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ESTABLISHING *SODALIS SPECIES* AS A LABORATORY MODEL OF ENDOSYMBIONTS

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Abstract

Symbiosis is defined as the close and long relationship between two organisms. Establishment of new symbioses, or redefining relationships underpins much of the ecological diversity found in the natural world. Microbial symbionts, being some of the longest living organisms on the planet with the largest distribution, offer the best opportunity to understand the complex mechanisms behind host-symbiont interactions and evolutionary processes. The insect kingdom, comprised of over 1.2 million described species, is an ideal sample group in symbiont research as over 50% of them harbour microbial symbionts. *Glossina* spp., the viviparous, obligate blood feeding (tsetse) flies that populate sub Saharan Africa, are of interest within symbiont research as they play host to at least four bacterial symbionts, with diverse phenotypes: *Wigglesworthia*, *Wolbachia*, *Spiroplasma* and *Sodalis*. *Sodalis glossinidius* – a secondary endosymbiont - is interesting as sequencing of its genome suggests *S. glossinidius* has undergone less genome reduction than its primary symbiont counterparts such as *Wigglesworthia,* and therefore has a more recent association with its host than the other symbionts. The benefit of this reduced rate of genome reduction is the ability to culture *S. glossinidius* in vitro, a feature that most bacterial symbionts lack. Culture of two *Sodalis* species – *S. glossinidius* and a related species, *S. praecaptivus*, was performed to compare the viability of *S. glossinidius* to free living bacteria to determine its potential as a laboratory model of symbiosis. This was studied via growth curves in different laboratory media, resistance to oxidative stress, antibiotic susceptibility and survival in an experimental host – *Galleria mellonella*. The difficulties in culturing bacterial endosymbionts is highlighted; the ability, with care, to culture *S. glossinidius*, and the potential to compare to closely-related, free-living species

such as *Sodalis praecaptivus* is vital as a research model for studying symbiosis and host-interaction.

Introduction

The importance of symbiosis in nature

Symbiosis is defined as “the close and long-term relationship between two organisms”. The term “symbiosis” is often misconstrued or misused by scientists and the public alike. Most conflate “symbiosis” with “mutualism”, that is to say that both organisms benefit as a consequence of their close association, however it is more appropriate to define symbiosis as above. Symbiosis is split into three or four broad sub-definitions, which includes parasitism (where one symbiont is harmed), commensalism (where one symbiont benefits, while the other is essentially unaffected in any significant way) and mutualism (as described above). A fourth definition: neutralism – is controversial and defined as neither symbiont affecting the other in any way, however these are unlikely to exist in any meaningful way in nature (Martin and Schwab, 2012).

Together these definitions come together on the “symbiosis spectrum”, as shown in figure 1, below:

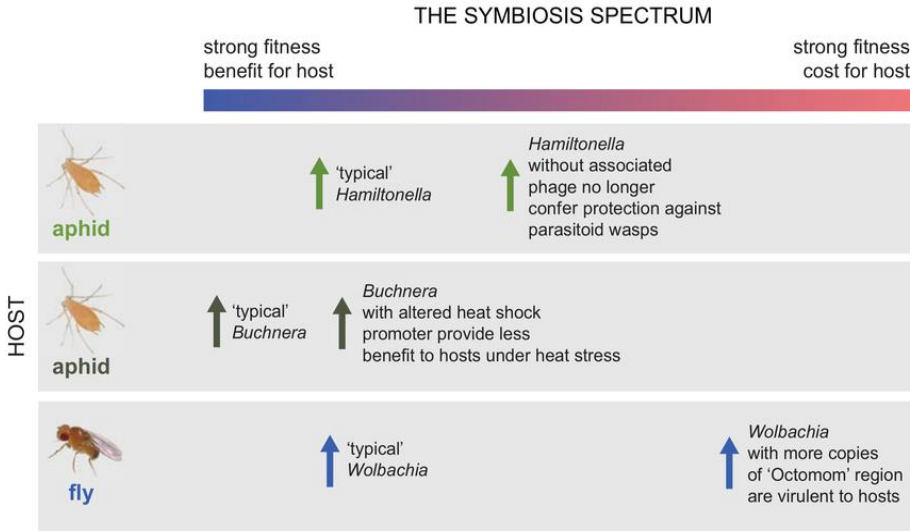

Figure 1-The symbiotic spectrum which describes the effects of symbiotic bacteria in relation to the fitness advantages/disadvantages to the host- taken from Gerardo (2015)

These classifications are very fluid; one organism can interact differently depending on the other organism it is in symbiosis with. *Wolbachia,* for example, is a symbiotic bacterium that has an association with both arthropods and nematodes, but its effects on the host species vary. Within arthropods, *Wolbachia* largely acts a parasite which manipulates the reproductive capabilities of the host through four phenotypes; male killing, feminisation, parthenogenesis and cytoplasmic incompatibility (Warren et al, 2008). In nematodes, however, *Wolbachia* act in mutualistic association and provide their host with some fitness advantage necessary for reproduction and survival if the nematode (Foster *et al.*, 2005). This was further proven in a further study by Taylor *et al.*(2005) where nematode worms given doxycycline (200mg daily over an eight week period) experienced detrimental effects in fertility and viability.

Symbiotic relationships can be the driving force for evolutionary novelty and ecological diversity found on the planet and is therefore an important area in evolutionary research (Wernegreen, 2004). Microbial symbionts, due to the wide distribution of bacteria through various ecosystems and long-standing ancestry (~4 billion years), have had a catalytic effect on the evolution of many organisms (Wernegreen, 2004).

A good example of the effect of microbial symbionts are the *Rhizobia* species and their relationship with leguminous plants. *Rhizobia* are Gram negative nitrogen-fixing bacteria that reside within soil and are the only nitrogen-fixing bacteria to form a symbiotic relationship with legumes. The symbiotic relationship formed between the two organisms involves the signal exchange of flavonoids secreted from the roots of the host plant which lead to the accumulation and attachment of *Rhizobia* to root hair cells (Maj *et al.*, 2010). The flavonoids trigger the secretion of nod factors by *Rhizobia* which causes developmental

changes within the root hairs, leading to the formation of root nodules (Gage, 2004). Within the root nodules, *Rhizobia* fix atmospheric nitrogen into ammonium which is used to synthesise amino acids in their host. In return, the legumes provide carbohydrates to the bacteria as well as oxygen via leghaemoglobins for cellular respiration. The mutualism between the two species has developed over 66 million years (Zharan, 1999) to the point where *Rhizobia* cannot express the genes used in nitrogen fixation without the presence of the legumes.

The relationship between coral and *Symbiodinium* (symbiotic dinoflagellates) is interesting from the point of symbiosis. Coral reefs exist in many marine ecosystems and are home to millions of species (Knowlton, 2001). It is widely known that *Symbiodinium* plays a key role in the survival of coral in harsh marine environments (Liu *et al*, 2018). *Symbiodinium* species colonise the tissue of the coral and photosynthesise and provide the photosynthates (water, glucose and oxygen) to the coral. The coral will metabolise the photsynthates to form a calcium carbonate skeleton strong enough to withstand harsh conditions. The waste inorganic nutrients and CO_2 generated by the coral is recycled by the dinoflagellates (Muscatine and Porter, 1977). Similar to the leguminous plants and *Rhizobia*, the interaction between the coral and *Symbiodinium* is initiated by the secretion of chemical signals by the coral which attract free living dinoflagellates (Davy, Allemand and Weis, 2012). The coral undergoes dynamic remodeling of its cytoskeleton and membrane to allow entry of the dinoflagellate via phagocytosis (Davy, Allemand and Weis, 2012). The association between the two is very interesting as it was thought to be a 'pure' symbiotic relationship, meaning that the two were reliant on each other for survival, however some research has found evidence to the contrary. Wooldridge (2010) found that the dinoflagellates are capable of survival outside of their host, however, when they enter their host, there is a negative

impact on their reproductive success and in essence become “trapped” within the coral. The coral, on the other hand, seem to pay no cost for this relationship and as such, it can be asserted that the relationship between the two is not a mutualistic one, but rather a more parasitic relationship in favour of the coral.

The aforementioned examples of symbiosis are instances where there is one bacterial symbiont present, however, there are many host organisms that form symbiotic relationships with several microorganisms. This can be said to be most prevalent within the insect kingdom.

Symbiosis and Insects

The insect kingdom is comprised of over 1.2 million described species, and of that number, over 50% of them are estimated to harbour microbial symbionts, dubbed endosymbionts. (Hirose, Panizzi and Prado, 2012).

