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Nygaard Jensen, Annette; Hald, Birthe

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Campylobacter* contamination level in houseflies after exposure to materials containing *Campylobacter

A. N. Jensen^{1*} and B. Hald¹.

National Food Institute, Technical University of Denmark, Kemitorvet, Bygning 202, DK-2800 Kgs. Lyngby, Denmark; anyj@food.dtu.dk

Although houseflies have been found to carry *Campylobacter jejuni*, little is known about the quantitative campylobacter level in naturally contaminated houseflies and their ability to contaminate surfaces. This study aimed to elucidate how houseflies' previous exposure to campylobacter-contaminated material (faeces or liquid) for 1 or 4 h affects the acquired campylobacter level in houseflies and their contamination potential.

Cups of 250-ml were added 5 g of chicken faeces or 1 ml liquid and spiked with approximately 3, 4, 5 or 7 Log₁₀ CFU *C. jejuni*. Sixteen houseflies were added to each cup. After 1 h of exposure, four houseflies were removed from the cup for enumeration of campylobacter in each fly by plate spreading. Another four houseflies were transferred onto Abeyta-Hunt-Bark (AHB) agar plates (9 cm) to assess possible contamination of surfaces. After 1 h on the AHB plate, each fly was tested for level of campylobacter. This procedure was repeated after approx. 4 h of exposure for the remaining eight houseflies.

The *C. jejuni* acquisition in houseflies increased with exposure dose and was higher after liquid exposure compared with faeces exposure, while there was no significant effect of exposure time (1 vs. 4 h). For faeces, 90.0% (n=80), 48.4% (n=64), 6.3% (n=48) and 0% (n=16) of houseflies were campylobacter-positive when exposed to 7, 5, 4, and 3 Log₁₀ CFU with a mean ($\pm$ SE) of 2.0 $\pm$ 0.1, 0.8 $\pm$ 0.1, 0.3 $\pm$ 0.0 and 0 Log₁₀ CFU recovered per campylobacter-positive fly, respectively. For liquid, 95.7% (n=47), 91.4% (n=47), 20.8% (n=48) and 6.3% (n=16) of houseflies were campylobacter-positive with a mean of 3.3 $\pm$ 0.2, 2.0 $\pm$ 0.1, 0.8 $\pm$ 0.2 and 0.3 $\pm$ 0.0 Log₁₀ CFU. The surface of the AHB plates was only contaminated by houseflies previously exposed to >4 Log₁₀, but the *C. jejuni* number found on the AHB surface did not correlate with the number found in the corresponding fly.

Introduction:

The bacterial load and species diversity varies between different insect species, where houseflies (*Musca domestica*) are found to harbor a broad range of species including host-species specific bacterial taxa (Bahrndorff *et al.* 2017; Gupta *et al.* 2012; Gill *et al.* 2017; Junquera *et al.* 2017). Houseflies feed on decaying organic matter, carcasses and faeces, a feeding behavior implying a substantial exposure to various bacteria incl. human pathogens. Among human pathogens detected in houseflies are *Campylobacter* spp. incl. *C. jejuni*, which is the most common cause of foodborne gastrointestinal infections in humans and therefore are flies hypothesized to play a role in transmission of *Campylobacter* (Ekdahl *et al.* 2005; Nichols, 2005). The reported *Campylobacter* spp. prevalence in flies is between 0.2% and 11% depending on season and environment (Hald *et al.* 2004, 2008; Hansson *et al.* 2007; Royden *et al.* 2016; Szalanski *et al.* 2004). The *Campylobacter* spp. contaminated flies are shown to be a possible transmission vehicle for introduction of *Campylobacter* spp. from the environment into poultry facilities, as the *Campylobacter* spp. prevalence were reduced in poultry flocks where fly screens prevented flies physically from entering the poultry facility (Hald *et al.* 2007, Bahrndorff *et al.* 2013). Under experimental conditions, Shane *et al.* (1985) also found that houseflies became contaminated with *C. jejuni* when confined in an isolator with *C. jejuni* infected chicks, and following, these houseflies transmitted *C. jejuni* to a new batch of specific-pathogen-free (SPF) chicks. However, it is uncertain if this *C. jejuni* transmission to chicks occurs indirectly due to houseflies' contamination of the environment

