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## Role of egg-laying behavior, virulence and local adaptation in a parasitoid's chances of reproducing in a new host

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## 1. Introduction

Co-evolution, which occurs when species have a reciprocal effect on each other's evolution, is one of the major processes explaining biodiversity (Thompson, 1999). According to Thompson, 1994, parasites have a special place in the study of co-evolution because theirs is the most prevalent lifestyle and they exhibit the most extreme degree of specialization to other species. Among parasites, parasitoids are particular. Indeed, parasitoid females lay their eggs in or on another arthropod to ensure the development of their progeny, which results in the death of the host, whereas most parasites do not kill their host. Hence parasitoids are considered as intermediate between parasites and predators (Godfray, 1994).

Parasitoid reproductive success lies on a wide spectrum of adaptations to their host. Notably, it depends on many traits, including virulence, which indicates the ability of the parasitoid to overcome host immune response. In endoparasitoids, which inject their eggs in their host haemocoel, the main host immune defense with which they must comply is the cellular response called encapsulation (Lavine and Strand, 2002; Salt, 1968). To avoid or suppress encapsulation, parasitoids have developed a large arsenal of weapons. The most studied are venoms produced by eponymous glands, teratocytes formed from the membranes that envelop parasitoid eggs and polydnavirus (Asgari and Rivers, 2011; Beckage and Drezen, 2012; Strand, 2014). The latter is a nice example of mutualistic viruses. The polydnviridae family groups viruses from multiple origins which were domesticated and integrated in several lineages of Ichneumonoidea. Due to their integration into parasitoid genomes, polydnvirus are vertically transmitted (Fleming and Summers, 1991). They are produced in the form of viral particles containing double-stranded DNA segments, in specialized cells of the calyx, a tissue located in the upper part of the lateral oviducts (Marti, 2003; Wyler and Lanzrein, 2003). These particles are secreted into the lumen of the lateral oviducts and thus injected in the host with the eggs. After oviposition, viral particles infect host cells where virulence genes carried by DNA segment are expressed. Translated virulence proteins disturb host development and suppress its immune responses, allowing the growth of parasitoid eggs (Beckage and Gelman, 2004; Glatz et al., 2004). Polydnvirus differentiation is involved in the evolution of host range (Herniou et al., 2013), as shown in the *Cotesia* genus member of the Microgastrinae family (Braconidae), which harbors polydnvirus from the genera Bracovirus (Branca et al., 2017, 2011; Jancek et al., 2013).

Like virulence, oviposition strategy can evolve in response to constraints in developmental resources. This is well illustrated by clutch size evolution, a classical topic in ecology, notably in birds (Lack, 1947). Its study is especially relevant in the case of host-parasitoid interactions where host resource is often variable. Many factors such as host size, host quality, host availability, host previous parasitism, parasitoid egg load or experience can influence clutch size, suggesting that females are able to combine information to adjust it (Godfray, 1994; Ikawa and Okabe, 1985; Pexton and Mayhew, 2005; Quicke, 1997; Rosenheim and Rosen, 1991). Beyond the plasticity of this trait, large numbers of injected eggs could also be adaptive, allowing the parasitoid to overcome host resistance by saturating its immune system (Blumberg and Luck, 1990; Kapranas et al., 2012; Rosenheim and Hongkham, 1996; Salt, 1968). The impact of the number of eggs injected on virulence may be addressed. For that purpose, species showing variation in clutch size among individuals is necessary. Such variation was described for *Cotesia typhae* (Fernández-Triana) (Hymenoptera, Braconidae) between two strains from different geographic origins namely Kobodo and Makindu which can interbreed and produce fertile offspring (Benoist et al., 2017).

*C. typhae* is a gregarious endoparasitoid specialized on caterpillars of *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera, Noctuidae) and was found in Kenya, Tanzania and Ethiopia (Kaiser et al., 2015). This species is prosynovigenic because females emerge with almost all their mature eggs. Thanks to molecular, ecological and morphological analyses, *C. typhae* was recently distinguished from *Cotesia sesamiae*, a generalist species composed of several populations with different host ranges. Local adaptation to *S. nonagrioides* appears to be the main factor responsible for the speciation of *C. typhae* (Branca et al., 2011; Kaiser et al., 2015, 2017a, 2017b).

1 In the context of biological control, it is necessary to understand the factors that drive the adaptation of the  
2 natural enemy to the targeted host. *C. typhae* is one of the sister species of *Cotesia flavipes* (Cameron),  
3 which is used worldwide for biological control against maize and sugarcane Lepidoptera stemborers (Postali  
4 Parra and Coelho, 2019). The strict specificity of *C. typhae* for *S. nonagrioides* makes it a good potential  
5 biocontrol agent against this important crop pest (Cordero et al., 1998; Eizaguirre and Fantinou, 2012),  
6 currently in expansion in France (Rousseau, 2009).

7  
8 In Benoist et al., 2017, the reproductive success of several strains of *C. typhae* from Kenya in a French  
9 population of *S. nonagrioides* was investigated. Among the strains used, those from Kobodo and Makindu  
10 localities were respectively the most and the least virulent against this new host population. Females from  
11 the Kobodo strain injected more eggs, despite an equal initial egg load, and induced a higher expression of  
12 two virulence genes in the first host encountered, in comparison to Makindu females. Given that viral  
13 particles are injected along with eggs and that Kobodo females inject more eggs at a time, it was  
14 hypothesized that they may also inject more viral particles in the host, which may explain the difference of  
15 virulence genes expression and thus the difference of virulence.

16  
17 The first objective of the present work is thus to test if the difference in parasitism success results from a  
18 difference in the number of viral particles injected, and if this number varies together with the number of  
19 eggs injected. More globally, the second objective is to characterize the reproductive potential of the  
20 parasitoid strains in a biocontrol perspective. The last objective is to investigate if the better pre-adaptation  
21 of the Kobodo strain to the French host may come from local adaptation to a more resistant host  
22 population.

23  
24 For these purposes, Kobodo and Makindu parasitoid females were allowed to oviposit in three successive  
25 hosts of the French population. At each oviposition rank, host acceptance, number of eggs injected,  
26 parasitism success (used as an estimate of the parasitoid virulence), offspring number and sex-ratio were  
27 measured. The relative quantity of viral segments injected was measured at the two first ovipositions  
28 (parasitism success collapsed at the third oviposition). Finally, egg-laying behavior, parasitism success and  
29 offspring traits of both parasitoid strains were measured on their African sympatric and allopatric host  
30 populations.  
31

## 2. Materials and Methods

### 2.1 Biological Material

The *C. typhae* strains were established from individuals emerging from parasitized larvae collected in two Kenyan field localities: Kobodo (0.679S, 34.412E; West Kenya; collected in 2013) and Makindu (2.278S, 37.825E; South-East Kenya; collected in 2010-2011), which were used to name the strains. They were reared as isofemale lines at the Evolution, Génome, Comportement et Ecologie (EGCE, Gif-sur-Yvette) laboratory from 2015.

Three strains of *S. nonagrioides* were used as host, one from southwest France and two from Kenya. The founder individuals of the two Kenyan strains originated from Kobodo and Makindu, and the founder individuals of the French strain originated from 3 localities in southwest France: Cudos (44.3897N, -2.2183W), Biarotte (43.5640N, 1.2550W) and Longage (43.3680N, 1.1926E).

### 2.2 Insect rearing and parasitism

The larvae of *S. nonagrioides* were reared on an artificial diet (adapted from Overholt et al., 1994) at 26°C, ca. 60% relative humidity (RH) under a photoperiod of 16:8 (Light:Dark). Three week old larvae from the Makindu host strain were used for the rearing of *C. typhae*, as both parasitoid strains showed similar high reproductive success on this host population. Larvae intended to be parasitized were retrieved and fed for at least 24 hours with fresh maize stems before parasitism to increase their acceptance by the *C. typhae* female. On the day of parasitism, the host larvae were placed individually under a 2 cm diameter plastic top with one *C. typhae* female until the ovipositor insertion was observed, insuring that each larva was parasitized only once. Parasitized larvae were then placed in a Petri dish with artificial diet at 27°C under rearing condition. Once the parasitoid larvae had emerged from the host and spun their cocoons, each parasitoid cocoon mass, corresponding to one progeny, was placed in a disposable plastic box (500 ml) with honey droplets and a water imbibed cotton wool ball under the same rearing conditions. When adults emerged, temperature was decreased to 24°C and the photoperiod changed to 12:12 to lengthen their life expectancy. In the plastic boxes, siblings were free to mate (mating was not controlled). Females were used for parasitism 1 day after emergence to allow time for mating.