Bacterial endosymbionts of insects can be categorised as either primary or secondary (Raina et al, 2005) and could be found intra- or intercellularly within the host body. Primary or P-endosymbionts have been in a long association with its host, forming an obligate association and displaying phylogenetic congruence with the host (Clark, Baumann and Baumann, 1992). P-endosymbionts provide nutrients that their host is unable to acquire themselves and can metabolise waste products generated by the host into less harmful substances (Baumann, 2005) Secondary or S-endosymbionts have a comparatively shorter evolutionary history with their host (Dale and Moran, 2006) and thus exhibits a more facultative relationship. S-endosymbionts are reported to confer a variety of functional benefits to the host. *Acyrthosiphon pisum* (pea aphid) holds interest as it is host to both primary and secondary endosymbionts. The P-endosymbiont of the pea aphid is *Buchnera aphidicola*. *B. aphidiciola is* found within specialised cells within *A. pisum* known as bacteriocytes (Clark *et*

al., 2000). The symbiotic relationship is primarily a nutritional one; the aphid's diet typically consists of plant phloem sap which is insufficient in essential amino acids ((Douglas, 2006). *Buchnera aphidicola* can synthesise and provide these amino acids for their hosts in return for the other nutrients their host provides (Baumann *et al.*, 1995).

One of the more intriguing S-endosymbionts of *A. pisium* is *Hamiltonella defensa*. *H. defensa* is distributed within the sheath cells and haemolymph. While it is not essential for host survival, *H. defensa* confers protection to their host from parasitoid wasps such as *Aphidius ervi* and *Aphidius eadyi* by preventing the larval development of the wasps within the host (Oliver, Moran and Hunter, 2005). Despite this benefit, *H. defensa* is found irregularly within *A. pisium* and its presence within its host is tied to the intensity of parasitoid pressure (Oliver *et al.*, 2008).

Symbiosis and the Tsetse Fly

Tsetse (*Glossina* spp.; Diptera: Glossinidae) are viviparous, obligate blood-feeding flies found across sub-Saharan Africa. Around 37 species of Tsetse exist across various ecological niches in Africa, ranging from savannah to tropical forest areas having the largest distribution. The adult flies of both sexes feed exclusively on largely sterile blood meals from livestock, wildlife and humans (Figure 2).

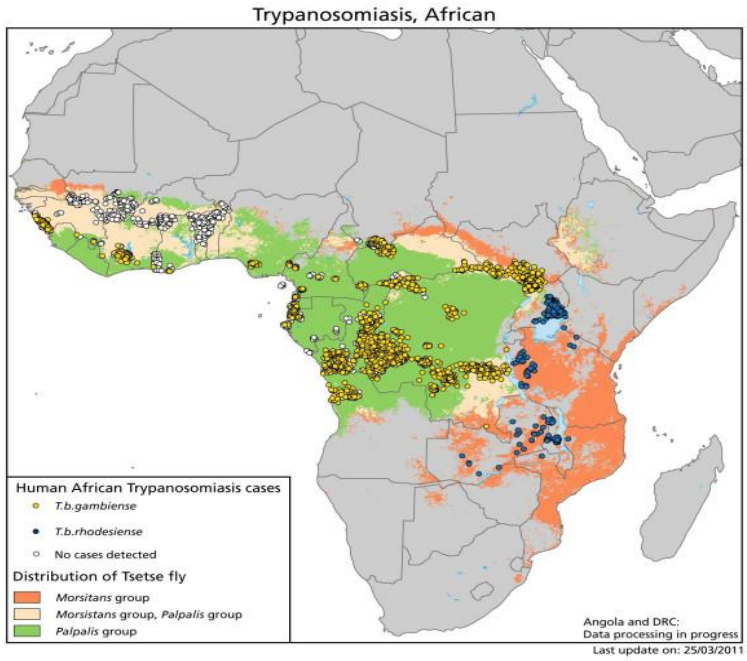

Figure 2- The distribution of the different species of tsetse fly across Africa. The prevalence of tsetse flies within the area is correlated to the spread of African trypanosomiasis. The distribution of the tsetse and areas where sleeping sickness is most prevalent are intrinsically linked, taken *from https://blog.wellcome.ac.uk/2012/03/01/developing-the-atlas-of-human-infectious-diseases/*

Tsetse Life Cycle

The life cycle of the tsetse fly is rather unusual; female tsetse flies after mating produce one egg which is retained within the uterus. The larva hatches from the egg and undergoes its first three developmental stages internally whilst feeding on the milk produced by the milk glands of its mother; this process is known as adenotrophic viviparity (Leak, 1999). The larva is then birthed on the ground and burrows into the earth to pupariate for around a month until eclosion.

Trypanosomiasis and the Tsetse Fly

Tsetse are of clinical and veterinary interest as they are the only arthropod vectors of African trypanosomes, responsible for human African trypanosomiasis (HAT) and animal African trypanosomiasis (AAT). The two common parasitic agents that cause trypanosomiasis are *Trypanosoma brucei gambiense* which is responsible for 98% of reported cases (WHO, 2019) and *Trypanosoma brucei rhodesiense* which has limited

geographical range and only affects East and South Africa (WHO, 2019). *T.b. gambiense* causes a chronic condition with can remain undetected for several months until symptoms are exhibited and it can be up to three years into the infection before death occurs (Brun *et al.*, 2010). *T.b. rhodesiense* causes the acute form of the disease; symptoms arise with weeks of infection and death with a few months (Kuepfer et al., 2011). HAT has a case mortality rate of virtually 100% (WHO, 2019) and is estimated that Africa loses $1.5 billion per year as a direct result of the disease (WHO, 2019). Drug treatment for the disease has a history of being ineffective and toxic to the recipients and although improvements have been made, more preventative measures such as vector control have taken the forefront (Kennedy, 2013). Tsetse fly control strategies such as the clearing of vegetation and aerial distribution of pesticides (Hocking, Lamerton and Lewis, 1963) were effective in 1960s but as HAT prevalence decreased, there was a lack of follow-up in the control efforts and as a consequence, tsetse and trypanosomiasis resurged (Simarro *et al.*, 2011). There has been some ruminations about looking into utilising the tsetse's natural endosymbionts in preventing the establishment of trypanosomes (Van Den Abbeele and Rotureau, 2013).

From a symbiosis point-of-view, tsetse flies are of particular interest, because of their unique reproductive strategy and similar to *A. pisum*, it is the host to multiple bacterial endosymbionts. Until recently the central dogma of tsetse symbiosis was that they harbour three main endosymbionts: *Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia pipientis*. However this has been recently revised to include *Spiroplasma* after its discovery in *Glossina fuscipes* (Doudoumis *et al.*, 2017); Figure 3).

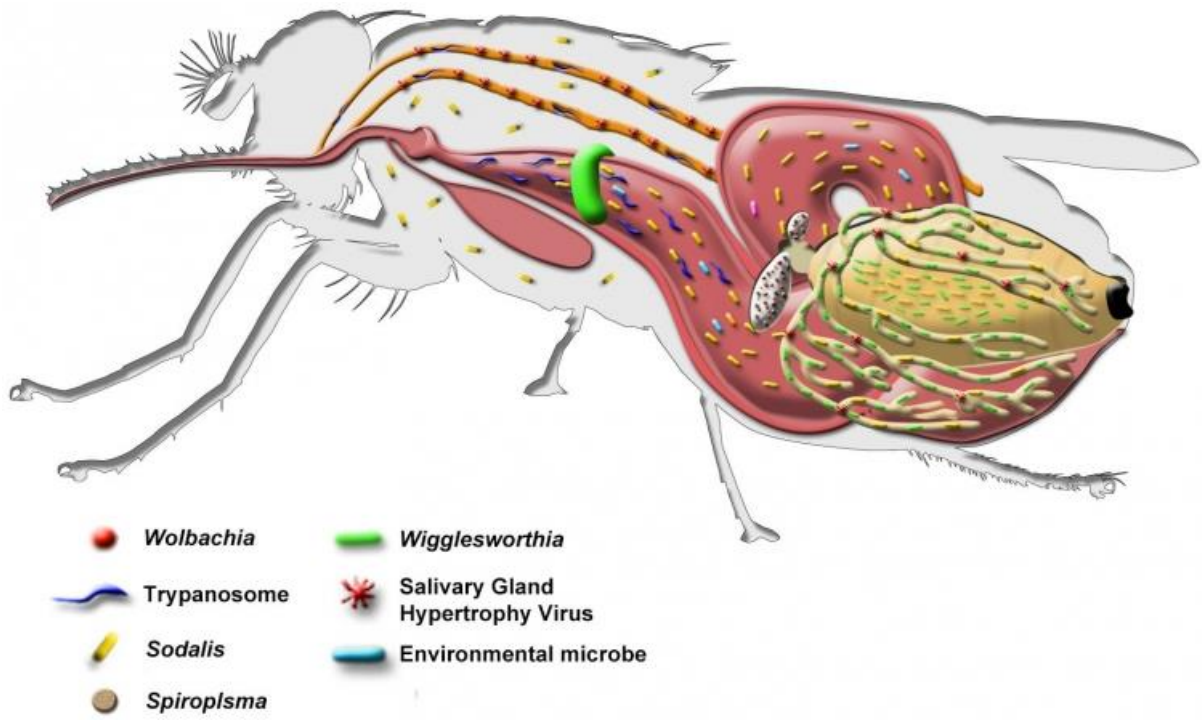

Figure 3- Visual representation of the tsetse fly microbiota and their localisation within their host, taken from https://www.iaea.org/newscenter/news/international-research-project-explores-novel-strategies-to-improving-the-sterile-insect-technique-toeradicate-tsetse-flies-through-enhancing-males-refractoriness-to-trypanosome-infection)

