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Behaviour of two fly species reared for livestock feed: optimising production and insect welfare

Y. Kortsmit[1#] iD, **M. van der Bruggen**[2#] iD, **B. Wertheim**[2] iD, **M. Dicke**[1] iD, **L.W. Beukeboom**[2] iD and **J.J.A. van Loon**[1*] iD

[1]Laboratory of Entomology, Wageningen University and Research, P.O. Box 16, 6700 AA Wageningen, the Netherlands; [2]Groningen Institute for Evolutionary Life Sciences, University of Groningen, P.O. Box 11103, 9700 CC, Groningen, the Netherlands; joop.vanloon@wur.nl; [#] these authors contributed equally to this work

REVIEW ARTICLE

Abstract

The mass rearing of insects as animal feed is a new and rapidly growing component of circular agriculture, which offers the opportunity to develop it in such a way that it promotes insect health and welfare. Behaviour is an important indicator of animal performance and welfare. In this review, we synthesise the current behavioural knowledge on two saprophytic dipteran species that are increasingly being used as mini-livestock, the black soldier fly (*Hermetia illucens*) and the housefly (*Musca domestica*). We evaluate which behaviours need to be considered to optimise insect production and welfare under mass-rearing conditions. We distinguish between the different life stages (adults and larvae), and describe their feeding behaviour, social interactions (adult mating, larval aggregation), oviposition behaviour and possible cannibalism. For each species, we review what is known about these behaviours in natural environments, and how this is affected by abiotic factors or interactions with conspecifics and heterospecifics. We also address how the flies' microbiome and pathogens can influence various aspects of behaviour. Notable differences in natural behaviours between the two species, such as their courtship and mating behaviour and the larval distribution within feed substrates are identified. These behavioural differences have important implications for how we should rear the two fly species in industrial settings, as a mismatch in mass-rearing conditions may induce environmental stress or compromise insect productivity and welfare. Escape behaviour, larval aggregation behaviour, possibly cannibalism in the larval stage and mating frequency and reproduction rate are identified as behaviours providing information on welfare of larval and adult flies. Finally, a number of aspects are identified for which behavioural knowledge is currently still sparse, while this may be important to safeguard insect welfare. We conclude with recommendations for future research to promote insect welfare.

Keywords: black soldier fly, *Hermetia illucens*, house fly, *Musca domestica*, health, stress factors

1. Introduction

Insects and insect-based products are used for a variety of economic purposes. Insects are used to control pest species, to pollinate crops in greenhouses, but also to produce food, such as honey (reviewed by Dicke, 2017; Francuski and Beukeboom, 2020). In recent years there is an increasing interest in the use of insects as animal feed. The larvae of saprophytic flies, such as *Hermetia illucens* L. (black soldier fly; BSF) (Diptera: Stratiomyidae) and *Musca domestica* L. (house fly; HF) (Diptera: Muscidae) feed on organic residual

streams (Liu *et al.*, 2019; Wang *et al.*, 2016) and have great potential as a novel, durable component of animal feed and their industrial production is rapidly growing (Van Huis, 2020). At the same time, the commercial use of insects and large-scale insect rearing is raising concerns about insect welfare (De Goede *et al.*, 2013; Van Huis, 2021). Being relatively new, this industrial sector offers the opportunity to incorporate insect welfare considerations early-on into the design of large-scale insect rearing systems. This may avoid high costs for system adaptations needed to safeguard animal welfare at a later stage of industrial development.

Behavioural changes can be used as an indicator of the ability to experience pain and therefore, behaviour is an important indicator of animal welfare (Horvath *et al.*, 2013). To assess insect welfare, it is important to recognise stress-induced behaviours under commercial mass-rearing conditions, and to be able to monitor and evaluate such behaviours (Horvath *et al.*, 2013; Mayhew, 2018). As insects display a diversity of species-specific and complex behavioural responses to the surrounding environment (Hoy, 2019), it is important to understand the relevance of such behavioural responses as indicators of insect welfare. In other words, behavioural responses can indicate 'discomfort' and can cause long-term behavioural changes (Sneddon *et al.*, 2014), and as such, behavioural traits can be used to evaluate insect welfare (Horvath *et al.*, 2013). Insect welfare is a recent concept and underexplored compared to welfare of other animals (Boppré and Vane-Wright, 2019; Van Huis, 2021). Research on insect welfare is in its infancy and ethical aspects such as moral status, intrinsic value, sentience, suffering and euthanasia are under debate. In the absence of factual knowledge application of the precautionary principle to sentience of insects has been advocated (Knuttson and Munthe, 2017). Therefore, no generally accepted standards are available. In view of the great diversity in insect lifestyles it is likely that species-specific requirements for welfare need to be designed. For the purpose of this review we use as an operational definition of insect welfare 'the expression of the full behavioural repertoire of all life stages as observed under natural conditions'.

Within the natural environment there are various factors that induce behavioural responses (Fordyce, 2006). These include abiotic factors such as temperature, humidity and feed substrate distribution, and biotic factors such as density of conspecifics and predator abundance. Examples of important insect behaviours in response to environmental changes include feeding, aggregation and reproductive behaviour (Cividini and Montesanto, 2020; Fouche *et al.*, 2018; Hoy, 2019; Mayhew, 2018). Insects are ectotherms and ambient temperatures influence body temperature and behaviours such as feeding, growth, locomotion, mating and courtship behaviour as well as on immune function and sensory input (Angilletta *et al.*, 2002; Willmer, 1991). When exposed to unfavourable abiotic conditions, insects can display specific behavioural strategies (Sugiura, 2020) such as escape behaviour that may be rapid but complex (Card, 2012). For instance, insects may increase their body temperature as a response to the presence of pathogens (Hunt *et al.*, 2011). This can be achieved by moving to an area with a higher temperature (Wojda, 2017) or by locally increasing temperature through aggregating (Heaton *et al.*, 2014). A well-known example is defence behaviour of honeybees towards attacking hornets, by increasing local temperature through forming clusters resulting in temperatures up to 47 °C, lethal to the hornet (Ono *et al.*, 1995).

Animal responses to environmental conditions are under natural selection to maximise an individual's fitness (Allen and Nowak, 2016). Environmental changes can be perceived by insects via a diversity of sensory systems and receptors (Cividini and Montesanto, 2020). Receptors include those that detect chemical (Boullis *et al.*, 2016; Stökl and Steiger, 2017), visual (Ferveur and Cobb, 2010; Oike *et al.*, 2017), tactile, vibrational and/or auditory cues (Cividini and Montesanto, 2020; Fouche *et al.*, 2018). Responses to these cues can affect long- and short-distance intraspecific and interspecific interactions (Buehlmann *et al.*, 2020; Claridge, 1985; Ferveur and Cobb, 2010; Oike *et al.*, 2017; Renou and Anton, 2020). Insects not only respond to environmental changes, they also use infochemicals to gather or convey information, such as aggregation pheromones (Wertheim, 2005), mating pheromones (Wicker-Thomas, 2007) or alarm pheromones (Napper and Pickett, 2008).

What is considered a stressful environmental condition may differ between distinct life stages in holometabolous insects. Insects in orders such as the Hymenoptera, Coleoptera and Diptera undergo a complete metamorphosis during development (Truman, 2019; Figure 1). These distinct life stages have different requirements and often use different habitats and resources and display stage-specific behavioural responses (Kingsolver and Buckley, 2020). This is especially the case for larvae that feed on other diets than the adults. Larvae of many dipteran species feed on decaying organic substrates exposing them to a microbe-rich environment, whereas the adults are rarely involved in the decomposition process and presumably less exposed to parasites and pathogens (Skevington and Dang, 2002). Therefore, it is important to differentiate between life-stage specific behaviours and behavioural changes when entering a next stage in their life cycle. In addition, behaviours such as reproduction and dispersal by flight only occur in the adult stage.

In this review, the term 'rearing' refers to raising larvae to the (pre-)pupal stage, the growth and developmental processes relevant for production of biomass, whereas 'breeding' refers to reproduction by adult insects.

