

## Morpho-colorimetric characterization of the embryonic development of *Chrysoperla externa* (Neuroptera: Chrysopidae): Approach for its use for biological control strategy

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### Caracterización morfo-colorimétrica del desarrollo embrionario de *Chrysoperla externa* (Neuroptera: Chrysopidae): Enfoque para su uso en control biológico

**RESUMEN.** El Manejo Integrado de Plagas (MIP) es un enfoque de control de plagas que considera todas las estrategias disponibles y su posterior integración para el control de plagas. Entre sus estrategias se destacan el Control Biológico (CB) mediante el uso de enemigos naturales. El control biológico por depredadores generalistas ha cobrado relevancia en la última década. Esta estrategia por aumento exige la cría masiva de estos organismos. En la Región Neotropical, *Chrysoperla externa* (Neuroptera: Chrysopidae) tiene gran importancia por su alta voracidad y su amplia gama de hospederos. El objetivo de este estudio fue describir los cambios externos de los huevos, especialmente en color y forma, asociados al desarrollo intracoriónico de embriones de *C. externa*, para caracterizarlos morfo-colorimétricamente. Se construyó una clave práctica a partir de la determinación de su desarrollo embrionario como herramienta para la técnica de cría masiva. Esta clave proporciona información valiosa que facilitaría la implementación de CB para programas de MIP en regiones en desarrollo, donde aún es incipiente.

**PALABRAS CLAVE.** Control biológico. Cría masiva. Fases embrionarias. Huevo. Manejo integrado de plagas.

**ABSTRACT.** Integrated Pest Management (IPM) is a pest control approach that considers all available strategies and their subsequent integration for pest control. The Biological Control (BC) of pests through the use of natural enemies is an outstanding strategy. Biological control by generalist predators has had a highlighted relevance in the last decade. This strategy by augmentation demands mass rearing of these organisms. In the Neotropical Region *Chrysoperla externa* (Neuroptera: Chrysopidae) is of great importance for its high voracity and its wide range of hosts. The aim of this study was to describe the external changes of the eggs, especially in colour and shape, associated with the intrachorionic development of *C. externa* embryos, to characterize them morfo-colorimetrically. A practical key from its embryonic development determination was constructed as a tool for mass rearing technique, providing valuable information that could facilitate BC implementation for IPM programs in developing regions.

**KEYWORDS.** Biological control. Egg. Embryonic phases. Integrated pest management. Mass-rearing.

## INTRODUCTION

Integrated Pest Management (IPM) is an agricultural pest control approach that includes all available pest control strategies and their subsequent integration (Food and Agriculture Organization of the United Nations [FAO] & World Health Organization [WHO], 2015). This type of management aims to keep pest population levels below the Economic Injury Level (EIL) and reduce the use of pesticides and other interventions to economically justified levels, minimizing risks to human health and the environment (FAO, 2019).

Biological Control (BC) is one of the strategies for pest control in IPM programs (Helyer et al., 2014). This type of control paradigm is very important when considering the transition to sustainable agriculture and for production systems that commercialize under the demands of good agricultural practices (Vázquez et al., 2008; León et al., 2018). Biological control is worked out by natural enemies, such as parasitoids, predators, and entomopathogens, which feed on or develop in phytophagous organisms (Van Driesche et al., 2007; Fischbein, 2012). This type of control is also known as natural control and is used to reduce populations of pest organisms to densities below EIL, either temporarily or permanently (Van Driesche et al., 2007). Classical, Neoclassical and Augmentative are the main types of BC strategies, where augmentation of natural enemies by inoculative form could have relevance in developing countries (Hajek & Eilenberg, 2018; Souza & Souza Bezerra, 2019).

Predators are among the most important types of natural enemies and are characterized by feeding on crop pest organisms (Helyer et al., 2014). Knowledge of not only their taxonomy, biology, specificity, and predation rates is essential for their successful use (Van Driesche et al., 2007) but also the knowledge of their life cycles (stages' development time, molting, reproduction, and behavior between other characteristics) is required prior to use in IPM programs.

