# TITLE

*Mappa mundi est non terranum*: The Search for the Role of Aquaporin in a Filamentous Fungi *Neurospora crassa*[1].

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# ABSTRACT

The aquaporin water channel within cell membranes has been heavily researched in the years following its official discovery in 1992. Much of the experimentation has been performed on organisms within the plant and animal kingdoms, with some results indicating that aquaporin can indeed increase cell water permeability under certain conditions. However, many of the results were derived from organisms with multiple aquaporin homologues and with more complex lifestyles. We decided to investigate the effects of aquaporin in *Neurospora crassa*, as it contains an efficient genome with only one aquaporin homologue, and a knockout mutant is available. The goal was to expose *Neurospora* to a range of environmental conditions that were associated with water in some way. Treatment with a hypo-osmotic solution caused the same percentage of lysis of the cell walls in both wildtype and the *aqp* mutant. When grown under controlled humidity, the appearance of hyphae changed as a physiological adaptation, but simple variation in the growth was enough to conceal any differences between wildtype and *aqp*. The viability of conidia after freezing proved to be slightly higher in wildtype and this was attributed to the ability of aquaporin to enhance dehydration of the cell to prevent internal ice crystallization. Germination rates of conidia produced under carefully controlled temperature and relative humidity were also examined. In distilled water, *aqp* mutant conidia exhibited higher germination rates than wildtype. This was unexpected since the aquaporin channel should enhance germination rates of wildtype. The cause of this effect may be strain differences in the physiology of the condiation pathway under controlled conditions of high humidity and elevated carbon dioxide in the closed containers.

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# OBJECTIVE

To determine the phenotype of a model organism, *Neurospora crassa*, when a putative water channel (aquaporin) is absent.

# INTRODUCTION

There is no molecule more indispensable to life than water. Living forms, exemplified by cells, simply cannot exist without the presence of water in their surroundings. As cells are composed mostly of water, it is this aqueous environment that a cell is dependent on for its diverse biological reactions and functions. Similarly, multicellular organisms must use intricate processes to enable water supply to their vital organs and the proper osmoregulation of their cells. So, it is not surprising that much attention is devoted to discoveries that have the potential to provide insight into the methods of water manipulation on the biological scale. The initial discovery of a protein channel capable of conducting water resulted in a Nobel Prize for Peter Agre [Knepper et al., 2004]. Since its discovery, the channel, named aquaporin, has been the subject of intensive investigation.

The aquaporin water channel has been found to be a member of the major intrinsic protein (MIP) family; integral membrane proteins that form specialized channels within the cell membrane [Agre, 2006]. Although, water is known to readily diffuse across a lipid membrane, it is hypothesized that in certain situations, an increase in water permeability is necessary for cell fitness and overall organism survival. Adding to the complexity, different studies have found that aquaporins can transport other small, uncharged molecules besides water [Yu et al., 2006]. Since their initial discovery, the characteristics and roles of aquaporins have steadily increased, as has the discovery of their omnipresence throughout the biological world.

Organisms containing one or more aquaporin genes have been found in all kingdoms of life [Agre et al., 1998]. Interestingly, these assorted genes share very similar homologies and well-conserved amino acid motifs [Borgnia et al., 1999]. This ubiquity suggests that they indeed have some sort of universal significance for the organisms that contain them. Since water has always been the main constitute of the cell, as well as governing the cell's existence, it seems evident that the aquaporin evolved with a set purpose. However, as the complexity of organisms has increased during evolution, so too has the complexity of aquaporin homologues. Many experiments show that aquaporins have a noticeable significance within the higher kingdoms of both plants and animals. It is here that aquaporins play a crucial role in osmoregulation of tissues and organs.

Although it has been established that aquaporins increase the water permeability of cell membranes, this does not imply that all organisms use them in the same manner, nor can

it be inferred that the aquaporin is simply for this task. Many organisms have multiple homologous genes and indeed the reasoning behind so many variants of aquaporin within the same organism seems to be that it exhibits more than one role. Even among closely related species of filamentous fungi and yeast whose genomes have been completely sequenced, the number of aquaporin genes varies significantly. Within the few examples that have been phenotypically analyzed, the characteristics have often been surprising and not always typical of a channel that simply increases water permeability. Motivated by such results as well as the need for more knowledge of aquaporin function in the fungal kingdom, we proposed to study the phenotypic properties of an aquaporin in the model organism *Neurospora crassa*.

*N. crassa* was chosen as the organism of study because it contains only one aquaporin gene and a knockout mutant is available. Experiments were performed that specifically target laboratory situations that might reveal a phenotypic difference. The intent was to expose *N. crassa* to the effects of water in its three phases. This was accomplished through treatments with solutions of varying osmotic gradients, incubating growing mycelia under controlled humidity, and exposing *N. crassa* conidial germlings to freezing temperatures. In addition to the work with the mycelia form of the fungus, tests were performed on its macroconidia with the reasoning that their smaller size and simpler morphology would show better diverging results among the control and the knockout aquaporin mutant.

# MATERIALS AND METHODS

**Strains.** Stock cultures of wildtype (WT) (St. Lawrence 74–OR23–1VA, FGSC 2489) and *aqp* (FGSC 12017, deletion mutant of NCU08021 [Colot et al., 2006]) were obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA) [McCluskey, 2003] and maintained on slants of Vogel's Minimal Medium [Vogel, 1956] plus 1.5% (w/v) sucrose and 2.0% (w/v) agar.

