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Trypanosomatid parasites infecting managed honeybees and wild solitary bees ☆

Verena Strobl [a,b,*], Orlando Yañez [a], Lars Straub [a], Matthias Albrecht [b], Peter Neumann [a,c]

[a] Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[b] Agroecology and Environment, Agroscope, Zürich, Switzerland
[c] Swiss Bee Research Centre, Agroscope, Bern, Switzerland

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ABSTRACT

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 one 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

☆ Note: Nucleotide sequence data reported in this paper are available in the European Nucleotide Archive (ENA) databases under the accession number(s), **PRJEB26880** (**ERP108906**).

* Corresponding author at: Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland.
E-mail address: verena.strobl@vetsuisse.unibe.ch (V. Strobl).

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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 *Lotmaria passim* (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 hemidesmosomes, and subsequently to damage of the intestinal cells (Hubert et al., 2017). These lesions can decrease the health of the host at the individual 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 *B. terrestris* hosts via a fecal-oral transmission route (Schmid-Hempel, 2001). 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; Cornman et al., 2012; Ravoet et al., 2013).

Recently, two common solitary bee species, *Osmia cornuta* and *Osmia bicornis* 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 cm^3) (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).

2.3. Crithidia mellificae cultivation

A *C. mellificae* (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).

2.4. Crithidia mellificae inoculation

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.

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

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 CO_2. Then, *C. mellificae* cells were quantified (Cantwell, 1970).

2.6. DNA extraction and PCR assays

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).

Crithidia 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 µmol/L) (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

Table 1
Primers used for PCR and quantitative PCR (qPCR) diagnosis of *Crithidia mellificae* and the 18S rRNA reference gene in individual *Apis mellifera* and *Osmia cornuta* bees.

Target	Primer	Sequence (5′–3′)	Size [bp]	Reference
C. mellificae	ITS1 IR1	GCT GTA GGT GAA CCT GCA GCA GCT GGA TCA TT	410	Ravoet et al. (2015)
	ITS1 5.8R	GGA AGC CAA GTC ATC CAT C		
C. mellificae	qCrFw1	TCC ACT CTG CAA ACG ATG AC	153	Runckel et al. (2011)
	qCrRev1	GGG CCG AAT GGA AAA GAT AC		
A. mellifera 18S rRNA	A. mel – 18S – 955F	TGT TTT CCC TGC CCG AAA G	62	Ward et al. (2007)
	A. mel – 18S – 1016R	CCC CAA TCC CTA GCA CGA A		

cycling profile was set following Tritschler et al. (2017). Purified PCR products of known concentrations (10^{-2}–10^{-6} 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 S2) was designed to amplify a 228 bp section that includes the 62 bp section amplified in the qPCR assay. After sequencing, the 18S rRNA gene identity in *O. cornuta* was confirmed and uploaded onto the European Nucleotide Archive (ENA), PRJEB26880 (ERP108906); https://www.ebi.ac.uk/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 doi: 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 ± 90,696.5 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 *Crithidia mellificae* infection experiments. *Apis mellifera*, and *Osmia cornuta* female (♀) and male (♂) body mass, as well as *C. mellificae* cell counts and copies are shown.