In the Neotropical Region, one of the most important predators is the generalist *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae), whose role as potential biological control agent controlling pests is relevant (Carvalho & Souza, 2009; Almeida, 2020). This species is widely cited in Central America and the Caribbean up to South America (Albuquerque et al., 1994; Morales et al., 2012). It is also described for Argentina, Colombia, Costa Rica, Guatemala, and Cuba, and even in the southern United States and Mexico (Montserrat & Freitas, 2005). In Argentina, *C. externa* is commonly associated with pome fruit trees in the upper valley of Río Negro (González et al., 2011), olive crops in La Rioja (González Olazo et al., 2012), and greenhouse peppers in La Plata, Buenos Aires (Haramboure et al., 2014). Its life cycle is known and has been well described (Palomares-Pérez et al., 2020). Briefly, it has three larval stages, where the first larval instar hatches from solitary eggs. Females have a high

reproductive performance (Schneider et al., 2009; Haramboure et al., 2016.).

Larvae of *C. externa* feed on different phytophagous organisms, many of which are considered pests of economic importance in different crops, such as aphids, whiteflies, and mealybugs, among others (Silva et al., 2006; Valencia Luna et al., 2006; Castro et al., 2016). They prefer small-size preys, with a soft body and fine cuticle (Ribeiro et al., 2013). Adults, however, are glycophagous, feeding on pollen, nectar, plant fluids and insects' sweet excretions commonly known as "honeydew" (Silva et al., 2006; Devetak & Klokočvnik, 2016).

Although the life cycle and biological aspects have been reported by several authors (Albuquerque et al., 1994; Fonseca et al., 2015; Almeida, 2020; Palomares-Pérez et al., 2020), to the best of our knowledge there is no information regarding the development of embryos inside of eggs. In this sense, bibliographic research about the embryonic development of *Chrysoperla* showed that studies published hither to only refer to the egg colour in a few species, without relating the stage of development to colour variations (Reguilón & Campero, 2006; González Olazo et al., 2009). Some studies also describe the external morphology of eggs and the type of laying (Smith, 1921; Da Costa Lima, 1943; Monserrat et al., 2001)

Conservation biological control as a pest control strategy is an optimal strategy in developing countries. In several countries of Latin America, it was implemented because other biological control techniques (classical or augmentative) have a higher economic cost. The use of Chrysopidae species as biological control agents in agroecosystems is worldwide developed, mainly in Europe and the United States (van Lenteren, et al., 2018). In Brazil, *C. externa* is used as a successful biological control agent, where larvae are used by augmentation (inoculative or inundative form), then larvae are produced and marketed by mass production companies or are reared by the farmers (Souza & Souza Bezerra, 2019). However, the use of eggs in the biological control strategy by inoculative augmentation could be relevant for developing countries due to the fact that it could be cheaper than larvae releasing, which requires large amounts of preys. In this sense, the inoculative biological control in the field could be implemented with eggs releasing.

The potential use of *C. externa* eggs for BC strategy by augmentation demands the knowledge of its embryos' development for further implementation of mass-rearing techniques of this predator and its field release as eggs.

The aim of this study was to describe the intrachorionic development of *C. externa* embryos and the external changes in colour and shape of the eggs to characterize them morpho-colorimetrically. With the data obtained, we constructed a practical key as a tool for the mass rearing of this predator for inoculative biological control using eggs for field releases in vegetal agroecosystems.

