ENDOSYMBIONT TRANSFECTION OF THE HIPPOBOSCID LOUSE FLY WITH BACTERIA FROM THE TSETSE FLY AND A PARASITOID WASP

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

There are two not mutually exclusive theories explaining the widespread presence of symbiotic bacteria belonging to the same *Arsenophonus* clade. Both the environmental progenitor model and lateral symbiont transfer theories have gathered some evidence. My aim was to find out whether experimental lateral transfer of secondary symbionts was possible between three different insect-bacterium systems.

The louse fly (*Pseudolynchia canariensis*), normally carrying *Arsenophonus arthropodicus,* was used as a test host and pupae were infected by microinjecting secondary symbionts of the tsetse fly (*Glossina sp.*) and the parasitoid wasp (*Nasonia vitripennis*). The co-injected Kanamycin-resistant strain of *A. arthropodicus* served as control and qRT-PCR was used to quantify bacterium cell numbers in pupae and flies.

I found that microinjection resulted in stable colonization of pupae. The exogenous bacteria survived eclosion and were propagated to the next (F1) generation. The microinjection method decreased survival rates of pupae, and injection of *S. glossinidius* in particular decreased survival even further, and shifted the sex ratio of eclosed flies. Native symbiont colony size changes were minimal. As exogenous symbiont colonization prevalence and size decreased greatly by the second generation (F2), the colonization was not self-perpetuating. Unless the new endosymbionts grant a very fitness advantage to the hosts, they

are not expected to survive any host defense mechanisms. Since colonization of injected pupae, eclosed flies and transmission to the first non-injected generation did occur, these results lend further experimental evidence that lateral symbiont transfer is not physiologically impossible, at least initially. The methods utilized in this study may be effectively used to further study lateral symbiont transfer and exogenous symbiont colonization of insects.

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1. INTRODUCTION

1.1 Primary and secondary symbionts

Insects are the most abundant class of the animal kingdom, representing possibly more than 90% of *Metazoan* species (Terry 1982). About 10% of species from the class Insecta maintain mutualistic associations (cooperative interaction between species) with one or more specialized symbiotic bacteria (Wernegreen, 2003). There is a broad spectrum of symbiotic relationships between insects and bacteria, ranging from strongly parasitic associations through commensalism to mutualism. At one end of the spectrum stand parasites, which are harmful for the insect host, whereas on the other end, mutualistic associations are essential or at least beneficial for host survival.

According to the strength of the association between the insect and the bacteria we distinguish two main types of symbionts. The more ancient group, primary symbionts, were first characterized by light microscopy in 1884 (Buchner 1965). They can be found usually in special insect organs and or cells (bacteriocytes or mycetometes), and by providing essential nutrients for the host they are not dispensable for host survival. During evolution, they often co-speciate with their insect host. They have taken their bacteria "with them"; therefore, their phylogeny is a mirror image of the host's (Figure 1.1A) (Dale and Moran 2006, Pontes and Dale 2006). Primary symbionts originated probably from a single infection event of the insect host ancestor by an ancestor bacterium, up

to 270 million years ago (Baumann 2005, Dale and Moran 2006). The host and the bacteria went through a process of co-speciation during millions of years, which had several consequences for both host and symbiont. Living in the host environment caused a massive genome reduction of the symbiont facilitating loss of biochemical processes needed for survival as a free-living bacterium. The result of this co-evolution is a reciprocal dependence between the host and the symbiont. Primary symbionts cannot persist outside the host and as a consequence, their investigation is much harder than that of secondary symbionts (Wernegreen 2002) , some of which have been cultured.

Secondary symbionts are less tightly connected to their hosts and they are not strictly necessary for host survival. Co-speciation with the host is not as tight as in primary symbionts (for an illustration of a hypothetical phylogeny of a secondary symbiont, see Figure 1.1B, Dale and Moran 2006, Pontes and Dale 2006), so they are not as specialized as the primary symbionts. The lack of congruence between the branch length of the phylogenies is thought to be evidence for a more recent association. Thanks to the lesser extent of their specialization, some of them can even be cultured, like *Arsenophonus arthropodicus*, *Arsenophonus nasoniae* and *Sodalis glossinidius*. Secondary symbionts can provide ancillary benefits for the host, like protection from certain natural enemies, or better tolerance of environmental stresses (Baumann 2005, Dale and Moran 2006).

1.2 The tsetse fly - *Sodalis glossinidius* system

The tsetse fly (Diptera: *Glossinidae*) is the sole vector of the African trypanosomes (*Trypanosoma brucei* subpp.), causing approximately 10000 deaths each year in Africa. The tsetse fly is also a good model organism to study symbiosis. It has a relatively short generation time and it harbors three types of symbiotic bacteria. Two of them are enteric and belong to the gamma-proteobacterial symbionts, and one is considered a facultative parasite in the insect. These bacteria are, the primary symbiont *Wigglesworthia glossinidia* (Aksoy, 1995), and two secondary symbionts, *Sodalis glossinidius,* and *Wolbachia pipientis* (Dobson, 2003). *Wolbachia* is a parasite, causing reproductive disorders (O'Neill et al., 1997) and the infection cannot be treated without eliminating the other two symbionts.

The tsetse fly has a special reproductive strategy called adenotrophic viviparity. This means that the eggs remain in the female body and the progeny go through three larval stages inside the uterus during which time they are fed with a protein and lipid-rich food by the mother via milk gland secretion (Ma and Denlinger 1974). Thus the fly lays not eggs but pupae, from which a perfectly developed fly emerges. The "milk" contains *W. glossinidia* and *S. glossinidius* that colonize the larvae; this way it is ensured that the progeny gets inoculated with the bacteria needed for their survival.

Sodalis glossinidius, the secondary symbiont of tsetse fly, is one of the few endosymbionts that can be maintained in liquid culture. Its function in the host is not clear yet, but its presence increases the tsetse fly's susceptibility to

trypanosome infection (a negative effect) while also increasing lifespan (a positive effect, Dale and Welburn 2001). It can be specifically eradicated from the tsetse fly, without harming the primary symbiont, using streptozotocin (Dale and Welburn 2001).

There has been limited success in determining the exact function that *S. glossinidius* plays in the tsetse fly's metabolism. It has been known for more than 80 years that the tsetse fly is not able to synthesize essential B vitamins (Sweetman and Palmer 1928, Craig and Hoskins 1957). This is interesting because the tsetse fly is a haematophagus insect (meaning they feed on blood and blood only), which lacks B vitamins (Edwards et al., 1957). It was thus logical to assume that symbiotic bacteria provide B vitamins for the host. With the publication of genomic data of the symbionts, a somewhat convoluted picture arises regarding thiamin (vitamin B1) production. Based on the genomic data, *W. glossinidia* is capable of thiamin synthesis (ie it possesses genes for the requisite enzymes), whereas *S. glossinidius* is not. On the other hand, the *S. glossinidius* genome has a putative thiamin ABC transport system. This system is used by the closely related bacteria *Salmonella typhimurium* to transport exogenous thiamin into the cells of the bacteria (Webb et al., 1998). In vitro experiments on *S. glossinidius* cells showed a higher growth rate in thiamin-containing medium with the ABC transporter's expression being driven by the presence of thiamin (Synder et al., 2010). This suggests that the primary symbiont may produce thiamin for both the host and the secondary symbiont. Though *W. glossinidia* certainly has the necessary gene set for thiamine synthesis, the actual presence

of this biochemical function and the use of thiamine by the secondary symbiont has never been measured directly.

1.3 The *Nasonia vitripennis - Arsenophonus nasoniae* system

The cosmopolitan wasp *Nasonia vitripennis* has a different lifestyle from the louse- and tsetse flies. It belongs to the genus of parasitoid wasps, *Nasonia*. *Nasonia vitripennis* is well known as a pest control agent in dairies for the house fly *Musca domestica* L. and in poultry houses for the stable fly *Stomoxys calcitrans* (Morgan et al., 1991). It can be also used to determine precisely the time of death in forensic cases, based on its temperature-dependent development in synanthropic flies (Grassberger and Frank 2003). This parasitoid wasp attacks various fly species by ovipositing in their pupae. It makes a hole in the puparial shell, and lays its eggs on the surface of the puparium. When the larvae eclose, they feed on the puparium which kills it. The larvae go through several stages of development in the puparium shell, and emerge from it as adult wasps (Grassberger and Frank 2003).

Nasonia vitripennis is also known to show occasional male-female ratio shifts. The decrease of male/female ratio is caused by the symbiotic bacteria *Arsenophonus nasoniae*, belonging to the family Enterobacteriacea. This bacterium can be transmitted both maternally and by novel infection. It decreases the survival rate of male embryos (male killing trait) through inhibition of the maternal chromosomes, which are required for early male development (Ferree et al., 2008). The physiological role of these bacteria – similar to *S. glossinidius* – has not been worked out yet. This *Arsenophonus* species can be found only in

the somatic tissues and in the interstitial fluid around germ cells (Huger et al., 1985). Similar to *S. glossinidius*, *Arsenophonus nasoniae* can be maintained in cell-free media, making it suitable for *in vitro* and controlled transfection experiments.

1.4 The louse fly - *Arsenophonus arthropodicus* system

The monophyletic clade of secondary symbionts belonging to *Arsenophonus* is present in many different kinds of insects, including aphids, whiteflies, hippoboscids, bees, lice, ticks and wasps (including the above mentioned parasitoid wasp). These insects have very different life styles. Some of them, like aphids, live on a plant sap diet, others are parasites or blood-suckers, like the louse fly (Novakova et al., 2009).

Similar to tsetse flies, louse flies are viviparous; thus, instead of laying thousands of eggs, they deposit just a single prepuparium at a time. This prepuparium represents a single fly that has passed all the three larval developmental stages inside the mother's body, where they are fed by a milk-like liquid containing secondary symbionts. The prepuparium wall then rapidly darkens and hardens to become a puparium, containing the real pupa (Bequaert 1953). Pupae are usually deposited by the flies in dark places under some kind of cover; thus in our case they were usually found under the layers of paper that was put on pans at the bottom of the cages of pigeons infected with flies. The fly that ecloses from the pupa after 3 weeks is fully developed and can reproduce in about a week. It needs, however, to feed (suck blood) soon after eclosion.

1.5 Endosymbiont acquisition theories

Bacteria of the *Arsenophonus* clade can survive in many different insects with different diets meaning that they seem to be capable of fitting in a wide range of biochemical niches. But how did these bacteria found their way into so many different hosts? One possibility is that lateral symbiont transfers have occurred, meaning the transfer of symbionts from one insect to another. In this case, either a direct or "vectorial" transmission between insects had to occur. Vectorial transmission requires transmission agents, for example parasitoid wasps, mites, or nematodes, which are able to carry bacteria between different insect hosts. Apart from lateral transfer, another possibility is that there could have been an environmental pool of bacteria that served as a common source of symbionts picked up by different host insects. These hypotheses are not mutually exclusive and there is some supportive evidence for both interspecific transfer (Weiss et al., 2006; Jaenike et al., 2007) and the common environmental pool dependent transmission (Husnik et al., 2011; Clayton et al., 2012). In particular, a new strain (designated "strain HS for Human Sodalis") of bacteria cultured from a tree branch-impaled wound of a patient was found to be closely related to the tsetse fly endosymbiont *Sodalis glossindius*. HS is also related to the endosymbionts of the chestnut weevil (*Curculio sikkimenis*) and stinkbug (*Cantao occelatus*) (Clayton et al., 2012), indicating that a *Sodalis*-allied pathogenic bacterial strain may be able to survive in a variety of organisms spanning both the plant and animal kingdoms. Clayton et al. (2012) also showed that this clade of endosymbiotic bacteria demonstrates distinct levels of disruptive gene

mutations in different insect species.This indicates different levels of gene inactivation and genome reduction - signs of long-term adaptation to a mutualistic relationship, also indicative of the different time elapsed since colonization. Based on genetic studies (Husnik et al., 2011; Clayton et al., 2012) it seems probable that there have been at least a few different, originally gut-related and/or pathogenic ancestors, giving rise to the current variety of primary and secondary *Enterobacteriaceae* endosymbionts. The goal of my study was to determine if it is possible to transfer symbionts from one host to another. Can bacteria that are already symbionts of one host organism survive and reproduce in another host organism?

Current experimental demonstrations of lateral transmission are few and have been shown only in closely related species, and never between phylogenetically distant insects with intact endosymbiont pools (Russel and Moran 2005, Weiss et al., 2006; Jaenike et al., 2007). In this study, I specifically addressed the question whether forced acquisition (by microinjection) of non-native endosymbionts will result in:

- survival and establishment of a bacterial colony
- transmission of bacteria to the nontransfected generation
- reliable transmission across multiple generations (ie start of a new host-endosymbiont system).

Artificial horizontal symbiont transfer, i.e., the man-made transfer of the symbiont of one host into a new host, has already been demonstrated in symbiont biology. The first studies of horizontal transmission were made on Wolbachia (not

necessarily a mutualist), and showed that it is possible to infect and colonize different insect hosts with heritable symbionts (Braig et al., 1994; Rigaud 2001; Sasaki 2002; Russel and Moran 2005). A recent phylogentic study (Novakova et al., 2009) revealed genetically close symbionts found among a diverse set of host species, suggestive of symbionts occasionally hopping horizontally between host species. The hypothesis was tested experimentally in aphids (Russel and Moran 2005). Secondary symbionts were transferred from three aphid species into the pea aphid, *Acyrthosiphon pisum*. The investigators took the different symbionts directly from donor insect hosts and injected them into young pea aphids. As a positive control, they injected the native symbiont of pea aphids *Candidatus Regialle insecticola* back into the pea aphids that were lacking this symbiont. Two of the four transferred symbionts survived and became established in the new host. Although these experiments provided evidence for the possibility of experimental symbiont transfer, they have a few shortcomings. First of all, as they took their bacteria samples directly from insects, they were not able to verify the presence and density of symbiont bacteria, thus introducing a level of uncertainty that was further increased by the injection's side effect of killing over half to two third of injected hosts. No actual control with saline injection was performed to ascertain the affect of the injection. Instead only the native bacteria were injected; however this is not a control for the method itself, as injecting even native bacteria may have deleterious effects on survival (due to for example the sudden increase in the bacterial load and/or the improper position of the bacteria).

A year later, the Aksoy lab (Weiss et al., 2006) conducted a similar experiment in tsetse flies to determine if the secondary symbiont *Sodalis glossinidius* from different tsetse species (*Glossina fuscipes fuscipes*, *Glossina morsitans morsitans*) can be transinfected into the other tsetse species. They found that ampicillin pretreated flies could be stably recolonized by *S. glossinidius* bacteria from different species (though these species belong to the same genus). It thus seems likely that horizontal transfer of symbionts is at least not impossible in nature. The work that has been done so far, however, is limited in the scope of the transinfection, i.e. pea aphid symbiont were injected into pea aphids and tsetse symbionts into other tsetse flies. Another shortcoming is the antibiotic treatment of hosts before injection of the bacteria. Although this seemed to be necessary, it does not mimic the natural situation, whereby a prospective endosymbiotic bacteria invading a new host needs to confront the resident (both primary and secondary endosymbiotic) bacteria and survive not only the host's immune system but compete with the resident bacteria. Thus while the above cited works do represent a useful initial experimental design, they do not necessarily allow for far reaching conclusions and they do not fully explain origin of the surprising variety of organisms hosting similar endosymbiotic bacteria.

The goal of my study was to determine if it is possible to transfer symbionts between more unrelated hosts. Can bacteria that are already symbionts of one host genus survive and grow in another?