**Sucrose Lysis Experiments.** To compare the membrane water permeability of WT with *aqp*, mycelial colonies were grown on 90 mm plastic Petri dishes containing 25 ml of Vogel's Minimal (VM) medium, biotin, 1.5% (w/v) sucrose and 2% (w/v) agar. Starter plates were prepared by incubating Petri dishes at 28ºC until the mycelium covered the entire surface. Thin agar blocks (dimensions of 0.5 cm x 3 cm) were sliced from the starter plates and inoculated at one edge of a new Petri dish containing VM, biotin, 2% (w/v) agar and different concentrations of sucrose depending on the experiment. The transfer of the agar blocks allowed for the mycelial growth to be approximately linear from time zero (otherwise a lag in growth would occur due to conidia germination). The colony grew towards the centre of the plate; after overnight incubation at 28°C, the colony edge was near the centre of the plate (this allowed easy scoring of lysis at the colony edge). The plates were exposed to room temperature and open air for ten minutes to minimize any possible effect of environmental shock on the fungal growing tips. The plates were then treated with solutions containing VM and sucrose of varying osmolarity. To score for hyphal tip lysis, the colony edge was scanned for hyphal tips using the 10X objective of the microscope. The hyphal tips were first scored solely for numbers present

and then scored again for lysis events. Only the colony edge up to 1 mm behind the edge was scanned (that is, possible lysis events 1 mm or more behind were not scored, even though they were occasionally observed). To be scored as a lysis event, the cytoplasmic debris had to be within 0.5 mm of the hyphal tip (to avoid scoring cytoplasmic debris that had floated away).

To minimize the possibility of observer bias, a single blind protocol was used in all experiments. The inoculated plates were given to a colleague, who labeled them using random numbers [Haahr, 2009] (these random numbers are derived from atmospheric noise picked up on a radio). The plates were then sorted from lowest to highest random number and returned for incubation overnight.

**Humidity Growth Experiments.** To determine if the relative humidity of the air that the fungi were grown in had any appreciable effect on growth of WT and the *aqp* mutant, slants were prepared containing Vogel's Minimal medium, biotin, 1.5% (w/v) sucrose and 2% (w/v) agar. After inoculation with slices of agar, the slants were placed in large laboratory media bottles containing different saturated salt solutions to produce the desired relative humidity [Greenspan, 1977]. A temperature/relative humidity datalogger was also inserted to provide assurance that relative humidity and temperature were maintained at constant values (Fig. 1).

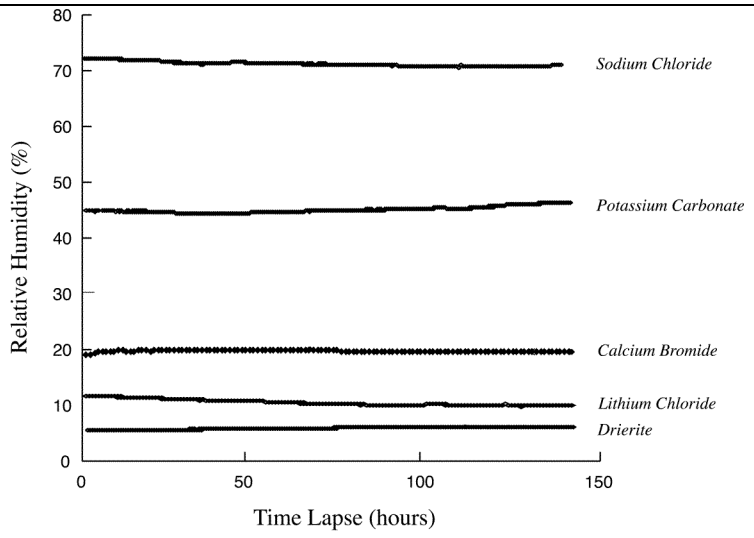



**Figure 1: Relative humidity produced by saturated salt solutions in closed containers.** The four saturated salt solutions and drierite were placed in large laboratory media bottles along with a humidity datalogger to verify that the humidity levels were stable over long periods of time.

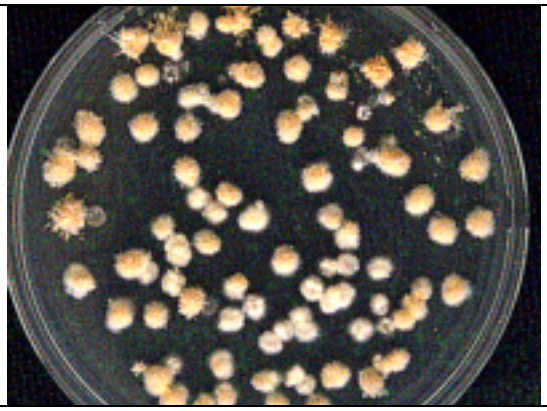
The bottles were sealed with parafilm and incubated at 28ºC for 7 days at which time the slants were removed and the fungal growth of WT and the *aqp* mutant compared.

**Conidial Germination Experiments.** To test if prior growth conditions had any effect on WT and *aqp*, the germination of the macroconidia grown at moderate relative humidity was examined. The conidia were harvested from slants grown in large sealed laboratory media bottles containing a saturated potassium carbonate salt solution to produce a constant relative humidity (about 50–60 %) for 7 days at 28ºC. The conidia were transferred to 30 mm Petri dishes and treated with double-distilled water. Conidia germination was scored using the X10 objective on the microscope. The scoring was performed every hour for 6 hours. Conidia were scored as germinated if a germ tube was evident or if an individual conidium had become definitively polarized and thus indicated the imminent appearance of a germ tube.

Two independent control experiments were also performed; one in which the fungi were grown in slants at room temperature and humidity and one in which the fungi were treated with a germination solution (in place of double distilled water) containing VM, 1.5% sucrose and biotin.