Experiments were conducted in the same conditions as parasitoid rearing. When successive ovipositions were performed, each *C. typhae* female was offered one host larva once a day. This was repeated three times at most (the mean life expectancy of *C. typhae* females is 3 days). Females were one-day old when offered their first host. They were kept individually in a plastic tube (height: 9.5cm, diameter: 2cm) with honey droplets and a piece of water-imbibed cotton wool at 21°C between each host exposure (temperature was decreased to lengthen parasitoid life expectancy).

### 2.3 Phenotypic traits measure

Several phenotypic traits were measured: host acceptance, number of eggs injected, parasitism success, offspring number, sex-ratio, and the quantity of viral segments injected (see next section for this last trait).

Host acceptance is the proportion of females that accepted to oviposit within 3 minutes of host exposure. In order to avoid counting females twice, a female was not considered for further oviposition if it refused to oviposit once. Parasitism success corresponds to the proportion of stung larvae from which parasitoid larvae emerge. To estimate the number of eggs injected at each oviposition rank, females were divided into four groups corresponding to the number of ovipositions they performed (zero to three), and we calculated the difference between the mean numbers of eggs in the ovaries of females of each group. For all dissections, we counted together mature oocytes and the few immature ones. The ovaries dissection protocol is available in Benoist et al., 2017. The offspring number corresponds to the total number of parasitoid larvae that emerged from one host larva (one progeny). The mean offspring number did not include cases without progeny and was estimated only from mixed-sex progenies. Indeed, in haplo-diploid

1 insects like *Cotesia*, unmated females give all-male progeny and in *C. typhae* offspring number varied  
2 significantly between mated and unmated females (Benoist et al., 2017). Similarly, sex-ratio was estimated  
3 only on mixed-sex progenies. The sex-ratio of *C. typhae* can be determined only at adult stage, so progenies  
4 with more than 20 % of larval mortality (cases where larvae died inside cocoons due to rearing conditions)  
5 were not taken into account for the estimation of sex-ratio.  
6  
7

#### 8 **2.4 Comparison of the quantity of viral segments injected in the host**

9 To compare the quantity of viral segments injected in the French host between each treatment (*C. typhae*  
10 strain x oviposition rank) we developed a relative qPCR assay. The *S. nonagrioides* larvae were weighed  
11 before parasitism. Only larvae with a weight comprised between 260 and 290 mg were kept to ensure that  
12 the quantities of hemolymph retrieved were homogeneous between samples. The larvae were dissected 2h  
13 post-parasitism. They were anesthetized by cold exposure (-20°C) for 20 minutes. Pro and true legs were  
14 cut to collect as much hemolymph as possible. Hemolymph samples were stored at -20°C until DNA  
15 extraction. Before DNA extraction, PBS (Phosphate-Buffered Saline) was added to samples to a final volume  
16 of 200 µl. DNA extractions were performed with the NucleoSpin Tissue kit (Macherey-Nagel) following the  
17 manufacturer protocol. A RNase (DNase free, Roche) treatment (20 min, 37°C) was included.  
18

19 In *Cotesia congregata* and *Cotesia vestalis*, the bracovirus is organized in 35 viral segments (Jancek et al.,  
20 2013). A similar number is expected in *C. typhae*. We decided to quantify 15 viral segments, representing  
21 about half the putative segment number. The segment-specific primer pairs were designed for qPCR, based  
22 on the sequence of *C. sesamiae* Kitale bracovirus segments published in Jancek et al., 2013 and available in  
23 GenBank (accession numbers: HF562906 to HF562931). The *S. nonagrioides* ribosomal protein gene *rps3*  
24 and mitochondrial gene *col* were used as references for normalization. The primers' efficiencies were  
25 calculated using a calibration curve with four 5-fold successive dilution points using a pool of DNA samples  
26 and were close to 100%. The primer sequences are presented in Table 1. PCR reactions were run on a  
27 CFX96 (Bio-Rad) in 10 µl comprising: 2.4 µl of water, 5 µl of 2X FastStart Universal SYBR Green Master  
28 (Roche), 0.3 µM of each primer, and 2 µl of sample DNA (3 µg/µl). A negative control was included for each  
29 primer pair and all samples were amplified in duplicate. The amplification protocol was the same for all  
30 primer pairs: 10 min of denaturation at 95°C, followed by 45 cycles of 10 sec of denaturation at 95°C and 30  
31 sec of annealing/elongation at 60°C followed by a melting curve step.  
32

33 For each primer pair a single peak was observed for all melting curves, indicating specificity and  
34 homogeneity of the amplifications. A single threshold mode was used to determine the quantification cycle  
35 ( $C_q$ ) values. If the maximum  $C_q$  standard deviation observed between duplicates was higher than 0.4 then  
36 the samples were removed. Relative quantities of viral segments were compared using the  $\Delta\Delta C_q$  method  
37 [ABI User Bulletin #2 (11-15)].  
38  
39

#### 40 **2.5 Data analysis**

41 All statistical analyses were performed using R Software (R Core Team, 2018) with *agricolae*, *fifer* and  
42 *emmeans* additional packages. The significance threshold was set at 0.05 p-value. Except for the viral  
43 segments relative quantities, all phenotypic traits were analysed using generalized linear model (GLM) to  
44 test the effect of each factor (*C. typhae* strain, oviposition/presentation rank, *S. nonagrioides* strain). The  
45 error family used in GLM was: binomial for the acceptance and the parasitism success; quasi-poisson for  
46 the number of eggs remaining in ovaries and the offspring number; quasi-binomial for the sex-ratio. When  
47 a significant effect was observed, a multiple comparison using Tukey's HSD test was performed on the GLM  
48 data. The viral segments' relative quantities were analysed using the Kruskal-Wallis test, followed by  
49 multiple comparison with Holm correction.  
50  
51  
52

1 **Table 1: Sequence of the segment-specific and reference genes primers used for qPCR.** The segment  
 2 numbers correspond to those from *Cotesia sesamiae* Kitale in Jancek et al., 2013.

Target	Orientation	Sequence
Segment 1	Forward	5'-ACGGAAGCACAGAAAGAACCT-3'
	Reverse	5'-ACCGAAGCTTTCAGGACACA-3'
Segment 2	Forward	5'-GTTTCGGGTGCGTTTCTGTC-3'
	Reverse	5'-ACTTTGCCATCACGCGTTT-3'
Segment 4	Forward	5'-TCCGCTGCTGTCTCACTTTT-3'
	Reverse	5'-GCCGGGTCCAATTGTTGTTC-3'
Segment 13	Forward	5'-AGAACCGATTTGCTCCCGTT-3'
	Reverse	5'-ACTGCTTTCTAGGTGCTTCAGA-3'
Segment 14	Forward	5'-AGCGTAGTTGATGGCGTTCA-3'
	Reverse	5'-TGCTTCTGAATATGGGCGC-3'
Segment 24	Forward	5'-TCGTCGTCGAAGCTTTCGGAA-3'
	Reverse	5'-CCGATCTCACTTGCGAACCT-3'
Segment 26	Forward	5'-TTGATGAGGGAGACGGGGAT-3'
	Reverse	5'-CCCAGGTGTCTTCTATCGGC-3'
Segment 28	Forward	5'-CGCAGGGTATGAAGAGTCCG-3'
	Reverse	5'-ACAACAGAGAGTCGACCAGC-3'
Segment 30	Forward	5'-CCAGGCTGCTGAACCAAAAC-3'
	Reverse	5'-AGCGTCCTGTGGCATAGAAA-3'
Segment 32	Forward	5'-GCGATTTAGCGTGCCAAGAC-3'
	Reverse	5'-TGACGTCAAGCAGCGAAAAG-3'
Segment 33	Forward	5'-CCCACACTCATTTCACCTCCA-3'
	Reverse	5'-GTTCTTACCAGTCGAGCCGG-3'
Segment 35	Forward	5'-TGTACGTCCCAGTAGCACCT-3'
	Reverse	5'-GGAGTGAAGAATCTGCCCC-3'
COI	Forward	5'-GGAGCCCCAGATATAGCATTTC-3'
	Reverse	5'-TCATCCTGTTCCAGCCCCAT-3'
RPS3	Forward	5'-GGGAGCTTGCTGAAGATGGC-3'
	Reverse	5'-AGACTGCTCGGGGATGTTGA-3'

3  
4

### 3. Results

#### 3.1 Reproductive success in new host population and correlation between oviposition behavior and virulence

To study the reproductive strategy and the mechanisms behind the parasitism success, females from Kobodo and Makindu *C. typhae* strains were allowed to oviposit several times in the French host. Different phenotypic traits were measured at each oviposition rank.

