

Sensing in our sisters: how choanoflagellates sense and respond to their complex environment

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Introduction

The choanoflagellates are a group of widely dispersed aquatic protists. Choanoflagellates feed on bacteria and are in turn predated on by plankton feeders, making them an important link in marine ecosystems (Leadbeater, 2015). Choanoflagellates are a group of over 300 species, found in nearly all bodies of water, and all species share a basic distinctive morphology characterised by an apical collar complex (Leadbeater, 2015; WoRMS, 2023) (fig. 1a). Choanoflagellates are of particular interest for research into the evolution of animals, since they are a sister group to animals. Choanoflagellate cells bear a striking resemblance to choanocyte cells found in sponges, one of the simplest animals. This similarity has sparked discussions about the relationship between choanoflagellates and animals ever since the first observations by James-Clark in 1866 (James-Clark, 1867). Modern phylogenetic methods have since confirmed that choanoflagellates are indeed the closest relatives to animals (Carr, 2008; Ruiz-Trillio, 2008).

Choanoflagellate morphology is characterised by a collar complex consisting of a single flagellum and a collar, used both for prey capture and motility. Although choanoflagellate species share this basic layout, they can have very different lifestyles. Some species are completely sessile, while others have complex life cycles that include various cell types, both free-living and colony forming, as well as sexual reproduction (Leadbeater, 2015; Levin & King, 2013). Interactions with bacteria have also been identified (Alegado, 2012). An important aspect of choanoflagellate biology is how they sense, respond to and interact with their environment. Choanoflagellates and other marine microorganisms encounter a whole range of environmental stimuli, including prey and predators, dissolved gases, pH gradients, heat and light (Stocker & Seymour, 2012). Research has revealed an increasing number of environmental cues that influence aspects of choanoflagellate behaviour. Although molecular mechanisms are often unknown, conserved pathways found in animals such as nitric oxide signalling have been shown to exist in choanoflagellates as well (Reyes-Rivera, 2022).

Understanding choanoflagellate biology and identifying similarities and differences to animals is key in elucidating how animals evolved. Many features that define animal life, such as multicellularity, sexual reproduction and heterotrophy have now also been identified in different choanoflagellates, raising the question if these could have been present in the ancestor of both choanoflagellates and animals. Some aspects of environmental sensing and interactions in pre-metazoan organisms that have been inherited in animals might have gained new functions, and certain specific environmental factors might have driven the transition to multicellularity and cellular differentiation

In this review, I aim to summarise key aspects of choanoflagellate morphology and behaviour and how these are affected by environmental stimuli.

Choanoflagellate motility and morphology

Choanoflagellate species share a basic morphology

All choanoflagellate species share a similar and distinctive morphology (Leadbeater, 2015). Choanoflagellate cells are highly polarised (fig. 1b), with on the apical side a collar complex consisting of a collar and a single flagellum. This flagellum has a dual function, in feeding and swimming. The beating of the flagellum both facilitates movement and generates a small current which draws in bacterial prey. The collar, consisting of microvilli, surrounds the flagellum, enabling choanoflagellates to capture and ingest prey. The Golgi apparatus is also localised at the apical side. On the basal side, cells have food vacuoles and filopodia (Carr, 2008; Leadbeater, 2015).

This basic cell layout is conserved within choanoflagellates, although some morphological differences can be noted. An important distinction can be made between loricates (*Acanthoecida*) and non-loricates (*Craspedida*) based on the presence of a lorica, an inorganic “basket-like” extracellular structure. Instead of a lorica, non-loricates can form an organic extracellular covering called a theca (Leadbeater, 2015). The majority of research in choanoflagellates is carried out in non-loricata species, in particular in model species *Salpingoeca rosetta* and *Monosigna brevicollis* (Hoffmeyer & Burkhardt, 2016) (fig. 1c-d).

The morphological similarities of choanoflagellates to sponge choanocytes have been well described, but the significance ascribed to these similarities varies. Some have interpreted this as evidence that the first animal cells must have shared a comparable morphology with choanocytes and choanoflagellates (Nielsen, 2008). It has also been suggested that sponge choanocytes and choanoflagellates merely represent similar, specialised filter-feeding cell types, and are not indicative of what an ancestral animal cell looked like (Maldonado, 2005). Interestingly, genomic analysis of different cell types in the sponge *Amphimedon queenslandica* revealed that not choanocytes but archaeocytes express relatively the most pre-metazoan genes, indicating that choanocytes might not reflect an ancestral animal cell that accurately. Archaeocytes function similarly to stem cells and have the capacity to quickly differentiate into different cell types, including choanocytes (Sogabe, 2019).