via excreta and regurgitates or via contact to feet and external body structures, or directly from ingestion of contaminated houseflies. Also, little is known about the specific mechanisms of *Campylobacter* spp. contamination of houseflies under natural conditions and the houseflies' ability to transmit *Campylobacter* spp. afterwards. An experimental campylobacter infection model in houseflies indicates that contaminated houseflies turn campylobacter negative within 24 h after ingestion and that houseflies mainly serve as a mechanical vector without amplification of *Campylobacter* spp. (Gill *et al.* 2017; Skovgaard *et al.* 2011). This is contrary to e.g. *Escherichia coli* and *Enterococcus faecalis*, which seem to proliferate in houseflies (Doud and Zurek, 2012; Kobayashi *et al.* 1999). Attempts have been made to assess the location of bacteria in houseflies after exposure. Shane *et al.* (1985) found that the recovery of *C. jejuni* was 69% from viscera and 19% from feet and ventral surface of the body (n=32) after exposure to a broth containing 2.8×10^8 CFU/ml. Gill *et al.* (2017) found that vomitus of inoculated houseflies was *C. jejuni* positive 4 h but not 8 h after ingestion of 2.1×10^6 CFU. They also found that 40% of rinse samples (n=5) from the inside of 1.5 ml Eppendorf tubes containing excreta and vomitus from an inoculated housefly was positive within 1 h and up to 4 h after ingestion but not after 8 h. For a better understanding of the *Campylobacter* spp. infection potential and contamination risk from houseflies, this study aimed to elucidate the quantitative level of *C. jejuni* acquired in houseflies after exposure to contaminated material (natural infection) and also houseflies' ability to contaminate surfaces following. The effect of exposure dose, duration of exposure and type of contaminated material was addressed.

Methods:

Rearing of houseflies.

The *Musca domestica* (houseflies) used in this study was obtained from a Danish dairy cattle farm in 1989 and reared continuously under laboratory conditions at 25°C and 80% relative humidity. Eggs were laid and developed in a medium consisting of wheat bran (24.6%), alfalfa (12.3%), yeast (0.26%), malt (0.9%) and tap water (61.6%). Adult houseflies were fed on tap water and a mix of sugar (1/3) and skimmed milk powder (2/3).

Preparation of C.jejuni suspension.

The *C. jejuni* strain used in this study, SC181, originates from poultry (Bang *et al.* 2003). For preparation of *C. jejuni* stock suspensions, cells were harvested in 1 ml Brain Heart Infusion (BHI) broth from blood agar (BA) plates (5% v/v calf blood) with *C. jejuni* subcultures incubated for 16-24 h at 42°C under microaerobic atmosphere (6% O₂, 7% CO₂, 7% H₂ and 80% N₂). The *C. jejuni* stock suspension was adjusted to an optical density (OD) of 0.600 measured at wavelength 600 nm (OD_{600 nm}) in a UV-mini 1240 spectrophotometer (Shimadzu Europe GmbH, Duisburg, Germany) to reach approximately 9 Log₁₀ CFU ml⁻¹. Subsequently, *C. jejuni* suspensions of specified cell concentrations were made by appropriate dilution of the stock suspension in BHI broth. For all *C. jejuni* stock suspensions, CFU ml⁻¹ was estimated from 10-fold dilution series in BHI broth, plated onto BA agar plates and incubated in microaerobic atmosphere at 42°C for approximately 48 h before colony counting.

Experimental design.

The quantitative *Campylobacter* uptake in houseflies after exposure to different contamination levels in faeces or liquid material was estimated. Houseflies were placed in a 250-ml cup containing a) 5 g of chicken faeces spiked with 1-ml *C. jejuni* suspension or b) a lid from a 50 ml CELLSTAR® Polypropylene Tube (Greiner Bio-One GmbH, Frickenhausen, Germany) added 1-ml *C. jejuni* suspension and covered with a metal grid to avoid drowning of the houseflies. The added 1-ml *C. jejuni* suspension was adjusted to