##### 3.1.1 Host acceptance

Overall, host acceptance varied significantly with the presentation rank (p-value =  $5.947 \times 10^{-14}$ ) but not between *C. typhae* strains (p-value = 0.085). Almost all females (>90%) accepted to oviposit at the first host presentation (Table 2). At the second host presentation, the acceptance dropped significantly to around 65 % for both strains. At the third host presentation a significant difference was observed between the two strains. Indeed, while the host acceptance by Kobodo parasitoid did not differ between second and third presentations, host acceptance by Makindu females increased to 78.5 %.

**Table 2: French host acceptance by *C. typhae* parasitoid strains.** n = number of *C. typhae* tested females. Conditions with the same letter are not significantly different (p-value > 0.05, Tukey's HSD test following binomial GLM).

Presentation rank	<i>C. typhae</i> strain	Host acceptance		
		% accepted	n	Statistical test
First host	Kobodo	91.10	123	ab
	Makindu	94.60	111	a
Second host	Kobodo	64.00	114	c
	Makindu	66.10	115	c
Third host	Kobodo	62.30	61	c
	Makindu	78.46	65	b

##### 3.1.2 Number of eggs injected

Fecundities of the two *C. typhae* strains were not significantly different: females from Kobodo and Makindu strains produced around 200 oocytes (Table 3). However, the parasitoid strain had a significant effect on the dynamic of egg depletion (p-values = 0.027). At the first oviposition Kobodo females injected around 110 eggs, when Makindu females injected around 72 eggs representing respectively 56% and 34 % of their egg load (Table 3). At the second oviposition, the number of eggs injected was reduced by more than half for Kobodo females, whereas only an 11% decrease was observed for Makindu ones. Hence, the number of eggs injected was almost similar for the two strains, around 50-65 eggs. At the third oviposition, Makindu females injected three times more eggs (around 26) than Kobodo ones. This highlighted a difference of oviposition behavior between Kobodo and Makindu females, Kobodo females injecting most eggs in the first host while Makindu females allocated their eggs more equitably along successive ovipositions. Despite these different allocation dynamics, the cumulated predicted number of eggs injected was similar between Kobodo and Makindu: Kobodo=167.87; Makindu=162.36.



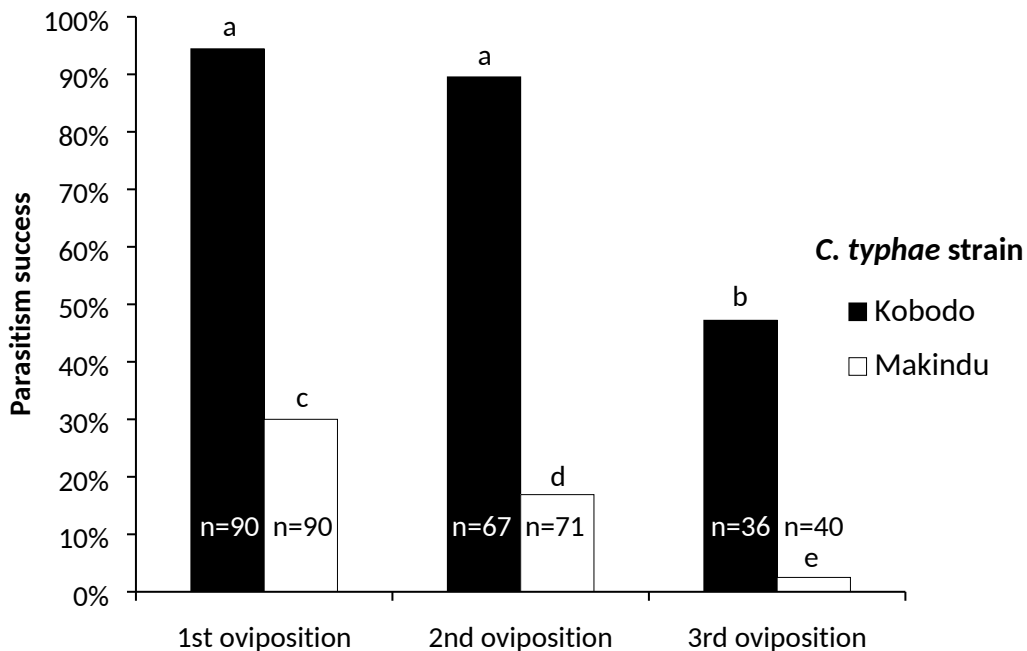
1 **Table 3: Number of remaining oocytes in ovaries and predicted number of eggs injected per host larva**  
 2 **after each oviposition rank of *C. typhae* parasitoid strains in the French host.** n = number of females  
 3 dissected; se = standard error. Conditions with the same letter are not significantly different (p-value >  
 4 0.05, Tukey's HSD test following quasi-poisson GLM). The number of eggs injected is deduced from the  
 5 difference between the mean numbers of eggs in the ovaries before oviposition or after one, two or three  
 6 ovipositions.  
 7

Oviposition rank	<i>C. typhae</i> strain	Number of oocytes in ovaries			Statistical test	Predicted number of eggs injected per host larva
		mean	se	n		
No oviposition	Kobodo	194.6	± 3.17	116	a	
	Makindu	212.3	± 6.58	44	a	
First oviposition	Kobodo	84.9	± 4.3	46	c	109.7
	Makindu	139.9	± 4.18	71	b	72.4
Second oviposition	Kobodo	34.9	± 2.48	52	e	50.0
	Makindu	75.7	± 5.05	36	c	64.2
Third oviposition	Kobodo	26.7	± 2.36	38	e	8.2
	Makindu	49.9	± 2.97	47	d	25.8

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 10  
 11  
 12

1 3.1.3 Parasitism success

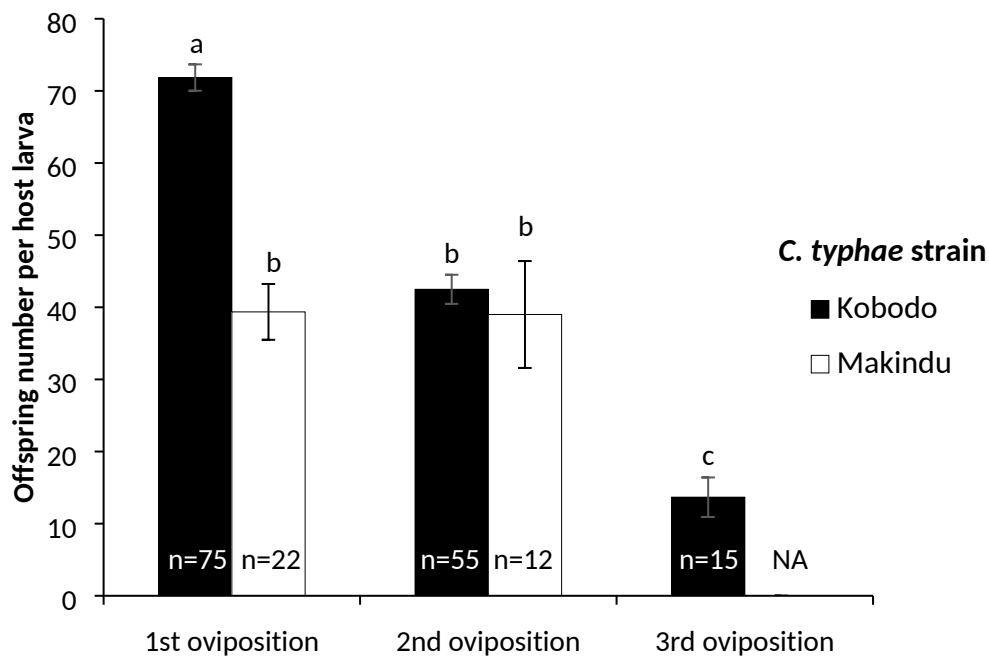
2  
3 *Cotesia* strain and oviposition rank had a significant effect on the parasitism success (respectively p-value <  
4  $2 \times 10^{-16}$  and p-value =  $6.744 \times 10^{-8}$ ). A similar pattern was observed at the first and the second oviposition  
5 rank: Makindu females had low parasitism success (<30%), while it was high for Kobodo ones (~90%) (Fig.  
6 1). The parasitism success decreased significantly at the third oviposition, to a level of 50% for Kobodo  
7 females and to less than 5% for Makindu ones. Globally, the parasitism success decreased along oviposition  
8 rank, and the Makindu strain is characterized by its low parasitism success whatever the oviposition rank.  
9 Comparison of these results with those from Table 3 showed that the difference of parasitism success  
10 between Kobodo and Makindu parasitoids in the French host could not be explained by a difference in the  
11 number of eggs they injected: Despite both strains injecting similar number of eggs at the second  
12 oviposition rank, their parasitism success is markedly different. The great majority of host larvae that were  
13 not successfully parasitized developed normally into pupae, and very few died (3 to 5 % of the total host  
14 larvae sample per treatment, as observed in unparasitized host larvae).



