Trypanosomatid parasites infecting managed honeybees and wild solitary bees

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A B S T R A C T

The parasite Crithidia mellificae (Kinetoplastea: Trypanosomatidae) infects honeybees, Apis mellifera. No pathogenic effects have been found in individual hosts, despite positive correlations between infections and colony mortalities. The solitary bee Osmia cornuta might constitute a host, but controlled infections are lacking to date. Here, we challenged male and female O. cornuta and honeybee workers in laboratory cages with C. mellificae. No parasite cells were found in any control. Parasite numbers increased 6.6 fold in honeybees between days 6 and 19 p.i. and significantly reduced survival. In O. cornuta, C. mellificae numbers increased 2–3.6 fold within cages and significantly reduced survival of males, but not females. The proportion of infected hosts increased in O. cornuta cages with faeces, but not in honeybee cages without faeces, suggesting faecal – oral transmission. The data show that O. cornuta is a host of C. mellificae and suggest that males are more susceptible. The higher mortality of infected honeybees proposes a mechanism for correlations between C. mellificae infections and colony mortalities.

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1. Introduction

Declines in wild bee species have been reported from several regions of the world (IPBES, 2016a,b), and pathogen spillover from managed honeybees (Apis mellifera) may contribute to these declines (Cameron et al., 2011; Szabo et al., 2012; Burkle et al., 2013; Fürst et al., 2014). Indeed, a variety of pathogens so far exclusively known for honeybees were detected in several wild bee species (e.g. Ravoet et al., 2014; McMahon et al., 2015; Dolezal et al., 2016). However, the detection of a pathogen in another species does not necessarily imply that this species can actually serve as a novel host. Indeed, individuals of certain species may simply carry pathogens, but not enable their replication (Ruiz-González and Brown, 2006a; Graystock et al., 2015). Without clear evidence that the pathogen is actually replicating, such observations only indicate that a host shift may have occurred. Controlled infection scenarios are required before deriving conclusions. Furthermore, being a novel host does not necessarily lead to the same progression of disease and intensity of clinical symptoms as in the original host and indeed it is well known that different sensitivities towards pathogens exist among, as well as within, species (Feng et al., 1990; Jensen et al., 2009). Life history traits (e.g. reproductive strategies and degrees of sociality), nutrition, other environmental traits and the genetic background of both host and pathogen usually determine the susceptibility towards pathogens and the outcome of infections (Fuxa and Tanada, 1987; Baer and Schmid-Hempel, 1999; Palmer and Oldroyd, 2003). For example, host genetic heterozygosity can enhance resistance to a pathogen (Penn et al., 2002), and higher genetic diversity in social groups can help reducing pathogen loads (Baer et al., 2001; Baer and Schmid-Hempel, 2001; Tarpy, 2003).

In the Hymenoptera, haploid males derive from unfertilized eggs (Gerber and Klostermeyer, 1970). The haploid susceptibility hypothesis predicts that such haploid males should be more susceptible to diseases compared with their diploid female counterparts, because they lack heterozygosity at immune loci (O’Donnell and Beshers, 2004). Indeed, male honeybees seem to be more susceptible to the microsporidian parasite Nosema ceranae compared with female workers (Retschnig et al., 2014). However, bumblebee (Bombus terrestris) males and workers did not differ in their susceptibility towards the trypanosomatid parasite Crithidia bombi (Ruiz-González and Brown, 2006b). It therefore appears that male susceptibility can vary substantially between species or...
populations, depending on the host species’ unique set of parasites and underlying genetics governing host resistance (O’Donnell and Beshers, 2004).