specific concentrations of approximately 3, 4, 5 or 7 Log₁₀ CFU ml⁻¹. Sixteen houseflies were anesthetized with CO₂ and placed in each cup covered with a cotton mesh cloth to allow aeration. After approximately 1 h of exposure to the contaminated material, four houseflies were removed from the cup for enumeration of *C. jejuni* in each fly. Another four single houseflies were each transferred to an Abeyta-Hunt-Bark (AHB) agar plate (9 cm Petri dish) with a sterile tweezer and left on the surface of the agar for 1 h before enumeration of *C. jejuni* in each fly. The AHB plates were following incubated at 42°C under microaerobic atmosphere for 48 h before visual inspection of *C. jejuni* contamination of the plates. After approximately 4 h of exposure, the procedure described above was repeated for the remaining eight flies in the cup. This experimental setup was replicated as follows for the different exposure doses, for faeces; 3 Log₁₀ CFU (n=1), 4 Log₁₀ CFU (n=3), 5 Log₁₀ CFU (n=4) and 7 Log₁₀ CFU (n=5) and for liquid; 3 Log₁₀ CFU (n=1), 4 Log₁₀ CFU (n=3), 5 Log₁₀ CFU (n=3) and 7 Log₁₀ CFU (n=3). Consequently, the total number of houseflies analyzed for each type of material (faeces or liquid) and exposure dose was as follows: Faeces; 3 Log₁₀ CFU (n=16), 4 Log₁₀ CFU (n=48), 5 Log₁₀ CFU (n=64) and 7 Log₁₀ CFU (n=80) and for liquid; 3 Log₁₀ CFU (n=16), 4 Log₁₀ CFU (n=48), 5 Log₁₀ CFU (n=48) and 7 Log₁₀ CFU (n=48). In total, *Campylobacter* uptake was estimated for 208 houseflies exposed to contaminated faeces and for 158 houseflies exposed to contaminated liquid (2 houseflies were omitted from analysis). The houseflies' ability to contaminate the surface of AHB plates was assessed for half of the houseflies, i.e. 184.

Enumeration of C. jejuni in houseflies.

At the time of analysis, each cup was flushed with CO₂ and placed on ice to immobilize the houseflies before transfer of individual houseflies to a sterile Eppendorf tube with a sterile tweezer. Likewise, the lid of the AHB agar plates was lifted carefully in one side to allow flushing with CO₂ before transfer of the fly to an Eppendorf tube. Each fly was added 200 µl BHI and crushed with a sterile pellet pestle of polypropylene (Kimble™ Kontes™, Sigma-Aldrich Denmark A/S. Copenhagen, DK) before vortexing. Ten-fold dilutions of the fly homogenate (in duplicate) were spot inoculated (3× 15 µl spots) onto Abeyta-Hunt-Bark (AHB) agar plates. Additionally, for each experiment, three single non-exposed houseflies were included as negative controls. Each fly was added 200 µl BHI and after homogenization the suspension was plated onto AHB.

All agar plates were incubated at 42°C under microaerobic atmosphere for approximately 48 h before counting and calculation of CFU recovered per fly and visual inspection of negative control plates.

Statistical analysis.

All statistical analyses were performed with GraphPad Prism, 5.0 (GraphPad Software Inc., La Jolla, CA, US). Generally, CFU recovered per fly was transformed by Log₁₀ to approximate Normality of data, but not all data passed the Shapiro-Wilk normality test ($P < 0.05$), so non-parametric one way analysis of variance (Kruskal-Wallis) has been undertaken for analyzing the effect of exposure length (1 h vs. 4 h) and level (Log₁₀ CFU) using 0.05 as level of significance. To see if the contamination level on AHB plates depended on the houseflies' preceding exposure dose, the proportions of AHB positive plates for 5 Log₁₀ CFU and 7 Log₁₀ CFU were compared by Fischer's exact test for contaminated faeces as well as liquid.

It was tested if the campylobacter contamination observed on an AHB plate was correlated to the number of campylobacter recovered from the corresponding fly by Spearman's rank

correlation coefficient, non-parametric. This was tested for 36 AHB campylobacter-positive plates (negative AHB plates were excluded from the analysis).

Results:

Quantitative level of campylobacter in houseflies after exposure

For houseflies exposed to either *C. jejuni* contaminated faeces or liquid, an increased length of exposure from 1 h to 4 h did not increase the campylobacter numbers found in the houseflies (analysis by Kruskal-Wallis) as shown in figure 1 and figure 2.

