Addictive manipulation: a perspective on the role of reproductive parasitism in the evolution of bacteria-eukaryote symbioses

Michele Castelli[1]*, Tiago Nardi[1], Michele Giovannini[2,3], Davide Sassera[1,4]

1. Department of Biology and Biotechnology, University of Pavia, Italy
2. Department of Biology, University of Pisa, Italy
3. Department of Biology, University of Florence, Italy
4. IRCCS Policlinico San Matteo, Pavia, Italy

* corresponding author: michele.castelli@unipv.it

Abstract

Wolbachia bacteria encompass noteworthy reproductive manipulators of their arthropod hosts. which influence host reproduction to favour their own transmission, also exploiting toxin-antitoxin systems. Recently, multiple other bacterial symbionts of arthropods have been shown to display comparable manipulative capabilities.

Here we wonder whether such phenomena are truly restricted to arthropod hosts. We focused on protists, primary models for evolutionary investigations on eukaryotes due to their diversity and antiquity, but still overall under-investigated.

After a thorough re-examination of the literature on bacterial-protist interactions with this question in mind, we conclude that such bacterial “addictive manipulators” of protists do exist, are probably widespread, and have been overlooked until now as a consequence of the fact that investigations are commonly host-centred, thus ineffective to detect such behaviour.

Additionally, we posit that toxin-antitoxin systems are crucial in these phenomena of addictive manipulation of protists, as a result of recurrent evolutionary repurposing. This indicates intriguing functional analogy and molecular homology with plasmid-bacterial interplays.

Finally, we remark that multiple addictive manipulators are affiliated to specific bacterial lineages with ancient associations with diverse eukaryotes. This suggests a possible role of addictive manipulation of protists in paving the way to the evolution of bacteria associated with multicellular organisms.

Overview and purposes

Multiple diverse bacteria live in association with a great variety of eukaryotic hosts [1–3]. Such symbiotic associations are widespread, exhibiting different shades of effects on the involved partners, ranging from mutualism to parasitism [4], with the same partnership varying depending on physiological states or on external conditions [5,6]. Along evolution, the functional properties of the symbiotic partners can be deeply influenced by the association [7].

A noteworthy and peculiar type of bacterial-host interaction is reproductive manipulation, exerted by some phylogenetically diverse bacteria (e.g., *Wolbachia*) on their arthropod hosts, with cytoplasmic incompatibility (CI) as the most distinctive instance [8,9]. As a result, the new host generation from an infected male cannot survive unless it receives the bacterium from the female (Figure 1). This tight association might superficially resemble an obligatory mutualism. However, it is due to the ability of the bacterium to make the host unable to get rid of it, namely to "addict" the host, rather than to the provision of benefits.

A recent work explored the concept of "evolutionary addiction" from the host perspective [10], proposing that, after prolonged associations with their microbiome, hosts may evolve dependence on the bacteria, thus becoming secondarily addicted (see Box 1).

Still, addiction may also be the consequence of active mechanisms exerted by the bacteria on their hosts, as in the case of CI. One could wonder whether such primary addictions are evolutionary oddities restricted to a few specific cases, or the phenomenon has wider evolutionary and ecological significance. Following this line of thought, here we explore the presence of addiction in host-bacterial interactions from the perspective of the bacteria, rather than sticking to a more "conventional" host-centric approach. We focus on unicellular eukaryotic hosts (i.e., protists), which constitute the vast majority of eukaryotes including the most ancestral lineages [11,12], thus being fundamental for understanding the eukaryotic features and their evolution [13]. Bacterial-protist symbioses are widespread [1], but neglected, and in most cases their foundations still await to be

understood. Given the distinctive and diverse physiology and ecology of protists [14–16], these associations only partly fit to “reference” models of bacterial-host symbioses, chiefly nutritional mutualists of animal hosts [1].

Here we reason on whether the origin and maintenance of some bacterial-protist associations could be explained by a process similar to the known cases of animals addicted by their bacterial symbionts, namely by an “addictive manipulation” of host reproduction. Therefore, we examine the literature on bacterial-protist associations looking for indications of potential addictive phenomena and mechanisms. According to several lines of evidence, we propose that addictive manipulation (Figure 1; Box 1) is quite common, though not properly recognised, among bacterial-protist associations, possibly being fundamental in the evolution of many such interactions.

We will start by presenting the most relevant features of well-studied addictive manipulators in arthropods, exemplified by *Wolbachia*. Then, we will move to bacterial-protist symbioses, reasoning on the expected features of addictive manipulation in those associations, and on why, in our view, available clues have not been properly recognised. Subsequently, we will focus on selected cases in which we found convincing signs of addictive manipulation, showing how their re-interpretation allowed us to draw an evolutionary framework that also accounts for possible underlying molecular mechanisms. We will then conclude with a general evolutionary perspective on addictive manipulation and its role in the evolution of bacterial lineages with evolutionarily conserved interactions with protists and other eukaryotes.

Wolbachia, a prototypical addictive manipulator

Reproductive manipulation is a quite well known phenomenon in arthropod hosts, which can be made addicted by multiple diverse bacterial symbionts, including *Rickettsiales* (*Wolbachia*, *Rickettsia* and *Mesenetia* - *formerly Mesenet* [17]) [8,9,18–23], *Mollicutes* (*Spiroplasma*) [24], *Cytophagales* (*Cardinium*) [25], and *Legionellales* (*Rickettsiella*) [26].

Wolbachia is the most studied, and noteworthy enough to deserve the title of "master manipulator of invertebrate biology" [8]. We will use this symbiont to delineate the major features of addictive manipulators. *Wolbachia* is widespread in insects and other arthropods [27,28], thanks to multiple strategies enhancing its vertical transmission through host generations, namely feminisation, parthenogenesis, male killing, and the intriguing CI [8]. CI makes crosses between infected males and non-infected females non-viable, thus favouring the fitness of infected females. In this way, since the symbionts' vertical inheritance relies solely on transovarial transmission from the mother to the offspring, the bacteria massively increase their own fitness (Figure 1). The effect of CI is so powerful that it is being successfully used for biocontrol of arthropod vectors of pathogens [29,30].

While reproductive manipulation has been known for a long time, its molecular mechanisms were elusive until recently [9,19,31,32]. A modification-rescue model had been proposed for CI [33], under which some bacterial-derived factor "poisons" the male gametes, leading to the unsuccessful development of the zygote, and can be counteracted only by a rescue factor present in the infected female gametes. Two *Wolbachia* proteins responsible for these mechanisms were recently discovered [34,35] and shown to form a complex, which can act by a toxin-antitoxin (or "toxin-antidote") regulation [36] (Figure 1). The toxic effect is probably dysregulation of ubiquitination [35,37,38], linked to defects in condensation of the male pronuclei [9,39]. Interestingly, the two involved genes are adjacent in the *Wolbachia* genome, within a putative phage-derived region, and their expression appears to be linked to prophage induction [34]. Several paralogs to these genes are present in different *Wolbachia* strains, and may account for mechanisms of reproductive manipulation other than CI, host specificities, and/or competition between strains [31,34,40]. Among the very few homologs of these genes outside *Wolbachia*, notable are those found in *Rickettsia* and *Spiroplasma* [2 2]. Taken together, these data indicate a spread of CI-inducing factors by horizontal gene transfer (HGT), possibly driven by phages, suggesting that other symbionts could, by molecularly homologous mechanisms, be analogous "master manipulators".

Addictive manipulation of unicellular eukaryotic hosts

Drawing an ideal parallel with the cases involving arthropod hosts listed above, one could wonder whether some bacterial symbionts associated with protists could exert addictive manipulation on their hosts, possibly exploiting analogous modification-rescue processes.

At first glance, it might seem surprising that, despite the diversity and abundance of protists and their bacterial symbionts, an actual addictive manipulation has never been clearly recognised and demonstrated. However, in our view, several aspects should be taken into account, in particular the strong bias in the hosts investigated in most studies. Indeed, despite valuable past (e.g., [41–46]) and recent (e.g., [5,47–58]) investigations, bacterial-protist partnerships are still profoundly under-investigated compared to symbioses involving bacteria and multicellular hosts.