Trypanosomatidae are unicellular eukaryotic flagellate parasites of invertebrates, vertebrates and plants. Dixenous (with two hosts in their life cycle) species are agents of a number of diseases in humans, domestic animals and plants, and monoxenous (with a single host) species are restricted to insects (for details see Lukeš et al., 2018; Maslov et al., 2019). In A. mellifera, a monoxenous trypanosomatid parasite was first identified as Leptomonas apis Lotmar and later described as Crithidia mellificae (Langridge and McGhee 1967; McGhee and Cosgrove 1980). A recent taxonomic re-examination, however, has revealed that the globally predominant trypanosomatid in honeybees is instead the newly described species Crithidia sp. (Schwarz et al., 2015). Both Crithidia and Lotmaria colonize the hindgut (primarily the rectum (Langridge and McGhee, 1967; Schwarz et al., 2015)) and interact with the host’s intestinal cells (Votýpka et al., 2015). Through their flagellum they attach to the host’s gut wall, where they form a single layer that leads to the formation of hemidemesomes, and subsequently to damage of the intestinal cells (Hubert et al., 2017). These lesions can decrease the health of the host at the individual level as well as colony level (Schaub, 1994; Boulanger et al., 2001; Brown et al., 2003). After oral ingestion, C. bombi cells multiply and are transmitted to novel hosts (Schaub, 1994; Boulanger et al., 2001; Brown et al., 2003). However, comparatively little is known about C. mellificae and its effects on honeybees (Morse, 1990; Bailey and Ball, 1991; Higes et al., 2016). Even though C. mellificae infections appear not to significantly reduce longevity of adult honeybee workers (Langridge and McGhee, 1967; Higes et al., 2016), positive correlations between C. mellificae infection levels and honeybee colony winter mortalities suggest possible pathogenic effects (Runckel et al., 2011; Cormann et al., 2012; Ravoet et al., 2013).

Recently, two common solitary bee species, Osmia cornuta and Osmia bicolor, have been proposed to constitute other hymenopteran hosts of C. mellificae (Ravoet et al., 2015; Schwarz et al., 2015). This conclusion was based on PCR detections of C. mellificae in field-sampled Osmia individuals in Belgium and in the USA (Ravoet et al., 2015; Schwarz et al., 2015). However, to evaluate the role of Osmia spp. as hosts of C. mellificae and the possible impact of a pathogen spillover from managed honeybees, controlled infection experiments are required.

Here, we challenged honeybee workers as positive controls and male and female O. cornuta with C. mellificae to examine whether this parasite is able to infect this solitary bee and to re-examine infection loads and mortality in their original host. We used host body mass, as well as survival and pathogen infection level, as measures of susceptibility (Retschnig et al., 2014). We hypothesize that (i) C. mellificae replicates in A. mellifera, but does not affect longevity, (ii) O. cornuta can serve as a host by showing C. mellificae cell replication, but infections do not significantly affect survival, (iii) C. mellificae infections significantly reduce body mass of A. mellifera as well as O. cornuta, and (iv) male O. cornuta are more susceptible in terms of reduced survival as predicted by the haploid susceptibility hypothesis.

2. Materials and methods

2.1. Study design

The study was performed in Bern, Switzerland between March and April 2017. Osmia cornuta cocoons (n = 150 females and 150 males) were purchased from WAB Mauerbienenzucht, Konstanz, Germany and kept at 4 °C until before being randomly allocated to treatments (exposed to C. mellificae cells) or uninfected controls. Male and female cocoons were separated (Bosch and Blas, 1994). Then, 25 cocoons of each sex were placed into cages (47.5 × 47.5 × 47.5 cm) BugDorm – Insect rearing cage, control: n = 2 cages, C. mellificae exposure: n = 4; Supplementary Fig. S1) and maintained in the laboratory at room temperature (RT; 25 °C) and in darkness. Four days later, emerged adult bees were counted, and cocoon skins and cocoons with non-hatched bees were removed. Bees were fed with 50% sucrose solution (w/v) ad libitum for 19 days.

