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Measuring titratable alkalinity by single versus double endpoint titration: An evaluation in two cyprinodont species and implications for characterizing net H^+ flux in aquatic organisms

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ABSTRACT

In this study, Na^+ uptake and acid–base balance in the euryhaline pupfish *Cyprinodon variegatus variegatus* were characterized when fish were exposed to pH 4.5 freshwater (7 mM Na^+). Similar to the related cyprinodont, *Fundulus heteroclitus*, Na^+ uptake was significantly inhibited when exposed to low pH water. However, it initially appeared that *C. v. variegatus* increased apparent net acid excretion at low pH relative to circumneutral pH. This result is opposite to previous observations for *F. heteroclitus* under similar conditions where fish were observed to switch from apparent net H^+ excretion at circumneutral pH to apparent net H^+ uptake at low pH. Further investigation revealed disparate observations between these studies were the result of using double endpoint titrations to measure titratable alkalinity fluxes in the current study, while the earlier study utilized single endpoint titrations to measure these fluxes (i.e.,. *Cyprinodon* acid–base transport is qualitatively similar to *Fundulus* when characterized using single endpoint titrations). This led to a comparative investigation of these two methods. We hypothesized that either the single endpoint methodology was being influenced by a change in the buffer capacity of the water (e.g., mucus being released by the fish) at low pH, or the double endpoint methodology was not properly accounting for ammonia flux by the fish. A series of follow-up experiments indicated that buffer capacity of the water did not change significantly, that excretion of protein (a surrogate for mucus) was actually reduced at low pH, and that the double endpoint methodology does not properly account for NH_3 excretion by fish under low pH conditions. As a result, it overestimates net H^+ excretion during low pH exposure. After applying the maximum possible correction for this error (i.e., assuming that all ammonia is excreted as NH_3), the double endpoint methodology indicates that net H^+ transport was reduced to effectively zero in both species at pH 4.5. However, significant differences between the double endpoint (no net H^+ transport at low pH) and single endpoint titrations (net H^+ uptake at low pH) remain to be explained.

1. Introduction

In freshwater fish, the linkage between Na^+ uptake and excretion of acid equivalents at the gill apical membrane has been recognized for 85 years (Krogh, 1937, 1939). Two mechanisms have been identified for Na^+ uptake at the apical membrane of the fish gill in dilute (≤2 mM Na^+) freshwater. Na^+ is either directly exchanged for H^+ via a Na^+/H^+ exchanger (NHE) or indirectly via a H^+-ATPase linked to a putative Na^+ channel (Hwang et al., 2011). Hence, in the characterization of Na^+ transport in fish, it is often useful to simultaneously characterize acid–base transport. Patrick and Wood (1999) took this approach with the mummichog (*Fundulus heteroclitus*), a euryhaline cyprinodont, measuring Na^+ transport and acid–base balance in response to exposure to amiloride and low pH (4.5). Exposure to amiloride inhibited both Na^+ uptake and net H^+ excretion. When fish were acutely exposed to pH 4.5 water, Na^+ uptake was strongly inhibited, with a corresponding reversal of apparent net H^+ transport from net secretion under control conditions to net uptake at pH 4.5. This effect was shown to be fully reversible upon return to control conditions. Subsequent gene expression studies support and refine these initial observations, indicating *F. heteroclitus* upregulates branchial expression of NHE-2 upon transfer from saline to freshwater, whereas branchial expression of V-type H^+-ATPase remains low and unchanged, and the already low branchial expression of NHE-3 decreases further (Scott et al., 2005).

We have been studying freshwater osmoregulation in another euryhaline cyprinodont, the pupfish *Cyprinodon variegatus variegatus*, which occurs along the Gulf and Atlantic coasts of North America and tolerates salinities ranging from freshwater up to 167 g L^{-1} (Nordlie, 2006). We

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recently demonstrated that like *F. heteroclitus*, *C. v. variegatus* appears to rely predominantly on an NHE for apical Na^+ uptake in 2 mM Na^+ freshwater and appears to lack a H^+-ATPase/Na^+ channel system (Brix and Grosell, 2012). These initial conclusions are based on studies using pharmacological inhibitors. In the current study, we initially sought to repeat the experimental approach of Patrick and Wood (1999) with *C. v. variegatus* to provide further support for the presence of an apical NHE. However, in initial experiments, we observed what appeared to be effectively the opposite results of those observed by Patrick and Wood (1999) with respect to apparent net H^+ transport. A detailed comparison of methods and results from the two studies suggested these discrepancies might be the result of methodological differences between the use of single endpoint titrations (used by Patrick and Wood 1999) and double endpoint titrations (used in the current study) to measure titratable acid (TA) flux. Consequently, we shifted our focus to explore the cause(s) for observed differences between the two studies.

