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Extracellular activity of NBAD-synthase is responsible for colouration of brown spots in *Ceratitis capitata* wings

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Running head: Brown wing spots tanning in absence of cells

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Abstract

After the emergence of the *Ceratitis capitata* imago, the pale and folded wings are expanded and sclerotized to acquire the definitive form and to stabilize the cuticle. The wings of this fly show a specific pattern of brownish and black spots. Black spots are pigmented by melanin, whereas there was scarce information about the development of the brownish spots. N-beta-alanydopamine (NBAD) is the main tanning precursor in *C. capitata* body cuticle, and we hypothesized that it may be responsible for the colouration of the brownish spots. We determined the topology and timing of NBAD synthesis and

deposition to attain the species-specific colouration pattern. We demonstrated that during the first hours the colour of the brownish spots was principally determined by the tanning of the hairs. Haemolymph circulation through the veins is required to tan the wings. We confirmed that soon after wing spreading, most of the wing epidermal cells disappeared. Thus, the tanning of the brown spots was accomplished when the wing lamina was devoid of cells. NBAD synthase (NBAD-S; Ebony protein in *D. melanogaster*) activity in wings was detected in pharate adults and lasted several days after the emergence, even after the end of the tanning process. This observation is in contrast to epidermal NBAD-S activity in the body, where it was nearly undetectable 48 hours post emergence. Our results indicate that NBAD-S was exported and deposited into the extracellular matrix of the brown spot areas before cell death and that tanning occurs through gradual export of NBAD precursors (dopamine and b-alanine) from veins.

1. Introduction

The integument of insect wings consists of a bilayer of epithelial cells attached at their basal laminae that secrete cuticle from their apical surfaces (Belalcazar et al., 2013). Dipteran wings are supported by a network of veins and are covered by hairs, each constructed by a single wing epithelial cell (Classen et al., 2005). Wings of newly emerged flies are highly folded, with a pale and pliable cuticle. Haemolymph then fills the narrow space between the dorsal and ventral epithelia. Changes in the haemolymph pressure are important for the expansion of the wings (Johnson and Milner, 1987; Zdarek and Denlinger, 1987; 1992), and the circulatory system is responsible for the distribution of hormones and metabolites that control post-ecdysial processes (Pass et al., 2015). During wing expansion, the two initially separate epithelial layers come together along a sharp interface and undergo several changes followed by programmed

cell death. Thus, after this process almost all of the epidermal cells become destroyed, which is a prerequisite for the fusion of the two cuticular layers (Kimura *et al.*, 2004; Peabody *et al.*, 2008). The resulting cell fragments are removed to a large extent through phagocytic uptake by certain hemocytes (Kiger et al., 2001; 2007).

The cuticle of the imagoes must be sclerotized soon after the ecdysis in order to avoid environmental injuries and also to provide the mechanical properties for muscle attachment and locomotion. Sclerotization stabilizes the cuticle through the incorporation of phenolic compounds, followed by the formation of quinones that crosslink proteins and chitin (Hopkins and Kramer, 1992; Rongda et al., 1997; Andersen, 2010; Sugumaran and Barek, 2016). As the imago is attaining the final body shape, the cuticle is getting coloured, hardened and stiffened by the processes of sclerotization and/or tanning.

Two dopamine derivatives, N-β-alanyldopamine (NBAD) and N-acetyldopamine (NADA), are central sclerotizing precursors which are involved in the tanning of the typical brown insect cuticles and in uncoloured cuticles, respectively (Hopkins and Kramer, 1992). When sclerotization is initiated, NADA and NBAD are actively transported from the epidermal cells into the cuticular matrix and oxidized to the corresponding ortho-quinones (Hopkins and Kramer, 1992). The black colour results from the incorporation of melanin that derives from DOPA and dopamine into the cuticle (True et al., 1999; Sugumaran, 2002). Several publications assessed the expression of genes and enzymes responsible for melanin wing pigmentation (True et al., 1999; Wittkopp et al., 2002; Fukutomi et al., 2017), but as *Drosophila melanogaster* does not contain any spots on the wings, NBAD metabolism was scarcely discussed. In spite of several studies (True et al., 1999; Wittkopp et al., 2002) many details of the developmental mechanisms of brown colour pattern formation in dipteran wings, other than *Drosophila*, remain unknown. *Ceratitis capitata* wings are pigmented with black and brownish spots.

Studies in *Drosophila biarmipes* reported that the Yellow protein is expressed in the pigmented area (where melanin is present), whereas Ebony is expressed where the the pigmentation does not occur (Wittkopp et al., 2002; Gompel et al., 2005). However, our studies in NBAD metabolism in *C. capitata* suggest that brownish spots in this fly would be tanned mainly by NBAD. We previously reported that the enzymatic activity responsible for the synthesis of NBAD in *C. capitata* epidermis is detected during narrow temporal windows, occurring during the sclerotization of larval cuticle to form the puparium, as well as during the pharate period and the first hours following imago ecdysis (Perez et al., 2004; 2011).

The aim of this work was to test if NBAD-S, the enzyme that conjugates dopamine with β-alanine, is involved in the colouration of the brown spots in *C. capitata* wings. Two main approaches were followed: **1-** to determine the topological and temporal pattern of NBAD synthase activity biochemically, and **2-** to correlate this pattern with the progressive pigmentation of wing brown spots.

2. Material and Methods.

2.1. *Chemicals*

Chromatographic acid alumina and DAPI were obtained from Sigma Chemical Co (St. Louis, MO, USA). [^{14}C]β-Alanine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). Optiphase “Hisafe” 3 was from Perkin Elmer. All the solvents used were of the highest purity available.

2.2. *Insects*

Colonies of 100 wild type *C. capitata* adults (strain “Mendoza”) were maintained in 3.75 L flasks with free access to sucrose: dry yeast (3:1) and 1% agar as food and water source, respectively. These flasks were kept in a chamber (CMP- 3244, Conviron,

Canada) at 23 ± 1°C, 50–60% relative humidity, and L16:D8 h photoperiod. Lights turned on at 8:00 h and turned off at 24:00 h. To collect the eggs, plastic fruits presented small holes to allow the female to insert the ovipositor and deposit the eggs. Eggs were laid during 4 h period and then were collected to start synchronized fly cultures. Larvae were reared in pumpkin-based medium as described in Pujol-Lereis (2006).