Wigglesworthia glossinidia is the P-endosymbiont of the tsetse and is found in 100% of tsetse flies. *W. glossinidia* provides nutrients to its host that are otherwise deficient in the blood (e.g. B vitamins) and in exchange, it is granted nutrients for its own survival, protection from the host's immune response and an efficient vertical transmission route to the tsetse's offspring (Bing *et al.*, 2017). In addition to its role in nutrition, *W. glossinidia* is essential for several essential physiological functions of the tsetse. A study conducted by (Weiss *et al.* (2011) discovered that the presence of *W. glossinidia* during larval development of the tsetse is beneficial to the maturation of the immune system in adult flies. *W. glossinidia* also plays a crucial role in the maintenance of the fecundity of the female tsetse fly. *W. glossinidia* can synthesise the vitamin B6 which acts as a co-factor for the enzyme AGAT in the tsetse fly which is responsible for the biosynthesis of proline from alanine (Michalkova *et al.*, 2014). Proline is utilised by the tsetse as an energy source and is

crucial during energy intensive processes during the tsetse's reproductive cycle such as the lactation period. Experimentally induced asymbiotic female tsetse flies were shown to have lower levels of vitamin B6 and exhibited hypoprolinemia which lead to a decrease in fecundity (Michalkova *et al.*, 2014).

Primary endosymbionts undergo genome reduction as they become more dependent on their host and therefore, there is inevitable loss of function required for free-living existence (Sloan and Moran, 2012). *W. glossinidia* has one of the smallest genomes of any living organism with a single chromosome of 700,000 base pairs and a singular plasmid of 5.2kbp (described further below) (Akman et al, 2002).

Secondary endosymbionts, compared to P-endosymbionts, have been in association with their host for a comparative shorter period, and as such, their relationship with their host is facultative (Wernegreen, 2012). A notable secondary endosymbiont is *Sodalis glossinidius* which is also harboured by the tsetse fly. *S. glossinidius* can be found in various places of the tsetse's anatomy; intracellularly and extracellularly within the gut lumen, milk glands, flight muscles, mouthparts, testes and ovaries (Cheng and Aksoy, 1999).

Wolbachia

Wolbachia, the second P-endosymbiont of the tsetse fly, are parasitic bacteria that are also prevalent in over 50% of arthropod species. Within the tsetse fly, *Wolbachia* reside within the reproductive tissue. The presence of *Wolbachia* infections have a significant negative impact on the reproductive capabilities on their host and are the driving force behind the cytoplasmic incompatibility within the tsetse fly. Cytoplasmic incompatibility occurs when a *Wolbachia* infected male mates with an uninfected female which leads to the development

arrest of the embryo. It also involves *Wolbachia* infected females which can mate with infected or uninfected males, which guarantee the production of viable, *Wolbachia*-infected offspring (Alam *et al.*, 2011). Along with effectively continuing its spread within the tsetse population through the female tsetse, *Wolbachia* may drive desirable phenotypes and other maternally-transmitted genes and symbionts (Jin, Ren and Rasgon, 2009) and effective lead to speciation (Werren, 1997). Due to these factors, there has been research into utilizing *Wolbachia* as part of a vector control strategy (Alam et al, 2011).

Spiroplasma

Spiroplasma is the third endosymbiont of the tsetse fly, recently discovered in 2017 within *Glossina fuscipes*. *Spiroplasma* has known associations with various plant and arthropod species. *Spiroplasma* species can live intracellularly within host tissue or systematically within haemolymph of the host (Doudoumis *et al.*, 2017). The role of *Spiroplasma* within the tsetse fly is thought to be protective and/or nutritional; higher densities of *Spiroplasma* were found in the gut tissue of larva and live female tsetse and because of this, it is suggested that it provides some form of fitness advantage (Doudomis et al, 2017).

Studying Microbiota using 16S Sequencing

Studies to investigate the bacterial communities of insects (including studies related to tsetse flies such as Doudoumis et al (2017)) have been enabled by the invention of high-throughput sequencing, and specifically 16S-based microbiota sequencing. 16S sequencing focuses on sequencing, in a high-throughput manner – all copies of the 16S rRNA gene present in a population. The 16S rRNA gene, ubiquitous across bacteria and archaea, is comprised of both conserved and hypervariable regions; conserved regions allow for the

design of 'universal primers' that can amplify the corresponding hypervariable regions, which in turn act to delineate the bacterial species from which they are derived, by providing ample phylogenetic information (i.e. a "barcode"; Pereira *et al*, 2010). Another advantage of of 16S rRNA gene is that is suggested to evolve at relative constant rates. The application of 16S sequencing to insect symbionts has allowed for rapid identification and characterization of bacterial symbionts and their function within insect species that would be otherwise undetectable through phenotypic methods. Betelman *et al.* (2017) utilised 16S sequencing in their study of the bacterial symbionts present within three species of filth fly parasitoids (*Spalangia cameroni, Spalangia endius* and *Muscidifurax raptor*). They discovered that there was a diversity in the species found; all of the flies contained *Wolbachia* strains, but they had also found that two of them contained *Rickettsia* and *Sodalis* species within them. Betelman and al (2017) suggest that *Rickettsia and Sodalis* were facultative symbionts of the parasitoids, moreso in the case of *Sodalis* as it was also found in samples that contained *Wolbachia* and *Ricksettia* with the implication being that *Sodalis* is dependent on the presence of other symbionts for vertical transmission.

A different study utilised 16S amplicon sequencing to study the microbiome of two invasive species of aphids (Zepeda-Paulo *et al.*, 2018) in comparison to other aphids native to that geographic location. They found that the cereal aphids *Sitobion avanae* and *Rhopalosiphum padi* had lower diversity of microbiota in comparison to other aphid species and more specifically, a difference in the prevalence of secondary endosymbionts among native and introduced *S. avenae* which suggests the association between aphids and endosymbiotic bacteria can vary across a geographic range.

16S sequencing has been crucial in regards to the understanding the association between tsetse and their endosymbionts. (Pais *et al.*, 2008) administered antibiotics (ampicillin,

carbenicillin, tetracycline and rifampin ranging between 20-60ng/ml) to female tsetse flies every two days over 60 days to selectively eliminate endosymbionts to better understand their function. The study found that tsetse lacking in *Wigglesworthia* negatively impacted the fertility of the female tsetse and the progeny produced from these females displayed a cost in longevity and a compromised ability to digest their blood meal. The use of 16S sequencing has also allowed for the comparison of the relative ages of association with the tsetse between the different endosymbionts. Aksoy (1995) compared the 16S rDNA pair differences of the P- and S-endosymbionts of two distantly related tsetse species. The data revealed that *Wigglesworthia* strains within the distant hosts had an 82 base pairs difference in 16S rDNA, *S. glossinidius* only had 4 base pairs different which indicated that *Wigglesworthia* symbiotic association is much older in origin than of *Sodalis.*

Extreme Genome Reduction in Symbionts

Since the advent of next-generation sequencing, with the release of the 454 Life Sciences GS20 and Solexa 1G (Schendure 2008), and the advent of rapid bacterial genome sequencing and annotation, our knowledge of bacterial genome structure and function has increased dramatically. Similarly, dramatically smaller genomes than were thought possible have been recovered from insect symbionts, and, in particular, obligate symbionts (McCutcheon and Moran, 2012).