**Viability after Freezing.** Ice crystal formation during freezing can kill cells, however as the extracellular media freezes, water movement out of the cell can prevent this. So, the effect of freezing on viability of the WT and *aqp* mutant strains was examined. Conidia were harvested from mycelia grown on Vogel's Minimal medium plus biotin, 1.5% (w/v) sucrose and 2% (w/v) agar in 50 mL Erlenmeyer flasks incubated at 28°C. The conidia were placed in a small volume of double-distilled water. Conidia were counted with a haemocytometer, and adjusted to approximately 1000 conidia per mL. An aliquot (0.2 mL, about 200 conidia) of the condial suspension was placed on 90 mm Petri dishes containing 25 ml of medium and spread evenly on the surface using a bacterial cell spreader. The Petri dishes contained VM, 2% (w/v) sorbose (to induce colonial growth), 0.05% (w/v) glucose, 0.05% (w/v) fructose and biotin. The glucose and fructose were used in place of sucrose to avoid the toxicity observed when sorbose and sucrose are mixed. Sorbose was used to induce colonial growth to allow individual colonies to be counted. (Fig. 2) The sorbose was autoclaved separately from the VM to avoid browning of the medium, which preliminary experiments showed to cause non-colonial growth.

**Figure 2: Colonial growth of *Neurospora crassa* in sorbose cultures.** Each colony represents the growth of a single conidium and thus the total number of conidia could be scored by macroscopically scoring the visible colonies.



# RESULTS

**The *aqp* Mutant and Wildtype Exhibit Similar Hyphal Tip Lysis Phenotypes.** Treatment of the growing edge of the fungal colony with a hypo-osmotic sucrose solution caused a percentage of the total hyphae to lyse. (Fig. 3) These lysis events were scored for WT and *aqp* and compared. Three different sucrose gradients were used; the gradient was based on the molarity of sucrose in the growing media (and hence the growing fungi) and the molarity of the sucrose in the treatment solution. As the gradient decreased, the percent lysis of the growing tips decreased as well. In each trial there was much scatter attributed to the variation in the hyphal tips, but overall the percent lysis was the same for WT and *aqp*.

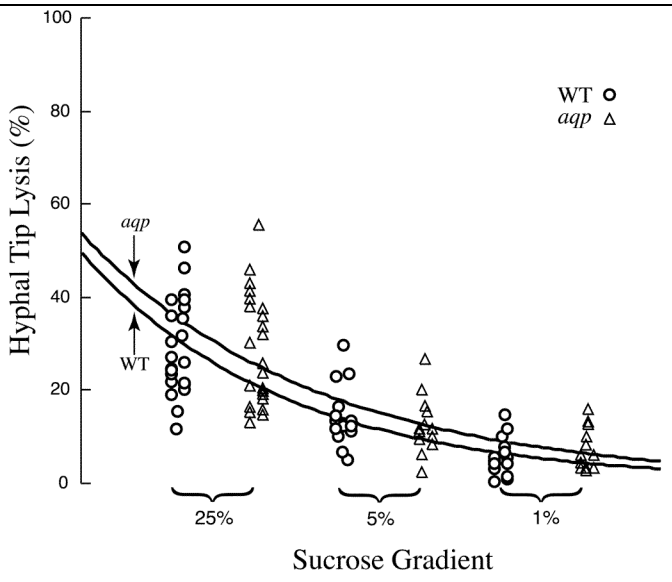



**Figure 3: Lysis percentage of hyphal growing tips of wildtype and *aqp* when treated with hypotonic sucrose solution.** Three tests were performed, each with a different osmolarity gradient between the substrate medium and the treatment solution. The lysis percentage of WT and *aqp* were similar in all three tests.

**The Physiological Appearance of Fungi Varies with Humidity Level Exposure.**
The saturated salt solutions within closed containers maintained a near-constant humidity although the growth of the hyphae raised the overall humidity higher due to transpiration. (Fig. 4) The hyphae grew at the same rate for each different humidity level, however, the appearance of the hyphae varied among each trial. At the low humidity levels produced by lithium chloride and calcium bromide saturated solutions, the hyphae sometimes took on a *cut*-like phenotype where the ends of the strands abruptly ended at the same aerial length. (Fig. 5) When isolated from each other, slants of *aqp* and WT increased the humidity level sharply at approximately the 24-hour mark. Both types exhibited the increase in humidity although the magnitude of the rise as well as the time period it took to correct the humidity level differed. (Fig. 6)