## MATERIAL AND METHODS

Specimens of *C. externa* used in this study, which were never exposed to pesticides, were obtained from permanent colonies of the Laboratory of Ecotoxicology, Pesticides and Biological Control of “Centro de Estudios de Parasitología y Vectores” (CEPAVE), “Universidad Nacional de La Plata”-CONICET, located in La Plata, Argentina. Mated adults (around 50-80 females) were placed in ventilated transparent plastic containers (5 l, 21 cm diameter, 25 cm high) covered with a fine mesh following the rearing method developed in our laboratory and described in Haramboure et al. (2016). Briefly, considering that chrysopidae adults have no predator behaviour, they were reared on an artificial diet based on honey, wheat germ, and brewer’s yeast, which is commonly used for adults of the species (Vogt et al., 2000) and tap water *ad libitum*. In addition, clean black cardboard (15 cm width x 15 cm high) was added inside containers (the walls of containers were lined) as an oviposition substrate and replaced periodically. Colonies and bioassays were maintained in a bioterium at  $25 \pm 5^\circ\text{C}$ ,  $70\% \pm 5\%$  of relative humidity, and under a photoperiod of L:D 16:8. When 60 eggs were obtained (commonly obtained after 4 h), all cardboards were extracted from containers and sections of these cardboards containing one egg were isolated into vented Petri dishes. Observations were made from eggs early laid and every 24 hours under a stereomicroscope (Leica®, Germany) to record changes in egg colour and embryonic development.

**Microscopic observation:** Observation of eggs followed the protocol developed by Schneider for predator eggs described in Mirande et al. (2010) and Fogel et al. (2016). Briefly, from 60 eggs that were insolate in Petri dishes as it was detailed above, six eggs were taken at each different moment of the study (24 h, 48 h, 72 h and 96 h after laying). The eggs were removed from the cardboard with a soft tweezer and placed in an eppendorf with Bouin fixative and followed stepwise dehydration using 20-80% dilutions of analytical grade alcohol (phosphate buffer PH 7) until reaching 100% alcohol analytical grade. The dehydrated material was placed on a slide and covered with a glass coverslip, using Hoyer as the mounting medium. The preparations were dried in a culture oven (Faeta S. A., Argentina) at  $50^\circ\text{C}$ , for 48 hours.

## RESULTS

### Description of the intrachorionic development phases of the *C. externa* embryos

The eggs were ellipsoid in shape, with a mean size of  $0.92 \pm 0.01$  mm (min: 0.92, max: 0.95)  $\times$   $0.46 \pm 0.07$  mm (min: 0.35, max: 0.53) and have a long hyaline pedicel, as in several Chrysopidae species. The external colour of the

eggs changed differentially throughout the days, which allowed to set categories associated with the stage of embryonic development. These categories, which are defined for the first time in this study, are described below:

**Recently-laid eggs.** Eggs laid within the first 24 hours. These are smooth and have a fluorescent green colour. The micropile can be observed at the apical end. Internally, the embryo cannot be distinguished (Fig. 1a, b, c). Under the microscope, the well-differentiated micropile with striated edges can be observed. At this stage, the chorion, vitelline membrane, and cytoplasmic material of the embryo can be clearly differentiated as a homogeneous cell mass (Fig. 2a, b, c).

**Eggs at an early development stage (48 h-development).** Notorious changes in colour can be observed, from fluorescent green to light green. The micropile has a deep green colour. Close to the pedicel, the colour became lighter until reaching a yellow-orange colour at the lower end where the embryo is located (Fig. 1d, e, f). Under the microscope, the embryo can be observed lying on one of the egg sides with clearly identified segments. The embryo is darker than the rest of the sac (Fig. 2d, e, f).

**Eggs at a medium development stage (72 h-development).** The light green colour is only maintained at the micropile end, whereas the rest of the egg acquired a whitish colour. The embryo is “C” shaped, reddish to orange in colour and occupies the entire egg. Ring-shaped segments can be seen in the embryo body (Fig. 1g, h, i). The presence of ocelli and the leg primordia in the embryo can already be observed in microscopic preparations (Fig. 2g, h, i).