1.6 Aims

The main aim of this study was to determine experimentally if lateral symbiont transfer can occur between different fly-symbiont systems. I wanted to repeat and improve upon earlier experiments by avoiding elimination of the original host endosymbionts, and by using unrelated donor hosts - testing the theory that lateral transfer is possible between hosts of different genera. By using *Sodalis glossinidius* from the tsetse fly (*Glossina morsitans*), and *Arsenophonus nasoniae* from the parasitoid wasp *Nasonia vitripenis*, I compared similarities between hosts (tsetse fly to louse fly), or similarities between the bacteria themselves (*A. nasoniae* to *A. arthropodicus*). Similarities between tsetse and louse flies include lifestyle (both are blood suckers), development (both are viviparous) and route of intergenerational bacterial transfer (maternal, through the milk). I injected *Sodalis glossinidius* (from the tsetse fly) into the louse fly, which normally harbors *Arsenophonus arthropodicus*. An advantage of this experimental design is that both *S. glossinidius* and *A. arthropodicus* can be cultured, and thus the amount of bacteria injected can be controlled. In addition, a genetically modified *A. arthropodicus* strain was available, with a Kanamycin resistance (Km) casette inserted into a pseudogene. This resistance gene served as a marker. By injecting the louse fly with both *S. glossinidius* and the *A. arthropodicus* bacteria I had both a positive control over the success of the injection (through the fate of *A. arthropodicus (Km)*) and the actually injected amount of bacteria. Additionally, I could compare the survival rate of endogenous *A. arthropodicus* to the injected *A. arthropodicus* pool. This way the flies did not

need to be treated with antibiotics to remove endogenous bacteria first, thus the recipient system was as close to its natural state as possible.

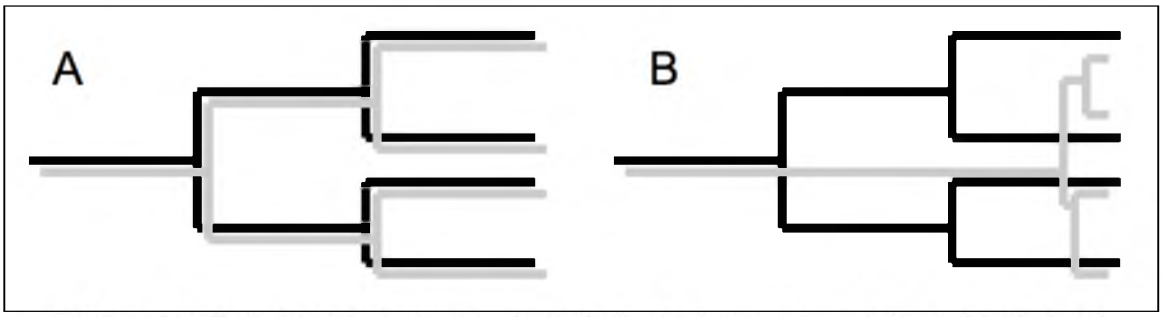

Figure 1.1 Relationship between *hypothetical phylogenies* of host (black tree) and their symbionts (grey tree). **A:** Primary symbionts, **B:** Secondary symbionts (adapted from Dale and Moran, 2006).

2. MATERIALS AND METHODS

In order to test the feasibility of a lateral symbiont transfer to the louse fly, I used 1:1 mixtures of *Arsenophonus arthropodicus/Sodalis glossinidius*, and *Arsenophonus arthropodicus / Arsenophonus nasoniae*. The particular *Arsenophonus arthropodicus* strain (Ars (Km)) used was a recombinant *Arsenophonus*, which has a pseudogene (alcohol dehyrogenase, AdhE gene) replaced by a Kanamycin (Km) cassette, developed by Kari Smith. The reason for injecting the other bacteria, in addition to Ars(Km) was three-fold. As *A. arthropodicus* is normally a native secondary symbiont in the pupae, the use of the Km-tag allowed me to:

i. test the feasibility of establishing a symbiont colony by injection. Since *Arsenophonus arthropodicus* is already a symbiont, the use of the tagged bacteria was a positive control to check if this particular method (injection) of entering the louse fly allows for the establishment of a symbiont colony.

ii. compare the relative number of native *A. arthropodicus* and the exogenously supplied *A. arthropodicus*, to test for competitive effects on establishment.

iii. quantify the outcome of the competition between *A. arthropodicus* and *S. glossinidius* or between *A. arthropodicus* and *A. nasoniae* in the two sets of experiments.

2.1 Animal housing

Louse flies were cultured on wild caught rock pigeons *Columba livia*. Pigeons were housed in wire cages inside climate controlled rooms (temperature: 23-26 ^{o}C, humidity: 65-75 %) and were fed ad libitum. The beaks of pigeons were trimmed and bitted in order to reduce their ability to kill the flies. The wire cages were surrounded by tightly woven wedding veil to prevent the flies from escaping.

2.2 Bacterial cultures

Arsenophonus arthropodicus, *Arsenophonus nasoniae* and *Sodalis glossinidius* cells were grown and maintained in liquid cultures (Mitsuhahsi and Maramorosch Insect Medium, MM; containing (g/L): CaCl2 0.15102; MgCl2 0.04685; KCl 0.2; NaCl 7.0; NaH2CO3 0.1739; Na2HCO3 0.12; D(+)-glucose 4.0; HEPES 7.1499; Lactalbumine hydrolysate 6.5; yeast extract 5.0) with Polymixin B (50 µg/ml from Sigma) at 28 ^{o}C , 28 ^{o}C and 26 ^{o}C respectively. They all originated from our frozen (at -80 ^{o}C) stock, and were verified at random intervals by PCR (see primer list in Table 2.1). To make a 1:1 mixture of these strains for the microinjection, liquid cultures of each were used at 0.01 OD_{600}. To obtain a density of 0.01 OD_{600}, cells were grown for 2 days in MM, centrifuged for 5 minutes at 8000g, and the supernatant aspirated to remove the antibiotic. This washing procedure was repeated following addition of and vortexing in 1x phosphate buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4). After washing, the pellet was re-suspended in 1 ml PBS buffer, followed by a measurement of its absorbance at 600 nm in a Thermo

Helios γ spectrophotometer. The solutions containing the different bacteria were then diluted to 0.01 OD_{600} each, and mixed together.

2.3 Microinjection

The injector used for microinjection was made from a 10 ml syringe, a plastic tube, and a glass micropipette. These three parts of the injector were interconnected with parafilm. Micropipettes were pulled, their tip cut back with a razor blade, and autoclaved before use. The tip was cut at a 45-60 degree angle to get a slanted tip able to penetrate the puparium. The micropipette was pushed only far enough into the wall so as to penetrate it with just the slanted end of the tip, then it was withdrawn slightly, and 1 uL of bacterium containing solution was deposited from the micropipette directly over the injured point on the outside of the puparium. The pupa sucked up the deposited bacteria mixture in a few minutes. The mechanism of the sucking action is not known; however, it may be due to a difference in osmolarity (ie higher osmolarity inside compared to the osmolarity of PBS).

For microinjection I used puparia less than 24 hours old. The microinjection was not performed at the prepuparium stage, which is characterized by a soft and light colored wall, but rather at an early puparium stage. This was necessary because the puparium hardens and becomes more brittle with age and the injection would cause more damage if done in older pupae. If the hole made by the micropipette was too big, it could not regenerate and the pupa died due to dehydration. The injection was done behind the posterior pole, distinguishable by its narrow shape and dark pigmentation, at

about 1/3rd of the length of the pupa as indicated on Figure 2.1 (see arrow).

2.4 DNA extraction, PCR, qRT-PCR

All pupae were frozen within an hour after injection, and adult flies were frozen when they were less than 1 day old (within 24 hours of eclosion). DNA of pupae and flies was extracted with the DNeasy Blood and Tissue Kit (Quiagen), and kept at -20 ^{o}C. The presence of bacteria in each pupae and fly was confirmed by PCR using the primers in Table 2.1. For regular PCR, I used an iCycler BioRad thermal cycler, with the following protocol: 5 minutes hot start at 95 ^{o}C, followed by 35 cycles of 95 ^{o}C for 20 sec, 55 ^{o}C for 30 sec, and 72 ^{o}C for 30 sec and a final extension / elongation at 72 ^{o}C for 7 minutes. I used this program for all bacteria, except the primary symbiont. For amplifying primary symbiont DNA, the annealing step at 55 ^{o}C was 1.5 minutes long, to accommodate for the amplified fragment's length (1500 bp compared to the less than 200 bp fragments amplified for the secondary symbionts). The amplified product composition and length was verified by agarose gel electrophoresis.

I used Quantitative Real-Time Polimerase Chain Reaction (qRT-PCR) to determine the number of bacteria in each individual pupa and fly. qRT-PCR was run on an iCycler iQ Multicolor Real-Time PCR detection system (Bio-Rad) with the following parameters: 10 minutes at 95 ^{o}C, than 40 cycles at 55 ^{o}C for 1 minutes, 95 ^{o}C for 1 minutes, and 55 ^{o}C for 1 minutes. The samples for the standard curve were deluted in Tris-EDTA (TE) buffer (100 mM Tris-HCl (pH 7.5) and 10 mM EDTA (pH 8.0)).

2.5 Statistical analysis

The statistical analysis was done with IBM SPSS Statistics. First a normality test was performed, and if a dataset did not pass, I used a non-parametric test (Chi square, Binomal test, Mann-Whitney and/or Kruskal-Wallis). The type of specific test conducted is noted for each result analyzed on the corresponding results page.

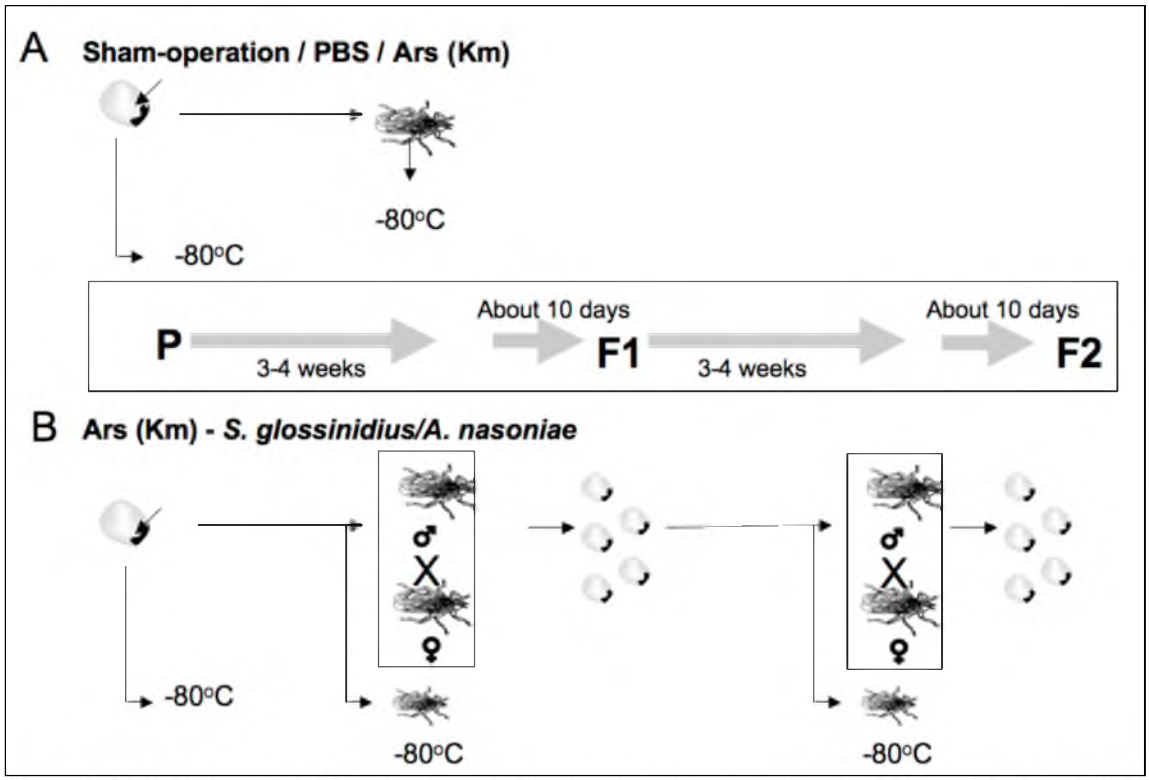

Figure 2.1 Scheme of experimental design to assess survival of injected *A. arthropodicus*, *S. glossinidius* and *A. nasoniae* and their propagation through generations of louse flies. **A**) As controls, less than 1 day old puparia were poked but not injected (Sham-operation), or were injected with PBS only. Kanamycin resistant *A. arthropodicus* („Ars (Km)“) were injected, the resistence gene serving as a way to distinguish this treatment from the native *A. arthropodicus* symbionts. Flies eclosing from these pupae were frozen within a day for later analysis. **B)** Less than 1 day old puparia were injected with a 1:1 mixture of either *A. arthropodicus / A. nasoniae* or *A. arthropodicus / S. glossinidius*. Flies eclosing from these pupae (parents, P) were then mated to give rise to the first generation (F1) of pupae, which were not injected. Flies from the F1 generation were mated to produce the F2 generation. Some flies from P, F1 and F2 were sacrificed for determination of endosymbiont pools using PCR and qRT-PCR.

Table 2.1 Primers for PCR and qRT-PCR* determination of endosymbiont composition in louse flies.

	Forward (5‘- 3‘)	Reverse (5‘- 3‘)
Primary symbiont	AACGGGGAAGCTATGCTTCTGC	GAGGTTTGCTAACTTTTGCAAGCT
A. arthropodicus (“WT”)	AAAACCTGCCATCGGTGTAG *	CTCTGACGCGCAAATAACAC *
A. arthropodicus (“Km”)	GGCACCTATCTCAGCGATCT *	TTCAATTTTGTTAGCTGTGCG *
A. nasoniae (“Nas”)	GTGGCTTGCCTGGAACAG *	GTCCAGCCTCGTGCTATAG *
S. glossinidius (“SG”)	GCAGGTCATATTCTTGATGG *	CGAACGCTACGGTATTCC *

Primers marked with * were used for both PCR and quantitative real-time PCR.

3. RESULTS

3.1 qRT-PCR quality control

Several primers were tried and extensively analyzed to find the most specific ones for each bacterium. By testing the primers with control template DNA (used in a range of 10^2 - 10^6 template DNA molecules/reaction), primer-dimer formation was noticed in the case of *Ars(Km)* (Figure 3.1) and *S. glossinidius* (Figure 3.2), but minimal-to-no primer-dimer formation in the case of *Ars (WT)* (Figure 3.3) and *A. nasoniae* (Figure 3.4) when the melt curves were analyzed. Because the Sybyr-green fluorescence based qRT-PCR measurement cannot distinguish between amplified primer dimers and the correct product, we were not able to avoid a false positive signal when no, or very few bacteria were present in the tested samples, giving a false "baseline" or "background" level for these measurements.

The lack or very low number of these bacteria in the aforementioned samples was ascertained using regular PCR with the same sets of primers. To correct for this baseline qRT-PCR error, first the level of this error was determined experimentally by injecting pupae with sterile PBS only (no bacteria added) and establishing the maximum "number" of these bacteria in the pupae and also in flies eclosed from these pupae.

PBS injected pupae, and adult flies all had numbers of bacteria below 1000 in the cases of *Ars (Km)* and *S.glossinidius,* and less than 10 in the case of

A. nasoniae. These numbers were considered the background noise of these measurements. Thus when a qRT-PCR reaction indicated that the number of bacteria was less than or equal to these levels, the result was considered false positive and set to zero.

Note that this makes the estimate of the average bacterium number in the samples somewhat conservative.

3.2 Survival rates

In our fly colony, over 90 % percent (331 out of 364) of nontreated pupae eclosed (Fig 3.5), while poking the puparium wall with a microinjector's needle (sham group) decreased survival to 75 % (60 out of 80). Injection of control vehicle (PBS) further reduced survival to 61.5 % (40 out of 65), whereas injection with *A. arthropodicus* (Ars (Km)) did not have a more adverse effect on survival rate (62.8 %, 32 out of 51). Coinjection with *A. arthropodicus* and *S. glossinidius,* however, further reduced survival rate to approximately 45 %.

