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Odour-mediated behaviour in codling moth, *Cydia pomonella*: Do fermentation odours affect the attraction and oviposition behaviour in codling moth?

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Påverkar jästdofter attraktions- och äggläggningsbeteende hos äppelvecklaren?

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Preface

This Degree project in Biology of 30 ECTS (Hp) is a part of the Agricultural Programme – Soil and Plant Science. It was executed at the Department of Plant Protection Biology, Chemical Ecology Unit, Swedish University of Agricultural Sciences (SLU), Alnarp. Supervisors were Dr. Paul G. Becher, Dr. Sébastien Lebreton and Professor Peter Witzgall.

I want to express my warmest gratitude towards my supervisors, Dr. Paul G. Becher, Dr. Sébastien Lebreton and Prof. Peter Witzgall. I'm so very grateful to Paul for your endless patience and for supporting me from beginning to end. I'm also thankful to Sébastien for your invaluable help with the statistical analysis and support during the experiments, and I also want to thank Peter for giving me the opportunity to carry out this degree project at Alnarp. Thank you!

I also want to thank the Jobackers, for welcoming me into their home during my time at Alnarp. I am thankful to my family and friends who has been cheering me on ever since I started working on this, and want to thank my brother, Peter Hansson, for supporting and encouraging me to complete the project. Finally I want to express my gratitude to one and all, who directly or indirectly, have helped me to complete this degree project.

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ABSTRACT

The codling moth, *Cydia pomonella* is a common pest in apple orchards and can be found worldwide. Feeding by codling moth larvae can cause a substantial decrease in apple yield. Control methods comprise for example the use of pesticides, viruses and pheromones. As a sustainable control method, pheromones have been successfully applied to disrupt moth mating behaviour. Other behaviours like foraging, search for mating sites or host finding could be potential targets for additional odour-mediated behavioural manipulation but need further investigations of the underlying odour stimuli and behavioural plasticity.

Many insects are attracted to the smell of fermenting fruit and associated microorganisms. Fermentation odours generally signal the presence of fermentable sugar and microbes as potential food resources for larval and adult insects, and hence might indicate a favourable site for adult feeding, mating or egg-laying. We hypothesized that fermentation odours might induce or influence attraction and oviposition behaviour in *C. pomonella* moths, and that the behaviour might be sexually dimorphic and modulated by mating state.

The result show a difference in the attraction of virgin and mated female moths. Virgin moths show an attraction towards unfermented odour whereas mated moths show a preference towards fermented odours. Female and male moths with the same mating status behave comparable, although the response is stronger in females. Furthermore, the oviposition assay indicated that yeast odour mediate significant oviposition behaviour, with an exception on fermenting apple juice.

The results indicate that virgin moths, both male and female, might search for unfermented fruit as suitable site to meet and mate, while mated moths might primarily seek for fermented site to oviposit. Knowledge on mechanisms underlying ecologically relevant behaviours and the response to fermentation odours might be significant for future development of control techniques based on behavioural manipulation.

Sammanfattning

Äppelvecklaren, *Cydia pomonella*, är en vanlig skadeinsekt i äppelodlingar och den förekommer i hela världen. Äppelvecklarens larver kan orsaka kraftiga skördeminskningar, men skadorna kan reduceras med hjälp av pesticider, virus och feromoner. Genom att använda feromoner kan äppelvecklaren kontrolleras på ett mer hållbart sätt genom att förhindra parning. Andra beteenden, så som födosökande, val av parningsplats eller värdväxt kan också vara potentiella områden där beteendet kan manipuleras med hjälp av dofter. Dessa metoder kräver dock vidare studier av bakomliggande doftstimulanser och beteendemönster.

Många insekter attraheras av dofter av fermenterande frukter och associerade mikroorganismer. Doften av jäsningsprocessen anger vanligtvis närvaro av fermenterbara sockerarter och mikrober, vilket kan vara en potentiell födokälla för larver och fullvuxna insekter. Förekomsten av jäst kan också innebära en fördelaktig plats för parning och/eller äggläggning. Hypotesen var att dofter som uppstår i jäsningsprocessen kan påverka attraktions- och äggläggningsbeteende hos *C. pomonella*. Dessutom var hypotesen att beteendet är olika hos honor och hanar (könsdimorfism), och beroende av parningsstatus.

Resultaten visar en skillnad i attraktion hos oparade och parade honor. Oparade honor attraheras av ofermenterade dofter medan parade honor attraheras av fermenterade dofter. Honor och hanar med samma parningsstatus uppvisar ett liknande beteende, dock är honornas attraktion mer påtaglig. Vidare visar äggläggningsförsöken att jästdofter har en påverkan på äggläggningen.

Resultaten indikerar att både oparade honor och hanar letar efter ofermenterad frukt där de kan para sig. Medan parade malar primärt söker efter en plats med fermenterande dofter där de kan lägga ägg. Kunskap om mekanismer och bakomliggande biologiskt relevanta beteenden och responsen på fermenterande dofter kan vara intressant för vidare utveckling av kontrolltekniker baserade på beteendemanipulering.

Sökord: *Cydia pomonella*, codling moth, apple, fermentation,

Innehållsförteckning

Introduction

Agricultural and horticultural production is constrained by pests and diseases. The codling moth, *Cydia pomonella*, is one of the dominant insect pests in apple orchards (Vallat & Dorn, 2005). The larva causes damage to the developing fruit, which makes the fruit unmarketable and the decrease in production causes large financial losses (Wearing, et al. 2001). The use of pesticides has been a key factor behind the major productivity increase in agriculture and application of insecticides is a conventional pest control technique for *C. pomonella* (Dunley & Welter, 2000; Thacker, 2002; Demain & Sanchez, 2009). However the use of pesticides is problematic as it may cause pesticide resistance, it can be a health risk for farmers and consumers and result in non-target effects e.g. on pollinators or birds (Palumbi, 2001; Aktar et al. 2009). As a consequence of the recorded problems, regulations and laws increasingly restrict the application of pesticides (Chandler *et al*. 2011).

