The puparium structure of the sheep nasal botfly (Oestrus ovis L.)

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SUMMARY

The structure, weight and size of nasal sheep bot fly (O. ovis L.) puparia were described by a SEM study, and the relationships of puparial measures with third-instar larvae and newly-emerged adult variables were estimated. Third instars (n=550) were collected from slaughtered goats in La Paz, Mexico, and reared in the laboratory until adult emergence (n=68). Puparial measures (Mean, SD) were: length (LP) 13.9±1.0 mm, width (WP) 6.4±0.7 mm, and thickness (TP) 6.3±0.6 mm. There were no differences in puparial measures or wall thickness (WT) between males and females. Average WT decreased significantly from the anterior segment (167.8±28.1 mm) to the middle (159.3±28.7 mm) and posterior segments (155.0±29.9 mm). Overall average WT was 160.3±26.6 mm. The deviation angle of the anterior end due to puparium curvature was 28.8±5.7 degrees. Third-instar weight was positively correlated with puparial measures but not with WT. Correlation between third-instar weight and empty puparium weight was significant (r=0.44). Weight of the empty puparium increased significantly as LP, WP and TP increased (r=0.71 to 0.78). Puparial WT at anterior, middle and posterior segments were highly correlated with puparial measures. The O. ovis puparium is a strong transitory structure highly specialized to protect the developing individual. Our results support the approach of reducing third-instar weight as a strategy to affect O. ovis free-living populations.

Keywords: Metamorphosis, puparial development, SEM, Oestridae, myiasis

RESUME

Structure du puparium d’Oestrus ovis.

La structure, le poids et la taille des pupes d’Oestrus ovis L. ont été déterminés par microscopie électronique à balayage. Les relations entre les mensurations des larves du troisième âge et les adultes qui ont éclos ont été recherchées. Des larves du troisième âge (n=550) ont été prélevées sur des chèvres abattues à La Paz (Mexique) et entretenue au laboratoire jusqu’à l’émergence (n=68). Les mensurations suivantes ont été effectuées et indiquent (Moyennes et Ecart-types) : longueur (LP) 13,9±1mm, largeur (WP) : 6,4±0,7mm, hauteur (TP) : 6,3±0,6mm. Aucune différence de mensuration ou d’épaisseur de la paroi (WT) n’a été relevée entre les mâles et les femelles. L’épaisseur de la paroi diminue significativement depuis les segments antérieurs (167,8±28,1 mm) aux segments de la partie moyenne (159,3±28,7 mm) aux segments postérieurs (155±29,9mm). L’épaisseur moyenne de la paroi des larves était de 160,3±26,6 mm. L’angle d’incurvation du puparium était de 28,8±5,7 degrés. Le poids des larves du troisième âge était positivement corrélé aux mensurations du puparium mais pas avec son poids. La corrélation entre le poids de la larve du troisième âge et le poids du puparium vide était significative (r=0.44). Le poids du puparium vide augmente significativement avec sa longueur, sa largeur et son épaisseur (r=0.71 à 0.78). L’épaisseur de la paroi du puparium au niveau des segments antérieurs, moyens ou postérieurs était aussi hautement corrélée aux mensurations. Comme le puparium d’O. ovis est une structure très spécialisée pour la protection de l’imago en développement, nos résultats confirment l’option du contrôle du parasitisme par réduction du poids des larves du troisième âge.