The aim of this article is to review behavioural knowledge on BSF and HF, two promising emerging dipteran species within the feed industry, in the context of animal welfare. We discuss the different life stages and evaluate which behaviours may be used to monitor performance and welfare of these flies under mass-rearing conditions. The interest of the use of these insects as components in animal feedstuff is a relatively new development. We would like to point out the differences in the levels of behavioural knowledge about BSF and HF. The behaviour of HF has been studied more extensively, mainly because it is a vector species that can disseminate human pathogens via its body surface, excretions, mouthparts and regurgitant (Förster
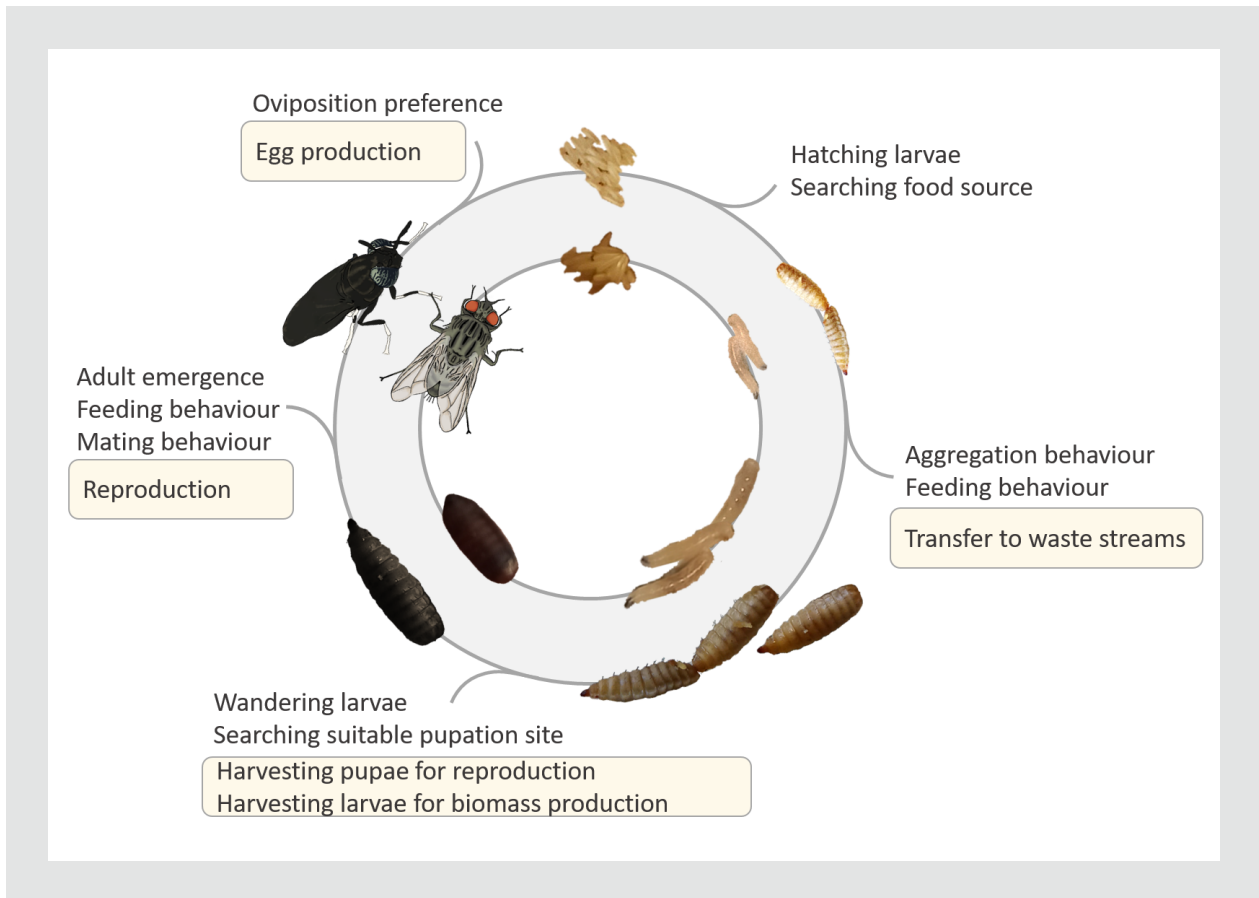
Figure 1. Lifecycle of black soldier fly and housefly with their major life stages and behaviours. Events or processes relevant for mass-rearing are indicated in boxes.

et al., 2007; Sarwar, 2015). Studies on BSF mostly focus on the use for feed (reviewed by Barragan-Fonseca *et al.*, 2017; Dörper *et al.*, 2020; Veldkamp and Vernooij, 2021), with some studies in the forensic area of expertise (Barros *et al.*, 2019; Pujol-Luz *et al.*, 2008), resulting in limited knowledge about its behaviour. If knowledge about particular aspects of BSF and/or HF is lacking in the literature, closely related species are discussed in this review. Behavioural knowledge may aid in developing welfare protocols and quality control of these flies in industrial settings.

2. Adult behaviour

Locating feed substrates and feeding behaviour

Knowledge about adult feeding behaviour benefits rearing systems. Such information can help identify risks and opportunities for welfare of the flies and improve yield of the mass-rearing.

Locating the food source is the first step of feeding behaviour. Adult HF can show active searching behaviour when offered a drop of sucrose (White *et al.*, 1984), where searching behaviour was defined by a decrease in locomotor activity and an increase in absolute turning rates. Interestingly, HF adjusts its feeding behaviour in response to unfavourable food substrates. Insecticide-contaminated food sources resulted in changes in feeding behaviour over generations due to the contact-dependent avoidance of contaminated sucrose bait traps (Hubbard and Gerry, 2021). During feeding, HF adults perform a scraping movement with the prestomal teeth, enabling the tearing and sucking up of cells (Kovacs Sz *et al.*, 1990). HF typically feed on decaying plant and animal material (Yamamoto and Jensen, 1967) and are able to discriminate between certain compounds. HF showed the strongest proboscis extension response to yeast hydrolysate in choice assays (Robbins *et al.*, 1965). The morphology of the prestomal teeth in HF males and females is similar, indicating that both sexes consume similar food sources (Sukontason *et al.*, 2003). Mostly female HF showed a strong clustering response to the presence of yeast hydrolysate (Robbins *et al.*, 1965).

Even though adult BSF are commonly believed not to feed, they do possess teeth-like structures within the mouthparts (Bruno *et al.*, 2019). Closely related stratiomyid adult flies feeding on agave can also be observed pressing mouthparts onto the plant material in a way that suggests feeding is

occurring (Alcock, 1990). Adult BSF have typical sponge-like mouthparts, suited to suck up liquid or liquified food (Bruno *et al.*, 2019; Oliveira *et al.*, 2016). Liquification of food might happen in a similar way as executed by HF and other fly species, by spitting out digested material on the food source (Grübel *et al.*, 1997). Bertinetti *et al.* (2019) suggest the natural food substrate of adult BSF to be pollen, nectar or honeydew. In addition, adult BSF possess a functioning digestive system (Bruno *et al.*, 2019). Even though feeding is not necessary for successful reproduction (Tomberlin and Sheppard, 2002), the capability of ingesting and digesting food can have advantages in mass-rearing because female BSF have a high reproductive output when being offered the chance to feed. Providing both water and sugar led to the highest egg production as well as an increase in oviposition period and adult lifespan compared to deprivation or only water (Macavei *et al.*, 2020). Egg counts were three times higher when females were offered a protein source and oviposition period could be increased by 10 days when offered milk powder (Bertinetti *et al.*, 2019). BSF females have larger maxillary palps than males (Pezzi *et al.*, 2021), but the functional significance of this sexual dimorphism is not known.

In conclusion, especially for BSF the benefit of feeding is known to have positive effects on egg yield and longevity of the females. However, for HF most information is provided from a pest control point of view. Knowledge of the positive effects of certain diets would be of interest for mass-rearing facilities to increase production and possibly also insect welfare.

Mating behaviour and mate choice

Insects can use acoustic, visual, tactile and chemical communication in courtship behaviour (Wicker-Thomas, 2007) and have evolved a variety of mating strategies (Shuker and Simmons, 2014; Thornhill and Alcock, 1983). Although behavioural studies of *Hermetia* species are rare, courtship of HF has been studied in detail (Meffert and Bryant, 1991; Wicker-Thomas, 2007), including the heritability of courtship traits and genetic architecture of mating behaviour (Aragaki and Meffert, 1998; Meffert and Hagenbuch, 2005). Even with limited information available for comparison, some notable differences in mating behaviour and male-male interactions associated with reproductive behaviour are evident between BSF and HF.

