Effective date: 15/10/15

Protocol established according to the ANA-I1.MOA.15_Rev03

This document describes a general protocol. Therefore neither reference nor names of equipments, reagents or consumables are given.

1. PURPOSE AND SCOPE

The small hive beetle (*Aethina tumida* Murray, 1867; Coleoptera: Nitidulidae) is an insect native to South Africa. Part of its reproductive cycle takes place in honey bee colonies and part in the soil, and less frequently, on rotten fruits. Reproduction in bumble bee colonies has been demonstrated in experimental conditions but not in nature. Female beetles drawn by the smell of the hive enter it and lay masses of eggs in wood crevices. These eggs hatch into larvae. The predatory larvae grow by feeding on bee brood (eggs, larvae), honey and pollen. This causes significant damage for beekeeping. In severe cases, there are entire bee colony and harvest losses.

The small hive beetle (SHB) has been introduced in various regions of the world over the past few years; in Egypt in June 2000, in Canada in 2002, 2006 and 2008-2012 and in Australia in November 2002 (Neumann, 2008; OIE, 2013). An introduction was reported in Portugal in 2004, following a bee queen importation from Texas, United States. Sanitary measures implemented allowed to avoid the spread of the beetle in this country. In September 2014, the presence of SHB was confirmed in the South of Italy. Sanitary measures were set up immediately, but visits of the apiaries in the same areas showed that the SHB has spread in the regions of Calabria and Sicily.
The infestation by SHB is ruled in Europe[1] and internationally (OIE, 2013). Moreover, the Regulation (EU) N° 206/2010 imposes the examination in laboratory of cages and of accompanying bees when importing queens and bumble bees from non EU countries[2].

The small hive beetle can be identified by examining the external appearance of adults and/or larvae. The identification method proposed below is a method based on the method described in OIE manual (2013). This method consists in the visual examination of individuals (adults and/or larvae) with recording of morphological characteristics and, if necessary, comparison of the sample to be identified with a reference sample or detailed photographs. The insects to be identified are collected in or near honey bee hives (for example, in beekeeping equipment or queen cages).

The morphological identification can be confirmed using a molecular method. For larvae, if the morphological identification leads to a positive or a non-interpretable result, a molecular identification will be systematically performed.

[1] COUNCIL DIRECTIVE 92/65/EEC of 13 July 1992 laying down animal health requirements governing trade in and imports into the Community of animals, semen, ova and embryos not subject to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC
[2] COMMISSION REGULATION (EU) No 206/2010 of 12 March 2010 laying down lists of third countries, territories or parts thereof authorised for the introduction into the European Union of certain animals and fresh meat and the veterinary certification requirements

2. CONTENT

2.1 Principle

The identification of *A. tumida* takes into account certain morphological characteristics of the adult insects and larvae. A distinction shall be made with other beetles belonging to the family of Nitidulidae, found in honey bee hives in Europe and with not known pathogenic effect on colonies:

- *Cychramus luteus* Fabricius, 1787 (Coleoptera: Nitidulidae), another member of the Nitidulidae family, found in Europe, mainly feeds on pollen (Neumann and Ritter, 2004).
- *Carpophilus lugubris* Murray, 1864 (Coleoptera: Nitidulidae), found in hives in Italy (Marini *et al*., 2013).

The larvae of *A. tumida* can also be mistaken for larvae of the lesser wax moth, *Achroia grisella* Fabricius, 1794 (Lepidoptera: Pyralidae), as well as for the honeycomb moth, *Galleria mellonella* Linnaeus, 1758 (Lepidoptera: Pyralidae). These lepidoptera are generally found in colonies and beekeeping equipment.

Note: Due to the sanitary risk implied by this exotic parasite, the analysis must be done immediately after reception of the sample, in order to confirm or not the suspicion and thus apply the official sanitary measures.

2.2 Materials

- stereomicroscope (and/or magnifier)
- tweezers
- glass, plastic, or porcelain evaporating dishes, or Petri dishes
- ethanol 70% (not denatured)
- capped tubes

2.3 Protocol

Note: Opening the samples received at the laboratory.