Variables	Groups	Treatments	Sample size	Mean	S.D.	Percentiles					Shapiro-Wilk's Test (*P*)	Distribution
						Min.	0.5	Median	0.95	Max.		
Body mass [mg]	*A. mellifera*	Control	25	107.48	17.81	87	87.6	103	148.8	150	0.008	Non-parametric
		C. mellificae-exposed	100	113.06	22.15	81	86.0	107	165.45	175	<0.001	Non-parametric
	O. cornuta ♀	Control	26	136.42	35.7	67	74.35	135	205.55	208	0.659	Parametric
		C. mellificae-exposed	51	128.75	21.67	80	90.8	132	161.2	188	0.578	Parametric
	O. cornuta ♂	Control	25	59.84	21.49	32	33.8	51	103.1	104	0.007	Non-parametric
		C. mellificae-exposed	40	56.7	18.64	28	30.65	49.5	96.9	98	<0.001	Non-parametric
C. mellificae cell counts [cells/bee]	*A. mellifera*	*C. mellificae*-exposed	26	342,788.5	311,552	12,500	16,875	268,750	936,250	962,500	0.002	Non-parametric
	O. cornuta ♀	*C. mellificae*-exposed	28	173,214.3	90,696.52	25,000	30,625	175,000	320,625	337,500	0.308	Parametric
	O. cornuta ♂	*C. mellificae*-exposed	27	220,833.3	220,003.3	37,500	47,500	175,000	905,000	1,125,000	<0.001	Non-parametric
C. mellificae quantification [copies/bee]	*A. mellifera*	*C. mellificae*-exposed	40	300,7910	5,982,086	409.38	452.75	4675.14	22,065,710	24,714,550	<0.001	Non-parametric
	O. cornuta ♀	*C. mellificae*-exposed	21	894,725.2	553,250.2	2678.04	31,567.7	707,992.4	2,109,669	2,156,369	0.443	Parametric
	O. cornuta ♂	*C. mellificae*-exposed	10	101,4543	924,401.7	129,542	129,542	708,845.3	2,717,425	2,717,425	0.027	Non-parametric

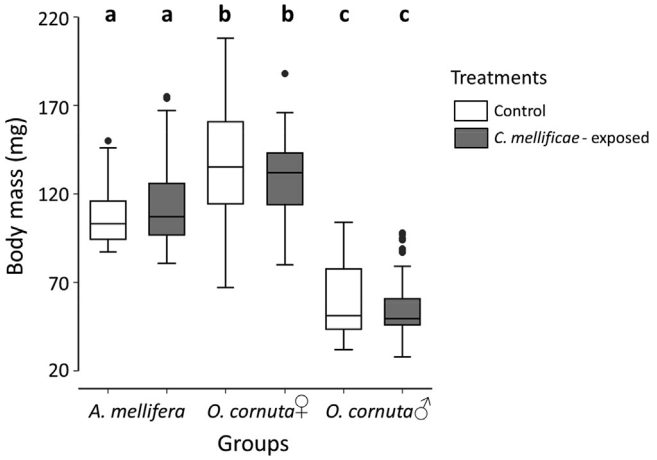

Fig. 1. Body mass of *Apis mellifera* and female (♀) and male (♂) *Osmia cornuta*. There was no significant treatment effect between control and *Crithidia mellificae* – exposed individuals in all groups of bees (Kruskal–Wallis One-Way ANOVA: all *P* values > 0.2). However, body mass between groups of 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. Crithidia mellificae quantification

No *C. mellificae* were detected in any of the controls, nor in any individuals examined on the day before inoculation (day 0). In honeybee workers, *C. mellificae* genomic equivalent copies ranged between 409.4 and 24,714,550 with a median of 4675 (956 (95% LCL) and 631,203 (95% UCL)) copies per bee on days 15 and 19 p. i. ($n = 40$). In *O. cornuta* females, *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 (±553,250 S.D., $n = 21$). *Crithidia mellificae* genomic equivalent copies ranged between 129,542 and 2,717,425 with a median of 708,845 (142,003 (95% LCL) and 2,563,281 (95% UCL)) copies per bee in male *O. cornuta* on day 15 p.i. ($n = 10$). A significant positive correlation between *C. mellificae* cell counts and *C. mellificae* genomic equivalent copies per bee was found in all groups of bees ($n = 43$, $P < 0.001$, Fig. 4).

4. Discussion

Our results provide clear evidence that the honeybee parasite *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 *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 *Crithidia*, because the proportion of *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 *C. mellificae* infection levels.