Mots-clés : Oestrus ovis, puparium, développement, SEM, Oestridae, myiasis

Introduction

Oestrus ovis is an obligate myiase-producing agent which is prevalent in sheep and goat populations in many regions of the world. On warm and sunny days, adult gravid females lay first-stage larvae on the hosts’ muzzles. Larvae spend a feeding period from five weeks to several months in the nasal and sinus cavities, molting twice until they become third instars [1]. They then return to the host’s nose to be ejected to the ground by violent sneezing, where they immediately burrow to form the puparium. Cyclorrhaphic dipteran species are unique through insect taxa because the puparium is formed from integumental structures of the third instars [21], a process known as tanning, which is driven by hormonal processes [20]. Therefore, the O. ovis puparium may be regarded as a transitory structure made of chitin, sclerotized proteins and minerals which protects the fragile tissues of the developing individual from chemical, physical, and biological damage. During the O. ovis life cycle, two phases are clearly distinguished, i.e. the feeding (larval), and the free-living (intrapuparial and adult). During the feeding phase, the entire nutrient reserves needed for pupal development and adult activities must be finally stored by the parasitic third instar since adults have their mouthparts atrophied and do not feed nor drink. Thus, mature third instar, puparium and adult weights are extremely important in the life cycle, since a strong relationship between mature larval weight and adult survivability has been reported for this species [4]. The puparial period usually lasts 3-5 weeks depending on the sex of the individual and surrounding temperature [3]. So far, the general characteristics of oestrid species puparia have been described [22] and reviewed [13]. Also, a study of intrapuparial development of O. ovis was conducted [5]. However, a more detailed description
of the puparium and morphological aspects of the puparial period in the sheep botfly are still missing. This knowledge would be valuable when searching for critical aspects of the life history useful to fight against the parasite, i.e. the intrapuparial period. Therefore, the objectives of this work were to describe structure, size and weight of the *O. ovis* puparium and estimate relationships of puparium sizes and weights with sizes and weights of the third instar and the newly eclosed adult.

**Material and Methods**

**LARVAL COLLECTION AND PUPAE REARING**

*Oestrus ovis* larvae (*n*=550) were collected from the heads of goats slaughtered at the municipal slaughter house at La Paz, Baja California Sur, Mexico. From these larvae, a total of 160 mature third instars were chosen to be transferred to individual terraria for pupariation. Selected mature third instars were previously washed with PBS and air-dried to be weighed in a microbalance. Then each third instar was identified and transferred to an individual terrarium with sterile sand to allow pupariation. Terraria were maintained in the laboratory at daily controlled temperatures alternating between 16°C (12 h) and 32°C (12 h) to enhance pupal survivability.

**PUPARIAL WEIGHTS AND MEASUREMENTS**

Important differences in weight between female and male mature third instars have been reported for *O. ovis* [4], which may result in puparial size differences. A total of 68 viable adults eclosed (males 19 days, females 21 days) and immediately were sexed, anesthetized with CO₂ gas and weighed. The empty puparia including the operculum cap were also weighed after adult eclosion and sizes (length, width and thickness) measured using a Vernier caliper (Mitutoyo absolute 500-197-20, Mitutoyo America Co., Illinois, USA). Length was measured as the distance between the prothoracic fringe and the anal tubercle, width (distance between right-left lateral sides at segment VIII), and thickness (distance between ventral-dorsal sides) were measured at segment VIII. Cross section cuts were made at segments V, VIII, and XI using micro scissors. The puparial wall was then measured on each segment using a micro scale under the stereoscope (Meiji EMZ-TR, Meiji Techno Co., Tokio, Japan). Dorsal, ventral and lateral measurements of the puparial wall were done on each cut line. To measure the deviation angle of the curved puparium, all puparia were individually photographed (lateral view) and the deviation angle due to curvature measured using an on-screen angle measuring scale. For this purpose, the puparial longitudinal axis was projected as a straight line drawn from the ventral anal tubercle to the dorsal area of the fifth segment. Deviation angle was then measured from the main longitudinal axis to the prothoracic fringe. It is noteworthy to mention that segmentation and structures present in the puparium of oestrid species are largely the same as those seen in the integument of third instars [13]. Thus, previous larval morphology studies using scanning electron microscopy (SEM) were used as references [7, 9, 10]. Nomenclature used here to describe *O. ovis* puparia was in accordance with Order Diptera authorities [16, 19]. Whitworth [19] pointed out that the first segment of the Protocalliphora puparium corresponds to the prothoracic adult segment while the last puparial (XI) segment is modified to fit the spiracular plate and the excretory openings.

**SEM PROCEDURES**

Eclosed puparia were dried and coated with gold (Denton Desk II Sputer coater, Denton Vacuum, New Jersey, USA) and observed and photographed using a scanning electron microscope (Hitachi Mod. S-3000-N, Hitachi Science Systems, Tokyo, Japan) at 25 kElectron volts.

**STATISTICAL ANALYSIS**

Descriptive statistics of measurement variables were calculated. Simple correlation coefficients among measurement variables were calculated. Paired *t* test comparisons between female and male puparium measurements were carried out. Wall thickness at segments V, VIII, and XI was compared by one-way ANOVA and post-hoc Duncan test. All statistical procedures were carried out with the Statistica software [15].