It is therefore important to find alternative and sustainable methods of pest control. Regardless of the control method it is important to understand the behaviour of the pest insects. Insects are known to respond to odours and use them e.g. in host and mate finding (Pinẽro & Dorn, 2009). Semiochemicals are successfully applied in monitoring and direct control of pest insects (Chandler *et al*. 2011). Pheromones e.g. are used to confuse or trap insects, which interferes with their reproduction and survival (Witzgall, et al. 2010). The codling moth sex pheromone, codlemone, is identified (Roelofs et al., 1971) and with manufactured codlemone, *C. pomonella* outbreaks can be monitored. However, mass-trapping of males can only partly control *C. pomonella.* Nevertheless, mating disruption has been proven to be effective and the techniques can still be improved (Charmillot et al. 2000). The development of new control methods require detailed knowledge of insect behaviours like host preference, foraging and mating.

Traditionally, semiochemical research and application has focused on insect and plant compounds. However, in this study the focus was to evaluate yeast volatiles as semiochemicals. Fermentation odours are semiochemicals that signal the presence of fermentable fruit and microbes as rich food resources for larval and adult insects (Ganter, 2006). Fermentation signal therefore might indicate a favourable site for adult feeding, mating or egg-laying. A number of insects, like the common fruit fly, *Drosophila melanogaster* and some noctuid moths (e.g. the spotted cutworm, *Amathes c-nigrum* the bertha armyworm, *Mamestra configurata* and the speckled cutworm moth, *Lacanobia subjuncta*) have shown attraction behaviour towards fermentation compounds like acetic acid (Landolt & Hammond, 2001; Landolt, 2001; Becher et al. 2010). It is therefore possible that similar mechanisms exist in other insects e.g. the codling moth. A better knowledge of the odour-mediated behaviour in *C. pomonella,* in response to fermentation products can possibly be used to further develop control methods.

Host selection by use of volatiles

All organisms release organic compounds, semiochemicals, which transmits "messages" within and between species (Law & Regnier, 1971). All plants emit blends of volatiles and the blends vary between species (Paré & Tumlinson, 1999). Chemosensory systems detect and process signals and allow animals to respond to the stimulation with the most adequate behaviour (De Bruyne & Baker, 2008). Insects generally use olfaction, when foraging, searching for mates or locating and selecting suitable sites to oviposit (De Bruyne & Baker, 2008; Pinẽro & Dorn, 2009; Davis, et al. 2013). For phytophagous insects, chemical signals emitted by plants are of ecological relevance as they indicate suitable hosts (Bruce et al. 2005). Choosing the most suitable host is of significant importance in the reproduction of herbivorous pests (Jaenike, 1970; Fraenkel 1969). Herbivores can therefore be assisted in the search for a suitable host by recognizing the specific blends emitted by a suitable host (Paré & Tumlinson, 1999, McCormick et al. 2012).

Insects and plants have co-evolved and have accordingly developed a large variety of either deleterious or beneficial interactions (Schoonhoven et al. 1998). Co-evolution has for example led to the emission of pollinator attracting volatiles by flowers, to the benefit of plant and insect (Schoonhoven et al. 1998; Ehrlich & Raven, 1964). In contrast, the emission of herbivore attracting plant volatiles rather benefits the insect only.

During the development, the plant changes in morphology, but also in volatiles emitted. A green, ripening apple has a different smell than a ripe or overripe fermenting apple. In a study by Vallant & Dorn (2005) it was shown that the volatiles emitted from apple trees change, both in composition and amounts, during the growing season. Apple odours are known to interest *C. pomonella*, e.g. α-farnesene that naturally occurs in the coating of apples (Huelin & Murray, 1964). α-farnesene is a component of the characteristic green apple odour and has been reported to attract gravid *C. pomonella* females (Wearing et al. 1973). It has also been indicated that α-farnesene has a significant oviposition-stimulating effect and stimulates hatching of *C. pomonella* larvae (Hern, & Dorn, 1999).

However, volatiles emitted from host plants are not only of phytochemical origin but also are produced by microorganisms associated with the plant. In relation to this, microorganisms can also contribute to host quality (Becher et al. 2012).

Insect microbe interactions

Interactions between insects and microbes are often essential for the survival of the insect. There is a large variety in interactions between insects and microbes and many phytophagous insects have a mutualistic relation with microbes (Janson et al. 2008). In many cases, the interaction provide nutrition to the insect, while the insect provides something in return e.g. the insect-body can offer protection (Ollerton, 2006). However, not all interactions request the insect to consume the microbes to get a nutritive benefit. One example is that of aphids and other hemipterans where the presence of specific yeast-like fungal endosymbionts are vital as they provide essential amino acids (Janson et al. 2008). The codling moth is an example of a phytophagous insect that can benefit from an interaction with yeast. The larvae benefit from hatching close to, or on maturing fruit, in return the mechanical damage that the insect cause when feeding is beneficial for the development of the yeast (Witzgall and Knight, 2013). In recent years, insect-microbe interactions received increasing attention in ecological research (British Ecological Society). However, the chemical signals underlying those interactions are rarely clarified (Biedermann and Kaltenpoth, 2014). In this study we investigate how fermentation odours influence attraction and host choice behaviour in *C. pomonella*.