2.2. Honeybee workers

Known age cohorts of freshly emerged workers without clinical symptoms of disease were randomly allocated to eight hoarding cages (80 cm²) (control: n = 3, C. mellificae exposure: n = 5; n = 32 workers each) and maintained in darkness at 30 °C and 60% relative humidity (RH) for 19 days (Williams et al., 2013). A. mellifera (ATCC ® 30254™) cell culture was purchased from ATCC® (American Type Culture Collection, Wesel, Germany, www.atcc.org). Following the manufacturer’s instructions, the cells were cultivated in ATCC® 355 medium (Supplementary Table S1) and culture tubes (SARSTEDT, Germany) were tightly sealed with the screw cap and incubated at 30 °C. On a daily basis, cell growth within the medium was visually investigated using light microscopy and density of living cells calculated using a Neubauer counting chamber (Hornitzky, 2008).

2.3. Crithidia mellificae cultivation

A 50 % (w/v) sucrose solution was prepared with a final concentration of 25,000 living C. mellificae cells/bee (assuming equal distribution via individual consumption) (Schwarz and Evans, 2013; Williams et al., 2013). Each cage was provided with 400 μl of the C. mellificae sucrose solution or only with sucrose (controls). Every 4 h, food consumption was checked, and as soon as bees had consumed the entire 400 μl, uncontaminated 50% (w/v) sucrose solution was provided ad libitum until the end of the experiment.

2.4. Crithidia mellificae inoculation

Survival was recorded every 24 h, dead individuals were removed from their cages and immediately stored at −80 °C. On the day before inoculation (day 0), and at time intervals p.i. (days 6, 10, 15 and 19), bees were investigated for living C. mellificae cells: bees were individually weighed to assess body mass and anesthetized with CO₂. Then, C. mellificae cells were quantified (Cantwell, 1970).

2.5. Survival, body mass and C. mellificae cell counts

DNA was extracted using routine protocols (Evans et al., 2013) and stored at −20 °C until use. PCR runs were performed for bees on day 0, and days 15 and 19 p.i. with C. mellificae by using the MyTaq® kit (BioLine, Germany) with 1 ng of the extracted DNA and following the manufacturer’s protocols. A pair of species-specific C. mellificae primers (Cr ITS1-IR1/5.8R; Table 1) and an established PCR protocol were used (Ravoet et al., 2015).

C. mellificae was quantified by quantitative PCR (qPCR) using KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems) with 15 ng of extracted DNA, 0.24 μl of forward and reverse specific primers (10 pmol/μl) (Table 1) and 6 μl of 2 × reaction buffer in a total of 12 μl final reaction volume (de Miranda et al., 2013). The qPCR
cycling profile was set following Tritschler et al. (2017). Purified PCR products of known concentrations \((10^{-2}-10^{-6}\text{ ng})\) were used as standard curves on each individual plate, together with non-template controls and 18S rRNA as a reference gene (Ward et al., 2007).

For *O. cornuta*, the primers designed for *A. mellifera* were used to quantify the 18S rRNA gene (Ward et al., 2007). To confirm the gene identity between *A. mellifera* and *O. cornuta*, a pair of primers (Supplementary Table 2) was designed to amplify the 62 bp section included in the qPCR assay. After sequencing, the 18S rRNA gene identity in *O. cornuta* was confirmed and uploaded onto the European Nucleotide Archive (ENA).

### 2.7. Statistical analyses

All variables were tested for normality by using Shapiro–Wilk’s Tests. Body mass, *C. mellificae* cell counts and *C. mellificae* copies were normally distributed (Shapiro–Wilk’s Test, \(P > 0.05\)) in *O. cornuta* females and values were therefore compared using a one-Way ANOVA. However, they were non-normally distributed (Shapiro–Wilk’s Test, \(P < 0.05\)) in honeybee workers and *O. cornuta* males, and were therefore analysed with a Kruskal–Wallis One-Way ANOVA. Post-hoc comparisons for body mass between groups of bees and *C. mellificae* cell counts over time were conducted using a multiple pairwise comparisons test (Bonferroni Multiple Comparison Test). Survival analyses were performed using Kaplan–Meier cumulative survival curves and Log-Rank values were calculated to determine differences amongst treatment groups. XY scatter plots and Spearman’s correlation coefficient were used to assess possible correlations between *C. mellificae* cell counts and *C. mellificae* copies. All statistical analyses and figures were performed using NCSS (NCSS version 12, Statistical Analysis Software, Kaysville, Utah, USA).