Making even a small hole in the puparium wall is detrimental to survival, although injecting PBS, *A. arthropodicus (Km) or A. nasoniae* did not make a significant difference in emergence rate. The presence of *Arsenophonus species* did not affect survival, whereas *S. glossinidius* significantly decreased it. *S. glossinidius* thus negatively affected the survival rate, compared to the mixture of *A. nasoniae* and *A. arthropodicus* (Km) ($\chi^2(5)=150.866$ $p<0.001$; Post hoc analysis for emerged pupae, standard residual values: Noninjected= 4.9; Sham=0.6; PBS=-0.8; Ars(Km)=-0.6; Ars(Km)-A. nasoniae=-1; Ars(Km)-*S. glossinidius*=-4.4).

3.3 Effect of experimental procedure and injected symbionts on the sex ratio after eclosion

We tested the effect of the experimental procedure and exogenous bacteria on the sex ratio of eclosed flies using the binomial test. The male to female ratio in un-injected wild type flies was not statistically different from 1:1 ($p=0.826$). Sham operation (poking the puparium wall) or injection of PBS buffer did not change the sex ratio significantly either ($p=0.366$ and $p=0.272$, respectively). Injection of *A. Arthropodicus (km)* and *A. nasoniae* did not have a statistically significant effect either ($p=0.597$ and $p=0.180$, respectively), whereas injection of *S. glossinidius* resulted in a higher ratio of males in the emerged flies ($p=0.018$). Since the sex ratio has already been determined in the puparium before the injection, and *S. glossinidius* did not increase general survival rate (see Fig 3.6), we can conclude that it may be decreasing female survival more than male survival.

3.4 Microinjection with *Arsenophonus arthropodicus* (Km) and *S. glossinidius*

3.4.1 Colonization of hosts

As illustrated in Figure 3.7, microinjection resulted in approximately 3-4 orders of magnitude smaller numbers of bacteria (Mann-Whitney test; $U=42.000$; $p<0.001$ for the *WT* vs. *Km* and $U=36.000$ $p<0.001$ for the *WT* vs. *SG* comparison, respectively), when compared to the native pool. The average of the number of injected *Km cells* ($2.67\text{x}10^4 \pm 5.94\text{x}10^3$) was not significantly different (Mann-Whitney test; $U=1619.000$; $p=0.017$) from the number of *SG* cells ($2.67\text{x}10^4 \pm 1.33\text{x}10^4$). Thus, optical density-based titration of the injected

bacterial mixture seems to be a usable method, allowing a good titration of injected bacteria.

Both injected bacteria established growing colonies in the pupae, increasing approximately 1000-fold in the first week (*Km*: Mann-Whitney test; U=184.000; p<0.001; *SG*: Mann-Whitney test; U=294.000; p=0.001) and also generally during the whole pupa development period (*Km*: Kruskal-Wallis test; chi2 (3)=29.092 p<0.001; *SG*: Kruskal-Wallis test; chi2 (3)=28.306 p<0.001). The presence of the exogenous bacteria significantly decreased the level of endogenous bacteria (Kruskal-Wallis test; chi2 (3)=51.543; p<0.001) over the 3 week period. Over the 3 weeks, exogenous *Km* and *SG* cell pools reached approximately the same size (Mann-Whitney comparison at week 1: U=88.000 p=0.294; at week 2: U=102.500; p=0.673; and at week 3: U=92.500; p=0.792). By the end of the third week the number of the two exogenous *bacteria (Km and SG)* were not significantly different from the endogenous *WT* (*Km*: Mann-Whitney test; U=86.500; p=0.591; *SG*: Mann-Whitney test; U=84.000; p=0.507)

One possible confounding factor in these measurements is the lack of control for the viability of the pupae. As approximately 40-50 % of pupae dry out and die after microinjection, the observed colonization might not have been a physiological process in some of the cases. However, this was probably not the case, as it will become evident from the next set of results (see Figure 3.9).

3.4.2 Individual flies carry different sets of endosymbionts, and exogenous endosymbionts survive eclosion

Next, adult flies that emerged from microinjected pupae were subjected to PCR to determine the presence or absence of the three bacterial strains, the native (WT), injected *A. arthropodicus* (Km) and the injected *S. glossinidius* (SG). Altogether 57 flies were analyzed, of which 4 (7%) did not contain a measurable level of the native *A. arthropodicus (WT)*. These flies did contain both injected bacteria strains. Altogether 41 flies (72%) contained both injected bacteria strains, 8 (14%) contained only *A. arthropodicus* (Km and WT) and 1 (2%) contained only *S. glossinidius* and the native *A. arthropodicus.* Seven (12%) flies contained only the native secondary symbiont.

Thus injection of pupae resulted in a high percentage (88%) of transformed adult flies, which means that bacteria that colonized the pupae survived in the pupae and the process of eclosion into adult flies. It is of note that the presence or absence of wild type secondary symbiont could not be ascertained in the pupae before microinjection. For this reason, the presence of flies lacking the wild type *A. arthropodicus* is not necessarily indicative of a negative effect of injected bacteria on the native symbiont.

3.4.3 Microinjected symbionts propagate from pupae to adult flies and can colonize flies

0 day pupae (left column set) were microinjected with *A. arthropodicus* (*Km*) and *Sodalis glossindius* (*SG*) at approximately 1:1 ratio. The number of injected bacterium cells was significantly lower than that of the native *A.*

arthropodicus (*WT*) symbiont. The amount of wild type bacteria did not change between injection and eclosion (Mann-Whitney test; U=2789.500; p=0.353). *Km* colony size increased (Mann-Whitney test; U=746.500; p<0.001), and was nearly identical ($5.16x10^7$) to the wild native symbiont count ($5.89x10^7$). *SG* also colonized the host (Mann-Whitney test; U=1462.000; p<0.001) but did not reach the colony size of the *Km* colony (Mann-Whitney test; U=2717.000; p<0.001). Thus through microinjection, colonies of exogenous symbionts that survived after eclosing in the adult flies was established successfully.

3.4.4 Transmission between generations

After finding that injected *A. arthropodicus* and *S. glossinidius* can successfully colonize injected pupae and persist in the adult flies, I mated flies previously injected as pupae. Females were fertile and deposited the first generation of pupae (F1) that themselves were not injected.

I ran qRT-PCR analysis on F1 pupae and I found that 15 (23.44 %) of 64 pupae contained SG and 45 (70.31 %) of 64 pupae contained Km. Thus, the transfer of the originally exogenous symbionts occurred with different efficiency for Km and SG. A qualitatively similar but quantitatively slightly different result emerged using PCR to test for the presence or absence of the studied three bacterial strains (Fig. 3.11). Both methods suggested a decrease of percent of flies carrying the exogenous endosymbionts with each generation, with hardly any flies harboring these bacteria two generations from the time of microinjection.

I mated the F1 generation to obtain the next generation of flies (F2), which I analyzed the same way. In this case, I found no (0 %) flies with *SG* and 2 flies

(4.26 %) out of 47 that contained *Km* bacteria. Figure 3.12 shows the average bacterium cell count in the fly population across 3 (1 injected parental and 2 descendent) generations of flies. These averages contain data from all flies tested, including those that did not harbor the exogenous symbionts.

Wild type *A. arthropodicus* (*WT*) cell count changed slightly, but significantly between the generations going down in F1 (Mann-Whitney test; U=2204.500; p=0.008) and up in F2 (Mann-Whitney test; U=587.500; p<0.001). *S. glossinidius* cell counts dropped across all generations (Kruskal-Wallis test; chi2 (2)=62.060; p<0.001) as well as between parental and F1 and between F1 and F2 generations (Mann-Whitney test; U=1566.500; p<0.001; and U=1200.500; p=0.002, respectively) with a total loss of this symbiont in F2.

Exogenous *A. arthropodicus (Km)* followed a similar pattern of decline (Kruska-Wallis test; chi2(2)=85.307; p<0.001) to *SG*, though it did not disappear in F2. When comparing the size of different bacteria pools, the native symbiont had the largest colony, followed by the exogenous *Km* with *SG* giving the smallest colonies (Mann-Whitney test, all pairs of statistical comparisons within each generation at p<0.001).

3.5 Injection with Ars (Km)-A. nasoniae

3.5.1 Colonization of hosts

Following successful microinjection of louse fly pupae with *S. glossinidius,* I repeated the same set of experiments with a different bacterium, *A. nasoniae (Nas)*. I strived to inject a 1:1 ratio of *A. arthropodicus (Km)* (Ars(Km) or simply *Km*) and Nas, however the spectrophotometric method for *Km/Nas* titration was

less precise than in the case of the *Km/SG* pair giving significantly different starting levels after injection (see "day 0", Mann-Whitney test; U=107.500; p<0.001).

Both injected bacteria established growing colonies in the pupae, increasing approximately 1000-fold in the first week. The presence of the exogenous bacteria significantly decreased the level of endogenous bacteria (Kruskal-Wallis test; chi2 (3)=21.596; p<0.001) at the end of the 3 week period. Over the 3 weeks, the pools of exogenous *Km* and *Nas* cells reached approximately the same size (Mann-Whitney comparison at week 1: U=99.000 p=0.490; at week 2: U=106.000; p=0.716; and at week 3: U=101.000; p=0.598). By the end of the third week the number of the two exogenous *bacteria (Km and Nas)* were still significantly lower than the endogenous *WT* (*Km*: Mann-Whitney test; U=6.000; p<0.001; *Nas*: Mann-Whitney test; U=2.000; p<0.001).

3.5.2 Microinjected *A. nasoniae* propagate from pupae to adult flies

After finding that microinjected bacteria established stable colonies in the pupae, I tested if these colonies survive the eclosion process. As shown on Figure 3.14, both injected bacteria were present in the freshly emerged flies. Similarly to what was found in the pupae (Kruskal-Wallis test chi2; (2)=54.353; p<0.001), the bacterium pools were of different sizes (Kruskal-Wallis test; chi2 (2)=163.351; p<0.001), with both Km and Nas pools being smaller than the number of native WT bacteria (Mann-Whitney test; *Km* vs. *WT*: U=1066.000; p<0.001 and *Nas* vs. *WT*: U=581.500; p<0.001), and they were not significantly different from each other (Mann-Whitney test: U=5527.500; p=0.366).

3.5.3 Transmission between generations

Lastly, I checked if the exogenous bacteria could be transferred between generations. I determined bacteria cell numbers with qRT-PCR in flies that were microinjected in their pupa stage, mated these flies to produce the first (F1) generation and mated F1 flies to produce F2 pupae and flies. As throughout this study, I used freshly eclosed flies to determine the number of different secondary symbiont cells. All bacteria propagated from the injected, parental generation to the next, however no flies containing *Km* bacteria were found in the following (F2) generation and average *Nas* cell count also dropped significantly (Mann-Whitney test; $U=62.500$; $p<0.001$). This drop is in contrast to little to no change found in colonization levels between the parental and F1 generations for either of the injected bacteria (Mann-Whitney test; *Km*: $U=2712.000$ $p=0.795$; *Nas*: $U=2353.500$; $p=0.118$ – neither of these p values are significant).

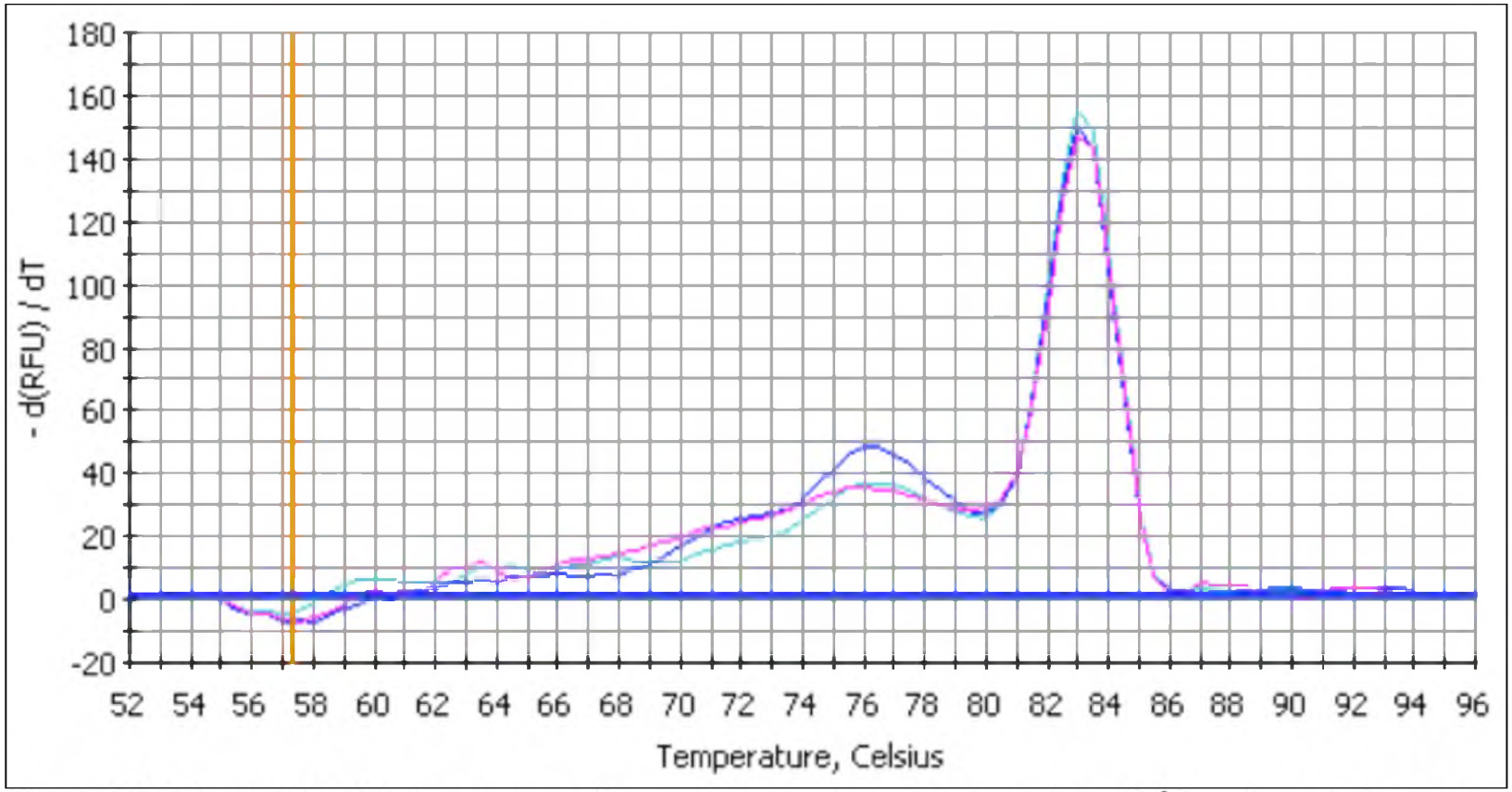

Figure 3.1 A typical *Ars (Km)* melt curve. Primer dimers give an approximately 76 °C melt point. The correct product melting point is at 83 °C.