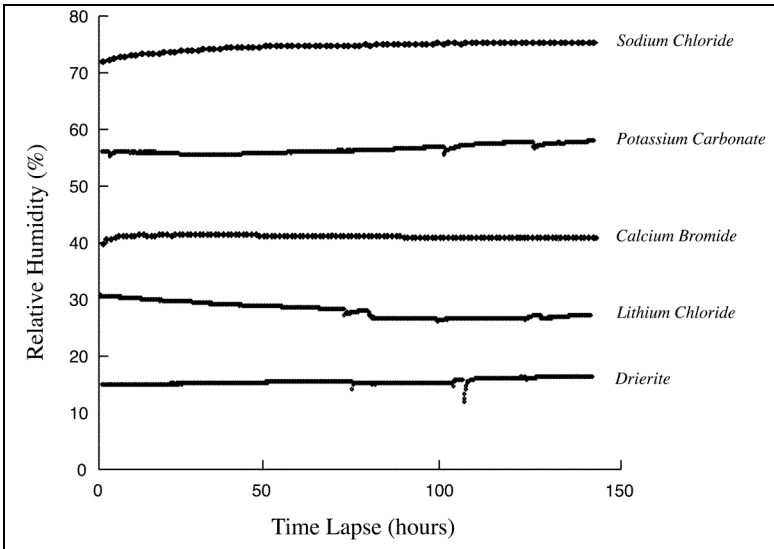
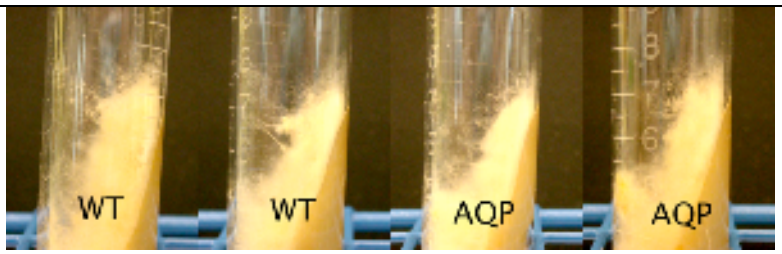
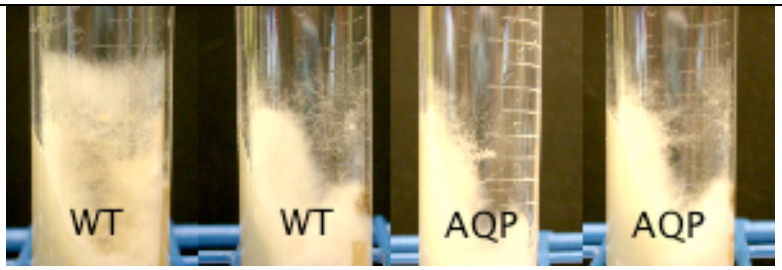



**Figure 4: The relative humidity within closed bottles containing saturated salt solutions and 4 to 6 agar slants with growing fungi.** The humidity values were recorded with a datalogger. The values show considerable more scatter as well as being shifted upwards compared to the control experiment without the presence of the agar slants. This shift was attributed to the transpiration of the fungi.



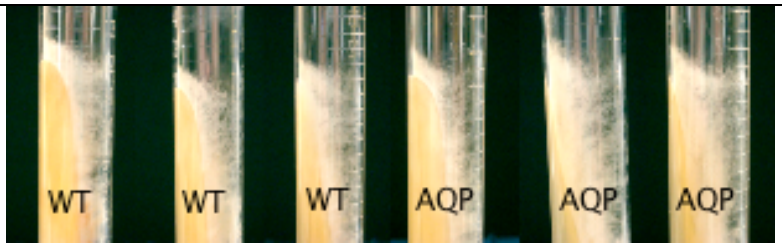

*Drierite*

*(16% RH)*
*($\Psi_{wv}$ =-255MPa)*



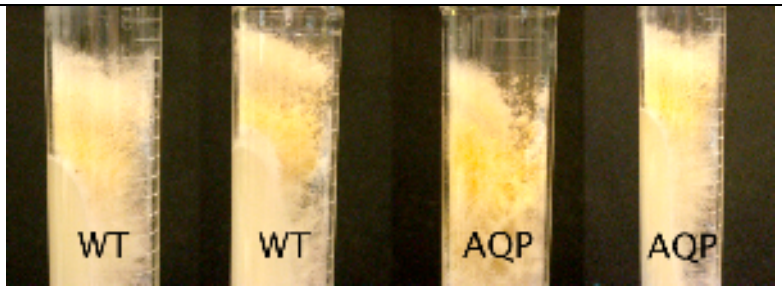

*Lithium Chloride*

*(29% RH)*
*($\Psi_{wv}$ =-172MPa)*



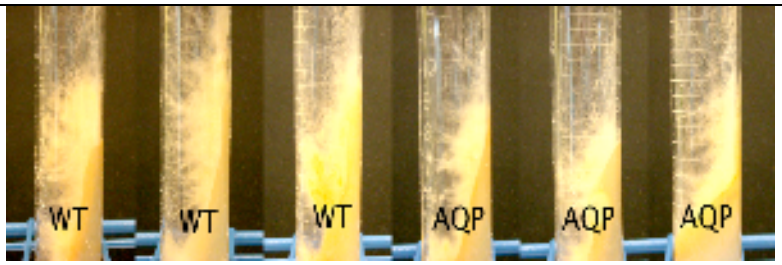

*Calcium Bromide*

*(40%RH)*
*($\Psi_{wv}$ = -127MPa)*




*Potassium Carbonate*

*(56%RH)*
*($\Psi_{wv}$ = -81MPa)*




*Sodium Chloride*

*(73% RH)*
*($\Psi_{wv}$ = -44MPa)*

**Figure 5: The appearance of hyphae after incubation at controlled humidity.** The fungi were grown on slants within sealed bottles containing different saturated salt solutions. The different relative humidity levels created a large range in water vapour potential, $\Psi_{wv}$. *Cut*-like growth of the hyphae was only seen at the humidity levels produced by lithium chloride and calcium bromide.