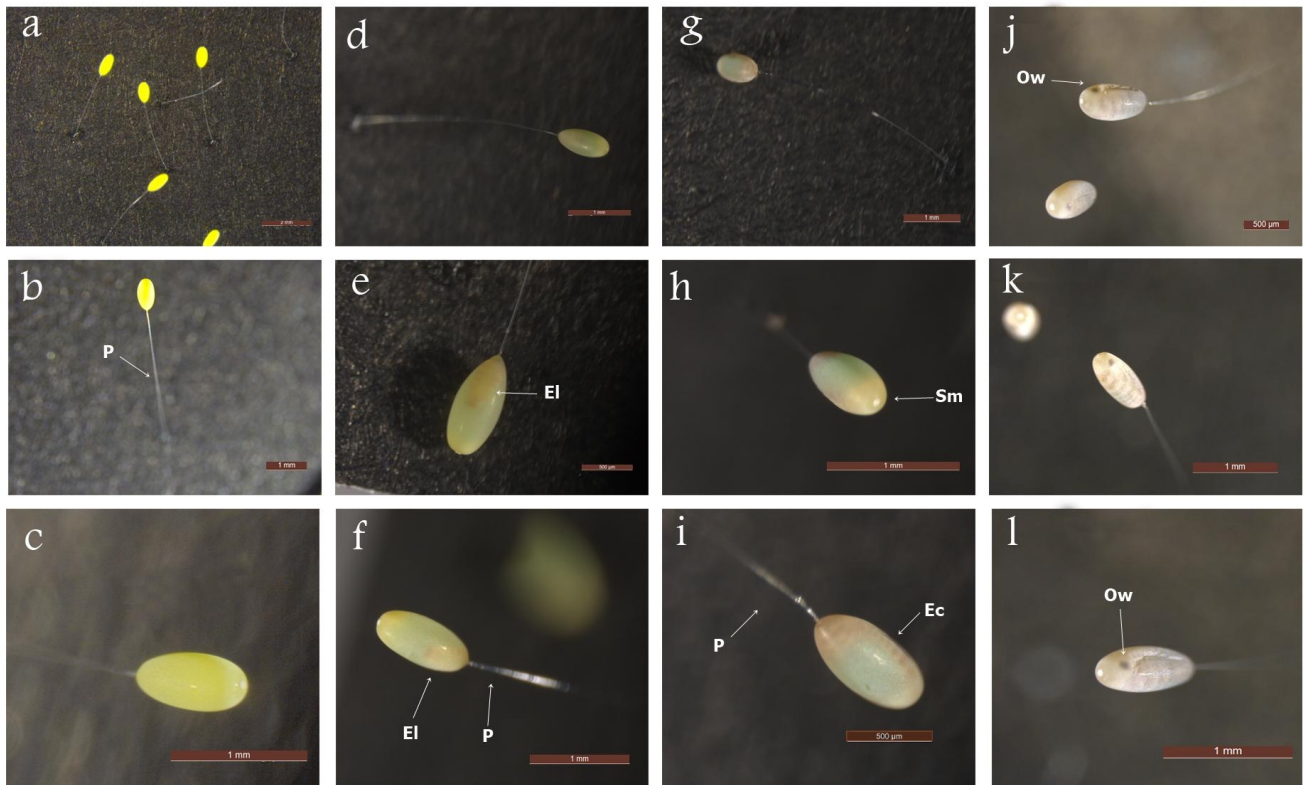
**Eggs at a late stage of development (96 h-development).** They have a white colour. The embryo becomes fully visible, with reddish ocelli located at the end close to the micropile. Ring-shaped segments, which are also reddish, are differentiated throughout the egg. This is the last stage before the insect emergence (Fig. 1 j, k, l). Under the microscope, the embryo occupies the entire egg and has well-defined ocelli and segments (Fig. 2j, k, l).

**Hatched eggs (eggs with larvae emergence).** They have a white colour and flattened appearance. The opening (made by the larva with its mandibles) through which it emerged can be observed at one of the ends (Fig. 3a, b).

