Of mice and flies
- How the age of a carcass affects wind tunnel attraction in the blowfly Calliphora vicina

Hvordan alderen til et kadaver påvirker attraksjon i vindtunnel for spyfluen Calliphora vicina

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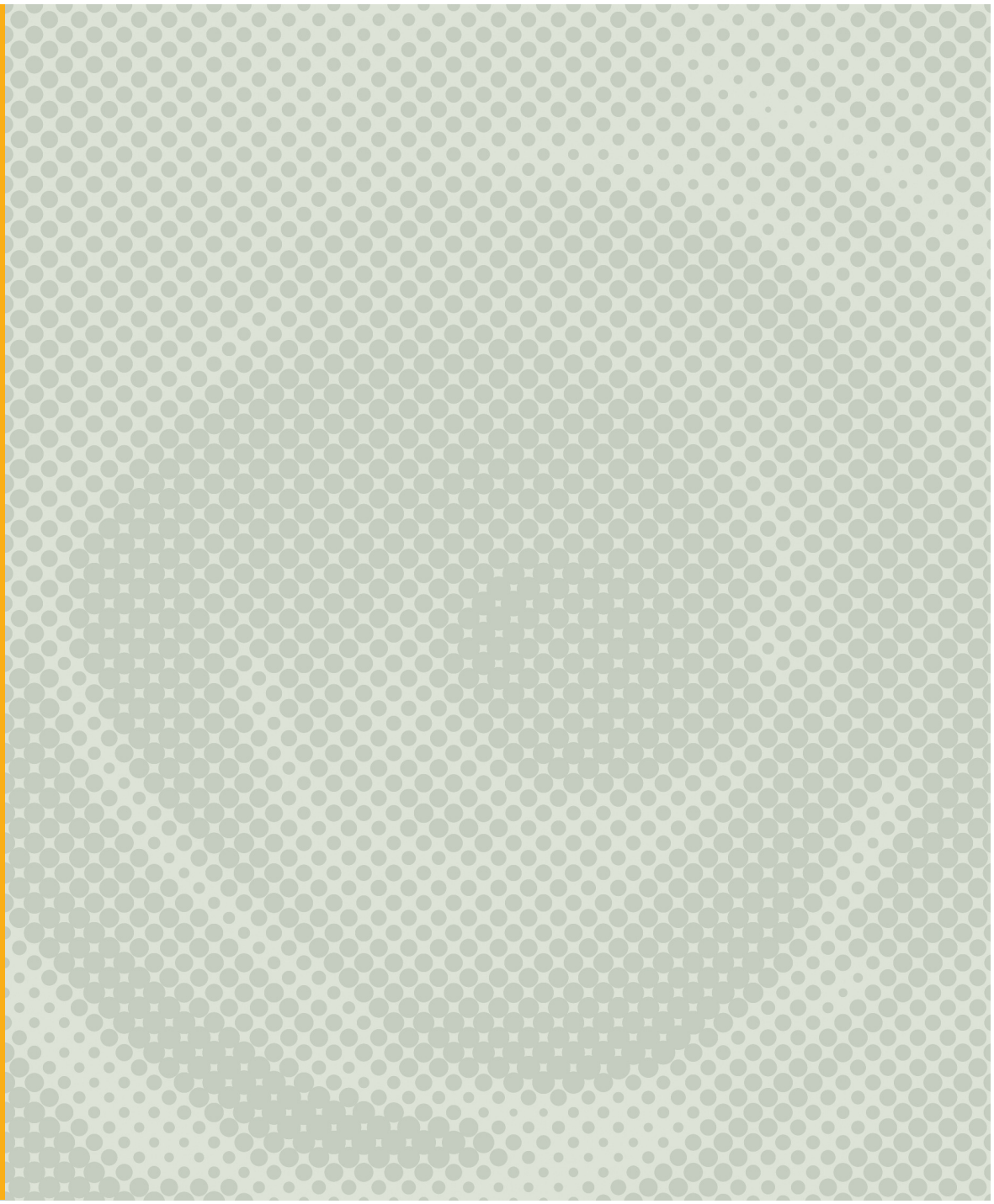

Forord

Denne masteroppgaven er skrevet ved Universitetet for Miljø- og Biovitenskap, Institutt for Naturforvaltning. Alt arbeidet med oppgaven har vi gjort felles, både i laboratoriet og skriftlig. Arbeidet med dette vindtunnelforsøket ble utført ved Bioforsk Plantehelse, som stilte vindtunnel, laboratorier og utstyr til rådighet. Bur og annet utstyr til flueoppdrett ble lånt fra Folkehelseinstituttet, og herfra kom også fluene brukt til å starte kulturen og musekadavrene benyttet i forsøkene.

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Sted/dato: Ås, 14.05.2011

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Hege Johansen	Marit Solum

Of mice and flies – how the age of a carcass affects wind tunnel attraction in the blowfly *Calliphora vicina*

Hege Johansen and Marit Solum

Abstract

Larvae of the saprophagous blowfly *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) cause substantial damage by infesting stockfish in Lofoten. A synthetic lure has been developed to control this pest, but the attraction towards this lure is still lower than towards natural odors. Better understanding of the succession of volatiles released from a carcass during decomposition could improve the lure by identifying missing components, or lead to the detection of antagonistic compounds.

This study is a wind tunnel experiment investigating how the volatiles from carcasses of different age affects how attractive they are to male and female *C. vicina*. Mice carcasses were used as natural odor sources. We predicted that flies would be more strongly attracted to carcasses in early stages of decay, and that females would show stronger preferences for early stages than males, since they depend on the carcass for oviposition. We also performed additional experiments investigating how conspecific larval secretion affects attraction.

Both male and female *C. vicina* showed variation in their response to volatiles released from mice carcasses of different age, with significantly higher attraction towards carcasses that were three, six, and nine days old. We found no differences between males and females regarding oriented flight responses, but observed an increase in landings for female flies at the most attractive carcasses. Larval secretion was not attractive when tested alone, and had little effect on attraction when added to a three day old carcass.

This study shows that *C. vicina* is capable of assessing an odor source from a distance, without relying on visual cues provided by the source. This highlights the importance of olfaction for blowflies when locating a resource, and indicates that they use the succession in the volatiles emitted from a carcass to locate suitable carrion. The interaction between gender and age of carcass observed for landings might point to a closer coupling of vision and olfaction in female blowflies compared to males.

Table of contents:

Introduction

We humans perceive the world mostly through vision, sound, and touch, and depend relatively little on our sense of smell. Insects, on the other hand, use olfactory cues in almost all aspects of their life, from location of resources or suitable mates to mediation of social interactions with conspecifics (Städler 1984, Keil 1999, Cardé and Millar 2004, Stensmyr 2004, Gullan and Cranston 2010). Their world is a complex mosaic of countless odors, out of which they must be able to sort out and respond to only the small fraction that is relevant (Hansson and Christensen 1999, Hartlieb and Andersson 1999, Salecker and Malun 1999, Larsson and Svensson 2004). To do so they have a well-developed olfactory system, often tuned to only a small number of compounds, or to specific blends (Mustaparta 1984, Stensmyr 2004). Olfactory signals are mostly detected by the antennae (Larsson and Svensson 2004), densely covered by sensory organs known as sensilla (Stensmyr 2004, Gullan and Cranston 2010). Odor molecules are able to pass the cuticle through tiny pores in these structures and interact with specific olfactory receptor neurons (Steinbrecht 1997, Keil 1999, Hansson and Christensen 1999). The signals from these neurons are transmitted to the antennal lobe, the main processing center of the central nerve system (Hansson and Christensen 1999, Stengl et al. 1999, Todd and Baker 1999). Here the axons of receptor neurons expressing the same receptors converge into spheroid structures called glomeruli (Anton and Homberg 1999, Todd and Baker 1999). Signals from the glomeruli transmit information to higher centers in the brain, which are in turn connected to motor centers (Strausfeld 1976).

Seemingly similar behavioral orientation mechanisms are employed by flying insects to locate a wide variety of odor sources, regardless of whether they respond to a sex pheromone (Kaissling 1997), host-plant odor (Zanen and Cardé 1996), or the odor of a host animal (Gibson et al. 1991, Cossé and Baker 1996). Odor strands travel in a more or less straight line away from a source (Todd and Baker 1999), creating a meandering plume. Turbulence within the moving air causes the odor plume to break up into filaments of high concentration packets of odor interspersed with pockets of clean air (Hartlieb and Anderson 1999, Schoonhoven et al. 2005, Murlis et al. 1992, Cardé and Willis 2008). The concentration of compounds in these filaments is fairly constant throughout the plume, whereas the density of odor filaments decreases as you move away from the source (Murlis and Jones 1981). Odor plumes can therefore not provide insects with the directional information necessary to locate the source

(Cardé and Willis 2008). The quickest way for an insect to locate an odor source is to head upwind when in contact with the plume, and switch to casting, zigzag motions perpendicular to the wind direction, if contact is lost (Gibson et al. 1991, Hartlieb and Anderson 1999). When contact with the plume is re-established, upwind flight can resume (Cardé and Willis 2008). To keep track of the wind direction, flying insects employ optomotor responses: they use visual cues to compute their displacement relative to their surroundings (Mafra-Neto and Cardé 1994, Vickers and Baker 1994). This combined system is called optomotor anemotaxis (Cardé and Willis 2008).