The customer sampling form should mention that every specimen suspected to belong to the species *A. tumida* must be sent dead to the laboratory. In case of doubt, the package must be opened in containment conditions.

If the specimens arrive alive to the laboratory, the sample is placed for one hour in a freezer at -80°C. Afterwards, the specimens are placed in a tube with ethanol 70%.

1. Lay-out of the work area:
Clean the work area before the analysis and prepare the material required.

2. General observation of the specimens and sampling for analysis:
- Place the specimens in a dish (Petri dish or similar).
- Directly examine all the beetles to verify the homogeneity of the sample. If necessary use a stereomicroscope (and/or a magnifier).
- Take at least 10 beetles, or all of them if less than 10, using entomological tweezers.

Note: Try to choose specimens that are not damaged.

3. Observation with the stereomicroscope:
- Examine the sample using different magnifications in order to appreciate the different criteria for the identification detailed in paragraph 2.4.
- Measure the size of the specimens.

Compare the samples with reference specimens if available.

4. Conclusion of the analysis:
- Observation of samples of adults:

If doubts remain (impossible to distinguish from other coleopterans, for example) or if the physical condition of the specimen makes morphological identification difficult or even impossible, molecular identification will be necessary.

- Observation of larval samples:

If the morphological examination of larvae leads to a negative result, it can be concluded the non-identification of *A. tumida*.
If the morphological examination of larvae leads to a positive result, a molecular test must be performed in order to confirm the identification.

In case of a positive result, the official sanitary authorities must be informed with no delay.

5. Results recording:
Write down the results on the result sheet.

6. Sample conservation:
Place the beetles in a capped tube with ethanol 70%.

2.4 Identification of the small hive beetle *A. tumida*

A. tumida belongs to the class of Insects, order of Coleoptera and family of Nitidulidae.

- **Guidelines for the identification of *A. tumida*, adult form**

(Marini *et al*, 2013; Neumann and Ritter, 2004; OIE, 2013 - Figures 1 and 2)

1.	Body divided in three parts: head, thorax and abdomen
2.	Three pairs of legs
3.	Presence of elytra
4.	Dimensions: length: 5 to 7 mm; width: 3 to 4.5 mm (approximately)
5.	Colour: reddish brown at birth, dark brown to black in adulthood Presence of a lighter band around thorax and abdomen (optional criterion)
6.	Club-shaped antennae
7.	Sharp latero posterior tips of the pronotum
8.	Elytra not covering the entire abdomen

Size is one of the indicator criteria that are used to identify *A. tumida* according to our conditions. Under no circumstances shall size be a sole criterion for identifying this beetle.

In the case *Cychramus luteus*: (Figure 3 - Neumann and Ritter, 2004)
- elytra completely cover the abdominal apex;
- antennal clubs are looser with detached segments;
- latero-posterior tips of the pronotum are not sharp;
- colour of the body is light-brown.

In the case of *Carpophilus lugubris*: (Figure 4 - Marini *et al*., 2013)
- body is brown; elytra have orange regions. Legs and antennae are orange (antennal clubs are dark orange);
- body length: 3.3 to 4.5 mm.

However, as for *A. tumida*:
- elytra don't cover the entire abdomen;
- club-shaped antennae have compact segments;
- latero-posterior tips of the pronotum are sharp.