### 2.8. Data accessibility

The complete raw data is available on the Dryad repository at [10.5061/dryad.ck2v06j](10.5061/dryad.ck2v06j).

### 3. Results

An overview of all descriptive statistics and normality tests regarding body mass assessment, *C. mellificae* cell counts and *C. mellificae* genomic equivalent copies is given in Table 2.

A total of 235 *O. cornuta* (138 males and 97 females) emerged within the first 4 days post–cage initiation. In total, five females and four males died before the beginning of *C. mellificae* exposure. Fifty-six cocoons did not emerge within the first 4 days and were therefore excluded from the experiment. The *C. mellificae* contaminated sucrose solution was entirely consumed within 24 h in all *C. mellificae*–exposed *A. mellifera*, as well as in the *O. cornuta* cages.

#### 3.1. Body mass and survival

The body mass of all groups of bees did not significantly change over time p.i. (all \(P > 0.05\)). Overall, there was no significant treatment effect on body mass between control and *C. mellificae*–exposed individuals (all \(P > 0.2\), Fig. 1, details are provided in Table 2).

By the end of the experiment (19 days p.i.), 75.5% of the control honeybees \((n = 83)\) and 63.2% of the *C. mellificae*–exposed honeybees \((n = 128)\) were alive, thereby showing a significantly reduced survival in *C. mellificae*–exposed individuals \((P = 0.006\); Fig. 2A). However, *C. mellificae* exposure did not significantly affect survival of *O. cornuta* females compared with their controls \((P = 0.318\); Fig. 2B). Here 80.7% of the control \((n = 25)\) and 68.1% of *C. mellificae*–exposed individuals \((n = 56)\) survived throughout the entire experiment. *Osmia cornuta* males showed the lowest survival of all groups of bees. By the end of the experiment 39% of the controls \((n = 43)\) and none of the *C. mellificae*–exposed individuals \((n = 81)\) were alive, thereby showing significantly reduced survival in *C. mellificae*–exposed *O. cornuta* males \((P = 0.032\); Fig. 2C).

#### 3.2. Crithidia mellificae cell counts

No *C. mellificae* cells were detected in any of the control bees, nor in any individuals examined on the day prior to inoculation (day 0; Fig. 3A–C). From a total of 80 individual honeybee workers sampled p.i. for *C. mellificae* counting, 32.5% of the bees showed an infection. Cell counts were conducted for 25% of the individuals on day 6 p.i., for 30% on day 10 p.i., for 50% on day 15 p.i. and for 25% on day 19 p.i. (Table 3). *Crithidia mellificae* cell counts ranged between 12,500 and 962,500 with a median of 268,750 (75,000 (95% lower confidence limit (LCL)) and 412,500 (95% upper confidence limit (UCL))) *C. mellificae* cells per bee \((n = 26)\) and increased 6.6 fold between days 6 and 19 p.i. \((P < 0.01\); Fig. 3A).