Even though previous laboratory studies suggested that *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

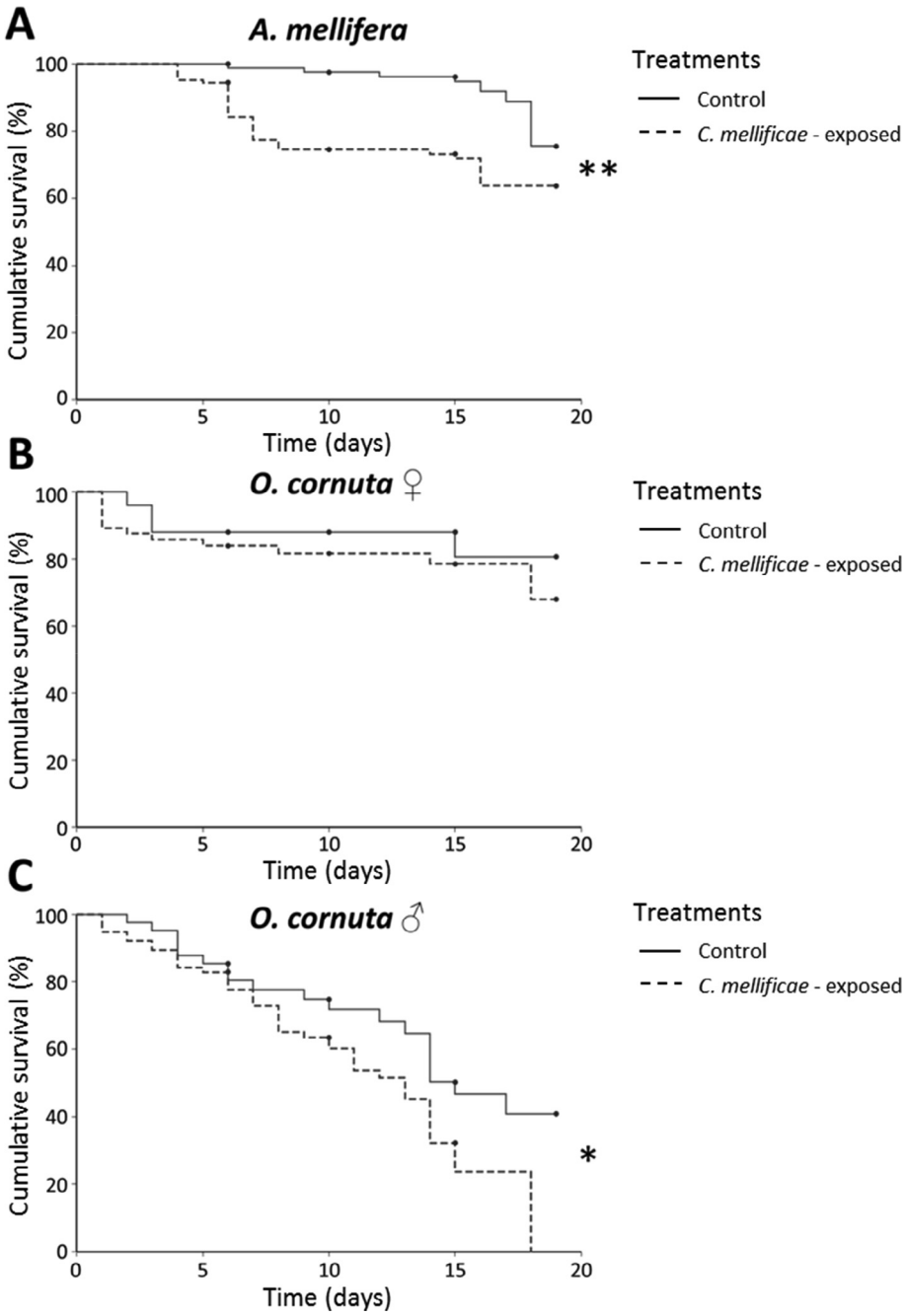

Fig. 2. Kaplan–Meier survival curves show the decline of the cumulative survival (%) over time. (A) Significantly reduced survival was found in *Crithidia mellificae* – exposed *Apis mellifera* ($n = 128$) compared with controls ($n = 83$) (Log-rank Test: $P = 0.006$). (B) *Osmia cornuta* females (♀) 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 ($n = 43$) (Logrank Test: $P = 0.032$). Black dots indicate censored data (bees taken for *C. mellificae* counting). Significant differences between treatment groups are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$.