The age within the puparium was measured in hours before imago emergence (h BIE). In our culture conditions, the stages within puparium last 12 days, and external morphological characters were used to age pharate adults, as previously described in Rabossi et al. (1992).

2.3. *Wing processing and microscopy*

Adult flies were collected just after emergence, sexed under CO_2 anaesthesia, and males were placed in flasks with food and drink as described previously. In each experiment, the wings were cut at the level of the hinges at specific times and placed into a 1.5 mL microcentifuge tube with 96% ethanol for 20 sec. After washing them twice with 0.1 M phosphate buffer pH 7.2, they were dried and placed on a slide with a drop of Gelvatol mounting medium (Cold Spring Harbor Protocols, 2006). For cell nuclear visualization, wings were incubated with a solution of 0,002 μg /μl DAPI in PBS for 15 min. After three washes with PBS, wings were mounted as described above. Images were taken using an Olympus BX60 microscope connected to an Olympus DP71 digital camera. Images were converted to greyscale and the brightness value of each section was measured using ImageJ software. The development of spots colouration was measured as the decline in image brightness, which is associated with increased light absortion by the cuticle in this section, due to the presence of pigment.

2.4. *Vein obstruction assay*

In order to block the haemolymph circulation in the wings, 15 minute-old adults were anesthetized at 0ºC for 10 min. The subcostal, radial 1 (R1), radial 2+3 (R2+3) and radial 4+5 (R4+5) veins of the right wing (see Fig 1 for vein names) were obstructed with an inoculation loop (made of an insect pin 0.4 mm rod diameter). The left wing of the same insect was left untouched and used as a control of brown spot tanning. Then, the flies were transferred to 23 ºC and after 3 h the wings were cut and processed, as described in section 2.3.

2.5. In vitro NBAD-synthase assay

Samples (wing- and leg-free beheaded bodies; whole wings or wing sections) were dissected and homogenized in 50 mM sodium borate buffer, pH 8.2, saturated with phenylthiourea and containing 10 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 2 mM dithiothreitol (DTT), 10% glycerol and a mixture of protease inhibitors (E-64, pepstatin A, and phenylmethanesulphonyl fluoride).

Cell-free NBAD-S activity was measured as previously described (Perez *et al.*, 2004). The reaction mixture contained 5 mM $MgCl_2$, 2 mM ATP, 0.1 mM dopamine; 0.01 mM β-alanine and 50,000 cpm of [1-^{14}C]β-alanine (55.0 µCi/µmol; 250 pmoles) in 50 mM sodium borate buffer, pH 8.3 to a final volume of 50 µL. The reaction was started by adding 3 µg of enzymatic homogenate and stopped with perchloric acid (2% final concentration). After centrifugation at 16,000 x g, the supernatant was immediately analyzed. Catecholamine derivatives were isolated by using 150 mg chromatographic acid alumina (Sigma) in 200 µL of 1 M Tris-HCl buffer, pH 8.7. The supernatant was added to the alumina slurry and the mixture was incubated for 15 min at ambient temperature with continuous shaking. The suspension was poured into small polyethylene columns, and the alumina was washed 10 times with 1.5 mL of 0.1 M Tris-HCl buffer, pH 8.7. The first percolate (1.5 mL) was recovered into scintillation vials.

Finally, bound catecholamines were eluted with 1.7 mL of 1 M acetic acid, recovered into a scintillation vial, and radioactivity was counted in a Rackbeta 1214 (Pharmacia) liquid scintillation counter, using Optiphase “Hisafe” 3 (Perkin Elmer) as a scintillant. At least 3 replicas of 10 beheaded (leg- and wing-free) bodies or 20 whole wings were analyzed for each developmental age. Protein concentrations were determined using Bradford reagent (Sigma-Aldrich) and bovine serum albumin (BSA) as a standard.

2.6. *Tissue NBAD-synthase activity in different wing regions*

Isolated portions of the wings were tested for *in vitro* NBAD-S activity, as described in section 2.5. Twenty-four hours after adult emergence, wings were dissected to obtain veins and intervein lamina sections. Anterior samples were taken from the upper part of the brown Discal crossband spot (White and Elson-Harris, 1992). For the purposes of this paper, the Discal crossband spot was named brown spot 1 (Fig. 1, black box 1). Anterior-distal samples were from the brown “costal band” (White and Elson-Harris, 1992); in this paper named brown spot 2 (Fig. 1, black box 2). The veins between the anterior part of the Discal crossband were collected (Fig. 1, white boxes). The samples in the grey lamina section “silver paint” according with Sivinski et al. (2004) were from the posterior region between M and CuA1, CuA1 and CuA2 veins (Fig 1, black box 3 and 4). Thus, veins, brown spots lamina and silver paint lamina portions of wings were tested separately for NBAD-synthase activity.

2.7. *NBAD-synthase immunoprecipitation and activity assay*

Immunoprecipitation studies were performed on whole wing extract of 2 h-old adults using rabbit anti-NBAD-S serum (Wittkopp *et al.*, 2002), kindly provided by Sean B. Carroll (University of Wisconsin-Madison, Wisconsin USA). As a control, the same extract was incubated with preimmune rabbit serum. The enzyme preparation, as

described in section 5, was incubated with the serum (final volume 35 μL) in microcentrifuge tubes for 2 h at 4ºC with agitation. After this incubation, 15 μL of protein A-Sepharose CL-4B (Pharmacia) (enough to approximately precipitate 10 μg of antibodies) was added, and the mixture was incubated for 40 min at 4ºC with agitation. Then, the mixtures were centrifuged at 10,000 rpm for 4 min. The supernatants were removed and assayed for NBAD-S activity, as described in section 5.

2.8. *Statistical analysis*

One-way ANOVA with Tukey's post test was performed, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

3. Results

3.1. Male wings tan after emergence

Newly emerged adults undergo physiological events leading to the final body shape and full expansion of their wings (1 h 45 min after ecdysis). The mature wings of *C. capitata* show brownish and black spots within a pale grey background (or “silver paint“) (Fig 1A and D, 72 h). In newly emerged flies, the colour of the wings appeared clear or pale-grey (Fig 1C, 30 min) and remained grey in the non-spot area of the mature insect (Fig 1D, since 72 h). Areas of future black spots were evident as dark grey areas soon after emergence (Fig 2, 1h) and developed a black coloration 48 h after ecdysis (Fig 2). In the area of future brown spots, the colour was faint yellow (Fig 1C, 30 min) and turned to reddish brown in mature insects (Fig 1D, 72 h).