We hypothesized three possibilities could be causing the observed discrepancies. First, it was possible that when performing this type of experiment at low pH (4.5), fish are releasing a buffer (e.g., mucus, phosphate) that leads to errors using the single endpoint methodology. Second, at low pH, the double endpoint methodology could be incorrectly accounting for the contribution of ammonia to net TA (see Discussion for detailed explanation). Finally, we hypothesized that *C. v. variegatus* and *F. heteroclitus* simply respond differently with respect to net H^+ transport when exposed to low pH. This paper describes our initial experiment which identified the apparent discrepancy between the two methods, a series of experiments to test these hypotheses, and discusses the implications of experimental results for measuring titratable alkalinity flux, and therefore net H^+ transport in aquatic organisms.

2. Methods and materials

2.1. Animal holding

Adult *C. v. variegatus* were collected from a small pond on Key Biscayne, FL that is intermittently connected to Biscayne Bay. Salinity in this pond ranges seasonally from 12 to 39 g L^{-1}. Fish were held at the University of Miami in 110-L glass aquaria under flow-through conditions with filtered natural seawater from Bear Cut, FL. Adult fish were bred and F_1 offspring were hatched and raised in seawater until the late juvenile stage (~2 months old; 200–400 mg). Fish were fed *Artemia* nauplii for the first 2 weeks and then over a 1 week period gradually switched to flake food.

F_1 fish were then acclimated to near freshwater conditions (0.3 g L^{-1}; 7 mM Na^+) for a minimum of 2 weeks prior to testing. City of Miami tapwater (~1.0 mM Na^+, 1.0 mM Cl^-, 0.5 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.5 mM SO_4^{2-}, 0.8 mM HCO_3^-, pH 7.9) was mixed with filtered natural seawater to achieve the desired salinity (~7.0 mM Na^+, 8.0 mM Cl^-, 0.5 mM Ca^{2+}, 0.8 mM Mg^{2+}, 0.8 mM SO_4^{2-}, 0.8 mM HCO_3^-, pH 7.9). This water has a higher NaCl concentration than that used by Patrick and Wood (1999) which had 0.7 mM NaCl, but is similar in other respects. The higher NaCl concentration was necessary as preliminary experiments indicated this was the lowest salinity at which *C. v. variegatus* could be maintained and successfully reproduce, consistent with previous studies (Dunson et al., 1998). Fish were not fed for 2 days prior to experimental use.

Experiments on *F. heteroclitus* were performed on adult fish obtained from Aquatic Research Organisms Ltd. (Hampton, NH, USA). Fish were initially held at the University of Miami in 110-L glass aquaria under flow-through conditions with filtered natural seawater from Bear Cut, FL and fed flake food. One month prior to experimental use, fish were acclimated to City of Miami tapwater. Fish were not fed for 2 days prior to experimental use.

2.2. Low pH (4.5) experiment with C. v. variegatus

For *C. v. variegatus*, juvenile fish (175–390 mg) were exposed for 3 h to control conditions (pH 7.9, 7 mM Na^+) followed by 3 h in water adjusted to pH 4.5 with 1 N H_2SO_4, and then another 3 h recovery period again under control conditions. Eight fish were used for each 3-h treatment. This experiment was performed twice, once to characterize Na^+ uptake and once to characterize acid–base balance. In each experimental run, fish were maintained individually in 29 ml of test solution.

