank test: $\chi2$: 39.1; p-value < 0.0001; Fig. 1a), these results indicate that chestnut medium favoured pupal development of this species (median survival time for Chestnut and Standard: 22 and 18 days, respectively). Accordingly, also survival curves for *P. variegata* pupae showed significant difference in the use of the two media (log-rank test: $\chi2$: 7.4, p-value: 0.007; median survival time of 21 days in Chestnut-reared pupae and 16 days in Standard- reared pupae, Fig. 1b). Likewise, the survival probability curves of *P. oldenbergi* adults showed a significant difference between the two media, with the median survival time of the Chestnut-reared flies that is almost doubled as compared to the Standard-reared ones (34 and 19 days, respectively; log-rank test: $\chi2$: 14.9; p-value: 0.0001; Fig. 2a). Similarly, survival probability is also higher for *P. variegata* Chestnut-reared adults (Chestnut: 28 days, Standard: 15 days; log-rank test: $\chi2$: 9.3; p-value: 0.002; Fig. 2b).

Table 3
Mean time of development from egg to adult (days ± 1) of *Phortica oldenbergi*, *Phortica semivirgo* and *Phortica variegata* per generation (F1, F2, F3) and feeding medium (Chestnut, Standard). na: data not available.

Species	Medium	F1 (days ± 1)	F2 (days ± 1)	F3 (days ± 1)
Phortica oldenbergi	Chestnut	14 (min = 11, max = 33, SD = 2.7)	23 (min = 16, max = 30, SD = 6.1)	na
	Standard	12 (min = 4, max = 49, SD = 5.9)	15 (min = 4, max = 30, SD = 7.2)	20 (min = 11, max = 30, SD = 8.6)
	overall	12 (min = 4, max = 49, SD = 5.6)	16 (min = 4, max = 30, SD = 7.5)	20 (min = 11, max = 30, SD = 8.6)
Phortica semivirgo	Standard	15 (min = 7, max = 21, SD = 8.3)	na	na
Phortica variegata	Chestnut	13 (min = 7, max = 21, SD = 3.3)	na	na
	Standard	13 (min = 3, max = 46, SD = 9)	12	na
	overall	13 (min = 3, max = 46, SD = 8.4)	12	na

Discussion

The breeding protocol here presented indicated the importance of optimizing parameters such as density, humidity and diet in successfully breeding *Phortica* species. In fact, a F3 has been obtained for the first time, with a substantial improvement as compared to previous attempts. To date, a single laboratory breeding protocol for *P. variegata* has been described [6], in which the authors successfully bred this species up to the second generation adopting a simple approach based on feeding *ad libitum* with fresh apple all development stages of the flies into a cage of 30cm^3 with a high relative humidity. As compared to previous protocols [6], in which 0.5 F1 adults/female were obtained, this study shows that limiting the fly numbers per pot, the modulation of relative humidity during the life cycle, with drier conditions for pupal phase, and optimal feeding based on chestnut flour and liquid dietary supplement, provides higher performance in terms of adult progeny for both F1 and F2 (*P. variegata*: 4.5 F1/f, 1.8 F2/f; *P. oldenbergi*: 13.5 F1/f, 1.4 F2/f; Table 1).

Moreover, this rearing protocol allowed to breed for the first time *P. oldenbergi*, providing first data on its life history traits. Also, this species has demonstrated to be more adapted to insectary conditions as compared to *P. variegata*, encouraging its employment as a potential model for challenge studies and trials of veterinary products, also considering that its vector competence for *T. callipaeda* has been recently demonstrated (Bezerra-Santos *et al.*, submitted).

The optimal artificial diet here proposed was chosen based on the assumption that *P. variegata* is closely associated with turkey oak forests [21], where walnuts are supposed to be one of the potential feeding sources for larvae. Therefore, a chestnut-based medium could approximate natural feeding conditions, with better performance than a standard *Drosophila* medium based on corn flour, but also as compared to fresh fruit. In addition, the liquid dietary supplement containing sodium chloride and mucin proteins (snail extract) might have partially compensated the deficiency of salts and proteins consequent to the lack of lachryphagy under laboratory breeding conditions. Indeed, it has been shown that fertility and survival rates in some lachryphagous lepidopteran species (*Thymelicus lineola*; Ochsenheimer, 1808) are closely related to the right amount of sodium that the male offers to the female as a nuptial gift, which is essential for egg maturation and oviposition [22].

Despite these encouraging results, the low initial number of wild females used in this study, especially the low number reared with the Chestnut medium, might have affected the possibility to obtain a stable colony. This is probably a consequence of the low initial genetic variability of the laboratory population [23] as well as an intrinsic low oviposition rate of the field collected *Phortica spp.* (*P. oldenbergi*: 33.9 eggs/female, *P. variegata*: 22.6 eggs/female). Comparing data herein obtained about oviposition rates with those of species belonging to the *Drosophila* genus (> 2500 eggs/female; [24]), the low progeny numbers per generation may account for the biological limitation of *Phortica spp.* in obtaining a stable colony. New attempts will be conducted with a higher starting number of field collected females to overcome these limitations and try to obtain a stable colony.