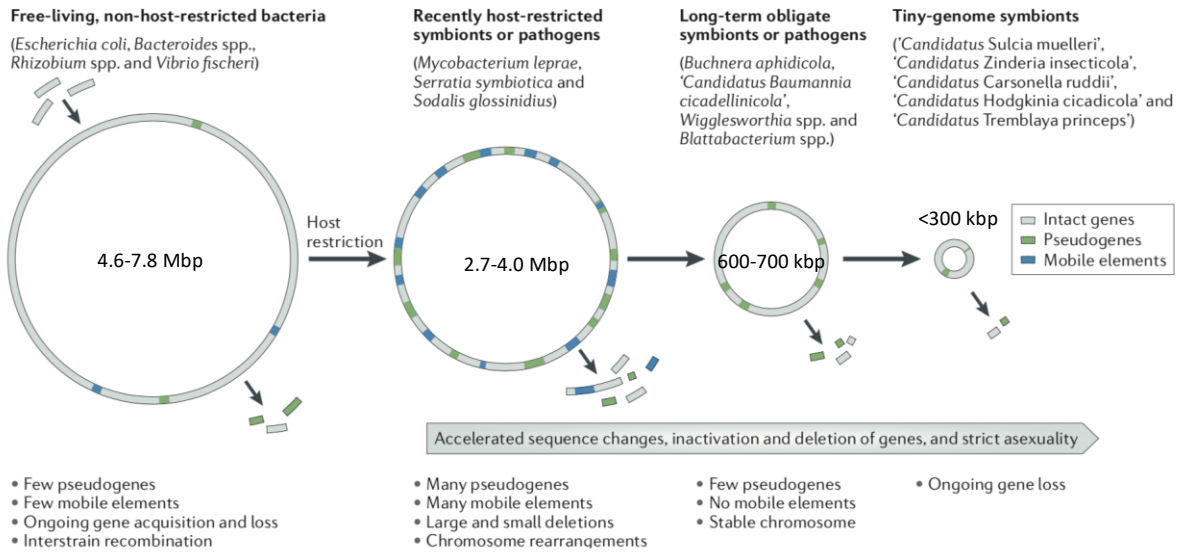

Figure 4- Stages of the genome annotation in host-restricted bacteria for which small population sizes result in mutation fixation. Taken from McCutcheon and Moran (2012)

McCutcheon and Moran (2012) discuss the effects of reduced genome size in the bacterial symbionts and how they challenge the notion of a minimal genome. They describe a minimal genome as the 'gene set that is sufficient for life under nutrient rich and stress-free conditions' or 'gene set required for axenic growth in rich media'. From 2006, drastically reduced genomes have been recovered by bacterial endosymbionts and were discovered to have genomes so small that it changed all previous views thought possible; four members of the *Candidiatus* genus were found to have genome sizes of less than 300 kb (McCutcheon and Moran, 2012). Mutualistic bacterial endosymbionts are thought to undergo rapid genome evolution as they shift towards functional integration with their host (Wernegreen, 2015) and often have distinct phylogenetic lineages. Bacterial endosymbionts such as *Wigglesworthia glossinidia* (genome size ~700kb (Haines *et al.*, 2002)) *and Buchnera aphidicola* (genome size ~618kb (Van Ham et al, 2003)) share similar roles within their respective hosts, however, their gene repertoire different in terms of host nutrition and cellular functions such as replication initiation and DNA repair (Aksoy, 2002) (van Ham *et al.*,

2003). Primary symbiotic bacteria are curiously shown to retain genes for the synthesis of GroES and GroEL, chaperonin molecules that associated with the folding of many proteins. It is found to account 10% of the protein with *B. aphidicola* (Poliakov *et al.*, 2011) and is thought to be the most abundant protein within *W. glossinidia* (Haines *et al.*, 2002). Endosymbiotic bacterial genomes tend to be rich in AT base content (Wernegreen, 2015). There are a couple of models which theorise the reasoning behind this; Muto and Qsawa (1987) suggest that there is an extreme mutational bias against GC base pairs whereas an alternative model proposes a selection bias towards the selection of AT due to the lower associated energy cost and relative abundance of ATP (adenine) within intracellular niches (Rocha and Danchin, 2002).

There have been various studies to underline the processes behind genome reduction within endosymbiotic bacteria but underpinning the evolutionary mechanisms has proven difficult. Some research suggest that it is neutral gene loss as a result of a relaxed selective pressure within an intracellular niche and bacterial genes that are not actively maintained during selection will eventually be deleted (Moran and Mira, 2001). Genetic drift is also often attributed to the gene loss found in microbial endosymbionts (Giovannoni, Cameron Thrash and Temperton, 2014). Moran and Mira (2001) reconstruct the gene deletion events that occurred within *Buchnera* species using comparative 16S rDNA analysis of *B. aphidicola* and a larger ancestral genome. They suggest that *Buchnera* species, soon into the establishment into the endosymbiotic lifestyle, lost many genes through the fixation of large deletions, a process which does not fall in line with the principle of graduation genome reduction through neutral gene loss (Wernegreen, 2011)

To fully understand the driving forces behind endosymbiont evolution, it is practical to study secondary endosymbionts as their facultative association with their host imply a

comparatively recent association with their host than the primary counterparts. This would suggest that S-endosymbionts have undergone less genome degradation and thus can functionally serve as analogues of P-endosymbionts within the early stages of their host association.

Why is *Sodalis* so interesting?

Sodalis glossinidius, the facultative secondary endosymbiont of the tsetse fly, is a key subject of interest. *S. glossinidius* was the first and, currently one of the few, insect endosymbionts to be successfully culture *in vitro*, initially isolated from *Glossina morsitans morsitans* (Dale and Maudlin, 1999) and further characterized by Matthew *et al.* (2005)

One of the most interesting aspects of *S. glossindius* is its genomic structure. *S. glossinidius* possesses one circular chromosome of approximately 4Mb (Toh *et al.*, 2006). In comparison to free-living bacteria such as *E. coli* (4.6Mb) (Serres *et al.*, 2001), the genome size is *S. glossinidius* is small, however, not to the extreme extent of obligate endosymbionts such as *W. glossinidia (*700kb) (Aksoy, 2002). As, previously discussed, genome reduction is typical of endosymbiotic relationships, as bacteria transition from free-living existence and start to become adapted to their host, yet, when compared to the P-endosymbiont *Wigglesworthia*, *S. glossinidius* appears to be in the early stages of its symbiotic relationship with the tsetse. Despite this, *S. glossinidius* has a reduced coding capacity of approximately 51%, possessing only 2,472 protein-coding genes, a trait that is unusual for bacterial genomes. Regarding these genes, *S. glossinidius* has retained genes involved in the synthesis of nucleic acids and amino acids, as well as those associated with transcription, translation and regulatory processes. The retention of these functions are most likely associated with the endosymbiont's ability to be cultured outside of its host (Welburn, Maudlin and Ellis, 1987). Akin to other insect symbionts, *S. glossinidius* has started to accumulate pseudogenes. Pseudogenisation is defined as the

process of gene silencing via one or more deleterious mutations (Goodhead and Darby, 2015). *Sodalis glossinidius* possesses approx. 972 pseudogenes, and through hybridisation of its DNA to gene macroarrays, it was discovered that some of the genes were orthologous to genes in *E. coli* involving the metabolism of carbohydrates and inorganic ions, as well as defense mechanisms. The accumulation of pseudogenes infer the bacteria's adaptation to the energy-rich environment of the tsetse due to singular diet of blood (Akman *et al.*, 2001). Accumulation of pseudogenes is an indication of recent adaption of endosymbionts to their host whereby genes unnecessary for viability within the host are selectively silenced (Darby and Goodhead, 2015), further suggesting the notion of a recent introduction of *S. glossinidius* to the *Glossina* species.

S. glossinidius is thought to provide benefits similar to an obligate endosymbiont, i.e. provision of metabolites and vitamins to the tsetse (Douglas, 1989), however, it is also thought to increase susceptibility of the tsetse fly to trypanosome infection. The exact mechanism behind this interaction is unknown, it is postulated that the production of N-acetyl-d-glucosamine by *S. glossinidius* inhibits the trypanocidal nature of the flies' midgut (Farikou *et al.*, 2010).

Laboratory Models of Symbiosis, including Experimental Evolution

Current laboratory models of bacterial symbiosis within insects are currently very limited by the very nature of the bacterial endosymbionts. Due to the extreme genome reduction outlined, studies of P-endosymbionts have been generally restricted to genomic and molecular analysis (Gil and Latorre, 2019). The ability to study endosymbiotic bacteria *in vitro* would allow for a better understanding of the molecular mechanisms that underpin the interaction between insect and microbe however due to the close association of the

two, P-endosymbionts are generally unamenable to axenic culture within a cell-free medium. Current laboratory models typically focus on the cultivation and maintenance of endosymbiotic bacteria with insect cell lines. One notable cell line is that of *Aedes albopictus* (Asian tiger mosquito) which has been utilised in the cultivation of *Wolbachia pipentis* (O'Neill *et al.*, 1997) and two aphid S-endosymbionts (Darby *et al.*, 2005). *Drosophila melanogaster* as both a common laboratory model and host to endosymbiotic bacteria has also be shown to cultivate *Wolbachia pipentis* (Andrianova *et al.*, 2010).