For the Na^+ uptake component of the experiment, ^{22}Na was added only to the control treatment for the first 3 h sampling period. At the end of the control flux, control fish were terminally sampled. Water in the flux beakers was replaced with pH 4.5 water for the remaining two treatments (pH 4.5 and recovery), fish were allowed to settle for 5 min and then ^{22}Na was added to the pH 4.5 treatment. At the end of the 3-h exposure to pH 4.5, fish from this treatment were terminally sampled for analysis of ^{22}Na, while water in the recovery treatment was replaced with control water and ^{22}Na was added for the last flux period, at the end of which fish were terminally sampled. Water samples were collected at the beginning and end of each 3 h period for measurement of Na^+ and ^{22}Na in the treatment where ^{22}Na was added.

For the acid–base balance component of the experiment, the same 8 fish were used for the entire experiment. Water samples were collected at the beginning and end of each flux period for measurement of total ammonia and titratable alkalinity. Samples for total ammonia were frozen at −20 °C and analyzed within 1 week of collection. Titratable alkalinity samples were refrigerated at 4 °C and analyzed within 18 h of collection.

2.3. Evaluation of single versus double endpoint titrations for measuring titratable alkalinity

Given the disparate results between the single and double endpoint measurements for titratable alkalinity in the low pH experiment (see Results section), we investigated potential reasons for observed differences. We hypothesized three possible reasons for the observed discrepancy between the two methods.

First, we hypothesized that when fish are exposed to low pH, they increase mucus production or they release some other buffer to the water. To test this hypothesis, we repeated the low pH experiment and collected water samples at the beginning and end of each 3-h flux period (control and low pH) for protein analysis which served as a surrogate for mucous production. Due to the small size of the fish, it was not practical to collect urine to evaluate its potential contribution to changing buffering capacity. Instead, we attempted to quantify any change in the buffering capacity of pH 4.5 water after a 3 h flux period as a way to determine whether an unknown buffer was being released to the water. To accomplish this, double endpoint titratable alkalinity was measured at the beginning and end of a 3 h flux period with *C. v. variegatus* at pH 4.5 as previously described. However, in this analysis, during the back titration with NaOH, the pH was recorded after every 10 µl of 0.02 N NaOH addition and titration curves were developed based on these data.

A second hypothesis was that ammonia excreted by the fish was not being properly accounted for using the double endpoint method (see Discussion for detailed explanation). To test this hypothesis, we conducted two experiments. First, ammonia (as NH_4Cl—i.e., as NH_4^+) was spiked into dechlorinated City of Miami tap water at nominal ammonia concentrations of 10, 50, and 100 µM. Samples were then analyzed using single and double endpoint methods (both titrated to pH 3.8) to evaluate the effects of increasing ammonia on titratable alkalinity measurements. Second, ammonia (as NH_4OH—i.e., as NH_3) was spiked into dechlorinated City of Miami tap water adjusted to pH 4.5 to see if it could be detected using either single or double endpoint techniques. For this experiment, the nominal NH_4OH concentration

spiked was 10 μM which, in preliminary experiments, was the maximum concentration that could be added while maintaining pH <5.0.

A final hypothesis was that there are simply differences between *C. v. variegatus* and *F. heteroclitus* with respect to net H^+ transport and response to low pH water. To test this, we repeated part of the experiment by Patrick and Wood (1999) testing the effect of low pH exposure on acid–base flux. The experiment was performed using small adult *F. heteroclitus* (1.00–1.82 g) in 139 ml of water dechlorinated City of Miami tapwater. Otherwise, the conditions and experimental procedure were the same as described above for *C. v. variegatus* except that Na^+ uptake was not characterized.

2.4. Analytical methods and calculations

Water pH was measured by a combination glass electrode (Radiometer pHC4000-8, Cedex, France) connected to a pH meter (Radiometer PHM201, Cedex, France). Total Na^+ in water samples was measured by atomic absorption spectrophotometry (Varian SpectraAA220, Mulgrave, Australia). Water and fish samples were measured for ^{22}Na activity using a gamma counter with a window of 15–2000 keV (Packard Cobra II Auto-Gamma, Meriden, Connecticut). Total ammonia (T_{amm}) in water was measured by a micro-modified colorimetric method (Verdouw et al., 1978). Proteins in water samples were concentrated 10× by vacuum centrifugation at room temperature and then analyzed for protein content by colorimetric assay (Micro BCA Protein Assay, Thermo Scientific, Rockford, Illinois).

