CHAPTER 1

General introduction

Cooperation as an evolutionary driving force

Evolution is often regarded as a brutal struggle for existence as organisms compete for limited resources. Yet, wherever we look in nature we see cooperation between species, or mutualisms. Bees pollinate flowers in exchange for nectar (Filella et al., 2013), cleaner fish keep their 'clients' free of parasites (Bshary & Grutter, 2002b), animals depend on gut bacteria to digest food in return for nutrients and shelter (Ley et al., 2008; Walter & Ley, 2011), even our very own cells are an ancient symbiotic merger of different microbes (Sagan, 1967; Embley & Martin, 2006; de Duve, 2007). Almost all organisms need to cooperate with other species to thrive (Bronstein, 1994; McFall-Ngai et al., 2012; Berendsen et al., 2012): mutualisms are key factors in organisms' development (Moran, 2007; Oldroyd et al., 2009), drove species diversification (Bascompte & Jordano, 2007; Joy, 2013; Weber & Agrawal, 2014), enabled the colonisation of entirely new habitats (Heckman et al., 2001; Brundrett, 2002; Dubilier et al., 2008) and transformed global nutrient cycles (Houlton *et al.*, 2008; Vitousek *et al.*, 2013; Batterman *et al.*, 2013a).

Consequentially, without cooperation, life on our planet would look very differently (Herre *et al.*, 1999). From a biological perspective, a behaviour or trait is cooperative if it benefits another individual, and if it evolved at least partially because of this benefit (West *et al.*, 2007b). A mutualism is an interaction where the beneficiary of this behaviour belongs to another species, and both species benefit from the interaction (Bronstein, 1994; Leigh, 2010). Although cooperation, both mutualistic and within species[1], is ubiquitous in nature, individuals have the potential to 'cheat' or 'defect' by obtaining benefits without contributing to the partnership (Bao & Addicott, 1998; Sachs *et al.*, 2010; Ghoul *et al.*, 2014). The reason for this is that, all else equal, it is beneficial for an individual to defect from cooperation and gain the benefits of cooperation without paying its cost (West *et al.*, 2007b; Ghoul *et al.*, 2014). As a consequence, successful mutualisms can break down (Sachs & Simms, 2006; Merckx & Bidartondo, 2008). Precisely this ever-present tension between cooperation and conflict is what makes mutualisms such a fascinating topic of study. How has nature solved – at least for now – this tension? Human societies have invented rules, norms and authority to prevent defection and solve the problem of cooperation. How is this achieved in biology, often even in organisms without cognition like plants or microbes? Why do species evolve to cooperate? Will the partnership remain stable? It is within this wider framework that I will study belowground cooperation between plants and root microbes in this thesis.

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1. Although 'mutualism' and 'mutualistic' is sometimes (but rarely) defined in a more general sense to also include reciprocal cooperation within a species (*e.g.* West-Eberhard, 1975; Clutton-Brock, 2002), I will here use it in its common meaning of cooperation between actors belonging to different species (West *et al.*, 2007b).

How can mutualistic cooperation be stable?

Whereas many cooperative behaviours within species, like parental care, collaborative hunting or collective defence, can be explained by the benefits they provide to related individuals (Hamilton, 1964a,b; Gardner *et al.*, 2011; Liao *et al.*, 2015), cooperation among different species (mutualism) is more challenging to explain from an evolutionary perspective. Within a species, a way this can be prevented is when cooperation preferentially benefits relatives sharing the same genes as the cooperating individual (Hamilton, 1964a,b; Gardner *et al.*, 2011; Liao *et al.*, 2015). However, if the partner is another species, individuals should be favoured to defect and take the benefits from a partnership without contributing to it. Nevertheless, mutualistic cooperation persists, and some mutualisms remain stable over dozens or hundreds of millions of years (Remy *et al.*, 1994; Soltis *et al.*, 1995; Currie, 2006; Kaltenpoth *et al.*, 2014).