There is benefit in the of culture of endosymbiotic bacteria within insect cell lines, namely the indefinite maintenance of a single taxon under uniform conditions (Darby et al, 2005), however, the ability to culture bacterial endosymbionts in axenic and cell free culture will broaden the experimental possibilities available in the research of said symbionts. As previously mentioned, rapid improvement in and ready availability of sequencing technology and molecular biology techniques, there has been a significant increase in knowledge on the genome structure of bacterial symbionts (McCutcheon and Moran, 2012) and their various effects on host phenotypes (Feldharr, 2011). Conversely, there is limited research on the phenotypic capabilities of bacterial symbionts and the phenotypic consequences associated with host-symbiont interactions. The *in vitro* culture of insect endosymbionts has the potential to provide a fresh perspective and insight into the mechanisms that drive host-symbiont interaction that has remained elusive over several decades.

There has been some successful attempt in recent years to cultivate both primary and secondary symbionts in cell-free growth medium; Masson *et al.* (2018) managed to maintain the *in vitro* culture of the *Spiroplasma poulsonii* with an optimized commercial media, two individual groups respectively cultured S-endyosymbiont *Serratica dosymbiotica* (Sabri *et al.*,

2011) and *Hamiltonella defensa* (Brandt *et al.*, 2017) and Dale *et al.* (2006) isolated in pure culture Candidatus *Arsenophonus Arthopodicus,* a secondary endosymbiont of *Pseudolynchia canariensis* (hippoboscid louse fly).

Sodalis glossinidius has been selected as the endosymbiotic bacterial model of choice for the basis of this thesis because its culture dynamics have been previous explored (Matthew et al, 2006) and *S. glossinidius* has a related free-living bacterium known as *Sodalis praecaptivus* which provides the opportunity for comparison in terms of *in vitro* viability. *Sodalis praecaptivus* is a Gram-negative bacterium, like *S. glossinidius*, but possesses several different characteristics. *S. praecaptivus* possesses a larger genome of 5.16 Mb and does not possess any traits of genome reduction (Clayton *et al.*, 2012). It remains viable temperatures up to 37°C and grows aerobically (Chari *et al.*, 2015). Chiari *et al.* suggests that *S. praecaptivus* acted as an evolutionary precursor to the Sodalis-allied lineage of insect endosymbionts.

Aims and Objectives

This thesis presents *Sodalis glossinidius* alongside *Sodalis praecaptivus* as a viable experimental model of endosymbiotic bacteria in the exploration of their phenotypic capabilities. The two bacterial species will be investigated via growth dynamics under ten different laboratory media chosen due to their availability within the laboratory and their specific properties that were amenable to *S. glossinidius* growth (Table 1 for list of media) , resistance to oxidative stress, antibiotic resistance and viability *in vivo* with an experimental host *Galleria mellonella* (waxworm), a readily commercially available model organism that is easy to inoculate and can generate results with a 24-48 hour period (Kavanagh *et al*, 2018).

Methods

Culture of Sodalis glossinidius

Sodalis glossinidius used was cultured from stocks provided from the University of Liverpool- the strain is GMMB4, originally isolated from *Glossina morsitans morsitans*. 5µl loops were used from the stock aliquots (stored at -80°C in 25% v/v glycerol) and streaked onto Columbia agar plates (Sigma-Aldrich) supplemented with 5% v/v horse blood. The plates were incubated at 25°C under microaerobic conditions generated by Campygen Atomsphere Generation 2.5L sachets inside of sealed anaerobic jars for 72-96 hours where individual colonies became visible. Individual colonies were taken from the blood plates and inoculated in 5ml of Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% v/v fetal bovine serum and placed into ThermoScientific 15ml falcon tubes and incubated at 25°C for 72 hours.

Culture of Sodalis praecaptivus

Sodalis praecaptivus was cultured from stocks from University of Liverpool- the strain and isolation origin are unknown. 5ml loops were taken from the stock and streaked onto LB agar plates. The plates were incubated under normal atmospheric conditions at 25°C for 24 hours where individual colonies were visible. Individual colonies were taken from the LB agar plate and inoculated in 5ml of LB media in ThermoScientific 15ml falcon tubes and incubated at 25°C for 24 hours.

Table 1- List of laboratory media used to test the growth dynamics of Sodalis species, their general function and key components

Laboratory Media	Primary Culture Usage	Key Components per Litre (if available)
Schneider's Insect Media	Insect Cell Lines	Not Available from Sigma Aldirch
M9 Minimal Salts Media	*Escherichia coli*	33.9g disodium phosphate, 15g potassium monophosphate, 5g ammonium chloride, 2.5g sodium chloride
Luria-Bertani Broth (LB)	General bacterial culture	10g Select Peptone 140, 5g Select Yeast Extract, 5g Sodium Chloride
Brain-Heart Infusion (BHI) Broth	Fastidious Pathogens	5g beef heart, 12.5g calf brains, 2.5g disodium hydrogen phosphate, 2g D(+)-glucose, 10g peptone, 5g sodium chloride
Mueller-Hinton Broth	Antibiotic Susceptibility	2g beef infusion solids, 17.5g casein hydrolysate, starch 1.5g
Iso-sensitest Broth	Antibiotic Susceptibility	11g hydrolysed casein, 3g peptone, 2g glucose, 3g sodium chloride, 1g starch, 2g disodium hydrogen phosphate,1g sodium acetate
Tryptone Soya Broth (TSB)	Aerobes and Facultative Anaerobes	16g pancreatic digest of casein, 3g enzymatic digest of soya bean, 5g sodium chloride, 2.5g dipotassium hydrogen phosphate, 2.5g glucose
Nutrient Broth	General bacterial culture	1g 'Lab-Lemco' powder, 2g yeast extract, 5g peptone, 5g sodium chloride
Malt Extract Broth	Mold and yeast	17g malt extract, 3g mycological yeast

Growth dynamics of *S.glossinidius* and *S.praecaptivus* within various laboratory media

Individual colonies were taken from *Sodalis glossinidius* and *S. praecaptivus* and inoculated into 5 ml of ten different culture media (Table 1). 200µL of each inoculum was pipetted into a 96 well plate with eight replicates for each growth medium. These plates were incubated at 25°C under normal atmospheric conditions for a seven-day period. Absorbance at 600nm for the plates and colony forming units were taken using the Miles Misra method (Hedges, 2002) at daily intervals.

Antimicrobial resistance in *S.glossinidius* and *S. praecaptivus*

Swabs of liquid culture from *S. glossinidius* and *S. praecaptivus* were taken using aseptic technique and used to form a bacterial lawn on Columbia agar plates supplemented with 5% v/v horse blood (*S. glossinidius*) and LB agar plates (*S. praecaptivus*). An antibiotic disc of penicillin G (10µg), meropenem (10µg), linezolid (10µg), ceftazidme (30µg) and sulphamethorazole (25µg) were each placed respectively on the spread plates of *S.glossinidius* and *S. praecaptivus,* 3 replicate plates per antibiotic per bacteria plus a negative control plate with no antibiotic for each (32 plates in total). These five antibiotics were chosen as they were readily available in the laboratory and classed as broad-spectrum antibiotics that are known to act on a wide range of bacteria. The plates were incubated at 25°C under microaerobic conditions with Campygen Atomsphere Generation 2.5L sachets inside of sealed anaerobic jars for 72 hours. The LB plates were incubated under normal atmospheric conditions at 25°C for 24 hours. The diameter of inhibitory zones was measured in millimeters (mm).

Culture dynamics of *S. glossinidius* and *S. praecaptivus* under oxidative stress

Schneider's Insect Medium was supplemented with 30% w/v hydrogen peroxide to create a 1mM stock solution. The stock was serially diluted with Schneider's Insect Medium to give the final concentrations of 100µM, 10µM, 1µM and 0.1µM. 4.5ml of each concentration (including 1mM) was placed into ThermoScientific 15ml falcon tubes. 500µL of liquid *S. glossinidius* culture was inoculated into each concentration of hydrogen peroxide and left to incubate at 25°C over the duration of seven days. OD measurements at 600nm and colony forming units were counted using the Miles Misra method (Hedges, 2002).