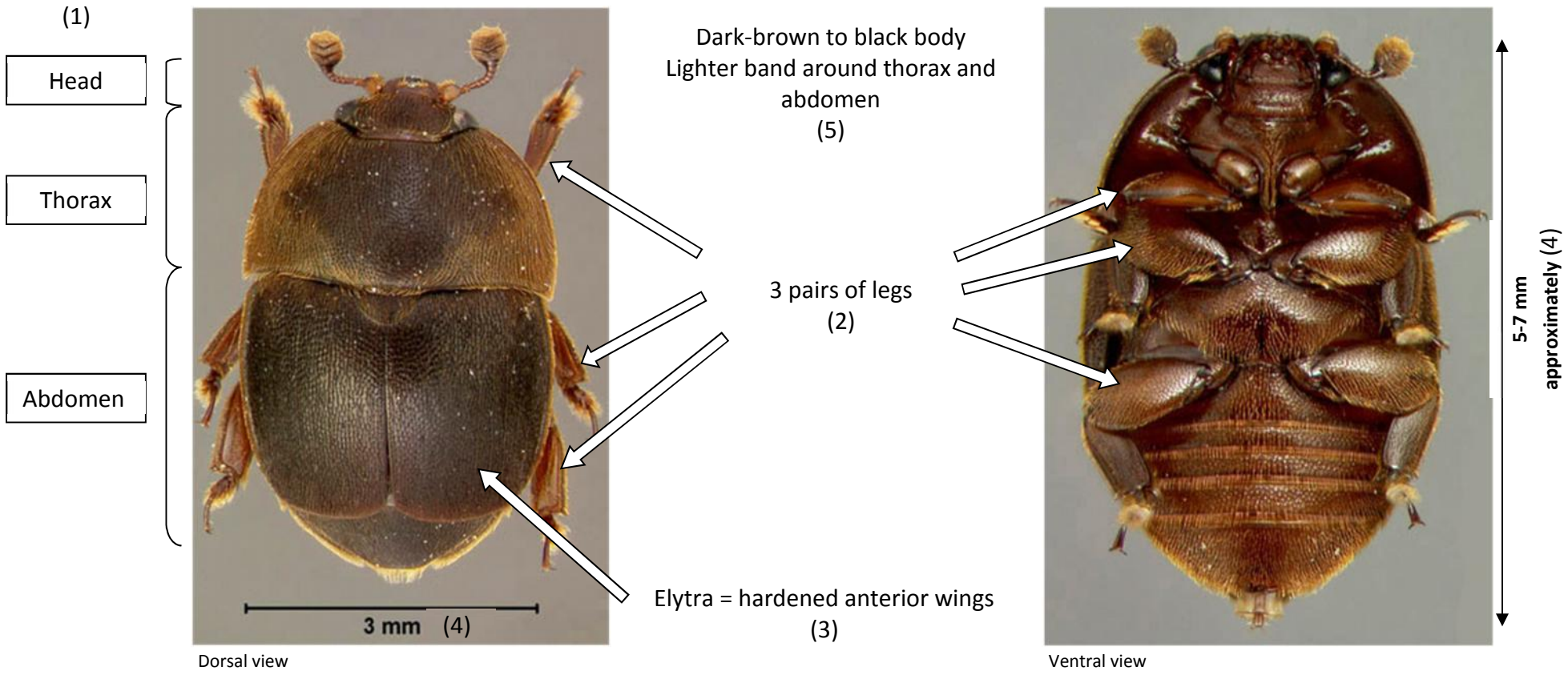
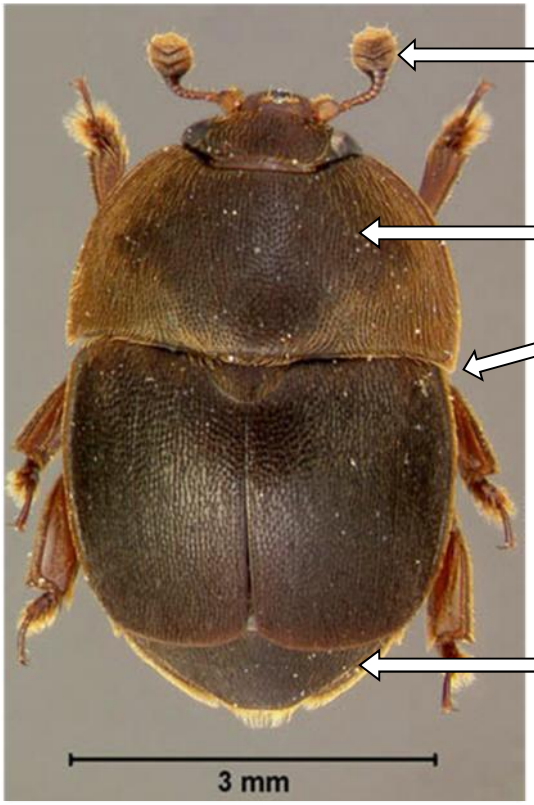