Moreover, studying such associations present multiple inherent limitations, making any hint of addictive manipulation difficult to detect and likely disregarded. In metazoan hosts, vertical transmission is accomplished during sexual reproduction, allowing researchers a clear observation of the effects of potential addictive manipulation exerted by the symbionts (particularly, distortion of sex ratio in the progeny). On the other hand, unicellular eukaryotes most frequently (though not exclusively, see also Box 2) reproduce asexually by cell division, which may nuance and completely "hide" the effect of addiction, such as, plausibly, the death of daughter cells that did not receive the bacteria. Indeed, this is inherently hard to distinguish from a primary obligatory mutualism, in which the host is "simply" dependent on the bacteria (see Box 2 for potential proof-of-principle experiments).

Actually, while a number of bacterial-protist partnerships appear to be transient and unstable [1,59,60], several others have been stably maintained, even for decades [49,61,62], with targeted attempts to remove the bacteria frequently unsuccessful [47,63–65]. These data clearly indicate the presence of a "bond" between those bacteria and their hosts, which in some cases could be assimilated to "true" mutualisms [56,57]. However, multiple other cases display additional and differential features, which, we argue, are suggestive of ongoing addictive manipulation. Closely related bacteria,

even belonging to the same species [61,62,66], are hosted by phylogenetically, physiologically and ecologically diverse hosts. For instance, the *Rickettsiales* bacterium *Megaera (formerly, Megaira* [67]) *polyxenophila* can be associated with heterotrophic protists such as ciliates, multiple photoautotrophic algae, and even cnidarians [49,51,52,68]. Although the bacteria may be in principle able to provide universal mutualistic benefits to such host arrays, it seems meaningful to consider a potential involvement of addictive manipulation, which could enable tight associations to diverse hosts thanks to effector molecules with broad specificity on eukaryotic targets.

Moreover, protists that have been repeatedly found as hosts for stably-associated bacteria (e.g., *Paramecium aurelia*, *Paramecium caudatum*, *Acanthamoeba*) are also commonly found devoid of any bacterium[41,65] [41,65]. This reminds of *Wolbachia* present in multiple diverse arthropod species, with variable prevalence [27]. Eventually, many bacteria could be experimentally removed from their protist hosts by elaborate but potentially fluky approaches [69,70], with the hosts then surviving and often thriving [65,70]. This is sharply different from a primary dependence on the bacteria, being instead reminiscent of addictive manipulators, which are not required by their hosts inherently.

Addictive manipulative mechanisms are also unlikely to be “all-or-nothing” phenomena in every condition (Figure 2; Box 2). Even for *Wolbachia*, reproductive manipulation does not show full penetrance, being dependent on host genetic background [71] and age [72], as well as on external factors [73], so that in some hosts it was initially completely overlooked [9].

Thus, the best indications for an “elusive” trait such as addictive manipulation in protist hosts likely need comprehensive comparative investigations aimed at evidencing general trends, as herein.

Bacteria addictively manipulating protist hosts

Here we highlight those cases showing, in our view, the most distinctive and convincing signs of addictive manipulation of protist hosts exerted by associated bacteria.

The first instance pertains to *Legionella jeonii* (initially termed "X-bacteria" [43]), on which an interesting set of experiments was performed decades ago [74]. When introduced in symbiont-free *Amoeba* cells, it repeatedly produced harmful effects (reduced size, fragility, poor clonability, slower growth, or even death) [42]. However, after some time, surviving subpopulations of amoebas became healthier and, surprisingly, dependent on the symbiont [75], so that antibiotic treatments led not only to bacterial death, but also to demise of the host [76]. In principle, these findings could be interpreted as the consequence of an experimentally induced mutualism (or an evolutionary addiction *sensu* Hammer [10]).

The observed effects were partly correlated with specific pairings of nucleus and cytoplasm (containing the bacteria), as experimental combinations of nuclei from infected cells with cytoplasms from non-infected ones were mostly unviable. However, such combinations survived in a minority of cases, thus not presenting an absolute "all or nothing" outcome, as would be most probable in an "idealised" obligatory mutualism.

Even more remarkably, the same series of effects were observed when *L. jeonii* was transferred to other amoeba cells, which in turn eventually became dependent on the bacteria [75,77]. These data strongly indicate that the factor(s) leading to the non-breakability of the association are derived from *L. jeonii*. The mechanism of this interaction is unknown, but was tentatively linked to a plasmid-encoded 29 kDa protein [43], which can influence host gene expression [78] after being translocated to the host cytoplasm and nucleus [79].

To summarise, available data point to *L. jeonii* possessing the ability to manipulate its *Amoeba* host, making it addicted through context-dependent gene regulation involving plasmids, and resulting in host epigenetic mechanisms (Figure 1).

Other noteworthy and long-time known cases are those of *Caedibacter taeniospiralis* (*Thiotrichales*) and *Caedimonas varicaedens* (*Holosporales*) [80,81], which, although phylogenetically unrelated, were originally grouped together in a single genus for their many shared traits [82]. These bacteria are

typically intracellularly hosted by ciliate protists of the genus *Paramecium*, and are able to confer them a “killer trait”.

Under certain conditions such as starvation, part of the bacteria arrest their replication and produce R-bodies, i.e. large proteinaceous elements shaped as coiled ribbons [44]. Some bacteria are released extracellularly, and, if endocytosed by *Paramecium* cells lacking the symbiont, the acidification of the digestive vacuoles causes the unrolling of the R-bodies and the release of a still uncharacterised toxin [81]. This leads to *Paramecium* cell death by multiple alternative mechanisms, depending on the bacterial and host strain/species, namely hump killing, spin killing, vacuolisation, and paralysis [44,81]. These multifaceted lethal effects are reminiscent of the multiple reproductive manipulation phenomena by *Wolbachia* in arthropods. The *Caedimonas*/*Caedibacter* bacteria are assumed to produce an antitoxin that rescues the toxicity, thus protecting their natural hosts. R-bodies and possibly also toxin-antitoxin genes are encoded into plasmids that also bear phage genes [45,46], and the presence of R-bodies was associated with prophage induction [83].

The killer trait was proposed to provide a competitive advantage to the *Paramecium* hosts towards non-infected conspecifics, thus being indicative of mutualism [81]. In addition, we propose that it is an addictive manipulation phenomenon, in which the host that loses the symbionts is “punished” indirectly, thanks to the probable close presence of “sister cells” still bearing the bacteria (Figure 1). One could say that *Caedimonas*/*Caedibacter* kills paramecia that have lost it pretty much as *Wolbachia* sterilises females that do not have it. From an evolutionary perspective, competitive advantages would then represent an exaptation of a pre-existing control mechanism acting on the host cells, further strengthening the association.

Interestingly, in the past decades several other bacteria were found to cause killer effects in protists hosts [84]. Among them, more recent molecular and phylogenetic characterisations revealed that *Lyticum* spp. are part of the *Rickettsiales* [85], which also encompass *Wolbachia* and other addictive manipulators of arthropods.

Mechanisms and evolution of addictive manipulation

The cases exposed above present common molecular traits, all involving modification/rescue mechanisms and mobile elements (plasmids and phages), which equate them to addictive manipulators of arthropods (Figure 1).

Accordingly, we posit that modification/rescue mechanisms, mediated by toxin-antitoxin systems, could lie behind these and potentially many other cases of addictive manipulation of protist hosts. In the broadest sense [86], multiple types of molecules could be involved through various mechanisms, such as post-transcriptional and/or post-translational regulation. In bacteria, toxin-antitoxin systems are also involved in the addictive control exerted by plasmids [86,87]. Moreover, they were shown to be active on eukaryotic cells [88,89], and are thus plausible candidates for "exaptation" towards addictive manipulation of eukaryotic host cells in general, as already hypothesised for some specific cases [81,90]. Multiple independent events of development/exaptation of molecular determinants of addictive manipulation could be envisioned in different bacterial symbionts of protists. Noteworthy is the *Holosporales* bacterium *Bodocaedibacter*, which expresses toxin and antitoxin genes, and whose suppression by antibiotics leads to death of its host, the flagellate *Bodo saltans*, thus suggesting an addictive role and its determinants [47].