The above method was repeated for *S. praecaptivus*.

qPCR Analysis of the effects of oxidative stress in *S. glossinidius*

Primer design for oxidative stress analysis

Figure 5 and *Sodalis glossinidius* (strain GMMB4) genome annotation provided by my supervisor (genome annotation accessed through Artemis genome browser and annotation tool from the Sanger Institute website) were used to identify genes related to oxidative stress. Base pair sequences for the target genes were downloaded in FASTA format. Base pair sequences were inputted into PRIMER3 with the parameters for product size range of 100-150bp and T_M of 55-60°C. See Table 2 for primer sequences.

Table 2- Primers designed for the study of the effect of oxidative stress on Sodalis glossinidius

Enzyme/Protein	Primer Name	Forward/Reverse F/R	Primer Sequence (s)	Product Size (bp)	Annealing Temperature (°C)
DNA gyrase A	GyrA	F	GTCTCCGAGGTAAGCATCGT	117	59
		R	ATCGTCGTCATCGACCTGTT		59
Catalase	Cat1	F	CAGGGTAACTGGGATGTGGT	119	59
		R	GGGGATTTCAGATTCGTCGC		59
Superoxide Dimutase	SodA	F	ACGCTACCTTCCCTGCTTTA	120	59
		R	CAAGGCGCCATTGGTGTTAT		59
Peroxiredoxin	OxyR5	F	TATCCGCGACCTCAAGTTGT	120	59
		R	TTCTCTTCCAGGCGCTGAAT		59
N-acetylglucosamine deacetylase	1pxC	F	TGAGGAGCTTAACAGTGCCA	112	59
		R	GAAATCGAGCGTAAAGCCGT		59

RNA extraction

After day 7, RNA was extracted from three samples of each concentration of hydrogen peroxide containing *Sodalis glossinidius* through the Qiagen RNeasy Mini Kit, following the manufacturer's instructions. Extracted RNA samples were tested for yield and quantity using Nanodrop equipment.

cDNA synthesis and qPCR of samples

RNA samples were converted into cDNA using the Qiagen QuantiTect Reverse Transcription Kit, following the manufacturer's instructions. The cDNA was then prepped for qPCR via the Qiagen QuantiTect SYBR Green RT-PCR kit following manufacturer's instruction based on the use of the Qiagen Rotor-Gene Q.

Viability of *S. glossinidius* and *S. praecaptivus* within *Galleria mellonella* larva (waxworm)

Galleria mellonella stock (purchased from Amazon) was stored at approximately 4°C in low-light conditions until required.

OD measurements at 600nm were taken from established liquid cultures of *S. glossinidius and S. praecaptivus* and diluted with sterile phosphate-buffered saline (PBS) solution to make a 1ml inoculum of each at an OD of 1.0. 5µL of each respective inoculum was injected into the right proleg of the waxworm (Harding et al., 2013). This was repeated until there were 20 worms; 10 of each inoculated with either *S. glossinidius* or *S. praecaptivus*. Two groups of *Galleria mellonella* were used as negative controls; 10 worms were injected with PBS solution; 10 worms were not injected. The worms were incubated without food at 25°C within Petri dishes over 7 days.

Live and dead *Galleria* larva, as well as larva that had begun to pupate from the two infected and two control groups were extracted from the Petri dish and homogenized using a pipette tip within 2ml micro centrifuge tubes containing 1ml sterile PBS solution. The homogenate was vortexed and 500µl of each homogenate was inoculated into 4.5ml of Schneider's Insect Medium and left to incubate for 24 hours at 25°C.

60µL of each sample was used to check for bacterial cell viability via colony forming units via the Miles Misra method.

DNA was extracted from the samples infected with *Sodalis* species and both control groups via the ZymoResearch Quick DNA^{TM} Miniprep Plus Kit, following the manufacturer's instruction regarding bacterial cells.

The extracted DNA was then specifically amplified through Polymerase Chain Reaction (PCR), using a reaction mixture of Bioline Mitaq Red and primers to amplify the *groEL* gene of *Sodalis glossinidius (*forward primer 5'- CCA AAG CTA TCG CTC AGG TAG G-3', reverse

primer 5'-TTC TTT GCC CAC TTT CGC CAT A-3', taken from Matthews, PhD thesis, 2005). The PCR products were then analysed through gel electrophoresis run on a 1.5% v/v agarose gel and external 16S sequencing. DNA extracted from *Sodalis praecaptivus* was also sent for 16Ssequencing (primer sequences: 515FB - 5'-GTG YCA GCM GCC GCG GTA A-3' (Caporaso *et al.*, 2011; Parada, Needham and Fuhrman, 2016) and 806R - 5'-GGA CTA CNV GGG TWT CTA AT-3' (Apprill *et al.*, 2015).

Results

Growth dynamics of *Sodalis* species within various laboratory media

Table 3- Table of OD600 and c.f.u.ml^{-1} of Sodalis species within different growth media after 5 days incubation

Media	Sch	M9	LB	BHI	MH	ISo	TSB	NB	MEB
OD_{600} *S. glossinidius (D*ay 0)	0.088	0.016	0.021	0.013	0.060	0.010	0.023	0.012	N/A
OD_{600} *S. glossinidius* (Day 5)	0.266	0.252	0.400	0.344	0.140	0.057	0.359	0.028	N/A
c.f.u. µl^{-1} S. glossinidius (Day 0)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
c.f.u. µl^{-1} *S. glossinidius* (Day 5)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OD_{600} *S.praecaptivus* (Day 0)	0.058	0.062	0.046	0.065	0.056	0.078	0.047	0.630	N/A
OD_{600} *S.praecaptivus (Day 5)*	2.84	2.93	2.67	2.77	3.04	3.12	2.74	2.56	N/A
c.f.u. µl^{-1} *S.praecaptivus* (Day 0)	1.2×10^8	1.4×10^8	2.2×10^8	3.3×10^8	6.7×10^7	1.3×10^8	1.6×10^8	7.0×10^8	N/A
c.f.u. µl^{-1} *S.praecaptivus* (Day 5)	4.5×10^6	3.3×10^6	4.2×10^6	N/A	3.8×10^6	1.0×10^7	6.2×10^7	3.5×10^6	N/A

From Table 3, both *Sodalis species* exhibited growth within all laboratory media except for malt extract broth. The growth of *S. glossinidius* is slower in comparison to *S. praecaptivus* in all media used over the five-day incubation period. From Table 3, it is shown that that there is no difference in growth based on the optical density measurements between the different media for either species. Colony forming units were obtained from *S. praecaptivus* except from the brain-heart infusion broth and malt extract broth. It was not possible to obtain colony forming units from *S. glossinidius* due to culturing issues to be discussed.

Culture dynamics of *Sodalis* species under oxidative stress

The qPCR of *S. glossinidius* showed no quantitative expression of any of the genes selected for, including the housekeeping gene GyrA.

Optical density measurements and cell viability counts for *Sodalis glossinidius* were unavailable due to culturing issues to be discussed.

Antimicrobial susceptibility of *Sodalis* species

Table 4- Antmicrobial susceptibility data from anitimicrobial disc diffusion assay vs five common antibiotics

Antibiotic	Average Inhibition Zone Diameter (mm)	Average Inhibition Zone Diameter (mm)
	Sodalis glossinidius	*Sodalis praecaptivus*
Penicillin G (10µg)	N/A	26
Meropenem (10µg)	N/A	51
Linezolid (10µg)	N/A	33
Ceftazidme (30µg)	N/A	23
Sulphamethoxazole (25µg)	N/A	37

Antibiotics are shown to have an effect on *Sodalis praecaptivus*; it appears to be the most resistant to penicillin G and the least resistant to meropenem. It was not possible to obtain antimicrobial resistance data from *Sodalis glossinidius* due to culturing issues.