An accurate sense of smell coupled with good flying abilities enable blowflies to quickly locate food, oviposition sites, and other resources. They are among the earliest colonizers of carrion, and are therefore important decomposition agents, often used in forensic investigations (Rognes 1991, Byrd and Castner 2009). But they are also capable of locating resources meant for human consumption, thereby becoming pests. The blowfly species *Calliphora vicina* Robineau-Desvoidy (Diptera, Calliphoridae) is a problem for the stockfish industry in northern Norway. Stockfish is made by hanging cod (*Garrhus morhua*) out to dry on racks from February to the end of May (Aak et al. 2010a), and female *C.vicina* inflict substantial damage by ovipositing on the drying fish – during years of high damage levels, losses can amount to 2 million € (Aak 2010). Application of pesticide could be a solution (Sømme and Gjessing 1963, Walker and Donegan 1984), but is undesirable due to pesticide residue in the fish. Harsh weather conditions prevent the use of physical barriers such as insect nets, whereas indoor drying is too expensive and reduces the quality of the product (Aak et al. 2010a).

A possible way of reducing the blowfly population is mass trapping, a technique where pest insects are caught with baited traps. Many criteria need to be fulfilled for this technique to be successful, and a highly attractive lure is of utmost importance (Karg and Suckling 1999). Experiments with *C. vicina* have shown strong attraction to the odor of freshly dead carrion (Stensmyr 2004, Aak et al. 2010b, Aak and Knudsen 2011). However, the use of natural baits is problematic due to handling difficulties and variable attraction over time (Muirhead-Thomson 1968). These problems may be circumvented by using a synthetic lure. An attractive lure has been developed for *C. vicina* (Aak et al. 2010b), and has been used for successful mass trapping at stockfish production sites in Lofoten (Aak 2010). However, in a wind tunnel

study Aak et al. (2010b) found higher attraction towards natural odors (fish, mice carcasses), indicating that the lure can be improved – for instance by adding missing attractive components from natural sources.

Such compounds might be identified by studying the succession of chemicals in the odor plume of a carcass. Decomposition of animal tissue is a complex process, and carcasses are constantly changing throughout decay (Vass 2001, Gullan and Cranston 2010). Improved knowledge of the decay process can yield valuable information about the compounds produced in the different stages, and how they affect blowfly behavior. This can lead to the identification of missing components that can be added to the existing lure, as well as potential antagonistic compounds detering blowflies from an odor source.

Blowfly species with saprophagous larvae, such as *C. vicina*, use dead animals both as food for the adults, and as a resource for the developing larvae (Rognes 1991, Archer and Elgar 2003a). Male flies attend carrion to obtain food and matings (Shorey et al. 1969), whereas females also depend on carrion as a suitable oviposition site (Archer and Elgar 2003a). Locating fresh carrion is therefore likely to be more crucial for female blowflies, and a sexual bias in selection pressure might have lead to females becoming more attracted to odors emitted by carrion in early stages of decay. Oviposition is to a large extent guided by cues from the oviposition site, but cues from conspecifics can also be important (Hartlieb and Anderson 1999). The larvae of *C. vicina* empty their alimentary tract prior to pupation, and this secretion might be a signal to females of a less suitable oviposition site. It could also be a signal to both genders of a food resource of poorer quality. If such antagonistic compounds exist, they could be very useful for blowfly control.

The present study is a wind tunnel experiment investigating how volatiles from carcasses of different age affect how attractive they are to male and female *C. vicina.* Mice carcasses in different stages of decay were used as natural odor sources. We also tested the effect of adding larval secretion to an attractive carcass. We predicted that:

- Flies would be more strongly attracted to early stages of decay than to late stages
- Females would show stronger preferences for earlier stages than males
- Addition of larval secretion would reduce blowfly attraction

Materials and methods

Study species

The blowfly *Calliphora vicina* Robineau-Desvoidy (Diptera, Calliphoridae) (Fig. 1) belongs to a large family of flies, consisting of more than 1000 species (Stensmyr 2004). It has an almost circumpolar distribution (Byrd and Castner 2009), and is very common all over Fennoscandia (Rognes 1991). Larval development in animal flesh is characteristic of blowflies (Stevens 2003), but *C. vicina* can also complete development in a wide selection of other materials (Sømme and Gjessing 1963, Rognes 1991, Stensmyr 2004). *C. vicina* has three larval instars, and the rate of development is very dependent on temperature (Rognes 1991). Prior to pupation, the third instar larvae empty their alimentary tract and enter a migratory stage. After emergence, the adults feed on carbohydrates, but females also require protein to complete maturation of the ova (Rognes 1991).

Figure 1: The study species *Calliphora vicina* (Diptera, Calliphoridae). Photo: A. Aak, illustration: Hallvard Elven. ©The Norwegian Institute of Public Health

C. vicina is an important decomposition agent (Rognes 1991). It is among the first colonizers of fresh carrion (Stensmyr 2004), and is therefore commonly used to estimate the time of death in forensic investigations (Greenberg 1991, Byrd and Castner 2009). It is normally not considered an important pest, but as seen at stockfish production sites in northern Norway, it can cause problems by infesting food products (Sømme and Gjessing 1963, Rognes 1991, Mallis and Hedges 1997, Aak et al. 2010a). It is also an accidental myiasis agent: it may infest living animals by ovipositing in necrotic wound tissue (Rognes 1991).

Rearing of insects

The flies used in the experiments originated from a laboratory population at the Norwegian Institute of Public Health. This population was founded by flies collected at a stockfish production site in Lofoten, but has been bred in the laboratory for five generations. We formed a culture using 130 flies, and used the sixth generation for our experiments. Females were given a petri dish with small pieces of fish and beef on which they could lay their eggs. After oviposition the petri dish was placed in a clear plastic box (30x22x6 cm) with a mesh cover, and the larvae were provided with a surplus of meat to feed on. The box was lined with moist paper, and humidity was kept high by spraying with water every second day. To ensure similar age for all flies in one batch, the larval boxes were examined every day to check for pupae. All developed pupae were removed and put in a separate plastic box (250 mL). They were allowed to develop in room temperature for one day, and were then stored at 4°C to arrest development. Approximately one week before the desired emergence time, development was allowed to resume by returning pupae to room temperature. They were placed in a plastic hatching box (250 mL) with coarse sawdust, mixed with 30 mL of water to prevent desiccation. This box was placed inside an insect cage (25x20x30 cm), and kept in a climate chamber at a 16:8 (light:dark) light cycle, with 65 % relative air humidity and a temperature of 20ºC. Most of the flies emerged during the course of a single day, and the hatching box was subsequently removed to ensure that all flies in one cage were the same age. Adult flies were fed with a continuous supply of sugar and water, and were given ground meat as a protein source. The meat was removed after ten days to prevent oviposition and induce protein deprivation. Flies were 16-18 days old when used in the experiments.

The wind tunnel

The distinctive behavior displayed by flying insects orienting towards an odor source can be readily observed using a wind tunnel (Fig. 2).

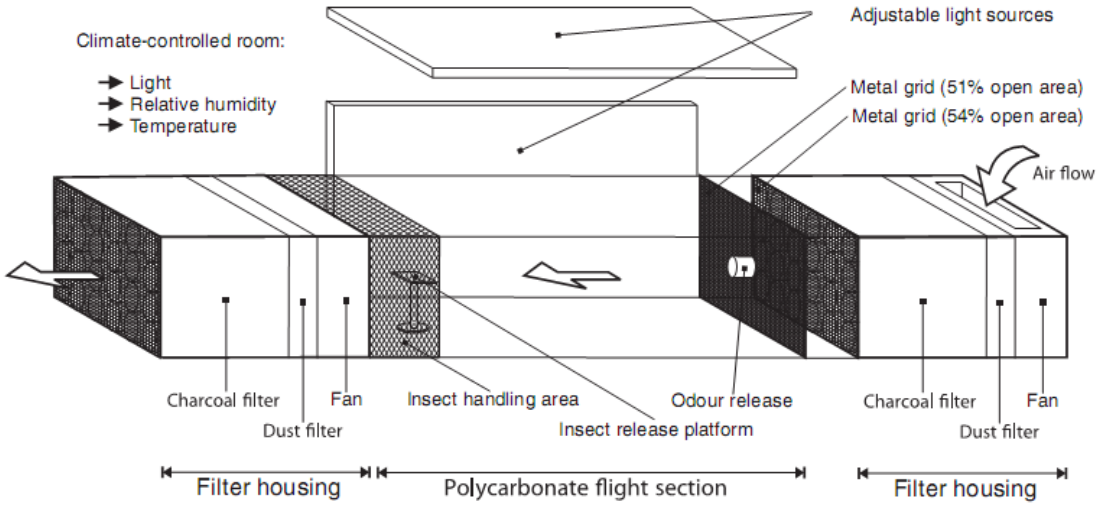


Figure 2: Schematic drawing of the wind tunnel at Bioforsk, Ås (after Aak et al. 2010b).