The time course of colour changes during *C. capitata* wing maturation is shown in Fig 2. We observed that the colouration of the wings began first in the anterior regions, and then continued to the posterior regions, thus following the direction of haemolymph circulation (Fig. 1B, Fig. S1). The development of colour intensity was quantified as the

decline in image brightness where the brown spots form (Fig. 1A, sections 1 and 2), as well in two sections of the pale grey background (sections 3 and 4). The image brightness in sections 1 and 2 decreased rapidly from adult emergence until 24 h (Fig. 1E), and then barely changed until 48 h when the brown spot tanning was completed (Fig. 1E). No changes were registered in the image brightness of the two grey sections (sections 3 and 4) following adult emergence (Fig. 1E).

3.2. The brownish spot colour is mainly determined by wing hairs

Analyzing the brown spots in wings of different ages, from 30 min (pale brown) to 24 hours (reddish brown) after emergence, we ascertained at high magnification that the brownish colour is determined first by the tanning of the hairs and sockets and then, following 4 h also by the lamina (Fig 3). In wings of young flies, less than 2 h post emergence, the hairs showed a faint yellowish hue, allowing a very easy visualization of the uncoloured lamina between hairs (Fig 3, 30 min, 1 h, and 2 h). As the tanning of the hairs increased, the colour progressively intensified (Fig 3, 24 h). The spot visualization merged the dorsal and ventral surfaces and thus, under low magnification the appearance of a uniform brownish pattern seems to be the result of an ~~optic~~ optical effect (Fig 2 and Fig 3, 24 h). Hairs with colouration other than brown were also observed in the wings of *C. capitata.* In grey regions, hairs and sockets are black (Fig 3). There are other regions, where the hairs are black and the sockets are dark brown (Fig 3), so there would be a mixed situation where black and brown melanization occur. As we are interested in NBAD metabolism, we focused our work in the brown spots.

3.3. Brown spots are coloured gradually from the veins to the intervein lamina

The progress of brown spot colouration spread spatially from the veins to the lamina (Fig. 4). In spot 1, the colour first appeared close to the veins R1, R2+3, R4+5 (Fig. 4, 2h) and then continued to the posterior laminar region up to M, CuA1 and CuA2 veins

(Fig 4, 4 h). The developmental timing of colouration of the brown spot 1 coincided with that of the neighbouring black spot (Fig 4, dashed arrow). Four hours after the emergence, the typical brownish colour was evident, and 24 hours later, the final colour was almost attained (Fig 4).

3.4. Haemolymph circulation is necessary to tan the wings

After the emergence of the adults, the wings are spread by the pressure of the haemolymph pumped into the veins by abdominal muscles. In order to assess if the haemolymph carries the precursors for NBAD synthesis or NBAD itself, an obstruction was made in the proximal anterior region of the right wing veins close to the hinge, thus blocking haemolymph circulation (Fig 5B, arrow; Fig. S 2B). The left wing of the same individual was left untouched as a control for the tanning progress (Fig 5A). After 3 h the right wing without hemolymph circulation was scarcely pigmented (Fig 5D). At this time, the spots in the left wing started to develop their normal brownish colour (Fig 5C), thus confirming that haemolymph is required for tanning of the wings spots. In agreement with the observation that the transport of substrates through the veins is critical to complete the melanin pattern in *D. melanogaster* and *Oncopeltus fasciatus* (Kiger et al., 2007; Liu et al., 2014), our results demonstrated that the substrates for NBAD-synthesis or NBAD itself must be carried by the haemolymph and confirmed that the tanning process progresses from the veins to the spot regions.

3.5. *The epidermal cells of the wings completely disappeared before 2 h post-emergence*

C. capitata wing epidermal cells began to die soon after the emergence. Thirty min after ecdysis, a great number of cells from the lamina disappeared, and at 1 h, practically no cells were observed (Fig. 6A), which is in agreement with previous reports in *D.*

melanogaster (Kiger et al., 2007). Extracted soluble proteins from the wings steadily diminished after emergence in coincidence with the decrease in the number of cells and the tanning of the cuticle (Fig. 6B, C). On the other hand, the cells of the veins were clearly noticed during all the analyzed ages (Fig. 6A). Therefore, the development of the brown spots occurs after the death of the wing lamina cells.

3.6. *NBAD-S activity in wings*

The epidermal NBAD-S activity in wing- and leg-free beheaded bodies was maximal at the time of adult emergence, then decreased and was barely detectable after 48 h (Fig. 7A). The measurement of NBAD-S activity in isolated wings of different ages demonstrated that the enzymatic activity is present in pharate adults, increased until 1 h after emergence, and then persisted for several days (Fig. 7B). This is a striking result because it means that the NBAD-S activity remained after the death of most of the epidermal cells. The presence and activity of NBAD-S in 24 h-old medfly wings was confirmed by an immunoprecipitation assay. After precipitation of the complex of prot A-antibody-NBAD-S, only 27% of the activity remained in supernatants of samples pre-incubated with the specific antibody, whereas controls pre-incubated only with buffer or pre-immune serum did not change the synthase activity levels, as compared to the standard reaction (not shown).

In order to discriminate whether NBAD-S activity is present in the whole wing or restricted to the tanned areas, such as veins and brown spots, we dissected wings of 24 hour-old adults and separated the veins, the brown spots and the silver-paint lamina. These three isolated portions were tested for NBAD-S activity. As expected, the veins showed NBAD-S activity, whereas the grey lamina lacked this activity (insert to Fig. 7B). Surprisingly, the brown spots showed a considerable amount of NBAD-S activity (insert in Fig. 7B).

4. Discussion

In this work we report a novel long-lasting extracellular NBAD-S activity present in medfly wings which presents a different temporal and spatial pattern compared with the transient epidermal activity that participates in the tanning of body cuticle (Walter et al., 1996; Perez et al., 2004; Schachter et al., 2007; Perez et al., 2010; Perez et al., 2011; Noh et al., 2016).