**Results**

Descriptive measures of the puparium and a general view are shown in Table I and Figure 1. The pupariation process takes about 12h in *O. ovis* (5), during this period the individual shrank longitudinally from 22 to 13.9 mm which represents 63% of larval length, eventually acquiring the typical puparial shape (Figure 1). When the burrowing third instar reached the desired depth into the pupariation substrate, it turned the anterior end 180° to be in an upward position, ready for adult emergence. Complete tanning and sclerotization of the puparium is usually completed by 24 h postpupariation. The eclosion operculum (fenestra) is a fingernail-shaped plate clearly formed on the dorsal
surface from Segment I to Segment IV, which coincides with
the anterior end of the puparial curvature. The deviation
angle due to the puparium curvature at the anterior end
was 28.8±5.7°. The anterior end (Segments I, and II)
was somewhat flattened and usually empty in the living
puparium, because the pharate adult occupied the puparial
cavity from Segments III to XI. On the inner ventral surface
of the puparial remains of the proencephalon were located,
as well as the cephalopharyngeal skeleton, mouth hooks and
the exuvia derived from the larval anterior digestive system
(Figure 2-B).

The widest size of the puparium was actually located at
Segment VIII, where the cross section area is almost round-
shaped (mean width 6.4±0.7, thickness 6.3±0.6 mm, Table I).
There were no statistical (P>0.05) differences in puparium
length between males (13.7±1.0 mm) and females (14.1±0.9
mm) or for any other size measured (data not shown in
Tables). Weight of empty puparium was 29% weight of live
puparium during the third week.

Because of the integumental shrinkage and wrinkle
formation that occurs during the pupariation process,
puparial wall thickness was very irregular along the puparial
surface. Nevertheless, thickness decreased significantly from
the anterior to the posterior end (Table I), except for the
operculum fissure which was about a third thinner than the
rest of the wall (Figures 1-A, B). Likewise, dorsal and ventral

<table>
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<th>Size/weight of the individual</th>
<th>Mean</th>
<th>SD</th>
<th>Confidence interval of mean</th>
<th>Range</th>
</tr>
</thead>
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<tr>
<td>Weight (mg)</td>
<td></td>
<td></td>
<td>−95%</td>
<td>+95%</td>
</tr>
<tr>
<td>Mature third instar</td>
<td>498.6</td>
<td>103.7</td>
<td>464.6</td>
<td>532.7</td>
</tr>
<tr>
<td>Live puparium</td>
<td>226.7</td>
<td>29.9</td>
<td>208.7</td>
<td>244.8</td>
</tr>
<tr>
<td>Empty puparium</td>
<td>64.8</td>
<td>20.5</td>
<td>58.2</td>
<td>71.5</td>
</tr>
<tr>
<td>Emerged adult</td>
<td>86.4</td>
<td>23.4</td>
<td>78.7</td>
<td>94.0</td>
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<table>
<thead>
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<tbody>
<tr>
<td>Length</td>
<td>13.9</td>
<td>1.0</td>
<td>13.6</td>
<td>14.2</td>
</tr>
<tr>
<td>Width</td>
<td>6.4</td>
<td>0.7</td>
<td>6.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Thickness</td>
<td>6.3</td>
<td>0.6</td>
<td>6.1</td>
<td>6.5</td>
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</table>

<table>
<thead>
<tr>
<th>Thickness of puparial wall (µm)</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Anterior</td>
<td>167.8*</td>
<td>28.1</td>
<td>160.8</td>
<td>174.4</td>
</tr>
<tr>
<td>Middle</td>
<td>159.3b</td>
<td>28.7</td>
<td>152.4</td>
<td>166.2</td>
</tr>
<tr>
<td>Posterior</td>
<td>155.0c</td>
<td>29.9</td>
<td>147.8</td>
<td>162.2</td>
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<tr>
<td>Dorsal</td>
<td>158.4e</td>
<td>27.1</td>
<td>151.9</td>
<td>165.0</td>
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<tr>
<td>Ventral</td>
<td>160.0f</td>
<td>29.7</td>
<td>152.8</td>
<td>167.2</td>
</tr>
<tr>
<td>Lateral</td>
<td>162.1g</td>
<td>29.1</td>
<td>155.0</td>
<td>169.1</td>
</tr>
<tr>
<td>Overall</td>
<td>160.3</td>
<td>26.6</td>
<td>153.9</td>
<td>166.8</td>
</tr>
</tbody>
</table>