Many fly species demonstrate lekking behaviour, which means that aggregations of males are formed to engage in competitive displays and courtship rituals. This results in a form of male-male competition (Benelli, 2014). BSF males are known to exhibit lekking behaviour (Tomberlin and Sheppard, 2001) as has been described for other stratiomyid flies as well (Alcock, 1990). These lekking sites,

tend to be chosen based on landmark positions, rather than useful resources for females (Alcock, 1990). Within lekking sites, males generally wait for females to arrive and aggressively fight off rivalling males entering their lek site (Kotzé *et al.*, 2019). Male BSF have been seen to vertically spiral with the territorial intruder above the territorial site, where the winner returns to the lek site (Tomberlin and Sheppard, 2001). Larger males generally have a higher chance of winning this territorial fight and maintaining the favourable site within the lek for a longer period of time (Alcock, 1990, 1993). Male-to-male aggression rates were higher among larger males (Jones and Tomberlin, 2020). Additionally, male-male sexual interactions are not uncommon in BSF. During these same-sex courting attempts, males are grabbed in mid-flight, land on their back and interactions are only ended when courted males manage to escape (Giunti *et al.*, 2018). In contrast, male-male sexual interactions in HF only seem to happen when the males have been isolated from females for some time (Shin *et al.*, 2003). Courted males do not show defensive behaviour like females, but simply try to move away (Ragland and Sohal, 1973). Unlike females, courted males do not spread their wings and do not kick courting males away with the hind legs, a behaviour that would cause more wing damage to the courted female than to the courting male (Ragland and Sohal, 1973). There is some evidence that pheromones on the male's cuticle inhibit male-male sexual interactions in HF (Schlein and Galun, 1984).

Mate finding and female choice happen in very different ways in BSF and HF. Female stratiomyid flies, such as BSF, find mates by visiting lekking sites, where males attempt to grab any passing female to mate with (Barbosa, 2009; Tomberlin and Sheppard, 2002). Female flight activity is thought to be more important than female pheromones to induce male courtship behaviour (Giunti *et al.*, 2018). Females stop beating their wings when grabbed in mid-flight by males and spiral down while the male initiates the mating sequence (Tomberlin and Sheppard, 2001). After landing, the male starts wing fanning followed by moving back onto the female while tapping the female's abdomen with the tarsi, trying to achieve genital contact (Giunti *et al.*, 2018; Jones and Tomberlin, 2020). Failed mating attempts can be identified by not being followed by copulation (Jones and Tomberlin, 2020), because non-accepting females will try to dismount the male by moving away or moving their wings (Giunti *et al.*, 2018). Even when being grabbed in flight, females of lekking species may still be able to display female choice in an earlier stage of mating. Not only do lekking species engage in intrasexual competition, they can also show female choice based on male phenotype, territory, lek site and copying other females' mate choice (Balmford, 1991). In the closely related species *Hermetia comstocki* Williston (Diptera: Stratiomyidae), females seem to distinguish between male territorial sites, resulting in an increased mating success when a good lekking site is

selected by the male (Alcock, 1990). Whether BSF females have a similar method of recognising territorial sites remains to be studied.

Interestingly, Giunti *et al.* (2018) found a female preference in BSF for shorter male wing fanning durations, a behaviour not reported in the mating sequence of other stratiomyid flies (Barbosa, 2009; Eberhard, 1988). This may be hinting towards possible female choice in a later stage of the mating sequence. However, more research is needed to confirm female choice in BSF where male size may affect female choice behaviour. Even though *H. comstocki* females do not seem to discriminate male size within the lekking site (Alcock, 1993), there could still be female choice in a later stage of the courtship sequence including cryptic female choice. However, research is needed to confirm if female and cryptic female choice occurs in BSF.

In contrast to BSF, muscid flies, such as HF, do not display lekking behaviour: males opportunistically approach females through so-called strikes (Goulson *et al.*, 1999). Before initiating courtship, the male moves towards the female and taps with the forelegs on the female (Meffert and Hagenbuch, 2005). Male courtship consists of a series of behaviours that include: the male jumping on the female's dorsum, wing-fanning behaviour and jumping over the head of the female (Carrillo *et al.*, 2012; Colwell and Shorey, 1975; Meffert and Bryant, 1991). When the male is accepted by the courted female, she extends her wings and raises her metathoracic legs up to behind her wings (Colwell and Shorey, 1975), after which the male attempts to copulate (Meffert and Hagenbuch, 2005). Female choice in HF is much more obvious than in BSF, as females can avoid males by flying off, thus moving away (Murvosh *et al.*, 1964) or resisting the mating by kicking the male with the metathoracic legs whereby males often suffer wing damage (Carrillo *et al.*, 2012; Ragland and Sohal, 1973). There is some effect of HF size on mating success, where females prefer larger, heavier males and males prefer larger sized females (Goulson *et al.*, 1999; Shin *et al.*, 2003). However, laboratory populations may differ from field populations: male HF are known to show a more aggressive mating behaviour when reared under laboratory conditions (Ragland and Sohal, 1973).

There is little information in the literature about the mating frequency of BSF and HF females. HF females normally mate only once (Riemann *et al.*, 1967), but additional mating can take place until 10 days of age. Re-mating is not restricted to laboratory conditions: field collection of older females suggests that re-mating also occurs in natural populations (Butler *et al.*, 2009). However, systematic study of female re-mating frequency in natural populations has not been performed yet. Male re-mating could be size-related because additional matings in HF were mostly observed in smaller males in laboratory populations

(Baldwin and Bryant, 1981). Regarding BSF there is some debate about the mating frequency of females. Whereas Kotzé *et al.* (2019) suggest BSF to be polyandrous, Giunti *et al.* (2018) claim females to be monogamous due to the lack of female re-mating with virgin males. The spheroid shape of the three spermathecae found in female BSF reproductive tracts also indicate a correlation with monogamous species (Malawey *et al.*, 2019).

Generally speaking, fertilisation is not guaranteed when mating is completed. Sperm structure and function, sperm competition and cryptic female choice can affect fertilisation success post copulation (Kotzé *et al.*, 2019). Females may influence offspring parenthood by promoting or discouraging the movement of sperm in the reproductive tract via muscle contractions (Wedell *et al.*, 2002). However, female behaviour can also be influenced by seminal fluid proteins transferred during mating (Carrillo *et al.*, 2012). These proteins can influence female re-mating frequency, oviposition rate and activity, such as female flight and feeding behaviour (Avila *et al.*, 2011). Seminal fluids are known to contain many different types of accessory gland proteins, such as sex peptides (Ferveur and Cobb, 2010). In HF, seminal fluids have been shown to inhibit female re-mating (Butler *et al.*, 2009). The effect of seminal fluids may depend on male body size as well as on the number of previous matings of males. In HF, smaller males have a shorter copulation duration and female mating partners re-mate more frequently (Baldwin and Bryant, 1981). Unlike HF, female stratiomyid flies may have fewer options for female choice (Alcock, 1990; Barbosa, 2009), making it more likely that cryptic female choice plays a role in male mating success. In the soldier fly *Merosargus cingulatus* Schiner (Diptera: Stratiomyidae), the female oviposits directly after copulation when males perform post-copulatory courtship but postpones oviposition when she has copulated with males that do not perform this behaviour, resulting in lower reproductive success of the latter males (Barbosa, 2009). Even though not much is known about female choice and cryptic female choice, the morphology of the female reproductive tract may give more information. BSF females have three spermatheca capsules four times smaller than the flagellum. This suggests a more effective block of the tract preventing sperm storage from future matings (Malawey *et al.*, 2019). However, whether this is a method used by BSF males to ensure mating success still needs to be determined. Another method of preventing remating in the female is mate guarding. Mate guarding can occur as an increase in aggression towards other intruder males (Baxter *et al.*, 2015). However, whether such behaviour is present in BSF and HF is unknown.

Chemical communication via sex pheromones can play an important role in courtship and mating behaviour of dipteran species (Wicker-Thomas, 2007). A common method for a variety of flies is to disseminate pheromones

by wing fanning (Wicker-Thomas, 2007). Wing fanning occurs as part of mating in both HF and BSF and in BSF no copulation was observed without wing fanning (Giunti *et al.*, 2018). HF males are known to be attracted to females by olfactory and visual cues (Shin *et al.*, 2003). The involvement of sex-related volatile compounds in HF has been known for a long time (Rogoff *et al.*, 1964). The major sex pheromone has been isolated from the female cuticle and faeces and identified as (*Z*)-9-tricosene (Carlson *et al.*, 1971). This pheromone component induces striking behaviour in males at the beginning of the mating sequence (Butler *et al.*, 2009). The major component (*Z*)-9-tricosene together with the minor components (*Z*)-9, (*Z*)-14-tricosene-10-one, 10-epoxytricosane and a complex mixture of methyl alkanes make up the pheromone blend of HF females (Tang *et al.*, 2016). When in close contact with females, male HF are triggered in producing volatile components, resulting in more attraction from females (Schlein and Galun, 1984). In BSF, female volatiles seem not to be involved in inducing male courtship behaviour (Giunti *et al.*, 2018) and remain to be investigated in more detail. Interestingly, more than half of the BSF genes coding for olfactory receptors are thought to encode species-specific pheromone receptors (Zhan *et al.*, 2019), which does point towards a role of chemical communication in BSF mating. Pheromones could be used in a variety of different behaviours, ranging from sex pheromones used for mating (Wicker-Thomas, 2007), to the marking of an oviposition site (Jiang *et al.*, 2002). However, if BSF uses male sex pheromones influencing female choice, anti-aphrodisiacs or chemical signals for oviposition site marking is unknown.