We studied the NBAD metabolism in adults during wing development and maturation. In *C. capitata,* we determined that final body shape and wing spreading are attained 2 hours after emergence (Bochicchio et al., 2013). Sclerotization and pigmentation of wing cuticle began during pharate adult stage, but at the moment of emergence the colour of the wings was very faint. Here we demonstrated that immediately after the emergence of the adult, the tanning of the spots progressed gradually (mainly during the first 24 h) from the veins towards the intervein lamina until the acquisition of the full colour 48 hours after emergence (Fig 2 and 4). We confirmed that the haemolymph circulation through the veins is necessary for the tanning of the wings in agreement with *Drosophila* (True et al., 1999), since when the veins were obstructed, the spots 1 and 2 in crossbands remained untanned.

Here we demonstrated that the colour of the brownish spots was first determined by the tanning of the hairs and then by the intervein lamina. This was easily observed at the beginning of the tanning (Fig. 3), when the hairs started to acquire colour, whereas the surrounding lamina was still light grey. With the progress of tanning, the hair colour was intensified and the lamina acquiered a brownish hue.

We confirmed that soon after wing spreading most of the wing epidermal cells disappeared (Fig. 6, 1h after emergence), which is in agreement with previous reports in flies (Seligman and Doy, 1973; Seligman *et al.*, 1975; Kiger *et al.*, 2007). Thus, when the

tanning of the brown spots began to be noticeable, the wing lamina was deprived of cells. This raises the question of how the brown tanning is achieved. Our results indicated that the development of the tanning might be achieved according with two possibilities: NBAD is exported out of the veins towards the spot, or it is synthesized by NBAD-S in the spots. To better approach this question, we measured NBAD-S activity in wings of pharate adults and imagoes, and we observed that the activity remained during several days after the emergence (Fig. 7B), even after the end of the tanning process. This is in contrast to epidermal NBAD-S activity in beheaded bodies, where it was high during the first 2 h after the emergence and almost disappeared 48 hours post emergence (Fig. 7A). This result demonstrated that NBAD-S activity is present in wings, but it does not discriminate whether the activity is in the whole wing or it is restricted to some areas. To ascertain this, we cut equivalent portions of wing regions (veins, interveins crossband, and grey lamina) and measured NBAD-S demonstrating that the enzyme is present only in the brown spots and veins (insert to Fig. 7B). This is the first report showing the production of NBAD in the wing brown spots due to presence of the enzyme in a spatially restricted and cell-free environment, and with an extended life activity.

Our data strongly indicate that the NBAD-S was exported to the extracellular matrix before cell death or, remained attached to cuticle in the brown spot region together with cellular remnants close or within the hair sockets. As far as we know, no putative signal peptide has been reported in *Drosophila* NBAD-S protein (named Ebony). As *C. capitata* NBAD-S gene (*niger*) has not been sequenced yet, we were unable to determine whether or not it contains a signal peptide. However, there are many non-classical mechanisms of protein secretion in eukaryote cells (Nickel, 2003), thus NBAD-S might be secreted to the extracellular matrix by a non canonical mechanism.

The long lasting extracellular activity is very surprising because NBAD-synthase activity decays fast in *in vitro* preparations (Pérez et al., 2010), unless 20% or more glycerol and beta-mercaptoethanol were added to the homogenate buffer. This implies that in wings the enzyme should be in an environment (cuticle, extracellular matrix) that stabilizes the protein structure and thus the enzymatic activity.

It is known that bursicon plays an important role during the exarate adult emergence. Several studies highlight the essential role of the neurohormone bursicon to initiate wing expansion, cuticle tanning of the expanded wings, and apoptosis of the wing epidermal cells (Fraenkel and Hsiao, 1965; Seligman and Doy, 1973; Seligman *et al.*, 1975; Kimura *et al.*, 2004; Peabody *et al.*, 2008). Seligman and Doy (1973) demonstrated the disappearance of the epidermal cells of the wing lamina after the eclosion in flies, and the control of this process by bursicon. We do not analyzed the regulation of NBAD–S expression by bursicon and therefore we do not exclude that this neurohormone may be involved in the brown wing spots generation. However, our main goal was to show the role of NBAD-S as well as to demonstrate the new temporal and spatial expression pattern of this enzyme. The long lasting and extracellular activity of wing NBAD-S is completely different to what is currently known for the epidermal enzyme that sclerotizes the body cuticle. As far as we know this is the first report showing this kind of expression. Further studies must be done to determine the involvement of bursicon in the regulation of this process

The brown wing pigment pattern process in *C. capitata* here described seems to agree with the two-step process of melanin pigmentation for *D. melanogaster* proposed by (True et al., 1999) and *Oncopeltus fasciatus* proposed by (Liu et al., 2014). First, the enzymatic activity is spatially prepatterned in the wings during pharate adult development. Then, the substrates dopamine and b-alanine gradually diffuse out from

veins and are conjugated to NBAD in order to accomplish the tanning of the spots, producing the specific colour pattern of medfly wings.

Our results allow us to propose that there is a conserved mechanism for brown and black wing spot pigmentation that implies the secretion of specific enzymes before cell clearance, and their location in the cuticle layers where the pigmentation pattern forms. It has been previously proposed that the Yellow protein is secreted to the extracellular matrix (Kornezos and Chia, 1992) and here, our results suggest that the same occurs with the NBAD-S.

The NBAD-S in medfly wings has dual effects which are the tanning of the veins and the specific pattern of spot colouration. The former is important to give support to wings, whereas the latter is important for intraspecific and interspecific communication, such as sexual attraction and predator deterrent activity (Rao N., 2012).

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Figure legends

Figure 1. Time course of brightness changes in wings of *Ceratitis capitata*.

(A) The naming of the colour banding pattern is taken from White and Elson-Harris (1992). Two rectangular sections (black box 1 and 2) of the brown spots and two sections (black box 3 and 4) of the grey lamina were analyzed. (B) Groundplan of *C. capitata* wing venation and circulation pattern (black arrows). (C) Images of 30-minute and (D) 72 h-old wings. (E) The colour changes were quantified by measuring image brightness of the above mentioned sections. Images were converted to greyscale and the brightness value of each section was measured using ImageJ software.