Figure 1 - Small hive beetle, *Aethina tumida* Murray
Source: University of Florida; OIE, 2013



Dorsal view
Source: University of Florida; OIE, 2013

Club-shaped antennae, with compact segments (6)

Pronotum (pro-thorax dorsal side)

Sharp latero-posterior tips of the pronotum (7)

Abdomen partially covered by elytra: visible apex (posterior tip of the abdomen) (8)

Defense position: SHB retracts its appendages beneath its body, leaving nothing extended for bees to grasp.

Dorsal view
Source: Anses, Sophia Antipolis

Figure 2 - Small hive beetle, *Aethina tumida* Murray – Guidelines to distinguish *A. tumida* from other Nitidulidae species present in the hive

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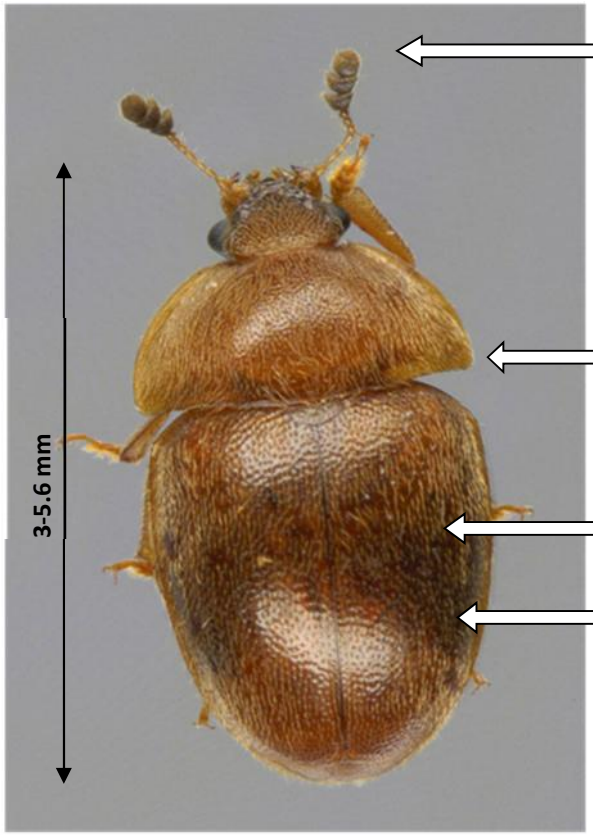

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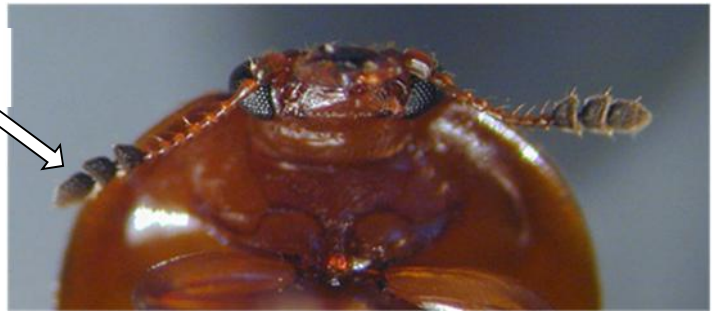

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Figure 3 - *Cychramus luteus* Fabricus (Neumann and Ritter, 2004)

