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The odd behaviour of carbonic anhydrase in the terrestrial cyanobacterium *Nostoc flagelliforme* during hydration–dehydration cycles

Changpeng Ye,[1,2] Kunshan Gao[3] and Mario Giordano[4]*

[1]*Marine Biology Institute, Science Center, Shantou University, Guangdong 515063, China.*
[2]*Institute of Hydrobiology, Jinan University, Guangdong 510632, China.*
[3]*State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China.*
[4]*Dipartimento di Scienze del Mare, Facoltà di Scienze, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy.*

Summary

The terrestrial cyanobacterium *Nostoc flagelliforme*, inhabiting arid areas, withstands prolonged periods of dehydration. How dehydration and occasional wetting affect inorganic C acquisition in this organism is not well known. As inorganic C acquisition in cyanobacteria often involves carbonic anhydrases (CA), we studied the effect of cycles of hydration and dehydration on the extracellular and intracellular CA activities, at the pH values presumably associated with dew or rain wetting. The external CA of *N. flagelliforme* (or of the microorganismal consortium of which *N. flagelliforme* is the main component) is activated by hydration, especially at low pH, and it may facilitate inorganic C acquisition when *N. flagelliforme* colonies are wetted by dew. Internal CA is present in dry colonies and is rapidly inactivated upon rehydration, therefore an anaplerotic role for this enzyme is proposed.

Introduction

The terrestrial cyanobacterium *Nostoc flagelliforme* (Berk. and Curtis) Bornet and Flahault is widely distributed in arid or semiarid areas of the world (Gao, 1998). In China, where it is a traditional medicament and food, the overexploitation of *N. flagelliforme* has reduced its abundance in the field, which resulted in a deterioration of the environment and in a change in the structure of the prevailing biological communities (Gao, 1998). The conservation of *N. flagelliforme* and an understanding of its physiology is thus of paramount environmental, scientific, economic and cultural relevance. In nature, *N. flagelliforme* is subjected to formidable environmental stresses. In the area of Inner Mongolia where this organism is found, precipitation is minimal (50–300 mm year^{-1}) and it mostly (70%) occurs in June, July and August. The evaporation rate is 10–20 times higher than rainfall, and temperature excursions, on both a daily (~16°C excursion for surface temperature; ~20°C excursion for air temperature) and yearly (~95°C excursion for surface temperature; ~70°C excursion for air temperature) basis, are extreme (Gao, 1998). When water becomes available, *N. flagelliforme* recovers full metabolic activity within a few hours (Scherer *et al*., 1984; Gao *et al*., 1998). In the field, hydration of *N. flagelliforme* may be caused by dew or rain: the wetting by dew, possibly because there is no water-phase continuity between the soil and the dew around the filaments, does not cause significant dissolution of the alkaline (pH 8.0–9.5) soil usually inhabited by *N. flagelliforme* (Gao, 1998). This is likely to make the pH around the colonies slightly acidic, owing to the insufficient buffer capacity afforded by the reduced metabolic activity of the partially hydrated *Nostoc* filaments (even if photosynthetic and respiratory activity may somewhat modify the pH in the close proximity of cells). The release of acidic polypeptides that typically occurs from the dry colonies of *Nostoc* when they are re-hydrated (Hill *et al*., 1994) could also contribute to keep the pH low, if the polypeptides are released with protons as counter ions and the amount of wetting water is insufficient to wash the polypeptides away. By contrast, copious rainfall solubilizes the alkalis in the soil and this mode of hydration is therefore associated with a high pH (Gao, 1998). Photosynthesis of *N. flagelliforme* at alkaline pH possibly relies on HCO_3^- as the main source of inorganic C (Ci; Gao and Zou, 2001). The utilization of HCO_3^- in cyanobacteria requires a variety of parallel or serial transport and enzymatic processes that generate CO_2, the only Ci substrate for Rubisco, in close proximity of this enzyme (Giordano *et al*., 2005). Carbonic anhydrases (CA) are often involved in these processes, either at the surface of the cell or intracellularly (Giordano *et al*., 2005

Received 30 April, 2007; accepted 7 November, 2007. *For correspondence. E-mail m.giordano@univpm.it; Tel. (+39) 071 220 4652; Fax (+39) 071 220 4650.