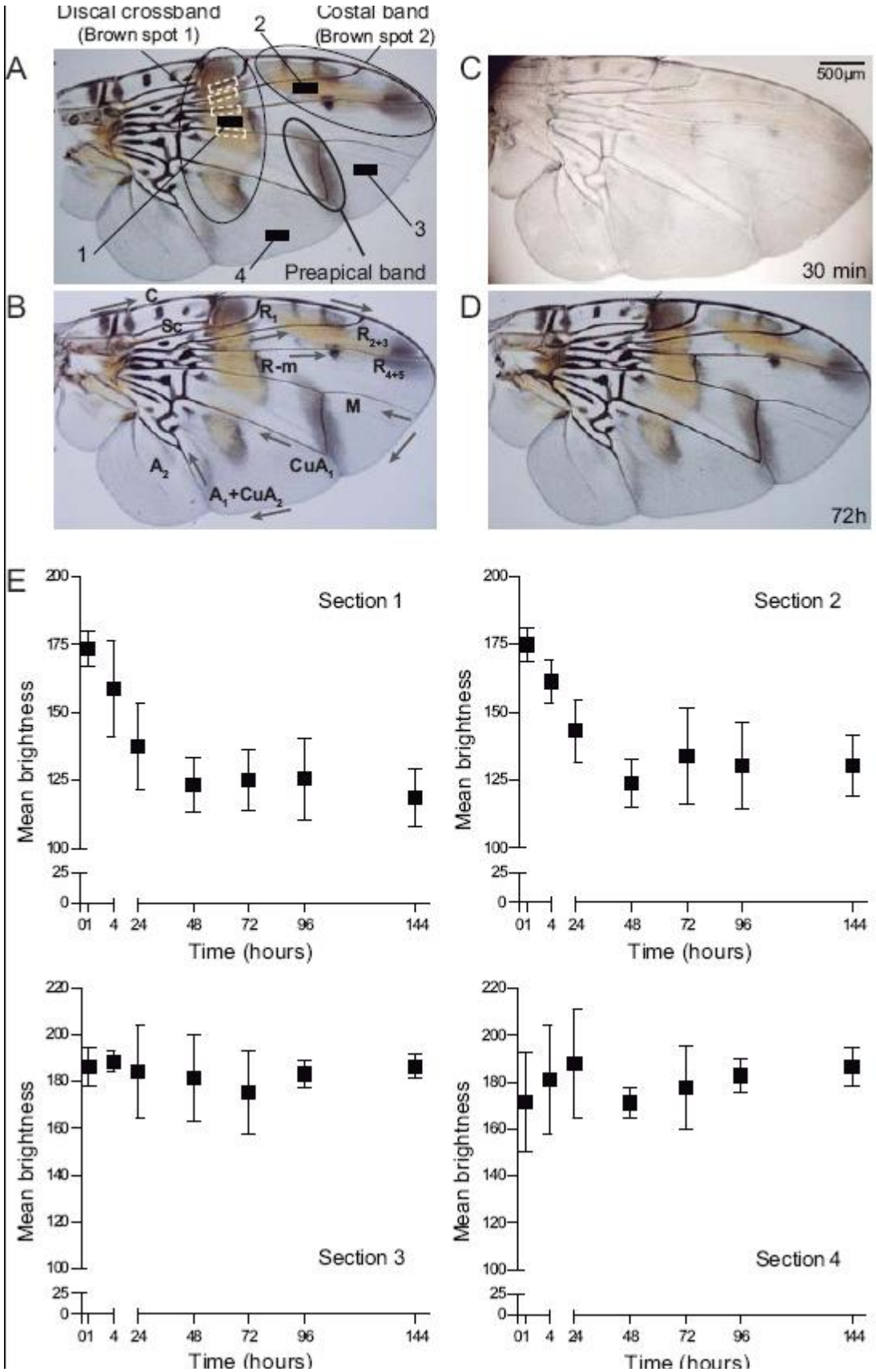

A
Discal crossband
(Brown spot 1)
Costal band
(Brown spot 2)
Preapical band
B
C
500µm
30 min
D
72h
E
Section 1
Section 2
Section 3
Section 4
Mean brightness
Time (hours)

Figure 2. Time course of wing colour progression in *Ceratitis capitata*. Images of medfly wings showing the progress of colouration after the emergence from 1 h to 480 h. At ~~72~~ 48 h the definitive colour intensity was attained.

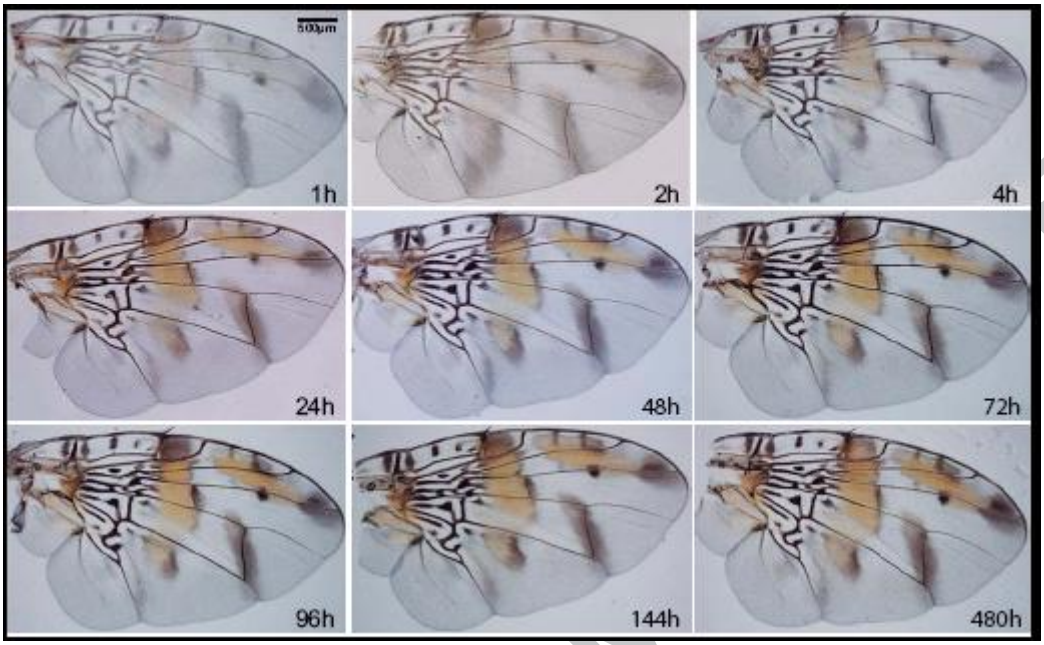


Figure 3. During the first hours after emergence the black and brownish colour of the spots are determined by the tanning of the hairs and sockets. The intensity of hair colouration at different ages, from 30 min to 24 hours after emergence is shown at high magnification. From 24 h onwards, the colour is also determined by the tanning of the lamina.