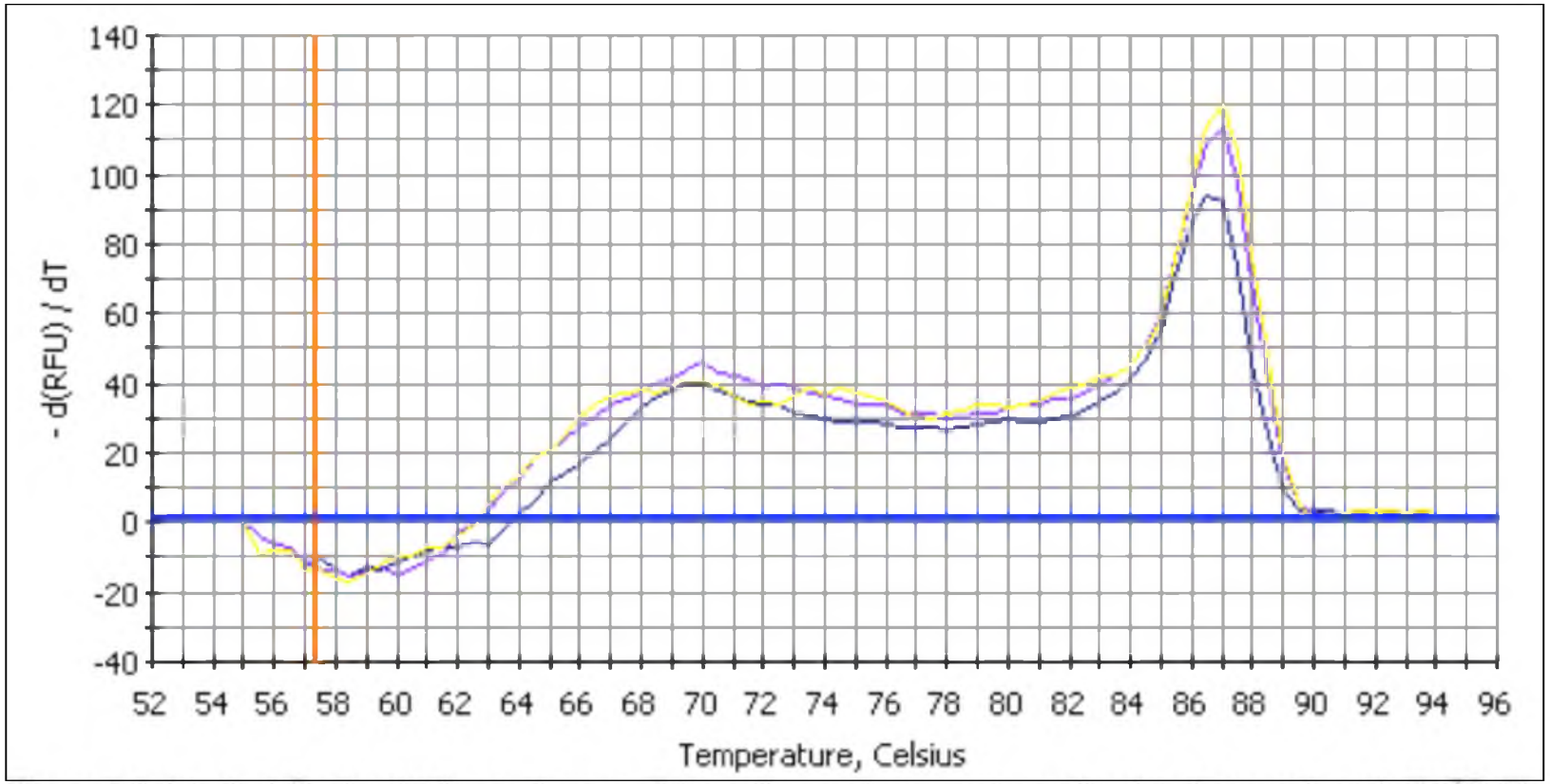

Figure 3.2 A typical *S. glossinidius* melt curve. Primer dimers show up at a melt point of approximately 70 °C. The PCR product melting point is at 87 °C.

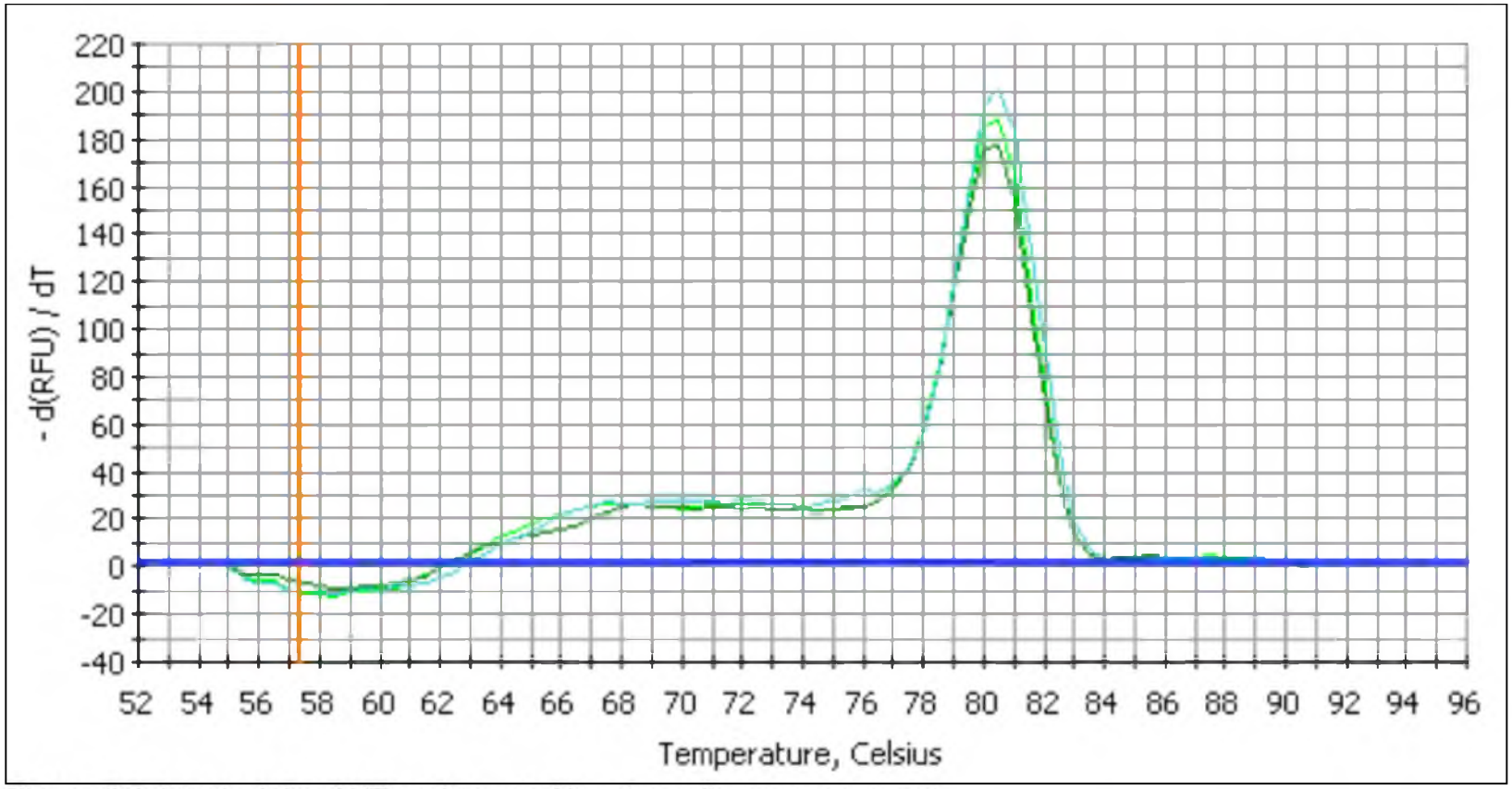

Figure 3.3 A typical *Ars (WT)* melt curve. No primer dimers are present.

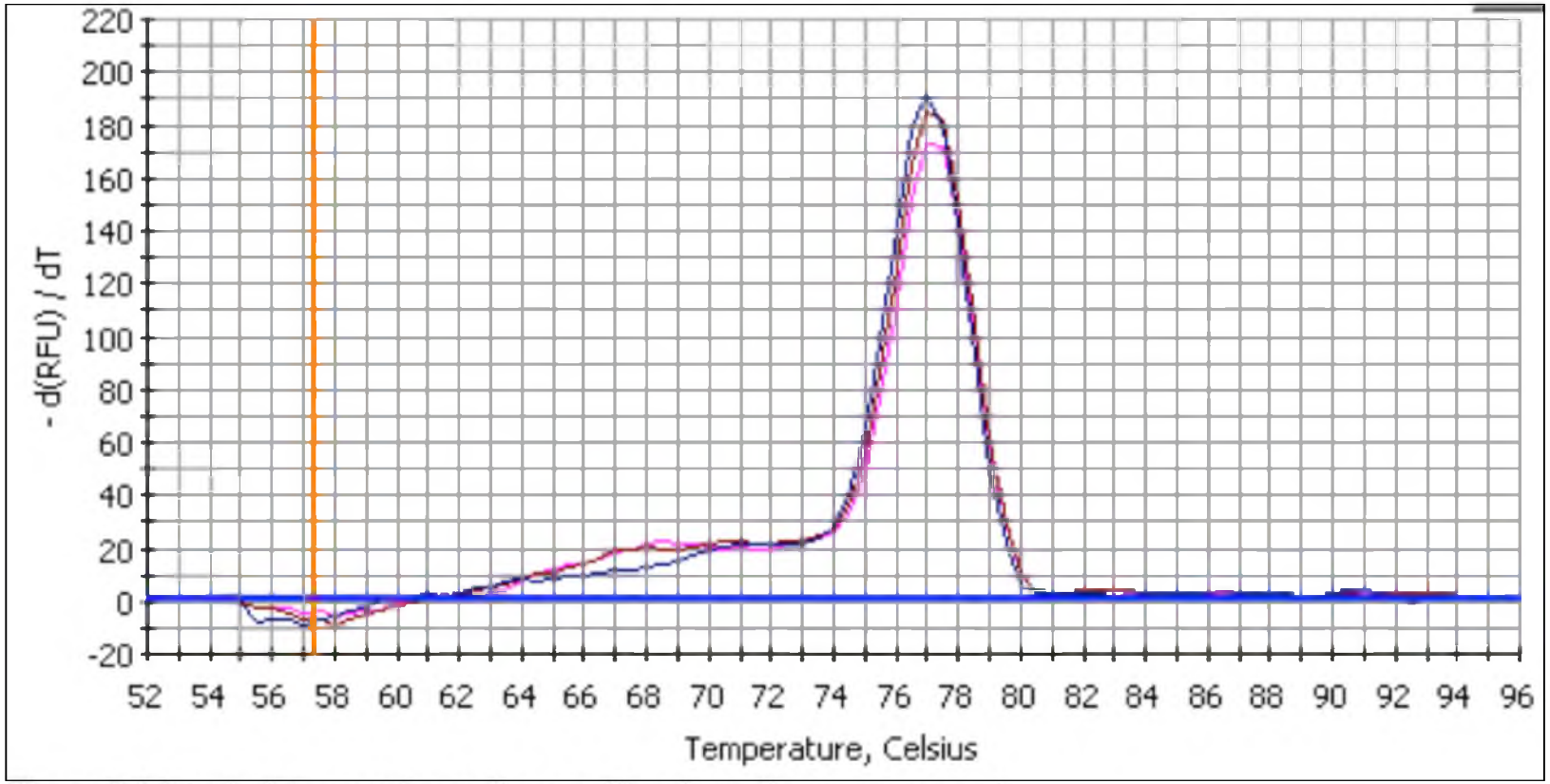

Figure 3.4 A typical *A. nasoniae* melt curve. No primer dimers are present.

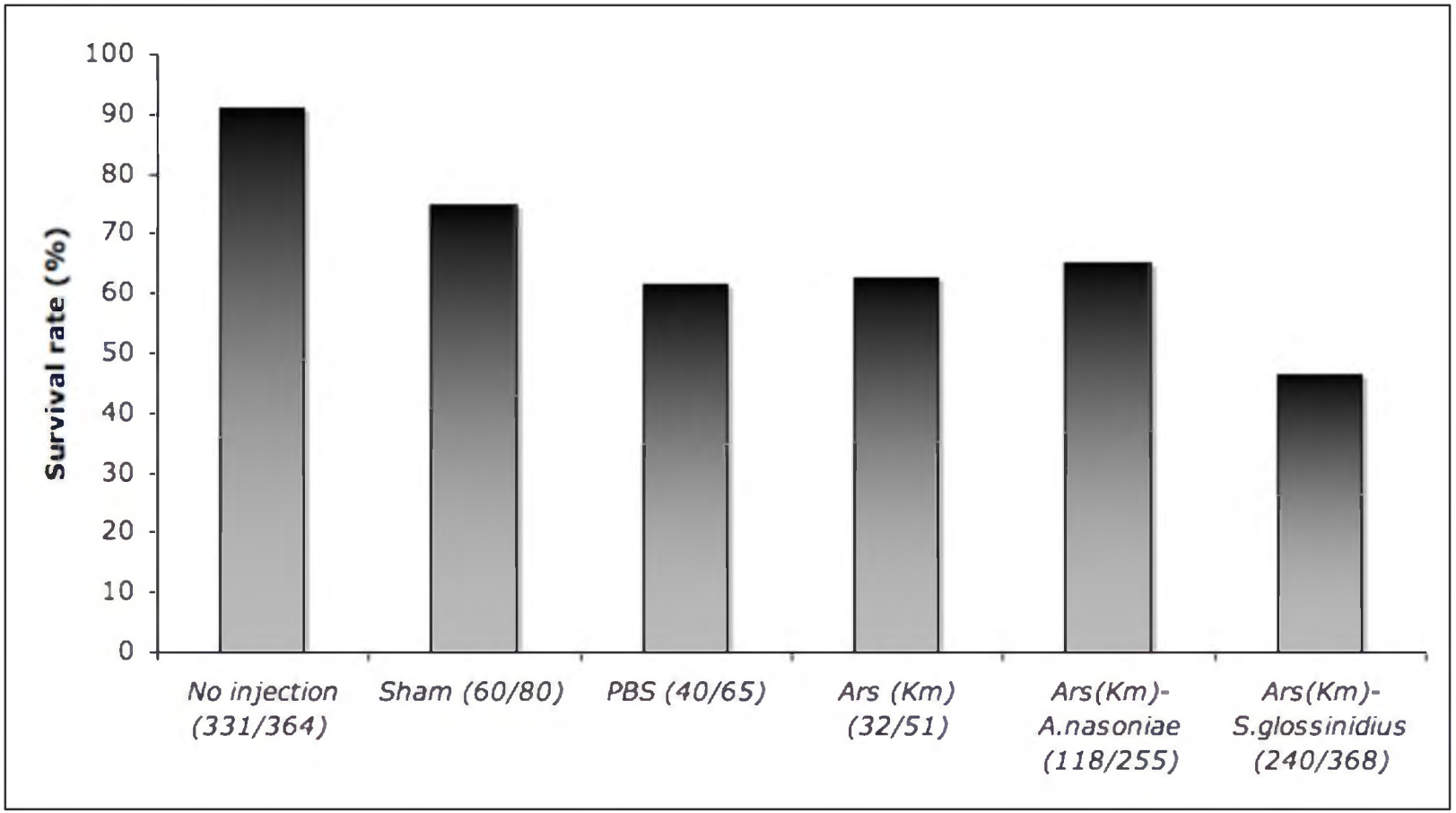

Figure 3.5 Overall survival rates after injection, measured as % of flies eclosing from pupae.