This bioassay allows for the control of light, relative humidity, temperature, and wind speed. It creates an artificial environment in which the odor source is the only variable. Odor filaments moving away from the release point creates a plume in the center of the tunnel. Optomotor anemotaxis in flying insects can be studied by observing the characteristic zigzag flight within the plume, resulting from a combination of upwind surge and casting, followed by landing close to the release point.

Experiments were conducted in the wind tunnel at Bioforsk, Norwegian Institute for Agricultural and Environmental Research (Ås, Norway) (Fig. 2). This tunnel consisted of a polycarbonate flight section (67x88x200 cm). Before entering the tunnel, air blown by a fan (model D640/E35; Fischbach GmbH, Neunkirchen, Germany) passed through a filter housing containing a dust filter and 24 active charcoal filters (Camhill Farr, Trosa, Sweden). To even the flow, there was a 30 cm section between the filter housing and the flight section, with perforated metal grids on each side (upwind pore size 8 mm with 54 % open area; downwind pore size 3 mm with 51 % open area). At the end of the tunnel, the air passed through a similar filtering system before release back to the room. The wind speed was calibrated to 30 cm/s (SwemaAir 300; Swema AB, Stockholm, Sweden).

The odor source was placed in a 2L glass jar closed with a grounded glass fitting, positioned inside the 30 cm section upwind of the flight arena. Pressurized and charcoal-filtered air passed over the odor source at approximately 1.10 L/min and entered the tunnel through a glass pipe 30 cm above the ground (Fig. 3, left). Directly below this release point a glass plate (18x10 cm) was mounted. Together with the adjacent area on the metal grid, this platform served as a predefined landing area (Fig. 3, right). To avoid odor contamination all glassware was replaced between treatments, and heated to 300°C for a minimum of 8 hours using a drying oven (model FD 53; Binder, Tuttlingen, Germany).

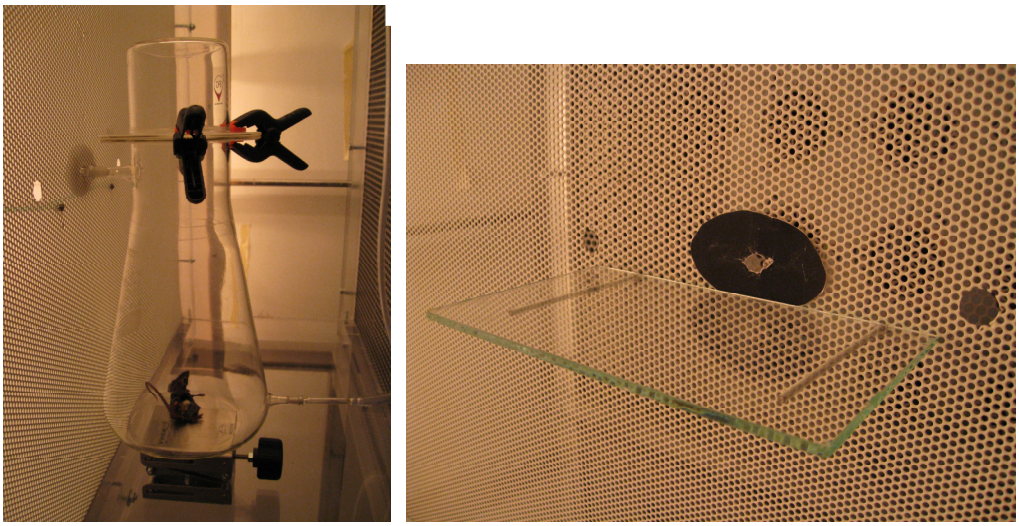


Figure 3: Details from the wind tunnel experiments. Left: the glass jar containing the odor source. Air enters the jar from the right, passes over the carcass at approximately 1.10 L/min, and is released into the tunnel. Right: the release point of the odor plume, with landing platform and visual stimuli. Photo: M. Solum.

To enable the flies to keep track of wind direction, visual cues were provided in the form of blue dots of different size (from 6 to 12 cm in diameter) along the tunnel floor (Fig. 4). *C. vicina* has been shown to require a vertical visual contrast in order to successfully initiate landing (Aak and Knudsen 2011). As the odor source provided no visual cues, a black paper oval was placed directly above the landing platform to provide this type of stimuli. A hole in the middle allowed the glass pipe from the jar to enter the tunnel (Fig. 3 and 4).

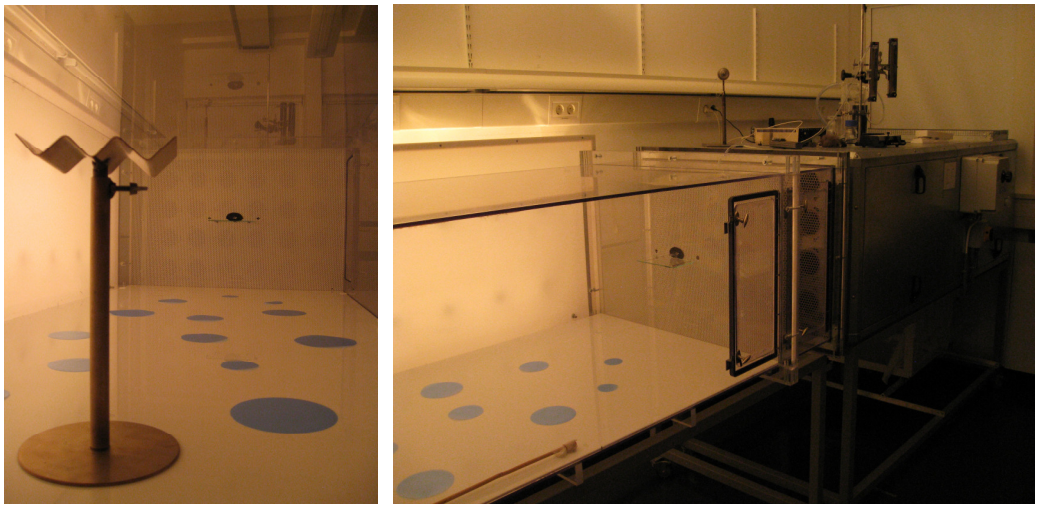


Figure 4: Visual cues provided in the wind tunnel. Left: The tunnel seen from the downwind end. Right: The upper half of the wind tunnel, with filtering system for incoming air. Photo: M. Solum

Experimental protocol

Wind tunnel experiments were conducted between 09.00 hours and 18.00 hours. The light intensity was 150 lux. Temperature inside the tunnel ranged from 20 to 24°C, and relative humidity from 47-79 %. Individual flies were placed in glass tubes (2.8x13 cm) covered with gauze at one end and a plastic cap at the other. They were allowed to adjust to the conditions in the wind tunnel room for at least one hour before the experiments. The odor source was placed in the glass jar just before the experiments started, and positioned inside the wind tunnel.

Flies were introduced into the tunnel one at a time by placing the glass tube on a starting platform 30 cm above the ground, 180 cm downwind of the odor release point (Fig. 4, left). The plastic cap was removed, and the flies were given four minutes to respond. Random flight was recognized as high-speed, nondirectional movements followed by landing outside the odor plume (floor, roof, tunnel walls). Oriented flight was recognized by the characteristic search pattern of flying insects displaying optomotor anemotaxis. Behavior was scored through personal observation as four predefined categories in accordance with well defined optomotor anemotaxis definitions: Take-off; oriented flight in the back half (0-90 cm) of the wind tunnel (OF-1); sustained oriented flight into the upper half (90-180 cm) of the tunnel (OF-2); and landing in the predefined landing area, following oriented flight. Flies were scored in a successive manner to only one of these categories. If orientation was sustained into the upper half of the tunnel, they were scored as OF-2 only (not as OF-1). Landing was

the maximum score for attraction, and further testing of flies scored to this category would give no new information. They were therefore removed from the tunnel. If the flies landed outside the odor plume, they were returned to the starting position. All flies showing no take-off were tested to see if they could fly. Flies that were not able to fly, were replaced by new individuals. Each fly was used only once. After the experiments, flies were killed by freezing them for at least one hour at -18°C. Females were dissected to assess their egg development stage. They were placed into four different categories: I – visible follicles, but no developed eggs; II – developed, circular eggs, still in an early stage; III – more developed, ellipse-shaped eggs; IV – fully elongated eggs ready for oviposition. After each treatment, headspace from the odor source was sampled for later chemical analyses. The methodology used is described in Appendix 1.