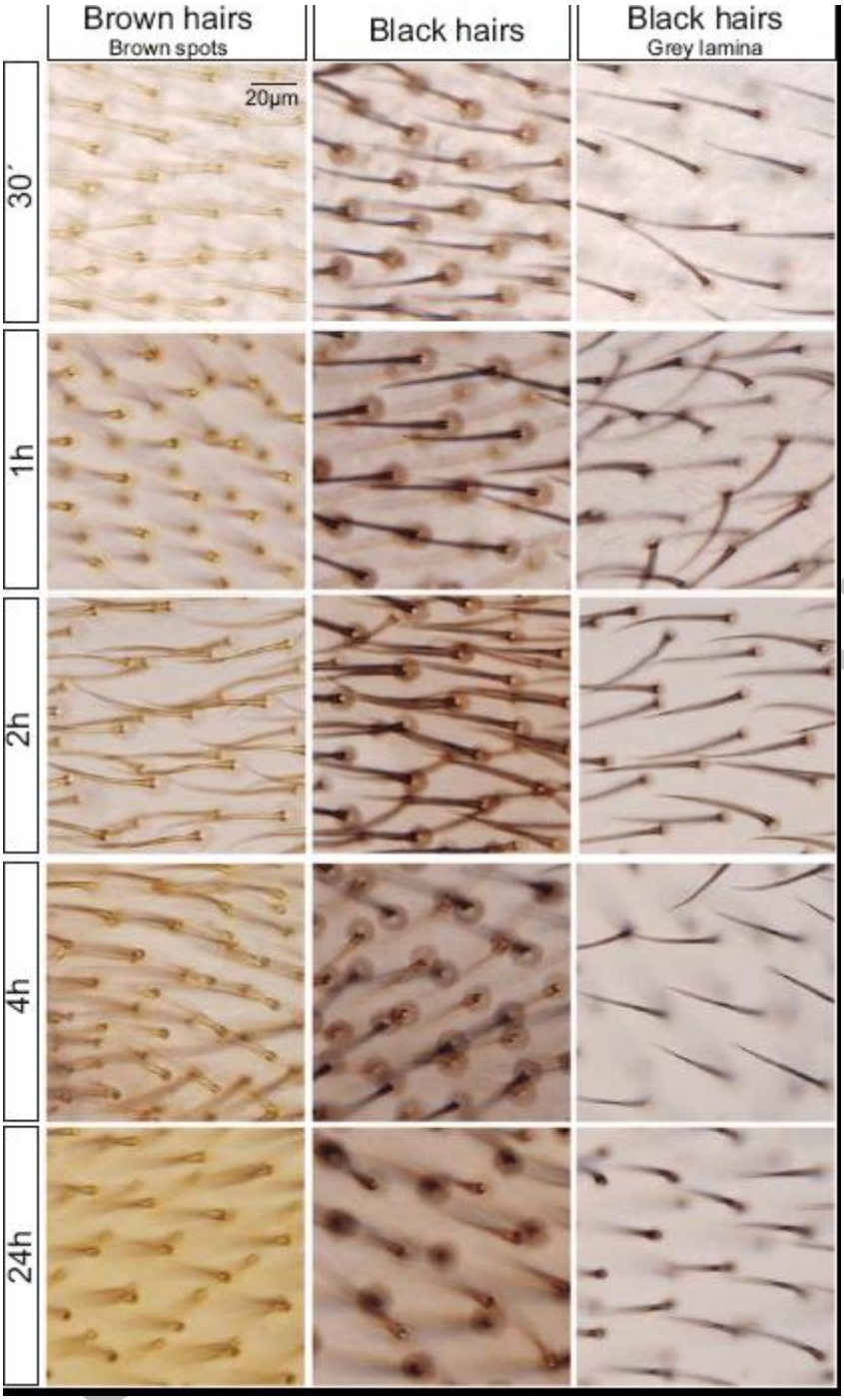


Figure 4. Progression of brown spot colour. The colouration occurred gradually from the veins to the lamina. Pictures were taken in the brown spot 1 (as indicated in Fig. 1) from 1 h to 24 h after emergence. Arrows show the progression of the brown spot from the veins. Dashed arrows indicate the progression of the black spot near the R-m vein.