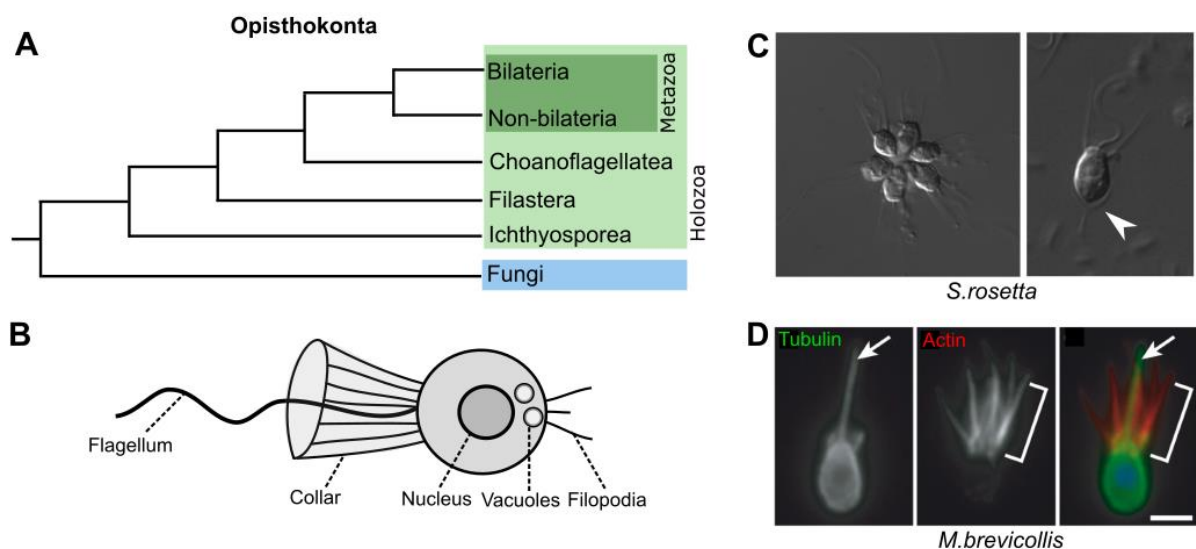


Figure 1. A. Phylogeny of the Opisthokonta group, which includes fungi, metazoa, choanoflagellates, filastera and ichthyospores. Based on analysis by Torruella *et al.* (2015). B. Overview of choanoflagellate morphology. C. *S. rosetta* rosette colony (left) and thecate cell type (right). The theca is indicated with an arrowhead (Adapted from Dayel, 2011). D. *M. brevicollis* cell, stained for tubulin (green) and actin (red) (Adapted from King *et al.*, 2008)

Motility and feeding

Choanoflagellates can be free-swimming as well as sessile, through attachment to a substrate. Different choanoflagellate species vary in their motility, with some species being attached most of the time and only utilising a free-swimming form for dispersal (Leadbeater, 2015). Swimming of choanoflagellate cells is facilitated by beating of its flagellum. The broad mechanism of flagella undulation in choanoflagellates is shared with other eukaryotes, including animal flagella (sperm cells) and cilia (e.g. epithelial cells). With little variation, eukaryotic flagella consist of 9 radially arranged doublets of microtubules and 2 central doublets (9 + 2 axoneme). Dynein motor proteins slide on the microtubules, applying force to them resulting in bending. Coordinated bending of microtubules leads to beating of the flagellum and displacement of water, allowing the cell to move (Mitchell, 2007; Pinsky, 2022).

The beating of flagella in choanoflagellates and the associated water current have been the subject of various modelling studies. Nguyen (2019) found that cell shape, flagella length and collar size all affect swimming performance. Increasing collar size negatively impacts swimming speed, probably due to cells experiencing more drag. A small collar, however, reduces prey capture (Nguyen, 2019). Choanoflagellate morphology thus seems to require a trade-off between efficient swimming and efficient feeding.

Some choanoflagellates have a life cycle that includes cells of different morphologies. The colony forming species *S. rosetta* has a life cycle of at least five distinct cell types (Dayel, 2011). Based on different conditions, cells differentiate into swimmers, thecate cells or colonial cells. Swimmers are characterised by a small collar, while thecate cells have a larger collar and are attached through a theca. This allows *S. rosetta* to emphasize either swimming or feeding, depending on what fits the environment (Kirkegaard & Goldstein, 2016). These different cell types can be cultivated in the lab and provide valuable insights in *S. rosetta* response to environmental conditions.

Colony formation

Choanoflagellates of diverse branches have shown the ability to form colonies. Different colony shapes have been described, including linear colonies, branched colonies and radially arranged rosette colonies (Fairclough, 2010;). Similar to animal development from a zygote, colony formation in choanoflagellates is currently thought to only arise through cell division, rather than aggregation of cells. However, no evidence of synchronised divisions in choanoflagellate colonies has been found, which is a characteristic of animal embryos (Larson, 2020). Although all cells in a colony are clones, some disparity between cells in colonies has been described. *S. rosetta* rosette colonies include cells of various shapes but whether such cells could have different functions is unknown (Naumann & Burkhardt, 2019; Laundon, 2019). The potential of some differentiation in multicellular choanoflagellates makes them an interesting model system to study the relationship between multicellularity and differentiation.

In addition to colony shape, the manner of attachment between cells also varies between species. In *Choanoeca perplexa*, neighbouring cells are held together by connections in the microvilli that shape the collar (Leadbeater, 1983). Intracellular bridges between cells, arising through incomplete abscission after division, have been identified in multiple species (Laundon, 2019). In *S. rosetta*, rosette colonies are connected by a secreted extracellular matrix (ECM) that fills the gap between cells (Larson, 2020).

The process of colony formation has only been studied in detail in *S. rosetta*. Interestingly, genes identified to be essential for rosette formation are all related to ECM regulation (Levin, 2014; Wetzel,

2018). *S. rosetta* encodes a C-type lectin, rosetteless, that is necessary for rosette formation (Levin, 2014). Biophysical studies of ECM formation in rosette colonies showed that it is the main contributor to colony stability, rather than intracellular bridges or cell-cell adhesion, and ECM properties such as stiffness were also shown to influence colony shape and packing (Larson, 2020).

Interactions with bacteria

Bacterial interactions shape the biology of eukaryotes

As prominent bacterivores, choanoflagellates have a predator-prey relationship with bacteria. Drawn in by a water current generated through beating of the flagellum, bacteria are captured on the collar surface and ingested (Leadbeater, 2015). Phagocytosis is initiated by pseudopodia that arise from the collar and target captured prey, a process that is poorly understood. Feeding through use of pseudopodia, however, is found in sponges choanocyte cells as well, so potential mechanisms could be conserved (Leys & Eerkes-Medrano, 2005). Choanoflagellates do not show a high degree of selectivity when filter feeding, as evidenced by their uptake of various particles that land on their collars including microspheres of different sizes and yeast (Dayel, 2014; Leadbeater, 2015). However, experiments in *Salpingoeca amphoridium* that were fed coated microspheres showed that this choanoflagellate preferred hydrophobic particles to hydrophilic particles for capture and ingestion (Pettitt, 2002). It is possible that choanoflagellates can specifically sense and selectively ingest prey, but the mechanisms behind this are not understood.