Inbreeding may quickly result in genetic detorioration and extinction of fly populations. Houseflies (Meffert and Bryant 1991; Meffert *et al.*, 1990; Reed and Bryant 2001a,b), and to a lesser extent BSF (Rhode *et al.*, 2020), have been used to experimentally test inbreeding effects on individual fitness and behaviour. These studies reveal severe inbreeding depression effects, including reduction of female mating propensity and alteration of courtship behaviours. Effects depend on population size, frequency of genetic bottlenecks and environmental stress levels. Effects of inbreeding on larval behaviour have not been studied thus far.

Oviposition choice and behaviour

In colony-reared BSF, mating takes place during the first five days and oviposition between the third and sixth day after eclosion. Both behaviours are mostly restricted to one day each within these time frames, 69% of matings occur on day two, while 70% of oviposition takes place on day four after eclosion (Tomberlin and Sheppard, 2002). Oviposition by BSF is mediated by time of day, with more clutches being oviposited later in the day (Tomberlin and Sheppard, 2002). Booth and Sheppard (1984) found in a field experiment

in August/September in Georgia (USA) that BSF has an oviposition activity peak within a 24 h cycle: most eggs are laid between 14:00 and 15:00 h. Furthermore, oviposition only took place during the day between 09:00 and 19:00 h, not during the night, with 87.5% of oviposition occurring between 12:00 and 17:00 h, when the ambient temperature was above 30 °C. HF females were also observed to only oviposit during the daytime (between 05:00 and 23:00 h) in wild populations in Texas (USA) (Baldridge *et al.*, 2006).

Light is an important factor for oviposition in BSF. When not making use of sunlight in large scale artificial rearing of BSF, oviposition rates are low (Zhang *et al.*, 2010). Zhang *et al.* (2010) discovered that a quartz-iodine lamp (500 W, 153 μmol/m^2/s light intensity) stimulated oviposition (Zhang *et al.*, 2010). In addition to light intensity, spectral composition influences oviposition behaviour in BSF. At 'warm' colours (red-yellow hue; 3,000 K) the oviposition peak occurs on day 1, whereas with 'cool' colours (blue hues; 6,500 K) the peak occurs 2.3 days later (Klüber *et al.*, 2020). Oonincx *et al.* (2016) designed an illumination system for indoor breeding of BSF using UV, blue and green light-emitting diodes (LEDs). The LED wavelengths were based on electrophysiological measurements of the ommatidia of the compound eyes that contain photoreceptor cells sensitive to UV, blue and green, allowing trichromatic vision. The LED illumination resulted in the production of a similar number of egg clutches as illumination from fluorescent tubes used for colony rearing. However, significantly more larvae hatched from the eggs under the triple LED array, suggesting a beneficial effect on BSF mating frequency and hence fertilisation rate. Oviposition by BSF occurs later when the adults are kept under LED light than under fluorescent tube illumination (Oonincx *et al.*, 2016). BSF adults avoid feeding in areas with a blue background and prefer a white background. In contrast, they prefer blue for oviposition, as demonstrated by significantly larger egg clutches and weights (Romano *et al.*, 2020). To our knowledge, light effects on oviposition behaviour have not been investigated for HF.

Oviposition substrate

The choice of oviposition substrate with specific biophysical properties is important for the development of BSF and HF larvae. The moisture content, pH, and microbial communities of a substrate affect oviposition attraction and the availability of nutrition for the larvae (Machtinger *et al.*, 2014). BSF is more attracted to plant-based substrates than a carrion resource when choosing an oviposition site and larger egg masses were oviposited on plant-based substrates (Kotzé and Tomberlin, 2020). This may have been influenced by the strain of BSF included in the research, they were from a population that had lived on a grain-based diet for more than 100 generations. As no carrion resources had been introduced during this time, the results

might not reflect the natural oviposition behaviour of wild populations. Although oviposition on wet surfaces is possible for BSF, they prefer dry sites (Booth and Sheppard, 1984). However, a higher humidity, exceeding 60%, is preferred by BSF for oviposition (Tomberlin and Sheppard, 2002). HF will oviposit in a diverse range of substrates, but prefer fresh (manure) substrates and those including plant material (Machtinger *et al.*, 2014).

Next to abiotic factors, conspecific oviposition cues and the microbiome within the substrate are important factors influencing oviposition by both BSF and HF. To prevent resource depletion or cannibalism due to age disparity, it is beneficial for female HF to oviposit on a substrate at the same time as other flies are ovipositing on the same (Lam *et al.*, 2007). Cannibalism has not been reported for HF, yet does occur in other Diptera species, as will be discussed later in this review. Synchronous oviposition becomes possible through cues that first induce oviposition and over time start inhibiting oviposition on that same site (Lam *et al.*, 2007). Bryant and Hall (1975) observed that, female HF are attracted to oviposition substrates that have been conditioned by HF larvae for up to 12 h. However, they would avoid oviposition in substrates that had already been fed on for more than 24 h (Bryant and Hall, 1975), which prevented the continuous addition of new larvae to the system.

Cues to oviposit at a certain location and time can have different origins. They can originate from the female HF themselves. Gravid HF females are attracted to substrates in which dissected ovaries of other gravid females are present (Jiang *et al.*, 2002). When these ovaries are present in fermented wheat bran instead of wet wheat bran, this adds to the attraction. Gravid HF females also showed a preference for fermented wheat bran to which ovary extracts were added (Jiang *et al.*, 2002). The microbiome might also have a role by providing cues to HF females. For example, the egg-associated bacterium *Klebsiella oxytoca* (Enterobacterales: Enterobacteriaceae) can act as a cue that changes over time in concentration or content (Lam *et al.*, 2007). This bacterium proliferates over time and when its density reaches a certain threshold it inhibits further ovipositing, overriding any oviposition-inducing cues (Lam *et al.*, 2007). Symbiotic bacteria associated with oviposition and present on the eggs contribute to HF larval development, likely as a diet supplement (Lam *et al.*, 2009). This is true for both oviposition-inducing as well as oviposition-inhibiting bacteria (Lam *et al.*, 2009). The presence of fungi can also influence oviposition. HF avoids laying eggs on substrates from which fungal volatiles are emitted, to prevent competition between the larvae and fungi (Lam *et al.*, 2010).

In contrast to HF, BSF does not seem to have a preference for oviposition in conditioned substrate (Tomberlin and

Sheppard, 2002). No difference was found in oviposition when female BSF were given a choice to oviposit in either substrate with or without 5-day-old BSF larvae. However, bacteria from various life stages and species of insects are able to significantly influence oviposition preference by gravid BSF females (Zheng *et al.*, 2013). Zheng *et al.* (2013) isolated bacteria from both conspecifics as well as competing species, added this to decomposing materials and tested the effect on BSF oviposition. Isolated bacteria from conspecifics attracted females to oviposition, while their response to isolated bacteria from competing species was mixed. BSF avoided substrates with specific bacteria from *Cochliomyia macellaria* Fabr. (Diptera: Calliphoridae) and *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae) in a choice test, while this avoidance was not observed with bacteria from *Chrysomya rufifacies* Macquart (Diptera: Calliphoridae) and a significant olfactory response was found to *Gordonia sp.* isolated from BSF eggs (Zheng *et al.*, 2013). These results can be explained because the former two species compete for the same resources, whereas the latter species is a newly introduced species to North America which does not compete for the same resources as BSF (Zheng *et al.*, 2013).

3. Larval behaviour

Aggregation behaviour

Aggregation behaviour is common in fly larvae and can occur in a variety of different substrates (Heaton *et al.*, 2014). The presence of conspecifics can have several benefits. Larval aggregations can reduce environmental risks (Broly *et al.*, 2013) such as a decrease in individual predation, or an increase in food intake and digestion resulting in faster growth and development (Gregg *et al.*, 1990; Gruszka *et al.*, 2020; Heaton *et al.*, 2018; Wertheim, 2005). Increased temperature within a larval aggregation can result in shorter development time (Fouche *et al.*, 2018; Johnson and Wallman, 2014). Within larval aggregations body temperature can increase rapidly and is strongly influenced by volume of larval mass within the aggregation (Heaton *et al.*, 2014; Slone and Gruner, 2007). Within large larval aggregations there is a thermal gradient whereby larvae move between the hot centre and cooler outline (Heaton *et al.*, 2018). Moreover, heat emission from aggregations is age-dependent due to the positive relationship between larval age and larval size. Smaller larvae emit less heat than older, larger larvae (Charabidze *et al.*, 2011). In some species, larvae were found to move towards species-specific temperature optima for development and growth (Aubernon *et al.*, 2016). Increased temperature within an aggregation could also result in protection against pathogens. This strategy for combatting pathogens is known as behavioural fever (Hunt *et al.*, 2011) and will be discussed later in this review. The increase in aggregation size can create conditions of overcrowding,

making the thermoregulating properties of aggregations costly. Overcrowded conditions can cause thermal stress resulting in reduced feeding and growth, developmental delay, malformations and decrease in weight (Podhorna *et al.*, 2018). These overcrowded aggregations can also lead to competition between individuals (Rivers *et al.*, 2011) and with the increasing size of aggregations, predation rate, disease and parasite transmission risks increase as well (Gruszka *et al.*, 2020). Largely overcrowded aggregations could also cause problems in industrial rearing crates, as aggregating BSF larvae can become trapped against crate walls (Shishkov and Hu, 2020).