The codling moth, Cydia pomonella

The codling moth, *C. pomonella*, is a member of the large lepidopteran family Tortricidae. A number of moths in the tortricid family are known as agricultural and horticultural pests and *C. pomonella* is described as one of the key pest in orchards worldwide (Wearing, et al. 2001).

Despite presence of natural enemies, damages of up to 15 % occur in the south of Sweden (Sjöberg & Hillbur, 2010) and as much as 30-80 % worldwide. One reason for the substantial damage is that females oviposit on a large number of apples within the tree, spreading the chances for survival of the larvae, but also spreading the damage (Wearing, et al. 2001).

The newly hatched codling moth larvae is approximately 2 mm long, while the fully grown larvae is 13-19 mm long (Agnello & Kain, 1996). The larvae is pale coloured with a yellowish-brown head, often with darker brown patterns on the thoracic shield (Wearing et al. 2001). The adult codling moth is a moderately small pale greyish-brown tortrix moth, with light grey and copper striped wings (wingspan approx. 17 mm). The codling moth overwinters as a fifth-instar larva in the bark of its host tree or in the ground-vegetation or even under the ground (Wearing et al. 2001). The larva pupates in the spring and the emergence of adults continues throughout the summer. Adults are able to mate 24 h after emergence (Gehring & Madsen, 1963). According to Geier (1963) and Wearing & Ferguson (1971) the average number of eggs laid varies between 50-100 eggs per female and most eggs are laid within the first day after mating. Some studies indicate that about 70-80 % of the eggs are laid within the first two hours after mating (Wearing & Hutchins, 1973; Knight & Light, 2001).

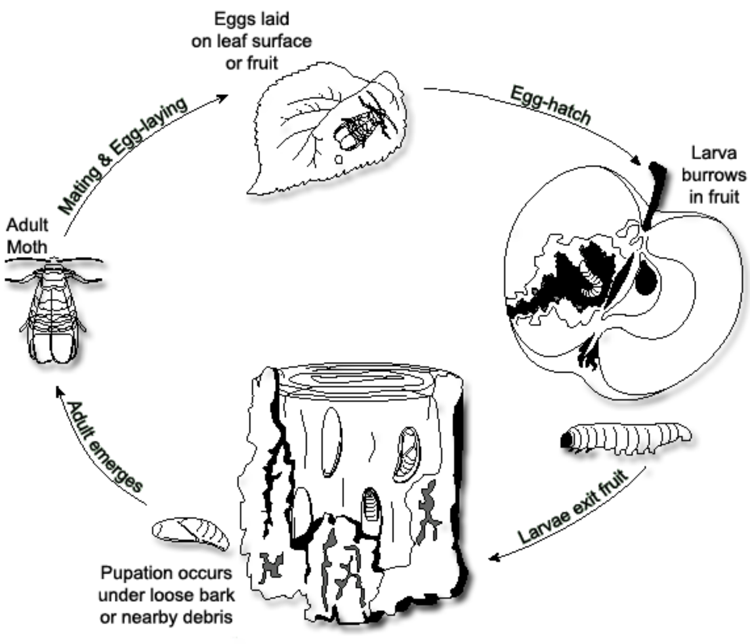

Figure 1. *Cydia pomonella* – life cycle (oksir.org).

Specific goals and implementation of the study

In 2010 when this project was conducted the significance of fermentation odours for host-finding, foraging and oviposition in *C. pomonella* had been studied to a limited extent. Preliminary studies on *C. pomonella* had however indicated that volatiles produced during yeast fermentation affect attraction and oviposition in *C. pomonella* (unpublished).

This project was a pilot study preceding the subsequent development of a novel, yeast-based control technique for the codling moth based on research on insect-yeast mutualism (Witzgall & Knight, 2013). The aim of this study was to move towards an understanding of the significance of yeast fermentation in the attraction and oviposition for *C. pomonella.*

The hypothesis was that fermentation odours induce or influence attraction and oviposition behaviour in *C. pomonella* moths, and that the behaviour might be sexually dimorphic and modulated by mating state.

The main questions we asked were:

- How does yeast odours affect attraction behaviour of *C. pomonella*?
- Does mating state influence attraction and do we see sex-specific differences?
- How does yeast fermentation odours affect oviposition behaviour of *C. pomonella*?

To answer these questions, attraction and oviposition bioassays have been conducted. The codling moth, *C. pomonella* was tested towards different odour sources, with and without the presence of fermentation-volatiles.

Materials and methods

Materials

All experiments were conducted during July and September 2010 at the Swedish University of Agricultural Sciences (SLU) at the Department of Plant Protection Biology, Chemical Ecology Unit, Alnarp.

Codling moth, Cydia pomonella

Larvae of the codling moth, *C. pomonella* were reared on an artificial wheat germ-based diet (Witzgall *et al.* 2012). The pupae of *C. pomonella* were kept in plastic containers (20 x 30 x 15 cm), at 20 ± 2°C and a 65 ± 5% RH and in an 18 h: 6 h – light: dark-photoperiod. All moths were moved after emergence, from the climate chamber to the room where the oviposition bioassay was conducted.

Odour samples – preparation

All odour sources, except the apples, were liquid samples. Minimal medium was used as a control, minimal medium is a growth medium that contains only the minimal requirements needed for an organism to grow (Todar, 2012). In this study, the medium was prepared as described by Merico *et al.* (2007). The apples were picked at the university orchard at Alnarp and washed in tap water and cut into smaller pieces.