Interactions between eukaryotes and bacteria often extend beyond predator-prey relationships. Bacterial associations widely vary in function (nutrient uptake, defence, metabolism), character (mutualism-parasitism, obligate non-obligate) and intimacy (ecto- and endosymbiosis). Most animals have a microbiome, a selection of associated microorganisms on which they rely for processes such as efficient nutrient uptake or development (Fraune & Bosch, 2010; Bosch & McFall-Ngai, 2021). A relevant example of developmental symbiosis is the sponge *Amphimedon queenslandica* (Song, 2021). During its larval stage, *A. queenslandica* requires bacterially produced arginine for its production of nitric oxide (NO). In turn, NO signalling plays a key role in the initiation of settlement and morphogenesis of the larvae. Although less widely studied than microbial interactions in animals and plants, nearly all supergroups of protists show evidence of symbiosis with prokaryotes (Husnik, 2021). Since bacterial symbiosis is so widespread in protists and also found in animals, it is very likely that the ancestor of animals also had such interactions. As the closest relatives to animals, identification of interactions between choanoflagellates and bacteria are therefore highly relevant.

Bacterial symbionts have not been widely reported in choanoflagellates, which possibly reflects the challenge of preserving such interactions during sampling. Nonetheless, two instances of endosymbiosis have been noted in choanoflagellates. Wylezich (2012) reported the presence of uncharacterised endosymbiotic Gram-negative bacteria in the cytoplasm of *Codosiga balthica*, a choanoflagellate collected from hypoxic water in the Baltic sea (Wylezich, 2012). Although the identity and function of this endosymbiont remained a mystery, it might support anaerobic metabolism in oxygen-depleted water. Culturing *C. balthica* for several years did not lead to loss of the bacteria, suggesting it is an obligate symbiont. Evidence of obligate symbiosis was also found in *Bicosta minor*, a highly abundant loricate choanoflagellate (Needham, 2022). The endosymbiont was revealed to be a novel bacterial species, *Candidatus Comchoanobacterales*, related to the human pathogen *Coxiella*. Its genome showed a marked reduction of genes related to metabolic pathways, which is a characteristic of obligate symbiosis. From analysis of the genome of

C.comchoanobacterales it is not directly clear whether this symbiosis is beneficial to *B.minor* and could represent an adaptation to harsh environments, like in *C.balthica*.

Hake (2021) report the isolation of a novel colonial choanoflagellate species, *Barroeca monosierra*, that has a microbiome. *B.monosierra* forms large spherical colonies and live bacteria were shown to occupy the lumen of these colonies, often associating with the ECM. The microbiome of *B.monosierra* includes diverse bacteria, with Gammaproteobacteria being found in nearly all colonies. This finding shows that stable associations between choanoflagellates and bacteria exist, aside from endosymbiosis. Whether *B.monosierra* and its microbiome exchange nutrients or metabolites, and to what extent *B.monosierra* needs these bacterial interactions for survival remains unclear currently (Hake, 2021).

Secreted bacterial factors influence *S.rosetta* colony formation

Although there is thus little evidence to suggest bacterial symbiosis is widespread in choanoflagellates, it has become clear in recent years that bacterial secreted factors regulate an increasing number of known aspects of choanoflagellate behaviour. Alegado (2012) first observed that rosette colony formation in cultured *S.rosetta* is dependent on the presence of prey bacterium *Algoriphagus machipongensis* (Alegado 2012). Various bacterial species were evaluated and several members of the Bacteroides phylum were found to induce colony formation in *S.rosetta*, including nearly all *Algoriphagus* species and several other species such as *Cyclobacterium marinum*. A sulfonolipid from *A.machipongensis* was isolated that had the capacity to induce colony formation, termed Rosette Inducing Factor 1 (RIF-1). Since then, multiple factors have been identified that influence colony formation in *S.rosetta*. A further sulfonolipid (RIF-2) and two lysophosphatidylethanolamines (LPE) were added to the list of bacterial compounds that modulate rosette formation (Woznica, 2016). Bacterial LPE's by themselves do not induce rosette formation but work synergistically with RIFs to form stable, tightly packed colonies. Colonies formed in the absence of LPE's are disorganised and more vulnerable to shear forces, suggesting that *S.rosetta* requires RIFs to initially induce colony formation and LPE's for maturation of rosettes (Woznica, 2016). Intriguingly, RIF-like sulfonolipids from other bacteria, including colony inducing *C.marinum*, did not have a rosette-inducing effect, suggesting the existence of additional secreted colony inducing factors (Leichnitz, 2022).

Interestingly, *A.machipongensis* also was shown to secrete an inhibitor of colony formation (IOR-1) (Woznica, 2016). The inhibitory effect of IOR-1 was found to be counteracted by a combination of RIFs and LPEs, explaining how addition of live bacteria manages to induce colony formation. In addition to IOR-1, abundant membrane components sulfobacin D and F were also found to be inhibitors of colony formation, probably due to competition for the same cellular target as RIF (Leichnitz, 2022). The nature of this target and the mechanism of action are, however, unknown. Modification of the ECM seems like a promising target, since this is the main factor contributing to colony stability and cultures lacking LPE's or essential ECM modifiers both show a similar phenotype of vulnerability to shear forces (Larson, 2020; Woznica, 2016).