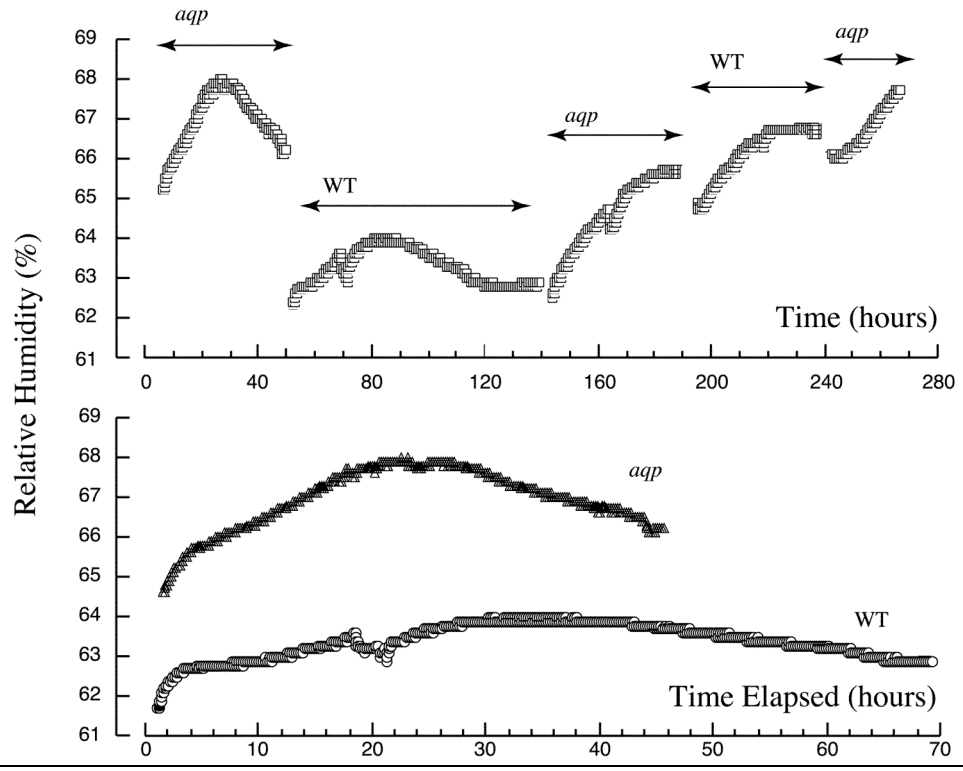



**Figure 6: The increase in relative humidity due to wildtype and *aqp* transpiration.** The top panel compares the humidity peaks for several trials of each strain. The gaps between each peak signify where a new trial begins. The bottom panel shows an expanded view of the first two peaks. The magnitudes of the humidity increase, as well as the timeframes, vary, but it was the presence of the peak in general that was deemed significant.

**The Viability of *aqp* Conidia was Lower than WT After Freezing.**
Conidia were grown until they were macroscopically visible in the form of a colony after freezing them for 24 hours. The numbers of *aqp* and WT were scored and compared to the control plates that were not frozen. (Fig. 7) After freezing, the number of conidia that were able to grow into colonies decreased for both types however the decrease was more pronounced in *aqp* conidia. Statistically, the *aqp* frozen conidia were different than the *aqp* control 4/5 times while the WT frozen conidia were different than the WT control 1/5 times.

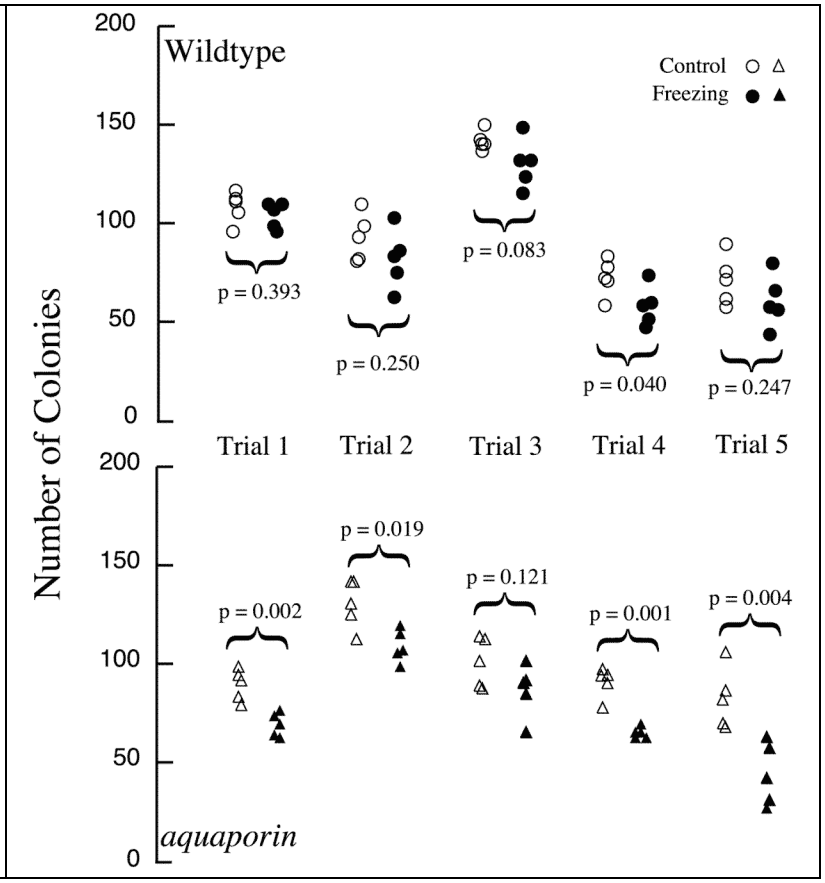



**Figure 7: The viability of wildtype and *aqp* conidia after freezing compared to a control without freezing.** Five trials were performed for each strain. The probability of divergence from the control based on chance alone was calculated using a standard *t*-test. Chance was ruled out if the probability values were less than 0.05. Thus, wildtype diverged from the control 1/5 times while *aqp* diverged 4/5 times.

**The Germination Rate of Conidia Increases After Controlled Incubation.** After the hyphae were grown in closed containers at a controlled humidity produced by saturated potassium carbonate solution, the conidia were placed in distilled water. In addition, hyphae were grown to the conidiation stage at ambient humidity with aeration and these conidia were used in two control experiments. The conidia of WT and *aqp* grown in the sealed container developed a significantly higher germination rate than the control conidia in distilled water (Fig. 8) (2-tail *t*-test, $P = 0.004$ at 6 hours for WT, $P < 10^{-6}$ for *aqp*). And, the *aqp* conidia (produced in the sealed container) exhibited a significantly higher germination rate compared to the WT conidia (2-tail *t*-test, $P < 10^{-5}$ at 6 hours). The control experiment in germination solution was used to assure the overall viability of the conidia, which was similar for both strains ($P = 0.563$ at 6 hours).