Titratable alkalinity was measured by double endpoint titration to pH 3.8. Samples (10 ml volume) were sparged with N_2 for 30 min, initial pH recorded and the samples were titrated with standardized acid (HCl) to pH 3.8, sparged with N_2 for an additional 15 min and then titrated back to the initial pH with standardized base (NaOH). Thus the initial part of this protocol simultaneously provided a measurement of single endpoint alkalinity. Titration acid and base (0.02 N) was dispensed using 2 ml Gilson microburettes. Acid and base solutions were normalized against each other and all measurements corrected accordingly.

Rates of unidirectional Na^+ uptake, as measured by the appearance of ^{22}Na radioactivity in the fish (in nmol g^{-1} h^{-1}), were determined as described in Brix and Grosell (2012). Net titratable acid and ammonia transport were calculated as described in Patrick and Wood (1999).

2.5. Statistical analysis

All values are expressed as means ± SEM throughout. Comparison data were analyzed by two-tailed Student's *t*-test, except for the spiked ammonia experiments which used a paired Student's *t*-test. In cases of unequal variance a Mann–Whitney rank sum test was performed. Means were considered significantly different at $p<0.05$.

3. Results

3.1. Low pH (4.5) experiment

Exposing *C. v. variegatus* to low pH (4.5) for 3 h resulted in a significant and robust (82%) reduction in Na^+ uptake relative to the control. After fish were returned to control conditions, Na^+ uptake completely recovered and was significantly higher (41%) during the next 3 h than under initial control conditions (Fig. 1).

With respect to whole animal acid–base fluxes, as assessed by double endpoint titrations, exposure to low pH significantly inhibited apparent titratable acid uptake while T_{amm} excretion remained unchanged, resulting in a significant increase in calculated net H^+ excretion. Upon return to control conditions at the end of the 3 h acid exposure, both titratable acid uptake and net H^+ excretion returned to levels similar to the control (Fig. 2A). The results for titratable acid uptake, as analyzed using the double endpoint titration method, are essentially the opposite of those reported by Patrick and Wood (1999) for *F. heteroclitus*, using the single endpoint titration method. This observation prompted analysis of our data using the single endpoint method, data which were necessarily collected during double endpoint titrations. Analysis of single endpoint data resulted in observations qualitatively similar to those observed for *F. heteroclitus* by Patrick and Wood (1999). Titratable acid uptake (by single endpoint) was significantly increased upon exposure to low pH, and a corresponding reversal of the calculated net H^+ flux

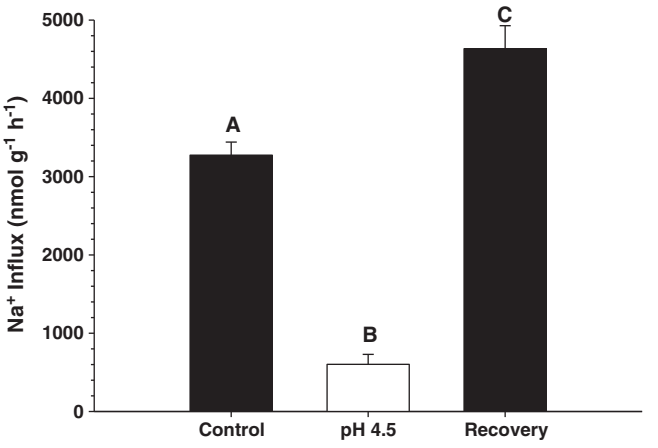

Fig. 1. Effect of exposure to low pH (4.5) and return to control conditions on Na^+ uptake in *C. v. variegatus*. Mean ± SEM (n = 8). Different letters indicate statistically significant differences between treatments ($p<0.05$).