Within aggregations the digestive and food intake abilities of individual larvae can increase. Fly species such as HF liquify food through the secretion of enzymes produced by the salivary glands (Espinoza-Fuentes and Terra, 1987). Within clusters this external digestion helps surrounding larvae to digest food, which is called 'social digestion' (Gregg *et al.*, 1990). Social digestion can help other larvae within the aggregation to digest food and minimise energy investment (Louis and de Polavieja, 2017). Even though this may be the case in HF, Kim *et al.* (2011) suggest it is not the salivary gland, but the gut that is the major source of digestion enzyme activity in BSF, which questions the occurrence of social digestion in BSF.

Aggregation behaviour can be mediated by responses to different cues such as visual, tactile and chemical cues (Boulay *et al.*, 2019; Fouche *et al.*, 2018). In over 300 species of non-social arthropods, including HF, information on the use of aggregation pheromones is known (Wertheim *et al.*, 2005). The chemical cues involved in attraction of conspecifics are most likely present on the larval cuticle (Fouche *et al.*, 2018). Aggregation cues may lead to responses by heterospecifics on shared resources, initiating mixed species aggregations (Boulay *et al.*, 2016; Fouche *et al.*, 2018).

In nature, mixed-species aggregations may occur, e.g. when different species show the same resource requirements. Such mixed species aggregations can occur in the dipterans *Lucilia sericata* Meigen (Diptera: Calliphoridae) and *Calliphora vomitoria* L. (Diptera: Calliphoridae) that are important in forensic science (Boulay *et al.*, 2016) as well as in different *Drosophila* species (Jaenike and James, 1991). However, species with similar resource requirements can negatively affect each other's survival. For example, HF oviposition is inhibited by the presence of BSF larvae in the substrate (Bradley and Sheppard, 1984). This inhibiting effect is also observed when recently hatched BSF larvae are placed on the substrate eight days before recently hatched HF are placed on the substrate (Miranda *et al.*, 2019) which was suggested to be caused by BSF larvae reducing the levels of *Escherichia coli* (Enterobacterales: Enterobacteriaceae), essential to HF development. In industrial settings, BSF

aggregation behaviour is frequently observed as the tendency of the larvae to aggregate within crate corners (Shishkov and Hu, 2020).

In conclusion, larval aggregations can yield interesting benefits in terms of thermoregulation and feeding that could be exploited within rearing systems. Chemical cues may play an important role in the formation of aggregations. Because overcrowded conditions can, on the other hand, cause some negative developmental effects, such as trapped larvae in crate corners, this is an important aspect to take into account in mass-rearing facilities.

Feeding behaviour

Both BSF and HF larvae commonly aggregate within their food substrates. Even though larvae may feed continuously as reported for *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Sewell *et al.*, 1974), this may not be the case for all dipteran species as for example *L. sericata* (Charabidze *et al.*, 2013). Discontinuous feeding behaviour may allow larvae to feed more efficiently via short feeding periods alternated with non-feeding intervals during which digestion and nutrient absorption takes place in high-competition circumstances (Rivers *et al.*, 2011). Interestingly, larval foraging behaviour can have a genetic basis, such as known from *D. melanogaster* 'rover' and 'sitter' alleles of the *foraging* gene (De Belle *et al.*, 1989). Whether such foraging polymorphisms occur in HF and BSF is not known.

Feeding behaviour in acephalic dipteran larvae consists of rhythmic protraction and retraction of the cephalopharyngeal skeleton, resulting in the digging movement of the mouth hooks (Schoofs *et al.*, 2009). Four phases of food intake have been described: relaxed larva as starting point for the food intake cycle, extension and tilting of the cephalopharyngeal skeleton, full extension of the cephalopharyngeal skeleton and digging movement of the mouth hook and lastly, the withdrawal of the cephalopharyngeal skeleton and mouthparts ending in relaxing of the mouthparts (Schoofs *et al.*, 2009). Similar phases of food intake are likely to be exhibited by HF as well. The morphology of BSF mouthparts differs from those of HF, since BSF larvae lack mandibular hooks. Stratiomyid larvae possess a fused mandibular-maxillary complex that is used for sweeping of food into the oral cavity rather than for chewing (Gobbi, 2012). Beneath the pharynx at the posterior end of the head capsule, two heavily sclerotised plates are present that likely function as 'mortar and pestle' for grinding up food material (Gobbi, 2012). Detailed information on the mechanical and sensory functions of BSF larval mouthparts is lacking.

Feeding behaviour can be influenced by factors such as larval age, substrate choice, presence of pathogens, competition,

temperature, etc. Temperatures below 21 °C resulted in an unsatisfactory feeding rate of BSF for waste consumption (Kalová and Borkovcová, 2013). The feeding rate of HF larvae is especially influenced when fed with the bacterium *Brevibacillus laterosporus* (Bacillales: Paenibacillaceae), leading to a decrease in feeding and locomotor activity (Ruiu *et al.*, 2012). This could be a common response of larvae to harmful pathogens. There is no information on changes in feeding or locomotion behaviour by BSF upon infection with pathogens.

Even though larvae are not given a substrate choice within commercial rearing facilities, preference behaviour has been observed. BSF larvae prefer pig manure over a cereal-based mass-rearing diet (Parodi *et al.*, 2020). This preference could be based on the preference for the microbial community and pH of the substrate or due to chemical cues (Parodi *et al.*, 2020). Substrate preference studies are needed for HF and BSF as they could indicate larvae preferring food with the highest nutritional value as in *Drosophila* (Sokolowski *et al.*, 1983) or the presence of social cues in the food that mediate chemical communication between individuals, like aggregation pheromones (Durisko and Dukas, 2013).

Differences in behaviour of larval instars

The various larval stages of flies are associated with different behaviours, the most dramatic changes occur when larvae are in the last instar, reaching the pupal stage. During this period, aggregation levels decrease when feeding larvae leave food substrates and develop into wandering non-feeding prepupae (Denlinger and Zdarek, 1994; Durisko *et al.*, 2014; Rivers *et al.*, 2011). Migrating BSF prepupae show a wandering pattern by leaving substrates, a convenient property for self-harvesting within rearing systems (Sheppard *et al.*, 1994; Wang and Shelomi, 2017). Wandering larvae do not only stop feeding but can also show differences in locomotor activity and abiotic preferences.

Within BSF locomotor activity, 6th instar larvae have an increased anterior arching and use the head more extreme as a pin for forward movements compared with prepupae that show more of a sinusoidal pattern with increased middle body arching (Giannetti *et al.*, 2022).

In *D. melanogaster* the preference for locations to pupate is shown to be influenced by light, surface texture, substrate consistency and humidity (Godoy-Herrera *et al.*, 1989). Substrate moisture is also a factor influencing the selection of a pupation site in late instar larvae. The avoidance of high-moisture surfaces can be quite drastic, such as in HF where prepupae show a strong avoidance of wet surfaces (Hafez, 1953). This behaviour is different from feeding HF larvae which show avoidance of dry surfaces (Hafez, 1950). BSF showed a similar pattern where prepupae were

crawling more frequently out of high moisture substrate compared to 6th instar larvae, this pattern was even more evident at low light intensity (Giannetti *et al.*, 2022). This switch in preference for moist surfaces is also observed in *D. melanogaster* (Godoy-Herrera *et al.*, 1984), hinting to a general change in preference that can be applied to many fly species. Substrate moisture levels are also affecting the location of the prepupae within the substrate. In dryer environments, larvae tend to dig deeper resulting in the pupae located within rather than on the substrate surface (Godoy-Herrera, 1994). The choice of the pupation site can also be influenced by air humidity levels. As lower sensitivity to relative air humidity levels above 75% was observed when comparing HF feeding stages with prepupal stages (Hafez, 1950, 1953), the sensory systems could be altered in this later stage in larval development. This could affect the responses to moisture or humidity change in later larval instars. The olfactory response to substrate odours also changes in later instar larvae. The significant decline in olfactory response could be explained by the hormonal changes when pupation is initiated (Cobb, 2007). Hafez (1953) described a similar decline in olfactory response to odours in prepupating HF. However, as found in *D. melanogaster*, these age-dependent changes in behaviour may differ among strains (Cobb, 2007).