In the olfactometer bioassay apple juice and apple juice inoculated with yeast were tested. The samples used in the oviposition bioassay were apple juice, apple juice inoculated with yeast, minimal medium, minimal medium inoculated with yeast, fresh apple and damaged apple.

The different odour samples were prepared in the laboratory in a clean, but not completely sterile environment. The unfermented samples were kept in refrigerator while the apple juice and minimal medium that were to be used as the fermented samples were inoculated with baker's yeast (*Saccharomyces cerevisiae*) was kept at room temperature for 24 hours. On the day of the experiment, the unfermented minimal medium was removed from the refrigerator and the apple juice was poured into clean petri dishes.

Assays

Two different bioassays with the codling moth, *C. pomonella* were conducted, one olfactometer bioassay and one oviposition bioassay. To study if the moths' attraction behaviour changes depending on mating status or sex, tests were performed with male or female moths of known mating state. An oviposition bioassay was conducted to investigate whether presence of fermentation-volatiles had a stimulating effect on oviposition in *C. pomonella*-females.

At emergence the moths were separated by sex and transferred into clean containers. Approximately 50 % of the moths were transferred into a larger cage where they were allowed to mate while the remaining moths were kept separated and virgin. Tests were conducted within a maximum of 36 h after emergence of the moths. All moths were starved, from emergence and throughout the tests. Each moth was only used in one of the tests, either in the olfactometer or oviposition bioassay, and only tested once.

Olfactometer assay

To test the behaviour of the codling moth towards different odours, an olfactometer bioassay was set up, modified from Vallat and Dorn (2005). Moths of different sex and mating status, mated or virgin, were tested. The olfactometer that was used was a rectangular glass-box (18 x 13 x 2 cm) with a separating wall halfway down the box from the short side. On each side of the separating wall an air stream (0.25 ml/s) was blown down the olfactometer. The olfactometer was custom-made at Humi-Glas AB, Södra Sandby. A sketch of the olfactometer can be found in Appendix 1.

A two-choice test was conducted to determine preference towards different odours. The odours tested were apple juice versus the apple juice inoculated with yeast. The moths were divided into four groups; virgin females, mated females, virgin males and mated males and all moths were starved prior to the test. Each moth was tested individually; each test lasted 3 minutes and the moths were not exposed to the odour prior to the test. A single moth was inserted into the olfactometer where it could choose the odour of preference by selecting either side. The air stream that ran through the box made the moth walk up-wind towards the odour source. All moths movement was documented twice, first when the moth passed the separating wall the first time (first choice), and then at the end of the test (final choice). The moths that never passed the separating wall into either side and remained in the common area, and therefor did not make an active choice was also documented.

After each moth the odour sources was removed and the olfactometer was ventilated. To assure that there was no bias in preference unrelated to the odour source, the two odour sources were switched randomly. When the method was tested, it was found that moths that had not made a choice within 3 minutes, most likely would not show a preference to either side at longer test duration. Table 1 show the number of moths tested.

Table 1. Olfactometer – number of replicates, C. pomonella

	total	*males*	*females*
Virgin	22	12	10
Mated	45	21	24

Oviposition assay

To test if different odours has an effect on oviposition of the codling moth, an oviposition bioassay was set up. The newly emerged moths (0-24 h) were removed from the cages when mating, and put pairwise into glass-cylinders (3 cm Ø, 15 cm), where they were allowed to finish mating and to oviposit. The ends of the cylinders were covered with a mesh to avoid the moths from escaping the cylinder. Six cylinders were then placed into a box (22 x 30, 7 x 5 cm) with an odour source, Table 2. The odour source was placed in a petri-dish (94 mm Ø, 16 mm) at approximately 5 cm distance from the cylinders. In the liquid samples, approximately 75 ml of the odour source was used and about half an apple (approx. 70 g) cut in smaller pieces.

The boxes were kept at room temperature (approx. 22 °C, 65±5 % relative humidity (RH)) under 18:6h. light:dark rhythm (L:D) for around 72 hours. In total six different odour sources were used, including the control (minimal media), the odour sources are presented in Table 2.

Table 2. Odour sources used in the oviposition bioassay, C. pomonella with number of replicates

Odour source	*Number of replicates*
1. Apple juice	16
2. Fermented apple juice	15
3. Minimal medium	18
4. Fermented minimal medium	16
5. Apple - fresh	23
6. Apple1,2 – infested with codling moth larvae – new infestation	16
7. Apple1,2 – infested with codling moth larvae – older infestation	15

1. Apple – infested with codling moth larvae – new infestation (6) and Apple – infested with codling moth larvae (7) – older infestation are added in results.

2. M. domestica 'Aroma', picked at university orchard (Alnarp, Sweden)

When the tests were planned and conducted, the damaged apples used as odour source were divided into two different treatments; *new infestation* (6.1) and *old infestation* (6.2). However, it was discovered during the first set of replicates that it was difficult to correctly determine the age of the infestation. Treatment 6.1 and 6.2 was therefore merged when the results were analysed. The treatment was named, *Apple- damaged* (6) in the results.

Results

Attraction behaviour of the codling moth towards different odours.

The response of *C. pomonella* in the olfactometer bioassay is presented in Figure 2. No significant difference between mating status or sex was found when looking at the final choice of responding moths. That some moths responded while others remained passive is unrelated to both mating status and sex (GLM, 0.2034, p>0, 1). Overall the insects' response is higher than the non-response.