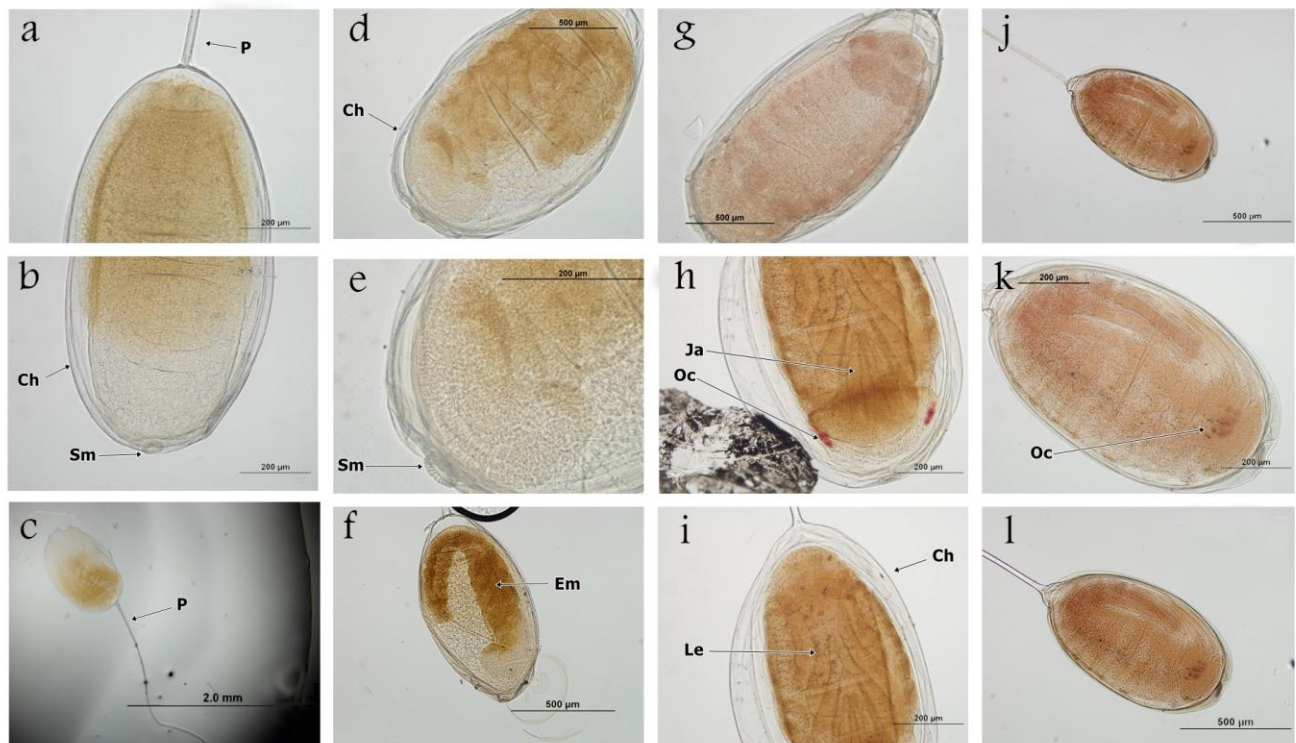
**Non-viable eggs (non-fertile eggs recently laid in which the embryonic development did not occur).** They are similar to hatched eggs in appearance but have a bright green colour (Fig. 3c).

### Practical key

The following practical key was constructed to differentiate the egg categories according to the external egg shape and colour associated with the internal stage of embryonic development.



**Fig. 1. Eggs of *Chrysoperla externa* at 25°C under the stereomicroscope.** a-b-c: eggs of 24 hours of development. d-e-f: eggs of 48 hours of development. g-h-l: eggs of 72 hours of development. j-k-l: eggs of 96 hours of development. The arrows indicate P: pedicel; El:embryo located in the basal part; Sm: striate micropile; Ec: c-shaped embryo occupying all the internal space; Ow: well-developed ocelli.