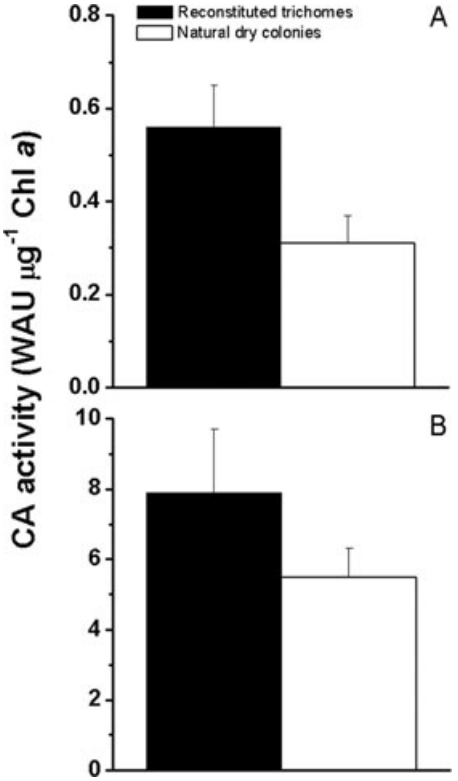

Fig. 1. External (A) and internal (B) carbonic anhydrase activity in *Nostoc flagelliforme* natural dry colonies (□) and reconstituted trichomes cultured in liquid medium at pH 8 (■). The error bars show the standard deviations ($n = 4$).

and references therein). In the light of the above, we set out to determine if the pattern of CA activity over hydration–dehydration cycles was compatible with a role of this enzyme in Ci acquisition, under conditions that mimicked the wetting of *N. flagelliforme* in the field.

Results

The hydration kinetics of dry natural colonies showed a biphasic behaviour: the initial (from time 0–0.5 h) hydration rate was equal to 44 mg H_2O (g DW)$^{-1}$ min^{-1} (s = 0.007); the subsequent (0.5–9.0 h) rate was one order of magnitude slower; at the end of this phase, 95% (s = 3.0) hydration was achieved. The hydration curve then followed a saturation kinetics that was completed 10–15 h after the beginning of hydration. The water content of fully hydrated colonies was equal to 27.2 g H_2O (g DW)$^{-1}$. Even the 'dry' colonies stored at room temperature contained an appreciable quantity of water, equal to 1.85% (s = 0.00) of the water content of fully hydrated cells.

Both external CA (eCA) and internal CA (iCA) activities were detected in the dry colonies of *N. flagelliforme* collected from the field and stored dry (except for the H_2O they could acquire from air) for over 3 years (Fig. 1). The iCA activity was more than one order of magnitude higher than that of eCA. Interestingly, when the dry colonies were fragmented and trichomes allowed to reconstitute and grow in liquid medium for a prolonged time (Gao and Ye 2003), iCA activity was not significantly different from that of the dry colonies, while eCA was almost two times higher (Fig. 1).

When the dry colonies were hydrated, eCA activity increased (Fig. 2). This increase was modest (1.6-fold) and transitory, if the pH of the hydration medium was set at 8. At this pH, eCA activity started to decline after 3 h of hydration and continued to decline during the remaining hydration time and for the first 40 h in the absence of water. At the end of this period, eCA activity was statistically indistinguishable from its value at time zero. However, when dehydration was prolonged for further 32 h, the activity increased again to about the maximum level attained during hydration, regardless of whether the cells were illuminated or not. The eCA activity of cells hydrated at pH 6 increased for the entire hydration phase, and after 9 h in water it was almost threefold higher than in dry natural samples. This level of activity was main-