Experimental design

Mice carcasses in different stages of decay were used as odor sources in the experiments. The mice came from a laboratory culture at the Norwegian Institute of Public Health, and were stored at -18°C. Before the experiments they were placed in a plastic box (250 mL) lined with moist paper. Boxes were kept in a vented hood in room temperature to start decomposition, and the abdomen of the mice was cut open to expose blood and intestines. Water was added every second day to prevent desiccation. In the early stages of decay, bacteria and other microorganisms found in the soil are of great importance (Vass 2001). Therefore each age of decay was tested with and without addition of soil bacteria. Bacteria were added by mixing rich soil, sampled from a deciduous forest, with water. The mix was filtered through a small-meshed sieve, and the carcass soaked in the resulting solution. We defined seven different decay treatments for our main experiment, based on age of carcass: 0 (fresh), 1, 3, 6, 9, 20, and 33 days old (Table 1). As control, we tested attraction towards an empty glass jar.

Table 1: The setup for the main experiment investigating the effect of carcass age on wind tunnel attraction in male and female blowflies. Numbers are total number of flies tested per treatment. For the different treatments, we used mice carcasses of different age (D = days). Each treatment was conducted with and without added soil bacteria. Treatments were replicated five times for each gender, using ten flies for each replicate. As control, attraction was observed when no odor source was present.

Decay treatment		0D	1D	3D	6D	9D	20D	33D	Control
Females	+ bacteria	50	50	50	50	50	50	50	
	- bacteria	50	50	50	50	50	50	50	50
Males	+ bacteria	50	50	50	50	50	50	50	
	- bacteria	50	50	50	50	50	50	50	50

Larval secretion for the additional experiments was collected by placing third instar larvae in sealed petri dishes lined with filter paper. Ten larvae were used for each petri dish. When they had emptied their alimentary tract the larvae were removed, and the paper stored at -18°C. We tested the behavioral response to this secretion in the wind tunnel, both alone and when added to an attractive carcass (Table 2). To confirm attraction, the carcasses used were tested as an odor source in the wind tunnel with male and female flies directly before adding larval secretion. Secretion was added to the carcass by placing the filter paper next to it in the glass jar.

Table 2: The setup for the additional experiments investigating how larval secretion affects wind tunnel attraction in male and female blowflies. Numbers are total number of flies tested per treatment. An attractive mouse carcass was used as an odor source. Larval secretion was presented as a filter paper with secretion from ten blowfly larvae, and was added to the carcass by placing the paper next to it. Treatments were replicated five times for each gender, using ten flies for each replicate.

Treatment	Larval secretion	Attractive carcass	Carcass + secretion
Females	50	50	50
Males	50	50	50

Each treatment was repeated five times testing ten individual flies each time. Two or three treatments, in random order, were conducted each day. The treatments were conducted with males and females on the same day, using the same odor source, and the order of gender was randomized by drawing. The overall order of treatments was also initially randomized by drawing. After all treatments had been replicated once, a new order was assigned by moving

the last treatment in the previous replicate to the start of the next. This process was repeated at the end of each series of replicates. In total, 1800 flies were used in the experiments: 1500 flies (750 males and 750 females) to investigate the effect of carcass age, and 300 (150 males and 150 females) for the additional experiments with larval secretion.

Statistical analysis

Statistical tests were done with Minitab (Version 15). We used t-tests for comparing two groups, and ANOVA for multiple comparisons. If data were not normally distributed, and we were unable to normalize them by transformation, we used Mann-Whitney Rank Sum tests to compare two groups and Kruskal-Wallis ANOVA on Ranks for multiple comparisons. Paired t-tests were used to compare attraction before and after added larval secretion. To investigate the effect of several factors on blowfly attraction, as well as any interactions between them, we ran a multiple nominal logistic model using JMP (Version 9). Single factors were first tested as predictor variables. To simplify the model, factors with a p-value ≥ 0.5 were removed. A new model with interactions was then run for the remaining factors. Figures were made using SigmaPlot (Version 12).

Results

Potential confounding factors

In order to assess the effect of carcass age on blowfly attraction, we wanted to keep potentially confounding factors constant across treatments. Age of flies have been shown to affect attraction in wind tunnel bioassays (Crnjar et al. 1990, Aak and Knudsen, manuscript). The mean age of all 1500 flies used in the main experiments was 17.4 (± 0.1) days. There was no significant difference in age between the seven decay treatments, neither for females (Kruskal-Wallis ANOVA on Ranks: H = 4.23, p = 0.753) nor for males (Kruskal-Wallis ANOVA on Ranks: H = 2.61, p = 0.918). The mean age was 17.3 (± 0.1) days for female flies, and 17.4 (± 0.1) days for males. When these means were compared by pooling data for all treatments, we found no significant differences in age between genders (Mann-Whitney Rank Sum: W = 5532.0, p = 0.6251) (Fig. 5).

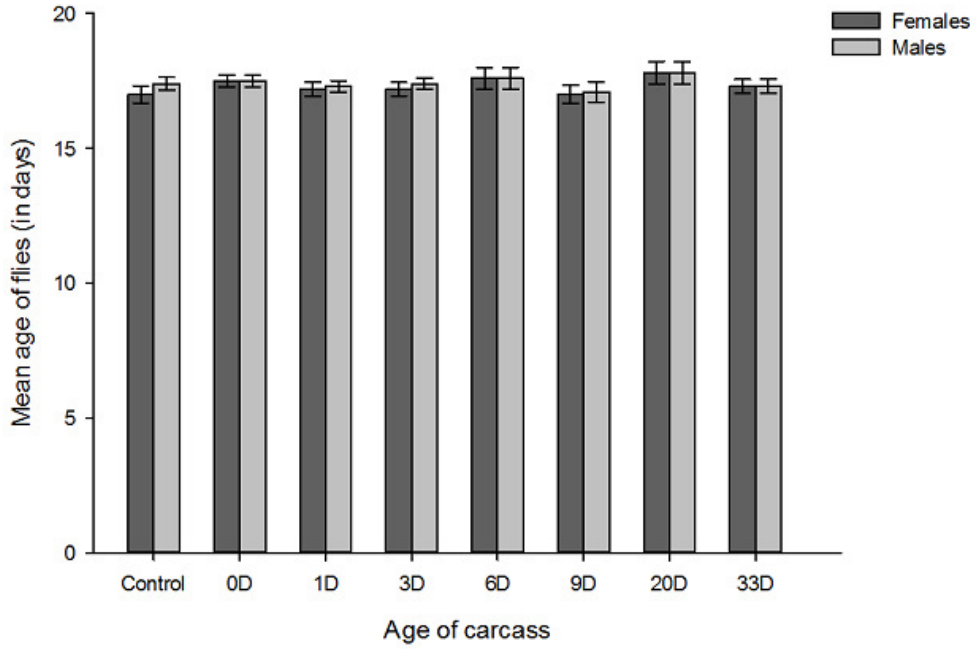


Figure 5: Mean age (± SE) of all blowflies used in the wind tunnel experiments for different ages (D = days) of mouse carcass.

The developmental stage of eggs influence female flight behavior (Campan 1977, Aak and Knudsen, manuscript). Our dissections of the ovaries showed that most flies had eggs in late stages of development (stage I = 1.6 %, II = 6.8 %, III = 23.3 %, IV = 68.3 %). Mean egg development was 3.6 (± 0.0) and there was no significant differences in egg development between decay treatments (Kruskal-Wallis ANOVA on Ranks: H = 7.79, p = 0.351) (Fig. 6).

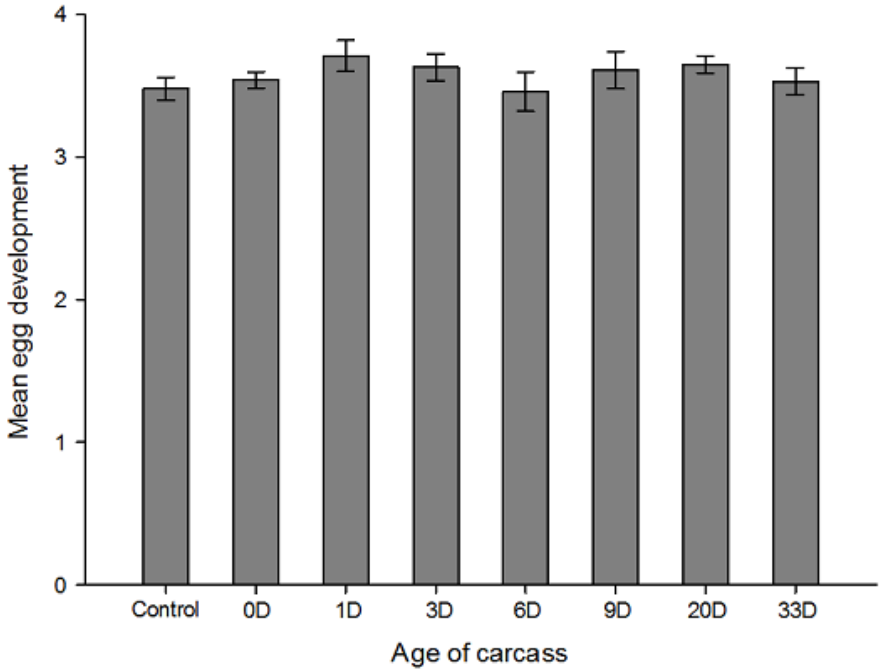


Figure 6: Mean egg development (± SE) for female *Calliphora vicina* for different ages (D = days) of mouse carcass. Egg development was scored using a continuous scale from 1-4, where 1 = barely developed eggs and 4 = fully developed eggs.

