CHAPTER 10

General Discussion
Life, as we currently know it on Earth, would not exist in the absence of symbiosis between different species (Bronstein 2015). Symbiotic interactions have, over time, generated a large diversity of species and have created complex organisms which are able to inhabit novel environments (Henry et al. 2013, Moran 2007). The success of symbiotic interactions can be attributed to organisms complementing each other with specialization in different resources and services (Selosse & Le Tacon 1998; Moran 2007; Leigh 2010). For example, many eukaryotes have evolved intricate partnerships with microbes which can increase their stress resistance, protect against pathogens, or provide their eukaryotic hosts with nutrients. This has been a key evolutionary innovation and it is clear that the symbiosis between eukaryotes and microbes has been prominent in shaping the eukaryotic evolution, however, how symbiotic partnerships operate remains poorly understood. How do symbiotic partners communicate? What strategies do the partners use to keep their benefit in the mutualistic relation? How flexible are these strategies and can organisms change their strategies depending on the biotic and abiotic conditions? In this thesis I have tried to answer these questions.

**Communication is important in symbiotic interactions**

While the molecular cross-talk between plant roots and microbes is well studied (Guttman et al. 2014), the evolutionary origin and stability of communication is less understood. My aim was to explore these evolutionary aspects, mainly trying to understand when communication is vulnerable to exploitation (chapter 2). Signals are evolved to change the behavior of the intended receiver, and depending on the reliability of the signal, can be very robust against dishonesty (McLinn & Stephens 2006). However, parasitic organisms can eavesdrop on signals and use them as a cue to harm the sending individual. Plant roots evolved relatively robust mechanisms to distinguish between parasites, pathogens and mutualists. To attract mutualists, roots emit certain molecules, which are released into the environment and function as a signal for mutualistic microbes to grow towards the root, but also as a cue for parasites (reviewed in Brewer et al. 2013). Roots are able to identify symbionts by the signaling molecules of the symbiont and distinguish between mutualistic and parasitic symbionts (Bonfante & Genre 2015). However, the signaling molecules of the parasitic organisms can co-evolve with the mutualistic partners and can, over time, evolve the same signaling molecules and enter the root unnoticed. The precise molecular mechanisms of partner recognition by the plant root and how the roots prevent to be parasitized by the ever co-evolving parasites remains unknown. More empirical and theoretical research is needed to answer the question how plants and mutualistic microbes maintain stable communication.

**Understanding fungal diversity**

Underground, plant roots are surrounded by a huge diversity of soil organisms of different species (Bardgett & Van Der Putten 2014), many which are fungi. While it is known that there are millions of fungal species, a framework to understand why so many different fungal species have evolved and are maintained remains unknown. I have reviewed the literature to search for tools used to understand the diversity of macro-organisms and asked whether these tools could help us understand fungal diversity (chapter 3). Methods relying on traits and trade-offs exist, for example for plants, but while these frameworks have some elements which may be applicable to fungi, many do not. Fungi are a very different group of organisms and have features that are unlike most macro-organisms. For example, some fungi have the ability to rapidly switch guilds, from symbiotic to saprotrophic when the environment changes. Also, data on fungal traits is lacking for many fungal species. Future research should focus on describing more fungal species, gaining more trait data on these fungal
species, and modeling how the traits and fungal species have evolved using comprehensive phylogenetics.

**Quantum-dot apatite**
I studied the arbuscular mycorrhizal symbiosis to answer questions about the functioning of symbiosis and trade between symbiotic partners. Many of the experiments in this thesis used quantum-dot apatite to study resource trade in the arbuscular mycorrhizal symbiosis (chapter 4). I have used quantum-dot apatite because it brings the potential to visually study the transfer of nutrients over time and across space, following the nutrients from different locations across the fungal network. This overcomes the limitations of using radioactive phosphorus isotopes, which is limited to only two isotopes, with half times of only a few weeks (Lal et al. 1957). The quantum-dot apatite I helped to develop can fluoresce in numerous colors depending on the chemistry in the core and has no toxic side effects. I have shown that quantum-dot apatite can be recognized by the arbuscular mycorrhizal fungi and transported to the host root. In the host it is transported to the growing tissue, for example the growing leaves. While the exact uptake mechanisms are not known yet, it is suggested that arbuscular mycorrhizal fungi can take up the particles by endocytosis, as has been shown to occur in other fungi (Epp et al. 2013; Lu et al. 2016). The particles are then transported in vacuoles to the plant where they are released apoplastically (Schwab et al. 2016). In this thesis I have shown that the plant transports the quantum-dot apatite in the roots and in the shoots (chapter 4–7,9). While the use of quantum-dots has advantages over the use of phosphorus isotopes, it has also its disadvantages. Tracing the transport and usage of phosphorus via quantum-dot apatite, only works as long as the phosphorus is attached to the quantum-dot. This limits the study of where the phosphorus is eventually used by the plant and the fungus because the phosphorus cannot be used by the plant and the fungi without losing the fluorescent tag. Theoretically, when this happens, a shift in emission spectrum could be observed, however, the shift would be so small that it would not be noticeable for our measurements within the plant root or the fungal hyphae. The usage of quantum-dot apatite is promising, but future research should focus on the exact uptake mechanisms and the usage of phosphorus from the quantum-dot apatite by the plants and fungi, perhaps by combining phosphorus isotopes and quantum-dots.