Secreted bacterial factors induce mating

Sexual reproduction is found throughout all eukaryotic lineages and is therefore thought to have been present in the last eukaryotic common ancestor. For many protists however, a sexual cycle has never been demonstrated. The first evidence pointing to sexual replication in choanoflagellates was noted by Carr (2010), who found that *Monosigna brevicollis* has several genes that are known to function in meiosis. Of 19 "meiosis toolkit detection" genes *M.brevicollis* was shown to possess 18,

including topoisomerase *spo11* and meiosis-specific cohesion *rec8*, more than the number found in the worm *C.elegans* and fly *D.melanogaster* which reproduce sexually exclusively (Carr, 2010, Schurko & Logsdon, 2008). Direct confirmation of sexual reproduction in choanoflagellates was found by Levin & King (2013), who demonstrated that starvation induces mating in *S.rosetta* cultures. Cultures grown on high nutrient medium produced haploid cells, that transitioned to diploid cells once transferred to low nutrient medium for a period of time. This shift from haploidy to diploidy occurred along with observations of cell fusion and heterozygous inheritance of polymorphism, indicating that *S.rosetta* has a sexual life cycle (Levin & King, 2013). However, pairing events were only observed in a very small subset of cells, perhaps due to limited contact between potential partners. Such a switch to sexual reproduction under limited nutrient conditions has been reported previously, most notably in fission yeast. It is thought to represent a protective strategy against accumulation of DNA mutations. Conditions of stress (such as starvation) can lead to an increase in mutations, and sexual reproduction can be a strategy to reduce adverse effects of these mutations in offspring (Wright, 2004; Bernstein & Johns, 1989). In the fission yeast *Schizosaccharomyces pombe*, decreased intracellular concentration of cyclic adenosine monophosphate (cAMP) in response to nutrient availability can trigger sexual reproduction (Mochizuki & Yamamoto, 1992). How choanoflagellates might sense nutrient availability and whether a similar mechanism involving second messenger compounds might trigger the production of haploid cells is unknown.

Sexual reproduction can also be induced through sensing of pheromones. Pheromones can be diverse compounds and originate from sexual partners as well as the environment (Karlson & Lüscher, 1959). Release of pheromones represents a way for organisms to coordinate release of gametes or induce swarming, to enhance the chance of mating (Levitan, 2004). Pheromones originating from the environment can also inform organisms of best conditions for mating, such as high nutrient availability. Woznica (2017) found that pheromones also play a role in the sexual cycle of *S.rosetta*. Exposure of *S.rosetta* cultures to *Vibro fischeri* bacteria induced swarming of large numbers of cells within half an hour. In these swarms, fusion of cells was observed and clear evidence for transfer of genetic material and genetic recombination was found. The active compound was found to be a chondroitin lyase, termed EroS, and the enzymatic function of EroS is crucial for its induction of mating. The target of EroS in choanoflagellates is chondroitin sulfate, an ECM component. Chondroitin lyases from other bacteria were also found to be capable of inducing swarming (Woznica, 2017). Since nothing is known about how *S.rosetta* mating is triggered in its habitat, whether choanoflagellates can also induce mating by themselves is unknown. Comparison of starvation-induced mating and EroS-induced mating reveals major differences, which could suggest that these are different processes. While EroS induces swarming and mating in a manner of minutes, *S.rosetta* colonies were starved for 11 days before mating was observed (Levin & King, 2013; Woznica, 2017). Under starvation only a small subset of cells started mating and no swarming was observed, while in EroS-induced mating a large number of cells aggregated. It is also noteworthy that the fusion events observed in nutrient-limited cultures only showed mating between a larger ovoid cell and a smaller round cell, distinctly resembling female and male gametes. In EroS-induced mating, cells appeared of roughly similar shape and size. Understanding how meiosis can be triggered in *S.rosetta* could perhaps help explain these differences and whether *S.rosetta* mostly relies on bacterial factors for its sexual reproduction.

Interestingly, exposure of *S.rosetta* colonies to cues for both colony formation (RIF) and mating (EroS) led to production of more colonies, that are also larger and contain more cells (Ireland, Woznica & King, 2020). Cultures treated with EroS and RIF required a lower concentration of RIF compared to cultures treated only with RIF in order to start colony formation, indicating that EroS enhances the effect of RIF. A possible explanation is that the chondroitinase activity of EroS, degraded components

of the ECM which leads to a more efficient delivery of RIF into choanoflagellate cells. The chondroitinase activity could also loosen the constraints of the ECM around rosettes, possibly allowing for larger colonies that incorporate more cells.

Based on the findings in *S.rosetta*, it is clear that choanoflagellates and bacteria have a more complex relationship than simply prey and predator. In order to assess whether bacteria shape choanoflagellate behaviour in species other than *S.rosetta*, the microbiome of choanoflagellate species needs to be accurately determined. This is challenging due to sampling, and even in *S.rosetta* claims about bacterial interactions in its natural habitat remain uncertain because *S.rosetta* has only been isolated once (Booth & King, 2022). Aside from the currently unknown targets of bacterial secreted factors, it is also unclear how bacterial factors reach choanoflagellates. Are choanoflagellates very sensitive to such dilute signals, or are relatively stable interactions between bacteria and choanoflagellates necessary?

Lastly, the ecological function of these interactions remains uncertain. It is possible that choanoflagellates can integrate secreted bacterial cues to determine conditions favourable to mating or colony formation. Research has indicated that *S.rosetta* colonies might feed more efficiently than single cells, so if a single cell encounters a high concentration of bacteria it might be beneficial to form a colony (L'Etoile & King-Smith, 2019). However, secreted bacterial factors might also represent a strategy on part of the bacteria to modulate choanoflagellate behaviour and reduce feeding efficiency.

The finding that bacterial factors induce such major behavioural shifts in a choanoflagellate has led to speculation that bacterial interactions could have driven multicellular animal development (Alegado & King, 2014). It will have to become clear whether a closely associated microbiome that modulates behaviour is a feature of choanoflagellates or whether this is more specific to certain species.