The proportion of flies initiating flight ranged from 84 % to 100 % when comparing mean take-off frequency for all decay treatments. In our experiments there was no significant difference in take-off between decay treatments, neither for females (Kruskal-Wallis ANOVA on Ranks: H = 10.46, p = 0.164) nor for males (Kruskal-Wallis ANOVA on Ranks: H = 4.49, p = 0.722). Mean take-off rate across all treatments was 93.7 (± 1.1) % for females and 96.0 (± 0.7) % for males. When these means were compared, there was no significant difference in take-off between genders (Mann-Whitney Rank Sum: W = 5439.0, p = 0.4019) (Fig. 7).

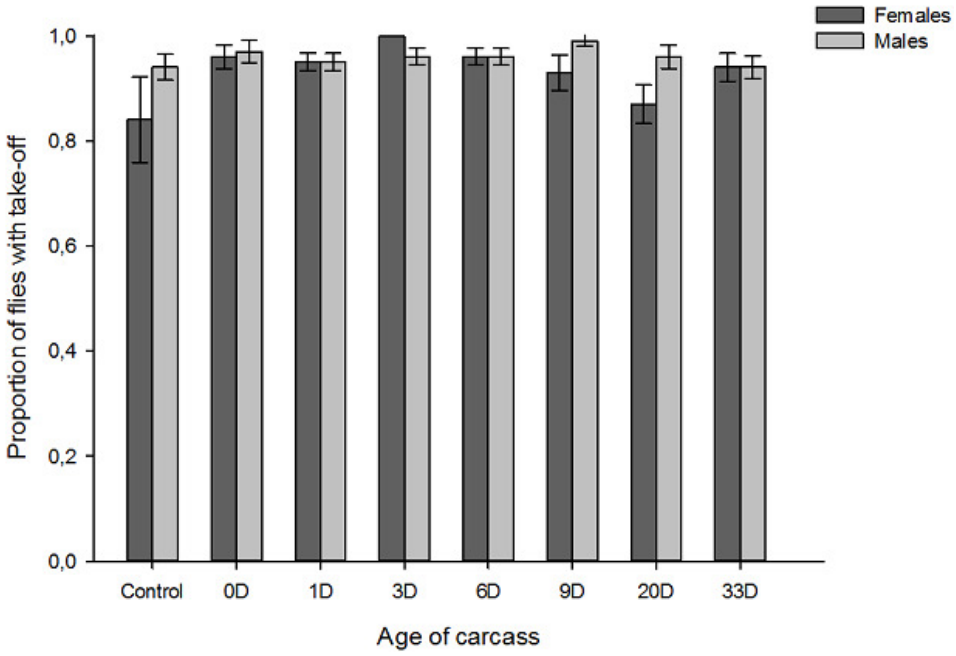
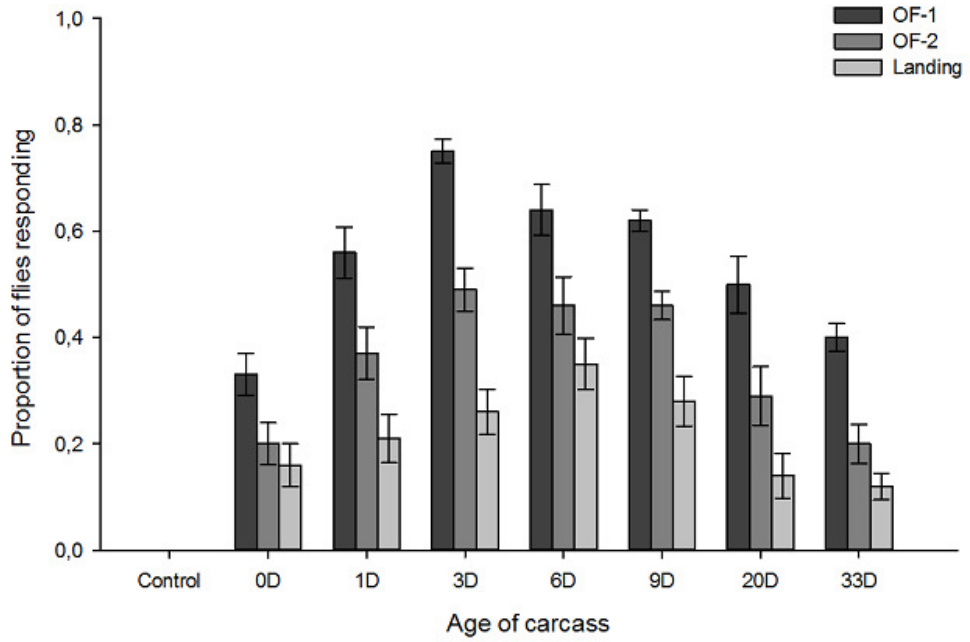


Figure 7: Proportion (± SE) of blowflies with take-off (initiated flight) in the wind tunnel for different ages (D = days) of mouse carcass.

Effect of decomposition on attraction

For all three behavioral categories (OF-1, OF-2, landing), we observed variations in blowfly attraction to the different decay treatments. This was evident both for females (Fig. 8A) and males (Fig. 8B).

A) Females



B) Males

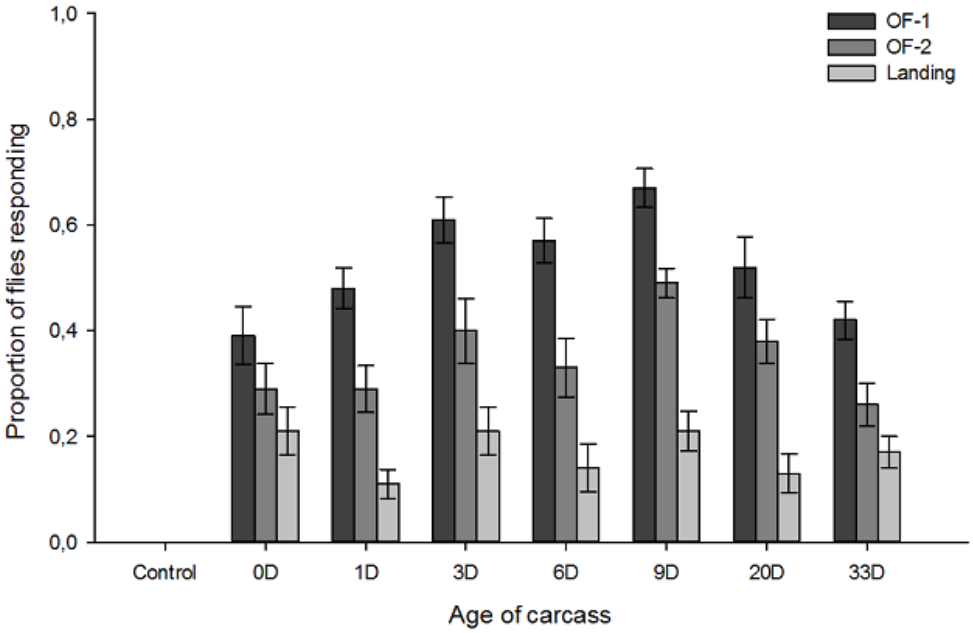


Figure 8: Wind tunnel attraction (± SE) for female (A) and male (B) *Calliphora vicina* to different ages (D = days) of mouse carcass. Attraction was scored in three categories: OF-1 (oriented flight in the back half (< 90 cm) of the wind tunnel), OF-2 (oriented flight in the upper half (> 90 cm) of the tunnel), and landing (orientation followed by landing in a predefined area).

To investigate differences between treatments (age of carcass), a multiple nominal logistic model was used to assess the effect of decay and other single factors (gender of flies, added soil bacteria) on blowfly attraction. The results of the model are presented in Table 3.

There was a significant effect of age of carcass in all three behavioral categories (multivariate logistic model: Chi = 70.7, df = 6, $p < 0.0001$ (OF-1); Chi = 50.8, df = 6, $p < 0.0001$ (OF-2); Chi = 18.2, df = 6, p = 0.0058 (landing)). There was also a significant effect of gender for landing (multivariate logistic model: Chi = 5.4, df = 1, p = 0.0203).