16 **Fig. 1: Parasitism success at each oviposition rank of *C. typhae* parasitoid strains in the French host.**  
17 Parasitism success corresponds to the proportion of stung larvae from which parasitoid larvae emerged. n =  
18 number of host parasitized. Conditions with the same letter are not significantly different (p-value > 0.05,  
19 Tukey's HSD test following binomial GLM).  
20

21  
22  
23 3.1.4 Offspring number

24  
25 The offspring number decreased significantly along the oviposition rank (p-value <  $2 \times 10^{-16}$ ) (Fig. 2). It  
26 followed more or less the same dynamic as the number of injected eggs, with again a significant effect of *C.*  
27 *typhae* strain (p-value =  $7.629 \times 10^{-11}$ ). At the first oviposition, Kobodo females had a higher offspring  
28 number than Makindu females. The two strains had a similar offspring number, near 40, at the second  
29 oviposition. At the third oviposition the offspring number of Kobodo females was low, and that of Makindu  
30 could not be estimated due to the lack of progeny development, despite a high rate of host acceptance for  
31 oviposition (the only progeny observed in Fig. 1 was discarded because only composed of males).  
32



1  
2 **Fig. 2: Offspring number per host larva at each oviposition rank of *C. typhae* parasitoid strains on the**  
3 **French host.** n = number of progenies; error bar = standard error; NA = No progeny in this case. Conditions  
4 with the same letter are not significantly different (p-value > 0.05, Tukey's HSD test following negative  
5 binomial GLM).  
6  
7

### 8 3.1.5 Sex-ratio

9 The sex-ratio did not vary significantly with the *C. typhae* strains, while a significant effect of oviposition  
10 rank was detected (p-value =  $0.037 \times 10^{-5}$ ): The proportion of females in the progeny decreased significantly  
11 along ovipositions. The sex-ratios of the progeny were female-biased at the two first ovipositions with  
12 about two-thirds to three-quarters being females (Table 4). It notably decreased at the third oviposition  
13 rank for Kobodo females, for which a balanced sex-ratio was observed. However, pairwise comparisons  
14 were not significant, which was explained by the low number of replicates.  
15

16 **Table 4: Sex-ratio at each oviposition rank of *C. typhae* parasitoid strains in the French host.** n = number  
17 of progenies; se = standard error. Conditions with the same letter are not significantly different (p-value >  
18 0.05, Tukey's HSD test following quasi-binomial GLM).

Oviposition rank	<i>C. typhae</i> strain	Sex-ratio (%females)			
		mean %	se	n	Statistical test
First oviposition	Kobodo	68.41	± 2.15	70	a
	Makindu	76.01	± 3.24	16	a
Second oviposition	Kobodo	62.66	± 3.03	43	a
	Makindu	62.91	± 8.71	8	a
Third oviposition	Kobodo	45.74	± 6.81	8	a
	Makindu	No progeny in this case			

19  
20

1 3.1.6 Viral segment quantification  
2

3 Given that the number of eggs injected in the host caterpillars did not explain the difference of parasitism  
4 success between Kobodo and Makindu parasitoids in the French host, a relative quantification of viral DNA  
5 segments injected was performed on several segments to see if it could explain this difference.  
6

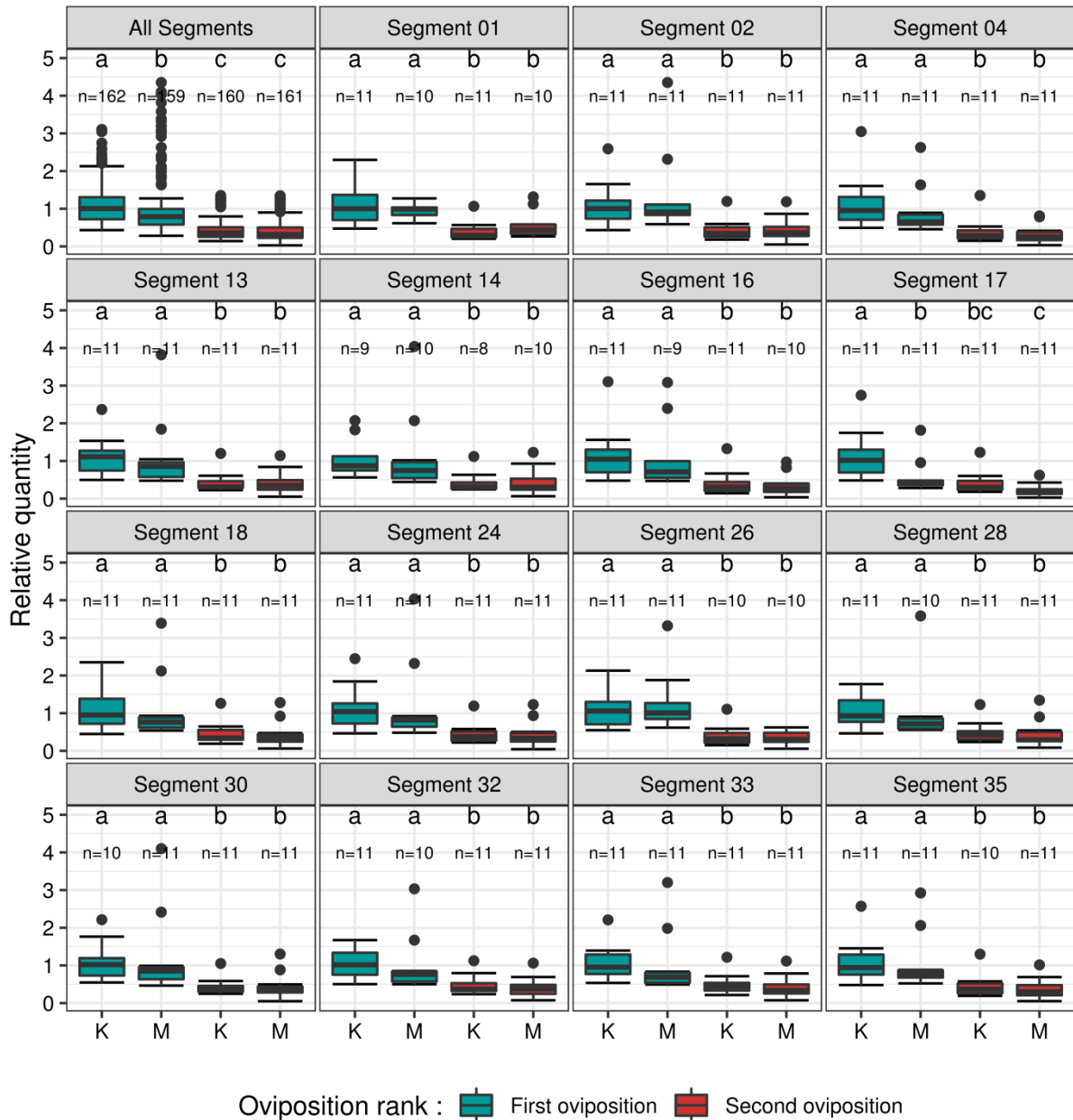
7 First of all, as injected quantities of all 15 segments showed a similar pattern (Fig. 3), we may assume that it  
8 is also the case for the other non-quantified segments. For the two *C. typhae* strains, the quantity of each  
9 viral segment injected decreased significantly by a factor of 2 to 3 between the first and at the second  
10 ovipositions. Regarding the difference between the parasitoid strains, it appeared that it was not significant  
11 when analysing segments independently, except for the viral segment 17. However, when analysing all  
12 segments together, it appeared that during the first oviposition, Kobodo females injected significantly more  
13 segments than Makindu females as observed for the number of eggs (Table 3).  
14