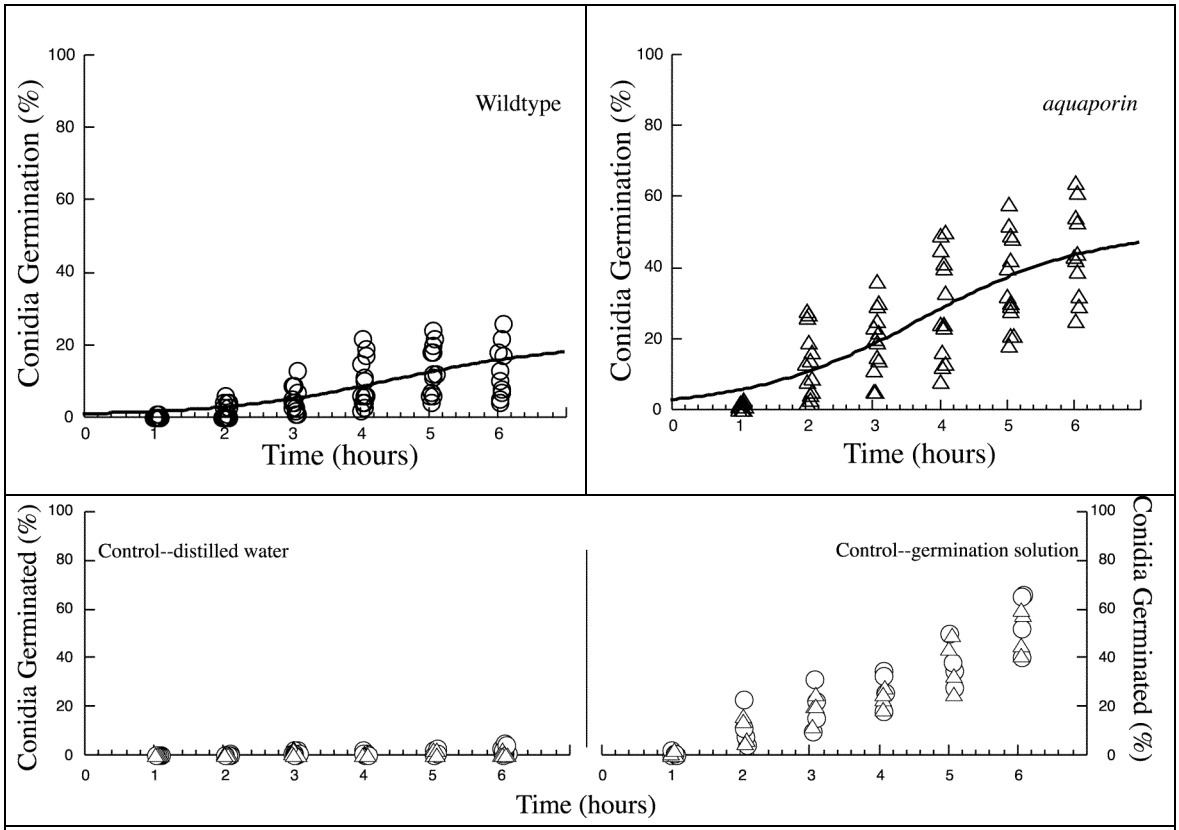



**Figure 8: The percentage of conidia germinated over time.** In the top two panels, the conidia were placed in distilled water. The *aqp* conidia show a higher germination rate than wildtype conidia. The bottom panel shows two control experiments using conidia grown at ambient humidity and aeration; the left side is conidia placed in distilled water and the right side is conidia placed in a germination solution containing VM, sucrose and biotin. The potassium carbonate results are compiled from 4 independent experiments. The controls were single replicates.

# DISCUSSION

The aquaporin has already been established as a protein channel capable of producing noticeable increases in water permeability for some organisms. The preliminary goal at the onset of our experimentation with *Neurospora crassa* was to determine if its aquaporin homologue exhibited much of the same behavior. One of the simplest ways to test this was to expose the growing fungal hyphal tips to an osmolarity gradient between the internal cytoplasmic environment and the external environment. The goal was to focus on the growing hyphal tips since this is where the cell wall is being newly expanded and is temporarily weakened. Osmotic potential differences could thereby cause the walls to lyse and it was these lysis events that were scored. The water flow through the cell membrane can be modeled as follows: the water flow ($J_v$) is equal to the hydraulic flow (driven by the hydrostatic pressure gradient ($L_P$•P) *minus* the osmotic flow (driven by the osmotic pressure gradient ($L_P$•$\sigma_s$•R•T•($C_s^i - C_s^o$). Simplifying:

$$J_v = L_p \bullet \left[ P - \sigma_s \bullet R \bullet T \bullet (C_s^{\,i} - C_s^{\,o}) \right],$$

where $L_P$ is the water permeability of the membrane, P is the hydrostatic pressure, $\sigma_s$ is the membrane selectivity to sucrose, R and T are the gas constant and absolute temperature, and ($C_s^i - C_s^o$) is the difference in concentration of sucrose on either side of the membrane. If $C_s^o < C_s^i$, then R•T•($C_s^i - C_s^o$) is positive, so then the osmotic pressure gradient ($L_P$•$\sigma_s$•R•T•($C_s^i - C_s^o$)) is negative, so that water will flow into the cell if the osmotic gradient term is greater than the hydrostatic pressure term ($L_P$•P) [Nobel, 1974].