**Resource variation influences symbiotic trade**
It is important to consider environmental conditions, such as the availability of resources, when studying symbioses because many species interactions are highly context-dependent (Hoeksema et al. 2010; Chamberlain et al. 2014). One aim in my thesis was to study how the trading strategies of arbuscular mycorrhizal fungi change with varying levels of resource inequality across a fungal network (chapter 4). I exposed fungal networks to rich and poor phosphorus patches, with either the same amount of phosphorus in each patch of the fungal network (50:50) or an unequal amount of phosphorus in each patch of the fungal network (70:30 or 90:10). I found that more resources were transferred to the roots when the fungal network was exposed to higher resource inequality. I found that the fungus translocated phosphorus from the nutrient rich patch in the network to the nutrient poor patch in the network. The idea is that because resources are scarce on the resource poor side, the plant demand is higher, and the fungus is able to fetch a higher price from the plant. This suggests that the fungus is able to change its trading strategies considerably depending on the spatial distribution of resources and is able to manipulate the trade in such a way that it fetches a higher benefit than under equal resource distributions. Arbuscular mycorrhizal fungi are able
to store phosphorus rather than to trade it with the plant partner (Hammer et al. 2011), I have shown that fungi can use that as a strategy to gain a higher benefit from the trade with plants (chapter 4, 5 and 7).

However, the fungus cannot always change its trading strategies in ways to create a greater fungal benefit. I have studied the effect of extreme weather events on the arbuscular mycorrhizal symbiosis. I found that the fungi were not able to help the plant to negate the negative effects of flooding or heat, and that the total amount of resource trade was not influenced by the extreme weather events. The fungi did not change their trading strategies under the stressing conditions, likely because the fungi themselves were also exposed to the stressing conditions (chapter 6).

**Predicting nutrient trade using a biological market framework**

While it is demonstrated that the nutrient trade between arbuscular mycorrhizal fungi and plants can vary with context (chapter 4, Bachelot and Lee 2018; Walder et al. 2012), it is an open question whether these trading patterns can be predicted (Wyatt et al. 2014, 2016; Noë & Kiers 2018). One framework to predict trading patterns is the biological market theory. The arbuscular mycorrhizal symbiosis is often thought to function like a biological market because it involves the exchange of nutrients between multiple plants and multiple fungi simultaneously (Cowden & Peterson 2009; Wyatt et al. 2014; Kiers et al. 2016). Many theoretical studies have used the biological market theory to study the arbuscular mycorrhizal symbiosis as a biological market (Schwartz & Hoeksema 1998; Kummel & Salant 2006; de Mazancourt & Schwartz 2010; Grman et al. 2012; Wyatt et al. 2014; Werner & Kiers 2015), however, many questions remain unanswered. In this thesis, I have studied the nutrient trade in the arbuscular mycorrhizal symbiosis empirically to test if I could predict the trade according to the rules of a biological market (chapter 5). The hypothesis was that the ‘price’ of phosphorus, i.e. how much carbon was exchanged by the plant for phosphorus, would drop when the supply of phosphorus increased and that the ‘price’ would rise if the phosphorus supply decreased. I tested these hypotheses by mimicking an economic ‘boom’ by injecting extra phosphorus to one part of the fungal network, or to an economic ‘crash’: a decrease in phosphorus availability by severing one part of the fungal network, restricting the access to phosphorus from that part of the fungal network. To follow phosphorus over the fungal network into the host root, I added quantum-dot apatite of three different colors as a phosphorus source, to the three different compartments: the manipulated fungal compartment, the stable fungal compartment and the root compartment. This allowed me to study from were across the fungal network the exchanged phosphorus originated. Contrary to the predictions, we found that the fungi in the boom treatment capitalized on the increased supply of phosphorus and gained more carbon benefit than the fungi in other treatments. We found that fungi in the crash treatment were able to compensate for resource loss by transferring more phosphorus from different parts of the network. These results suggest that the arbuscular mycorrhizal symbiosis does not function like a free biological market. However, a biological market requires the plant and fungi to choose with whom to trade, and in this setup, we only used one plant and one fungal ‘individual’: The fungus was probably able to monopolize the flow of phosphorus because the plant was not able to choose between competing fungi.