1 Weight of the live puparium recorded during the 3rd week of the puparial period.
2 Width and thickness were measured at the VIII segment.
3 Comparisons of means of thickness of puparial wall with superscripts a, b, c indicate statistical significant differences (P<0.05). Means with d, e, f (P=0.07), n=68.
puparial layers tended (P=0.07) to be thinner than the lateral ones.

Correlation coefficients shown in Table II indicate that mature weight of the third instar was positively correlated with all puparium sizes but not with puparial wall thickness. This means that the puparial wall is a protective layer in which thickness is synthetized by the prepupa independently of weight of the third instar. Correlation between larval weight and empty puparial weight was low but significant (r=0.44, P<0.05). Weight of empty puparia increased significantly as length, width and thickness increased (r=0.71-0.78, P<0.05). Puparial wall thickness at the anterior, middle and posterior puparium were highly and positively correlated among them and with dorsal, lateral and ventral thickness.

In addition, a series of 5-7 micropores aligned on each dorsal segmental band of the maturing third instar were detected. These micropores (about 20 µm diameter) remained detectable in the hard-dark puparium (Figure 3). These micropores are distributed symmetrically and are not associated with puparial thickness. They are, therefore, considered to be due to the puparium curvature, which is more pronounced and probably are involved in insect respiration during intrapuparial development.

**Discussion**

The puparia of many dipteran genera have been described, particularly, extensive studies have published for Protocalliphora parasites of birds [11, 19] and various calliphorids and phorids of forensic importance [8, 18]. In our knowledge, this is the first SEM description of the puparium structure for an oestrid species. Nilssen [13] published an extensive review of literature on pupal biology of the oestrid flies. However, studies therein described dealt mainly with intrapuparial metamorphosis and gonotrophic development. On this regard, it is clear that weight economy during the metamorphosis is very important in oestrids. In our study, average weight losses in O. ovis were: third instar-puparium (-54.5%), live puparium-adult (-61.9%) and third instar-adult (-82.7%). These figures are clearly greater than those reported (-24.5%, -34.0%, -50.0%, respectively) for Ctenocephalides felis by Bennett [2]. Likewise, mature third instar weight was positively related with puparium weight and size, and with adult weight. Nilssen [12] reported similar significant positive correlations (r=0.34-0.44) between third instar weight and newly eclosed adult weight in the reindeer oestrid parasites Hypoderma tarandi and Cephennemys trompe. More recently, Rivers et al. [14] demonstrated a significant effect of weight of the larvae on pupal size in six species of forensic importance (the calliphorids Lucilia illustris, Lucilia sericata, Phormia regina, Protaphormia terraenovae, the sarcophagid Sarcophaga bullata, and the phorid Megaselia scalaris), and this effect was found to be species-dependent. Differences in puparium wall thickness among the anterior, middle and posterior segments may be due to the puparium curvature, which is more pronounced and probably are involved in insect respiration during intrapuparial development.

**Table II**: Simple correlation coefficients$^1$ of variables of *O. ovis*, including larvae, puparia and adult sizes and weights.