3.4 Viability of *Sodalis* species within *Galleria mellonella*

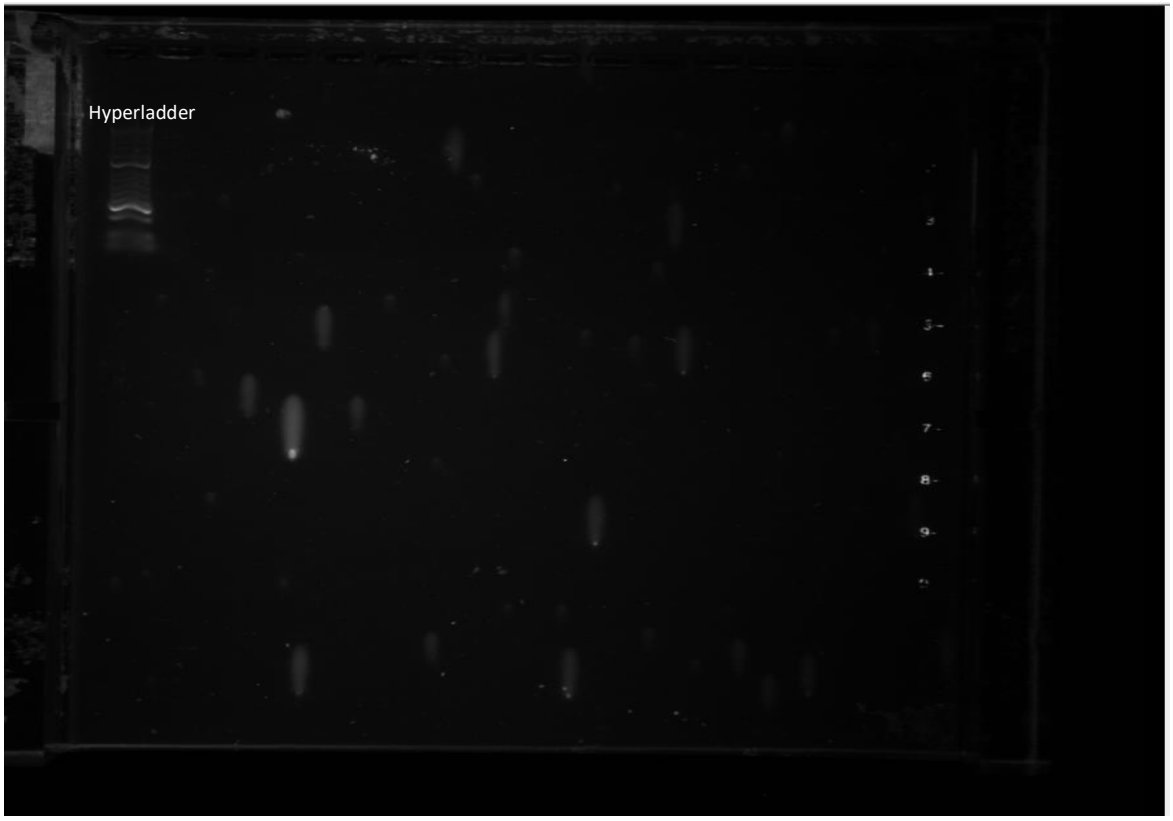

Figure 5- Gel electrophoresis (1.5% agarose, 5µl gel red) of Galleria mellonella homogenates that were inoculated with Sodalis glossinidius

Cell viability counts were unable to be obtained from the homogenates of *Galleria mellonella* inoculated with *Sodalis glossinidius* and *Sodalis praecaptivus* as individual colonies were not visible for the count. The upper range for colony forming units for both *Sodalis* species from the homogenates was ~10^7 c.f.u. ml^{-1}.

Gel electrophoresis was inconclusive, there are no visible bands of DNA, including the positive control for *S. glossinidius* and negative water control.

16S sequencing of the samples revealed there was no *Sodalis* species present within *Galleria mellonella* after the week of incubation.

Sequences for *Staphylococcus* species and *Enterococcus casseliflavus* were found in the samples meant to contain *Sodalis glossinidius* and *Sodalis praecaptivus.*

Discussion

The aim of this thesis was to establish *Sodalis glossinidius* as a reliable laboratory model of insect endosymbiosis via comparative phenotypic experimentation involving *Sodalis praecaptivus* and ultimately provide the framework for the *in vitro* study of the other cultivable endosymbionts. As the result show, not enough data was collected on the phenotypic capabilities of *Sodalis glossinidius* due to complications during the project, and thus it has been not feasible to discuss the difference in phenotypic ability between the two *Sodalis* species. Challenges in consistent axenic culture of *S. glossinidius* have occurred over the duration of the experiment. One of the largest barriers to progression in this study was the repeated contamination of bacterial cultures of *Sodalis glossinidius* with contaminant species. While there have been some instances of axenic cultivation, the presence of contaminant species and the lack of competitive characteristics on the part of *S. glossinidius* has created several setbacks. Some cultures of *S. praecaptivus* were also affected. Proper aseptic technique and increased countermeasures such as implementation of UV light, utliisation of Class II laminar flow hood during inoculation and the liberal use of sterilizing agents such as ethanol and Chemgene on all surfaces, equipment and containers had minimal impact on the rate of contamination. Possible solutions to this issue would involve the application of selective media and the use of antibiotics and antifungals through experimentation as an added precaution against contamination.

There was a lack in consistency in cultivation of *S. glossinidius* from stock aliquots and existing cultures within the culture media. During experimentation, *S. glosssinidius* was erratic in its growth dynamics; it is well-established that *S. glossinidus* grows optimally on agar plates supplemented with blood and under microaerophilic conditions (Matthews et al, 2006), yet on several occasions, *S. glossinidius* exhibited no growth under these conditions.

There were similar problems within liquid culture where *S. glossinidius* struggled to grow in liquid culture media such as Schneider's Insect Medium where there had been no prior issue. A plausible reason to this occurring has been discussed by Sridhar and Steele-Mortimer (2016). They suggest that there can be inherent variance within growth media, especially in less chemically defined media can affect bacterial interaction. The exploration the culture dynamics of *Sodalis* through inoculation in various media was in the attempt to understand what key components *Sodalis* species would require to produce a chemically defined media suitable for axenic culture. A recent paper utilised *in silico* modelling and refinement of a previously published genome metabolic model to generate an entirely defined growth media dubbed 'SMG11' which was reported to cause an endpoint increase the growth of *S. glossinidius* (Hall *et al.*, 2019) . Some of the major findings by Hall *et al.* (2019) were that *S. glossinidius* is dependent on N-acetyl-D-glucosamine (GlcNAC) as a primary carbon source, that the thiamine produced by the tsetse is important in the production of biomass and L-glutamate is essential in its TCA cycle. SMG11 is comprised of M9 minimal media supplemented with the aforementioned nutrients alongside others. Further experimentation involving the use of the aforementioned SMG11 or the generation of a similar defined media supplemented with GlcNAC, L-glutamate, thiamine and other essential nutrients specific to *S. glossinidius* should improve the consistency in which *S. glossinidius* can be established and maintained in liquid culture and thus advocate the use of metabolomics in the generation of tailored growth media for the study of other bacterial endosymbionts.

In regards to experimental design, the use of *Galleria mellonella* larva within the experiment was interesting but in future experimentation, the use of other *ex vivo* models should be explored. *Galleria mellonella* larva are quite frequently used as models for *in vivo* toxicology

and pathogenicity of prominent bacterial and fungal human pathogens (Cook and McArthur, 2013). There are several key reasons for the popularity; there are low in cost and commercially available, they can be inoculated with relative ease and generate results typically within 24-48 hours (Kavanagh *et al.*, 2018). *Galleria mellonella* larva, however has been described as not be suitable for some microbial species. Based on the sequencing results provided from the inoculated larva, the maintenance of *Sodalis* species within *Galleria mellonella* appears to be not possible, or at the very least, difficult. One possible explanation for this, in terms of *Sodalis glossinidius*, is the larva's lack of an exoskeleton. The primary carbon source for *S. glossinidius* is N-acetyl-d-glucosamine (GlcNAc) (Dale and Welburn, 2001) and it can be obtained by the breakdown of the chitin (polymerized GlcNAC) within the exoskeleton of the tsetse fly via the enzyme b-N-acetylglucosaminidase (Dale and Maudlin, 1999).

The use of a model that possesses a chitinous exoskeleton with more similarity to the tsetse fly should be considered in an expansion of the experiment. The most obvious choice would be another commonly used model within research, *Drosophila melanogaster*. The reasons behind the use of *Drosophila melanogaster* as a model organism is similar to that of *Galleria mellonella*; its care and cultivation within laboratories is not resource intensive, it has a short generation time (~10 days), and high fecundity and is amenable to genetic modification (Jennings, 2011).

There has been some investigation into the infection within the midgut of *Drosophila* with *Sodalis glossinidius* (Stevens, unpublished). Stevens found that there was no successful establishment of *Sodalis glossinidius* and suggested two plausible barriers to successful *Sodalis* infection; the alkalinity of the *Drosophila* midgut and competitive pressure from the commensal and transient bacteria within the *Drosophila* (Buchon, Broderick and Lemaitre,

2013). The use of axenic *Drosophila* would be ideal in further experimentation into infection with *Sodalis* species (Stevens, unpublished). Another change in the experimental design would be the use of the microdilution broth method over the disc diffusion assay for antibiotic susceptibility testing within *Sodalis* species. The disc diffusion method was chosen due to the immediate availability within the laboratory and the ease of and set-up. The disc diffusion assay does provide visual, qualitative data of the effect of antibiotics via the zone of inhibition. The assay, however, is better suited for clinically relevant bacterial species. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) database from which minimum inhibitory concentration (MIC) are ascertained from the diameter of the zone of inhibition does not have standards for *Sodalis* species.