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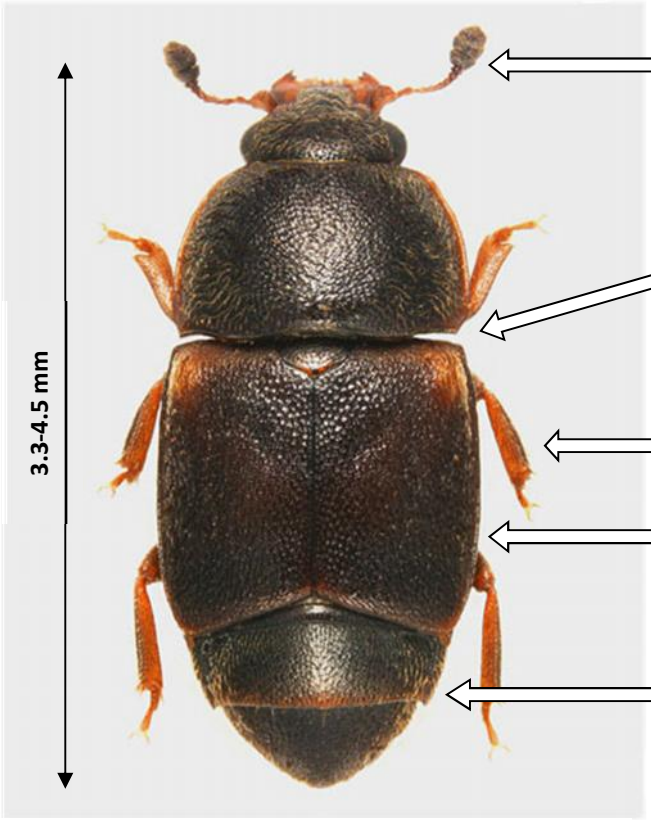

Dorsal view
Source: Marini *et al*., 2013

Club-shaped antennae, with compact segments

Source: Encyclopedia of life,
http://eol.org/data_objects/22780791
web page accessed on 28th May 2014

Sharp pronotum latero-posterior tips

Orange legs and antenna (the antennal clubs are dark orange)

Brown body with orange regions on the elytra

Abdomen partially covered by elytra: only the last two segments of the abdomen are visible

Figure 4 - *Carpophilus lugubris* Murray (Marini *et al*., 2013)

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- **<u>Guidelines for the identification of *A. tumida*, larval form</u>** (Marini *et al*, 2013; Neumann and Ritter, 2004; OIE, 2013 - Figures 5, 6, and 7)

Larvae of the SHB have a light beige body. The cephalic capsule (head of the larva) is brown.

The body lenght at maturity is about 1 cm (1.2 cm maximum length). The length depends on feeding. The width is about 1.6 mm.

1.	3 pairs of legs, one on each of the anterior segments
2.	Two dorsal spines on each segment (these spines are thicker in the last segment)
3.	No false legs (pseudopods) on the ventral side of the posterior abdominal segments

To distinguish *A. tumida* larvae from Lepidoptera larvae (lesser wax moth, *A. grisella* and honeycomb moth, *G. mellonella*), frequently present in honeybee hives:

- The Lepidoptera larvae present pseudopods on the ventral side of the abdominal segments.
- There are two bare segments between the last segment with legs and the first segment with pseudopods (figure 7).
- Besides, the Lepidoptera larvae can make a silky web, cocoons, and dark faeces (these webs and faeces can be observed in the sample containers received by the lab).

The identification of the small hive beetle larva is always confirmed by PCR, except when the results are negative (see paragraph 4. Results).