Table 3: Results from a multivariate logistic model with blowfly attraction in the wind tunnel as the response variable and age of a mouse carcass, gender of flies, and added soil bacteria as explanatory variables. Attraction was measured as three behavioral categories: OF-1, oriented flight < 90 cm; OF-2, oriented flight > 90 cm; landing in predefined area.

	Factor	**d.f.**	**Chi**	**P**
OF-1	Age of carcass	6	70.7	< 0.0001*
	Gender	1	0.7	0.4097
	Bacteria	1	0.4	0.5454
OF-2	Age of carcass	6	50.8	< 0.0001*
	Gender	1	0.1	0.7
	Bacteria	1	0.1	0.7
Landing	Age of carcass	6	18.2	0.0058*
	Gender	1	5.4	0.0203
	Bacteria	1	0.5	0.4945

* denotes significance at a 0.05 significance level

Bacteria had no significant effect on the behavior of the flies in any of the three categories (multivariate logistic model: Chi: 0.4, df = 1, p = 0.5454 (OF-1); Chi = 0.1, df = 1, p = 0.7318 (OF-2); Chi = 0.5, df = 1, p = 0.4945 (landing)). Applying our $p \geq 0.5$ criteria, bacteria was therefore excluded as an explanatory variable. The new model was then run with interactions for the two remaining explanatory factors (behavioral response = age of carcass + gender + age of carcass*gender), and the results are presented in Table 4.

Table 4: Results from a multivariate logistic model with blowfly attraction in the wind tunnel as the response variable and age of a mouse carcass, gender of flies, and the interaction of age of carcass and gender as explanatory variables. Attraction was measured as three behavioral categories: OF-1, oriented flight < 90 cm; OF-2, oriented flight > 90 cm; landing in predefined area.

	Factor	d.f.	Chi	P
OF-1	Age of carcass	6	71.4	< 0.0001*
	Gender	1	0.5	0.4599
	Age*gender	6	7.4	0.2840
OF-2	Age of carcass	6	51.0	< 0.0001*
	Gender	1	2.2	0.1381
	Age*gender	6	11.0	0.0886
Landing	Age of carcass	6	16.5	0.0114*
	Gender	1	0.8	0.3619
	Age*gender	6	14.5	0.0243*

* denotes significance at a 0.05 significance level

The age of a carcass had a significant effect on flight behavior: all three measured behavioral responses were significantly influenced by decay (multivariate logistic model: Chi = 71.4, df = 6, $p < 0.0001$ (OF-1); Chi = 51.0, df = 6, $p < 0.0001$ (OF-2); Chi = 16.5, df = 6, p = 0.0114 (landing)). The multivariate model also identified significant differences between the different stages of decay (Fig. 8; Appendix 2): For OF-1, attraction increased significantly from fresh carcasses (0 days of decay) to one day old carcasses, while for OF-2 and landing, there were no significant difference between these treatments. For all three behavioral measurements, attraction increased significantly from one day old carcasses to three day old carcasses. After this the attraction remained the same for carcasses that were six and nine days old, but decreased significantly for 20 day old carcasses. A further decrease was found for 33 day old carcasses for OF-1 and OF-2, but not for landing.

Sex differences in attraction

The model also showed that there was no effect of gender during oriented flight (multivariate logistic model: Chi = 0.5, df = 1, p = 0.4599 (OF-1); Chi = 2.2, df = 1, p = 0.1381 (OF-2)) (Table 4). However, a significant interaction between gender and decay treatment was observed for landing (multivariate logistic model: Chi = 14.5, df = 6, p = 0.0243), indicating

that carcass age influence landing behavior differently in male and female flies. This interaction can be interpreted through the profile of landing response across the different stages of decay (Fig. 9). Males show a flatter, more fluctuating profile, whereas females have a distinct peak performance at six day old carcasses.

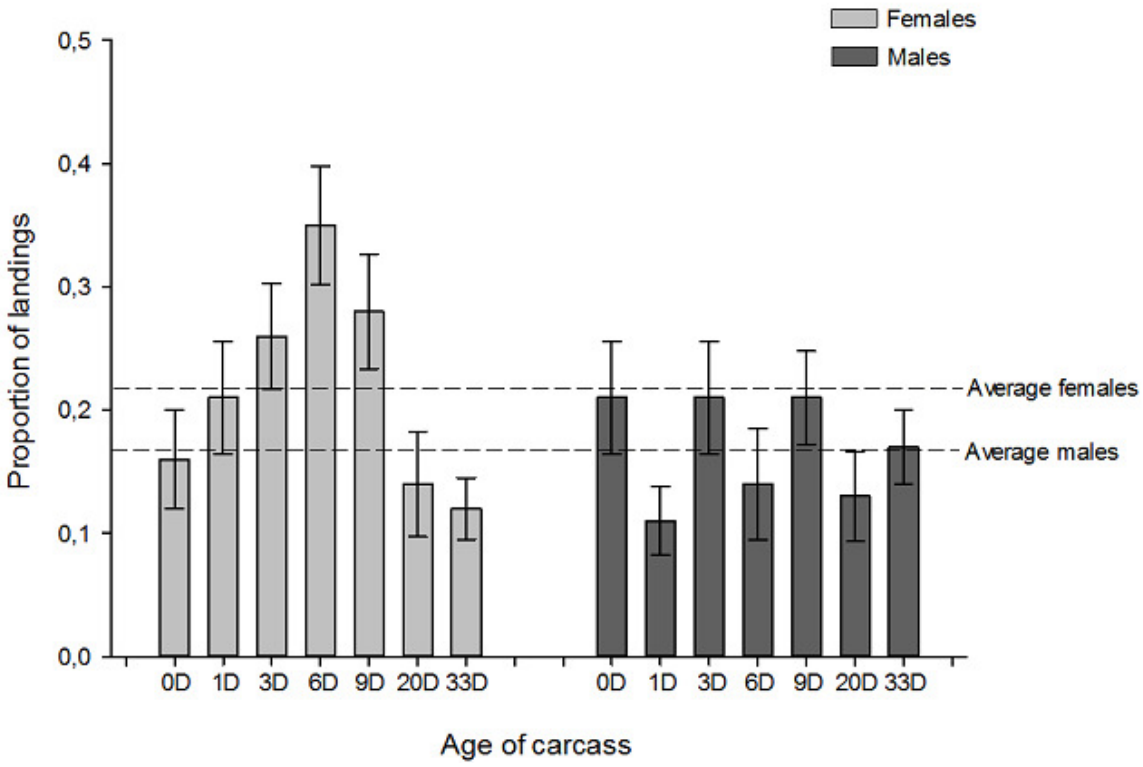
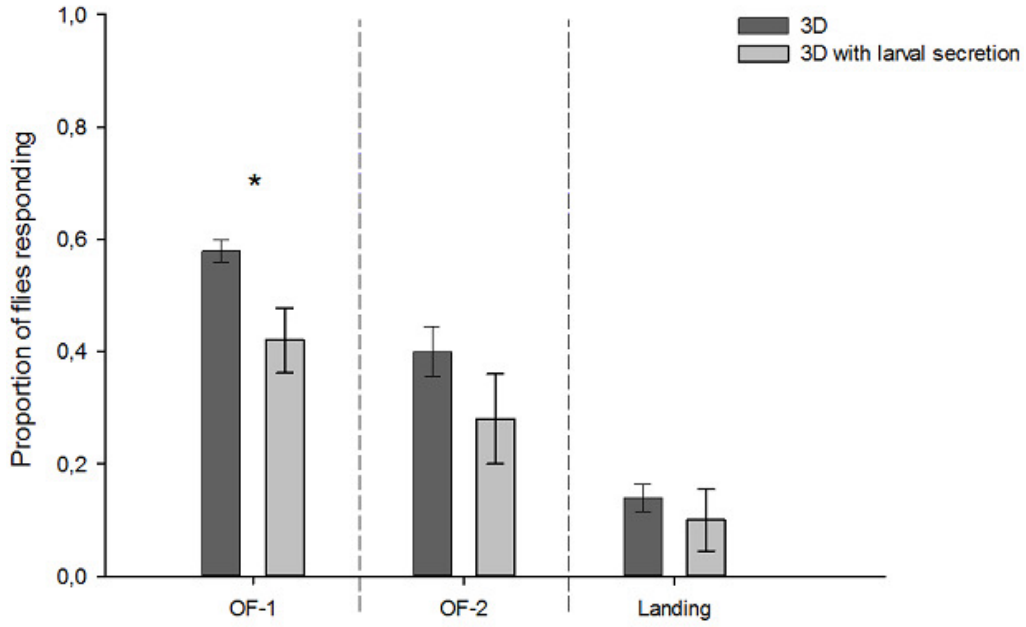


Figure 9: Proportion (± SE) of landings in a predefined landing area for male and female *Calliphora vicina* for different ages (D = days) of mouse carcass.