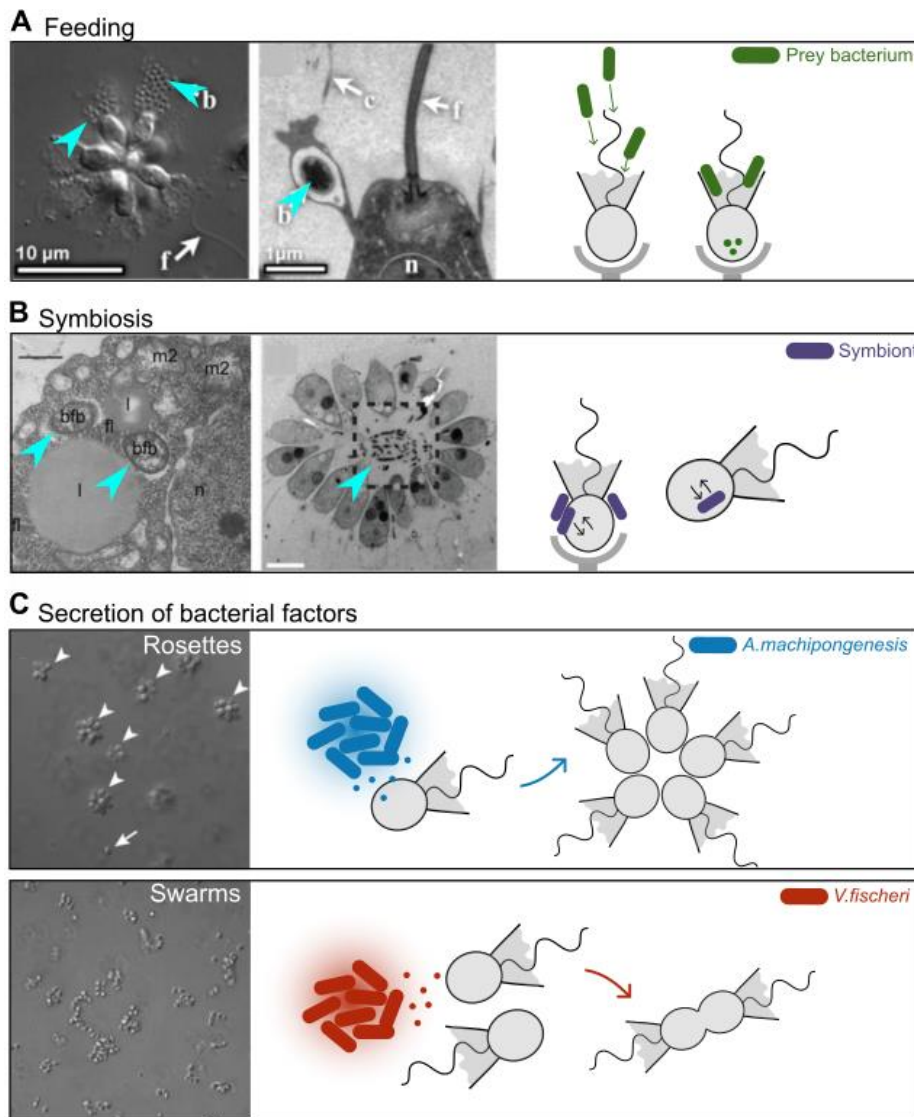


Fig. 2. Bacterial interactions Overview of different interactions between bacteria and choanoflagellates. Cyan arrowheads indicate bacteria. **A.** Sessile and free-swimming choanoflagellates feed on bacteria by capturing them on their collar. Adapted from Dayel & King, 2014. **B.** Endo- and ectosymbiosis of choanoflagellates and bacteria. Adapted from Welzch *et al.*, 2012 and Hake *et al.*, 2021. **C.** Bacterial factors RIF and LPE induce colony formation in *S. rosetta* while EroS induces swarming and mating. Adapted from Alegado *et al.*, 2012 and Woznica *et al.*, 2017.

Sensing of chemical stimuli

The ability to sense beneficial as well as harmful environmental conditions is an important feature of most living forms. Organisms can respond to a wide range of chemical cues such as pH, toxins and dissolved gases. The response to gradients of chemical attractants and deterrents, chemotaxis, has been described in many organisms, both unicellular and multicellular (Wadhams & Armitage, 2004; . Multicellular eukaryotes can show tactic behaviour as a whole organism (e.g. slime molds) and in mobile cell types (e.g. animal sperm cells). Chemotaxis in unicellular eukaryotes is less widely studied, but the amoeba *Dictyostelium discoideum* is a notable exception. *D. discoideum* has been shown to have tactic responses to oxygen (aerotaxis), lysophosphatidic acid, cAMP, folic acid as well as light (phototaxis) and electric fields (galvanotaxis) (Hirose, 2021; Jalink, 1992; Wang, 1988; Hong, 1981; Li, 2018). The life cycle of *D. discoideum* includes both a unicellular and a multicellular stage

and a tactic response to cAMP has been shown to trigger the transition from unicellular amoeboid cells to a multicellular organism.

pH-sensing in choanoflagellates

Chemotaxis is deployed by various organisms as a way to identify nutrients. It is unclear which strategies choanoflagellates use to find patches of bacteria and nutrients in their natural habitats. The potential of a chemotactic response in choanoflagellates to bacteria or bacterial metabolites has thus far only been evaluated in *S. rosetta*. Miño (2017) found that *S. rosetta* does not have a tactic response to live prey bacteria or bacterial medium, but responds to areas of low pH instead. From a range of tested conditions, *S. rosetta* selectively migrated into areas with an intermediate low pH (pH 6-7), which is markedly lower than average pH in ocean water (pH 8.18) (Miño, 2017). Moreover, only fast swimmer cell types showed pH-taxis, while slow swimmers and colonies did not respond to pH gradients. Although it is not directly clear why unicellular *S. rosetta* preferentially migrates into areas of low pH, pH-taxis could be related to nutrient identification. Areas of relatively low pH can be indicative of high bacterial presence, since bacterial metabolites can acidify water and bacterial biofilms are often found to be of a lower pH than surrounding areas (Solé, 2000; Liermann, 2000). The fast swimmer *S. rosetta* cell type is often regarded to be a dispersal form, which would fit with the ability to scope out areas high in nutrients. Possibly, identification of beneficial nutritional environment by fast swimmer through pH-taxis is coupled with differentiation into a cell type more suited to efficient feeding, such as a colony or a thecate cell (L'Etoile & King-Smith, 2019).

