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Technical Note

A Method to Study Extracorporeal Development of Early *Planococcus Citri* Embryos: a New Schedule for Facultative Heterochromatinization

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Abstract:

Study of imprinted heterochromatinization of the paternal chromosome set in male mealy bugs is made difficult because it takes place at the blastula stage within the ovary. We describe here a method that allows for the bulk preparation of staged early embryos that develop normally outside the mother. We define an accessible experimental window encompassing 48 to 72hours post-mating in which regulation of heterochromatinization of the paternal chromosome set can now be investigated outside the confines of the ovary.

Keywords: Heterochromatin; mealy bugs; parent-of-origin effects; genomic imprinting

Introduction:

The citrus mealy bug *Planococcus citri* (*P. citri*) is a damaging pest of many agricultural and horticultural crops and is also a vector for several plant viruses (Viggiani, 1975). In part motivated by the commercial need to manage such infestations the life cycle of *P. citri* has been studied in some detail (Figure 1). The species reproduces sexually and is oviparous. After fertilization, a considerable amount of development takes place within gravid females before the eggs are laid on day 4 post-fertilization with egg-laying continuing for another 9 to 11 days (Brown and Nelson-Rees, 1961). The amber-yellow eggs are laid in a fluffy posterior ovisac that is about equal in length to the body of the mother who subsequently shrivels up and dies. At the time of oviposition the eggs contain embryos at around the gastrula stage (Schrader and Hughe-Schrader, 1931). It is during early development within the mother that a remarkable cytological system operates in the nuclei of the blastula stage embryos (reviewed in (Hughes-Schrader, 1948)).

At fertilisation, both parental chromosomal sets are euchromatic and indistinguishable. In male embryos one haploid set, that of paternal origin, becomes heterochromatic after the 7th cleavage division in the blastula; in female embryos both parental sets of chromosomes remain euchromatic (reviewed in (Prantera and Bongiorni, 2012)). The *P. citri* chromosome cycle is a classical example of genomic imprinting, an epigenetic phenomenon found in many animals, including in humans (reviewed in (Singh et al., 2019)). A key question yet to be resolved concerns the nature of the epigenetic “mark” present on the paternally-derived chromosomes that directs selective heterochromatinization of the paternal chromosome set at the blastula stage in male embryos. Further, how environmental factors such as rearing temperature, food deprivation and age of mating (Ross et al., 2011) can change sex ratio are unknown but most likely affect early events that regulate the heterochromatinization. Addressing these questions has been hampered by the lack of a method to easily access and study early mealy bug development outside the mother. There is a need for a reliable method to investigate extracorporeal development of early embryos. Here, we present such a method for the generation of synchronized *P. citri* embryos in bulk, which will enable cytological, molecular and biochemical analyses of early developmental stages that have hitherto been difficult to obtain and investigate in large numbers.

Results:

In order to control for environmental factors that could affect reproducibility we first synchronized embryos using a previously published protocol (de la Filia et al., 2021; Ross et al., 2011) with a few modifications. Briefly, 5-10 fertilized females were placed on freshly sprouted potatoes kept a 26ºC and at least 60% humidity. They were allowed to lay eggs for 24h and were then removed by hand and placed on sprouted potatoes in a new container. The eggs left behind in the first container were kept at 26ºC and again at at least 60% humidity. This procedure was repeated 5-6 times to produce 5-6 sets of embryos that were laid in successive 24h periods.

After a week on the potatoes, the eggs hatched producing 1st instar nymphs (Figure 1). Around 2 weeks later the nymphs reached the 2nd instar. The males and females can be distinguished morphologically at this stage. Female nymphs are slightly bigger, are shaded yellow and have more ellipse-shaped bodies. Male nymphs are smaller, narrower and with more transparent grey-colored bodies (Figure 2). Based on the morphological differences seen at the 2nd instar presumptive females were collected and placed in separate containers with sprouted potatoes and allowed to continue their development to adulthood. Adult females obtained this way were approximately of the same age and considered synchronized.