Under this framework, mobile elements, found in multiple bacterial symbionts of protists [44,91–93] , could play a fundamental part, due to their well-recognised role in HGT [94], including specifically in protist-associated bacteria [50,95]. A single protist cell is frequently co-infected by different bacteria, which could easily exchange genes [96,97], thereby acquiring determinants for addictively manipulating their hosts. Accordingly, we can expect the presence of multiple alternative determinants in the same bacterium, with even significant variations between closely related bacteria. Such patterns could account for broad host ranges and their variation (which may be also explained by the molecular specificity of toxins towards targets in different hosts), as well as for competition among symbionts, such as in the case of *Wolbachia* [90]. Therefore, it seems highly intriguing the

discovery of plasmid-encoded R-bodies, possibly linked with an addictive killer trait, in several protist-associated *Holosporales* bacteria other than *Caedimonas* [98].

Additionally, it would be alluring to investigate the impact of potential HGT events from addictively manipulating bacteria towards their protist hosts, similar to known cases of *Wolbachia* in insects [99,100]. Indeed, in principle these events could provide the host with molecular determinants to modulate and counteract addiction.

Evolution of addictive manipulators

From the perspective of bacterial evolution, it is interesting that many of the bacteria with signs of addictive manipulation of different eukaryotes are phylogenetically akin. Particularly, it is remarkable to find multiple representatives of the *Rickettsiales*, the *Legionellales*, and the *Holosporales*. Along with other independent lineages, these phylogenetically unrelated bacteria share some peculiar functional and evolutionary traits making them noteworthy for the study of bacterial-eukaryotic symbioses in general, which also led some authors to categorise them as “professional symbionts” [1] . Their recurrent involvement in addictive manipulation suggests to examine them further.

The representatives of such “professional symbionts” live in association with eukaryotes, most likely since extremely ancient times (even over 1 bya) [54,101,102]. Each lineage collectively displays a broad host range, colonising diverse protists, as well as multicellular organisms [47,51,54,55,103–114]. The most thoroughly investigated representatives of each lineage are arthropod-borne pathogens [115–118]. However, the majority are hosted by aquatic protists, which are considered the ancestral hosts, with multiple independent secondary adaptations to multicellular hosts [103,104,108,119].

Despite being unable to multiply in the absence of host cells (though with few possible exceptions [93,120,121]), “professional symbionts” are not strictly host-confined. Indeed, along with vertical transmission, many of them can also perform horizontal transmission [122–125], even shifting between very different host species [104,126].

Consistently with their complex lifestyles, “professional symbionts” bear rich repertoires of still largely uncharacterised molecular effectors [92,127–131], enabling them to actively modulate, and possibly even “control” [1] those multifaceted interactions. In light of what is presented above, it seems intriguing to speculate that, among those molecular mechanisms, some capable of inducing addictive manipulation could be significant and widespread. Varied interactions with a wide array of eukaryotic hosts, as in the lineages of “professional symbionts”, would indeed be a plausible outcome for the descendants of hypothetical ancestral bacteria capable of addictive manipulation. Accordingly, addictive manipulation could have taken an active part in the evolution of these lineages, possibly even “determining” it. Variations in the repertoire and/or specificity of toxin-antitoxin modules would allow to achieve such a breadth and evolutionary variability of host ranges, including in particular shifts from protist to multicellular hosts.

Addictive manipulation and other interactions might concur in the establishment and maintenance of tight bacterial-host associations, and might repeatedly supersede each other over evolutionary times. Such alternative interactions include more conventional mutualisms, as exemplified by some *Wolbachia*, which have become necessary for filarial nematodes [8,20] and for some insects [132–136].

Concluding remarks

Through a targeted literature review and re-interpretation, here we propose a novel framework for the evolution and persistence of bacterial-protist associations, namely by addictive manipulation mechanisms enacted by many of those bacteria (Box 1), comparable to the reproductive manipulation in arthropods [18,19,22,24]. This would result in the death of those hosts that have recently lost the symbionts, through toxic activity exerted by the bacteria under those specific circumstances, rather than due to some inherent inability of the hosts to cope with the lack of the symbionts.

Such addictive manipulators of protists or other asexual hosts would behave as selfish addictive elements (Figure 2), with intriguing analogies with plasmid-bacteria interplays [137,138], especially

when considering the repurposing of the same kind of molecular determinants (toxin-antitoxin systems) [36,43,81,90] and the probable involvement of mobile elements in spreading such determinants among eukaryote-associated bacteria. Notwithstanding significant differences in sexual processes between animals and protists, e.g., conjugation in ciliates [139], it seems also worthwhile to consider that addictive manipulators may influence the relative frequency of sexual and asexual reproduction in protists, analogous to *Wolbachia* in arthropods [8,140].

Considering the inherent difficulties in distinguishing addictive manipulation from other interactions among bacterial-protist associations, we posit that the herein presented examples represent only the "tip of the iceberg" of a widespread phenomenon. Thus, we underline the need for dedicated research to elucidate the diffusion, mechanisms, impact, and evolutionary significance of addictive manipulation, in particular targeted experimental analyses (Box 2).

Given the fundamental roles of protists in a broad range of ecosystems [14–16], addictive manipulation likely has deep ecological impacts as well. As exemplified by *Wolbachia*, addictive manipulators can provide fundamental insights on the eco-physiology and evolution of each host [8], which may become the basis for innovative applications [29,30].

It is a quite accepted notion that, due to their antiquity, diversity and diffusion, protists may act as "Trojan horses" or "melting pots" for the evolution of bacteria associated with multicellular hosts [97,141]. Thus, it seems thought-provoking to examine the evolutionary significance of addictive manipulation of protists, in particular when considering the recurrent occurrence of addictive manipulators within lineages that encompass bacteria associated with both protists and multicellular organisms [93,104,119].