Data also allowed to clarify an open question about the population dynamics of *P. variegata* in the field. In fact, it is known that this species shows a switch of sex ratio along the breeding season, with an increase in lachryphagous males during late summer [25]. The F1 obtained by field collected females from May to October did not indicate any shift in progeny sex ratio along the season (Table 2). This led to the conclusion that the switch of relative proportion of males during the season is a consequence of a sampling bias due to their feeding behaviour instead of a physiological change of sex ratio in the population.

Conclusions

This novel breeding protocol of *Phortica spp.* allowed to investigate aspects of life history traits of these drosophilids, which are involved in the transmission of the zoonotic eyeworm *T. callipaeda*. Controlled climatic parameters and fly densities, together with the introduction of a proper feeding medium (i.e.,

considering the needs of *Phortica spp*. associated to oak forests) significantly improved the survival and fecundity of both *P. variegata* and *P. oldenbergi*. This standardized approach allowed to reach F3 generation for the first time, representing the basis for the establishment of stable colonies, which are an essential requirement for future behavioural/physiological studies on these vectors as well as pharmaceutical trials of veterinary and medical products.

Abbreviations

RH
relative humidity
GLM
Generalized Linear Model
F1
first generation
F2
second generation
F3
third generation.

Declarations

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author, MP, upon reasonable request.

Authors' contributions

DO, FB, JF, MP conceived and designed the study; CP, IB, MP, SM contributed in data collection; CP, IB, MP contributed to data analysis and interpretation; IB, DO, MABS, MP drafted the manuscript. All authors have contributed to draft revision and approved the submitted version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