Whether pH sensing by choanoflagellates is indeed a method of sensing bacteria and whether this method is applied by other bacterivores is unclear. The only other known example of pH-taxis in protists concerns the trypanosome *Trypanosoma brucei* (Shaw, 2022). As a parasite, *T. brucei* has been shown to use pH-taxis for navigation and coordinated motility inside host organisms. Signalling of cAMP was shown to be crucial for detection of pH gradients. How choanoflagellates might sense pH gradients, and whether cAMP signalling could play a role in its tactic response remains to be investigated.

Oxygen sensing in choanoflagellates

In addition to pH gradients, choanoflagellates have also been observed to respond to oxygen gradients (Fig. 3). Aerotaxis is found in many marine organisms, including different bacteria and algae (Hirose, 2021; Yu, 2002). Although species have been identified that prefer hypoxic environments, choanoflagellates show evidence for mostly aerobic respiration (Wylezich, 2012; Leadbeater, 2015). Locating areas of sufficient oxygen is therefore important for their survival. Kirkegaard (2016) shows that *S. rosetta* preferentially migrates towards areas of higher oxygen concentration. In contrast to pH-taxis, both *S. rosetta* single cells as well as colonies show a tactic response to oxygen gradient. Sensing of oxygen in animals occurs through the hypoxia-inducible factor (HIF) transcription factor pathway, which does not seem to be conserved in choanoflagellates so it seems likely that *S. rosetta* senses oxygen gradients through another mechanism (Rytkönen, 2011). It is possible that the ancestor of animals was also aerotactic, and rising oxygen levels have even been implicated in the origin of animal multicellularity (Kirkegaard, 2016).

The observation that colonies also migrate towards oxygen raised the question how tactic behaviour is organised in choanoflagellates. Studies on colony motility have shown that there is no coordinated beating of flagella in *S. rosetta* colonies, ruling out that colonies can actively steer and navigate towards attractants. An alternative mode of taxis is "run and tumble", a form of stochastic taxis whereby periods of swimming in a straight line (run) are alternated with a random change of

direction (tumble). The frequency of changes in direction is reduced as an organism gets closer to the attractant. This can be achieved by modulation of speed, where speed is increased as organism gets closer, or by modulation of direction, where the angle of direction change is reduced. Kirkegaard found that *S. rosetta* does not modulate its speed during taxis, but rather fits a model of modulation of direction. In addition, taxis in *S. rosetta* fits a model of logarithmic sensing best. In logarithmic sensing, the response of an organism increases logarithmically with the strength of the stimulus, rather than linearly (Kirkegaard, 2016). This helps explain how choanoflagellates can respond to small changes in concentrations, possibly to secreted bacterial factors as well.

Aerotaxis and pH-taxis are only two examples of chemical stimuli, so it is possible that choanoflagellates are sensitive to other chemical gradients. Perhaps choanoflagellates also secrete factors themselves that can be picked up by potential mates. The molecular mechanisms of sensing and response to chemical stimuli will have to be elucidated. Choanoflagellates do possess known receptors such as G-protein coupled receptors (GPCR), which could potentially play a role (de Mendoza, 2014).

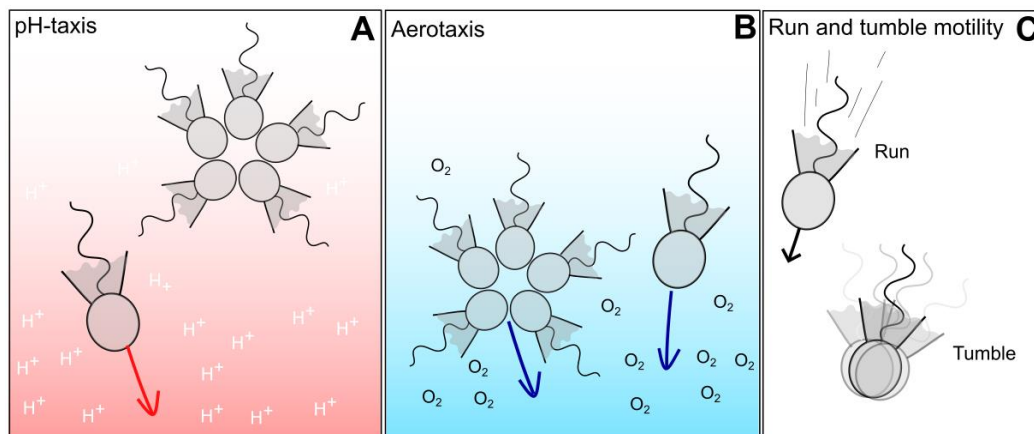


Figure 3. Chemotaxis in choanoflagellates. A. B. Directed movement of *S. rosetta* cells towards areas of low pH and high oxygen concentration. **C.** Schematic of run and tumble motility.