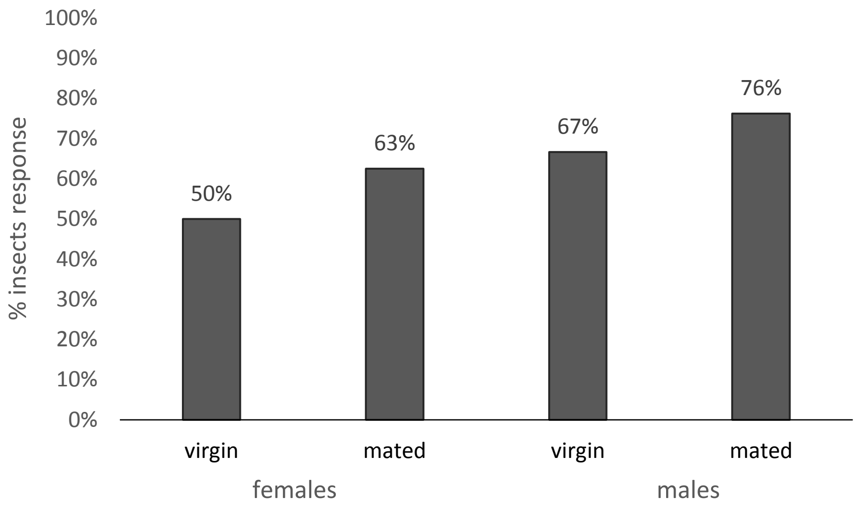

Figure 2. Mated and virgin, females and males, distribution of moths that show a response to either of the odour sources (%). No significant difference between mating status or sex can be found. Final choice.

In the olfactometer bioassay, the first choice was noted, as well as the final choice after 3 minutes. The first choice towards either apple juice (AJ) or fermented apple juice (AJY) is shown in Figure 3. The moths are separated by sex and mating status. As presented in Figure 3, 80 % of the mated females show a preference towards fermented apple juice, while virgin females show the opposite response with an 80 % preference towards the apple juice. Both virgin and mated males show a slight preference towards apple juice (AJ), hence there is no difference between mating status when looking at the male moths. Virgin males show a similar pattern of preference as virgin females, while mated males and mated females show an opposite preference pattern.

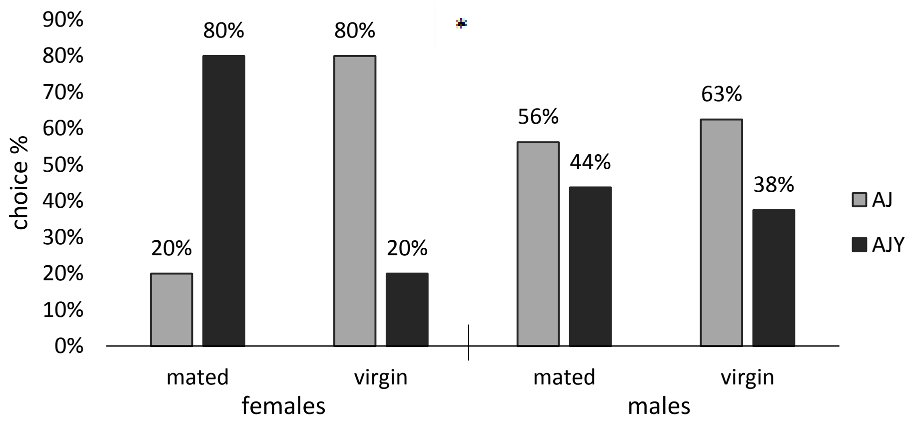

Figure 3. First choice. Females and males, mated & virgin. Distribution of tested moths that show preference towards either odour source (%). Apple juice (AJ) and fermented apple juice (AJY). The result was analysed with a general linear model-test, and there is an overall significant difference in preference, when comparing females and males (*, p < 0, 1). There is no significant difference between mating status (P= 0, 34).

In Figure 4 the final choice, after 3 minutes is shown. In the assay, 93 % of the mated females and 69 % of the mated males choose the fermented apple juice, while 80% of the virgin females and 75 % of the virgin males prefer unfermented apple juice. The result was analysed with a general linear model-test, and there is a significant difference when comparing mating status (***, p > 0,001). Some differences between sexes can be observed as a slightly larger number of mated females choose the fermented apple juice. However, there was no significant difference between moths of the opposite sex with the same mating status (GLM, p >0.1).

There is a slight difference between the first and final choice; whereas first and final choice correspond in females and virgin males, the final choice in mated males differ from the first choice. A number of the mated moths preferred the unfermented apple juice (AJ) at first, but ended up choosing the fermented apple juice (AJY) after 3 minutes.