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/beebook/). 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.

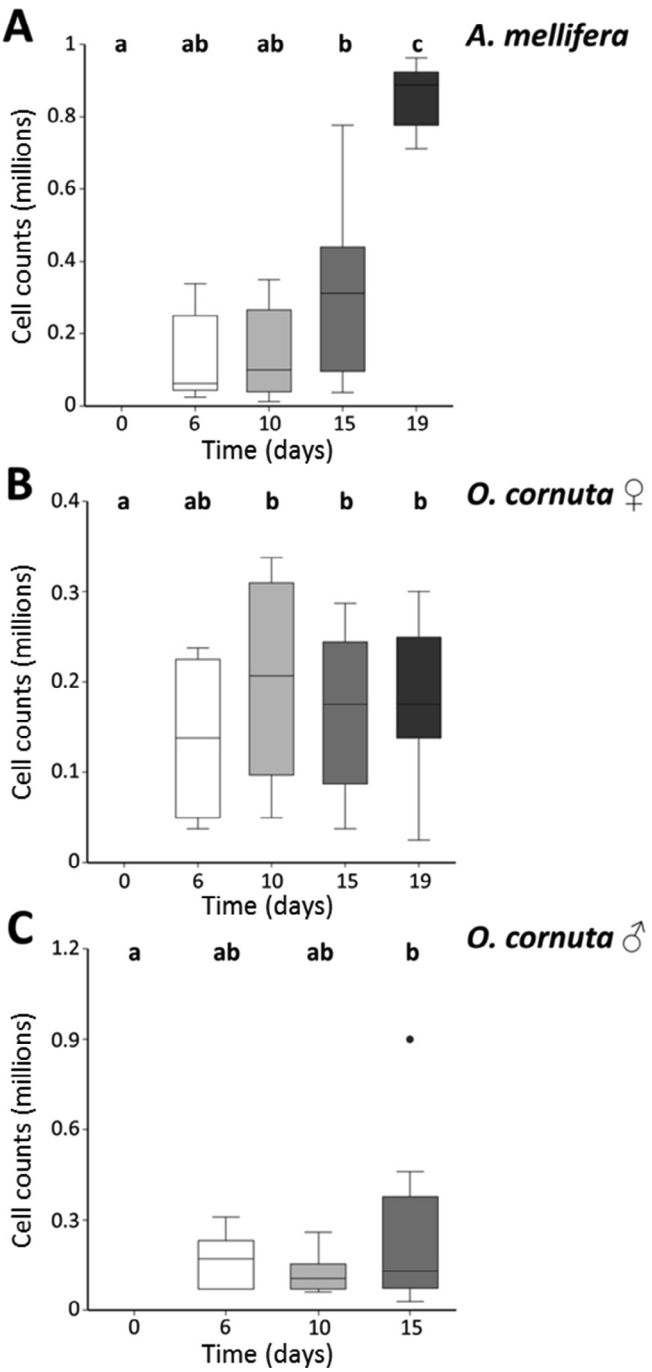

Fig. 3. *Crithidia mellificae* cell counts on specific days pre – infection and p.i. No *C. mellificae* cells were found in any bees on the day before inoculation (day 0). (A) In *Apis mellifera*, *C. mellificae* cell counts significantly increased over time p.i. (Kruskal–Wallis One-Way ANOVA with Bonferroni 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 (♂) (Kruskal–Wallis One-Way ANOVA with Multiple Comparison Test, $P > 0.05$). 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).