From the 41 *O. cornuta* females sampled p.i., 68.3% showed *C. mellificae* cells. The proportion of infected individuals increased over time p.i. (day 6: 40% infected, day 10: 60% infected, day 15: 90% infected and day 19: 90% infected; Table 3). *Crithidia mellificae* cell counts did not significantly change between days 6 and 19 p.i. \((P > 0.05\); Fig. 3B), and ranged between 25,000 and 337,500 *C. mellificae* cells per bee \((mean: 143,379.5 \pm 90,696.5 \text{ S.D., } n = 28)\). From the 30 individual male *O. cornuta* sampled over the entire p.i. period, 90% showed *C. mellificae* cell counts. Similar to females, the proportion of infected individuals increased over time p.i. (day 6: 70% infected, day 10: 100% infected and day 15: 100% infected; Table 3) and cell counts did not significantly change over time \((P > 0.05\); Fig. 3C). *Crithidia mellificae* cell counts ranged between 37,500 and 1,125,000 with a median of 175,000 (100,000 (95% LCL) and 212,500 (95% UCL)) *C. mellificae* cells per bee \((n = 27)\).

The sum of *C. mellificae* cell counts from individual bees deriving from the same *C. mellificae*–exposed cages resulted in a 2–3.6 fold increase in *C. mellificae* cell counts compared with *C. mellificae* cell numbers introduced into each cage at the beginning of the experiment (Table 4).
Table 2

Results of the infection experiments. Apis mellifera, and Osmia cornuta female (\( \$ \)) and male (\( # \)) body mass, as well as \( \text{C. mellificae} \) cell counts and copies are shown.

| Variables            | Groups | Treatments | Sample size | Mean S.D. | Percentiles | Distribution | Shapiro-Wilk’s Test (P) |
|----------------------|--------|------------|-------------|-----------|-------------|--------------|------------------------|
| Body mass \[mg\]     | A. mellifera | Control | 25          | 107.48    | 17.81       | 87.0 – 145.8 | Non-parametric         | 0.008                  |
|                     |        | C. mellificae-exposed | 100       | 113.06    | 22.15       | 81 – 150     | Non-parametric         | <0.001                 |
| C. mellificae cell counts \[cells/bee\] | A. mellifera | Control | 26          | 342,788.5 | 311,552     | 12,500 – 268,750 | Non-parametric         | 0.002                  |
|                     |        | C. mellificae-exposed | 28       | 173,214.3 | 90,696.52   | 25,000 – 320,625 | Parametric            | 0.308                  |
|                     | O. cornuta | Control | 25          | 59.84     | 21.49       | 32 – 104     | Non-parametric         | 0.007                  |
|                     |        | C. mellificae-exposed | 40       | 56.7      | 18.64       | 28 – 98      | Non-parametric         | <0.001                 |
|                     |        | O. cornuta | Control | 51          | 59.84       | 21.49       | Non-parametric         | 0.578                  |
|                     |        | C. mellificae-exposed | 10       | 101,454     | 924,401.7   | 129,542 – 2,717,425 | Non-parametric         | 0.027                  |

There was no significant treatment effect between control and \( \text{C. mellificae} \) – exposed individuals in all groups of bees (Kruskal–Wallis One-Way ANOVA: all \( P \) values > 0.2). However, body mass between groups of bees (\( A. mellifera: n = 125; O. cornuta \( \$ \): \( n = 77 \); \( O. cornuta \# \): \( n = 65 \)) significantly differed (Kruskal–Wallis One-Way ANOVA with Bonferroni Multiple Comparison Test: \( P < 0.001 \)). All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines above and beneath box), and outliers (black dots). Significant differences (\( P < 0.001 \)) between groups are indicated by different letters (a, b, and c).

3.3. \text{Crithidia mellificae} quantification

No \( \text{C. mellificae} \) were detected in any of the controls, nor in any individuals examined on the day before inoculation (day 0). In honeybee workers, \( \text{C. mellificae} \) genomic equivalent copies ranged between 409.4 and 24,714,550 with a median of 4675 (95% LCL) and 631,203 (95% UCL) copies per bee on days 15 and 19 p.i. \( (n = 40) \). In \( O. cornuta \) females, \( \text{C. mellificae} \) genomic equivalent copies on days 15 and 19 p.i. ranged between 2678 and 2,156,369 with a mean of 894,725 (\( \pm 553,250 \) S.D., \( n = 43 \); \( P <0.01 \)). \( \text{C. mellificae} \) genomic equivalent copies ranged between 129,542 and 2,717,425 with a median of 708,845 (142,003 (95% LCL) and 2,717,425 with a median of 708,845 (142,003 (95% UCL)) copies per bee in male \( O. cornuta \) on day 15 p.i. \( (n = 10) \). A significant positive correlation between \( \text{C. mellificae} \) cell counts and \( \text{C. mellificae} \) genomic equivalent copies per bee was found in all groups of bees \( (n = 43; P < 0.001, \text{Fig. 4}) \).