Drastic shifts in temperature preferences were observed in late third-instar larvae (Sokabe *et al.*, 2016), starting at 24 °C in early third-instar larvae and switching to a preference for 18-19 °C. This switch in temperature preference is also observed in HF, with a temperature preference switch from 20-25 °C for young larvae to 15 °C for prepupating larvae (Hafez, 1953).

In natural situations, the prepupal wandering period comes with predation risk. Rivers *et al.* (2011) speculate that the long wandering distance of necrophagous flies is thought to be an adaptation to avoid parasitoids, but these parasitoids have been seen hitchhiking on larvae, waiting for pupation to attempt parasitism. The larval and pupal stages of BSF and HF are attacked by several parasitoid species that may form a threat for production systems (Machtinger *et al.*, 2015; Maquart *et al.,* 2020). The various housefly parasitoids differ in depth at which they search for pupae in the substrate (Geden, 2002), and this may pose a selection pressure on larval pupation site choice.

Within mass rearing systems some other stress factors such as disturbance can still be present. In BSF it is shown that the response to disturbance can be instar specific, as prepupae show immobility after disturbance more frequently than younger larvae (Giannetti *et al.*, 2022).

When a suitable pupation site is found, the wandering stops. After the wandering period, most fly prepupae become immobile and create a hard pupal case (puparium) out of the

cuticle by means of rapid muscle contractions (Denlinger and Zdarek, 1994; Žďárek and Denlinger, 1991). Wandering behaviour of the two fly species seems similar; the prepupae tend to search for dryer areas to pupate, however, more behavioural studies are needed to confirm this.

Cannibalism

Cannibalism or intraspecific predation, by which a conspecific is killed and (partially) consumed is not limited to carnivores and has been demonstrated in many insect species in both laboratory and natural environments (Fox, 1975; Richardson *et al.*, 2010). Cannibalism may be an adaptive strategy (Richardson *et al.*, 2010). For example, it can either improve survival when food is scarce or regulate population density and reduce competition increasing the fitness of the cannibal (Richardson *et al.*, 2010). However, cannibalism can also be harmful for the cannibal if it decreases the population density below its optimum, brings physical harm as a result of defence of the prey, or causes pathogens to be transferred from prey to cannibal (Richardson *et al.*, 2010). Intraspecific predation by larvae has been observed in seven Diptera species in five families, including Muscidae. This mostly consists of larva-on-larva cannibalism, but larvae have also been found to cannibalise eggs or pupae (Richardson *et al.*, 2010).

Faria *et al.* (2004) studied the influence of prey size and larval density on cannibalism by third-instar larvae of *Chrysomya albiceps* Wiedemann (Diptera: Calliphoridae) under laboratory conditions. They scored cannibalistic behaviour as successful when the predator surrounded and pierced its prey and the pierced larva would struggle violently in response. They only observed cannibalism between third-instar larvae as predators and second-instar larvae as prey (Faria *et al.*, 2004). Cannibalistic *C. albiceps* larvae seem to seek out prey of intermediate size. Smaller prey (1st instar) are more difficult to find and give less nutrition, while larger prey (3rd instar) involve risks of role reversal and injury. Furthermore, third instars might be better able to escape. A higher density led to higher larval mortality. Yet the chance of escaping cannibalism is higher for individual larvae at higher densities, as the proportion killed is lower (Faria *et al.*, 2004). Larvae of *C. albiceps* only showed cannibalism if no other food source was available. Food deprivation in combination with increased foraging activity leads to more encounters and cannibalism opportunities (Faria *et al.*, 2004).

In *D. melanogaster* cannibalism occurs in crowded conditions, where young larvae will attack and consume pre-pupation larvae which are in the 'wandering-stage' (Vijendravarma *et al.*, 2013). Larvae of *D. melanogaster* and *Drosophila simulans* Sturtevant (Diptera: Drosophilidae) form aggregations mediated by chemical cues from the injured prey larvae (Kakeya and Takahashi, 2021). The larvae of *D. melanogaster* and *D. simulans* are able to sustain themselves, develop normally and reach adulthood on a solely cannibalistic diet, unlike other *Drosophila* species (Kakeya and Takahashi, 2021; Vijendravarma *et al.*, 2013). Therefore, cannibalistic behaviour is likely not a general feature of the whole *Drosophila* genus. *D. melanogaster* third-instar larvae show mouthpart plasticity resulting in approximately 20% more teeth on their mouth hooks when raised from the egg stage onwards on strictly cannibalistic nutrition versus a standard diet (Vijendravarma *et al.*, 2013). Vijendravarma *et al.* (2013) studied evolution over 118 generations of *D. melanogaster* kept under malnutrition conditions at the larval stage, and found an increased inclination towards cannibalism in later generations.

No reports on cannibalism in adult Diptera was found. Adult morphology, specifically of their mouthparts, makes it less likely that cannibalism occurs among adults. However, there is a lack of research that tested if certain species are cannibalistic, and few studies are available on cannibalism (Richardson *et al.*, 2010). At present, no instances of cannibalism among BSF or HF were found in literature.

Still, cannibalism is a common occurrence in many insect species (Faria *et al.*, 2004; Richardson *et al.*, 2010) and found in both laboratory and natural environments (Fox, 1975; Richardson *et al.*, 2010). For BSF larvae exposed to nutrient limitation indications for cannibalism have been observed (J.J.A. van Loon, unpublished data). If cannibalism does occur in BSF or HF this could have consequences for the yield and efficiency in mass-rearing systems. Moreover, when larvae start feeding on each other, this would clearly constitute a threat to insect welfare. As cannibalism increases at higher densities (Faria *et al.*, 2004), mass-rearing systems may cause an increase in density of BSF and HF compared to the natural situation. Therefore, it is important to take cannibalism under consideration in these systems.

Nociceptive behaviour

A potential indicator for lack of welfare could be pain or the experience of harmful stimuli. Nociception is the neural cognate of the perception of pain (Sulkowski *et al.*, 2011). Like other animals, insects can show nociceptive behaviour when experiencing a noxious stimulus. This behaviour often consists of avoidance of the stimulus or an escape response, which can prevent (further) damage to its tissues (Im and Galko, 2012). Although literature on nociception behaviour is lacking for BSF and HF, nociceptive behaviours have been extensively studied in *D. melanogaster* larvae and adults.

Larvae of *D. melanogaster* show a curling and rolling response, also referred to as a corkscrew-roll, to certain noxious stimuli (Dason *et al.*, 2020). Several types of stimuli can evoke such a reaction, including heat, chemical and

mechanical stimulation (Im and Galko, 2012). *Drosophila melanogaster* larvae do not show a thermal nociceptive response when a local heat probe with temperatures above 52 °C is used (Chattopadhyay *et al.*, 2012). Whether they are unable to sense higher temperatures, or cannot respond due to structural damage to nerves or muscles caused by higher temperatures, is unknown. The number of rolls indicating the amplitude of the response is determined by the duration of exposure, rather than the value of the temperature administered within the noxious range (Chattopadhyay *et al.*, 2012). A higher temperature does give a quicker response in both larvae and adults. When instead a heat plate for global exposure of the larvae to the noxious heat is used, nociceptive behaviours start at a lower temperature and more complex responses are observed. Behaviours not seen in local probing but seen with the heat plate method are whipping, seizure and paralysis (Chattopadhyay *et al.*, 2012). It might be that a critical mass of sensory neurons firing from more than one region of the body is needed to start these behaviours (Chattopadhyay *et al.*, 2012). Instead of curling and rolling, adult *D. melanogaster* show a characteristic jump response as nociceptive behaviour in response to a noxious thermal stimulus (Xu *et al.*, 2006). The nociception behaviour is completely different for cold noxious stimuli. When touched with a cold probe, *D. melanogaster* larvae responses include a robust full-body contraction, a 45-90° raise of the posterior segments, and a simultaneous raise of the anterior and posterior segments into a U-shape (Turner *et al.*, 2017).

As previously mentioned, nociceptive behaviour can also be a form of avoidance behaviour. *Drosophila melanogaster* avoids highly concentrated wasabi even without actual ingestion. Therefore they might be able to sense the wasabi through olfaction as well as through gustation (Ohashi and Sakai, 2015).