In theory, WT should lyse more than the *aqp* mutant because the presence of water channels would increase water permeability of the membrane ($L_P$) in WT. This would allow more water into the cell, raising the turgor to higher levels and causing more lysis. We dealt with small concentration gradients so that the osmotic water flow would be only slightly larger than the turgor. If there was to be any noticeable difference, the gradient would have to be slight otherwise the osmotic pressure gradient would be too large for any water channel to affect water flow and high percentages of lysis would result.

After testing three consecutively smaller osmotic gradients, there was no indication that WT had a higher water permeability. It is important to note however that there was extensive scatter in the data. This occurred possibly because of the variability of the fungi growing tips, which exhibit differences in size and also are known to contain different internal turgor pressures depending on their growth activity [Lew & Nasserifar, 2009]. As well, it is also possible that the hyphae can compensate for the osmotic gradient perhaps by actively transporting ions out of the cytosol, thereby reducing much of the osmotic potential. It is known that when *Neurospora* is subjected to hyperosmotic environments it increases its ion uptake to regulate its turgor [Lew & Nasserifar, 2009]. Although the fungus in this experiment was subjected to hypo-osmotic treatments, there is reason to assume that *Neurospora* follows the same methods to decrease its turgor.

Perhaps these are the limiting factors of the experiment that conceal the effects, if any, of the aquaporin.

There are also additional complications that arise from the biophysical properties of the fungal hyphae. As reported [Lew & Nasserifar, 2009], *Neurospora* exhibits a biphasic response to changes in osmotic pressures depending on the overall domain of pressure the organism is within. This is defined in terms of the modulus of elasticity; the modulus describes how the cell wall expands or contracts with changing pressure and is given by the relationship:

$$\Delta P = E \bullet \frac{\Delta V}{V_i},$$

where $\Delta P$ is the change in pressure, $\Delta V$ and $V_i$ are the change in volume and initial volume, respectively, and $E$ is the modulus of elasticity [Philip, 1958]. At the level of turgor normally exhibited by *Neurospora*, the modulus of elasticity of the cell walls has already reached a maximum. So, when treated with a hypo-osmotic solution, the water that flows into the cells increases the turgor but the walls cannot expand any further. This leads to lysis of the walls if the pressure gradient is not lessened through ion exchange or other means. Thus, the elastic modulus may conceal any small differences between the lysis of WT and *aqp* under constant conditions.

It has been reported [Lim & Lew, 2008] that there is no phenotypic difference in hypo-osmotic-induced lysis between the *aqp* mutant and WT when there was a high osmotic gradient. We tested lower gradients and also found no phenotypic difference. So, it is doubtful that the aquaporin has any effect on water permeability at any level discernable by this experiment. There may still be a very small range in gradients, not tested, where there is a phenotypic difference. However, even if such a range existed, it does not provide any insight into why *Neurospora* contains aquaporin for any physiological relevance. An evolutionarily built-in water channel that is only beneficial for a small range in gradients seems to be redundant considering the large range of osmotic gradients that *Neurospora* could come into contact with in the external environment.

The results of the lysis experiment convinced us that the *aqp* mutant of *Neurospora* does not exhibit the standard water permeability enhancement effects. We switched to an examination of a very different part of the life cycle of the fungus: conidia and young conidial germlings. An important part of these experiments was to also control the environmental influences as much as possible, especially humidity, an environmental factor that was predicted to have the potential to influence the growth and development of WT and the *aqp* mutant.

The saturated salt solutions in sealed containers proved very effective for stabilizing the humidity at various levels to control the relative humidity the growing fungus was exposed to. These experiments were based on work [Kuwana, 1953] that showed that the moisture content of the atmosphere that the fungus is exposed to influences the growth. At low levels of humidity, the WT form of *Neurospora* exhibited the same shortened

condial band at the top of slants that is seen in the osmosensitive *cut* mutant at normal relative humidity. The saturated salt solutions have different chemical activities and by a simple dilution effect (to a first approximation) [Nobel, 1974] the water activity is lowered. Therefore the water molecules have less of a tendency to leave the solution and the vapour pressure of the water in gas phase at equilibrium is lowered. When the relative humidity that the growing *Neurospora* is exposed to is low, there is a large vapour pressure difference between the water within the cells and the water in the air. Thus, much of the water is transpired from *Neurospora* when the air is dry, and turgor would decline, resulting in the termination of hyphal tips extension during conidial banding. In addition, the tight layer of conidia may protect the hyphae from further water loss, dessication, and death.

The *cut* phenotype was observed in both WT and the *aqp* mutant at the low humidity levels produced by calcium bromide and lithium chloride saturated solutions—but only sometimes. A lack of a *cut* phenotype when drierite was used may be because the humidity was so low that very little aerial hyphae were produced. At the very least, the experiment shows that simple variability in the organism is enough to mask any physiological effect of the aquaporin. Unexpected results were observed in the recorded humidity data. There was a temporary rise in humidity after about 24 hours of growth; a result credited to transpiration of the newly formed aerial hyphae. Based on these results, a subset experiment was performed to detect if both strains of *Neurospora* exhibited the effect or if it was restricted to the WT. Both WT and the *aqp* mutant did exhibit the temporary increase in humidity. Although the magnitude of the humidity change was slightly higher in the *aqp* mutant, insufficient replicates are available to determine whether the slight difference is significant; it probably is not.