30-40 synchronized virgin females were then put together with adult males collected from the main colony (0 hour timepoint in Figure 3) and allowed to mate. 3-4 adult males per female were placed in the mating containers. 24 or 48 hours after mating the females were dissected in PBS with ultra-fine forceps. The cuticle was gently removed to release the ovaries with embryos. The embryos were next separated from each other with fine needles and transferred to 1.5 ml Eppendorf tube in PBS using a glass pipette. Embryos were washed in PBS three times by brief centrifugation (Eppendorf 5424, 30 seconds, 1000g). Washed embryos were suspended in 80% glycerol in order to stop the embryos drying out in the steps that followed. Using a glass pipette the suspended embryos were gently placed as drops on the surface of 4% Bacto agar petri plates.

After a few hours on the agar plates the unfertilized embryos eventually dissolve in 80% glycerol. For embryos isolated 24h post-mating 80% were unfertilized and dissolved in glycerol and only 20% of embryos continued development to later stages. 80% of the embryos dissected 48h after mating survived and continued to develop (Figure 3). We stained embryos collected 48h after mating with DAPI to visualize DNA in nuclei (Figure 4A). In all embryos DNA was uniformly distributed in nuclei indicating that heterochromatinization of paternal chromosome in male embryos had not taken place. By contrast, when we stained embryos that had completed 72h of development post mating under 80% glycerol, large DAPI-positive heterochromatin blocks were observed in some embryos (Figure 4B). These were male embryos in which the paternal chromosome set had undergone heterochromatinization. Thus there is a 24h period (48-72h after mating) during which heterochromatinization takes place in the embryos that are programmed to become males (Figure 3).

Our method affords significant advantages for the study of early *P. citri* development. First, extracorporeal development of the embryos is now possible without any significant effect on normal development. Embryos isolated at 24h (20%) or 48h (80%) post mating were able to develop in 80% glycerol until the nymph stage. Second, each mated female contained about 200 embryos, which enabled the collection of relatively synchronized embryos in bulk. We could then stage the imprinted heterochromatinization of paternal chromosomes in males embryos. We could show that 80% of embryos are fertilized by 48h post mating and that heterochromatinization is clearly observed at 72h post mating. This places the key early events that regulate heterochromatinization of the paternal chromosome set in the window of 48h-72h post fertilization.

Discussion:

We describe a method that enables the bulk collection of staged early *P. citri* embryos and allows for the study of their development outside the mother. Our method is simple and efficient and allows normal pre-gastrula development in embryos dissected as early as 48h after mating.

We have used this method that provides an experimentally accessible window for the study of imprinted heterochromatinization of the paternal chromosome set in male embryos. Timing of heterochromatinization of the paternal chromosome set has been difficult because early stages of embryogenesis take place inside the ovary. Nevertheless, given the set of tools that were available, it was found that heterochromatinization is characterized by a "wave of heterochromatinization" that begins at one of the pole of the egg at around the 7th cleavage division in male embryos (reviewed by (Prantera and Bongiorni, 2012)). By our estimation this wave, at the 7th cleavage division, begins at around 48 to 72 hours post mating. We show using synchronized embryos outside the confines of the ovary that fertilization is largely (80%) complete at 48h and heterochromatinization in male embryos is observed at 72h post mating.

This provides a discrete window of 24h (between 48 to 72h) where key post-zygotic events take place that regulate heterochromatinization of the paternal chromosome set. Our method offers a tractable experimental approach for the investigation of the key events within this window.

Figure legends:

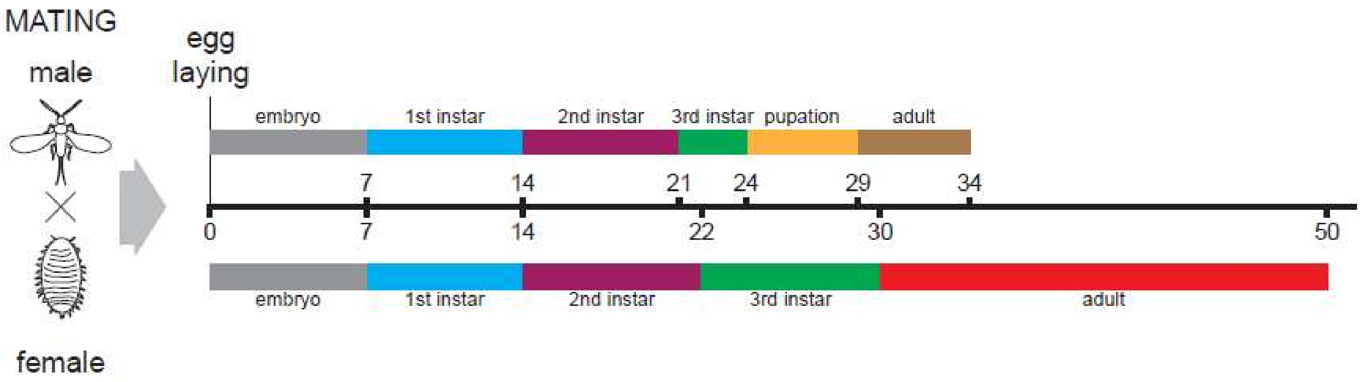

Figure 1 Planococcus citri life cycle. Embryonic development starts inside the ovary and continues for about 7 days after the eggs are laid. After hatching the 1st instar nymphs develop for 7 days and then molt into 2nd instar nymphs. Male nymphs molt again in 6 days and start pupation that continues for 5 days. Female nymphs molt into 3rd instar nymphs in 7 days and grow into adult females. Adult males live for 3-5 days, adult females live for 20-30 days. The duration of each stage is approximate because it is strongly affected by temperature, humidity, food source, and other conditions (Brown and Nelson-Rees, 1961; Mahmoud, 2017; Özgökçe et al., 2018; Reis da Silva, 2015).

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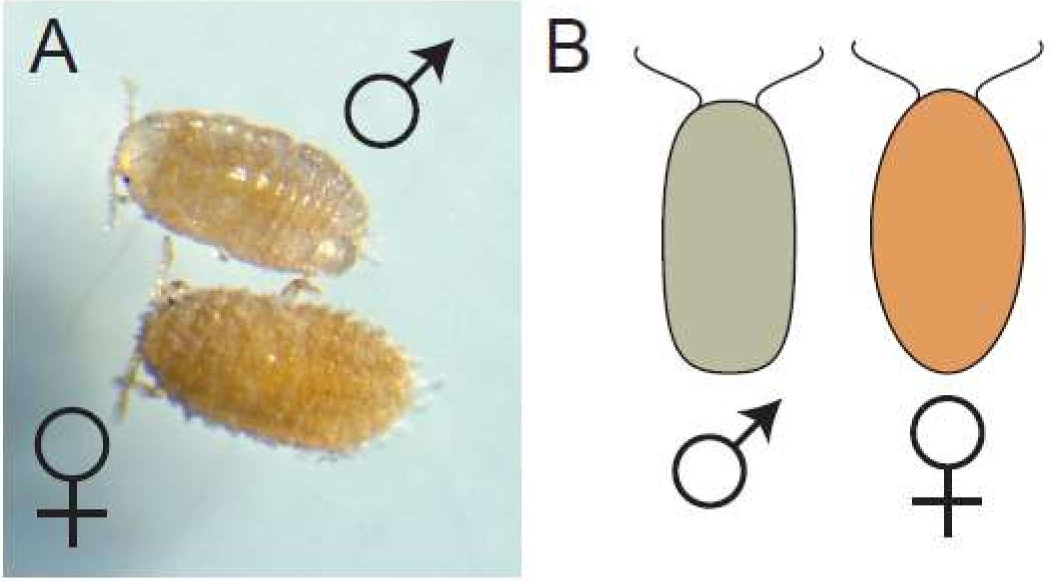

Figure 2. Male and female nymphs of P. citri. A – photograph of male (top) and female (bottom) nymphs, 21 days after mating. Male nymph has a narrower and slightly transparent body. Female nymph has an ellipse-shaped, non-transparent body of pinkish-yellow color. B – differences in male and female nymphs body shapes and pigmentation are shown schematically.