Figure 1. The number of *Campylobacter jejuni* recovered from positive houseflies (mean Log₁₀ CFU and S.E.) after exposure to contaminated faeces for 1 h (white bars) or 4 h (black bars).

Figure 2. The number of *Campylobacter jejuni* recovered from positive houseflies (mean Log₁₀ CFU and S.E.) after exposure to contaminated liquid for 1 h (white bars) or 4 h (black bars).

Consequently, the *C. jejuni* acquisition level in houseflies after 1 h and 4 h of exposure has been pooled, resulting in 90.0% (n=80), 48.4% (n=64), 6.3% (n=48) and 0% (n=16) campylobacter-positive houseflies after exposure to faeces containing approximately 7, 5, 4, and 3 Log₁₀ CFU, respectively. The mean ($\pm$ SE) CFU recovered per campylobacter-positive fly was 2.0 $\pm$ 0.1, 0.8 $\pm$ 0.1, 0.3 $\pm$ 0.0 and 0 Log₁₀ CFU for exposure levels estimated to be mean ($\pm$ SE) 7.3 $\pm$ 0.1, 5.4 $\pm$ 0.1, 4.3 $\pm$ 0.1 and 3.4 $\pm$ 0.0 Log₁₀ CFU respectively. A maximum of 3.6 Log₁₀ CFU was found in individual houseflies.

For houseflies exposed to campylobacter contaminated liquid, 95.7% (n=47), 91.4% (n=47), 20.8% (n=48) and 6.3% (n=16) of houseflies were campylobacter-positive when exposed to mean ($\pm$ SE) 7.2 $\pm$ 0.2, 5.2 $\pm$ 0.2, 4.6 $\pm$ 0.0 and 3.6 $\pm$ 0.0 Log₁₀ CFU with a mean ($\pm$ SE) of 3.3 $\pm$ 0.2, 2.0 $\pm$ 0.1, 0.8 $\pm$ 0.2 and 0.3 $\pm$ 0.0 Log₁₀ CFU recovered per campylobacter-positive fly. A maximum of 4.8 Log₁₀ CFU was found in individual houseflies. No *C. jejuni* was found in the negative control houseflies.

The *C. jejuni* recovery from the houseflies decreased with reduced levels of exposure and at 3 Log₁₀ CFU only 1 fly was positive (n=16) after exposure to contaminated liquid. Although the exposure dose in both faeces and liquid overall affected the *C. jejuni* acquisition level by houseflies significantly (Kruskal-Wallis, $P < 0.0001$), there was no significant difference between an exposure dose of 4 and 5 Log₁₀ CFU (Dunn's Multiple Comparison Test). A comparison between faeces and liquid contaminated at similar levels also showed a higher acquisition level from liquid than from faeces for 7 and 5 Log₁₀ CFU, while no significant difference was obtained for 4 and 3 Log₁₀ CFU.

Houseflies' contamination of surfaces after exposure

C. jejuni surface contamination of AHB agar plates by houseflies previously exposed to contaminated material was observed for 36 out of 184 plates (19.6%) and the AHB plates were only contaminated by houseflies previously exposed to at least 5 Log₁₀ CFU as shown in Table 1. Most AHB plates became contaminated by houseflies previously exposed to liquid (45%, n=48) compared with houseflies exposed to faeces (19% n=72).

Tabel 1. Number of AHB plates contaminated with *C. jejuni* by houseflies left for 1 h on the plate surface after previous exposure to *C. jejuni* containing faeces or liquid.

No. <i>C. jejuni</i> contaminated AHB plates ¹			
Exposure dose and material ²			
Log ₁₀ CFU	Faeces	Log ₁₀ CFU	Liquid
3.4±0.0	0 (n=8)	3.6±0.0	0 (n=8)
4.3±0.1	0 (n=24)	4.6±0.0	0 (n=24)
5.4±0.1	1 (n=32)	5.2±0.2	8 (n=24)
7.3±0.1	13 (n=40)	7.2±0.2	14 (n=24)

¹Abeyta-Hunt-Bark (AHB) agar plates (9 cm Petri dish), n= number of tested plates.

²Houseflies exposed (1-4 h) to either faeces (5 g) or liquid (1 ml) contaminated with *C. jejuni* at different levels.