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Figures and legends

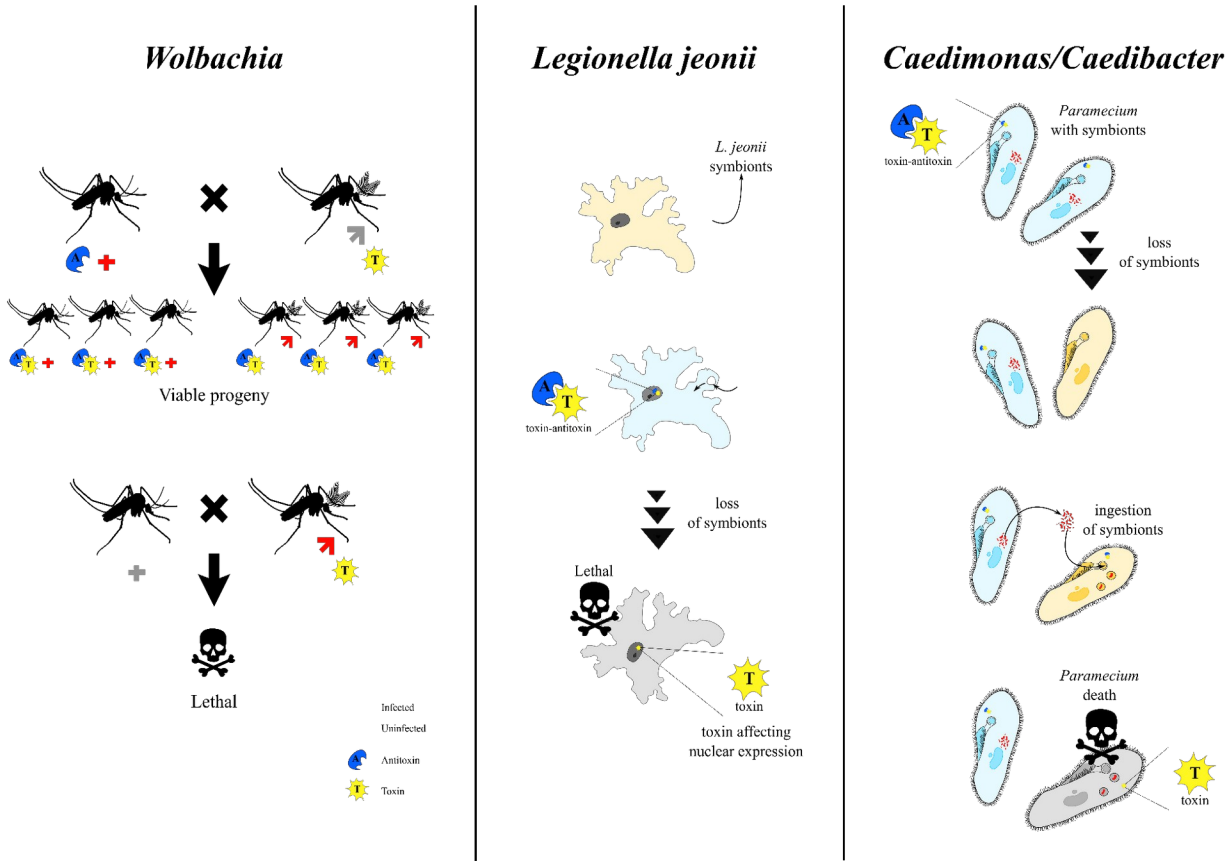

Figure 1. Addictive manipulation mechanisms exerted by bacterial symbionts on their diverse eukaryotic hosts, involving molecular determinants linked to mobile genetic elements. This way, the bacteria ensure their own proliferation by promoting their vertical transmission. *Wolbachia* (*Rickettsiales*) manipulates the reproduction of its vertebrate hosts by CI (and other mechanisms). The bacterium is vertically transmitted to the offspring only by the females. Gametes from infected males carry a prophage-linked toxin that kills the embryos, unless female gametes carry the bacterium with a cognate antitoxin, thus favouring the spread and maintenance of the bacterium in the host populations. Similarly, *L. jeonii* (*Legionellales*) manipulates the asexual life cycle of its unicellular eukaryotic hosts. When healthy amoebas get infected, they become unable to get rid of the bacteria. Most likely, a plasmid-encoded toxin by the bacteria epigenetically acts on host gene expression, a modification that persists after bacterial loss, and that can be rescued only in presence of live bacteria. *Caedimonas* (*Holosporales*) and *Caedibacter* (*Thiotrichales*) counteract their loss by *Paramecium* hosts by an indirect mechanism. The bacteria produce a plasmid-encoded toxin, against which their hosts are

protected by the cognate antitoxin. If a host loses the symbiont, it becomes sensitive to the toxin, and will be killed when ingesting symbionts released by its, still infected, sister cells.

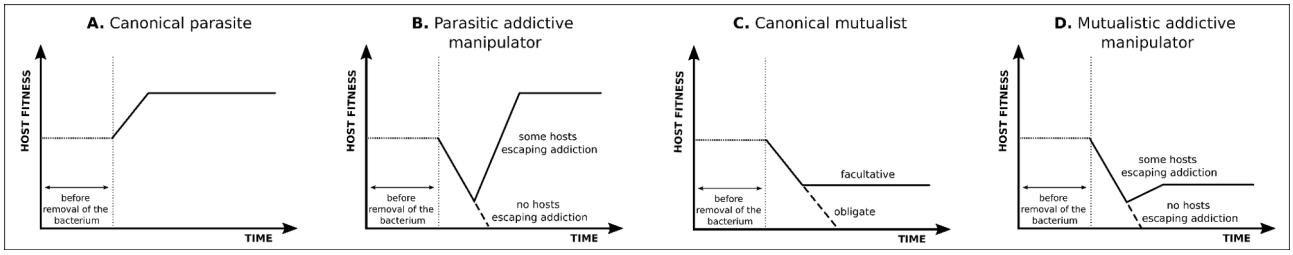

Figure 2. Comparisons of idealised fitness responses of a protist (or another asexually reproducing host) to the removal (dotted vertical line) of an addictive manipulator (B, D) in comparison to the removal of a canonical parasite (A) or a canonical mutualist (C). In turn, depending on other potential concomitant interactions, a manipulator may have overall detrimental or beneficial effects, respectively behaving as a parasitic (B) or mutualistic (D) addictive manipulator.

Box 1. Addictive symbiont-host interactions

Addictive symbiont-host interactions imply that the host receives damage, up to potential death, if symbionts are lost, regardless of direct benefits provided by the symbionts. As a consequence, the association results tightened, with potential advantages for the symbionts. The most thoroughly studied cases are those of reproductive manipulation exerted by *Wolbachia*, *Spiroplasma* and other bacteria on arthropods, through CI, male killing, feminisation, or parthenogenesis.

Additionally, the concept of evolutionary addiction was recently proposed, namely that coexistence with the symbionts will cause different evolutionary processes in the host, which would eventually result in dysregulation in case the bacteria are removed [10]. Specifically, according to Hammer, "adaptive accommodation" implies the irreversible accommodation of host regulatory mechanisms in the presence of bacteria, while "compensated trait loss" implies that the redundancy of certain metabolic and functional features in host and symbionts may result in the loss of the respective genes in the host, which would need compensation by the symbionts.

On the other hand, in case of reproductive manipulation, the addiction would depend directly on active properties exerted by the bacteria, specifically, in the experimentally validated cases of *Wolbachia* and *Spiroplasma*, by the action of toxins and antitoxins [9,19,31].

Here we propose the concept of "addictive manipulation", by generalising the case of reproductive manipulation of arthropods to other eukaryotes, in particular protists. Under this condition, the hosts are addicted to bacterial symbionts as a result of some active property evolved and exerted by the symbionts themselves, without directly implying any evolutionary change in the hosts. As in the specific cases of reproductive manipulators of arthropods, addictive manipulation likely takes place thanks to molecular toxin-antitoxin systems, and may consist in different phenomena depending on the physiology and ecology of host and symbionts (see also Box 2 "How to test addictive manipulation").

Accordingly, host-symbionts interactions in case of addictive manipulation expectedly result in complex interplays, which, to be fully delineated, should require accounting for several other features, such as the potential capability of symbionts to spread horizontally, and the interaction of host and/or professional symbionts with other organisms, including non-infected hosts (see the case of *Wolbachia* or *Caedimonas*/*Caedibacter*) [8,81].

At an evolutionary scale, we highlight the possibility that addictive manipulation could have had important consequences in the evolution of bacterial lineages with ancient and evolutionarily stable interactions with eukaryotic hosts (e.g., *Rickettsiales*, *Legionellales*, *Holosporales*, *Chlamydiae*).

Box 2: How to test addictive manipulation

The inherent complexity of addictive manipulation hampers its proper identification in protists. Possible approaches to discern it could involve modelling bacterial-host interactions in case of addictive manipulation, for instance by analogy with models of addiction of bacterial cells on plasmids [138], and then subject those models to experimental validation.

Herein, it seems appropriate to outline some simple general criteria as a starting ground, in particular by evaluating the effect of symbiont removal on the host. For this purpose, we assume that: i) the host is reproducing asexually, ii) host survival, reproductive success and/or well-being can be measured (here collectively termed as “fitness”), iii) a method for removing the addictive manipulator is available (e.g. antibiotics), iv) any addictive manipulation phenomenon is not 100% effective. The latter assumption seems reasonable based on the available knowledge on *Wolbachia*, *Caedibacter*/*Caedimonas*, and *L. jeonii*, for which the addictive manipulation mechanisms are conditionally regulated (e.g. by prophage inductions) according to physiological states or external factors such as temperature [9,43,81]. Although this may represent a confounding factor, it can also be instrumental in discriminating an addictive manipulator from a necessary mutualist (see below).