**Nutrient trade in a shared fungal network**

Arbuscular mycorrhizal fungi can form networks connecting the roots of several plants simultaneously (Montesinos-Navarro et al. 2012). Depending on biotic and abiotic factors
(Merrild et al. 2013; Walder et al. 2015; Weremijewicz et al. 2016), the fungus allocates resources differently to the different host roots. However, because it was difficult to study the relationship between host plant demand and fungal trade strategies across a shared network, it remained an open question which factors exactly determined the resource allocation by arbuscular mycorrhizal fungi over time. Using the quantum-dot apatite tagging technique, I studied the allocation of phosphorus in a shared fungal network over time. My aim was to understand the temporal dynamics of phosphorus allocation in a shared fungal network: when, where and how much phosphorus was allocated (chapter 7)? I used quantum-dot apatite of three different colors and injected each color at a different time point in a shared fungal network between an older and a younger host root with different nutrient demands. I found that the fungi transferred the nutrients over the shared fungal network from the older host root to the younger host root, depending on the nutrient demand of the younger host root. When the younger root had a higher phosphorus demand, the fungus transferred more phosphorus to the young root, but this pattern took time to emerge. Via confocal microscopy I was able to show that the fungus transferred the quantum-dot apatite from intraradical hyphae to the host root, also depending on the nutrient demand of the host plant: when the nutrient demand was higher, over time, more quantum-dot apatite was transferred from the intraradical hyphae into the host roots, and less was retained within the intraradical hyphae. These results demonstrate the importance of temporal dynamics in the study of resource transfer in mutualisms.

**Nutrient foraging of fungal hyphae**

Both plants and fungal partners in the arbuscular mycorrhizal symbiosis can actively change how much nutrients are allocated to the symbiotic partner (Burleigh 2001; Olsson 2002; Smith 2003; Maldonado-Mendoza et al. 2007; Hammer et al. 2011; Smith et al. 2011). However, it is unknown to which extent one partner can manipulate the nutrient allocation or growing patterns of the other partner to increase its own benefit. My aim was to study if a host plant can influence the growing patterns of the fungus. Is a plant, which is limited in phosphorus, able to manipulate the symbiotic fungus to grow towards phosphorus sources (chapter 8)? I created a fungal foraging area with nutrient rich patches and followed the growth of the fungal hyphae over time. Against expectations, the growth of fungal hyphae was not directed towards the phosphorus source when the host plant was phosphorus limited, but rather to the nitrogen source. I found more fungal hyphae and more spores near the nitrogen patch. I found no evidence that host roots are able to manipulate the fungi to specifically get the resources the plant needs. However, I found that the growing patterns of arbuscular mycorrhizal fungi change when the nutrient need of the plant changes. The fungi allocated more biomass to nitrogen sources when the phosphorus concentrations were low. It is generally known that low phosphorus conditions stimulate the arbuscular mycorrhizal symbiosis (Nouri et al. 2014) and most likely the fungi benefitted from the low phosphorus conditions by gaining more carbon from the host plant (Konvalinková et al. 2017). The fungi used this benefit to produce large amounts of spores, which requires nitrogen. The hyphae of arbuscular mycorrhizal fungi seem to forage to benefit the fungus, rather than the host plant.

While this experiment had promising results, the image acquisition of the fungal network required improvement to more accurately study the hyphal foraging patterns. We have designed a new imaging device that can automatically image the fungal networks of 40 Petri dishes across the duration of an in-vitro experiment, lowering the handling time of each Petri dish and increasing the resolution of the images. This would enable us to precisely follow the growth and development of the fungal network. Future studies should focus on the
fungal network architecture more closely to study fungal foraging patterns and how these are influenced by the nutrient need of the host plant.