4. Discussion

Our results provide clear evidence that the honeybee parasite \( \text{C. mellificae} \) can infect \( O. cornuta \) and further suggest that there are sex-specific differences in host susceptibility in this solitary bee species. While infected \( O. cornuta \) males showed markedly reduced survival, \( O. cornuta \) females showed only a slight, statistically not significant reduction in survival, in agreement with the haploid susceptibility hypothesis. There was no significant effect of \( \text{C. mellificae} \) infection on host body mass in any group of bees, probably because all bees were fed ad libitum. Furthermore, our results support a fecal – oral transmission route for \( \text{Crithidia} \), because the proportion of \( \text{C. mellificae} \) – infected \( O. cornuta \) individuals increased over time in cages with faeces, which was not the case in the honeybee cages without any faeces. Finally, infected honeybee workers showed reduced longevity, which may provide a mechanistic explanation for the observed correlations between overwintering colony mortalities and \( \text{C. mellificae} \) infection levels.

Even though previous laboratory studies suggested that \( \text{C. mellificae} \) does not affect longevity in infected adult worker honeybees (Langridge and McGhee, 1967; Higes et al., 2016), our data show significantly reduced survival compared with the...
controls. Differences in host and/or parasite genetics and different environmental impacts (e.g., host colony nutritional stage) could possibly explain the different findings (Australia: Langridge and McGhee (1967); Spain: Higes et al. (2016), Apis mellifera iberiensis: Higes et al. (2016), Apis mellifera carnica × Apis mellifera mellifera hybrids: this study). Susceptibility of honeybee larvae towards the fungus Ascosphaera apis (the causative agent of chalkbrood disease), can vary substantially within and between different subspecies (Jensen et al., 2009). Similarly, Bombus lucorum and Bombus terrestris differ in their susceptibility towards the microsporidian Nosema bombi (Rutrecht and Brown, 2009). Indeed, local adaptation and genotypic variations are well known to cause variation in host resistance (Kulincevic, 1986; Schmid-Hempel, 1998). The different experimental conditions call for standard protocols enabling comparison of studies (e.g., the COLOSS BEEBOOK, https://coloss.org/core-projects/beekbook/). By the end of the experiment 19 days p.i. (= post – emergence for A. mellifera), 75.5 % of control honeybee workers were alive. These control survival rates are in line with previous laboratory hording cage studies (Retschnig et al., 2014; Straub et al., 2016), thereby suggesting that the treatment mortalities actually reflect pathogenic effects of the trypanosomatid.

Fig. 2. Kaplan–Meier survival curves show the decline of the cumulative survival (%) over time. (A) Significantly reduced survival was found in Crithidia mellificae – exposed Apis mellifera (n = 128) compared with controls (n = 83) (Log-rank Test: $P = 0.006$). (B) Osmia cornuta females (♀) exposed to C. mellificae (n = 56) did not show a significant difference compared with controls (n = 25) (Log-rank Test: $P = 0.318$). (C) A significant difference was found between O. cornuta males (♂) exposed to C. mellificae (n = 81) compared with their controls (n = 43) (Log-rank Test: $P = 0.032$). Black dots indicate censored data (bees taken for C. mellificae counting). Significant differences between treatment groups are indicated by: * $P < 0.05$; ** $P < 0.01$. 