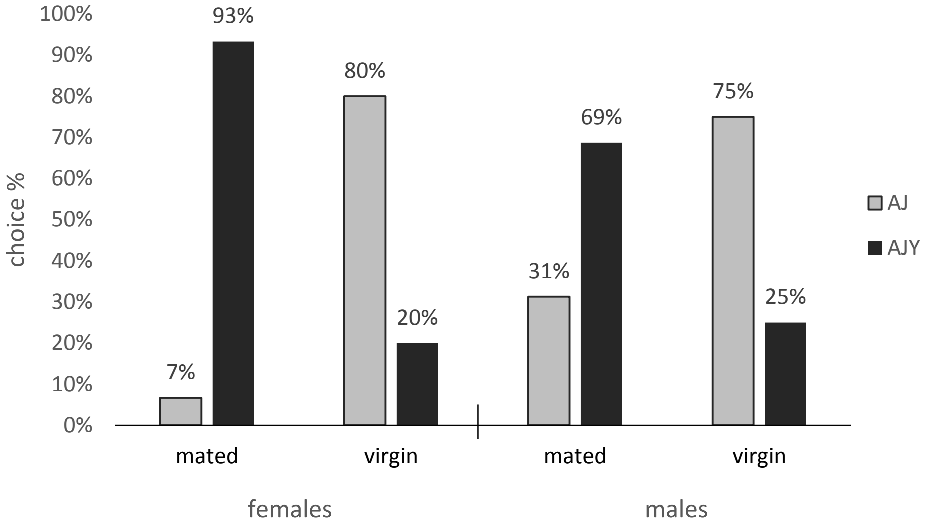

Figure 4. Final result, after 3 minutes. Females and males, mated & virgin. Distribution of tested moths that show preference towards either substrate (%). Apple juice (AJ) and fermented apple juice (AJY). The result was analysed with a general linear model-test, and there is a significant difference when comparing mating status (***, p > 0,001). Virgin moths show a preference towards unfermented apple juice while, mated moths show a clear preference towards the fermented apple juice.

Oviposition observations – effect of different odours

In Figure 4 and Table 3, the total number of eggs after 72 hours are presented. The largest number of eggs was oviposited in presence of the odour of fermented minimal medium, with 111 eggs/moth and in the test with the odour of apple juice, where 106, 2 eggs/moth were oviposited. Damaged apple (97, 7 eggs/moth), minimal media (96, 5 eggs/moth) and apple-undamaged (91, 2 eggs/moth) induced similar oviposition, while the fermented apple juice-treatment was the least attractive odour to mediate oviposition, with 73, 6 eggs/moth. Table 3 present the standard deviation and the median value in addition to the average number of eggs oviposited.

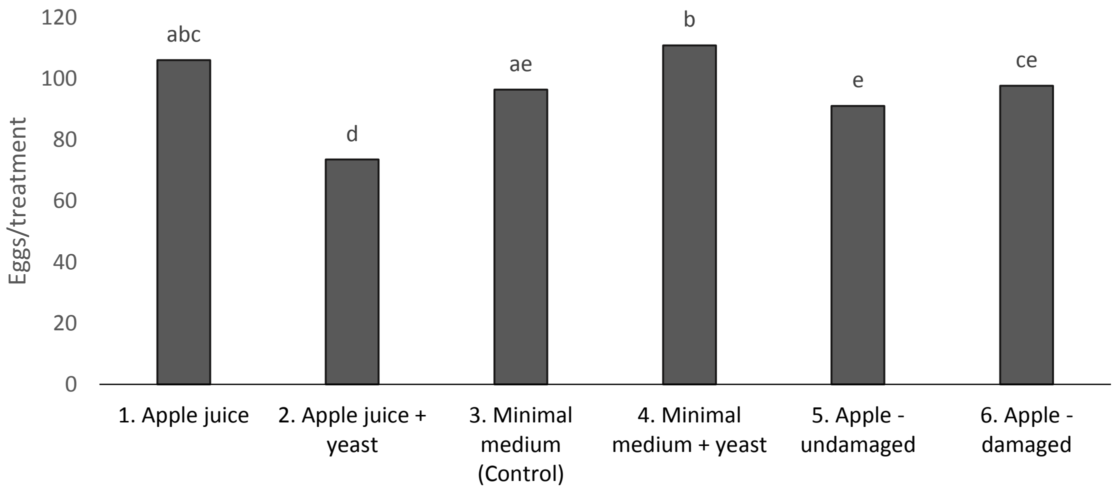

Figure 4. Average number of eggs/moth. All treatments compared with a linear model. Different lowercase letters in the figure indicate significant differences according to a GLM-test, with Poisson distribution ($P<0{,}001$). The result can also be found in table 3, with the standard deviation of the mean and median.

Table 3. Average number of eggs/moth, after day 3. Standard deviation and median-value

Odour source	*Average number of eggs/moth*	*Standard deviation*	*Median*
Apple juice	106,2	28	107,5
Fermented apple juice	73,6	36,5	71
Minimal media	96,5	40	95
Fermented minimal media	111	38,5	122
Apple – undamaged	91,2	35	96
Apple – damaged	97,7	41	101

Discussion

The codling moth *C. pomonella*, like other fruit-living insects, is associated with yeasts, which are a food resource and support larval development (Ganter, 2006; Witzgall *et al.* 2012; Becher et al, 2012). Yeast-produced fermentation odours consequently are expected to induce or influence *C. pomonella* attraction behaviour and oviposition.

In this study the behavioural attraction of *C. pomonella* towards odours of selected substrates, fresh or fermented, respectively, and the potential stimulating effect of fermentation odours on oviposition have been tested.

The result of the attraction bioassay indicate that mated codling moth females prefer fermented apple juice while virgin females prefer unfermented apple juice Furthermore, yeast-fermented minimal medium was inducing strong oviposition. Fermented fruit is known to have a stimulating effect on oviposition (Witzgall, *et. al.* 2012; Wearing et al. 1973; Hern & Dorn, 1999). Yeast adds essential food elements to the diet of fruit-associated insects (Baumberger 1919). Gravid *C. pomonella* possibly, according to the concept of "good mothers", choose their oviposition sites where the offspring has the best possible opportunities (Campbell & Reece, 2005; Wiese et al. 2008). We suggest that the observed female behaviours in response to fermentation odours is related to host location and egg-laying behaviour.