**Genetic relatedness between symbionts**

While abiotic environmental conditions, as nutrient availability, have large impacts on a symbiosis, also biotic conditions, as the presence of other symbionts, influence the symbiosis between plant and fungus (Roger et al. 2013). The genetic relatedness among a group of symbiotic microbes might affect the host positively or negatively (Frank 1996, 2006; West et al. 2002). I studied the effect of genetic relatedness between arbuscular mycorrhizal fungi on the trade between fungi and plants, to understand how fungal relatedness affects the network morphology and nutrient transfer in a shared fungal network formed between two host plants (chapter 9). I grew two plants in a single pot and inoculated the focal plant with a specific fungal strain while inoculating the partner plant with either the same fungus (selfing) or a different fungus (non-selfing), mediating the genetic relatedness between the two fungi. I studied phosphorus transfer through the fungal network by injecting quantum-dot apatite in one part of the network and observed how it was transferred over the fungal network to the focal plant. I found that the transfer of phosphorus, and thus plant benefit, lowered when the fungi were non-selfing and genetically less related. While it has been shown that inoculation with different fungi can have different effects, ranging from positive to negative (Van Der Heijden et al. 2006; Jansa et al. 2008; Long et al. 2010; Jin et al. 2013; Boyer et al. 2015; Lin et al. 2015), I have shown that these differences are likely due to the genetic relatedness between the fungal symbionts (chapter 9). When the genetic relatedness between fungal symbionts is low, the non-selfing fungi experience higher competition which has costs to the host. As competition increases, the fungal network may be less symbiotically efficient because the fungi allocate more energy into covering as much space as possible to compete with the other fungi, which trades off with the energy invested in phosphorus transfer to the host plant.

In many of the experiments in this thesis, I inoculated hosts with very simplified fungal communities – usually containing only one fungal partner - to be able to precisely study how environmental factors affected the trading behavior of the fungus. While this simplified model gives the opportunity to disentangle the different effects, it ignores the naturally occurring diversity in real fungal communities. The fungal diversity in nature is high (chapter 3) and all these different species have different functional traits and function differently under certain circumstances. Different arbuscular mycorrhizal fungi probably also provide their host with different benefits under changing conditions. In the future, I would want to work with multiple fungi simultaneously to mimic the natural environment more closely; I would expect very different outcomes compared to my monoculture experiments. For example, I expect that plants exposed to extreme weather events (chapter 6), would have benefited from inoculation with several fungi since one of these fungi might perform better under flooded conditions or high temperatures. Also, a monoculture can monopolize the phosphorus trade, thereby driving the price of phosphorus up, creating a benefit for the fungus and higher cost for the plant (chapter 5). A mixed culture would expose the plant to different trading partners to choose from, which could drive the price of phosphorus down (known as 'outbidding competition'), creating a benefit for the plant. However, I observed the opposite in chapter 9: monocultures were associated with higher phosphorus transfer, whereas mixed cultures transferred less phosphorus, creating a costly environment for the plant. Future experiments should test how a network of genetically different fungi would respond to variations in
nutrient availability across the network as I have tested in chapter 4, 5 and 7.

Conclusions

Symbiosis is fundamental to life on our planet. With this thesis, I have contributed to the knowledge on microbial symbiosis and symbiotic trade. I have shown that more research is needed on the evolution and maintenance of stable signals between symbiotic partners (chapter 2). I have shown that the diversity of fungi in natural systems is very high, but that we lack data and a framework to understand and predict how this fungal diversity functions (chapter 3). I have found that the trading behavior of arbuscular mycorrhizal fungi is largely influenced by environmental factors, as nutrient distribution through space and time (chapter 4, 5, 7) and the needs of the host plant (chapter 7, 8). I showed that the biological market theory is a useful tool to predict the outcome of the trading between partners in the arbuscular mycorrhizal symbiosis (chapter 5), but that the biological market framework does not always explain the empirical observations, due to limitations within the models used (chapter 5, 9). I have shown that the cooperation between plants and arbuscular mycorrhizal fungi is based on conflict between the two partners, in which the two partners always try to take the most benefit (chapter 4, 5, 7-9). I have shown that genetic difference within a network of arbuscular mycorrhizal fungi, can affect the competition between fungi in the fungal network, and can negatively affect the host plant (chapter 9).

The results of the empirical studies in this thesis can be used to support theoretical studies on the evolution and maintenance of symbiotic relations, and allows us to understand and predict the effect of abiotic and biotic factors on the trade in microbial symbiosis.

References