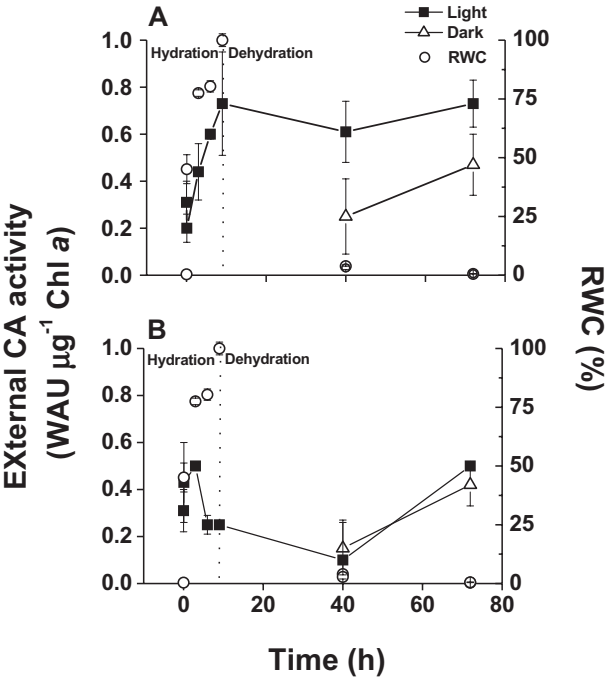

Fig. 2. External carbonic anhydrase activity (left axis) of *Nostoc flagellifomis* colonies during a hydration–dehydration cycle at pH 6.0 (A) and pH 8.0 (B). The 0 time value on a DW basis was 0.30 (s = 0.09) WAU (mg DW)$^{-1}$. The eCA activities per DW unit at 72 h, in the light, were 0.69 (s = 0.10) and 0.48 (s = 0.01) WAU (mg DW)$^{-1}$, at pH 6 and at pH 8.0 respectively. The eCA activities per DW unit at 72 h, in the dark, were 0.45 (s = 0.12) and 0.40 (s = 0.01) WAU (mg DW)$^{-1}$, at pH 6 and at pH 8.0 respectively. The open circles show the relative water content (RWC) of the colonies (right axis). The error bars show the standard deviations ($n = 4$). WAU = Wilbur-Anderson Units.

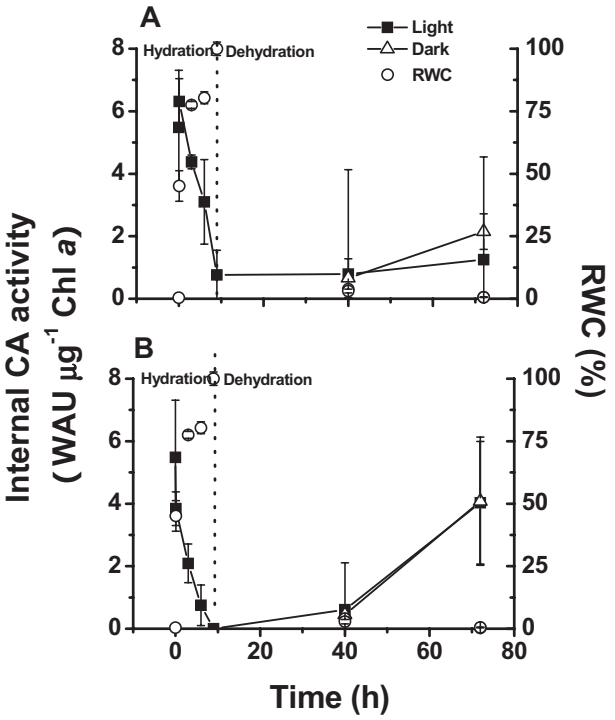

Fig. 3. Internal carbonic anhydrase activity (left axis) of natural colonies of *Nostoc flagelliforme* during a hydration–dehydration cycle at pH 6.0 (A) and pH 8.0 (B). The 0 time value on a DW basis was 5.50 (s = 1.75) WAU (mg DW)$^{-1}$. The eCA activities per DW unit at 72 h, in the light, were 1.20 (s = 0.50) and 3.86 (s = 1.01) WAU (mg DW)$^{-1}$, at pH 6 and at pH 8.0 respectively. The eCA activities per DW unit at 72 h, in the dark, were 2.06 (s = 0.55) and 3.83 (s = 1.90) WAU (mg DW)$^{-1}$, at pH 6 and at pH 8.0 respectively. The open circles show the relative water content (RWC) of the colonies (right axis). The error bars show the standard deviations (n = 4).

tained for the subsequent 72 h of dehydration, in the light. When colonies previously hydrated at pH 6 were allowed to dehydrate in the absence of light, however, eCA activity showed a trend of variation closely resembling that of cells at pH 8.