**Fig. 2. Embryonic development of *Chrysoperla externa* at 25°C.** Microscopic preparations of the eggs. a-b-c: 24 hour development embryos. d-e-f: 48 hour development eggs. g-h-i: 72 hour development eggs. j-k-l: 96 hour development eggs. The arrow indicates Sm: striate micropile P: pedicel; Ch: chorion; Le: legs; Ja: jaws; Oc: ocelli.



**Fig. 3. Raisin-like eggs of *Chrysoperla externa*. a-b: hatched eggs. c: non-viable egg.**

1. Eggs with a flattened appearance .....	4
1'. Eggs with oval shape and turgid appearance .....	2
2. Eggs of fluorescent green colour .....	<b>Recently-laid eggs</b>
2'. Eggs of a different colour .....	3
3. Presence of reddish round spots (ocelli) .....	<b>Eggs at a late stage of development</b>
3'. Absence of reddish round spots (ocelli) .....	6
4. Eggs of bright green colour .....	<b>Non-viable eggs</b>
4'. Eggs of a different colour .....	5
5. Eggs of white colour with an opening in the distal end of the pedicel .....	<b>Hatched eggs</b>
6. The embryo occupies only the basal part .....	<b>Eggs at an early development stage</b>
6'. The embryo has a "C" shape and occupies the entire egg .....	<b>Eggs at a medium development stage</b>

## DISCUSSION

Some authors previously described the eggs of *C. externa* (Smith, 1921; Da Costa Lima, 1943; Monserrat et al., 2001) referring to the external morphology and type of laying (solitary or in a group), without considering the relationship between egg colour, oviposition time and stage of embryonic development. Our results showed for the first time that there are changes distinguishable at the naked eye in the shape and colour of the eggs from oviposition to hatching, which can be related to the different phases of embryonic development. The four phases of intrachorionic development of the embryo, are easy to identify and can be recognized with the practical key proposed herein. Changes in the external colour of the chorion can be associated with the internal embryonic development (Canard & Volkovich, 2001; Boregas et al., 2003; Amaral et al., 2013), which is in turn directly related to temperature, being faster at 27 °C (72 hours) than at 25 °C (96 hours) (Griffoni et al., 2007).

Microscopy observations evidenced that 72 hours after oviposition at 25°C, the embryo already had the presence of legs, mouth apparatus and ocelli (Fig. 2), showing a great differentiation compared to the previous phases. This information might be of great relevance for other studies that analyse changes in the different phases of embryonic development in *C. externa*.

As previously described by Withycombe (1924), our results showed the presence of a striate micropile and a longitudinal opening of the chorion, which are characteristics shared by other species of Chrysopidae (Fig. 1 - Fig. 3). This study demonstrates that the stage of intrachorionic development of the embryo can be inferred through the observation of changes in the external shape and colour of *C. externa* eggs.

Embryos' development information as well as the key proposed in the present work could be useful for mass rearing companies of BC agents or farmers to improve the rearing and release techniques of Chrysopidae.

Besides, three species of *Chrysoperla* have been reported in Argentina: *C. externa*, *C. argentina* (González Olazo & Reguilón) and *C. asoralis* (Banks), which have a similar external egg morphology. In these species, eggs are laid singly; the eggs have long hyaline pedicels and a bright green colour that turns to brown when they hatch (González Olazo et al., 2009; Reguilón & Campero, 2006). Accordingly, the practical morpho-colorimetric key proposed in this work could be also useful for studies with these other *Chrysoperla* species. Moreover, our findings could be applied for field monitoring of Chrysopidae eggs in crops due to the fact that field monitoring of larvae is laborious, then the presence of these predators in the crops could be underestimated. However, the

development embryos and the key proposed under field conditions should be validated.

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