If an addictive manipulator is removed, we expect an initial reduction of host fitness, up to complete death, or followed by a subsequent recovery (by hosts escaping from non-100% effective addictive

389 manipulation) (Figure 2). The post-recovery fitness level would depend on whether the overall effect
390 of the addictive manipulator is mutualistic or parasitic. Notably, the end results would be
391 indistinguishable from canonical parasites or canonical mutualists, and, if taken alone, may mislead in
392 the classification of the interaction. This seems to be the case of *L. jeonii*, originally interpreted as a
393 necessary mutualist [76]. Rather, it is the temporal trajectory of the variation of fitness that matters, as
394 the fitness "reduction-recovery" process would be distinctive for an addictive manipulator (Figure 2).
395 Inevitably, such an approach is prone to confounding factors and to detection limits (in particular
396 relative to the speed of the process and the effect size). We put forward that identifying molecular
397 determinants could complement such limits, not only demonstrating the mechanism for addiction
398 manipulation of protists (or other asexual hosts), but also validating that it is actually taking place.
399 Additionally, it should be accounted that several protist hosts have relatively common and discernible
400 sexual processes, e.g., dictyosteliid amoebae and ciliates [139,142,143]. Thus, approaches more
401 comparable to those traditionally employed to investigate addictive manipulators of insects could be
402 attempted to investigate possible addictive manipulators of these protists (e.g., [41,81,85,143,144]).
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References

1. Husnik F, Tashyreva D, Boscaro V, George EE, Lukeš J, Keeling PJ. 2021 Bacterial and archaeal symbioses with protists. *Curr. Biol.* **31**, R862–R877.

2. Rosenblueth M, Martínez-Romero E. 2006 Bacterial endophytes and their interactions with hosts. *Mol. Plant. Microbe. Interact.* **19**, 827–837.

3. McFall-Ngai M *et al.* 2013 Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 3229–3236.

4. Sapp J. 2004 The dynamics of symbiosis: an historical overview. *Can. J. Bot.* **82**, 1046–1056.

5. Herrera P *et al.* 2020 Molecular causes of an evolutionary shift along the parasitism-mutualism continuum in a bacterial symbiont. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 21658–21666.

6. Regus JU, Gano KA, Hollowell AC, Sofish V, Sachs JL. 2015 Lotus hosts delimit the mutualism-parasitism continuum of Bradyrhizobium. *J. Evol. Biol.* **28**, 447–456.

7. Moran NA. 2007 Symbiosis as an adaptive process and source of phenotypic complexity. *Proc. Natl. Acad. Sci. U. S. A.* **104 Suppl 1**, 8627–8633.

8. Werren JH, Baldo L, Clark ME. 2008 *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751.

9. Shropshire JD, Leigh B, Bordenstein SR. 2020 Symbiont-mediated cytoplasmic incompatibility: what have we learned in 50 years? *Elife* **9**. (doi:10.7554/eLife.61989)

10. Hammer TJ. 2023 Why do hosts malfunction without microbes? Missing benefits versus evolutionary addiction. *Trends Microbiol.* (doi:10.1016/j.tim.2023.07.012)

11. Adl SM *et al.* 2019 Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. *J. Eukaryot. Microbiol.* **66**, 4–119.

12. Keeling PJ, Burki F. 2019 Progress towards the Tree of Eukaryotes. *Curr. Biol.* **29**, R808–R817.

13. O'Malley MA, Simpson AGB, Roger AJ. 2013 The other eukaryotes in light of evolutionary protistology. *Biol. Philos.* **28**, 299–330.

14. Caron DA, Countway PD, Jones AC, Kim DY, Schnetzer A. 2012 Marine protistan diversity. *Ann. Rev. Mar. Sci.* **4**, 467–493.

15. Geisen S *et al.* 2018 Soil protists: a fertile frontier in soil biology research. *FEMS Microbiol. Rev.* **42**, 293–323.

16. Burki F, Sandin MM, Jamy M. 2021 Diversity and ecology of protists revealed by metabarcoding. *Curr. Biol.* **31**, R1267–R1280.

17. Oren A. 2022 Candidatus List No. 4: Lists of names of prokaryotic *Candidatus* taxa. *Int. J. Syst. Evol. Microbiol.* **72**, 005545.

18. Hurst GDD, Frost CL. 2015 Reproductive parasitism: maternally inherited symbionts in a biparental world. *Cold Spring Harb. Perspect. Biol.* **7**. (doi:10.1101/cshperspect.a017699)

19. Chen H, Zhang M, Hochstrasser M. 2020 The Biochemistry of Cytoplasmic Incompatibility Caused by Endosymbiotic Bacteria. *Genes* **11**. (doi:10.3390/genes11080852)

20. Taylor MJ, Bandi C, Hoerauf A. 2005 *Wolbachia* bacterial endosymbionts of filarial nematodes. *Adv.*

Parasitol. **60**, 245–284.

21. Perlman SJ, Hunter MS, Zchori-Fein E. 2006 The emerging diversity of *Rickettsia*. *Proc. Biol. Sci.* **273**, 2097–2106.

22. Gillespie JJ, Driscoll TP, Verhoeve VI, Rahman MS, Macaluso KR, Azad AF. 2018 A Tangled Web: Origins of Reproductive Parasitism. *Genome Biol. Evol.* **10**, 2292–2309.

23. Takano S-I, Gotoh Y, Hayashi T. 2021 '*Candidatus* Mesenet longicola': Novel Endosymbionts of *Brontispa longissima* that Induce Cytoplasmic Incompatibility. *Microb. Ecol.* **82**, 512–522.

24. Pollmann M, Moore LD, Krimmer E, D'Alvise P, Hasselmann M, Perlman SJ, Ballinger MJ, Steidle JLM, Gottlieb Y. 2022 Highly transmissible cytoplasmic incompatibility by the extracellular insect symbiont. *iScience* **25**, 104335.

25. Nguyen DT, Morrow JL, Spooner-Hart RN, Riegler M. 2017 Independent cytoplasmic incompatibility induced by *Cardinium* and *Wolbachia* maintains endosymbiont coinfections in haplodiploid thrips populations. *Evolution* **71**, 995–1008.

26. Rosenwald LC, Sitvarin MI, White JA. 2020 Endosymbiotic *Rickettsiella* causes cytoplasmic incompatibility in a spider host. *Proc. Biol. Sci.* **287**, 20201107.

27. Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. 2015 The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc. Biol. Sci.* **282**, 20150249.

28. Sazama EJ, Bosch MJ, Shouldis CS, Ouellette SP, Wesner JS. 2017 Incidence of *Wolbachia* in aquatic insects. *Ecol. Evol.* **7**, 1165–1169.

29. Hoffmann AA *et al.* 2011 Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* **476**, 454–457.

30. Utarini A *et al.* 2021 Efficacy of *Wolbachia*-Infected Mosquito Deployments for the Control of Dengue. *N. Engl. J. Med.* **384**, 2177–2186.

31. Harumoto T, Lemaitre B. 2018 Male-killing toxin in a bacterial symbiont of Drosophila. *Nature* **557**, 252–255.

32. McNamara CJ, Ant TH, Harvey-Samuel T, White-Cooper H, Martinez J, Alphey L, Sinkins SP. 2024 Transgenic expression of cif genes from *Wolbachia* strain wAlbB recapitulates cytoplasmic incompatibility in *Aedes aegypti*. *Nat. Commun.* **15**, 869.

33. Werren JH. 1997 Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42**, 587–609.

34. LePage DP *et al.* 2017 Prophage WO genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. *Nature* **543**, 243–247.

35. Beckmann JF, Ronau JA, Hochstrasser M. 2017 A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat. Microbiol.* **2**. (doi:10.1038/nmicrobiol.2017.7)

36. Hochstrasser M. 2022 Cytoplasmic incompatibility: A Wolbachia toxin-antidote mechanism comes into view. *Curr. Biol.* **32**, R287–R289.

37. Terretaz K, Horard B, Weill M, Loppin B, Landmann F. 2023 Functional analysis of *Wolbachia* Cid effectors unravels cooperative interactions to target host chromatin during replication. *PLoS Pathog.* **19**, e 1011211.

38. Harumoto T. 2023 Self-stabilization mechanism encoded by a bacterial toxin facilitates reproductive parasitism. *Curr. Biol.* **33**, 4021–4029.e6.

39. Tram U, Ferree PM, Sullivan W. 2003 Identification of *Wolbachia*--host interacting factors through

cytological analysis. *Microbes Infect.* **5**, 999–1011.

40. Lindsey ARI, Rice DW, Bordenstein SR, Brooks AW, Bordenstein SR, Newton ILG. 2018 Evolutionary Genetics of Cytoplasmic Incompatibility Genes cifA and cifB in Prophage WO of *Wolbachia*. *Genome Biol. Evol.* **10**, 434–451.