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were exclusively fed with sucrose solution due to practical reasons. In our study, all bees had been shown to support honeybee immunocompetence and cell counts did not significantly change in C. mellificae cell counts significantly increased over time p.i. (Kruskal–Wallis One-Way ANOVA, $P < 0.01$). In Osmia cornuta females, C. mellificae cell counts did not significantly change over time p.i. (ANOVA with Bonferroni Multiple Comparison Test, $P > 0.05$). C. mellificae cell counts did not significantly change in O. cornuta males ($P > 0.05$). All boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines above and beneath box), and outliers (black dots). Significant differences ($P < 0.05$) are indicated by different letters (a, b and c).

**Fig. 3.** _Crithidia mellificae_ cell counts on specific days pre – infection and p.i. (A) In Apis mellifera, C. mellificae cell counts significantly increased over time p.i. (Kruskal–Wallis One-Way ANOVA with Bonferroni Multiple Comparison Test, $P < 0.01$); (B) In Osmia cornuta females (♀), C. mellificae cell counts did not significantly change over time p.i. (ANOVA with Bonferroni Multiple Comparison Test, $P > 0.05$); (C) _Crithidia mellificae_ cell counts did not significantly change in O. cornuta males ($P > 0.05$) (Kruskal–Wallis One-Way ANOVA with Multiple Comparison Test, $P > 0.05$). Both infected and control female O. cornuta males (♂) and control females did not show significantly different cell counts.

Host nutrition might also play a role, in particular protein supply. The significantly increased mortality of male O. cornuta compared with their controls and with both infected and control female O. cornuta hints at the haploid susceptibility hypothesis (O’Donnell and Beshers, 2004) and/or higher sensitivities towards stress under the given experimental conditions. Several cases have been identified where parasites impact individual host mortality only under stressful conditions (Schaub and Lüscher, 1989; Jaenike et al., 1995; Brown et al., 2000), presumably because hosts are in such poor condition that they cannot compensate for increased parasite-related defense costs (e.g. Moret and Schmid-Hempel, 2000). We therefore cannot exclude that the observed higher mortality of infected male O. cornuta may simply reflect more stressful experimental conditions, but it is rather unlikely. Moreover, it has been suggested that male honeybees are more sensitive to laboratory cage conditions than workers (Oertel, 1953; Roman et al., 2010; Retshnig et al., 2014), which could explain the observed low survival in the O. cornuta control males.

However, regardless of the treatment, on day 4 p.i., over 80% of male O. cornuta were alive. Male O. cornuta immediately mate with freshly emerged females that appear approximately 4 days after male cocoon eclosion (Bosch and Blas, 1994; Monzón et al., 2004). Although, to our knowledge, published information about the longevity of male O. cornuta in the field is largely lacking, the mortality rate seems to be sex – specific in monandrous species (O. cornuta is most likely monandrous (Seidelmann, 2014)) with males having a much shorter lifespan than females (Wiklund et al., 2003). Furthermore, reproduction and survival are generally negatively correlated (Harshman and Zera, 2006), and it therefore appears that male longevity is less relevant as long as mating has occurred successfully. Whether the reduced survival observed in this laboratory study would also occur in the field and to what extent it would have an influence on their reproductive success remains to be tested.

In contrast to N. ceranae (Retshnig et al., 2014) and C. bombi (Brown et al., 2003), C. mellificae infection did not have any signifi-
Table 3
Proportion of infected individuals on specific days post-inoculation (p.i.). The proportion of infected Apis mellifera workers, Osmia cornuta females (♀) and Osmia cornuta males (♂) are shown on days 6, 10, 15 and 19 p.i.

| Days p.i. | A. mellifera | O. cornuta ♀ | O. cornuta ♂ |
|----------|--------------|---------------|---------------|
| 6        | 25           | 40            | 70            |
| 10       | 30           | 60            | 100           |
| 15       | 50           | 90            | 100           |
| 19       | 25           | 90            | 100           |