15 To investigate the relation between the quantity of viral segments and the number of eggs injected, we  
16 normalized these estimates to the values obtained for Kobodo during the first oviposition (Table 5). Both  
17 quantities varied similarly according to parasitoid strain and oviposition rank, but with different amplitude.  
18 At the first oviposition, Makindu estimates were lower than Kobodo ones, but the difference in the egg  
19 number was more important than the difference in the viral segments. From the first to the second  
20 oviposition, the number of segments injected decreased more than the number of eggs; for instance, eggs  
21 injected by Kobodo were reduced by 1/2, whereas viral segments were reduced by 2/3.  
22

23 **Table 5: Comparison of the injected quantities of segments and eggs in the French host between *C.***  
24 ***typhae* parasitoid strains.** For both traits, the medians are given relatively to the Kobodo-First oviposition  
25 condition.

Oviposition rank	<i>C. typhae</i> strain	Relative quantity of segments injected (standardized median)	Number of oocytes injected (standardized median)
First oviposition	Kobodo	1	1
	Makindu	0.789	0.652
Second oviposition	Kobodo	0.359	0.506
	Makindu	0.313	0.562

26  
27 Most importantly, on average, Makindu females injected more viral segments at the first oviposition than  
28 Kobodo females at the second oviposition but parasitism success of Makindu females was lower (Fig. 1, Fig.  
29 3). Hence, neither the viral segments quantity nor the number of eggs injected in the host explained by  
30 itself the difference of parasitism success between Kobodo and Makindu females in the French host.  
31



1  
2 **Fig. 3: Relative quantification of the viral DNA segments in the French host hemolymph two hours post-**  
3 **parasitism by *C. typhae* parasitoid strains. K = Kobodo; M = Makindu; n = number of host dissected. “All**  
4 **segments” corresponds to data of all segments pooled. For all boxplots the Kobodo-First oviposition**  
5 **condition is used as reference. Conditions with the same letter are not significantly different (p-value >**  
6 **0.05, Multiple comparison with Holm correction following the Kruskal-Wallis test). Statistical tests were**  
7 **performed independently for each segment, and for the pool of all segments.**

### 3.2 Reproductive success in the natural hosts

In all previous experiments parasitism was studied in the French host. Higher success of Kobodo parasitoids in this host may originate from adaptation to a local host with a resistance level comparable to the French host. To test this hypothesis, the parasitism success of the two *C. typhae* strains was measured in their natural hosts from their native localities: Kobodo and Makindu (Table 6). The number of eggs injected, the offspring number and the sex-ratio were also estimated on these hosts.

Kobodo females parasitized all the exposed host strains with a high rate of success (~90%), whereas Makindu ones parasitized only their natural host strain with a high success (Table 6). The parasitism success of Makindu females in the Kobodo host corresponded to that observed in the French one. These two *S. nonagrioides* strains displayed a higher resistance to parasitism by the Makindu parasitoid strain (56 and 59 percent of host larvae developed into pupae) than the *S. nonagrioides* strain from Makindu (3.5 percent of pupae). Unlike females from the Kobodo strain, females from the Makindu strain had a different offspring number depending on the host strain parasitized. Indeed, they had around 63 offspring in the two Kenyan hosts, while their offspring number was around 40 in the French host. The comparison of the number of eggs injected confirmed that Kobodo females laid more eggs in the first host encountered than Makindu ones whatever the host strain considered. The two parasitoid strains injected fewer eggs in the host from Kobodo than in the other ones. The sex-ratio was neither impacted significantly by the parasitoid strain nor by the host strain and varied from  $68.41 \pm 2.15$  to  $76.01 \pm 3.24$  percent of females.

**Table 6: Comparison of phenotypic traits between *C. typhae* parasitoid strains in the sympatric and allopatric host populations.** n = sample size;  $\pm$  = standard error. Conditions in the natural host are in bold. Data in the French host correspond to those presented in previous experiments. Conditions with the same letter are not significantly different (p-value > 0.05, Tukey's HSD test following binomial GLM (Parasitism success) or quasi-poisson GLM (Offspring number)).

Phenotypic trait	<i>C. typhae</i> strain	<i>S. nonagrioides</i> strain		n	
Parasitism success	Kobodo	<b>Kobodo</b>	92.77%	83	a
		Makindu	88.24%	119	a
		French	94.44%	90	a
	Makindu	Kobodo	30.12%	83	b
		<b>Makindu</b>	93.91%	115	a
		French	30.00%	90	b
Offspring number	Kobodo	<b>Kobodo</b>	$75.96 \pm 2.17$	70	a
		Makindu	$68.91 \pm 1.68$	88	a
		French	$71.85 \pm 1.83$	75	a
	Makindu	Kobodo	$65.79 \pm 4.86$	24	ab
		<b>Makindu</b>	$60.54 \pm 1.83$	83	b
		French	$39.36 \pm 3.87$	22	c
Predicted number of eggs injected	Kobodo	<b>Kobodo</b>	87.66		
		Makindu	108.87		
		French	109.66		
	Makindu	Kobodo	68.1		
		<b>Makindu</b>	78.48		
		French	72.42		

## 4. Discussion

Using the host specific *C. typhae* Braconidae as a biological model, the main objectives of this article were to explore the potential link between parasitoid oviposition behavior and virulence against the host, to investigate if higher parasitism success of one parasitoid strain on a new host originated from its local adaptation to a more resistant host, and to characterize the parasitic potential of two parasitoid strains on the new host population targeted for biological control.

Regarding the link between oviposition behavior and virulence, a previous study on the same species reported differences in the number of eggs injected and in the parasitism success in the French host between Kobodo and Makindu parasitoid strains, when ovipositing once (Benoist et al., 2017). The study showed that the expression of *Cystatin* and *Crv1*, two virulence genes known to inactivate the host immune response (Edson et al., 1981; Glatz et al., 2004), was higher after parasitism by Kobodo, which injected more eggs at a time. Because *C. typhae* females inject eggs and viral particles at the same time in the host, we expected that the quantities of viral segments injected by Kobodo females would be higher than those injected by Makindu ones, which would explain the higher gene expression and thus higher parasitism success.

However, the present study refutes part of this hypothesis. Indeed, although results showed that the injected quantities of viral segments and eggs varied in the same direction (but not proportionally, Table 5), they showed that the quantity of viral particles injected does not explain the level of virulence because at the second oviposition Kobodo had a much higher success than Makindu despite the similar quantities of eggs and viral segments injected (Fig. 1 & 3).

To explain the difference of virulence between Kobodo and Makindu we assumed a difference of virulence gene expressions (Benoist et al. 2017). This hypothesis cannot be either validated or rejected as yet. If true, it would imply that the virulence gene expression and the quantity of viral segments injected are not correlated, because we showed that Kobodo females have a higher parasitism success at the second oviposition than Makindu females at the first one (Fig. 1), although Kobodo females inject fewer viral segments in this case (Fig. 3). This absence of correlation between the quantity of viral segments injected and virulence gene expression was reported for instance in *Microplitis demolitor* and *Cotesia plutellae* (Beck et al., 2007; Kim and Kumar, 2018) and so is likely in our case. As an alternative to the gene expression hypothesis, the difference of virulence between Kobodo and Makindu strains may result from the efficiency of their virulence proteins, due to possible mutations in the coding sequence. Experiments of gene expression on a higher gene number in larvae parasitized at the second rank, and sequencing of virulence genes of both strains will help validate the right hypothesis.