The various larval instars may exhibit different nociceptive behaviours. Before the third instar *D. melanogaster* larvae are unable to perform the full corkscrew-roll (Sulkowski *et al.*, 2011). The first instar larvae only show the first partial rolling behaviour in response to a noxious heat stimuli 40 h after eclosion (Sulkowski *et al.*, 2011). Therefore, the corkscrew-roll behaviour cannot be used to study nociceptive behaviour in first and second instar larvae. Research on BSF and HF nociception behaviour is needed to determine whether it corresponds to the nociceptive behaviour of *D. melanogaster*.

4. Microbiome and behaviour

An insect's microbiome consists of all microorganisms living either inside or on an insect and can be beneficial, neutral or pathogenic (Lewis and Lizé, 2015). There is a vast literature showing that an insect's microbiome can influence various behaviours, including mating, oviposition,

cannibalism and dispersal behaviour (Lewis and Lizé, 2015). In many dipterans, the microbiome is important in the choice of oviposition substrate because fly larvae are unable to develop in a sterile environment. This is, for example, evident in the stable fly *Stomoxys calcitrans* L. (Diptera: Muscidae) which cannot develop on a sterilised substrate (Romero *et al.*, 2006). Sterilised food also causes shift in microbiota composition and larvae performance, whereas disinfection of BSF eggs show no difference (Schreven *et al.*, 2021). Moreover, stable flies lay more eggs on a substrate with a suitable active microbial community that promotes offspring development. Gravid females were able to detect these suitable substrates via microbe-derived stimuli (Romero *et al.*, 2006). In HF, bacteria are transferred from the gut of larvae to the substrate, while feeding. These bacteria help to degrade and utilise the polysaccharides in the cell wall of wheat bran in the medium (Zhao *et al.*, 2017).

The microbiome can also influence other aspects of behaviour, such as activity patterns, although studies differ in their conclusions as to the importance of this. Gut bacteria in *D. melanogaster* females regulate locomotor behaviour (Schretter *et al.*, 2018). Depletion of the gut bacteria through antibiotic treatment led to increased exploratory and hyperactive behaviour. More research is needed to determine the pathways that lead to this change in behaviour (Schretter *et al.*, 2018). Selkrig *et al.* (2018) compared two generations of germ-free raised *D. melanogaster* to flies with an intact microbiome and found no large influence of the microbiome on behaviour. Anxiety related wall following, sleep (any event of 5+ minutes of consecutive inactiveness), locomotion, and courtship behaviour were only slightly affected. The lab conditions experienced for multiple generations may have altered the original microbial diversity, which could have had an impact on the results (Selkrig *et al.*, 2018). Hence, much needs to be learned on the role of the microbiome in insect behaviour and effects may depend on the species considered and environmental conditions.

5. Pathogens and behaviour

Infections with harmful pathogens decrease insect welfare. The focus here will not be on the physiological effects of an infection, but rather on how flies respond to it behaviourally. There are very few pathogens known to infect BSF, and research about the relation of pathogens and BSF behaviour is lacking. No occurrence of natural infection or major pathogen outbreak has been reported for BSF (Joosten *et al.*, 2020). One of the pathogens able to infect adult BSF is *Beauveria bassiana* KVL 03-122 (Lecocq *et al.*, 2021). In contrast, there are various pathogens known to infect HF. For example, the salivary gland hypertrophy virus (MdSGHV) negatively impacts the life span of HF as well as mating behaviour and reproduction (Lietze *et al.*, 2007). Mating attempts become less successful after infection, and

males become less motivated to attempt copulation with healthy females. The morphology of reproductive structures or ability to successfully copulate is not affected by the virus. Therefore, altered morphology cannot be the cause of this change in behaviour (Lietze *et al.*, 2007). Oviposition in flies can also be affected by pathogens. In HF, infection with the entomopathogenic fungus *Metarhizium anisopliae* (Hypocreales: Clavicipitacea) significantly reduces the rate of oviposition during the third and fourth day after exposure (Baker *et al.*, 2018).

A well-studied pathogen in HF is the fungus *Entomophthora muscae* (Entomophthorales: Entomophthoraceae). Houseflies show highly stereotyped unidirectional locomotor behaviour that is specific for an infection with *E. muscae* right before their death and ends in a specific body posture. This behaviour starts with the last locomotory movement, after which the proboscis is extended towards the substrate and attached. Then, the wings are moved upwards until they reach the maximal upward extension. In this final body posture the legs are spread and the abdomen is angled away from the substrate. This behaviour occurs at the time environmental conditions favour transmission of the pathogen (Krasnoff *et al.*, 1995). Male HF are attracted to and try to copulate with dead female HF infected with *E. muscae* (Møller, 1993). This manipulation of HF male sexual behaviour enables the fungus to spread through a population (Møller, 1993). This effect of the fungus is apparently not mediated through an alteration of cuticular hydrocarbons (Zurek *et al.*, 2002). *Entomophthora muscae* reduces the amount of sex pheromone produced by young virgin females, but has no effect on sex pheromones of old virgin females (Zurek *et al.*, 2002). Dead HF adults infected with *E. muscae* present a swollen abdomen, increased in size by almost 30% on average, caused by the growth of fungal hyphae (Møller, 1993). This enlargement mimics the appearance of increased fecundity in females and increases sexual attractivity (Møller, 1993). Møller (1993) experimentally exchanged abdomens of infected and uninfected individuals. The results showed that it is the swollen abdomen and not the whole infected body of the female that attracts the males. Still when presented with an uninfected individual and infected individual with the same abdomen size, the males preferred the infected individual (Møller, 1993). This points towards other factors being involved in infected individuals' attractiveness.

6. Behaviour related to immune responses

Although the microbiome and pathogens can influence fly behaviour, the opposite can also occur. HF can combat an infection by altering their behaviour; increased cleaning behaviour of HF adults when exposed to *Pseudomonas aeruginosa* (Pseudomonadales: Pseudomonadaceae) bacteria has been reported (Jacques *et al.*, 2017). This apparently enables HF to remove the bacterium from their body surface. However, this does not seem to be effective against all bacteria as the flies were unable to remove *E. coli* (Jacques *et al.*, 2017). A specific behaviour in response to pathogen infection is known as behavioural fever. This is defined as changes in thermoregulatory behaviour in response to infection, whereby the insect moves to a location with a higher temperature. Behavioural fever is an indicator of initial infection by pathogens; actual infection status needs to be confirmed by diagnostic methods (Maciel-Vergara *et al.*, 2021). HF show a behavioural fever response when challenged with a fungal infection both in laboratory (Anderson *et al.*, 2013a,b; Watson *et al.*, 1993) and natural populations (Kalsbeek *et al.*, 2001). By moving to a higher environmental temperature when infected, HF are able to combat the infection. Anderson *et al.* (2013a) demonstrated that adult HF balance the costs and benefits of behavioural fever by moving to different temperatures for different severities of infection. They placed adult HF individuals infected with *B. bassiana* (Hypocreales: Cordycipitaceae) in a thermal gradient box with a 6 h daily heated period. HF infected with *B. bassiana* showed a specific activity pattern. At the start of the heating period they would move to the hottest areas (40-42 °C) for the first hour, after which they gradually moved to the cooler temperatures (35-38 °C) again (Anderson *et al.*, 2013a). Three adaptive explanations have been proposed for this apparent activity pattern. First, the cycle of moving to warmer areas at the start of each day and cooler areas as the day progresses could be caused by exogenous immune elicitors, pathogen signal metabolites that trigger the immune system. By moving to the warmer area these immune elicitors are reduced or removed from the haemolymph. A decrease in immune elicitors causes the flies to move to cooler areas again during the day. During the night the fungus can then grow at its optimal temperature, causing the cycle to restart and continue the next morning. Secondly, residing in the high temperature environment costs more energy, which might lead the flies to only be able to sustain the peak fever temperatures for a short time in the morning. The third hypothesis is that the circadian rhythm influences the behavioural fever pattern (Anderson *et al.*, 2013a). A periodicity was observed by Watson *et al.* (1993) as well, after four to six hours of inducing a behavioural fever, adult HF infected with *E. muscae* would move back to cooler temperatures. Therefore, periodicity and the possibility of alternating between different temperatures could be an important factor for expressing behavioural fever. Although these studies focused on adult behaviour, larvae could potentially show behavioural fever as well considering their aggregation behaviour. Johnson *et al.* (2014) found that *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) larvae within a maggot mass move to temperatures higher than their preferred temperature for development, which had been previously determined by a temperature gradient experiment.