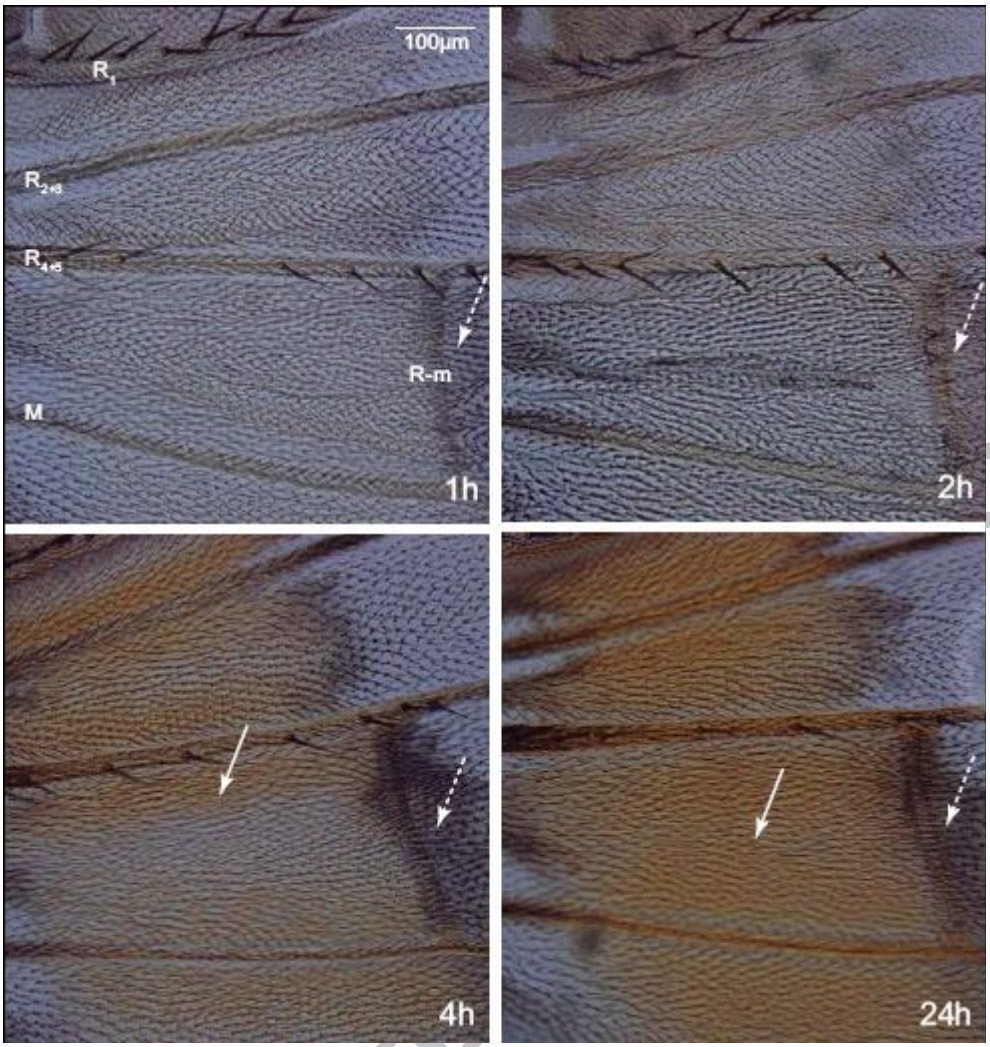


Figure 5. Obstruction of haemolymph circulation in the fly wing inhibited the brown spot colouration. Fifteen minutes old adults were anesthetized and the Subcostal, Radial 1 (R1), Radial 2+3 (R2+3) and Radial 4+5 (R4+5) veins of the right wing were blocked (B). Left wing was left untouched as a control (A). After 3 h the wings were cut and processed as described in section 3. C and D show the progress colouration in the control wing (C) and the obstructed wing (D).

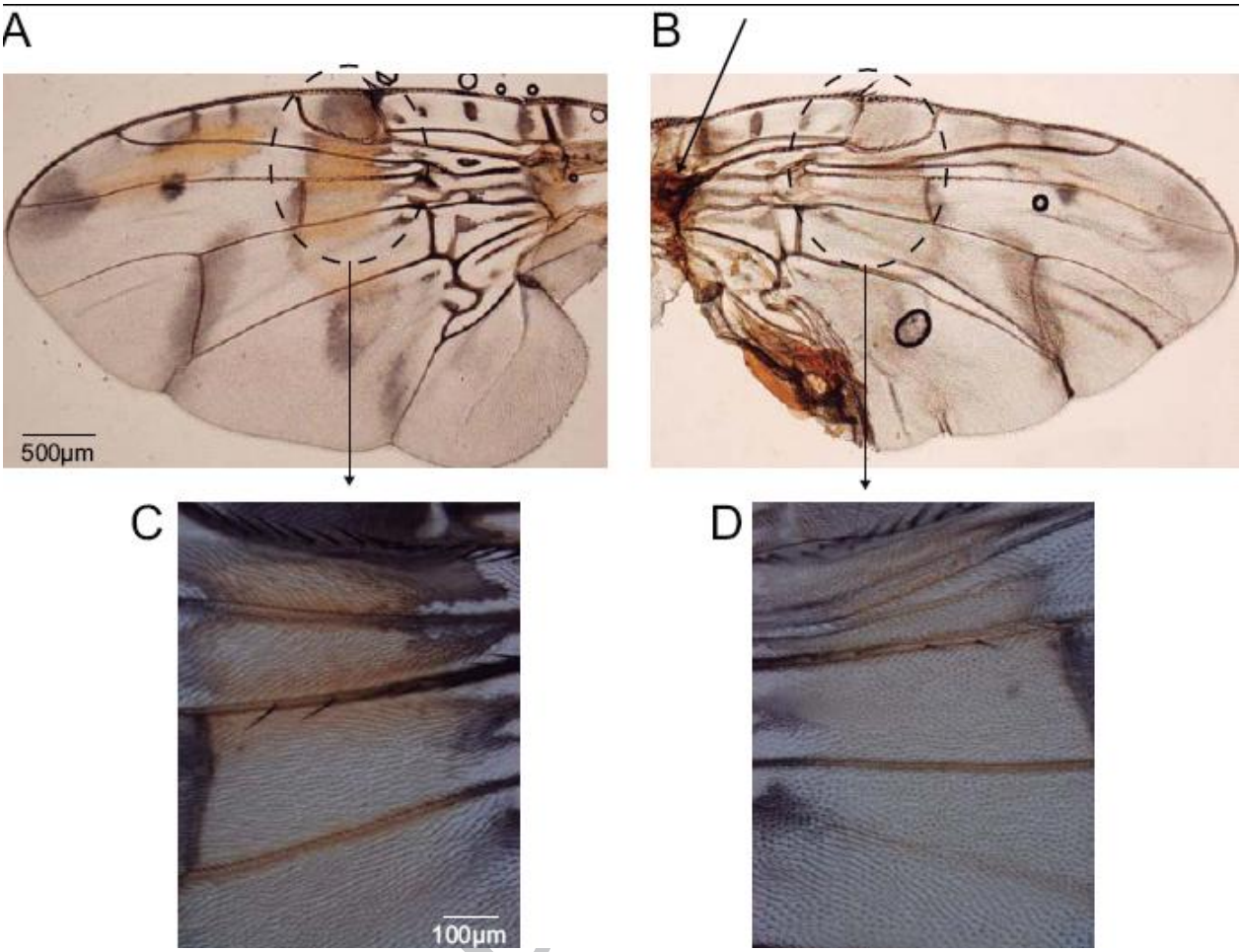


Figure 6. Epithelial cells disappeared from the wing lamina soon after wing spreading. A) DAPI staining photographies showing the reduction of cell number in the lamina from 15 min to 1h. B) Soluble protein extraction and quantification from wings of late pharate adult up to 20 days old flies (480h). C) Protein pattern of the soluble extracted protein in 10% SDS-PAGE.