Surprisingly, oviposition in response to fermented apple juice was lower than in resonse to unfermented juice. However, the samples were not strictly controlled for spontaneous fermentation, it can therefore not be ruled out that smaller amounts of fermentation volatiles were also present in non-inoculated samples. The low oviposition in presence of fermented apple juice does not meet the result of the olfactometer bioassay, where a significant attraction of mated females towards fermented apple juice can be observed. The contradiction between the weak effect of the fermented apple juice and the stimulating effect of fermented minimal medium requires further investigation. However, that the fermented apple juice was not the most attractive odour may also be a result of the uncontrolled fermentation that might have lead to negative effects by secondary metabolites.

When comparing the treatments *damaged (=infested) apple* and *fresh apple*, a larger number of eggs are oviposited in the *damaged apple*-treatment, which is consistent with other studies (Witzgall, et al. 2012). Infested apples are known to contain yeasts which most likely had a positive effect on oviposition (Witzgall et al. 2012). Also, that the second most attractive odour is apple juice is expected, since it is well-known that the codling moth is attracted to α-farnesene, which can be found in the outer coating of apples (Huelin & Murray, 1964).

The oviposition bioassay was set-up as a no-choice assay, which resulted in the moths ovipositing in all tested treatments. This indicates that the yeast has a stimulating effect, but is not a requirement. Previous studies show that although there is an advantage in ovipositing on the most suitable site, oviposition at a less optimal site can be amended by the movement of larvae (Ehrlich & Raven, 1964). Furthermore, the effect of the oviposition decisions has been investigated and the result show that although some oviposition sites might be preferable, the result indicate that females oviposit randomly (Paukku & Kotiaho, 2008). This shows that the urge to oviposit and thus reproduce, is of higher importance than finding the optimal site to oviposit. It is possible that there are some odours that partly or completely inhibit the oviposition, but no such odours were tested here.

The attraction assay showed a slight difference between virgin females and males, but both sexes show the same tendency of preferring unfermented to fermented juice. Interestingly, although not clear in the measure of the first choice, mated males similar to mated females prefer fermented apple juice to unfermented juice. Altogether, codling moths of the same mating status show the same preference towards the odours independent off sex. Prefered attraction to unfermented apple juice in virgin males and females might be linked to foraging or reproductive behaviour.

Both the codling moth larvae and the adult moth are attracted to, and feed on pomme fruit (Knight & Light, 2001). Why virgin females show the direct opposite tendency from the mated females is not obvious, since it is likely that adult moths prefer to feed on the same site (Knight

& Light, 2001). However it is also likely that the basic drive of the virgin female is to find a suitable mating site and not food. In this study we did not test feeding behaviour; odour mediated feeding and modulation by mating would be interesting to study in future experiments. In a study by Vallat & Dorn (2005) it is presented that mated females are attracted to apple fruit volatiles during midseason (mid July to mid August) while the attraction change towards twigs and leaves later in the season. Not only are the apples more attractive, twigs and leaves are in fact repelling during midseason. This indicates that females are attracted to fruit when mated, because they oviposit on maturing fruit. To which extent fermentation odours might further increase the attraction of mated females towards maturing fruit under field conditions is not clear.

When combining the olfactometer- and oviposition bioassays, it is clear that the behaviour shows plasticity along with the change in mating status, while sex seems to be of lesser importance. It is also clear that some volatiles have a stimulating effect on oviposition in the codling moth. The results indicate that the mating status has an influence on reproductive behaviour of the codling moth – a mated moth search for a site to oviposit, while the virgin moth are in pursuit to mate. The result in other studies investigating the noctuid moth *Spodoptera littoralis*, show that females respond different after mating and that the olfactory response switches to a desire to find a suitable place to oviposit (Saveer *et. al.* 2012). Furthermore, modulation at the olfactory system and odour-mediated behaviour was also shown in male *S. littoralis*, where attraction towards reporoducition-related sites is temporally down-regulated after mating, while foraging cues stay highly attractive independent from mating state (Kromann et al. 2015). In *C. pomonella*, virgin females show a clear preference towards the unfermented substrate, thus away from the potential oviposition and feeding site. When mated, the moth switches preference to the fermenting odours (Witzgall, 2012), hence, when the response switches, the moth will most likely move to a suitable site to oviposit.

A majority of the male moths show the same tendency of attraction as the female moths, when looking at the final result. A mating site separated from the oviposition site might benefit virgin males to find virgin females as there, the operational sex ratio of females might be higher. It is of uttermost importance that virgin males and virgin females are able to find each other to mate. The female codling moth uses sex pheromones to communicate when searching for mates. However, the male moth is known to be roaming the host area prior to when the female starts calling (Witzgall *et. al*. 2008). That indicates that the male moth must be at, or close by, the mating site beforehand which might explain why the virgin males behave similar to the virgin females, similar behaviour results in that both males and females end up at the same type of host site.

A number of moths did not make a clear choice, as many as 50% of the female virgin moths did not make a choice at all, compared with 38 % of the mated females. The fact that a large number of moths did not make a choice is interesting, however, a generalized linear model-test showed that there is no significant differences between sex and mating status when looking at the moths that did not make a choice. That indicates that the large number of moths that are indifferent to the odours depends on other factors than mating status or sex. It could be a natural deviation; all populations have individuals that deviate from the rest of the population. A larger number of replicates will lower the impact of possible deviations. Moths are able to mate 24 hours after emergence (Gehring & Madsen, 1963), but there are deviancies and testing the moths later might have given another result. Kronmann et al. (2015) showed that behavioural modulation by mating is reversible and thus time-dependent. Reasons for the indifference in some moths still needs further investigation.