The pattern of iCA activity in response to hydration and dehydration was remarkably different from that of eCA (Fig. 3). Relatively high iCA activity was present in the dry colonies; hydration caused it to become statistically indistinguishable from the background, regardless of the pH treatment. At pH 6, no appreciable variation was observed during subsequent dehydration, whereas, at pH 8.0, dehydration induced a recovery of the intracellular CA activity. The presence of light did not affect iCA activity during dehydration at either pH. Interestingly, the dry colonies, showed substantial O_2 consumption rates (16.9 μmol O_2 h^{-1} μg^{-1} Chl, s = 1.10) in the O_2 electrode (colonies were hydrated for the few minutes necessary for the measurement); these were about 75% of the respiration rates after 9 h of hydration at pH 6.0 (21.94 μmol O_2 h^{-1} μg^{-1} Chl, s = 1.09) and pH 8.0 (22.35 μmol O_2 h^{-1} μg^{-1} Chl, s = 6.92). No new data on photosynthesis are included here, because they are fully available in papers previously published by some of the authors (Gao, 1998; Gao and Zou, 2001; Gao and Ye, 2003).

Discussion

The pattern of CA activity in dry colonies of *N. flagelliforme* subjected to hydration and subsequent dehydration is rather unusual. While at the present stage we cannot explain all the phenomena we observed, some intriguing hypotheses can be formulated on the basis of our results:

(i) External CA activity is required to increase Ci availability and/or supply rate for *N. flagelliforme* photosynthesis, especially when cells are hydrated. The very low eCA activity of natural dry colonies and the results of the hydration/dehydration experiments (Figs 1 and 2) are compatible with the hypothesis that eCA expression and/or activation are stimulated by (and possibly require) hydration; the effect of hydration is greater if the pH of the liquid phase around the cells is acidic (dew wetting).

(ii) Internal CA activity is required by the cells in their dehydrated state (Fig. 1) to supply Ci to anaplerosis. Its regulation by hydration/dehydration (Fig. 3) may reflect the oscillation between photosynthetic and non-photosynthetic metabolism.

External CA

Cyanobacteria are traditionally assumed to lack periplasmic CA. However, the analysis of complete cyanobacterial genome sequences revealed the presence of genes encoding putative periplasmic CAs (e.g. So and Espie, 2005). In *Synechocystis* PCC6803 and *Synechococcus* PCC7942, protein encoded by these genes are indeed localized in the periplasm, albeit they seem to be catalytically inactive (Soltes-Rak *et al*., 1997; So and Espie, 2005). Within the family *Nostocaceae*, all the *Nostoc* and *Anabaena* strains sequenced (see http://www.jgi.doe.gov/) contain putative extracellular CA genes; whether these genes encode for active proteins remains to be ascertained. It should be noted that most cyanobacterial eCA activity measurements conducted so far were effected on strains maintained for substantial lengths of time in relatively luxurious laboratory conditions, with altered selection pressure. Mutations in the eCA genes may have accumulated in these organisms with little detriment. This idea is also confirmed by the fact that Kupriyanova and colleagues (2003) and Dudoladova and colleagues (2004) reported that catalytically active α-CAs are present in the glycocalix of some recently isolated cyanobacterial strains. It may very well be that the products of the eCA genes are