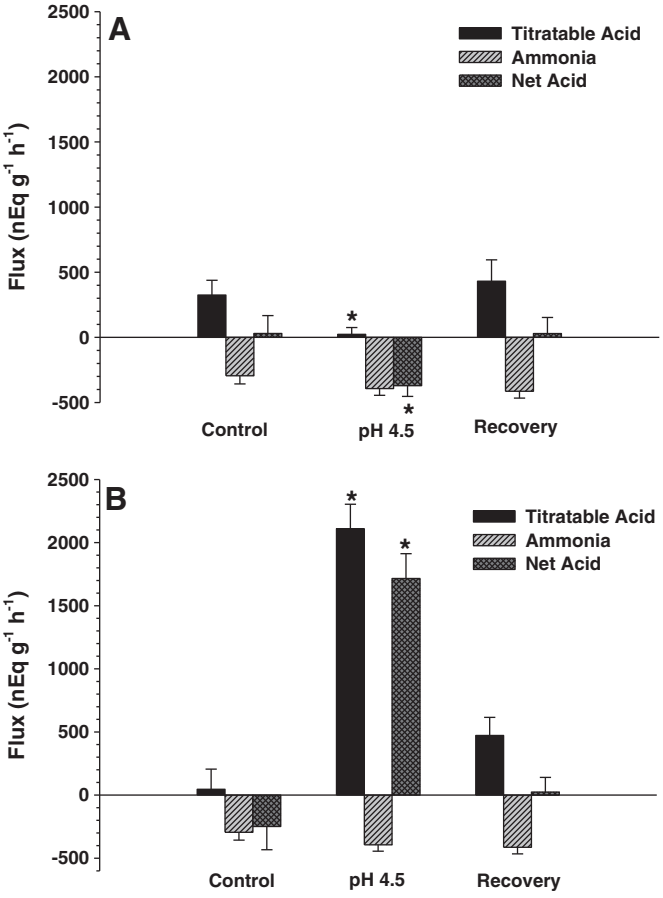

Fig. 2. Effect of exposure to low pH (4.5) and return to control conditions on acid–base fluxes in *C. v. variegatus*. A. Results based on using double endpoint titrations to pH 3.8. B. Results based on using single endpoint titrations to pH 3.8. Mean ± SEM (n = 8). * = significant difference compared to the control.

from net H^+ excretion during the control period to net H^+ uptake during the pH 4.5 exposure period occurred (Fig. 2B). Return to control conditions resulted in significant though incomplete recovery of titratable acid uptake at the end of 3 h, such that calculated net H^+ excretion was near zero.

Our repetition of the experiment with *F. heteroclitus* produced the same pattern of responses just described for *C. v. variegatus* in terms of both response to the pH 4.5 challenge and discrepancy between single and double endpoint titration methodologies (Fig. 3). By double endpoint titration, titratable acid uptake was reduced to zero, such that calculated net H^+ excretion significantly increased during exposure to pH 4.5, whereas by single endpoint titration, titratable acid uptake significantly increased, such that there was a calculated net H^+ uptake rather than excretion during this treatment. The results for *F. heteroclitus* were also qualitatively similar to those previously obtained by Patrick and Wood (1999). The only exception to this, both in comparison to *C. v. variegatus* and the earlier study on *F. heteroclitus*, was a significantly reduced T_{amm} efflux in pH 4.5 water relative to control conditions.

3.2. Evaluation of single versus double endpoint titrations for measuring titratable alkalinity

Protein secretion (measured as a surrogate for mucus secretion) rates in *C. v. variegatus* under control conditions and at pH 4.5 were significantly different with fish at pH 4.5 excreting ~50% less than control fish (Fig. 4). The fine scale titration of pH 4.5 water before and after a 3 h flux with *C. v. variegatus* revealed no significant difference in the water buffering capacity although there was a trend of higher buffer capacity in the water at the end of the 3 h exposure to pH 4.5 over the pH range of 5.0 to 8.0 (Fig. 5).

Additions of NH_4^+ (as NH_4Cl) resulted in measured T_{amm} concentrations of 2.4, 55, and 137 μM. When these samples were analyzed by single endpoint titration, a small (14 μM) but statistically significant reduction in titratable alkalinity was measured at the highest two ammonia concentrations (Fig. 6). This approximate 1% reduction in titratable alkalinity corresponds to the amount of dilution anticipated to result from spiking the solution with the ammonia stock solution. When the same samples were analyzed by double endpoint titration, the results were less consistent with a significant (14 μM) reduction in titratable alkalinity measured in the 55 μM NH_3 treatment, but not the 137 μM treatment. Overall, these changes are considered to be negligible.