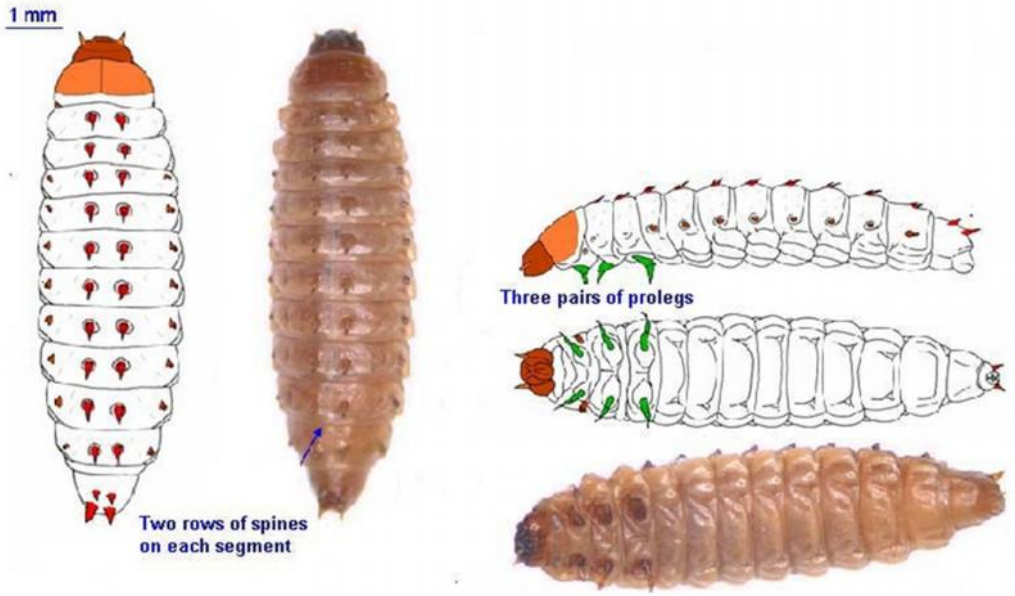
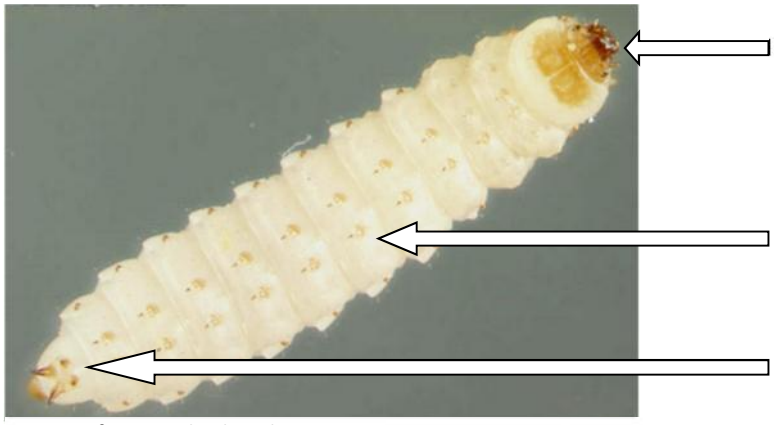


Figure 5 - Larva of *Aethina tumida* Murray
Source: Boeking, 2005.

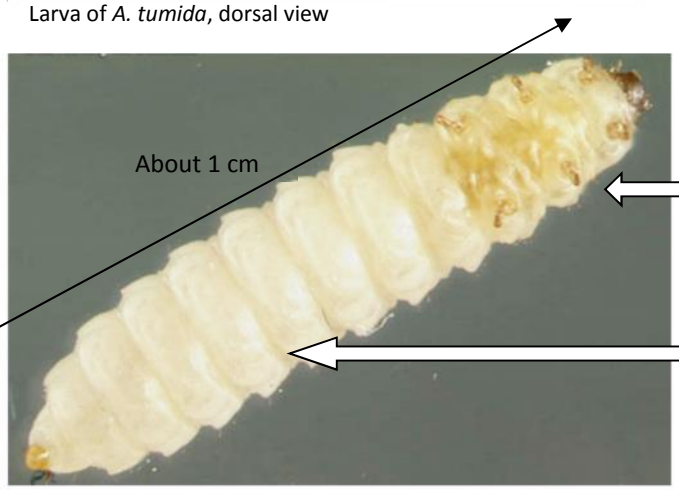


Light-beige body
Brown cephalic capsule

Two dorsal spines on each segment (2)

Two thick dorsal spines on last segment (2)

Larva of *A. tumida*, dorsal view



3 pairs of legs, one on each of the anterior segments (1)

No pseudopods on the ventral side of the posterior abdominal segments (3)

Larva of *A. tumida*, ventral view

Figure 6 – Larva of *Aethina tumida* Murray - University of Florida
Source: http://entnemdept.ufl.edu/creatures/misc/bees/small_hive_beetle.htm, web page accessed on 28th May 2014)