active in natural cyanobacteria as the one used for this study (G. Espie, pers. comm.). Alternatively, the eCA activity detected in this study may derive from other microorganisms that participate in a microbial consortium with *N. flagelliforme*, or by both *N. flagelliforme* and other members of the consortium. Microscope observations and the absence of obvious bacterial or fungal contamination in liquid cultures derived from the natural samples (reconstituted trichomes, in which eCA is also present; Fig. 1) lead to believe that microorganisms different from *N. flagelliforme*, if present, constitute a very small component of the consortium. Since the presence of eCA seems to be a general feature of *Nostoc* natural colonies, the production of active eCA from other components of the consortium would also be of great interest, because it would indicate that one or more eCA producing members of the consortium are selected. Regardless of its biological source, eCA could play a number of functions in *N. flagelliforme*. In absolute terms (moles per unit of time), the uncatalysed rate of interconversion of HCO_3^- and CO_2 is, other things being equal, proportional to the volume of water in which the reaction is taking place and, because of the impact of salinity on the reaction constants, approximately inversely proportional to the salt content of the solution. Thus, cells wetted by a small amount of dew may require a CA to speed up the supply of Ci for photosynthesis. Moreover, while the pH of the soil is alkaline, that of dew deposited on the colonies, which presumably does not solubilize soil components to a large extent, is not. It is thus possible that the Ci acquisition of dew-wetted colonies occurs at relatively low pH, at which eCA showed the highest activity. *Nostoc flagelliforme* is a terrestrial organism; it therefore derives Ci mainly from atmospheric CO_2 and/or from the respiratory activity of the heterotrophic component of the microbial consortium. Because of the intrinsically slow CO_2 solvation ($CO_{2(g)} \rightarrow CO_{2(aq)}$) and hydration ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$) reactions, the almost instantaneous equilibrium between CO_2 and HCO_3^- afforded by eCA would effectively trap atmospheric CO_2, which would be useful if HCO_3^- is directly used. This would be especially true if the surface to volume quotient of the water layer surrounding the cell is small. At pH 6, at which CO_2 and HCO_3^- are present in similar proportion (in freshwater, at 25°C, CO_2 = 55% Ci and HCO_3^- = 45% Ci), this could essentially double Ci availability at the cell surface. External CA may also trap the CO_2 generated by respiration and/or by leaky CO_2 concentrating mechanisms (CCM) in *Nostoc* cells and may therefore reduce the dependence of cells from the solvation reaction. It should be noted, however, that *N. fragelliforme* was suggested to acquire Ci as HCO_3 at high pH (Gao and Zou, 2001), but this has not been verified at low pH. If this was not the case, the Ci entrapped as HCO_3^- would have to be reconverted to CO_2 prior to its acquisition, making the trap futile. At pH 8, at which *N. flagelliforme* appears to take up Ci as HCO_3^- (Gao and Zou, 2001), the eCA-based Ci trap would not be needed, as the same function could be plaid, at least in part, by the alkaline pH. This agrees with the limited induction of eCA activity at this pH. Photosynthesis could also contribute to the entrapment of CO_2 by increasing the pH around the cells.

Internal CA

Natural dry colonies do not show photosynthetic activity (Qiu and Gao, 2001). The need for an iCA that is active when cyanobacterial cells are not photosynthetically competent may be associated with an enhanced contribution of the respiratory metabolism to basal biosynthetic activity. This notion is supported by recent findings that indicate that a fully functional, although modified, TCA cycle is present in cyanobacteria (Cooley *et al*., 2000; Vermaas, 2001). The ability of cyanobacteria to survive prolonged periods of photosynthetic inactivity by autophagy was already demonstrated for *Phormidium autumnale* (*Nostocales*, *Oscillatoriaceae*; Montechiaro and Giordano, 2006; Montechiaro *et al*., 2006). The necessity to maintain the cells functionally homeostatic, under these conditions, may require a (relatively) intense anaplerosis. The potential for an anaplerotic role for intracellular CA was shown for *Chlamydomonas rehinardtii* mitochondrial CA (Giordano *et al*., 2003) and may be hypothesized for *N. flagelliforme*. All this requires that *N. flagelliforme* retains sufficient intracellular water to allow metabolic functions. Our results show that, even when stored dry for several years, the colonies of *N. flagelliforme* retain appreciable amounts of water; in the field, their water content may be higher than in the stored samples, because of the daily dew deposition (see also Potts, 1999). It should also be considered that several microalgae living in osmotically challenging environments express CAs active at very low water potential (e.g. Booth and Beardall, 1991; Fisher *et al*., 1996; Bageshwar *et al*., 2004; Dudoladova *et al*., 2004; Premkumar *et al*., 2005). Thus, the amount of water required for iCA to play an anaplerotic role in *N. flagelliforme* may be rather small. In the presence of ambient water, photosynthesis resumes activity and new photosynthate becomes the main source of C for biosynthesis; the demand for replenishment of the respiratory pools may therefore be substantially reduced, eliminating the need for the expression of an anaplerotic CA. It is interesting that the reconstitution of the iCA activity during re-dehydration of the cells only occurs when the external pH is alkaline; while the reason for this remains somewhat elusive, it is noteworthy that this is exactly the situation in which *N. flagelliforme* is commonly found in nature for most part of the day (except after rare rain episodes and dew deposition in the early morning;

Gao, 1998). The presence of an active CA in the cytosol would short-circuit the CCM. Consequently, it is possible that the rapid decline of iCA during rehydration reflects a re-engagement of the CCM associated with the recovery of photosynthesis, which closely matches the decline of iCA activity (Qiu and Gao, 2001).