Also, the proportion of AHB contaminated plates were lower for houseflies exposed to faeces containing 5.4±0.1 Log₁₀ CFU *C. jejuni* than houseflies exposed to faeces containing 7.3±0.1 Log₁₀ CFU ($P=0.002$, Fischer's exact test). Contrary, for houseflies exposed to contaminated liquid, the exposure dose had no significant effect on the proportion of AHB contaminated plates ($P=0.147$, Fischer's exact test).

Moreover, for the 36 contaminated AHB plates (n=184), the *C. jejuni* Log₁₀ CFU observed on the plate (mean 0.8±0.1 Log₁₀ CFU and maximum 2.2 Log₁₀ CFU) was not correlated (Spearman r, $P=0.28$) to the numbers of *C. jejuni* recovered from the fly contaminating that plate (mean 2.8±0.2 Log₁₀ CFU), see Figure 3. Out of the 148 flies that did not contaminate the AHB plates, 66 flies were tested *C. jejuni* positive (mean 1.6±0.1 Log₁₀ CFU).

Figure 3. The number of *Campylobacter jejuni* deposited on the surface of 36 contaminated AHB agar plates (n=184) by houseflies previously exposed to *C. jejuni* containing material vs. the number of *C. jejuni* recovered from that respective fly.

Discussion:

In this study, it was found that the quantitative *C. jejuni* acquisition in houseflies after exposure to contaminated material depended on the previous exposure dose and type of material. The lack of a significant effect between the lower exposure doses was probably because of too few observations as only campylobacter-positive houseflies were incl. in the analysis. After exposure to 7.3±0.1 Log₁₀ CFU in 5 g faeces, the acquired *C. jejuni* level was 2.0±0.1 in the 90% of *C. jejuni* positive houseflies. Although the 10% negative houseflies were found among the houseflies exposed for the shortest time, the current exposure time (1 h vs. 4 h) did not exhibit a significant effect on the acquired *C. jejuni* level. For shorter exposure times of 10 s and 30 s, Pace *et al.* (2017) found that longer exposure times increased the uptake of *E. coli* O157:H7 and *Salmonella enterica* serovar Enteritidis, but also that the uptake level differed between the bacterial species. This may indicate that the bacterial uptake in houseflies occurs upon their first meal shortly after exposure (within 30 s) and may explain why no difference in acquisition level was observed between 1 h and 4 h of exposure in the current study. However, the thirst of houseflies may differ between studies due to various pre-experimental conditions influencing their feeding behavior. Still, even the houseflies treated similarly exhibited quite different feeding behavior during the current exposure study, as some houseflies went straight for the contaminated material while others appeared to sit still on the uncontaminated walls of the cups. No accumulation of *C. jejuni* seemed to take place

over time either as a consequence of feeding frequency or a continuous excretion and/or regurgitation of ingested bacteria. The latter is supported by findings by Gill *et al.* (2017) that found that vomitus and excreta were *C. jejuni* positive within the first 4 h after ingestion (inoculation). Also, the excretion interval in houseflies determined by their eosin coloring of filter paper has been calculated to be 6.5-32.6 min depending on sex and egg developmental stage based on the total excretion over 24 h (Sasaki *et al.* 2000).