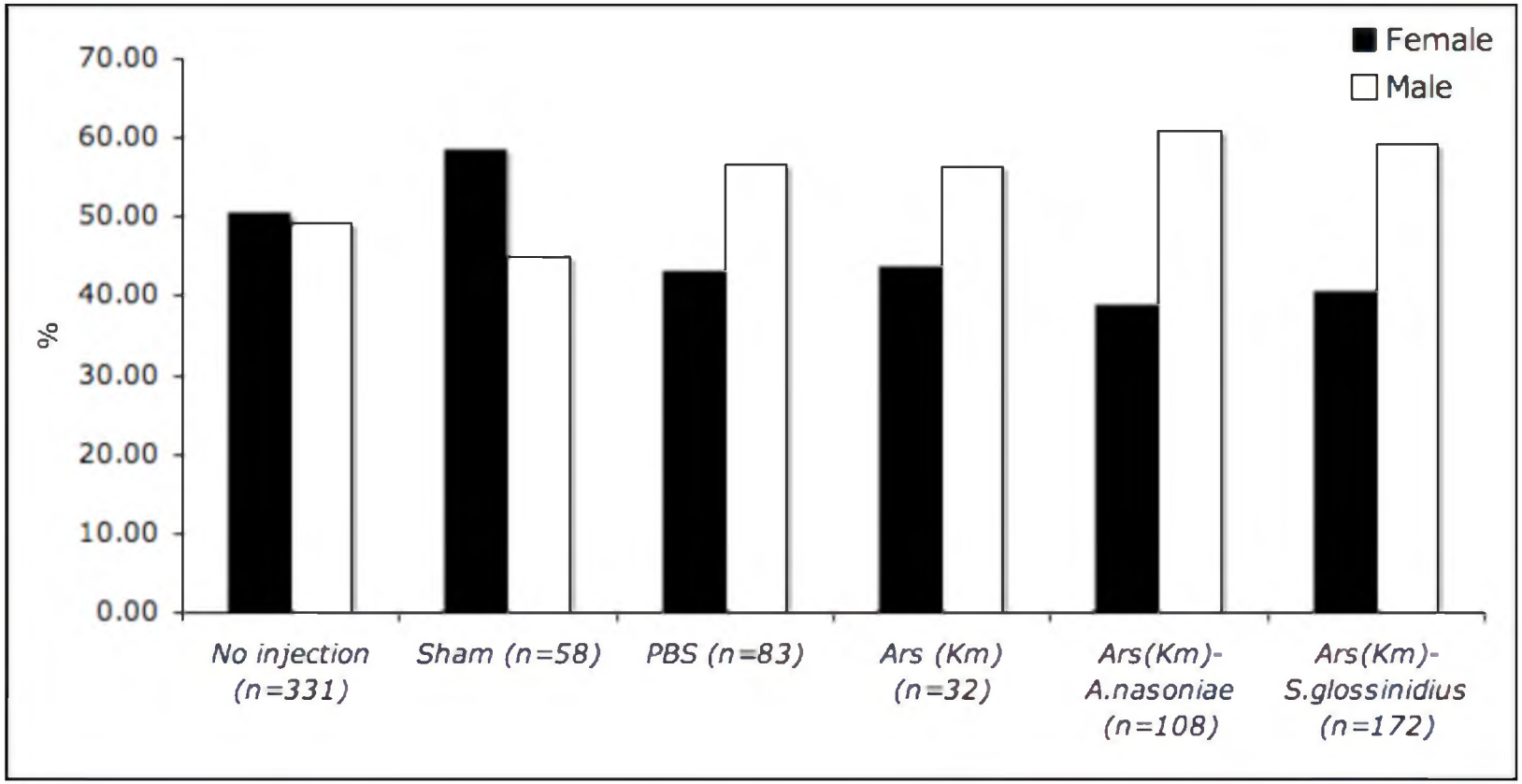

Figure 3.6 Effect of experimental procedure and injected symbionts on the sex ratio after eclosion

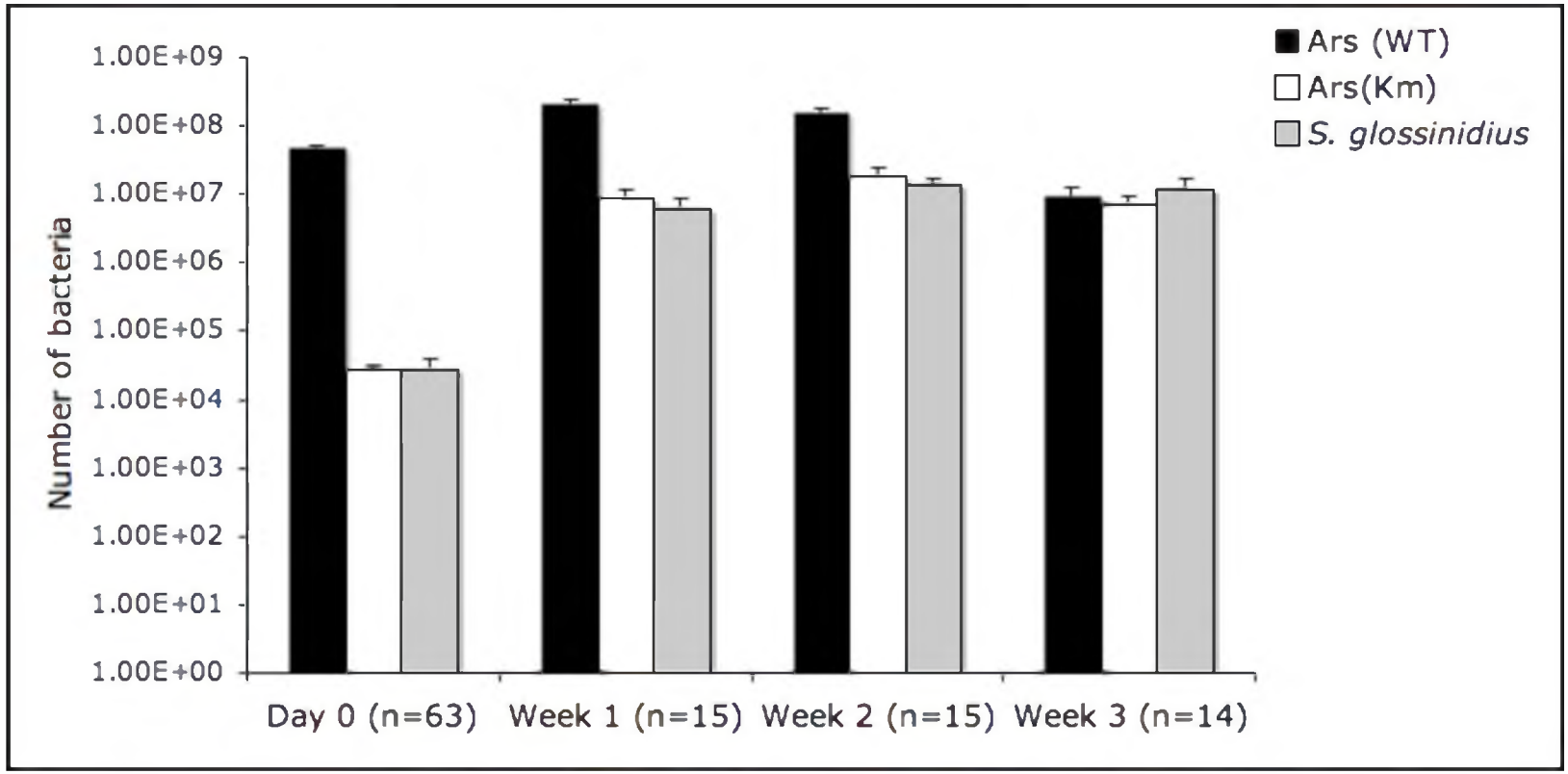

Figure 3.7 Temporal profile of colonization level of louse fly pupae microinjected with symbionts. Pupae were sacrificed at each time point for determination of native and exogenous bacteria, using qRT-PCR. Native: *Arsenophonus arthropodicus* (Ars (WT)), exogenous bacteria: *Arsenophonus arthropodicus* (Ars (Km)) *and Sodalis glossinidius (S. glossinidius)*. „0 day" samples (N=69) were frozen immediately after injection, then pupae were frozen 1 week, 2 weeks and 3 weeks later (N=15, 15, and 16 pupae for each time point, respectively).

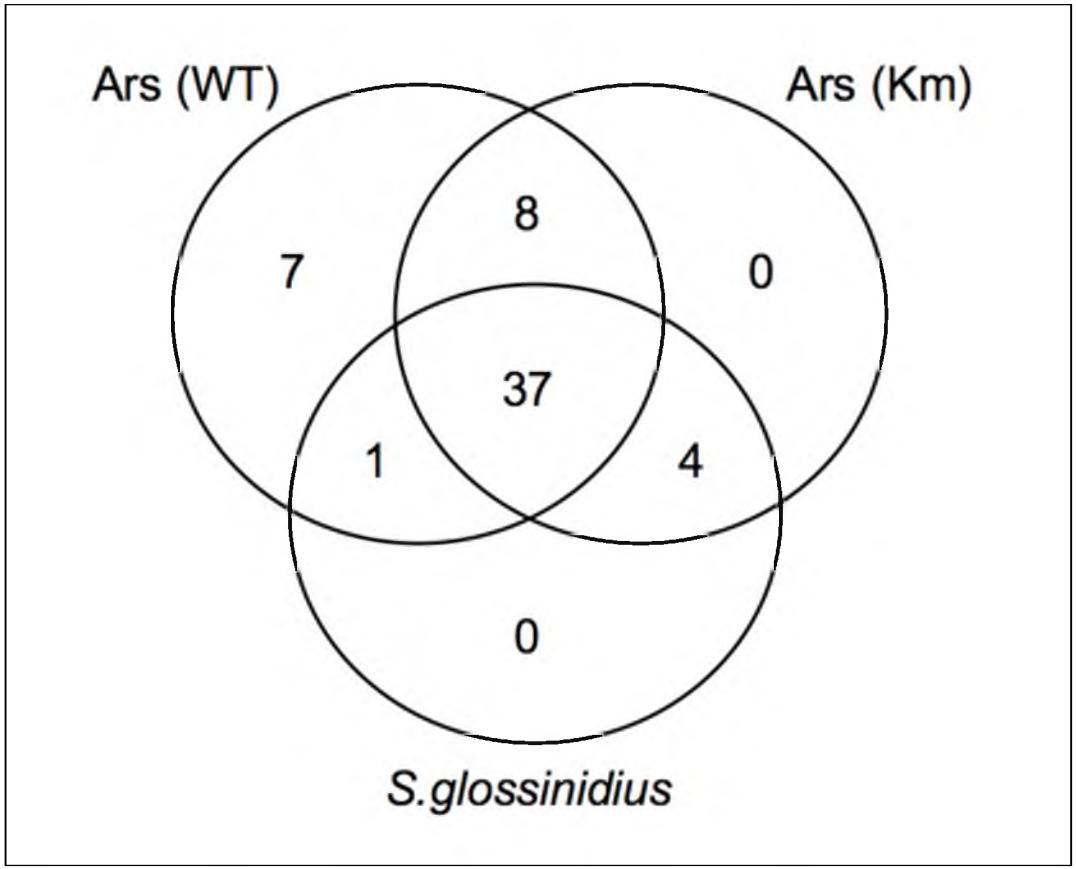

Figure 3.8 Number of adult flies (aged up to 2 weeks) carrying different sets of endosymbionts, following microinjection of pupae. „Ars (Km)“: injected Kanamycin resistant *Arsenophonus arthropodicus*; „Sg“: *Sodalis glossinidius*; Ars (WT): native *Arsenophonus arthropodicus*. A large percentage of all flies contained both injected bacteria strains.

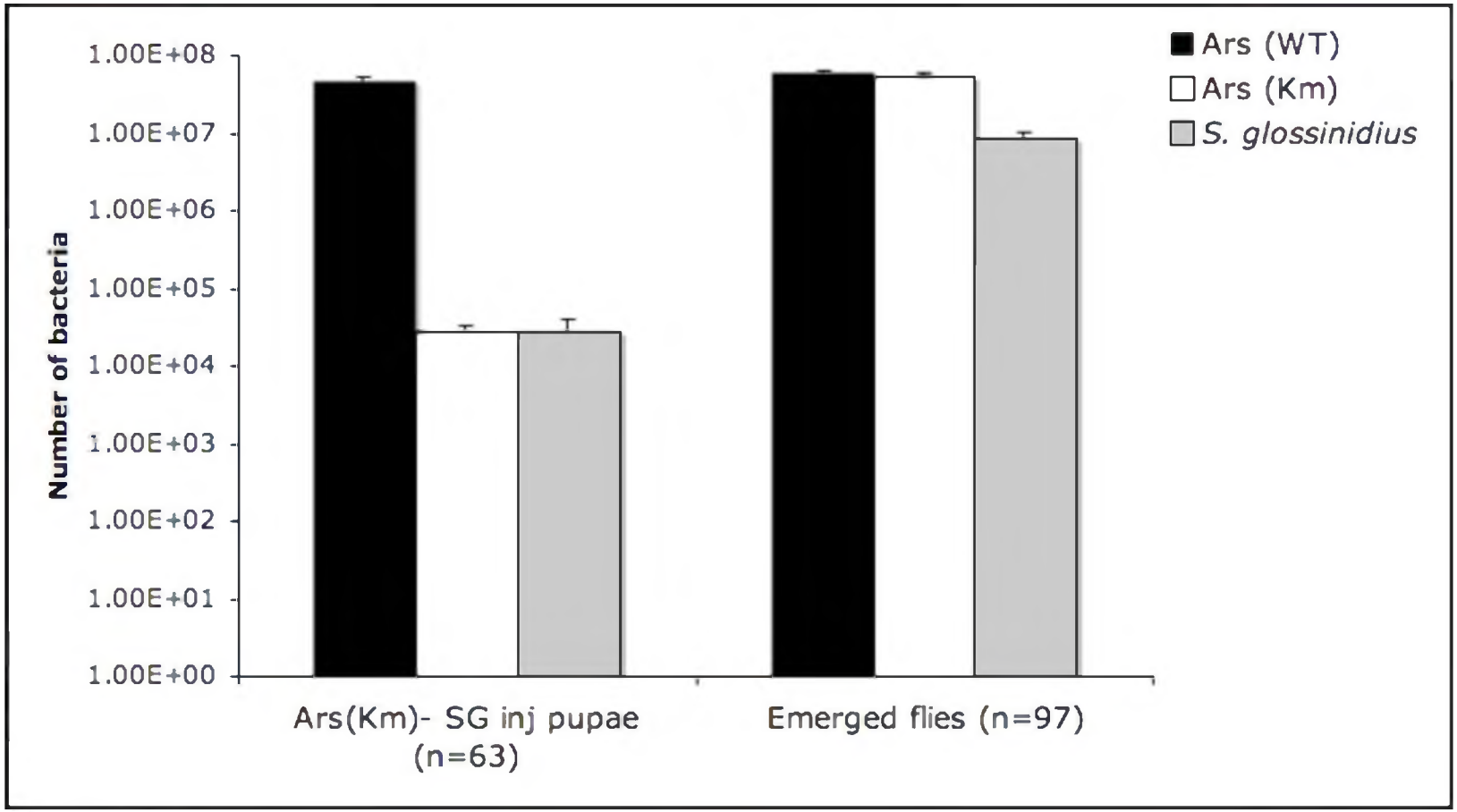

Figure 3.9. RT-PCR determination of colonization level in flies eclosed from microinjected pupae.