Additions of NH_3 (as NH_4OH) to pH 4.5 dechlorinated tap water resulted in a measured T_{amm} concentration of 10.5 μM. The double endpoint method did not detect the addition of NH_3, with a measured titratable alkalinity of 10.8 ± 0.6 μM in unspiked water and 11.5 ± 1.0 μM in NH_4OH spiked water. Using the single endpoint methodology, measured titratable alkalinity was 145.5 ± 5.6 μM in unspiked water and 154.5 ± 2.0 μM in NH_4OH spiked water (Fig. 7). Although the difference between unspiked and spiked waters using the single endpoint method closely approximated the expected difference based on the measured ammonia concentration, measured titratable alkalinity was not statistically different between the two waters.

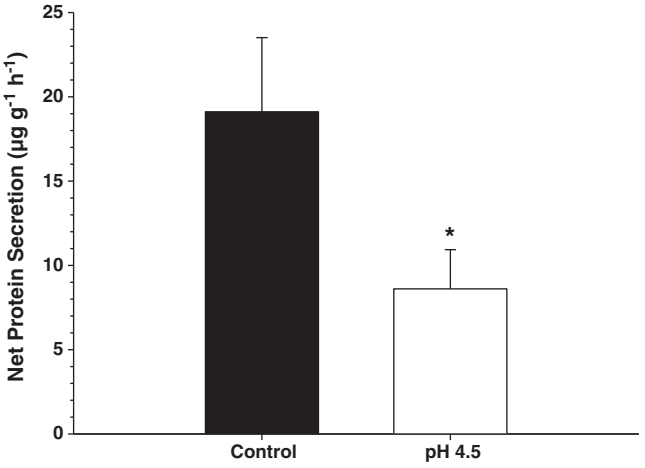

Fig. 4. Effect of exposure to low pH (4.5) on net protein secretion in *C. v. variegatus*. Mean ± SEM (n = 8). * = significant difference compared to the control ($p<0.05$).

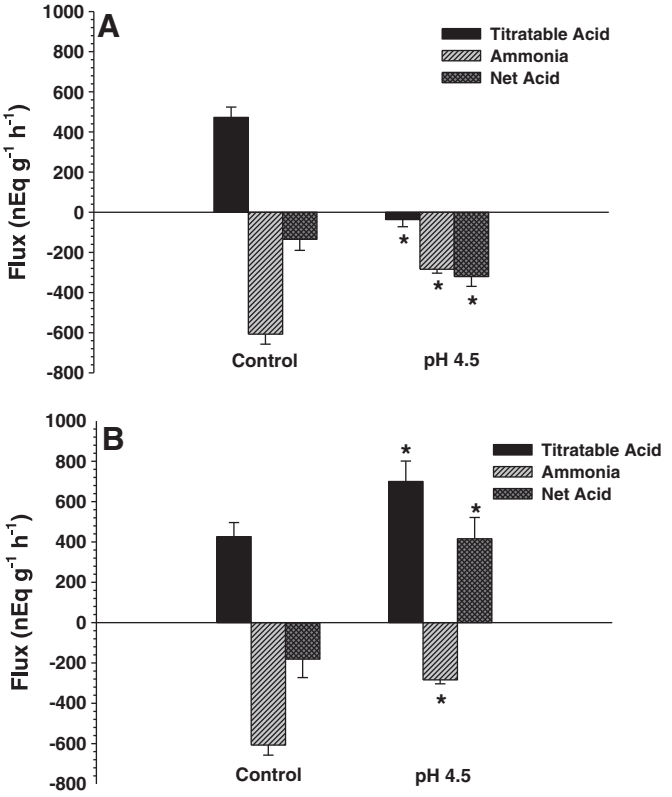

Fig. 3. Effect of exposure to low pH (4.5) on acid–base fluxes in *F. heteroclitus*. A. Results based on using double endpoint titrations to pH 3.8. B. Results based on using single endpoint titrations to pH 3.8. Mean ± SEM (n = 8). * = significant difference compared to the control.