Conclusion

When looking at the olfactometer and the oviposition bioassays combined, the result strongly indicates that gravid codling moth females prefer the fermenting substrate while the virgin moths prefer the unfermented substrate. This pilot study does not allow to draw definite conclusions which would require a higher number of replicated tests, however, there is a strong indication that the response in codling moth towards fermenting odours does change depending on mating status, when looking at females. Males show the same tendency with an exception of the result after the first choice, where the mated males show the opposite preference. However, the final choice is clearly comparable with the mated female. There is also an indication that fermentation has a positive effect on the oviposition in the codling moth, *C. pomonella,* although the presence of fermented substrates is not an absolute requirement.

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Pictures

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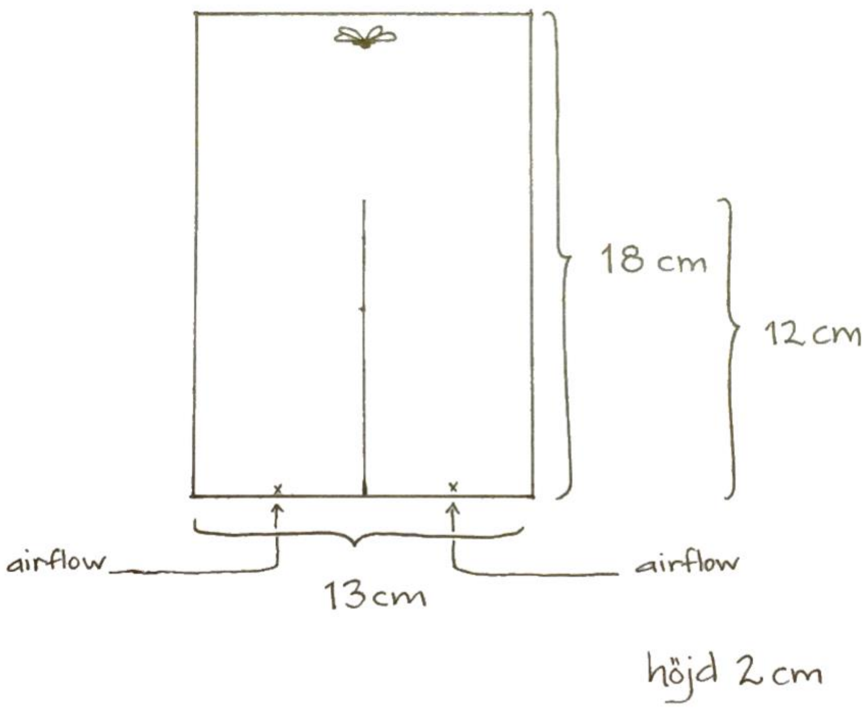
Appendix 1.

Basic-data, olfactometer



Appendix 2.
Statistical analysis, Olfactometer bioassay

First choice:

Choice vs No choice

	Df	Deviance Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			3	3.3191	
mating	1	0.52379	2	2.7953	0.46923
sex	1	0.02023	1	2.7751	0.88690
mating:sex	1	2.77506	0	0.0000	0.09574

Choice 1 vs Choice 2

	Df	Deviance Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			3	5.8977	
mating	1	0.9013	2	4.9965	0.34245
sex	1	4.5034	1	0.4931	0.03383*
mating:sex	1	0.4931	0	0.0000	0.48257

Final choice:

Choice vs No choice

	Df	Deviance Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			3	2.24035	
mating	1	0.62143	2	1.61891	0.4305
sex	1	1.61754	1	0.00137	0.2034
mating:sex	1	0.00137	0	0.00000	0.9705

Choice 1 vs Choice 2

	Df	*Deviance Resid.*	*Df*	*Resid. Dev*	*Pr(>Chi)*
NULL			*3*	*16.4583*	
mating	*1*	*13.1748*	*2*	*3.2836*	*0.0002837 ******
sex	*1*	*1.7671*	*1*	*1.5165*	*0.1837434*
mating:sex	*1*	*1.5165*	*0*	*0.00000*	*0.2181539*

Statistical analysis, Oviposition bioassay.

GLM with Poisson distribution

Fit: glm(formula = eggs ~ substrate, family = poisson, data = cydiaovtot)

Linear Hypotheses:

	Estimate	Std. Error	z -value	Pr(>|z|)
AJ - AD == 0	0.08288	0.03031	2.734	0.0674 .
AJY - AD == 0	-0.28369	0.03515	-8.070	<0.001 ***
AU - AD == 0	-0.06956	0.02841	-2.449	0.1374
MM - AD == 0	-0.01221	0.03009	-0.406	0.9986
MMY - AD == 0	0.12720	0.02988	4.256	<0.001 ***
AJY - AJ == 0	-0.36656	0.03866	-9.482	<0.001 ***
AU - AJ == 0	-0.15244	0.03264	-4.670	<0.001 ***
MM - AJ == 0	-0.09509	0.03412	-2.787	0.0585 .
MMY - AJ == 0	0.04432	0.03394	1.306	0.7791
AU - AJY == 0	0.21412	0.03718	5.759	<0.001 ***
MM - AJY == 0	0.27147	0.03849	7.054	<0.001 ***
MMY - AJY == 0	0.41089	0.03833	10.721	<0.001 ***
MM - AU == 0	0.05735	0.03244	1.768	0.4829
MMY - AU == 0	0.19676	0.03225	6.102	<0.001 ***
MMY - MM == 0	0.13941	0.03374	4.132	<0.001 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Adjusted p values reported -- single-step method)

AJ=apple juice; AJY= apple juice + yeast; MM= minimal media; MMY= minimal media + yeast; AU= apple undamaged; AD= apple damaged