41. Fokin SI. 2012 Frequency and biodiversity of symbionts in representatives of the main classes of Ciliophora. *Eur. J. Protistol.* **48**, 138–148.

42. Jeon KW, Lorch IJ. 1967 Unusual intra-cellular bacterial infection in large, free-living amoebae. *Exp. Cell Res.* **48**, 236–240.

43. Jeon KW. 1987 Change of cellular 'pathogens' into required cell components. *Ann. N. Y. Acad. Sci.* **503**, 359–371.

44. Pond FR, Gibson I, Lalucat J, Quackenbush RL. 1989 R-body-producing bacteria. *Microbiol. Rev.* **53**, 25–67.

45. Quackenbush RL, Burbach JA. 1983 Cloning and expression of DNA sequences associated with the killer trait of *Paramecium tetraurelia* stock 47. *Proc. Natl. Acad. Sci. U. S. A.* **80**, 250–254.

46. Jeblick J, Kusch J. 2005 Sequence, transcription activity, and evolutionary origin of the R-body coding plasmid pKAP298 from the intracellular parasitic bacterium *Caedibacter taeniospiralis*. *J. Mol. Evol.* **60** , 164–173.

47. Midha S, Rigden DJ, Siozios S, Hurst GDD, Jackson AP. 2021 *Bodo saltans* (Kinetoplastida) is dependent on a novel *Paracaedibacter*-like endosymbiont that possesses multiple putative toxin-antitoxin systems. *ISME J.* **15**, 1680–1694.

48. Castelli M *et al.* 2019 Deianiraea, an extracellular bacterium associated with the ciliate Paramecium, suggests an alternative scenario for the evolution of Rickettsiales. *ISME J.* **13**, 2280–2294.

49. Lanzoni O, Sabaneyeva E, Modeo L, Castelli M, Lebedeva N, Verni F, Schrallhammer M, Potekhin A, Petroni G. 2019 Diversity and environmental distribution of the cosmopolitan endosymbiont '*Candidatus* Megaira'. *Sci. Rep.* **9**, 1179.

50. Castelli M, Lanzoni O, Nardi T, Lometto S, Modeo L, Potekhin A, Sassera D, Petroni G. 2021 '*Candidatus* Sarmatiella mevalonica' *endosymbiont* of the ciliate *Paramecium* provides insights on evolutionary plasticity among Rickettsiales. *Environ. Microbiol.* **23**, 1684–1701.

51. Davison HR, Hurst GDD, Siozios S. 2023 '*Candidatus* Megaira' are diverse symbionts of algae and ciliates with the potential for defensive symbiosis. *Microb Genom* **9**. (doi:10.1099/mgen.0.000950)

52. Hess S. 2017 Description of *Hyalodiscus flabellus* sp. nov. (Vampyrellida, Rhizaria) and Identification of its Bacterial Endosymbiont, '*Candidatus* Megaira polyxenophila' (*Rickettsiales*, *Alphaproteobacteria*). *Protist* **168**, 109–133.

53. Arthofer P, Delafont V, Willemsen A, Panhölzl F, Horn M. 2022 Defensive symbiosis against giant viruses in amoebae. *Proc. Natl. Acad. Sci. U. S. A.* **119**, e2205856119.

54. Dharamshi JE, Köstlbacher S, Schön ME, Collingro A, Ettema TJG, Horn M. 2023 Gene gain facilitated endosymbiotic evolution of *Chlamydiae*. *Nat Microbiol* **8**, 40–54.

55. Paight C, Hunter ES, Lane CE. 2022 Codependence of individuals in the *Nephromyces* species swarm requires heterospecific bacterial endosymbionts. *Curr. Biol.* **32**, 2948–2955.e4.

56. Boscaro V, Syberg-Olsen MJ, Irwin NAT, George EE, Vannini C, Husnik F, Keeling PJ. 2022 All essential endosymbionts of the ciliate *Euplotes* are cyclically replaced. *Curr. Biol.* **32**, R826–R827.

57. Boscaro V, Husnik F, Vannini C, Keeling PJ. 2019 Symbionts of the ciliate *Euplotes*: diversity, patterns and potential as models for bacteria-eukaryote endosymbioses. *Proc. Biol. Sci.* **286**, 20190693.

58. Maita C *et al.* 2018 Amoebal endosymbiont *Neochlamydia* protects host amoebae against *Legionella pneumophila* infection by preventing *Legionella* entry. *Microbes Infect.* **20**, 236–244.

59. Gast RJ, Sanders RW, Caron DA. 2009 Ecological strategies of protists and their symbiotic relationships with prokaryotic microbes. *Trends Microbiol.* **17**, 563–569.

60. Nowack ECM, Melkonian M. 2010 Endosymbiotic associations within protists. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **365**, 699–712.

61. Potekhin A, Schweikert M, Nekrasova I, Vitali V, Schwarzer S, Anikina A, Kaltz O, Petroni G, Schrallhammer M. 2018 Complex life cycle, broad host range and adaptation strategy of the intranuclear *Paramecium* symbiont *Preeria caryophila* comb. nov. *FEMS Microbiol. Ecol.* **94**. (doi:10.1093/femsec/fiy076)

62. Schweikert M, Meyer B. 2001 Characterization of intracellular bacteria in the freshwater dinoflagellate *Peridinium cinctum*. *Protoplasma* **217**, 177–184.

63. Mironov T, Sabaneyeva E. 2020 A Robust Symbiotic Relationship Between the Ciliate *Paramecium multimicronucleatum* and the Bacterium "*Ca*. Trichorickettsia mobilis". *Front. Microbiol.* **11**, 603335.

64. Mironov T, Yakovlev A, Sabaneyeva E. 2022 Together forever: Inseparable partners of the symbiotic system *Paramecium multimicronucleatum*/"*Ca*. Trichorickettsia mobilis". *Symbiosis* (doi:10.1007/s13199-022-00854-z)

65. Flemming FE, Grosser K, Schrallhammer M. 2021 Natural Shifts in Endosymbionts' Occurrence and Relative Frequency in Their Ciliate Host Population. *Front. Microbiol.* **12**, 791615.

66. Senra MVX, Dias RJP, Castelli M, Silva-Neto ID, Verni F, Soares CAG, Petroni G. 2016 A House for Two--Double Bacterial Infection in *Euplotes woodruffi* Sq1 (Ciliophora, Euplotia) Sampled in Southeastern Brazil. *Microb. Ecol.* **71**, 505–517.

67. Oren A, Garrity GM, Parker CT, Chuvochina M, Trujillo ME. 2020 Lists of names of prokaryotic *Candidatus* taxa. *Int. J. Syst. Evol. Microbiol.* **70**, 3956–4042.

68. Schrallhammer M, Ferrantini F, Vannini C, Galati S, Schweikert M, Görtz H-D, Verni F, Petroni G. 2013 '*Candidatus* Megaira polyxenophila' gen. nov., sp. nov.: considerations on evolutionary history, host range and shift of early divergent rickettsiae. *PLoS One* **8**, e72581.

69. Bella C, Koehler L, Grosser K, Berendonk TU, Petroni G, Schrallhammer M. 2016 Fitness Impact of Obligate Intranuclear Bacterial Symbionts Depends on Host Growth Phase. *Front. Microbiol.* **7**, 2084.

70. Pasqualetti C, Szokoli F, Rindi L, Petroni G, Schrallhammer M. 2020 The Obligate Symbiont '*Candidatus* Megaira polyxenophila' Has Variable Effects on the Growth of Different Host Species. *Front. Microbiol.* **11**, 1425.

71. Walker T *et al.* 2011 The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* **476**, 450–453.

72. Layton EM, On J, Perlmutter JI, Bordenstein SR, Shropshire JD. 2019 Paternal Grandmother Age Affects the Strength of -Induced Cytoplasmic Incompatibility in *Drosophila melanogaster*. *MBio* **10**. (doi:10.1128/mBio.01879-19)

73. Ross PA, Ritchie SA, Axford JK, Hoffmann AA. 2019 Loss of cytoplasmic incompatibility in *Wolbachia* -infected *Aedes aegypti* under field conditions. *PLoS Negl. Trop. Dis.* **13**, e0007357.