<table>
<thead>
<tr>
<th>Variable$^2$</th>
<th>TW</th>
<th>EW</th>
<th>AW</th>
<th>LP</th>
<th>WP</th>
<th>TP</th>
<th>TA</th>
<th>TM</th>
<th>TS</th>
<th>TD</th>
<th>TV</th>
<th>TL</th>
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<tbody>
<tr>
<td>TW</td>
<td>1</td>
<td>0.87</td>
<td>0.41</td>
<td>0.86</td>
<td>0.83</td>
<td>0.9</td>
<td>0.11</td>
<td>0.17</td>
<td>0.13</td>
<td>0.15</td>
<td>0.13</td>
<td>0.16</td>
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<tr>
<td>EW*</td>
<td>*</td>
<td>1</td>
<td>0.43</td>
<td>0.76</td>
<td>0.71</td>
<td>0.78</td>
<td>0.03</td>
<td>0.08</td>
<td>0.05</td>
<td>0.07</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>AW*</td>
<td>*</td>
<td>1</td>
<td>0.36</td>
<td>0.46</td>
<td>0.32</td>
<td>0.15</td>
<td>0.17</td>
<td>0.17</td>
<td>0.19</td>
<td>0.16</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>LP*</td>
<td>*</td>
<td>*</td>
<td>1</td>
<td>0</td>
<td>0.71</td>
<td>0.75</td>
<td>-0.02</td>
<td>0.07</td>
<td>0.01</td>
<td>0.04</td>
<td>0.01</td>
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<tr>
<td>WP*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>1</td>
<td>0</td>
<td>0.77</td>
<td>0.44</td>
<td>0.51</td>
<td>0.47</td>
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<td>0.47</td>
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<td>TP*</td>
<td>*</td>
<td>*</td>
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<td>*</td>
<td>1</td>
<td>0.04</td>
<td>0.11</td>
<td>0.05</td>
<td>0.08</td>
<td>0.06</td>
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<tr>
<td>TA n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>1</td>
<td>0.91</td>
<td>0.88</td>
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<td>0.91</td>
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<tr>
<td>TM n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
<td>1</td>
<td>0.97</td>
<td>0.98</td>
<td>0.99</td>
<td>0.98</td>
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<tr>
<td>TS n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
<td>1</td>
<td>0.97</td>
<td>0.99</td>
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<tr>
<td>TD n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
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<td>0.98</td>
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<tr>
<td>TV n.s.</td>
<td>n.s.</td>
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<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
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<td>1</td>
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<tr>
<td>TL n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>1</td>
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</table>

$^1$Values above the diagonal are correlation coefficients among variables. Statistical significance is shown below the diagonal (* P<0.05, n.s. not significant, n=68).

$^2$Variable codes: TW =Third instar weight, EW=Empty puparium weight, AW= Adult weight, LP=Length of puparium, WP= Width of puparium, TP=Thickness of puparium, TA=Thickness of anterior puparial wall, TM=Thickness of middle puparial wall, TS= Thickness of posterior puparial wall, TD=Thickness of dorsal puparial wall, TV=Thickness of ventral puparial wall, TL=Thickness of lateral puparial wall.

**Figure 3**: A. Micropores aligned (arrows) on the VII dorsal segment of Oestrus ovis puparium, Bar=500 µm. B. The selected area was magnified to show the external ultra structure of the micropore (arrow), Bar=100 µm.
at the anterior puparial end. Integumental wrinkling is therefore enhanced at the anterior end of the puparium, and this thickening probably gives more resistance to mechanical forces when it is located in the pupariation substrate, or into the ground.

Respiration of individual *O. ovis* developing inside the puparium is an intriguing question. Amphipneustic dipteran species protrude spiracular structures to provide respiratory via to the developing insect. The horn fly (*Haematobia irritans*) and stable fly (*Stomoxys calcitrans*) pupae are clear examples of this mechanism [17]. According to Colwell [6], prothoracic spiracles have been so far described in the subfamilies Gasterophilinae and Cuterebrinae and inconsistently in Oestrinae. Without any doubt, third instar *O. ovis* are metamepneustic and prior to beginning the pupariation process, the intestine of the pupariating larva is emptied and the anal tubercle collapses over the spiracular plate [5]. Soil particles are often caked with the sticky fluid secreted through the anus, probably sealing the posterior spiracles, which may interfere with gas exchange. Therefore, transpuparial wall respiration (simple diffusion of gases throughout the puparial wall) has been hypothesized for *O. ovis* [5]. However, dorsal micropores here reported for *O. ovis* puparium may be important for intrapuparial respiration but their precise role remains to be investigated.

**Conclusion**

In conclusion, our results indicate that *O. ovis* puparium is a strong transitory structure highly specialized to protect the developing insect under a variety of conditions. Relationships of third instar weight and puparium dimensions support the approach of reducing the mature weight of third instar as a strategy to reduce adult populations. Such a weight reduction should be designed as a part of integrated pest management directed to affect the free-living phases of *O. ovis*.

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**References**