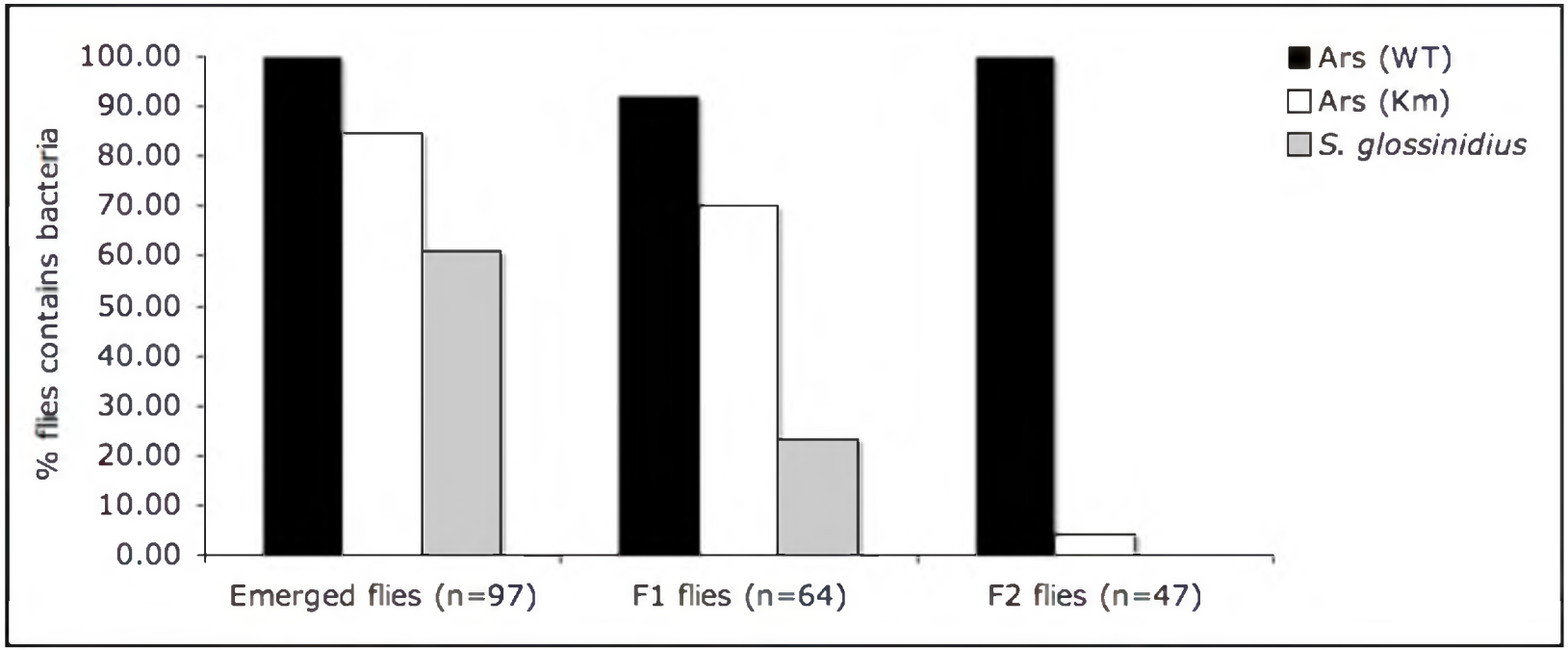

Figure 3.10 Exogenous endosymbiont propagation between generations of flies, using qRT-PCR. Bars show the percent of flies harboring (above the noise level) the indicated bacteria strains. **"**Ars (Km)": injected Kanamycin resistant *Arsenophonus arthropodicus*; "Sg": *Sodalis glossinidius*; Ars (WT): native *Arsenophonus arthropodicus*.

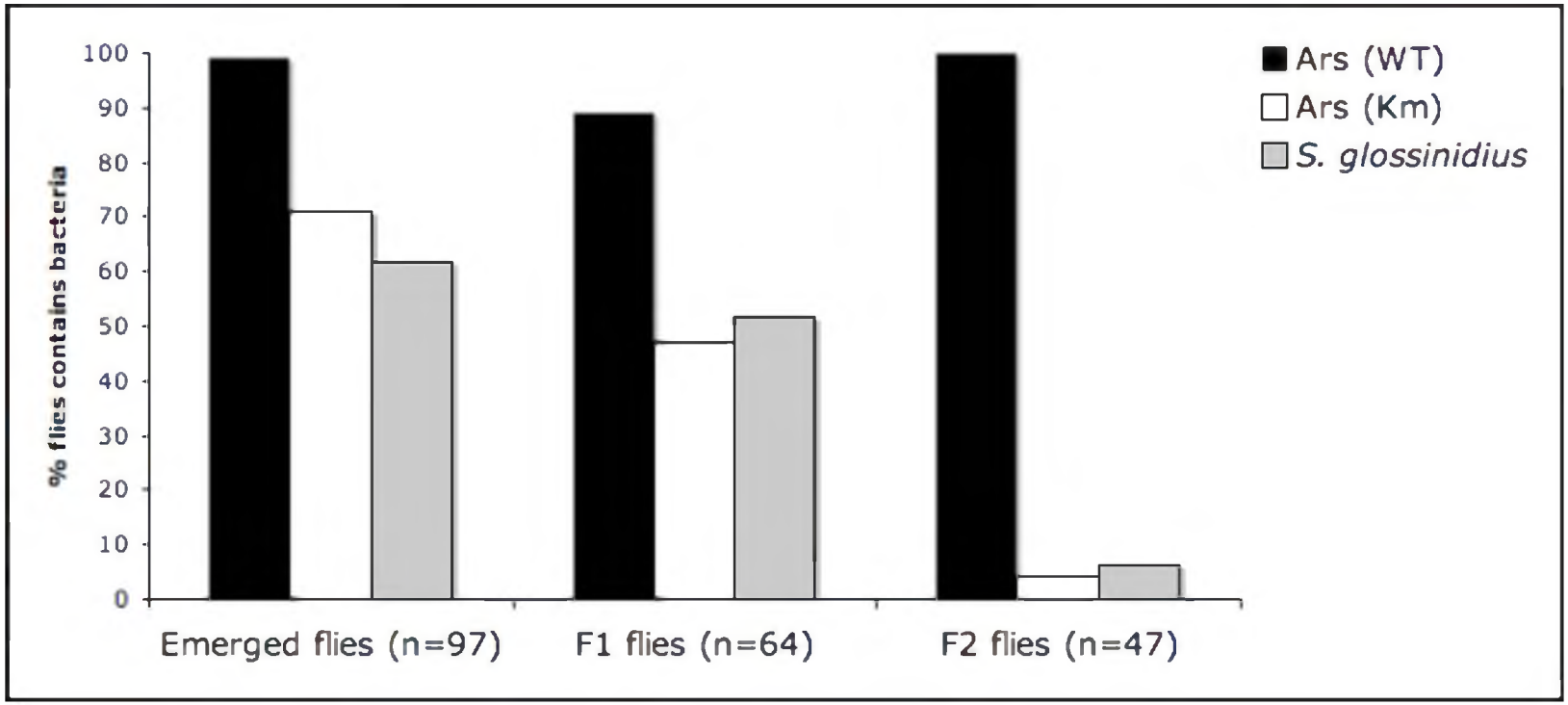

Figure 3.11 Analysis of exogenous endosymbiont propagation between generations of flies, using PCR. Bars show the percent of flies harboring (above the noise level) the indicated bacteria strains. **"**Ars (Km)": injected Kanamycin resistant *Arsenophonus arthropodicus*; "Sg": *Sodalis glossinidius*; Ars (WT): native *Arsenophonus arthropodicus*.

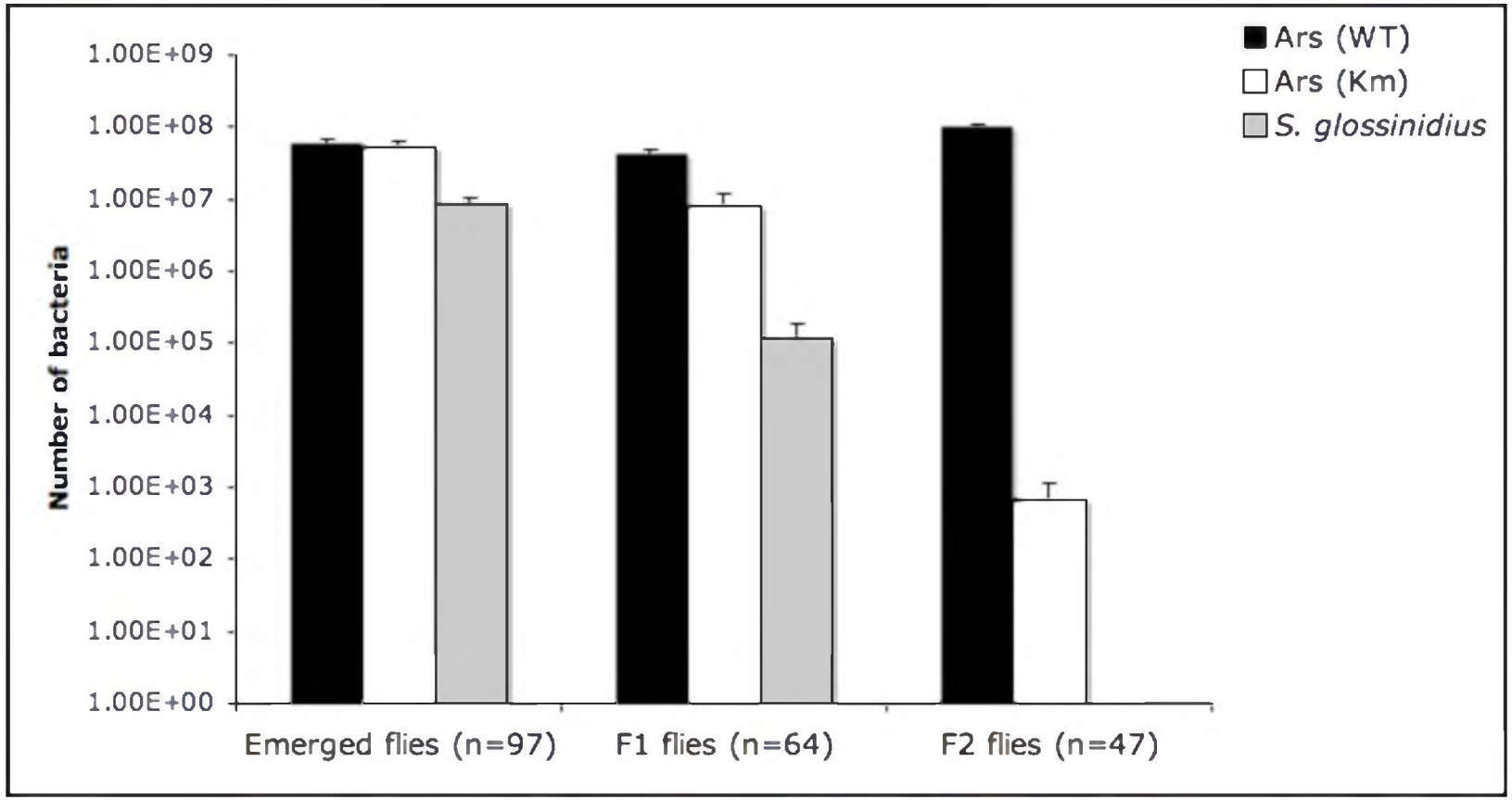

Figure 3.12 Multigenerational analysis of microinjected endosymbiont survival and propagation. **"**Ars (Km)": injected Kanamycin resistant *Arsenophonus arthropodicus*; "Sg": *Sodalis glossinidius*; Ars (WT): native *Arsenophonus arthropodicus*. Symbiont cell count was measured with qRT-PCR in three generations of flies, starting with the parental generation that was microinjected with *Km* and *SG* in their pupa stage.

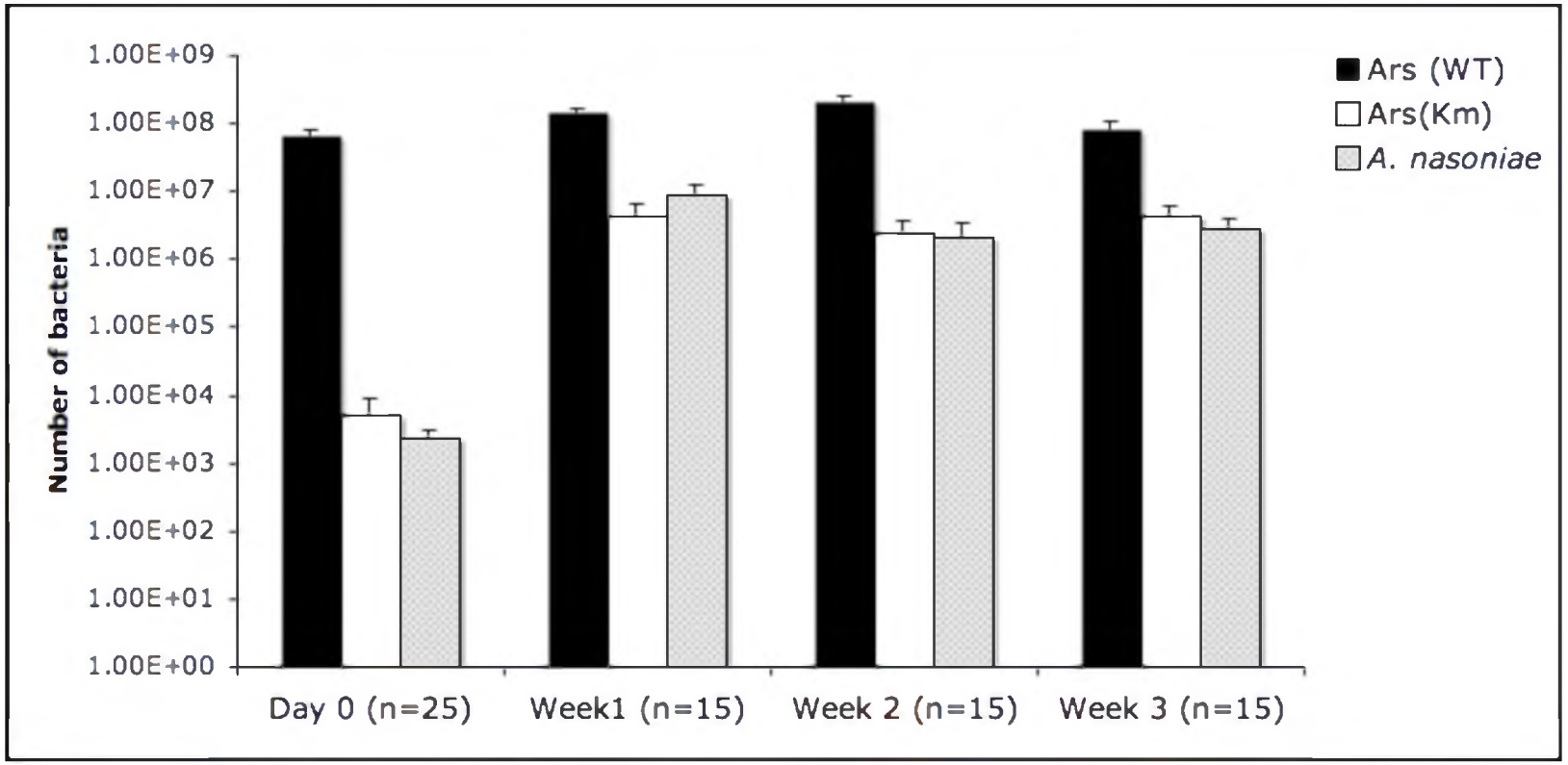

Figure 3.13 Temporal profile of colonization level of louse fly pupae microinjected with symbionts *A. arthropodicus (Km)* (Ars(Km)) and *A. nasoniae*. Bacterium cell count was determined with qRT-PCR. Colonization occurred within 1 week.