Effect of larval secretion

There was no attraction towards larval secretion when used alone as an odor source. Since they had been shown to be attractive, three day old carcasses were used to test the effect of adding larval secretion. When adding secretion to a carcass, we found a significant reduction in attraction for males in oriented flight in the back half of the wind tunnel (paired t-test: $t = 3.14$, $p = 0.035$ (OF-1)), but no difference in attraction for the two other behavioral categories (paired t-tests: $t = 1.39$, $p = 0.235$ (OF-2); $t = 1.00$, $p = 0.374$ (landing)) (Fig. 10A). For females, there were no significant differences in attraction for either of the behavioral categories (paired t-tests: $t = 2.24$, $p = 0.089$ (OF-1); $t = 0.00$, $p = 1.000$ (OF-2); $t = -0.78$, $p = 0.477$ (landing)) (Fig. 10B).

A) Males



B) Females

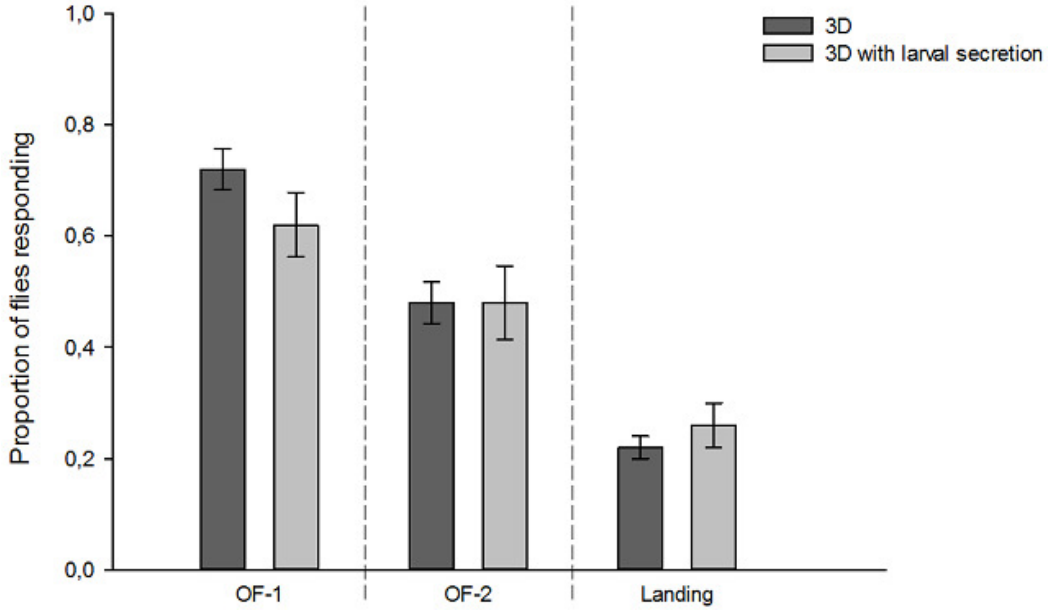


Figure 10: Proportion (± SE) of flies showing a behavioral response to a three day old carcass, before and after adding larval secretion. Wind tunnel experiment performed on male (A) and female (B) *Calliphora vicina*. Attraction was scored in three categories: OF-1 (oriented flight in the first half (< 90 cm) of the wind tunnel), OF-2 (oriented flight in the second half (> 90 cm) of the tunnel, and landing (orientation followed by landing in a predefined area). In a pairwise comparison within the same behavioral category, * denotes significant difference.

Discussion

This wind tunnel study demonstrated that both male and female *C. vicina* showed variation in their response to volatiles released from mice carcasses of different age, with significantly higher attraction towards carcasses that were three, six, and nine days old (Fig. 8). Interestingly, we found no differences between males and females in their oriented flight response, but we observed an increase in landings for female flies at the most attractive carcasses (Fig. 9). Our experiments excluded confounding elements such as age of flies, egg development, and variation in take-off rates, allowing us to conclude that the observed differences are a result of the odors presented to the flies. Addition of larval secretion to an attractive carcass had little apparent impact on attraction (Fig. 10), but as males on one occation responded negatively to relatively small amounts of secretion, we can not rule this out as a factor regulating behavior.

Observations from the field have identified *C. vicina* and other Calliphoridae as early colonizers of fresh carrion (Archer 2002, Stensmyr 2004, Gullan and Cranston 2010), and the results from our experiments, conducted in a controlled laboratory setting, confirm these observations. Laboratory trials allow more detailed observations of behavior than field studies, and may give valuable information about the mechanisms behind observed biological phenomena. Our study also demonstrates that *C. vicina* is capable of assessing a carcass from a distance, without visual or gustatory information provided by the actual resource. This indicates that *C. vicina* is strongly dependent on released volatiles when locating a carcass.

Effect of decomposition on attraction

Decomposition of animal tissue is a complex process consisting of many consecutive stages, and the volatile profile of a carcass changes throughout decay (Vass 2001, Gullan and Cranston 2010). This succession of olfactory cues can provide blowflies with a way of locating suitable carrion (Morris et al. 1998). In initial stages of decay, there is a lot of flesh present on the carcass (Archer and Elgar 2003a). Protein breakdown mostly occurs during these early stages, leading to the formation of sulphurous compounds (Brown 1982, Archer 2002, Statheropoulos et al. 2007). Oligosulphids have been shown to be highly attractive to carrion flies (Brown 1982, Ashworth and Wall 1994). Both male and female blowflies attend fresh carrion to obtain food, and released oligosulphids from carcasses in early stages of decay can be a signal that flesh is still abundant.

During later stages of decomposition, less flesh is present on the carcass, and bacterial activity might be reduced due to desiccation. The result can be a reduction in the amount of oligosulphids released, and this might explain decreased attraction towards older carcasses. However, an antagonistic effect of volatiles produced later in the decomposition process can also explain the decreased attraction. Degradation of fat during late stages of decay leads to the formation of volatile fatty acids (Vass et al. 1992, Gill-King 1997), and these compounds might be a signal to the blowflies of a food resource of poorer quality.

Sex differences in attraction

We predicted that females would be more responsive to the volatiles from early stages of decay than males, since female flies are dependent on carcasses for oviposition. Suitable oviposition sites are scarce, leading to strong intra- and interspecific competition for fresh carrion (Fuller 1934, Ullyett 1950). The ability to quickly locate a suitable carcass is therefore of vital importance for female blowflies (Spradberry 1979, Hayes et al. 1999), and it is likely that they have become more selected for locating carcasses in early stages of decay than males, who primarily attend carrion to locate potential mates (Shorey et al. 1969).

The equal orientation response for males and females found in this study seems to contradict previous results for saprophagous calliphorids, suggesting that female flies are more attracted by carcass odors than males (Stoffolano et al. 1990, Archer and Elgar 2003b). Most of these studies have been conducted in the field, where the capture rate of male and female flies have been used to assess attraction. In a wind tunnel the mechanisms behind this observed behavior can be investigated. The interaction of gender and age of carcass found in this study indicates that higher catches of female flies in the field might be a result of an increase in landings for females at attractive odor sources.

For many diptera, vision is important for foraging and oviposition (Prokopy et al. 1983a, Prokopy et al. 1983b, Brevault and Quilici 1999, Pinero et al. 2006), and Aak and Knudsen (2011) demonstrated the importance of visual feedback for *C. vicina* when locating an odor source. They found that female blowflies showed the highest response rate to a combined stimuli of an attractive odor source and visual cues. Our results, with a higher proportion of landings for female flies on the most attractive carcasses, point to the same conclusion. When a fly approaches an object, the image projected across its retina expands. This visual cue is

used to avoid collisions, either by turning away or by landing on the object (Tammero and Dickinson 2002, Srinivasan and Zhang 2004). Attractive odor sources seem to allow female flies to suppress avoidance behavior and initiate landing. As an increase in landing was not found in males, there seems to be a stronger connection between olfaction and vision in female blowflies. This might be a result of different selection pressures: males could be less dependent on landing on the carcass since they are able to locate and approach females in the air (Boeddeker et al. 2003). Specific neurons responding to rapidly moving targets have been found in male flies (Hausen and Strausfeld 1980, Gilbert and Strausfeld 1991), but are small or absent in females (Srinivasan and Zhang 2004). Another indicator of different use of vision is differences in the eye structure of male and female flies (Strausfeld 1976).

Effect of larval secretion

Compounds produced by larvae have been shown to have an antagonistic effect on conspecific adults for several species, for instance in leaf beetles (Blum 1994, Schindek and Hilker 1996) and in the Egyptian cotton leaf worm *Spodoptera littoralis* (Anderson et al. 1993). Mature larvae of *C. vicina* produce a larval secretion when they are done feeding on a carcass, prior to pupation. This could be a signal to conspecific females that the carcass in question is too old, or that the precense of other larvae will lead to strong competition. We found no apparent effect on attraction when adding secretion to a carcass. However, males did respond negatively on one occasion, even though only a small amount of secretion was added. We can therefore not rule out larval secretion as a potential factor affecting behavior.