Table 4
Crithidia mellificae cell counts per individual Osmia cornuta cage. For each C. mellificae exposed cage, the sum of individual C. mellificae cell counts and the number of infected individuals are shown. On the day of inoculation, a total of 1,000,000 C. mellificae cells were inserted into each cage.

| Cage | Sum of C. mellificae cell counts | Number of infected individuals |
|------|----------------------------------|-------------------------------|
| 1    | 2,287,506                        | 13                            |
| 2    | 3,662,507                        | 18                            |
| 3    | 2,837,509                        | 14                            |
| 4    | 2,025,006                        | 10                            |

Fig. 4. Crithidia mellificae cell counts and C. mellificae genomic equivalent copies on days 15 and 19 p.i. No C. mellificae cells were found by visual counting or quantitative PCR (qPCR) on day 0, the day before C. mellificae exposure (data not shown). There was a significant positive correlation between cell counts and genomic equivalent copies per bee in all groups of bees (Spearman \( r = 0.65, P < 0.001; n = 43 \)).

significant effect on body mass in any group of bees, probably because the bees were fed ad libitum. Even though body mass appears to be a good proxy for the overall health status of a bee (e.g., Bosch and Vicens, 2002), the elevated adult mortality in infected male O. cornuta and A. mellifera workers unequivocally shows the virulence of this trypanosomatid. We found a positive correlation between cell counts and genomic equivalent copies deriving from qPCR in all groups of bees. Therefore, the less costly visual counting appears to be an efficient method for quantification. Crithidia mellificae cell counts in the positive honeybee controls significantly increased over time p.i., thereby unequivocally showing infectivity of the pathogen. However, C. mellificae cell counts in individual Osmia bees did not significantly change over time p.i., but C. mellificae cells could be found up to 19 days p.i. (i.e. the end of the experiment). We did not quantify C. mellificae in infected bees that died during the experiment. This might help explain why we did not see an increase in C. mellificae cell counts over time in O. cornuta. However, total cell numbers per Osmia cage were 2–3.6 fold higher than the total number of cells introduced into each cage during exposure. This provides clear evidence for C. mellificae replication and therefore a positive infection. In contrast, other trypanosomatid parasites (e.g. Leptomonas seymouri) can colonize transient hosts (e.g. sand flies (Diptera)) without causing an infection which is reflected in very low parasite numbers in the transient host as well as in a decreasing proportion of infected individuals over time p.i. (Kraeva et al., 2015).

While the proportion of infected A. mellifera bees did not increase over time, this was clearly the case for O. cornuta. This is in line with the previously reported faecal – oral transmission of C. mellificae (Langridge and McGhee, 1967; Schwarz et al., 2015). Indeed, O. cornuta defecated frequently in their cages, which was never observed in any of the honeybee cages. Therefore, our findings provide indirect support for the faecal – oral route of C. mellificae transmission. The previously reported higher C. mellificae infection levels in dying overwintering honeybee colonies (Kavoet et al., 2013) may therefore be due to the presence of faeces in such colonies. Given that holds true, beekeepers should be advised to clean hives to limit C. mellificae infections in colonies.

Our study clearly shows that the honeybee trypanosomatid parasite C. mellificae can infect O. cornuta in the laboratory, which therefore constitutes another hymenopteran host. Male O. cornuta individuals may be more susceptible compared with females, in agreement with the haploid susceptibility hypothesis. The faecal – oral route seems to be the transmission route of C. mellificae. Field studies are now required to test for spillover potential from managed to wild bees or vice versa. The reduced survival of C. mellificae – infected honeybee workers contributes to our understanding of the positive correlation between this trypanosomatid parasite and honeybee colony mortalities. Even though L. passim seems to be more abundant globally, C. mellificae could nevertheless pose a higher risk than previously thought and further investigations of this long known, but still understudied pathogen, appear prudent.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.03.006.

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