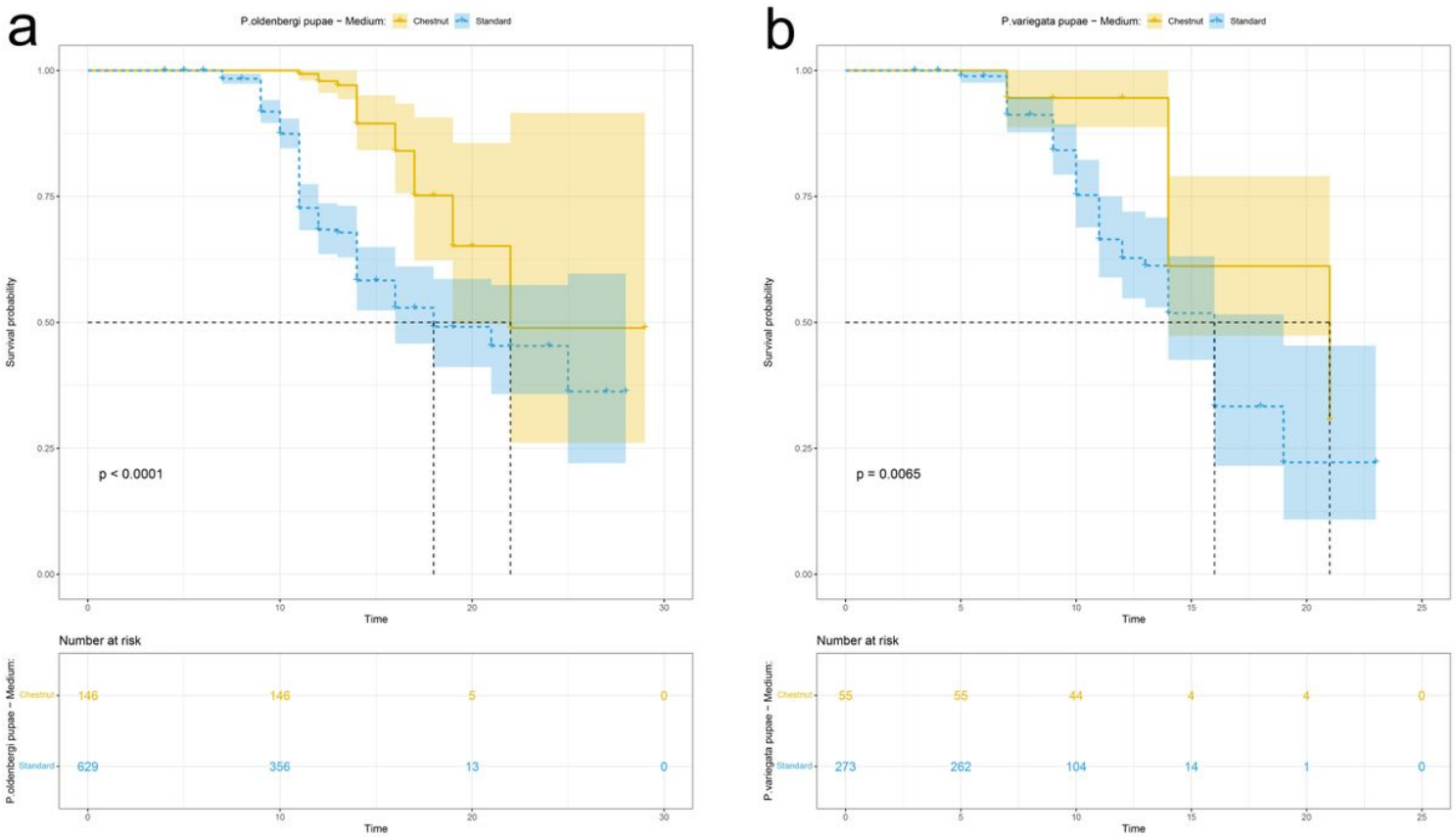

Figure 1

Survival probability curves of *Phortica oldenbergi* (**a**) and *Phortica variegata* (**b**) pupae treated with different media (Chestnut, Standard).

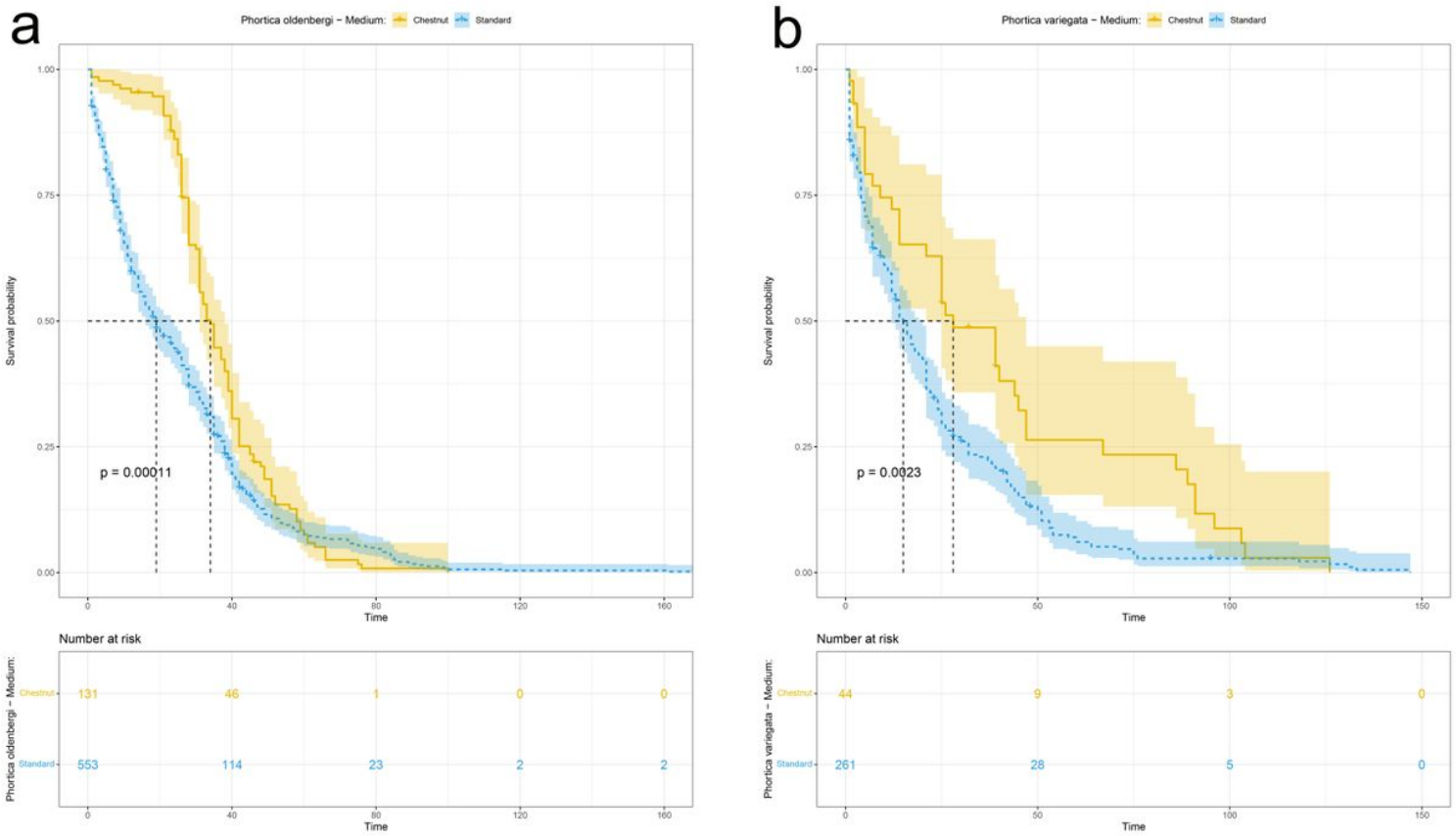

Figure 2

Survival probability curves of *Phortica oldenbergi* (**a**) and *Phortica variegata* (**b**) adults treated with different media (Chestnut, Standard).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- graphicalabstract.jpg