74. Park M, Yun ST, Kim MS, Chun J, Ahn TI. 2004 Phylogenetic characterization of *Legionella*-like

endosymbiotic X-bacteria in *Amoeba proteus*: a proposal for '*Candidatus* Legionella jeonii' sp. nov. *Environ. Microbiol.* **6**, 1252–1263.

75. Jeon KW. 1972 Development of cellular dependence on infective organisms: micrurgical studies in amoebas. *Science* **176**, 1122–1123.

76. Jeon KW, Hah JC. 1977 Effect of chloramphenicol on bacterial endosymbiotes in a strain of *Amoeba proteus*. *J. Protozool.* **24**, 289–293.

77. Jeon KW, Ahn TI. 1978 Temperature sensitivity: a cell character determined by obligate endosymbionts in amoebas. *Science* **202**, 635–637.

78. Jeon TJ, Jeon KW. 2004 Gene switching in *Amoeba proteus* caused by endosymbiotic bacteria. *J. Cell Sci.* **117**, 535–543.

79. Pak JW, Jeon KW. 1997 A symbiont-produced protein and bacterial symbiosis in *Amoeba proteus*. *J. Eukaryot. Microbiol.* **44**, 614–619.

80. Kusch J, Görtz H-D. 2006 Towards an understanding of the killer trait: *Caedibacter* endocytobionts in *Paramecium*. *Prog. Mol. Subcell. Biol.* **41**, 61–76.

81. Schrallhammer M, Schweikert M. 2009 The killer effect of *Paramecium* and its causative agents. In *Endosymbionts in Paramecium*, pp. 227–246. Berlin, Heidelberg: Springer Berlin Heidelberg.

82. Schrallhammer M, Castelli M, Petroni G. 2018 Phylogenetic relationships among endosymbiotic R-body producer: Bacteria providing their host the killer trait. *Syst. Appl. Microbiol.* **41**, 213–220.

83. Preer LB, Rudman BM, Preer JR, Jurand A. 1974 Induction of R bodies by ultraviolet light in killer paramecia. *J. Gen. Microbiol.* **80**, 209–215.

84. Görtz H-D, Fokin SI. 2009 Diversity of Endosymbiotic Bacteria in *Paramecium*. In *Endosymbionts in Paramecium*, pp. 131–160. Berlin, Heidelberg: Springer Berlin Heidelberg.

85. Boscaro V *et al.* 2013 Rediscovering the genus *Lyticum*, multiflagellated symbionts of the order *Rickettsiales*. *Sci. Rep.* **3**, 3305.

86. Jurėnas D, Fraikin N, Goormaghtigh F, Van Melderen L. 2022 Biology and evolution of bacterial toxin-antitoxin systems. *Nat. Rev. Microbiol.* **20**, 335–350.

87. Harms A, Brodersen DE, Mitarai N, Gerdes K. 2018 Toxins, targets, and triggers: An overview of toxin-antitoxin biology. *Mol. Cell* **70**, 768–784.

88. Yeo CC, Abu Bakar F, Chan WT, Espinosa M, Harikrishna JA. 2016 Heterologous expression of toxins from bacterial toxin-antitoxin systems in eukaryotic cells: Strategies and applications. *Toxins* **8**, 49.

89. You S *et al.* 2023 A toxin-antidote system contributes to interspecific reproductive isolation in rice. *Nat. Commun.* **14**, 7528.

90. Beckmann JF, Bonneau M, Chen H, Hochstrasser M, Poinsot D, Merçot H, Weill M, Sicard M, Charlat S. 2019 The Toxin-Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications. *Trends Genet.* **35**, 175–185.

91. Wang Z, Wu M. 2015 An integrated phylogenomic approach toward pinpointing the origin of mitochondria. *Sci. Rep.* **5**, 7949.

92. George EE, Husnik F, Tashyreva D, Prokopchuk G, Horák A, Kwong WK, Lukeš J, Keeling PJ. 2020 Highly Reduced Genomes of Protist Endosymbionts Show Evolutionary Convergence. *Curr. Biol.* **30**, 925–933.e3.

93. Castelli M, Nardi T, Gammuto L, Bellinzona G, Sabaneyeva E, Potekhin A, Serra V, Petroni G, Sassera D.

2024 Host association and intracellularity evolved multiple times independently in the *Rickettsiales*. *Nat. Commun.* **15**, 1093.

94. Haudiquet M, de Sousa JM, Touchon M, Rocha EPC. 2022 Selfish, promiscuous and sometimes useful: how mobile genetic elements drive horizontal gene transfer in microbial populations. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **377**, 20210234.

95. George EE, Tashyreva D, Kwong WK, Okamoto N, Horák A, Husnik F, Lukeš J, Keeling PJ. 2022 Gene Transfer Agents in Bacterial Endosymbionts of Microbial Eukaryotes. *Genome Biol. Evol.* **14**. (doi:10.1093/gbe/evac099)

96. Gomez-Valero L, Buchrieser C. 2019 Intracellular parasitism, the driving force of evolution of *Legionella pneumophila* and the genus *Legionella*. *Microbes Infect.* **21**, 230–236.

97. Wang Z, Wu M. 2017 Comparative Genomic Analysis of *Acanthamoeba* Endosymbionts Highlights the Role of Amoebae as a 'Melting Pot' Shaping the *Rickettsiales* Evolution. *Genome Biol. Evol.* **9**, 3214–3224.

98. Giovannini M, Petroni G, Castelli M. 2024 Novel evolutionary insights on the interactions of the *Holosporales* (*Alphaproteobacteria*) with eukaryotic hosts from comparative genomics. *Environ. Microbiol.* **26**, e16562.

99. Kondo N, Nikoh N, Ijichi N, Shimada M, Fukatsu T. 2002 Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 14280–14285.

100. Dunning Hotopp JC *et al.* 2007 Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. *Science* **317**, 1753–1756.

101. Wang S, Luo H. 2021 Dating *Alphaproteobacteria* evolution with eukaryotic fossils. *Nat. Commun.* **12**, 3324.

102. Hugoson E, Guliaev A, Ammunét T, Guy L. 2022 Host Adaptation in *Legionellales* Is 1.9 Ga, Coincident with Eukaryogenesis. *Mol. Biol. Evol.* **39**. (doi:10.1093/molbev/msac037)

103. Castelli M, Sassera D, Petroni G. 2016 Biodiversity of 'Non-model' *Rickettsiales* and Their Association with Aquatic Organisms. In *Rickettsiales*, pp. 59–91. Cham: Springer International Publishing.

104. Duron O, Doublet P, Vavre F, Bouchon D. 2018 The Importance of Revisiting *Legionellales* Diversity. *Tr ends in Parasitology*. **34**, 1027–1037. (doi:10.1016/j.pt.2018.09.008)

105. Muñoz-Gómez SA, Hess S, Burger G, Lang BF, Susko E, Slamovits CH, Roger AJ. 2019 An updated phylogeny of the *Alphaproteobacteria* reveals that the parasitic *Rickettsiales* and *Holosporales* have independent origins. *Elife* **8**. (doi:10.7554/eLife.42535)

106. Carrier TJ, Leigh BA, Deaker DJ, Devens HR, Wray GA, Bordenstein SR, Byrne M, Reitzel AM. 2021 Microbiome reduction and endosymbiont gain from a switch in sea urchin life history. *Proc. Natl. Acad. Sci. U. S. A.* **118**. (doi:10.1073/pnas.2022023118)

107. Guidetti R, Vecchi M, Ferrari A, Newton ILG, Cesari M, Rebecchi L. 2020 Further insights in the Tardigrada microbiome: phylogenetic position and prevalence of infection of four new *Alphaproteobacteria* putative endosymbionts. *Zool. J. Linn. Soc.* **188**, 925–937.