The acquired *C. jejuni* level per fly (mean $2.0 \pm 0.1 \text{ Log}_{10} \text{ CFU}$) after exposure to mean $7.3 \pm 0.1 \text{ Log}_{10} \text{ CFU}$ in this study indicated a rather low uptake volume from faeces calculated to be $<0.1 \mu\text{l}$. This apparent uptake capacity of the houseflies implies that their chance of becoming campylobacter positive at lower exposure doses would be markedly reduced. However, as up to $3.6 \text{ Log}_{10} \text{ CFU}$ was found in individual houseflies (exposed to faeces) it indicated an uptake volume up to $2.5 \mu\text{l}$, which is similar to the 2-4 μl reported by Skovgaard *et al.* (2011). Moreover, another study showed that female and male houseflies differed in their acquisition of *Salmonella* Typhimurium from manure but not *Escherichia coli* (Thomson *et al.* 2017). In the current study, the houseflies were allocated randomly to the cups without determination of sex so it is unsure to what degree the obtained results may have been influenced by the sex of the houseflies. However, regardless of the potential uptake volume, even at a *C. jejuni* exposure dose approximately $2 \text{ Log}_{10} \text{ CFU}$ lower, i.e. $5.4 \pm 0.1 \text{ Log}_{10} \text{ CFU}$, nearly half of the houseflies was tested *C. jejuni* positive (48.4%) with a mean of $0.8 \pm 0.1 \text{ Log}_{10} \text{ CFU}$. This indicates that uptake of campylobacter is likely under natural conditions, as livestock animals and particularly poultry may shed up to $8 \text{ Log}_{10} \text{ CFU}$ per g faeces (Nielsen, 2002; Jensen *et al.* 2006; Rosenquist *et al.* 2006). So although the *Campylobacter* spp. prevalence in houseflies is found to be low in most studies (Hald *et al.* 2004, 2008; Hansson *et al.* 2007; Royden *et al.* 2016; Szalanski *et al.* 2004) and inoculated houseflies seem to turn campylobacter negative within 24 h (Gill *et al.* 2017; Skovgaard *et al.* 2011), the readily uptake of *C. jejuni* under natural exposure levels shown in this study supports that houseflies may play a significant role in transmission of *Campylobacter*. This is further backed up by an observed *C. jejuni* prevalence up to 50.7% in houseflies trapped in a chicken flock facility (Rosef and Kapperud, 1983).

When houseflies were exposed to approximately $4 \text{ Log}_{10} \text{ CFU}$ it was mainly houseflies exposed to a liquid suspension that became *C. jejuni* positive (20.8%). The lower *C. jejuni* acquisition in houseflies exposed to contaminated faeces versus liquid suspension may in part reflect that *C. jejuni* in the faeces set-up was 'hidden' in 5 g of faeces opposed to the presence in 1 ml of suspension in the liquid set-up. It may also reflect that houseflies must regurgitate to liquefy the faeces before ingestion reducing the actual uptake. However, it emphasizes the importance of avoiding puddles of contaminated water in the farm environment as they may enhance the risk of transferring *Campylobacter* spp. and also other zoonotic pathogens via contaminated houseflies.

The *C. jejuni* contamination of surfaces by houseflies required a previous exposure dose of minimum $5 \text{ Log}_{10} \text{ CFU}$, Table 1. Although the highest proportion of *C. jejuni* contaminated plates derived from houseflies previously exposed to liquid, there seemed to be no correlation between the contamination level found on the agar surface and the *C. jejuni* numbers recovered from the fly contaminating that plate, Figure 3. Another study showed transfer of *E. coli* O157:H7 onto spinach leaves tested 18 h after entry of houseflies that had been exposed to either inoculated manure or a lawn of bacteria in a Petri dish (Talley *et al.* 2009). Also, only 50% of the leaves tested *E. coli* O157:H7 positive when houseflies were exposed to manure reflecting a natural contamination level opposed to a bacterial lawn resulting in 100% positive leaves. This emphasizes the importance of assessing the effect of different levels of

exposure in naturally contaminated materials for a better understanding of the *C. jejuni* transfer potential by houseflies. Further, that the experimental set-up needs careful consideration for proper interpretation of implications. Also indicated by a preliminary study (data not shown) where agar surfaces became heavily campylobacter contaminated by houseflies, most likely because the houseflies had been placed directly on contaminated faeces more or less enforcing contamination and therefore not reflecting natural exposure conditions.

The observed campylobacter uptake in houseflies may also be of relevance in relation to rearing of fly larvae for feed purposes, as the adult flies will be involved in the reproductive cycles although not eaten themselves. However, a study by Bahrndorff *et al.* (2016) showed that while campylobacter was transmitted between larvae and pupal stages no campylobacter was transmitted further to the adults. This may indicate that 'recycling of infections' between stages can be prevented, but eggs from infected adults should be tested to clarify this.

In conclusion, the *C. jejuni* acquisition level in houseflies depended on the previous level of exposure and occurred down to 4 Log₁₀ CFU per 5 g faeces, which indicates a strong potential for contamination of houseflies in a natural farm setting. Consecutive surface contamination was only observed for houseflies exposed to at least 5 Log₁₀ but did not correlate to the *C. jejuni* numbers recovered from the houseflies.

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