Experimental procedures

Natural colonies of *N. flagelliforme* were collected in Sunitezuoqi prefecture (113.7°E, 43.85°N), Inner Mongolia, and stored dry, at room temperature, for 3 years, prior to their utilization. Hydration of dry colonies (0.4 g dry weight, DW) was conducted in growth chambers at 25°C, with a photon flux density (PFD, 400–700 nm) of 70 μmol photons m^{-2} s^{-1}, over 10 h. The dry colonies were transferred to Petri dishes (12 cm diameter, 2 cm deep) containing 100 ml of BG11 growth medium (Stanier *et al.*, 1971) buffered at either pH 6 (25 mM MES) or pH 8.0 (25 mM Tris). The colonies were then deprived of the medium and allowed to dehydrate at 25°C for up to 72 h. Dehydration was conducted at either a PFD of 70 μmol photons m^{-2} s^{-1} or in the dark. The hydration/dehydration kinetics was studied gravimetrically. The water content was derived from the weight difference between colonies desiccated at 100°C for 24 h (or until weight was stable) and the samples at different time intervals after addition of liquid medium. Chlorophyll a content was determined according to Vonshak (1997).

Reconstituted trichomes were obtained and cultured in liquid medium as described by Gao and Ye (2003), at pH 8 and 25°C. Three days prior to the experiments, exponentially growing trichomes were exposed to the same light regime used for the hydration/dehydration experiments.

External CA and total CA activities were measured on natural dry colonies, colonies hydrated for 5 min, 3 h, 6 h and 9 h, and colonies re-dehydrated for 40 and 72 h. A slight modification of the potentiometric method by Wilbur and Anderson (1948) was used. Measurements were taken over the pH interval from 8 to 7, at 4°C. For eCA, about 0.5 g (fresh weight) of colonies were quickly blotted on paper (except for the dry colonies) and added to 4 ml of assay buffer (25 mM phosphate buffer, pH 8.36, and 10 μM $ZnCl_2$; Giordano and Maberly, 1989). The reaction was initiated by the addition of 1 ml of cold (4°C) CO_2-saturated water, as soon as pH and temperature in the assay mix were stable (~30 s). For the extraction of iCA, 1.0 g fresh weight of colonies was re-suspended in 5 ml of an extraction medium containing 300 mM Tris-borate (pH 8.36), 5 mM EDTA, 2.5 mM DTT, 2% (w/v) PVP-40, 0.1% (w/v) Triton X-100, and 0.5% (w/v) BSA (Giordano and Maberly, 1989) and lysed with a Ten-Broek homogenizer, on ice. The slurry (100 μl) was added to 3.9 ml of assay buffer and the total activity (iCA + eCA) was measured as for eCA. Internal CA activity was calculated as the difference between total and eCA activities. Carbonic anhydrases activity was also determined on reconstituted trichomes in liquid culture (pH 8.0; Gao and Ye, 2003). In control experiments, the trichome suspension and cell extract were substituted with an equal volume of growth medium and boiled extract respectively. In order to ascertain that the proton production in the Wilbur-Anderson assay was due to CA activity, activity determinations were conducted in the presence of sulfonamides, specific inhibitors of CAs, in the conditions at which maximum activities were observed. Specifically, whole-cell activity and lysate activity were also measured in the presence of 100 μM dextran-acetazolamide and 100 μM ethoxyzolamide respectively (Giordano and Bowes, 1997). These measurements gave results statistically undistinguishable from the background ($P > 0.05$). Both eCA and iCA activities were expressed on a chlorophyll basis. Dry weight was measured only at the beginning and at the end of the hydration/dehydration cycle owing to the difficulties of obtaining concomitant DW determinations at intermediate time points. Respiration was measured in an O_2 electrode as described by Montechiaro and colleagues (2006). All measurements were replicated four times. The significance of difference was tested with the *t*-test. The level of significance was always set at 0.05 and all tests were performed on samples of the same size ($n = 4$).

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