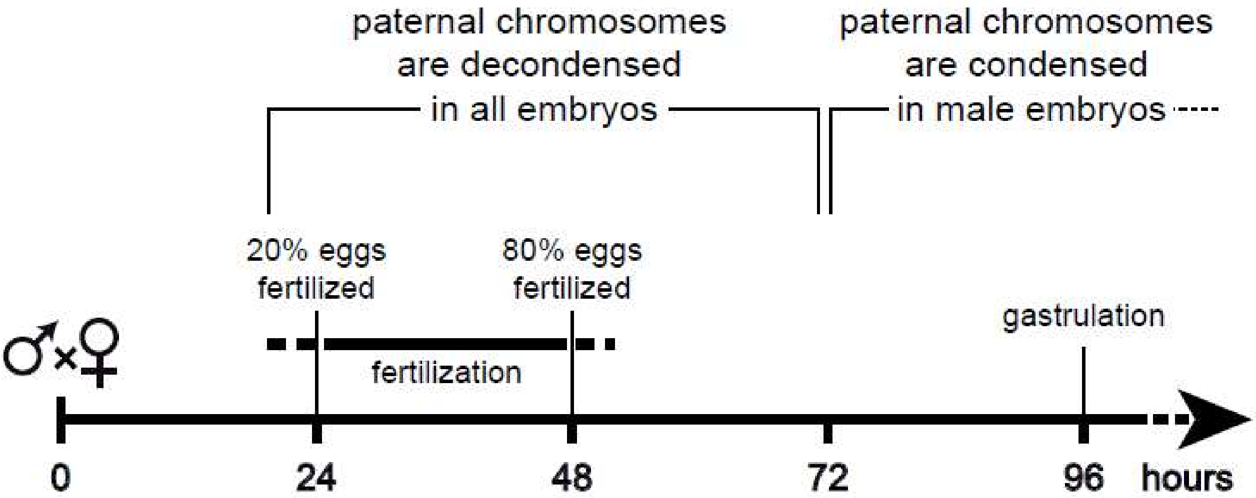

Figure 3. Timeline of the crucial events during the P. citri embryogenesis. Zero hours timepoint corresponds to the moment when males and females are put together in one container. Fertilization of the eggs occurs during the period around 24 to 48h. At 48h timepoint paternal chromosomes are diffuse in all embryos

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and do not differ from maternal chromosomes. Heterochromatin formation in male embryos is completed by 72h. Gastrulation occurs around 96 hours.

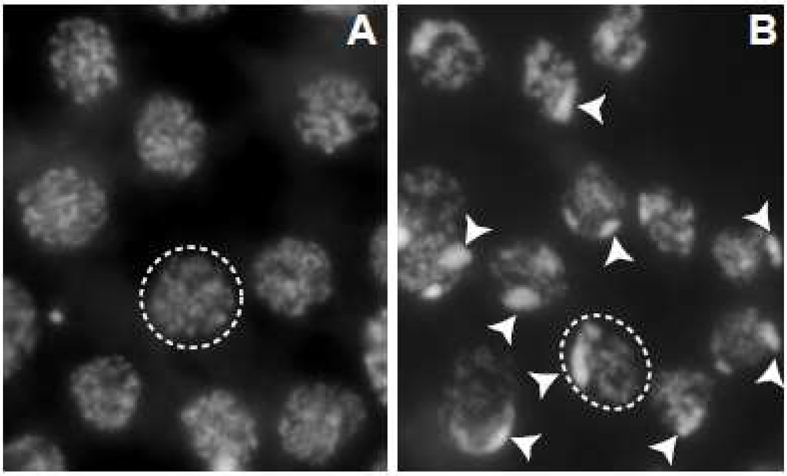

Figure 4. Embryonic nuclei before and after imprinted heterochromatinization of paternal chromosome set. A – typical picture of the embryonic interphase nuclei observed in all embryos at the 48 hour timepoint. The chromatin is diffuse and uniformly distributed in the nucleus. One nucleus is highlighted with the dotted line. B – example of the male embryo at 72 hour timepoint with large heterochromatin blocks formed by imprinted paternal chromosomes (shown with arrowheads). Embryos were stained with DAPI as described in (Bongiorni et al., 2007).

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