108. Szokoli F, Castelli M, Sabaneyeva E, Schrallhammer M, Krenek S, Doak TG, Berendonk TU, Petroni G. 2016 Disentangling the Taxonomy of *Rickettsiales* and Description of Two Novel Symbionts ('*Candidatus* Bealeia paramacronuclearis' and '*Candidatus* Fokinia cryptica') Sharing the Cytoplasm of the Ciliate Protist *Paramecium biaurelia*. *Appl. Environ. Microbiol.* **82**, 7236–7247.

109. Köstlbacher S, Collingro A, Halter T, Schulz F, Jungbluth SP, Horn M. 2021 Pangenomics reveals alternative environmental lifestyles among chlamydiae. *Nat. Commun.* **12**, 4021.

110. Potekhin A, Nekrasova I, Flemming FE. 2021 In shadow of *Holospora* – The continuous quest for new *Holosporaceae* members. *Protistology* (doi:10.21685/1680-0826-2021-15-3-3)

111. Gruber-Vodicka HR, Leisch N, Kleiner M, Hinzke T, Liebeke M, McFall-Ngai M, Hadfield MG, Dubilier N. 2019 Two intracellular and cell type-specific bacterial symbionts in the placozoan *Trichoplax* H2. *Nat Microbiol* **4**, 1465–1474.

112. Dittmer J, Bredon M, Moumen B, Raimond M, Grève P, Bouchon D. 2023 The terrestrial isopod symbiont '*Candidatus* Hepatincola porcellionum' is a potential nutrient scavenger related to *Holosporales* symbionts of protists. *ISME Commun* **3**, 18.

113. Halter T, Köstlbacher S, Collingro A, Sixt BS, Tönshoff ER, Hendrickx F, Kostanjšek R, Horn M. 2022 Ecology and evolution of chlamydial symbionts of arthropods. *ISME Commun.* **2**. (doi:10.1038/s43705-022-00124-5)

114. Galindo LJ, Torruella G, Moreira D, Eglit Y, Simpson AGB, Völcker E, Clauß S, López-García P. 2019 Combined cultivation and single-cell approaches to the phylogenomics of nucleariid amoebae, close relatives of fungi. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **374**, 20190094.

115. Chauhan D, Shames SR. 2021 Pathogenicity and Virulence of *Legionella*: Intracellular replication and host response. *Virulence* **12**, 1122–1144.

116. Renvoisé A, Merhej V, Georgiades K, Raoult D. 2011 Intracellular *Rickettsiales*: Insights into manipulators of eukaryotic cells. *Trends Mol. Med.* **17**, 573–583.

117. Elwell C, Mirrashidi K, Engel J. 2016 *Chlamydia* cell biology and pathogenesis. *Nat. Rev. Microbiol.* **14** , 385–400.

118. van Schaik EJ, Chen C, Mertens K, Weber MM, Samuel JE. 2013 Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*. *Nat. Rev. Microbiol.* **11**, 561–573.

119. Dharamshi JE, Tamarit D, Eme L, Stairs CW, Martijn J, Homa F, Jørgensen SL, Spang A, Ettema TJG. 2020 Marine Sediments Illuminate *Chlamydiae* Diversity and Evolution. *Curr. Biol.* **30**, 1032–1048.e7.

120. Schön ME, Martijn J, Vosseberg J, Köstlbacher S, Ettema TJG. 2022 The evolutionary origin of host association in the *Rickettsiales*. *Nat Microbiol* **7**, 1189–1199.

121. Singh S, Eldin C, Kowalczewska M, Raoult D. 2013 Axenic culture of fastidious and intracellular bacteria. *Trends Microbiol.* **21**, 92–99.

122. Rizzoli A *et al.* 2014 *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: New hazards and relevance for public health. *Front. Public Health* **2**, 251.

123. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA. 2010 The natural history of *Anaplasma marginale*. *Vet. Parasitol.* **167**, 95–107.

124. Huigens ME, de Almeida RP, Boons PAH, Luck RF, Stouthamer R. 2004 Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc. Biol. Sci.* **271**, 509–515.

125. Dantas-Torres F, Chomel BB, Otranto D. 2012 Ticks and tick-borne diseases: a One Health perspective. *Trends Parasitol.* **28**, 437–446.

126. Modeo L *et al.* 2020 "*Ca.* Trichorickettsia mobilis", a *Rickettsiales* bacterium, can be transiently transferred from the unicellular eukaryote *Paramecium* to the planarian *Dugesia japonica*. *PeerJ* **8**, e8977.

127. Gillespie JJ, Kaur SJ, Rahman MS, Rennoll-Bankert K, Sears KT, Beier-Sexton M, Azad AF. 2015 Secretome of obligate intracellular *Rickettsia*. *FEMS Microbiol. Rev.* **39**, 47–80.

128. Meir A, Macé K, Lukoyanova N, Chetrit D, Hospenthal MK, Redzej A, Roy C, Waksman G. 2020 Mechanism of effector capture and delivery by the type IV secretion system from *Legionella pneumophila*. *Nat. Commun.* **11**, 2864.

129. Betts-Hampikian HJ, Fields KA. 2010 The Chlamydial Type III Secretion Mechanism: Revealing Cracks in a Tough Nut. *Front. Microbiol.* **1**, 114.

130. Merhej V, Royer-Carenzi M, Pontarotti P, Raoult D. 2009 Massive comparative genomic analysis reveals convergent evolution of specialized bacteria. *Biol. Direct* **4**, 13.

131. Gillespie JJ *et al.* 2016 The *Rickettsia* type IV secretion system: unrealized complexity mired by gene family expansion. *Pathog. Dis.* **74**. (doi:10.1093/femspd/ftw058)

132. Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T. 2010 Wolbachia as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 769–774.

133. Jaenike J, Stahlhut JK, Boelio LM, Unckless RL. 2010 Association between *Wolbachia* and *Spiroplasma* within *Drosophila neotestacea*: an emerging symbiotic mutualism? *Mol. Ecol.* **19**, 414–425.

134. Mahmood S, Nováková E, Martinů J, Sychra O, Hypša V. 2023 Supergroup F *Wolbachia* with extremely reduced genome: transition to obligate insect symbionts. *Microbiome* **11**, 22.

135. Dedeine F, Vavre F, Fleury F, Loppin B, Hochberg ME, Bouletreau M. 2001 Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6247–6252.

136. Kremer N, Voronin D, Charif D, Mavingui P, Mollereau B, Vavre F. 2009 *Wolbachia* interferes with ferritin expression and iron metabolism in insects. *PLoS Pathog.* **5**, e1000630.

137. Rodríguez-Beltrán J, DelaFuente J, León-Sampedro R, MacLean RC, San Millán Á. 2021 Beyond horizontal gene transfer: the role of plasmids in bacterial evolution. *Nat. Rev. Microbiol.* **19**, 347–359.

138. Rankin DJ, Turner LA, Heinemann JA, Brown SP. 2012 The coevolution of toxin and antitoxin genes drives the dynamics of bacterial addiction complexes and intragenomic conflict. *Proc. Biol. Sci.* **279**, 3706–3715.

139. Miyake A. 1974 Cell interaction in conjugation of ciliates. *Curr. Top. Microbiol. Immunol.* **64**, 49–77.

140. Wedell N. 2020 Selfish genes and sexual selection: the impact of genomic parasites on host reproduction. *J. Zool.* **311**, 1–12.

141. Barker J, Brown MR. 1994 Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiology* **140 (Pt 6)**, 1253–1259.

142. Flowers JM, Li SI, Stathos A, Saxer G, Ostrowski EA, Queller DC, Strassmann JE, Purugganan MD. 2010 Variation, sex, and social cooperation: molecular population genetics of the social amoeba *Dictyostelium discoideum*. *PLoS Genet.* **6**, e1001013.

143. Mather RV, Larsen TJ, Brock DA, Queller DC, Strassmann JE. 2023 symbionts isolated from induce bacterial carriage in other species. *Proc. Biol. Sci.* **290**, 20230977.

144. Noh S, Peck RF, Larson ER, Covitz RM, Chen A, Roy P, Hamilton MC, Dettmann RA. 2024 Facultative symbiont virulence determines horizontal transmission rate without host specificity in *Dictyostelium discoideum* social amoebas. *Evol Lett* **8**, 437–447.