Viral particle production is initiated in wasp ovaries during late pupal development and can continue throughout the adult stage (Beckage and Drezén, 2012; Marti, 2003; Pasquier-Barre et al., 2002). In several solitary parasitoid species the amount of virus present at adult emergence seems to be enough for all ovipositions during female adult life (Beck et al., 2007; Kim and Sanghoon, 2007; Marti, 2003). To our knowledge the quantity of viral segments injected has never been measured along successive ovipositions in a gregarious parasitoid before this work. For both Kobodo and Makindu females the quantity of viral segments injected decreased at the second oviposition, suggesting a depletion of particle stock (Fig. 3). So in *C. typhae* viral segments production could be insufficient to afford an equal amount of virus for multiple ovipositions, so we can expect that this amount decreases at each new oviposition. At the third oviposition, parasitism success decreased in each experimental combination (Fig. 1). The same phenomenon was observed for the very close species *C. flavipes* that parasites *Diatraea saccharalis* (Scaglia et al., 2005). Even if our results show no correlation between the quantity of viral segments injected and parasitism success, a minimum amount of virus could be necessary to allow successful parasitism, and such a threshold would not be reached at the third oviposition of *C. typhae* due to stock depletion.

1 Inactivating the host immune system is not the only way parasitoids have to ensure the development of  
2 their offspring (reviews in Pennacchio and Strand, 2006; Salt, 1968; Vinson, 1990). For example, in the  
3 facultative gregarious endoparasitoid *Metaphycus flavus*, laying multiple eggs decreased the encapsulation  
4 rate of the host *Coccus hesperidum* and so increased parasitoid larval survival (Kapranas et al., 2012). Since  
5 the number of eggs injected differed between Kobodo and Makindu females (Benoist et al., 2017), an  
6 alternative hypothesis to explain the difference of parasitism success in the French host, irrespective of the  
7 quantity of viral segments, could be that Kobodo females exhausted the host immune system by laying a  
8 large amount of eggs. However, successive ovipositions did not support this assumption because the  
9 parasitism success of Kobodo females was higher than that of Makindu ones even with fewer eggs injected  
10 (Fig. 1 & Table3).

11  
12 Our results also indicated that inactivating the host immune system is not an all or nothing process. Indeed,  
13 at the first oviposition, Makindu females got one third less offspring from the French than from its natural  
14 host, although they laid almost as many eggs in both hosts. This reveals a lower egg-larval survival rate in  
15 the French host. So the difference in offspring number may come from higher encapsulation rate of the  
16 eggs and larvae. We observed capsules in this condition, like those described by Gitau et al., 2007, in  
17 another noctuid host species parasitized by the sister species *C. sesamiae*, but too few could be observed  
18 for reliable counting, due to probable embedding in the host fat body with the same whitish appearance.  
19 Thus, even when host larvae allow parasitic development, their immune response is not totally inactivated.

20  
21 Regarding *C. typhae* behavior, host acceptance by both strains exceeds 90% at the first host exposition,  
22 decreases significantly at the second but not at the third (Table 2). Egg load was shown to be an important  
23 stimulus for ovipositional activity in several parasitoid species (Collins and Dixon, 1986; Donaldson and  
24 Walter, 1988). So, we expected that host acceptance would decrease along ovipositions due to egg  
25 depletion. However, no decrease was observed at the third oviposition, suggesting that either the  
26 relationship is not linear or other factors drive the motivation to oviposit.

27  
28 In Benoist et al., 2017 the number of eggs injected was measured only at the first oviposition. On the basis  
29 of the observed difference, it was supposed that Kobodo females would inject the majority of their eggs in  
30 the first host encountered, while Makindu ones would distribute them more equally. Here, results confirm  
31 this hypothesis. Kobodo females lay more than half their eggs in the first host encountered, about 30% in  
32 the second and 5 % in the third. In Makindu females, the proportions of egg injected in the first and the  
33 second host are close (near 30%) and, unlike Kobodo ones, the proportion at the third oviposition remains  
34 high (almost 15%). Finally, after the third oviposition, Makindu females have around two times more  
35 remaining eggs in their ovaries (more than 40 eggs) than Kobodo ones (Table 3), suggesting that the  
36 behavioral strain difference would also appear upon a fourth oviposition. Thus, the oviposition behavior of  
37 Kobodo females differs from that of Makindu ones, despite close initial fecundities. This difference in egg-  
38 laying behavior was not an artifact due to the use of a new host population. Indeed, for all hosts tested  
39 Kobodo females injected more eggs at the first oviposition rank than Makindu ones (Table 6). Estimating  
40 the number of opportunities to oviposit in the field could give information about the causes of this  
41 differentiation of oviposition behavior. Indeed, the egg allocation strategy of Makindu females will increase  
42 their fitness only if they can oviposit multiple times. Thus, we expect that in the field Makindu females have  
43 more opportunities to oviposit than Kobodo, which could have resulted in the differentiation of the  
44 oviposition behavior by selection. This is in accordance with accessibility to host resource in their natural  
45 habitat.

46  
47 Indeed, Makindu and Kobodo are located in ecologically contrasted regions (Le Ru et al., 2006). The  
48 Makindu field location consists in a well limited and dense settlement of *Typha domingensis* localized on  
49 the banks of a small stream (B. Le Ru *personal communication*) and surrounded by dry habitat (Somalia-  
50 Masai Acacia-Commiphora deciduous bushland and thicket vegetation mosaic, White, 1983). In this habitat  
51 *T. domingensis* hosts *S. nonagrioides* as an almost exclusive stemborer (Kaiser et al., 2015). In contrast, the  
52 Kobodo region consists of a continuum of wetland located in the mosaic of East African evergreen bushland



1 and secondary Acacia wooded grassland vegetation mosaic, White, 1983), with diversified plant-stem-borer  
2 species associations (B. Le Ru *personal communication*). So *S. nonagrioides* larvae should be more difficult  
3 for the parasitoid to find at the Kobodo locality. Since *C. typhae* are short-lived and thus time-limited,  
4 constraints on the probability of finding a host may have favored a higher reproductive investment in the  
5 first encountered host.

6  
7 The reciprocal comparison of the parasitism success of Kobodo and Makindu females in their natural host  
8 populations gives information on local host adaptation. While Kobodo females have a high success on both  
9 hosts from Kobodo and Makindu, the parasitism success of Makindu females is high only in its sympatric  
10 host (Fig. 1 & Table 6). Like the French host, the host from Kobodo resists *C. typhae* females from Makindu  
11 but does not resist the local *C. typhae*. This suggests that the French host population has a level of  
12 resistance equal to that of the Kobodo Kenyan host population. Thus, local adaptation may explain the  
13 ability of Kobodo females to parasitize the French host successfully: the higher resistance of the Kobodo host  
14 population could have exerted a higher selective pressure on *C. typhae* virulence, which might pre-adapt  
15 Kobodo females to the French host.

16  
17 In southern Europe, *S. nonagrioides* and *Ostrinia nubilalis* are the major pests of maize. Unlike *O. nubilalis*  
18 against which *Trichogramma brassicae* are used, no biocontrol agent is available for *S. nonagrioides*.  
19 Regarding the potential use of *C. typhae* as a biocontrol agent of *S. nonagrioides* in Europe, this work  
20 confirms that Kobodo locality offers an efficient strain against this pest. The performances of this *C. typhae*  
21 strain are comparable, for instance, to those of *C. flavipes*, which is used massively in Brazil against the  
22 sugarcane pest *Diatraea saccharalis* (Trevisan et al., 2016; Vacari et al., 2012) and are promising for the  
23 control of *S. nonagrioides*. The current work shows that Kobodo females could parasitize more than one  
24 larvae efficiently. Such data are important for mass rearing and for adjusting parasitoid densities for field  
25 release.

26  
27

## 28 **5. Conclusions**

29 The *C. typhae* / *S. nonagrioides* system is an attractive system for documenting the mechanisms of  
30 adaptation to a host population because it offers differentiation of several reproductive traits between  
31 parasitoid strains, and differentiation of resistance between host populations. We addressed mechanisms  
32 causing pre-adaptation of a geographical parasitoid strain to an allopatric host pest population targeted for  
33 biocontrol. Regarding behavioral and physiological causes, we found that neither the number of eggs  
34 injected nor the number of viral particles injected explained the difference of success between strains.  
35 Regarding evolutionary causes, our data indicate that pre-adaptation of one strain to the allopatric host  
36 population may come from adaptation to its local host. This work also suggests that virulence and  
37 oviposition behaviour would have evolved separately, the first in response to the level of host resistance,  
38 and the second to environmental variables at play in host availability.