Further tests focused on the conidial germlings of *Neurospora*, since they are a much simpler life phase than the mycelial colony. Based on a report [Tanghe et al., 2006] that showed aquaporin has a noticeable effect on survival of the yeast *Saccharomyces cerevisiae* after freezing, we tested the viability of *Neurospora* conidial germlings after freezing. Sorbose (a non-metabolizable sugar that restricts growth) was added to the medium to allow individual colonies arising from single conidium to be scored. Most cells do not freeze unless the temperature falls below –5.5°C [Seki et al., 2009]. Water within the cell that is below its freezing point is considered to be supercooled and this supercooled water has a higher vapour pressure than that of the water in equilibrium with the ice outside of the cell. Thus, the resulting chemical potential difference will force water to leave the cell and freeze externally, causing dehydration of the cytosol. The rate of dehydration depends on the hydraulic conductivity $L_P$, and the cooling rate (which was kept constant). The rate of loss of cytoplasmic water depends on the difference in chemical potentials of intracellular and extracellular water expressed as the ratio of vapour pressure outside to the vapour pressure inside, $p_o/p_i$ :

$$\frac{dV}{dt} = \frac{\left[ L_p \bullet A \bullet R \bullet T \bullet \ln(\frac{p_o}{p_i}) \right]}{v_w},$$

where A is the cell surface area, R and T are the ideal gas constant and absolute temperature, respectively, and $v_w$ is the molar volume of water (volume occupied by one mole of water at a given temperature and pressure) [Seki et al., 2009]. So, with everything else held constant, an increase in $L_p$ would result in a higher rate of dehydration that would limit the occurrence of internal ice crystallization.

In five independent trials, the wildtype survival compared to the control (no freezing treatment) was significantly different only once. The survival of the *aqp* mutant was significantly less than the control in 4/5 trials. Thus, aquaporin does improve conidia viability by allowing the dehydration to occur rapidly enough to prevent ice nucleation within the cells. However, the difference in survival is still slight. A potential reason for this is that $L_P$ decreases with falling temperature. Perhaps this effect dominates over any effect of the aquaporin on the water permeability of the membrane. In addition, the drop in temperature used in this experiment was moderate. It was certainly faster than any drop the conidia would be exposed to in the external environment, but less than the cooling rate used in experiments with yeast that revealed more dramatic differences in survival of wildtype compared to an aquaporin mutant [Tanghe et al., 2006].

The germination viability of conidia is known to drop if the spores are stored at high humidity for several days [Schmit & Brody, 1976]. To test for such spore viability, the fungus was grown to the conidiation stage in the same experimental setup used to study the effects of humidity on growth. The saturated potassium carbonate system was used to create a constant relative humidity. Surprisingly, conidia of both strains germinated in distilled water. Even more surprisingly, the *aqp* mutant had a higher germination percentage than the WT strain.

The cause of this result remains unclear although there are at least two effects happening. The first is that the incubation period is somehow changing the physiology of the conidia or perhaps the specialized aerial hyphae during aerialogenesis such that the germination percentage in distilled water in the first six hours is increasing. The second effect is based on the presence of aquaporin since *aqp* conidia show a higher germination rate than WT. A possible explanation is that the increased humidity produced by the potassium carbonate humidity container reduced the vapour pressure gradient and limited the ability of water to escape during normal dehydration of the conidia in preparation for dormancy. With an increased water concentration it is perhaps possible that the conidia are not fully dehydrated and thus less dormant than normal. When they are put in contact with distilled water, the absence of dormancy results in higher germination. This would have an even greater effect in *aqp* since there might be higher levels of contained water that could account for the higher levels of germination. This explanation is possible because it has been discovered that an aquaporin is involved in sporulation for *Saccharomyces cerevisiae* [Sidoux-Walter et al., 2004].

Another factor to consider is the increased concentration of carbon dioxide in the sealed containers. Neurospora has been shown to be highly sensitive to a lack of aeration [Sargent et al., 1972], an effect that was evident in this experiment as colonies in the sealed containers had limited conidia production. However, it is unlikely that the

increase in carbon dioxide due to the saturated potassium carbonate solution had any effect on the internal pH of the cells. A test of the pH of 100mM potassium carbonate solution was found to be 11.62. At such a high alkalinity, degassing of the carbonate as carbon dioxide should be negligible to nil. On the other hand, respiration will increase $CO_2$ levels. Interestingly, carbon dioxide has been shown to gate an aquaporin in *Arabidopsis thaliana* [Chaumont et al., 2005], so it appears that carbon dioxide could be affecting multiple physiological pathways that lead to conidiation.

# CONCLUSION

It seems quite evident that the aquaporin homologue in *Neurospora* does not enhance water permeability in any simple theoretical way. The lack of a phenotypic difference between WT and *aqp* for such basic experiments involving hypo-osmotic treatments, suggests a complexity beyond relatively simple biophysical characteristics of cell membranes. Instead, aquaporin may be involved in more elaborate processes like conidial germination, possibly related to ambient conditions that include air levels of carbon dioxide. It is perhaps in the early conidia and germling stages where aquaporin has the most noticeable effect. The results of this investigation seem to signify that the physiological role of aquaporin is much more complex than originally predicted. *Mappa mundi non est terranum* (the map is not the terrain).

It is worthy to note that many of the positive results in experiments with other organisms obtained so far were based on genetic manipulation of the cells in which the aquaporin was *over*-expressed. Consequently, this does not reveal the true significance of aquaporin at levels found normally in the cell and the effects of aquaporin would be less obvious in many cases. Still, the ubiquity of aquaporin simply cannot be overlooked and further experimentation should be focused on the organisms that are able to exist with only limited copies of aquaporin homologues. Thus, for elucidating a role of aquaporin in general, *Neurospora crassa* remains an excellent candidate for future studies.

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