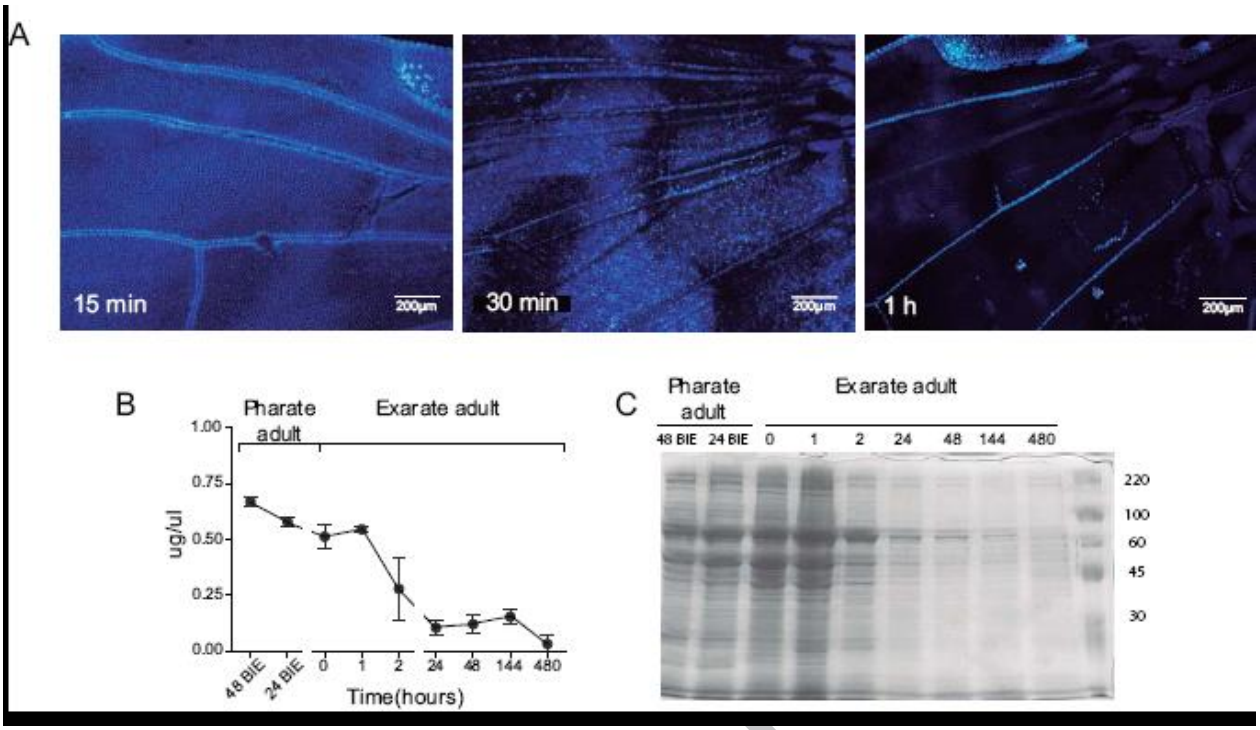
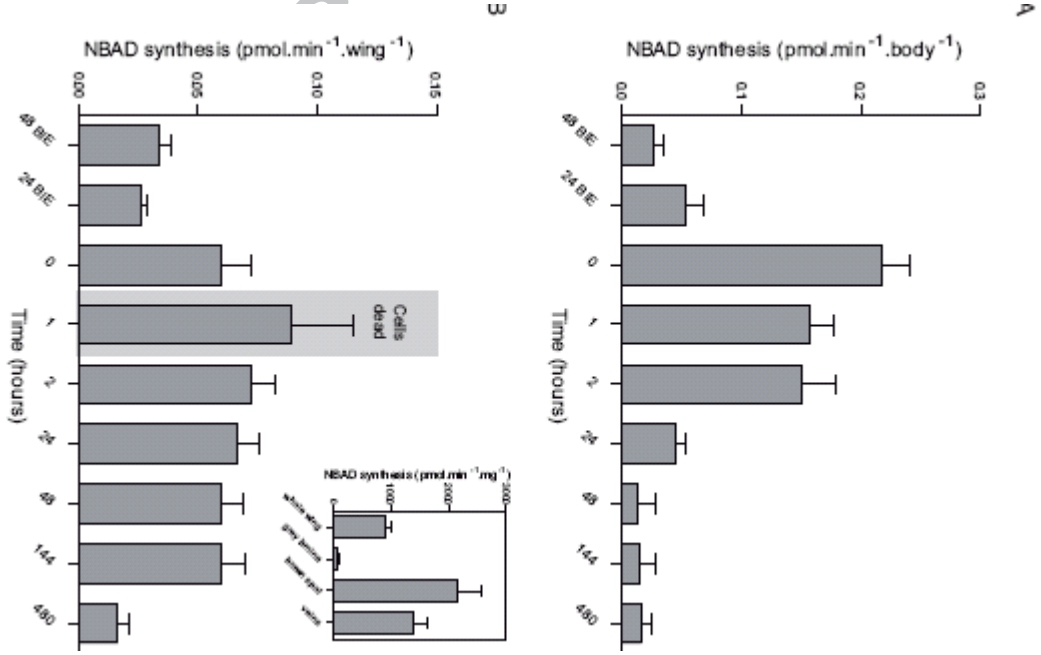


Figure 7. NBAD-S activity during late pharate and exarate adults of *C. capitata.* Beheaded body extracts (A) and wing isolated extracts (B) were assayed as described in M&M section 5. Data are mean ± SEM. The insert to the figure B shows the NBAD-synthase specific activity of veins, brown spots and grey lamina portions of the wings. BIE, before imago emergence.



Supplementary Data

Supplementary Figure 1: (A) Magnified view of the Discal crossband, showing the difference in colour intensity in the proximal and distal region. Arrows indicate the sense of hemolymph circulation. (B) Quantification of colour brightness in the proximal (1 and 2) and distal regions (3 and 4). The proximal region showed higher decline in brightness intensity than the posterior region (One-way ANOVA; $p < 0.05$).

Supplementary Figure 2: Magnified image showing the wing region where the veins were blocked in Figure 5. (A) control; (B) obstructed veins.

- Tanning of the spots was accomplished when the wing lamina was devoid of cells
- Synthesis of NBAD occurred in a cell-free environment
- Extracellular NBAD-S activity in wings remained several days after the emergence