Host nutrition might also play a role, in particular protein supply has been shown to support honeybee immunocompetence and the capability to fight diseases (e.g. Rinderer and Elliott, 1977; Alaux et al., 2010; Tritschler et al., 2017). In our study, all bees were exclusively fed with sucrose solution due to practical reasons. Bee guts full of pollen would have compromised *C. mellificae* cell counting. A previous study has shown that higher pollen quantity increases *N. ceranae* intensity, but also enhances the survival of honeybees (Jack et al., 2016). However, previous studies on *A. mellifera* testing the impact of *C. mellificae* on worker survival, similarly exclusively fed those sugar water (honey candy) (Langridge and McGhee, 1967; Higes et al., 2016). Therefore the reduced survival of *C. mellificae* – exposed honeybee workers is unlikely to result from protein deprivation and potentially offers a mechanistic explanation for the negative correlation between *C. mellificae* infections and colony winter survival (Runckel et al., 2011; Ravoet et al., 2013).

In contrast to *O. cornuta* males and *A. mellifera* workers, *C. mellificae* – infected *O. cornuta* females did not show significantly reduced survival. Individual honeybee workers usually do not reproduce and function as replaceable units; hence the death of a single and even many workers does not necessarily compromise colony fitness due to superorganism resilience (Straub et al., 2015). However, in *O. cornuta* each and every individual directly contributes to reproduction (or not). Furthermore, due to the female's essential role in egg laying and the capability of many insect species to store the sperm of males long-term in dedicated organs called spermathecae (including *Osmia* spp. e.g. Raw and O'Toole (1979); Seidelmann (2015)), female solitary bees per se should display a more efficient defense against a variety of stressors (including pathogens) compared with workers of the social insects. Exposure of *Drosophila melanogaster* to the trypanosomatid parasite *Jaenimonas drosophilae* causes activation of numerous immune genes as well as a reduction in female fecundity (Hamilton et al., 2015). Trypanosomatid infections and bee immune system interaction should be addressed in the future.

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 Lösch, 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; Retschnig 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* (Retschnig et al., 2014) and *C. bombi* (Brown et al., 2003), *C. mellificae* infection did not have any signif-

Table 3
Proportion of infected individuals on specific days 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.			
		6	10	15	19
Proportion of infected individuals [%]	*A. mellifera*	25	30	50	25
	O. cornuta ♀	40	60	90	90
	O. cornuta ♂	70	100	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

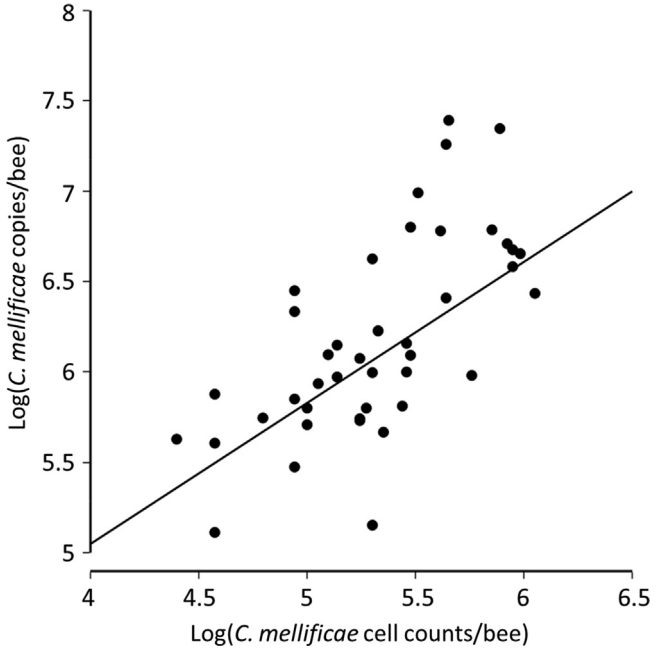

Fig. 4. *Crithidia mellificae* cell counts and *C. mellificae* genomic equivalent copies on days 15 and 19 p.i. No *C. mellificae* cells were found by visual counting or quantitative PCR (qPCR) on day 0, the day before *C. mellificae* exposure (data not shown). There was a significant positive correlation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

icant 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 (Ravoet 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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