There are many (theoretical) models to explain the occurrence of stable (costly) mutualisms (*e.g.* Frank, 1996, 1997; Doebeli & Knowlton, 1998; Mueller, 2002; Foster & Wenseleers, 2006; McNamara *et al.*, 2008; Johnstone & Bshary, 2008; Aanen *et al.*, 2009; Golubski & Klausmeier, 2010; Hom & Murray, 2014). Despite this diversity, conceptually they can be divided in two, not mutually exclusive, main classes[2]. In the first class, offspring of both partners are jointly transmitted to the next generation. This is called vertical transmission and stabilises mutualistic cooperation because it aligns the interests on both sides of a partnership (Ewald, 1991; Herre *et al.*, 1999; Bright & Bulgheresi, 2010). If your own progeny fails or thrives with that of your partner, evolutionarily, you have little choice but to help the partner. The ultimate evolutionary outcome of long-term perfect vertical transmission can be complete integration of the two previously independent organisms, which then cease to exist as separate species (Kiers & West, 2015; West *et al.*, 2015). This is how our complex eukaryote cells have been formed millions of years ago from a fusion of microbes (Sagan, 1967; Gould *et al.*, 2008; Keeling, 2010). To explain mutualism stability, some models rely on an increased likelihood of partners' offspring associating with each other, for instance due to limited dispersal or spatial structure (Frank, 1997; Doebeli & Knowlton, 1998). These models are sometimes referred to as 'partner fidelity feedback' models (Sachs *et al.*, 2004; Leigh, 2010; Archetti *et al.*, 2011; Kaltenpoth *et al.*, 2014), a term which is particularly used when referring to cases where transmission fidelity across generations is not perfect because there is no, or imperfect, physical integration of the two mutualistic partners. Conceptually, these models use a very similar logic to vertical transmission models, with perfect vertical transmission representing the most extreme case of a potential gradient (Bright & Bulgheresi, 2010; Ebert, 2013).

2. Arguably, a third class is represented by byproduct mutualisms (Sachs *et al.*, 2004). Here, benefits provided to a partner species are a byproduct of some other behavior directly beneficial to the actor (*e.g.* Bshary *et al.*, 2006). Since in this case cooperation is not costly to the individual and behaviours or traits may not always have evolved to facilitate the partnership, byproduct mutualisms do not represent an evolutionary puzzle to the same extent that costly cooperation does, and I will not further consider them in this thesis.

In the second class of mutualism models, organisms obtain partners from the environment (horizontal transmission) and are capable of some form of active selection, sanctions or preferential allocation to partners based on their quality as co-operators. Defecting individuals that do not pay the cost of cooperation can (typically) produce more progeny than co-operators. Because, due to horizontal transmission, this progeny is unlikely to associate with that of the partner, interests between partners are not aligned and investment in cooperation with the partner is not favoured (Ewald, 1991; Bright & Bulgheresi, 2010). Yet, horizontally transmitted mutualisms can be stabilised by various forms of partner choice (Bshary & Grutter, 2002b; Gubry-Rangin *et al.*, 2010; Regus *et al.*, 2014; Kaltenpoth *et al.*, 2014), sanctions against cheating partners (Pellmyr *et al.*, 1996; Bao & Addicott, 1998; West *et al.*, 2002b,a; Kiers *et al.*, 2006; Oono *et al.*, 2011; Wang *et al.*, 2014) or preferential allocation of resources to high-quality partners that contribute more to the mutualism (Bever *et al.*, 2009; Adam, 2010; Kiers *et al.*, 2011; Bever, 2015). While the precise terminology varies, all of these mechanisms have in common that they increase the relative fitness of a cooperating partner individual compared to a cheater, thereby favouring investment in the partner and counteracting the incentive for cheating or defection.

Selection of partners can drive cooperation and biological markets

If mutualists can detect individual contributions to a partnership and respond by rewarding high-quality partners, this can even lead to dynamics similar to those on human markets, often called 'biological markets' (Noë & Hammerstein, 1994, 1995). Cooperation is stabilised because individuals compete over access to benefits from partners through increasing their own investment in cooperation (Hammerstein & Hagen, 2005; Leimar & Hammerstein, 2010; Kiers *et al.*, 2011). On these biological markets, mutualists can thus be said to 'trade' a resource or service for a resource or service provided by the partner species. This enables both partner species to specialise in a particular mutualistic service or services, obtaining the traded service from their partner (Schwartz & Hoeksema, 1998; Hoeksema & Schwartz, 2003; Grman *et al.*, 2012). A range of different cooperative interactions, from grooming interactions and matings in primates (Gumert, 2007; Port *et al.*, 2009; Fruteau *et al.*, 2011a) and aphid-ant exchanges of aphid sugars (honey dew) for ant protection from herbivores (Völkl *et al.*, 1999; Stadler & Dixon, 2005) to cleaning services in the cleaning-fish mutualism (Bshary & Grutter, 2002b; Bshary & Schäffer, 2002) and bat-plant mutualisms (Schöner *et al.*, 2013), have been analysed in terms of biological markets, revealing phenomena like supply-demand induced variation in service 'price' (Fruteau *et al.*, 2009) and dropping prices in response to increased market competition (Bshary & Schäffer, 2002; Adam, 2010).