Some of the appeal of the broth microdilution method for determining antibiotic susceptibility is like that of disc diffusion in terms of cost and accessibility. The largest advantage to this method is the ability to determine MICs quantitatively relative to the microorganism being studied; this is useful due to the lack of comparative research available in antibiotic susceptibility of endosymbionts. The broth microdilution method allows for the testing of multiple antibiotics at a wider range of concentrations that can be easily modified during experimentation to accurately pinpoint the breakpoint MIC. The other advantage to microdilution method is the use of microtiter plates; they are more practical in terms of storage of samples, the generation of replicates and often, the results founds can be analysed via laboratory equipment (i.e. plate reader).

The discovery of antimicrobial resistant properties of *S. glossinidius* would be useful in the determination of a selective marker for positive identification of the bacteria and mitigation of the contamination issues faced. The potential of antimicrobial resistance expression in *S. glossinidius* would be a point for further exploration. *S. glossinidius*, as previously

mentioned, is in the process of pseudogene accumulation and genome reduction for adaptation to its host and as such, it would be unlikely that it would possess active genes for antimicrobial resistance. *S. glossinidius* does still possess genes for transcription and translation and possesses a plasmid (pSG1) which contains homologous regions to that of conjugative transfer pilus genes (Toh et al.,2006) which implies conjugation is possible. Horizontal gene transfer is thought to be a leading cause of the increase of antibiotic resistance (Gyles and Boerlin, 2013). In further experimentation, it would be intriguing to attempt to confer antibiotic resistance to *S. glossinidius* via conjugation or transformation through resistance genes carried on plasmids.

The difficulties associated with the research is highlighted by the challenges in establishing *Sodalis glossinidius* in consistent culture, but the experimental design of the project still holds some merit for innovation in how insect endosymbiont studies will advance and progress. The ability to understand how *Sodalis glossinidius* to react to oxidative stress, the natural defense mechanism of the tsetse fly against infection (Hao, Kasumba and Aksoy, 2003) would give some insight on whether endosymbionts retain the ability to protect

against their host defenses, and in terms, of paratransgenesis and vector control, defense mechanisms of other insect species (e.g. Figure 5, in axenic culture).

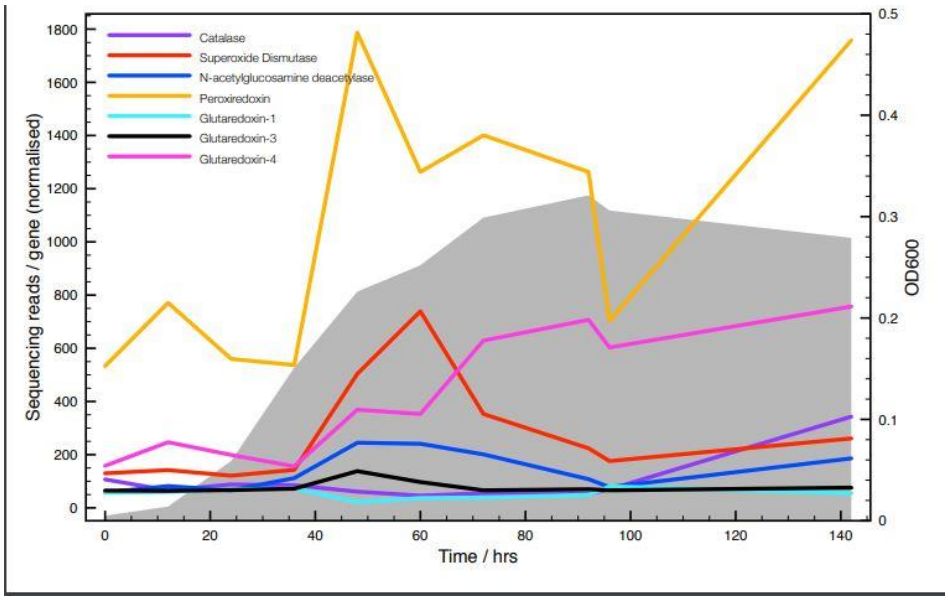

Figure 6- Plot of time (x) vs sequencing reads and OD600 (y) for the growth (grey area) of Sodalis glossinidius under no specific oxidative stress. The plot shows the expression of 7 genes related to activity under oxidative stress, provided by my supervisor Ian Goodhead

The application of using *in vivo* models such as *Galleria mellonella* or *Drosophila melanogaster* previously mentioned offers the opportunity to explore the concept of bacterial endosymbiont's viability – and the associated responses, both from a *Sodalis* and host-perspective - as part of vector control strategies for diseases such as HAT.

The core concept behind this thesis is that emphasis into the phenotypic capabilities of bacterial endosymbionts, especially those have the ability to be culture i*n vitro*, is required to advance the state of research into insect-bacteria. There are experimental techniques that are made accessible to the research of insect endosymbionts through the ability to culture said endosymbionts *in vitro.* The experimental evolution of bacterial endosymbionts would provide the ability to further understand their existing phenotypes, but also induce genomic and phenotypic change in real-time. Experimental evolution is the investigation of

evolutionary process through an experimental population and the conditions implemented on said population by the experimenter (Kawecki *et al.*, 2012). Experimental evolution has been utilised in various studies to understand evolutionary dynamics through the application of various manipulations to the environment the subject is exposed to, typically over an extended period. The most notable experimental evolution is the long-term *Escherichia coli* evolutionary experiment, spearheaded by Richard Lenski. This still on-going experiment tracked the genomic and phenotypic changes in 12 identical populations of asexual *E. coli* from. Over the duration of the experiment, Lenski and his team observed several interesting changes within the 12 populations; an increased growth rate in present populations in comparison to the ancestral strain (Lenski, 2003), a general increase in fitness from (Wiser, Ribeck and Lenski, 2013) and the ability of one population to grow aerobically on citrate, a trait previously unseen in *E. coli* (Blount, Borland and Lenski, 2008). There has been some research into applying experimental evolution into the study of animal-microbe interactions. (Gibson *et al.*, 2015) co-evolved *Caenorhabditis elegans* and the virulent bacterial parasite *Serratia marcescens*. They found that after 20 generations of co-evolution, *C. elegans* exhibited an increase in fecundity when allowed to exist and evolve in the presence of each other, leading to a reduction in previously established antagonism between the two. There have been some studies that utilised experimental evolution to understand insect-microbe interaction regarding the advantages in immunity granted by some endosymbionts. One such study explored the three-way interaction between *Drosophila melanogaster*, *Drosophila C* virus and *Wolbachia* which confers resistance to the virus to its host (Martinez *et al.*, 2016). They found that after nine generations of selection (infection of flies that were either harbouring or lacking in *Wolbachia* with *Drosophila C*), while resistance had increased within all populations, the frequency of an allele with effects

on resistance to *Drosophila C* was reduced in flies harbouring *Wolbachia*. This suggested that defensive endosymbiont presence can lead to a dependence and negatively impact the evolution of host's resistance genes (Martinez *et al.*, 2016).

Experimental evolution has been shown to be able the bridge the theoretical predictions provided by genomic data and empirical testing (Hoang, Morran and Gerardo, 2016) and its application towards the establishment of *Sodalis* species as laboratory models of symbionts would greatly improve our understanding of phenotypic ability of *Sodalis* species. The experimental methods described in the thesis (media testing, oxidative stress testing, antimicrobial susceptibility and viability within an experimental host) can be adapted in line with experimental evolution. The addition of these and other variables such as pH over a longer experimental period and across populations of *Sodalis* species will apply selective pressure which emulates the conditions experienced by primary and secondary symbionts within their host both bacterial species in the aim of inducing genomic changes within a laboratory setting. The successful establishment of this system would serve as a proxy for the adaptations that symbionts undergo as they move closer to symbiotic living and congruence with their host.

Conclusion

The *in vitro* study of the secondary endosymbiont *Sodalis glossinidius* and its relative, *Sodalis praecaptivus* has been difficult but the continuation of research into establishment of these, or similar, bacterial species as research models is necessary to further the understanding behind host-bacterial symbiont interactions. The advancement of genomic and molecular techniques has provided a lot of data in terms of the potential roles of symbionts and their adaptations to symbiotic existence, however the data presented can be described as hypothetical in nature. The implementation of phenotypic research through *in*

vitro culture of available symbionts through experimental evolution methods alongside current sequencing strategies will form a better picture and progress our knowledge of evolutionary mechanisms that underpin host-microbe interaction.

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