Sensing of light

A light to dark transition causes whole colony inversion

Many marine organisms have the ability to sense light and move to areas with optimal light exposure. Phototactic behaviour is widespread in eukaryotes, including cyanobacteria, sponges and dinoflagellates (Jékely, 2009). Although phototactic behaviour has not been demonstrated in choanoflagellates, Brunet (2019) found that the colony forming *Choanoeca flexa* has a striking response to light-dark transitions. Upon exposure to light, colonies adapt an flagella-in conformation which is inverted rapidly in the dark, leading to a flagella-out conformation (Fig. 4a). This light sensing response was shown to be mediated by a rhodopsin-phosphodiesterase receptor, which can convert light stimuli into a chemical signal through degradation of cyclic nucleotides (cGMP and cAMP). In *C. flexa*, cGMP, not cAMP, triggers the inversion response. Interestingly, since *C. flexa* lacks components of the rhodopsin pathway, it needs to obtain carotenoids from its diet in order to perform colony inversion (Brunet, 2019). When cultured with bacteria that do not synthesise carotenoids, *C. flexa* does not show an inversion response during light-dark transitions.

Inversions of colonies has an effect on the swimming efficiency of *C. flexa*. Flagella-in colonies have a relatively large surface area and are slow swimmers but efficient feeders. Inverted colonies are much smaller in surface area and are more efficient swimmers but poor feeders. Colony inversion thus

seems to be another example of the trade-off between an emphasis on feeding or swimming choanoflagellates can make. Because colony inversion is triggered in the dark, the net effect is a directed movement towards lighter areas. This photokinetic behaviour is different in mechanism from the *S. rosetta* response to oxygen and pH, and cannot be considered a true phototactic response.

The molecular mechanism behind inversion of *C. flexa* colonies relies on the constriction of an apical actomyosin ring at the bottom of the collar. Constriction of this ring effectively opens the collar, increasing the curvature and favouring an flagella-out arrangement of cells. Apart from light-dark transitions, other factors that induce colony inversion have been identified, such as mechanical perturbations and nitric oxide (NO) (Fig. 4a). The NO signalling pathway has been shown to be conserved in choanoflagellates, with several species possessing genes coding for nitric oxide synthase (NOS) and soluble guanylate cyclase (sGC). NO signalling plays an important role in the development of many animals (Ueda, 2016). Similar to rhodopsin-PDE signalling, NO signalling pathway also utilises cGMP as a second messenger. Interestingly, NO signalling does not seem to be the common mechanism behind all instances of colony inversion, since only light-dark induced colony inversion relied on active NO signalling (Reyes-Rivera, 2022).

Collar contractions in individual cells are conserved in various species of choanoflagellates, including *S. rosetta*, *M. brevicollis*, *Salpingoeca urceolata*, and *Diaphanoeca grandis*, representatives of main choanoflagellate lineages (Brunet, 2021). Collective contractions of apical actin rings could only be demonstrated in *C. flexa*, closely related *C. perplexa*, and in animals. In the sponge *Scypha ciliate*, whole embryos transition from a cilia-in to a cilia-out conformation during development (Franzen, 1988). The larvae of the sponge *Neopetrosia proxima* show a rapid transition from cilia-out to cilia-in as a response to increases in light intensity and an opposite response when light intensity is reduced, which was interpreted as a stress response (Collin, 2010). However, since *N. proxima* larvae were also shown to be positively phototactic, this inversion could perhaps also play a role in navigation towards lighter areas.

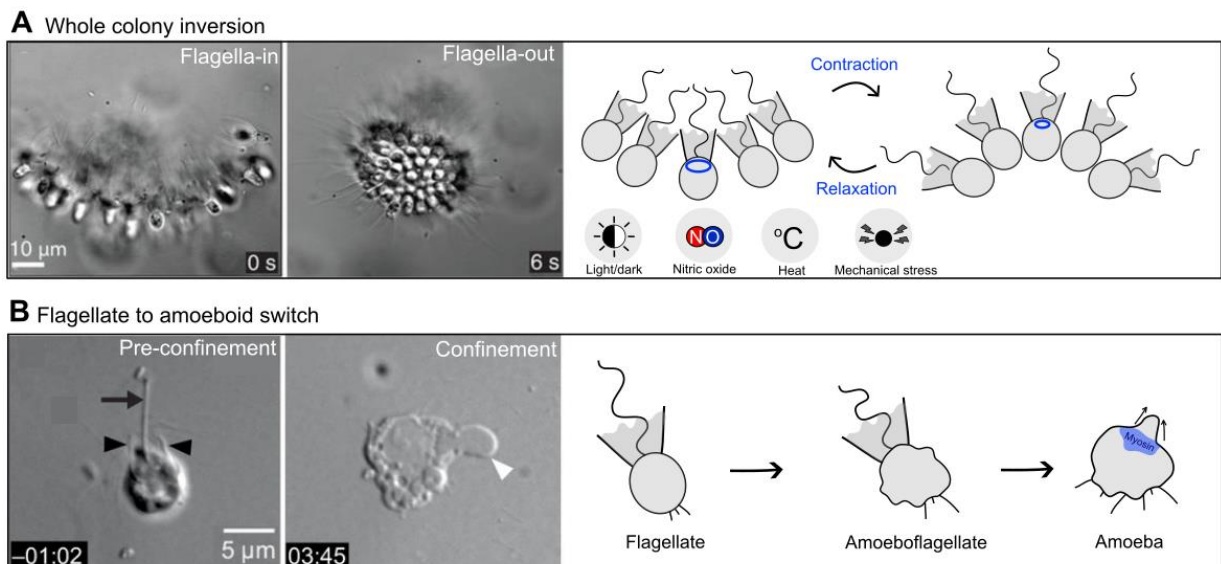


Figure 4. **A.** Colony inversion through collar contractions occurs as a response to various stimuli: light/dark transitions, nitric oxide signalling, heat shock and mechanical perturbations. Adapted from Brunet *et al.*, 2019. **B.** The switch from flagellate motility to amoeboid motility, through an intermediate amoeboflagellate state. Adapted from Brunet *et al.*, 2021.