However, while it is now well established that active selection of (potential) mutualistic partners can play an important role in some mutualisms, many general questions remain. For instance, while partner selection (in which I explicitly include both partner

rewards and sanctions) has been shown in a few well-studied and phylogenetically diverse taxa (Pellmyr & Huth, 1994; Bshary & Grutter, 2002b; Kiers *et al.*, 2003, 2011; Bever *et al.*, 2009), we do not know if and how widely it is spread outside these partner selection 'model systems'. How important are sanctions and rewards, relative to forces like vertical transmission or partner fidelity? Similarly, while we know that partner selection can work in controlled lab-settings, we have little idea about its strength and relevance in the field in the face of potential limitations to its efficiency. For instance, the potential for direct competition between mutualists (as opposed to indirect competition through providing fitness benefits and behaving cooperatively) could limit the efficiency of partner selection. Another pertinent question is how partner preferences are affected by fluctuations in external conditions affecting the value of exchanged goods and services. And can selection of partners be used not only to affect the level of investment in cooperation, but also the nature of the investment? Lastly, most studies of mutualisms in which partner selection plays a role are a-historical and study responses to cooperation and cheating in the present. But what is the evolutionary history of these systems? When and how did they evolve and remain stable? In my thesis I will (primarily) use plant-microbe root mutualisms to address these questions, asking how stable mutualisms evolve and are maintained.

Underground plant-microbial mutualisms are a puzzle

In this thesis, I study microbial mutualisms, or mutualisms where one or both partners are microbial, and in particular mutualisms between plants and soil microbes. Specifically, I focus on the mutualisms between plants and arbuscular mycorrhizal fungi (AMF), and between plants and N_2-fixing bacteria (both rhizobial and actinorhizal). These microbial mutualisms are among the oldest and most successful mutualism on our planet (Remy *et al.*, 1994; Soltis *et al.*, 1995; Doyle, 2011). For example, the majority of plant species partner with mycorrhizal fungi. Mycorrhizal fungi form extensive networks of fungal hyphae in the soil, exploring the substrate for nutrients and minerals, which they trade with plants in exchange for sugars (Parniske, 2008). This mutualism was likely crucial in facilitating the ancient evolution of land plants (Brundrett, 2002), and is still ecologically important for plant health and yield (Sawers *et al.*, 2008; Kahiluoto *et al.*, 2009; Hoeksema *et al.*, 2010; Lehmann *et al.*, 2012; Berendsen *et al.*, 2012). The symbiosis between some plant species (mostly legumes) and N_2-fixing is much rarer (in terms of number of host plant species), but is nevertheless ultimately responsible for a substantial proportion of nitrogen provisioning in many ecosystems, rendering it crucial to global nutrient cycles as well as being very important ecologically and economically (Peoples *et al.*, 1995; Vitousek *et al.*, 2013; Batterman *et al.*, 2013a).

Plant-microbe root mutualisms are interesting and useful models for mutualistic cooperation not only because of their ecological importance and success, but because they are not transmitted between generations vertically. This means that both partners have

the potential to defect from cooperation and that evolutionary interests of plants and microbes are not expected to be aligned. Why doesn't the mutualism break down? Recent research has shown that in the mycorrhizal mutualism, both plants and fungi can preferentially reward more cooperative partners, and this can help stabilise cooperation (Bever *et al.*, 2009; Kiers *et al.*, 2011; Fellbaum *et al.*, 2012, 2014). In the mutualism of plant and N_2-fixing bacteria, plants appear to be in control of the fitness of their symbionts. They have the capacity to identify root nodules containing bacteria that do not contribute nitrogen, or contribute at too low a level, and reduce resources to them (Kiers *et al.*, 2003; Simms *et al.*, 2006; Gubry-Rangin *et al.*, 2010). In this thesis, I analyse plant-microbe root mutualisms to (i) identify key open questions relating to partner selection in the mycorrhizal mutualism (Chapter 3), (ii) identify potential effects of environmental resource variations on the plant-mycorrhizal mutualism over multiple generations (Chapter 4), (iii) identify a potential limitation to the effectiveness of partner selection in the mycorrhizal mutualism (Chapter 5), (iv) study the evolutionary forces that shape *which* benefits symbionts offer (Chapter 6) and (v) study the 'deep', or hundreds of millions of years old, evolutionary history of the mutualism of plants and symbiotic N_2-fixing bacteria (Chapters 7 & 8).