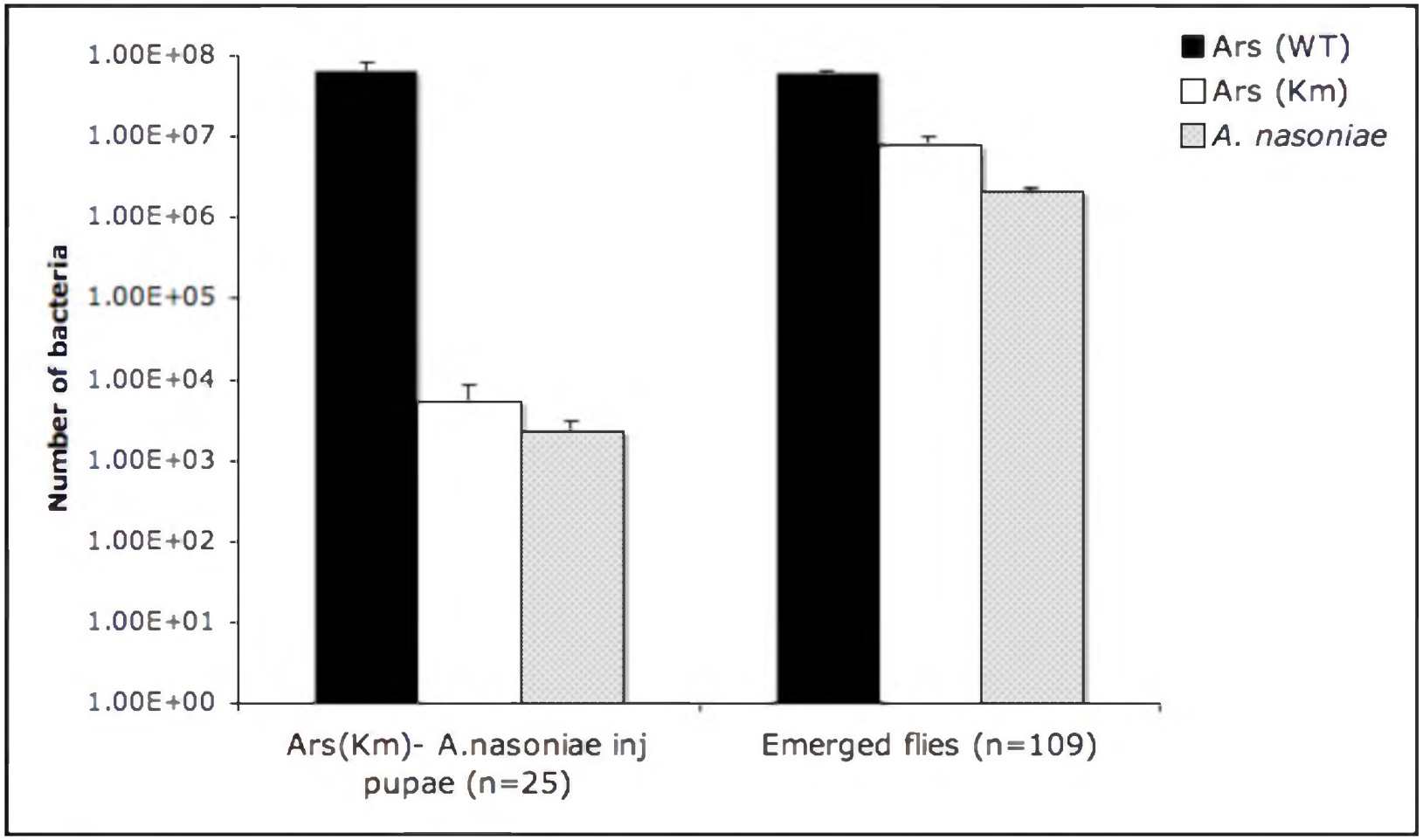

Figure 3.14 qRT-PCR determination of colonization level in flies eclosed from microinjected pupae.

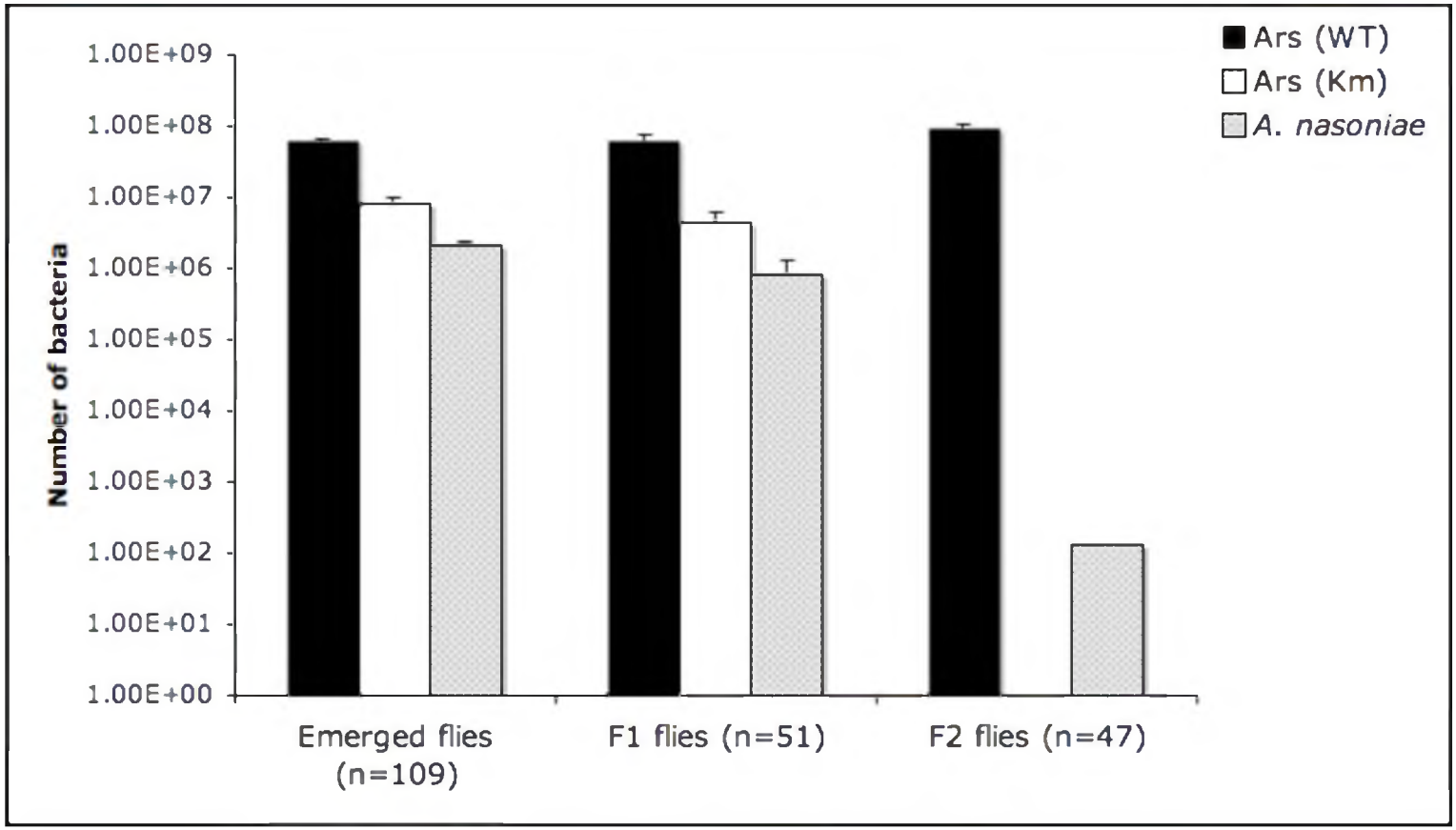

Figure 3.15 Multigenerational analysis of microinjected endosymbiont survival and propagation. **"**Ars (Km)": injected Kanamycin resistant *Arsenophonus arthropodicus*; "A. Nasoniae": *Arsenophonus Nasoniae (Nas)*; Ars (WT): native *Arsenophonus arthropodicus*. Symbiont cell count was measured with qRT-PCR in three generations of flies, starting with the parental generation that was microinjected with *Km* and *Nas* in their pupa stage.

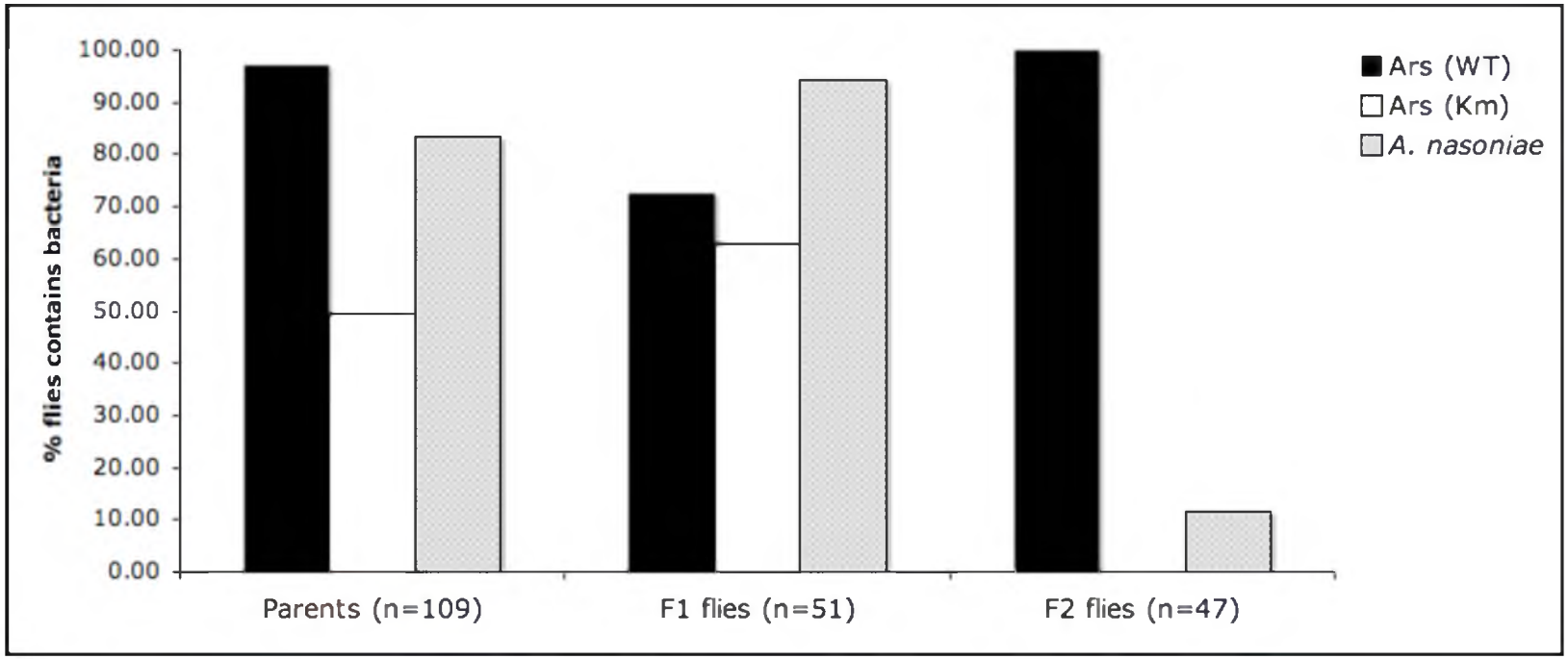

Figure 3.16. Analysis of exogenous endosymbiont propagation between generations of flies, using qRT-PCR. Bars show the percent of flies harboring (above the noise level) the indicated bacteria strains. "Ars (Km)": injected Kanamycin resistant *Arsenophonus arthropodicus*; Ars (WT): native *Arsenophonus arthropodicus*; *A. nasoniae: Arsenophonus nasoniae.*

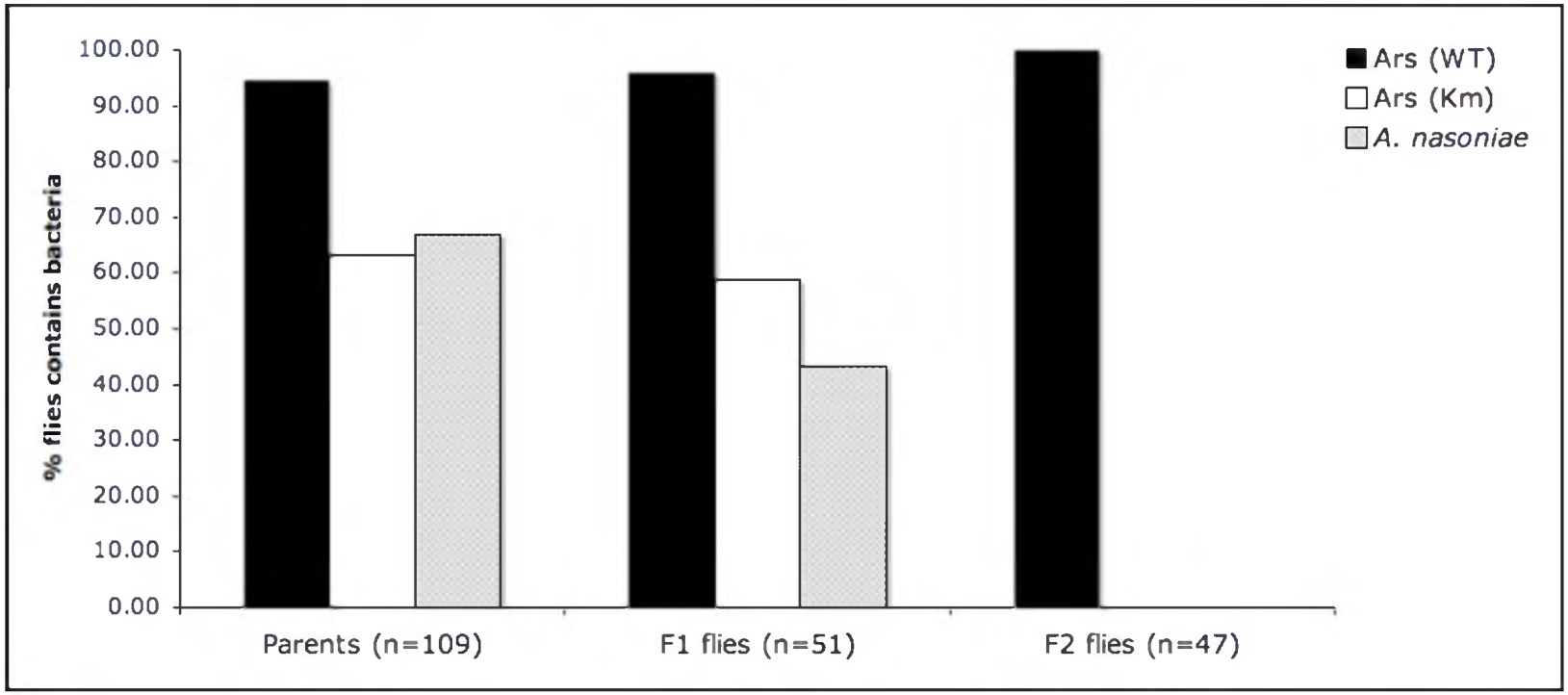

Figure 3.17. Analysis of exogenous endosymbiont propagation between generations of flies, using PCR. Bars show the percent of flies harboring (above the noise level) the indicated bacteria strains. **"**Ars (Km)": injected Kanamycin resistant *Arsenophonus arthropodicus*; Ars (WT): native *Arsenophonus arthropodicus*; *A. nasoniae: Arsenophonus nasoniae.*

Table 3.1 PCR and qRT-PCR analysis of PBS injected (control) pupae.