Sensing of mechanical stimuli

Aside from chemical and light stimuli, the environment of choanoflagellates contains a range of mechanical stimuli including heat, pressure and mechanical perturbations. Similarly to chemical stimuli, responses to mechanical stimuli are reported in many organisms. Mechanical sensing, mediated through mechanoreceptors, allows organisms to avoid harmful environments and underlies the animal senses of touch, movement and hearing (Delmas, 2011).

Response to mechanical perturbation

Choanoflagellates clearly have the ability to sense and respond to mechanical perturbations. Experiments in *C.perplexa* show collar contraction as a response to mechanical stress, induced by flow or a needle (Leadbeater, 1983). Contraction of the collar upon mechanical disturbance in *C.perplexa* has been shown to facilitate cells retreating into theca, which suggests collar contraction is a defensive response to mechanical stimuli (Leadbeater, 1977). A similar collective contraction response to light-dark transitions has been found in *C.flexa* upon gentle agitation of culture flasks and after heat shock (Reyes-Rivera, 2022) (Fig. 4a). In contrast to NO-mediated or light-mediated colony inversion, neither of these responses depend on active NO signalling (Reyes-Rivera, 2022). The mechanisms by which mechanosensing and thermosensing in choanoflagellates lead to defensive collar contraction thus remain to be discovered.

While *S.rosetta* does show collar contraction in individual cells, no collective response to light-dark transitions or mechanical disturbances has been found. *S.rosetta* rosettes are generally resistant to shear forces, but this depends on proper ECM maturation which in turn depends on bacterial factors (Larson, 2020; Woznica, 2016). Since *C.flexa* and *C.perplexa* are the only choanoflagellate species that show collective contraction as a response to mechanical stress, while many species have an individual response, it is possible that these two species possess intercellular signalling pathways that other choanoflagellates lack to coordinate responses.

In animals, an important factor in sensing of mechanical stimuli the cilium, with several receptors involved in sensing of auditory, mechanical, olfactory and light stimuli have been shown to localise to cilia (Johnson & Leroux, 2010). A role for the flagellum in sensing has also been described in bacteria (Harshey, 2016). This raises the possibility that the flagellum might have an additional function in choanoflagellates as a sensor.

Constriction reveals a conserved switch to amoeboid motility

A specific form of mechanical perturbation is constriction, where cells are squeezed between two surfaces and thereby deformed. Brunet (2021) reveals that upon constriction of *S.rosetta*, cells undergo a striking morphological change. Cells were shown to retract their flagellum, develop abundant membrane protrusions and adapt amoeboid, or crawling, motility (Brunet, 2021) (Fig. 4b). The transition from a flagellate to an amoeboid cell type was observed in all *S.rosetta* cell types, including rosettes and thecate cells. Moreover, the amoeboid switch is conserved in various representative choanoflagellate species, including *M.brevicollis* and *C.flexa*. Release from constriction restored the original flagellate morphology, with the flagellum being positioned near the location of the retracted flagellum, indicating that cells retain their polarity.

The membrane protrusions that facilitate amoeboid movement in choanoflagellates depended on F-actin and myosin II but not Arp 2/3, indicating that they are not likely pseudopodia but probably blebs. Bleb formation is mediated by actomyosin contraction in the cortex and blebs are initially empty of F-actin. Upon constriction of choanoflagellates, myosin II was observed localising to the

plasma membrane and F-actin only located to protrusions sometime after their formation. These observations suggest that amoeboid motility in choanoflagellates is indeed mediated by blebs. When only parts of the cell were subjected to confinement, myosin II only locally accumulated on the membrane, indicating that deformation of the cell can be sensed locally.

Crawling motility in cells is found in nearly all animal lineages, some ichthyosporeans and now also in choanoflagellates, making it highly likely that an animal ancestor possessed the ability to crawl (Brunet, 2021). Crawling as a response to confinement and mechanical stress is also found throughout the opisthokonta, indicating that this is a conserved response. For choanoflagellates, the switch to amoeboid crawling possibly enables cells to get out of tight spots, such as when encountered with predators. If external pressure that leads to local deformation is sufficient to trigger a switch to amoeboid motility, the question is raised how this works in colonies. In *S. rosetta* the ECM holds colonies together and exerts force, pushing cells against their neighbours. The forces cells experience in colonies are perhaps too small to trigger such a response or cells might have a way to suppress the amoeboid transition in colonies. Interestingly, there are observations in *S. rosetta* colonies of amoeboid-like cells that are expelled from the colony, suggesting that intra-colony forces could perhaps also trigger the switch to crawling movement (Dayel, 2011).

Conclusions

Recent choanoflagellate research has begun to reveal the diversity in choanoflagellate biology and key similarities and differences to animals. Current research suggest that choanoflagellates have a wide repertoire of responses to the various environmental cues that can be found in their marine habitats. Choanoflagellates have the ability to identify and navigate towards beneficial conditions (pH indicative of bacteria, oxygen) and possibly evade negative conditions (mechanical stress, heat shock), through different modes of motility, and with some behaviours being easier to interpret than others.

The interactions of choanoflagellates and bacteria remain somewhat elusive but are clearly important for the life cycles of at least some choanoflagellate species. Further study will have to reveal how widespread symbiosis between bacteria and choanoflagellates are and what character and function these interactions have. The discovery that specific bacterial factors regulate important behavioural changes in *S. rosetta* raises the possibility that bacterial factors have also been crucial in the transition from a unicellular organism to multicellular animals.

The specific receptors and signalling pathways that mediate how choanoflagellates interact with each other and the environment largely remain to be identified, but there is potential for conserved pathways between choanoflagellates and animals. Further research in choanoflagellates will help reveal which aspects of environmental sensing and response are pre-metazoan, and which specific environmental factors play a role in the transition to multicellular life.

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