For each replicate we used collected secretion from ten larvae, and this might not have been enough to deter females – especially since the carcass used was still fresh and undamaged. If the larvae had developed on the carcass, the production of larval secretion would have been accompanied by a reduction in available flesh. During experiments, flies did not have the opportunity to land on the actual carcass. Most oviposition-deterring pheromones are contact pheromones (Papaj 1993), and the effects of such compounds would not have been possible to assess with the present experimental setup.

Practical applications

The existing lure for *C. vicina* was developed by testing synthetic compounds known to attract other blowfly species (Aak et al. 2010b). By using authentic carcasses in our experiments, we found that six day old carcasses were the most attractive. This indicates that analyses of released compounds from these carcasses might lead to the identification of new components that can be added to the synthetic lure. Reduced attraction towards later stages of decay might also indicate that antagonistic compounds can be identified. Such compounds might be very useful in push-pull strategies, where they are combined with traps baited with the attractive lure. A lure with closer resemblance to odors released from an attractive carcass can improve the success of mass trapping at stockfish production sites in Lofoten. Blowfly infestation of drying fish is also a major concern in many countries in Asia, Africa, and the Pacific (Wall et al. 2001). Calliphorids are among the most common blowflies infesting fish in African countries (Walker and Donegan 1984, Walker and Wood 1985), and it is possible that attractive compounds identified for *C. vicina* could be used for population control of other saprophagous species in the same family.

The most important blowfly problem worldwide is myiasis – a form of parasitism where larvae develop in living animals (Hall and Wall 1995). Blowflies are divided into three groups based on their larval feeding habits: saprophagous species developing in decaying matter, facultative parasites living either as saprophages or initiating myiasis, and obligate parasites that only feed on the tissues of living animals (Stevens 2003). *C. vicina* normally develops in decaying matter, and only accidentally becomes a myiasis agent (Rognes 1991). However, field studies using the synthetic lure for *C. vicina* also resulted in high female catches of *Lucilia* spp. (Aak and Knudsen 2011), a genus that includes many facultative parasites (Stevens 2003). Several species of this genus are economically important myiasis agents in sheep (Wall and Smith 1997, Wall et al. 1992, Urech et al. 2009), and new attractive components identified for *C. vicina* might also improve existing lures for these species. Push-pull strategies using antagonistic compounds could be especially useful in this situation.

The presence of saprophagous flies is often used in forensic investigations (Greenberg 1991). Since *C. vicina* is an early colonizer of human corpses (Byrd and Castner 2009), detailed knowledge about the behavior of this species could be useful, for instance by improving the accuracy of time of death-estimates.

Possible limitations

A wind tunnel bioassay is an artificial setting, and results from laboratory experiments can not always be extended to the field. However, several results from laboratory work on *C. vicina* have been sustained in field trials (Aak et al. 2010b, Aak and Knudsen 2011), making the wind tunnel a valid tool for studying behavior in this species. A confounding factor in our experiments could have been that behavior was scored by two different observers. However, we believe that clearly defined behavioral categories prevented any effect of subjectivity.

Several factors might have had an impact on the decomposition of the carcasses used in these experiments. Due to the presence of decomposition agents, decay is likely to start earlier and proceed faster in nature, compared to what we observed in the laboratory. There might also have been an effect of using mice carcasses that had been frozen. It is possible that a slower artificial decomposition process might explain why we had relatively low attraction to one day old carcasses, while in the field these have been shown to be full of developing larvae (Archer and Elgar 2003b). However, since relative measurements of attraction are used to assess the effect of carcass age, it is likely that the relationship between different stages of decay will be the same in natural settings.

Future prospects

This behavioral study demonstrates variations in response to volatiles from carcasses of different age, but does not identify the actual compounds affecting blowfly behavior.
The evident course of action for future studies would therefore be to analyze the headspace volatiles sampled from each carcass. To do this, gas chromatography can be applied to separate the individual components of the collected volatiles by eludation. The gas chromatograph can be coupled with mass spectrocopy (GC-MS) to identify the components based on their mass. To identify the components affecting insect behavior, the compounds can simultaneously be passed over an insect antennae (GC-EAD).

The combination of these techniques would enable us to identify the components of the collected headspace that actually invoke a response in the flies. However, it tells us nothing about what kind of behavior these compounds elicit. This would have to be assessed separately, for instance by investigating how they affect behavioral responses in the wind tunnel.

To follow up our initial experiments on larval secretion, we would like to conduct more wind tunnel experiments. It would be interesting to investigate if adding a higher dose of secretion has an effect on attraction, or to add secretion to carcasses in later stages of decay.

Lastly, it would be interesting to investigate if the equal attraction found for male and female *C. vicina* in the laboratory can be observed in the field. If attraction is the same, we would expect to find an equal proportion of male and female flies around an odor source. But different responses in landing could lead to more females being present on the actual source, with a higher proportion of males found downwind of or around the odor source.

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Appendix 1: Methodology for collecting headspace

The odor source was placed in a 2L glass jar, closed with a grounded glass fitting. For collection of volatiles, we used traps made of 4x40 mm glass tubes containing 35 mg Super Q (80/100 mesh; Alltech, Deerfield, USA) held between glass wool plugs. A pure air stream produced by a zero air generator (model HPZA-3500-220; Parker-Balston, Haverhill, USA) was passed through a variable area meter (SHO-RATE series, model 1355; Brooks Instruments, Ede, the Netherlands) to create an air flow of 220 mL/min. A reduction valve ensured an air pressure of 1 bar. The air stream then passed through a charcoal filter and was pushed over the carcass from the top to the bottom of the jar and over the trap. The charcoal filter (incoming air) and the trap (outgoing air) were connected to the jar with grounded glass fittings. During sampling, the trap was wrapped in aluminum foil to protect it from light. Collections were done for 3 hours, at 20-22°C.

After sample collection, all further extraction procedures were conducted in a vented hood. Traps were rinsed with 0.3 mL of hexane to extract volatiles. The extracts were collected in 2 mL vials with a 100 µL glass insert. 500 ng 7-OAC heptyl-acetate (99.4 % pure; Chiron AS) and 500 ng 11-OAC undecyl-acetate (99 % pure; gift from Marie Bengtsson) were added as internal standard. The vial was then sealed with a crimp cap and stored at -80°C.

After use, traps were rinsed sequentially with 6 mL of laboratory grade n-hexane (> 99 % pure; Sigma-Aldrich), methanol (> 99 % pure; Sigma-Aldrich), and n-hexane again. All glassware was heated to 300°C for 8 hours between headspace collections. In addition to the headspace collections, we also conducted system controls by sampling volatiles from an empty jar (using the described procedures).

Appendix 2: Results from the multiple nominal logistic model

Variable	P-value	Category	% responding (n)	Odds ratio (95% CI)
		Oriented flight 1		
Treatment	< 0.0001	0D	36 % (200)	-
		1D	52 % (200)	0.51 (0.34 – 0.76)
		3D	68 % (200)	0.50 (0.33 – 0.75)
		6D	60.5 % (200)	1.41 (0.93 – 2.14)
		9D	64.5 % (200)	0.84 (0.56 – 1.27)
		20D	51 % (200)	1.75 (1.17 – 2.62)
		33D	41 % (200)	1.50 (1.01 – 2.23)
Gender	0.4599	Male	52.3 % (700)	-
		Female	54.3 % (700)	1.24 (0.70 – 2.23)
*Treatment*gender*	0.2840			
		Oriented flight 2		
Treatment	< 0.0001	0D	24.5 % (200)	-
		1D	33 % (200)	0.65 (0.42 – 1.01)
		3D	44.5 % (200)	0.61 (0.41 – 0.92)
		6D	39.5 % (200)	1.24 (0.83 – 1.85)
		9D	47.5 % (200)	0.72 (0.48 – 1.07)
		20D	33.5 % (200)	1.81 (1.21 – 2.72)
		33D	23 % (200)	1.83 (1.17 – 2.88)
Gender	0.1381	Male	34.9 % (700)	-
		Female	35.3 % (700)	1.63 (0.85 – 3.17)
*Treatment*gender*	0.0886			

Variable	P-value	Category	% responding (n)	Odds ratio (95% CI)
		Landing		
Treatment	0.0114	0D	18.5 % (200)	-
		1D	16 % (200)	1.24 (0.73 – 2.13)
		3D	23.5 % (200)	0.59 (0.35 – 0.99)
		6D	24.5 % (200)	1.03 (0.64 – 1.67)
		9D	24.5 % (200)	0.92 (0.57 – 1.48)
		20D	13.5 % (200)	2.06 (1.23 – 3.50)
		33D	14.5 % (200)	0.93 (0.53 – 1.65)
Gender	0.3619	Male	16.9 % (700)	-
		Female	21.7 % (700)	1.40 (0.68 – 2.90)
*Treatment*gender*	0.0243			

God in His wisdom

Made the fly

And then forgot

To tell us why

Ogden Nash