Supplementary file

Material and methods

The insects used in this experiment were from an inbred stock of the European grape berry moth, *E. ambiguella* (Lepidoptera, Tortricidae) reared at the INRA of Bordeaux (Aquitaine, France) for several years. This stock is based on a great number of caged adults (several thousand per week), to which wild adults are regularly added. Larvae were maintained in boxes (18 x 11.5 x 7cm) on a semi-artificial diet (described in Vogelweith et al., 2011) at a density of ca. 100 individuals per 300 ml of diet.

During the experiments, larvae were individually reared in centrifuge tubes containing 1.5 ml of rearing diet, which is sufficient for the larvae to complete development (Thiery and Moreau, 2005). The lids of the tubes were pierced with a needle to allow air circulation. Larvae were maintained until the 5th larval instar stage under standard laboratory conditions (22 ± 1°C, 70 ± 10% r.h., photoperiod: L16:D8).

Hemocytes were extracted from 5th larval instar stage *E. ambiguella* using the method described by Vogelweith et al. (2011; 2013a). Briefly, the larvae were anesthetized on ice for 20 min, and 1 µl of hemolymph was then collected and flushed into a micro-centrifuge tube containing 20 µl of sodium cacodylate/CaCl2 buffer (0.01M sodium cacodylate, 0.005M CaCl2; pH 6.5). With this sample, we measured the basal concentration of hemocytes in naïve larvae. Just after this first hemolymph collection, larvae were immune challenged in the posterior part of the ventral side of the abdomen with a sterile needle dipped in a concentrated suspension of heat-killed *Arthrobacter globiformis* (ca. 10^9 cells ml^{-1}). This bacterium is commonly used in the protocol testing of antimicrobial activity (Sadd and Schmid-Hempel, 2007; Vogelweith et al., 2011, 2013a, 2015). The larvae were then kept individually in micro-centrifuge tubes for 2, 6, 12, 24, 48 or 72 hours under standard conditions before a second sample of hemolymph was collected (Vogelweith et al., 2011). All the samples of hemolymph

were assessed immediately to avoid coagulation and desiccation of the hemocytes. Hemocyte concentration was estimated using an improved Neubauer hemocytometer counting chamber and phase contrast microscopy (magnification 400×).

We used the variation in hemocyte concentration (hemocyte concentration after – before immune challenge) in the results.

Results

The variation in hemocyte concentration was influenced by the time of the second hemolymph (One way ANOVA: $F_5 = 3.30$; $p = 0.006$). The concentration of hemocytes increase after the immune challenge until 6 h (Figure 1). This variation became stable 12 hours after the immune challenge (Figure 1).

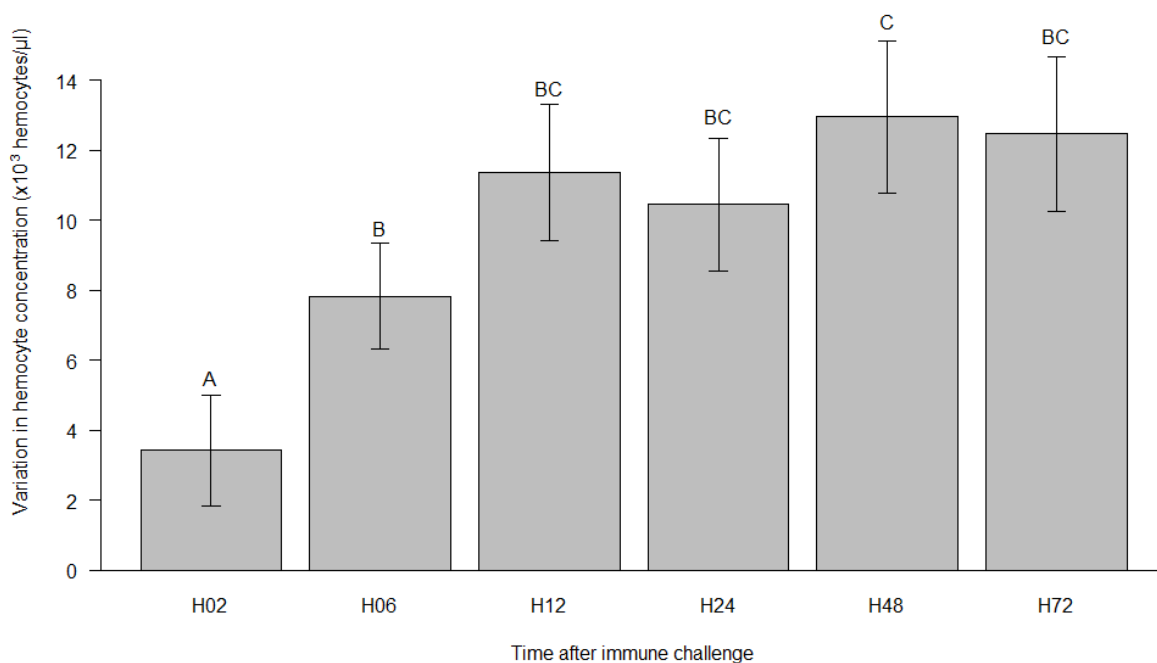

Figure1. Average variation in hemocyte concentration (after – before immune challenge) 2, 6, 12, 24, 48 or 72 hours after an immune challenge (± confidence interval 95 %). A same letter above the bars indicates no difference time (Tuckey HSD; $p > 0.05$).

Conclusion

We used these results to choose the timing after an immune challenge in our future experiments. Twenty-four hours was the timing selected because the increase of hemocyte concentration is stable at this time. Moreover, this timing is used in other study on insect immunity (Siva-Jothy 2000; Vogelweith et al. 2011).