39

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11

12  
13 **Author contributions**

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Benoist R.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing original draft

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## Manuscript Details

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### Abstract

Understanding the ability of parasitoid insects to succeed in new host populations is a relevant question for biological control and adaptive mechanisms. *Cotesia typhae* is an African parasitoid specialized on the moth *Sesamiae nonagrioides*, also called the Mediterranean corn borer. Two Kenyan strains of *C. typhae* differ in their virulence against a new host population from France. We explored behavioral and physiological hypotheses about this differentiation. *Cotesia* genus belongs to a group of Hymenoptera in which females inject a domesticated virus in their host to overcome its resistance. Since viral particles are injected along with eggs and since the strain with the higher virulence injects more eggs, we hypothesized that virulence could be explained by the quantity of virus injected. To test this assumption, we measured the injected quantities of eggs and viral particles (estimated by viral DNA circles) of each parasitoid strain along several ovipositions, to vary these quantities. Unexpectedly, results showed that virulence against the French host was not correlated to the injected quantities of eggs or viral circles, indicating that virulence differentiation is explained by other causes. The virulence against the respective natural hosts of the two *C. typhae* strains was also measured, and results suggest that local adaptation to a more resistant natural host may explain the pre-adaptation of one strain to the new host population. We also identified a differentiation of oviposition strategy and subsequent offspring number between the parasitoid strains, which is important in a biocontrol perspective.

<b>Keywords</b>	Parasitoid, <i>Cotesia typhae</i> , polydnavirus, oviposition behaviour, virulence, biocontrol
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<b>Suggested reviewers</b>	Michael Strand, Nicolas Ris, Jacques Brodeur, Guy Boivin, Anne-Nathalie Volkoff

## Submission Files Included in this PDF

### File Name [File Type]

Response to Reviewers - Benoist et al.docx [Response to Reviewers]

Graphical Abstract (Reviewer).tif [Graphical Abstract]

Benoist et al - virulence oviposition behaviour - reviewer JOIP.docx [Manuscript File]

Author statement - Benoist et al.docx [Author Statement]

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## Response to Reviewers

We thank both reviewers for their in-depth analysis of our work and for their constructive comments which were helpful to improve the manuscript. We carefully addressed all comments and questions and explained how we modified the manuscript when needed (text in blue following each comment/question).

### \*Reviewer 1\*

In this study, Benoist et al. examined parasitism of Mediterranean corn borer (MCB) from France and Africa by two strains (Kobodo, Makindu) of *Cotesia typhae* from Kenya. *C. typhae* is a gregarious species and like all microgastrine braconids relies on a bracovirus to parasitize hosts. The questions asked in this study was whether the Kobodo and Makindu strains of *C. typhae* differ in their ability to parasitize MCB that were collected from SW France and the Kobodo and Makindu regions in Kenya. The authors further examined whether successful parasitism correlated with the relative amount of *C. typhae* bracovirus (CtBV) that wasps injected into hosts. The overall message was that the Kobodo strain of *C. typhae* was more successful than the Makindu strain in parasitizing the French population of MCB. The Kobodo strain also exhibited similar efficacy in parasitizing Kobodo and Makindu MCB, whereas the Makindu strain of *C. typhae* was much more successful in parasitizing Makindu MCB than Kobodo or French MCB. A PCR based assay further generated no evidence that parasitism success was related to the relative amount of CtBV wasps injected into hosts.

My overall assessment is that studies to understand the host ranges of parasitoids are far and few between and from this perspective the manuscript contributes new information that documents differences in performance of two *C. typhae* strains in parasitizing different MCB populations. Approaches to the behavioral and oviposition studies performed are overall carefully done, properly analyzed and conclusions by authors are overall well supported.

- I am not very positive about the relative qPCR assay and approaches used for the studies done to assess CtBV segment abundance in the hemolymph of parasitized MCB larvae because a relative approach doesn't allow the authors to estimate the actual copy number of the viral segments present and how these numbers compare to the estimated total copy number of each segment in the calyces of wasps (which also would enable the authors to know whether wasps inject a large proportion of the virus they carry in each host they parasitized or a small amount). By not having any data on virus segment copy number in wasps, the authors also can't really draw any conclusions about whether reduced amounts of virus in successively parasitized hosts reflect constraints on the amount of virus available in wasps (as suggested in the Discussion). Real answers that are well supported experimentally require that the authors generate absolute copy number data and that they also generate absolute copy number estimates in the calyces of *C. typhae* females. The only thing the relative qPCR data supports is that the two strains of wasps exhibit no major differences in the amount of CtBV they inject into hosts and that relative copy number overall declines with successive oviposition.
  - [As written in the introduction \(page 3, lines 17-19\) "The first objective of the present work is thus to test if the difference in parasitism success results from a difference in the number of viral particles injected, and if this number varies together with the number of](#)



eggs injected”. In this objective of comparison between strains and oviposition ranks, absolute quantification is not required, so we preferred a relative quantification because it allows to normalize quantities among samples.

- We did not quantify the amount of CtBV segments in the calyx because our objective was not to estimate which proportion of the CtBV stock was injected at each oviposition. However, since the amount of injected CtBV decreased between the first and the second oviposition, we see no other explanation than a progressive stock depletion, as suggested in the discussion, where we replaced “appears to” page 14, line 45 by “could”.
- The main weakness of the study is it overall provides no mechanistic insights into why these strains of wasps differ in their ability to parasitize MCB from France and Africa.
  - Even if the study does not show what mechanism explains the virulence difference between the two parasitoid strains, it rules out an intuitive hypothesis, *i.e.* virulence being proportional to the amount of viral segment injected in the host. To our knowledge such relationship had not been investigated before.
- A second weakness is that the study also provides little or no information on the physiological interactions that occur in suitable versus non-suitable hosts as one might anticipate for manuscript submitted to a physiologically oriented journal like JIP.
  - As stated in the aim and scopes, JIP coverage includes reproduction and behavior and molecular approaches to physiological problems so we think that our study fits well with these fields.
- The authors allude to the possibility CtBVs from the Kobodo and Makindu strains could differ in transcription activity in different host backgrounds but did not examine this possibility in this study.
  - Given the high number of CtBV genes expected (> 200 based on the genome of *C. congregata*, Bézier et al. 2013), a next study will be devoted to such investigation which will be based both on comparative transcriptomics and on RT-qPCR on many genes.
- The authors also did not examine when wasp mortality occurs in parasitized hosts that are more unsuitable [...]
  - We begin to examine this point and observed some encapsulated eggs in host bodies. However, to fully address this question a comparative study is needed and it requires counting precisely the number of parasitoid into the host larvae throughout parasitoid development. For the moment, we are not able to count eggs in the host because they are hardly distinguishable from host fat body. To achieve that objective, methodological development of egg staining technique, for instance with antibody, is necessary. As the survey of parasitic development was not the main objective of our study, we have not yet proceeded to this development.
- [...] or provide any insights into how hosts wasps oviposited into respond physiologically (*i.e.* do they exhibit alterations in growth associated with successful parasitism but no or fewer wasp

progeny develop versus hosts exhibit few or no symptoms of parasitism which is associated with few or no wasp offspring successfully developing).

- In all conditions, most larvae that were not successfully parasitized developed normally into pupae and few died. We have added the data in the results (page 9, line 12-14; page 13, line 13-14).
- Minor points of wording include the suggestion that the authors not refer to the DNA segments that comprise the encapsulated portion of the CtBV genome as 'viral circles'. The PDV literature formally refers to these circularized BV DNAs as viral segments.
  - We replaced "viral circles" by "viral segments" in the manuscript.
- 'Acceptation' is not a word in English and could be changed to acceptance.
  - Done
- 'Ovocytes' should be changed to oocytes or better still eggs since the authors report that *C. typhae* is proovigenic.
  - We replaced "Ovocytes" by "oocytes". In reality *C. typhae* females are not proovigenic in a strict sense because adult females emerged with some immature oocytes in their ovaries. When we counted the oocytes, we did not distinguish mature and immature oocytes because it is tricky to separate both. So we preferred the term "oocytes" rather than "eggs". We clarified this point in the introduction (page 2, lines 45-46) and in the materials and methods (page 4 lines 48-49).
- Change 'experimentations' to experiments.
  - Done
- Table 3 and Figure 1 substantially overlap in the information they present. So one or the other should be deleted.
  - We discarded the Figure 1 and added the number of eggs injected in Table 3.
- Given the data presented the Koboto strain shows evidence of being able to successfully parasitize MCB across much geographic range than the Makindu strain but the authors largely don't reinforce this point enough in the Discussion
  - We did not reinforce this point in the discussion because to conclude that Kobodo stain is able to successfully parasitize MCB across a larger geographic range than the Makindu strain we think that investigation should be realized with host strains from various locations in the geographic range of MCB.
- nor do they raise the possibility that cryptic species could exist within *C. typhae* as has been discussed in the case of the work on *C. sesamiae*. It would help if the authors noted whether the Koboto and Makindu strain readily mate and produce offspring if crossed (which may be known in the literature but isn't mentioned here).