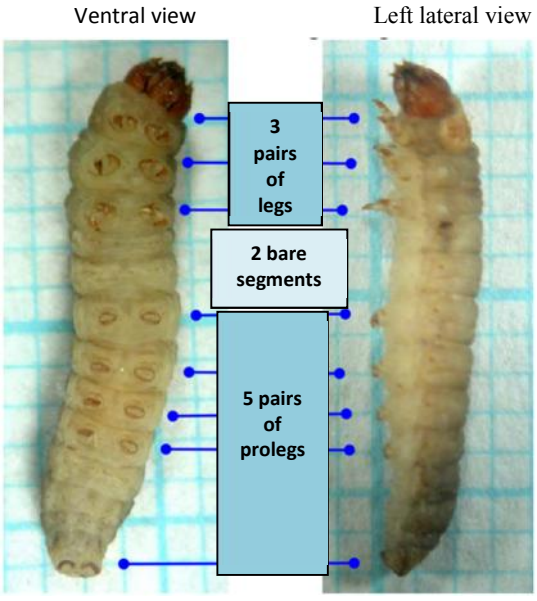


Figure 7 – Larva of *Galleria mellonella* (honeycomb moth)
Source: http://www.pir.sa.gov.au/__data/assets/pdf_file/0015/41262/apiary_shb_fact_sheet_2006.pdf

3. ANALYTICAL RESULTS

3.1 Adult forms

Analysis results	**Conclusion**
Criteria 1 to 8 confirmed for *A. tumida.*	Positive
Certain fundamental morphological characteristics of *A. tumida* are not present: • At least one out of the three criteria (1 to 3) not confirmed (in this case, the other observations are not realized). • Or at least one out of the five other criteria (4 to 8) not confirmed.	Negative
Impossibility to confirm the presence or absence of certain characteristics. →Molecular biology identification systematically realized.	Inconclusive

3.2 Larval forms

Analysis results	**Conclusion**
All criteria 1 to 3 confirmed. → Molecular biology identification systematically realized.	Suspicion
Criteria 1, 2 or 3 not confirmed.	Negative
Impossibility to confirm the presence or absence of certain characteristics. → Molecular biology identification systematically realized.	Inconclusive

4. BIBLIOGRAPHY

Boecking, O., Wagner, T., 2005. Der Kleine Beutenkäfer Aethina tumida, wichtige morphologische Bestimmungsmerkmale und Lebenszyklus. LAVES, Institut für Bienenkunde, Celle, Germany.
Available online at: http://www.laves.niedersachsen.de/portal/live.php?navigation_id=20139&article_id=73963&_psmand=23&cp=3_17111#list_17111

Encyclopedia of life, http://eol.org/. Web pages, accessed on 28 May 2014.

Government of South Australia, 2006. Small Hive Beetle, *Aethina tumida* Murray (Coleoptera: Nitidulidae). Information leaflet. FS 03/06, 13 pp.
Available online at: http://www.pir.sa.gov.au/__data/assets/pdf_file/0015/41262/apiary_shb_fact_sheet_2006.pdf.

Marini, F., Mutinelli, F., Montarsi, F., Cline, A., Gatti, E., Audisio, P., 2013. First report in Italy of the dusky sap beetle, *Carpophilus lugubris*, a new potential pest for Europe. *Journal of Pest Science* 86: 157-160.

Neumann, P., Ritter, W., 2004. A scientific note on the association of *Cychramus luteus* (Coleoptera: Nitidulidae) with honeybee (*Apis mellifera*) colonies. *Apidologie* 35: 665-666.

Neumann P., Ellis J.D., 2008. The small hive beetle (*Aethina tumida* Murray, Coleoptera: Nitidulidae): distribution, biology and control of an invasive species. *Journal of Apicultural Research* 47 (3): 181–183.

OIE, 2013. Small hive beetle infestation *(Aethina tumida). Chapter 2.2.5*. In: Manual of standards for diagnostic tests and vaccines for terrestrial animals, Paris.

University of Florida. Featured Creatures. Common name: small hive beetle, scientific name: *Aethina tumida* Murray (Insecta: Coleoptera: Nitidulidae), http://entnemdept.ufl.edu/creatures/misc/bees/small_hive_beetle.htm. Web page, accessed on 28 May 2014.