Thesis outline

In **Chapter 2**, I explore to what extent biological market models can be applied to microbial mutualisms (Werner *et al.*, 2014c). With some exceptions (*e.g.* Cowden & Peterson, 2009; Kiers *et al.*, 2011), biological markets models have so far mostly been considered in animal systems where cognition can explain partner discrimination. In this perspective paper, I analyse the requirements for biological markets to evolve and argue that immediate rewarding of cooperative behaviour can result in market dynamics in microbial mutualisms, even in the absence of any cognition. I identify microbial mutualisms which can be analysed in terms of biological markets, and discuss six main strategies employed by actors on microbial markets.

Having determined that biological market theory is suitable for microbial mutualisms in general, in **Chapter 3**, I direct my attention to partner selection specifically in the mutualism of plants and mycorrhizal fungi (Werner & Kiers, 2015a). I review the current evidence for partner selection in the mycorrhizal mutualism, and identify ecological factors that can affect the effectiveness of active selection of higher-quality partners in stabilising this mutualism. How do environmental conditions shape mycorrhizal partner selection and mycorrhizal biological markets? I subsequently set out to test two of these factors in the following two chapters.

First, in **Chapter 4**, I study the effect of variation in atmospheric CO_2-concentration on the long-term ability of plants to favour high-quality symbiont growth in their roots. Carbon is the main resource provided by plants to the exchange between plants and

AMF (Parniske, 2008). Since CO_2-concentration can drive the availability of carbon to the system relative to other exchanged resources like soil minerals, it is expected to influence the value of a unit of carbon on the plant-fungal market place and affect the intensity of partner selection (Wyatt *et al.*, 2014; Bever, 2015). I here test if fungal abundance patterns match this prediction in a multi-generational experiment where plants have the possibility of selecting either of two fungal species under depressed, normal and elevated CO_2-concentrations.

Second, in **Chapter 5**, using the same two fungal species I determine if so-called 'priority effects' can be a limit to the effectiveness of partner selection by plants (Werner & Kiers, 2015b). Priority effects occur when earlier arrival allows a species to become dominant in a habitat and exclude later arriving species, even though these may be superior in direct competition (Alford & Wilbur, 1985; Wilbur & Alford, 1985; Chase, 2003). I studied if early arrival gives AMF an advantage and allows for the exclusion of secondarily arriving AMF species. If so, this could be an important limitation to the effectiveness of partner selection in field settings.

Most mutualisms are specialised and provide their partners with only a single service or resource, while some mutualisms, like mycorrhizal fungi, provide a range of benefits to their hosts (Newsham *et al.*, 1995; Sikes *et al.*, 2010). We do not currently know what determines where along this spectrum mutualists are favoured to be in biological systems. In **Chapter 6**, I present the first game-theoretical model to study this question. Assuming a theoretical mutualistic exchange where competing populations of mutualists need to provide hosts with two different resources, I determine under which conditions the evolutionary stable strategy (ESS) is to provide both resources versus when a division of labour among two specialised mutualist populations arises.

In **Chapter 7**, I turn my attention to the ancient evolutionary history of another root mutualism (Werner *et al.*, 2014a). Here, I analyse the deep evolution of the mutualistic partnership between some plant species and N_2-fixing bacteria housed in specialised root nodules. In contrast to the mycorrhizal mutualism, this symbiosis is only found in a taxonomically relatively small subset of plant species (Soltis *et al.*, 1995; Doyle, 2011). I ask if its evolution was characterised by a single event, or if (multiple) intermediate steps were required that drove the rise of this interaction (Blount *et al.*, 2008, 2012). By compiling the world's largest database of symbiotic N_2-fixing plant species, and devising a novel framework for quantitative reconstruction of the evolutionary history of mutualisms, I was able to identify the ancient (~100-25 million years ago) key events that drove the evolution and current distribution of symbiotic nitrogen fixation among contemporary land plants.

Lastly, in **Chapter 8**, I further use these reconstructions to ask which (environmental) factors and other plants traits influence if the mutualism of plants and N_2-fixing bacteria

is stable, or persistent, over long time periods. A key question in the evolution of cooperation is not only how mutualism arise initially, but also how they persist, or not, over long time periods (Sachs & Simms, 2006). However, despite mutualism breakdown and persistence being key components of cooperation evolution, this question is only rarely addressed in mutualism research, particularly at a large phylogenetic scale. In this Chapter, I show how high plant nutrient demands in some species can be a factor driving increased persistence over dozens of millions of years of the plant-rhizobial mutualism