- We added in the introduction a sentence (page 2, lines 42-43) clarifying that Kobodo and Makindu strains interbreed and produce fertile offspring. So, we have no reason to raise the hypothesis that they might be a cryptic species in the discussion.

## \*Reviewer 2\*

The manuscript by Benoist and collaborators deals with the factors affecting parasitoids successful parasitism in the context of biological control using an introduced exotic parasitoid strain against a native pest, so a new host. Hence, the authors have previously identified two African strains of *Cotesia typhae* displaying different parasitism success rates on a new host, the French strain of *Sesamia nonagrioides*. In this work, they addressed the question whether the differences of the two strains were due or not to the quantity of laid eggs into the French host and/or to the quantity of injected polydnavirus particles. For that, they analyzed the initial egg load of the two strains, and followed the number of laid eggs (by dissecting the wasps after oviposition and counting the number of remaining ovocytes) and the quantities of viral DNA molecules injected into the caterpillars (by quantitative PCR) during successive ovipositions. They found that the differences in virulence were most probably not related to these two factors. Interestingly, this original work revealed different oviposition behavior when comparing the two parasitoid strains; the one chosen to be introduced (Mokodo strain, the most virulent in the French host) tends to lay half of its egg load in the first encountered host whereas the other strain (Makindu) tends to better distribute its eggs over the successive ovipositions. This different oviposition strategy could be related to the ecological constraints of the two parasitoid strains in their environment of origin; hence, the Kobodo region is more diversified and the parasitoid has less chance to find a suitable host than the one living in Makindu region. To assess the role of local adaptation on parasitoid successful parasitism on the French host, the authors also analyzed life history traits of the two parasitoid strains on their local host strain and alternative host strains. They found that virulence (parasitism success) of Makindu strain was high only on its own sympatric host strain (ie the Makindu host strain) whereas Kobodo parasitoid strain was successful on all the host strain that have been tested. The authors thus suggest that local adaptation of Kobodo parasitoid females to host resistant populations may explain its ability to parasitize successfully French borer populations.

This work brings new information on the factors underlying parasitoid successful development, on parasitoid population variability and deals with a parasitoid species of interest for biological control. The work is well introduced and discussed; experiments are straightforward and results well presented.

### Specific comments

#### Introduction

- P.2, l. 22. “a tissue located at the basis of the lateral oviducts”; replace by “a tissue located in the upper part of the lateral oviducts”  
➤ Done
- P.2, l.29. “which harbors the polydnavirus called Bracovirus”; replace by “which harbors polydnavirus from the genera Bracovirus”  
➤ Done

#### Materials and Methods

- P.4, l. 46-48. “The number of eggs injected at each oviposition rank was deduced from the difference between the mean numbers of eggs in the ovaries of females which had not oviposited or oviposited one, two or three times. The ovaries dissection protocol is available in Benoist et al., 2017.”; Not clear when exactly you dissected the females, neither if different females were used to count the number of ovocytes after 1st, 2nd and 3rd parasitism. I'd understood that the same female was used for the 3 successive parasitisms, so how can you have the number of eggs laid at the first parasitism?
  - We clarified the protocole in the Materials and Methods section (page 4, lines 46-47).
- P.4, l.50-52. “The mean offspring number was estimated only from mixed-sex progenies. Indeed, in haplo-diploid insects like *Cotesia*, unmated females give all-male progeny and in *C. typhae* offspring number varied significantly between mated and unmated females” ; Not clear why you didn't compare the data for mated and unmated females, rather than discarding unmated females.
  - We didn't compare the data because the majority of females were mated and they were very few offspring of unmated females. For instance, for the Kobodo strain they were 9, 3 and 0 offspring of unmated females for the first, second and third oviposition ranks respectively.
- P.5, l.10-11. “Pro and true legs were cut to collect as much hemolymph as possible.”; How did you make sure you didn't have eggs in your sample and/or that you didn't have wasp genomic DNA contamination?
  - We checked under binocular microscope several such hemolymph samples of parasitized caterpillars and we never found parasitoid eggs.
- P.5, l.17-18. “We decided to quantify 15 viral circles, representing about half the putative circle number.” What were the criteria to choose specifically these 15 circles, if any?
  - Some circles were specifically chosen: 13 because it includes *CrV1*; 1 and 26 because they are located on QTLs involved in parasitism success and offspring number traits (Benoist et al. in prep.) The others were chosen randomly among available sequenced circles in *C. sesamiae*.

## Results

- Figure 1 and Figure 3. Precise that numbers are given per host larva, if this is the case. Do these graphs mean for example that for Kobodo strain, about 100 eggs were laid/host at first oviposition, and from those 70 survived till mature larva stage?
  - Data from Figure 1 have been added to Table 3 and we added “per host larva” in both Table 3 and Figure 3 (which is now the Figure 2).
  - Yes, in average.
- Figure 1. Do the given numbers include the caterpillars that gave no wasp offspring?
  - No, we didn't include these data to decorrelate the offspring number from the parasitism success.

- We clarified this point in the materials and methods section (page 4, lines 51-52).
- P.11, l.11-13. “However, when analysing all circles together, it appeared that during the first oviposition, Kobodo females injected significantly more circles than Makindu females.”; Not stated clearly if there is or not a correlation between the number of eggs and the number of viral DNA copies.
  - The objective of Table 5 was to present the relationship between the number of eggs injected and the quantity of segments injected. Spearman’s test estimated a high correlation coefficient (0.8) but the correlation was not significant (p-value = 0.333). We decided not to include these results because we considered that such statistical correlation test is not valid with only four points. We think that the sentences we wrote in the result and discussion are clear enough “Both quantities varied similarly according to parasitoid strain and oviposition rank, but with different amplitude” and “results showed that the injected quantities of viral circles and eggs varied in the same direction (but not proportionally, Table 5)”.

## Discussion

- P.14, l.11-13. “The study showed that the expression of Cystatin and Crv1, two virulence genes known to inactivate the host immune response”; Are these genes on one of the circles tested?
  - The primers used to amplify segments were designed from available *C. sesamiae* bracovirus circles. *CrV1* is localized on the circle N°13 of *C. sesamiae*, included in our analysis, but *Cystatin* is not yet positioned on a *C. sesamiae* circle.
- P.14, l.32-34. “As an alternative to the gene expression hypothesis, the difference of virulence between Kobodo and Makindu strains may result from the efficiency of their virulence proteins, due to possible mutations in the coding sequence.”; Can you really exclude the hypothesis that you may have differences in a circle other than the 15 tested?
  - We cannot fully exclude this hypothesis. However, all the circles tested showed the same pattern so it is likely that it’s the same for all the circles.
- NB. You don't discuss at all the "waste" of eggs observed in this species, and particularly in Mokodo strategy (half of the egg load deposited at once, with only 60% survival.)
  - We did not discuss this point here because it was already addressed in a previous paper (Benoist et al. 2017).
- Graphical abstract: Some indication on the differences in virulence of the two parasitoid strains could be informative.
  - We added “highly virulent” and “weakly virulent” after strain names.

### Bio-geographic system



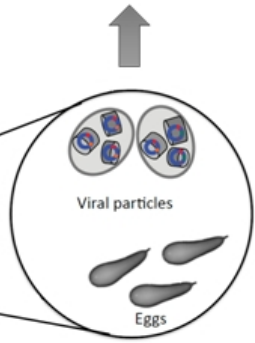
Ability of two strains of *Cotesia typhae* to parasitize a new host population

### Oviposition Behavior



Comparison of **Kobodo (highly virulent)** and **Makindu (weakly virulent)** strains on successive ovipositions

### Quantification



Relationship ?

### Parasitism Issue



Parasitoid success



Host resistance