PCR						qRT-PCR			
Sample ID	DNA	Ars (WT)	Ars (Km)	S. gloss	A. nas	Ars (WT)	Ars (Km)	S. gloss	A. nas
P1	+	+	-	-	-	4.95E+05	2.35E+01	1.24E+00	0
P2	+	+	-	-	-	3.95E+04	1.43E+01	1.82E+00	3.05E+00
P3	+	+	-	-	-	1.99E+04	3.54E+01	1.27E+00	0
P4	+	+	-	-	-	2.12E+05	1.93E+02	9.98E-01	2.41E+00
P5	+	+	-	-	-	1.68E+05	3.17E+02	7.56E+00	1.17E+00
P6	+	+	-	-	-	3.83E+05	1.12E+02	1.37E+01	5.88E+00
P7	+	+	-	-	-	1.05E+06	2.34E+02	3.33E+02	1.06E+00
P8	+	+	-	-	-	1.19E+06	9.55E+01	1.88E+02	0
P9	+	+	-	-	-	2.73E+06	1.55E+01	6.36E+01	0
P10	+	+	-	-	-	1.87E+06	3.22E+01	5.49E+01	0
P11	+	+	-	-	-	6.96E+06	8.30E+01	1.21E+01	1.50E+00
P12	+	+	-	-	-	1.24E+06	1.22E+02	2.43E+02	0
P15	+	+	-	-	-	3.04E+06	1.76E+02	1.08E+02	0

Table 3.1 continued

	PCR					qRT-PCR			
Sample ID	DNA	Ars (WT)	Ars (Km)	S. gloss	A. nas	Ars (WT)	Ars (Km)	S. gloss	A. nas
P16	+	+	-	-	-	9.09E+02	3.92E+02	4.73E+02	0
P17	+	+	-	-	-	4.19E+05	2.13E+02	3.18E+02	1.06E+00
P18	+	+	-	-	-	1.01E+06	2.11E+02	4.64E+02	0
P19	+	+	-	-	-	3.98E+06	1.16E+01	3.52E+02	1.05E+00
P20	+	+	-	-	-	6.21E+05	2.66E+01	6.34E+00	0
P21	+	+	-	-	-	6.24E+05	1.27E+01	1.24E+01	1.39E+00
P22	+	+	-	-	-	2.35E+06	9.92E+01	6.90E+00	0
P23	+	+	-	-	-	4.93E+05	1.46E+01	6.06E+00	4.12E+00
P24	+	+	-	-	-	1.51E+05	7.27E+01	7.75E+01	0

Both methods were used to detect (and quantify) the presence of Ars (WT), Ars (Km), *S. glossinidius* (S. gloss*)* and *A. nasoniae* (A. nas*).* The presence of primary symbiont DNA (DNA) was used as a measure of successful DNA extraction. PCR results showed that all samples were positive (+) for Ars (WT), and negative (-) for the other three bacteria: Ars (Km), S. gloss, A. nas. The number of exogenous bacterium cells was found with qRT-PCR to be always under 10 (for A. nas) or 1000 (for Ars (Km) and S. gloss).

Table 3.2 PCR and qRT-PCR analysis of adult flies, injected with PBS (control) during pupae stage.

Sample ID	PCR					qRT PCR			
	DNA	Ars (WT)	Ars(Km)	S. gloss	A. nas	Ars (WT)	Ars(Km)	S. gloss	A. nas
PF1	+	+	-	-	-	1.87E+05	2.03E+01	4.88E+00	0.00E+00
PF2	+	+	-	-	-	3.57E+04	2.98E+01	1.87E+00	3.95E+00
PF3	+	+	-	-	-	5.85E+04	8.48E+00	5.15E+00	1.99E+00
PF4	+	+	-	-	-	1.48E+05	1.04E+01	1.09E+01	1.43E+00
PF5	+	+	-	-	-	1.86E+05	3.40E+01	3.09E+00	0.00E+00
PF6	+	+	-	-	-	8.96E+04	1.27E+02	1.29E+01	0.00E+00
PF7	+	+	-	-	-	2.64E+06	5.48E+02	1.53E+04	0.00E+00
PF8	+	+	-	-	-	2.53E+06	5.56E+01	1.55E+02	3.95E+00
PF9	+	+	-	-	-	2.56E+06	5.68E+01	1.37E+02	0.00E+00
PF10	+	+	-	-	-	2.12E+06	4.81E+01	1.84E+02	0.00E+00
PF11	+	+	-	-	-	4.53E+06	1.26E+02	1.54E+02	0.00E+00
PF12	+	+	-	-	-	1.47E+06	1.13E+02	1.46E+02	0.00E+00

Table 3.2 continued

Sample ID	PCR					qRT PCR			
	DNA	Ars (WT)	Ars(Km)	S. gloss	A. nas	Ars (WT)	Ars(Km)	S. gloss	A. nas
PF13	+	+	-	-	-	6.24E+06	2.70E+02	2.59E+01	0.00E+00
PF14	+	+	-	-	-	1.13E+06	1.36E+02	1.69E+02	0.00E+00
PF15	+	+	-	-	-	2.31E+06	2.87E+01	2.94E+02	0.00E+00
PF16	+	+	-	-	-	1.92E+06	7.41E+01	1.39E+02	0.00E+00
PF17	+	+	-	-	-	2.42E+06	1.91E+02	4.33E+02	5.45E+00
PF18	+	+	-	-	-	3.03E+06	6.55E+01	2.47E+02	3.35E+00
PF19	+	+	-	-	-	1.36E+06	1.47E+01	1.05E+02	2.67E+00
PF20	+	+	-	-	-	1.55E+06	1.61E+01	8.93E+01	0.00E+00
PF21	+	+	-	-	-	2.97E+06	1.22E+02	1.28E+01	0.00E+00
PF22	+	+	-	-	-	1.85E+06	1.68E+02	2.75E+01	0.00E+00
PF23	+	+	-	-	-	2.48E+06	1.15E+02	1.61E+01	0.00E+00
PF24	+	+	-	-	-	1.31E+06	1.17E+02	4.64E+01	0.00E+00
PF25	+	+	-	-	-	4.09E+06	1.19E+02	9.36E+00	0.00E+00

Table 3.2 continued

Sample ID	PCR					qRT PCR			
	DNA	Ars (WT)	Ars(Km)	S. gloss	A. nas	Ars (WT)	Ars(Km)	S. gloss	A. nas
PF26	+	+	-	-	-	1.45E+06	3.83E+02	6.89E+01	0.00E+00
PF27	+	+	-	-	-	3.20E+06	2.19E+02	8.75E+01	0.00E+00
PF28	+	+	-	-	-	1.86E+06	1.15E+02	7.70E+01	0.00E+00
PF29	+	+	-	-	-	2.35E+06	4.14E+01	2.25E+01	0.00E+00
PF30	+	+	-	-	-	1.63E+06	6.20E+02	2.26E+01	0.00E+00
PF31	+	+	-	-	-	2.57E+06	6.20E+01	8.55E+00	0.00E+00
PF32	+	+	-	-	-	2.08E+06	5.92E+02	3.66E+01	0.00E+00
PF33	+	+	-	-	-	1.56E+06	1.31E+02	5.14E+00	0.00E+00
PF34	+	+	-	-	-	2.02E+06	1.38E+02	3.01E+01	1.47E+00
PF35	+	+	-	-	-	5.61E+06	7.27E+01	2.60E+01	0.00E+00
PF36	+	+	-	-	-	1.90E+06	1.08E+02	2.57E+01	5.60E+00
PF37	+	+	-	-	-	8.81E+05	1.10E+02	4.57E+01	1.12E+00
PF38	+	+	-	-	-	1.30E+06	1.39E+02	3.25E+01	1.93E+00

Table 3.2 continued

Sample ID	PCR					qRT PCR			
	DNA	Ars (WT)	Ars(Km)	S. gloss	A. nas	Ars (WT)	Ars(Km)	S. gloss	A. nas
PF39	+	+	-	-	-	8.10E+04	1.78E+02	4.30E+01	4.42E+00
PF40	+	+	-	-	-	1.86E+06	1.43E+02	2.94E+01	0.00E+00
PF41	+	+	-	-	-	5.65E+06	1.18E+02	4.56E+01	3.04E+00
PF42	+	+	-	-	-	5.72E+05	1.11E+02	3.17E+01	0.00E+00
PF43	+	+	-	-	-	8.05E+05	1.95E+02	3.19E+00	0.00E+00
PF44	+	+	-	-	-	1.12E+06	1.62E+02	2.48E+01	0.00E+00
PF45	+	+	-	-	-	7.26E+05	1.03E+02	3.27E+01	1.72E+00
PF46	+	+	-	-	-	2.59E+05	1.97E+02	5.80E+01	0.00E+00
PF47	+	+	-	-	-	2.02E+06	1.39E+02	1.72E+01	0.00E+00
PF48	+	+	-	-	-	2.62E+06	8.24E+01	2.85E+01	0.00E+00
PF49	+	+	-	-	-	1.04E+06	2.09E+02	4.17E+01	1.04E+00
PF50	+	+	-	-	-	5.24E+05	1.18E+02	3.75E+01	0.00E+00

Both methods were used to detect (and quantify) the presence of Ars (WT), Ars (Km), *S. glossinidius* (S. gloss) and *A. nasoniae* (A. nas).

Table 3.2 continued
The presence of primary symbiont DNA (DNA) was used as a measure of successful DNA extraction. PCR results showed that all samples were positive (+) for Ars (WT), and negative (-) for the other three bacteria: Ars (Km), S. gloss, A. nas. Accept for sample “PF7”, the number of exogenous bacterium cells was found with qRT-PCR to be always under 10 (for A. nas) or 1000 (for Ars (Km) and S. gloss).

4. DISCUSSION

The aim of the experiments described here was to establish if experimental lateral transfer of secondary endosymbionts was possible in the presence of the intact native primary and secondary symbiont colonies. I used the louse fly – *Arsenophonus arthropodicus* (Ars(WT) or "*WT*") system and tried to introduce two different bacteria, *Sodalis glossinidius* ("*SG*"; from the tsetse fly) and *Arsenophonus nasoniae* ("*Nas*"; from the parasitoid wasp). As an internal control, I always co-injected a kanamycin-resistant strain of *Arsenophonus arthropodicus (*Ars(Km) *or "Km").* By using regular PCR and qRT-PCR, I was able to determine the prevalence and approximate number of bacterial cells in each pupa and emerged fly and thus was able to compare colonization levels across different stages and between the different bacteria. I used the selected bacteria because one (*SG*) was coming from an insect with a similar lifestyle, diet and symbiont transmission but was from a different insect genus. The other (*Nas*) was from the same bacterial genus but was from an insect with a different (parasitoid) lifestyle and symbiont transmission (Gherna et al., 1991).

4.1 Method evaluation

I found that injecting pupae was a viable method to transfect louse flies, as all injected bacteria readily colonized the pupae, increasing the cell number greatly within 1 week after injection. Colony size changes after this initial period

were much smaller, indicating that whatever niche the newly injected symbionts found, it may have been imposing an upper limit on colony size. As was clearly demonstrated by the analysis of pupa survival, microinjection of pupae is an invasive method that inherently increases pupa death. The viability of the pupae is not easy to determine – the clearest indication of pupa death is when they do not eclose. Thus whenever "pupa bacterium content" is measured, the live/dead status of the pupa itself is not known. Interestingly, breaching the puparium wall, injecting PBS or PBS containing *Km* or *Nas* bacteria had the same deleterious effect on pupa survival rate; thus it seems that the presence of these bacteria did not affect survival. In contrast, *SG* decreased survival. Microinjection of *SG* was also the only experimental procedure that statistically significantly increased the percentage of males. Since sex of the flies is determined before microinjection, it seems that the presence of SG in the pupae preferentially decreased the survival rate of female flies.

Although injecting pupae with bacteria is an artificial and invasive method, I believe it is still a valid one as it is also a possible natural route of new bacterial infection, when the pupa wall is breached by natural causes.

4.2 Exogenous symbiotic bacteria in the louse fly

All of the studied bacteria injected in the pupae survived and colonized the pupae, then survived eclosion and were transmitted into the first, noninjected generation (F1). Although there were significant changes in the native secondary symbiont cell count, these changes were small in amplitude and did not seem to offset the growth of injected exogenous symbiont colonies. Also, these non-

native colonies never quite reached the colony size of native *A. arthropodicus*. It seems thus, that the capacity of flies to harbor secondary symbiotic bacteria is not limited to the endogenous pool. It is possible that at least some of the microinjected bacteria found a different part of the fly body to inhabit. The fly immune system was not able to destroy these new symbionts, although it may still have played a part in limiting and stabilizing the size of the symbiont colonies.

For lateral symbiont transfer to happen, three major requirements must be met. The first is that upon gaining entry into the insect, the bacteria need to be able to survive and establish a stable colony, without killing the host organism. As we have seen, this requirement was fully met for both *S. glossindius* and *A. nasoniae*. *SG* had a minor negative effect on survival rate, shifting the sex ratio but this effect was not so strong as to preclude lateral symbiont transfer. The second requirement is that the fertility of the host must not be diminished. I did not conduct specific measurements of fly fertility; however, it is safe to state that since I had no problem with propagating flies that were injected as pupae, fertility was most probably not affected in a major way.

The third major requirement is transmission into other flies either through infection or through the natural (for native secondary symbionts) transmission through milk. Although the path of transmission could not have been determined in these experiments, I have registered high percentage of transmission of all secondary symbionts into the first generation (F1, itself not injected). This

suggests that all major criteria were met and lateral transfer of secondary symbionts is definitely possible, if only for a very limited number of generations.

Why were not *S. glossindius* then transmitted to the second (F2) generation? As mentioned earlier, the presence of SG in pupae affected survival rate and decreased the percent of eclosed females – which should lower the chance of propagation but not in the experimentally controlled environment. Transmission of *SG* to F1 happened only in 18% of flies, while at the same time, transmission at this point was 100% effective for the injected Km. Thus it seems that their transmission had a relatively low probability compared to *A. arthropodicus* and in these controlled conditions (and absence of a positive selection of *SG*-containing flies) *SG* colonies were set to "die out" within a few generations, the number of which was dependent on the success rate of transmission. In the case of *A. nasoniae*, transmission was not as effective as that of the native or *A. arthropodicus*; however, it did not go extinct as *SG* did in F2. The level of *SG* colonization was decreased to below the level seen after microinjection, thus all the gains in bacterial cell number within the first pupae were lost in F2.

Interestingly, the exogenous *A. arthropodicus (Km)* colonies disappeared completely in this experiment in F2. This is seemingly in contrast with what I found in the experiments with *SG*, where Km remained in a large percentage of flies – although at a very significantly lower cell count. In fact the low average cell count was definitely mostly due to a small cell count per fly and to a much lesser extent the lack of flies carrying any of these bacteria. This later component only

reduced the average by approximately 10% whereas actual cell count decrease was responsible for the lion's share of the approximately10^4 decrease that brought cell counts below or close to the level seen right after injection. Thus it seems that although in the short term, lateral transfer through pupa infection is possible, the exogenous symbionts have a decreasing probability of long-term survival across generations in laboratory conditions. This is true even for the native *Arsenophonus* symbiont when it infects a pupa and is not transmitted through the regular pathway. The reason for probably not being able to utilize the normal transmission pathway may have been due to unnatural localization (thus not being able to "get there"), inhibition by the native secondary symbiont (because there could be limited capacity of transmission through milk), or inhibition by the immune system (again, "not being able to get there") or a combination of these factors. If the immune system played a major role in diminishing the exogenous endosymbiont colonies, it would have had to be able to differentiate between the native and exogenouse *Arsenophonus* bacteria. Thus it seems that the site and manner of infection/introduction and colonization site(s) of non-native symbiotic bacteria may be as important as the type of the colonizing bacterium in determining long-term survival and potential for transmission between generations. The next logical step in determining how lateral symbiont transfer may occur is the identification of the pathway through which exogenous bacteria may gain access to better propagation through the generations. This could be assessed through immunohistochemical and in situ hybridization (FISH) studies.

4.3 Summary

Based on the experiments described in this work, it is possible to transfect louse fly pupae with both the tsetse fly and the parasitoid wasp secondary symbionts. The transfection is:

1.) **Stable**, showing a strong colonization of pupae which means that the new endosymbiotic bacteria are not eliminated due to an immune response or native endosymbiont competition in either pupae or later in the adult flies.

2.) **Strong/substantial**, as the number of injected bacteria grew >1000 times.

3.) **Compatible**, as the presence of the non-native endosymbiont did not seem to inhibit development, fertility or adult survival of flies. It also did not decrease the pool size of the native secondary symbiont.

4.) **Declining across generations (not self-perpetuating),** as infected louse flies transmitted their new endosymbionts to their (F1) offspring but there was poor or no transmission to F2, which means that the new endosymbionts probably could not utilize the normal transmission pathways.

In summary, this study shows that interspecies transfer is not impossible between the studied host/symbiont systems. Based on these same studies however, the probability of a sustained, multigenerational colonization of the host by the transferred endosymbiont is very low and such a colonization would have to be repeated practically every other generation thus coevolution with the host would not be possible.

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