Behavioural fever has not only been observed under laboratory conditions. Kalsbeek *et al.* (2001) performed a field study on behavioural fever in adult HF infected with *Entomophthora schizophorae* (Entomophthorales: Entomophthoraceae) and *E. muscae*. They conducted a mark and release experiment in which 3,000 flies inoculated with *E. schizophorae* and another 3,000 flies as a control group were released in an enclosed swine farrowing barn. Infected flies were observed to spend more time on the warm surface of heat lamps, than non-infected flies (Kalsbeek *et al.*, 2001). They also collected HF on a dairy farm from a range of temperatures. Flies infected with *E. muscae* collected from higher temperatures zones tended to be in earlier stages of infection, while those collected at cooler temperature zones tended to be in later stages of infection (Kalsbeek *et al.*, 2001). Therefore, it is most likely that behavioural fever is a naturally occurring behaviour and not an adaptation specific to laboratory conditions.

Although behavioural fever can decrease the severity of an infection or delay its outcome, houseflies likely cannot completely rid themselves of an infection this way (Anderson *et al.*, 2013b). Behavioural fever can help the flies survive longer, increasing the time they have for reproduction and enabling them to lay more eggs. Still, there are costs linked to behavioural fever, for example the viability of the eggs is lower (Anderson *et al.*, 2013b). Anderson *et al.* (2013a) concluded that under the conditions of their experiment, where they made use of *B. bassiana* the costs and benefits of behavioural fever combined amounted to no net change in fitness. This raises the question why the flies would show this behaviour if it is not profitable. Since behavioural immune responses could be a potential marker for welfare, it is important to further research this behaviour in both BSF and HF (Vogel *et al.*, 2022).

7. Conclusions and perspectives

To identify and understand possible welfare issues in mass-rearing of BSF and HF an in-depth knowledge of the behaviour of the different life stages is essential. Adult behaviour has been studied relatively extensively for HF and, more recently, for BSF showing that the two species display very different mating behaviour. BSF requires specific light conditions for the highest mating frequency and substantial space for courtship and mating due to its lekking behaviour; this is either not the case or yet unknown for HF. Female and male sex pheromones are important in HF mating behaviour, whereas male BSF are visually triggered by flying conspecifics. Oviposition behaviour of both species shows similarities: BSF and HF oviposit only during the day, prefer plant material as oviposition substrate and respond to the presence of larvae in the substrate. Both species lay eggs in clutches and larvae show a strong tendency to aggregate. Taking behaviour under natural conditions as a reference for evaluating welfare

under mass-rearing conditions, this review reveals that information on larval behaviour under natural conditions is scant. Aggregation behaviour in larvae is pronounced, however, the factors triggering it remain unknown for both species. In other dipteran species advantages of aggregation behaviour have been documented, such as social digestion and reduced predation or parasitisation risk (Wertheim *et al.*, 2005). The occurrence of social digestion in BSF and HF larvae is unknown due to their strong tendency of feeding concealed inside the substrate, which has hampered detailed behavioural studies. In addition, cannibalism has not been documented in BSF and HF literature. Nociceptive responses of BSF and HF larvae have not been reported thus far. As larvae enter the prepupal stage, drastic shifts in preferences for abiotic conditions occur.

Stress factors in natural and mass-rearing conditions are similar

It is important to note that stress-induced behaviours have evolved under natural conditions and are inherent to the natural behavioural repertoire of insects. The most important stressors identified are low food quality, insufficient amount of food, e.g. due to competition under high larval or adult densities, suboptimal temperature and humidity, high-frequency sound, and infection by parasites and pathogens (bacteria, viruses and fungi). These stressors likely have negative effects on welfare that translate into prolonged development and reduced growth and reproduction rates. Mass-rearing systems aim to optimise growth and reproduction rates and therefore benefit from minimising stress levels in several ways. Compared to natural conditions characterised by highly variable abiotic conditions, current mass-rearing systems are operated under controlled temperature and humidity conditions. In addition, in mass-rearing systems insect densities are controlled, preventing competition for food and space. Moreover, current state-of-the-art mass-rearing facilities are closed systems, minimising the ingression of natural enemies, such as predators, parasites and pathogens.

Species and population differences

As the number of behavioural studies about BSF and HF increases, differences between these two species have become apparent such as the differences in mating and courtship behaviour discussed in detail above. In contrast to HF, BSF oviposition behaviour is also strongly affected by light conditions, such as UV-, blue- and green- light when natural sunlight is not present (Oonincx *et al.*, 2016). These factors translate into different rearing systems for both species to maximise yield and warrant welfare.

Concerning the larval stage, the differences in morphology of the mouthparts hints to different feeding methods between the species. However, details are missing due to the

lack of BSF and HF larval feeding studies. This information would be important for mass rearing facilities to optimally use the substrate that can be provided to the larvae. In addition, whether certain infochemicals are also used in the larval stages of BSF and HF is unknown. The possible use of aggregation pheromones upon moving through the substrate would be valuable information to understand the basics of aggregation behaviour. As this is a natural behaviour present in many fly larvae (Heaton *et al.*, 2018), this could be an indicator to gather welfare information from within industrial settings, as the frequency, size and occurrence of aggregations within rearing crates may change when exposed to stress; this information is currently lacking.

In testing potential stressors of mass-reared HF and BSF, population differences need to be considered. It has been shown that populations of HF respond differently to temperature stressors, most likely due to adaptive differentiation (Kjærsgaard *et al.*, 2015). For example, HF strains originating from warmer areas such as Spain, were better able to cope with heat stress than HF strains from colder areas such as Denmark and Switzerland (Kjærsgaard *et al.*, 2015).

Insect welfare is understudied and offers both fundamental and applied research opportunities

Welfare of vertebrate zoo animals, livestock and fish have received much attention over the past decades with a focus on the individual animal. In contrast, welfare of insects in mass-rearing conditions such as those prevailing over the past 50 years in large production facilities for application of the Sterile Insect Technique for flies such as the screwworm fly *Cochliomyia hominivorax* Coquerel (Diptera: Calliphoridae) or the medfly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Pérez-Staples *et al.*, 2021), has received little attention. It can be questioned whether knowledge and insights about welfare of vertebrates can be extrapolated to HF, BSF or other insects. A notable difference is that in nature BSF and HF occur in dense aggregations. Thus far information on larval behaviour under natural conditions is very limited for both fly species. This knowledge gap needs to be filled if behaviour under natural conditions is used as the reference for evaluating welfare. Researchers generally agree that the 'precautionary principle' should be applied when dealing with invertebrates in laboratory or mass-rearing settings until more is known about the ability of invertebrates to suffer, of which pain could be a component (Andrews, 2011). We concur with Boppré and Vane-Wright (2019) that this 'precautionary principle' applies to the insect mass-rearing industry as well.

Insect nociceptive behaviours are unexplored in mass-reared species

A generally accepted indicator of compromised animal welfare is the expression of nociceptive behaviours. Insects display such nociceptive behaviours when experiencing a noxious stimulus and respond by avoidance or escape (Im and Galko, 2012). Nociceptive behaviours have been extensively studied in the model species *D. melanogaster* for both larvae and adults, however, it is debatable whether extrapolation of this knowledge to BSF and HF is legitimate. Experimental studies are needed to address this crucial aspect of welfare.

Occurrence of cannibalism

No reports exist on possible cannibalistic behaviour of BSF or HF. To safeguard welfare it is crucial to verify whether it indeed is absent in both species. Three future research topics on cannibalism that could be of interest in the context of mass rearing are: behaviours limiting or promoting cannibalism, the influence of artificial environments on the incidence of cannibalism, and the effect of genetic relatedness on its occurrence (Richardson *et al.*, 2010). If cannibalism were to occur in BSF or HF this could have consequences for the yield and efficiency in mass rearing systems.

Behavioural indicators of compromised welfare

Three behavioural indicators of compromised welfare emerge from this review: (1) leaving the substrate prior to the prepupal stage, i.e. escape behaviour as a response to avoid nociceptive stimuli or unfavourable abiotic or biotic conditions prevailing in the food substrate; (2) larval aggregation behaviour leading to body temperatures above those optimal for growth ('behavioural fever'); (3) low mating frequency and reproductive output in adult flies.

In conclusion, BSF and HF are two insect species that are reared as feed component for aquaculture and livestock such as pigs and poultry. Their use has recently been approved by the European Commission (Regulation (EU) 2021/1372 of 17 August 2021 (EC, 2021)), thus opening unprecedented opportunities for the large-scale use of these insects in feed. To safeguard the welfare of these flies in mass rearing, knowledge on their behaviour is essential. Here, we present the state of the art on the behaviour of these flies for both adults and larvae. Our review identifies relevant topics for further study to allow insect production that pays due attention to the welfare of insects in mass rearing.

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Conflict of interest

The authors declare no conflict of interest.

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