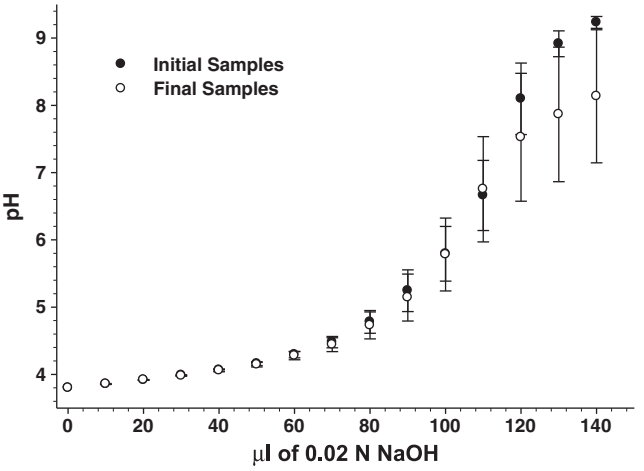

Fig. 5. The pH of water samples (10 ml) during back titration with 0.02 N NaOH at the beginning and end of a 3 h flux with *C. v. variegatus* in pH 4.5 water. Mean ± SEM (n = 4).

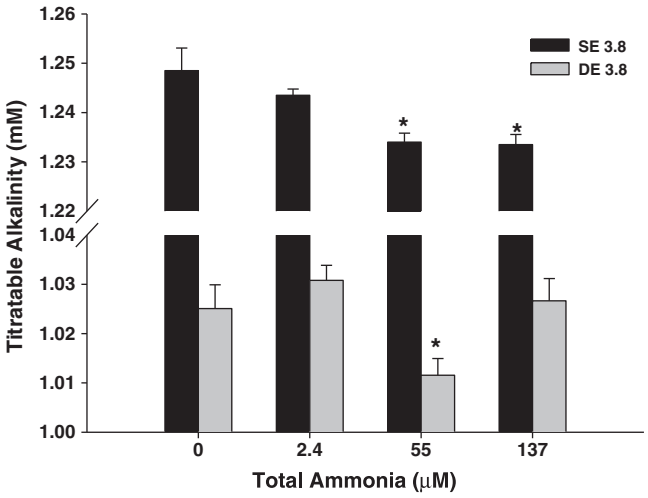

Fig. 6. Effect of experimentally increasing ammonia NH_4^+ concentration (by addition of NH_4Cl to water samples) on single and double endpoint titration methods for quantifying titratable alkalinity. *=significant difference compared to the 0 μM control ($p<0.05$). Mean ± SEM (n=4).

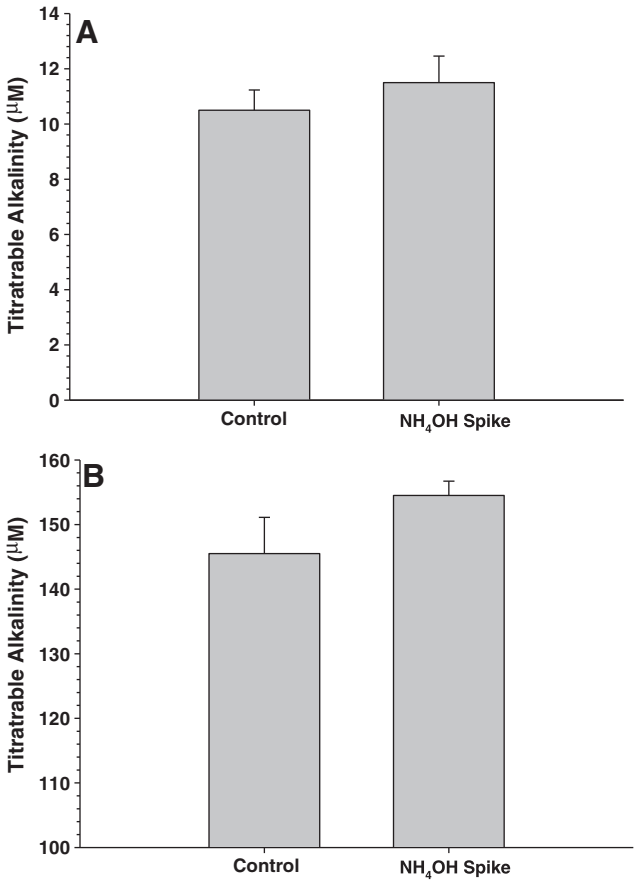

Fig. 7. Effects of experimentally increasing NH_3 concentration (by addition of NH_4OH to water samples adjusted to pH 4.5 with H_2SO_4) on single and double endpoint titration methods for quantifying titratable alkalinity. A. Measured by double endpoint titration. B. Measured by single endpoint tritration. Mean ± SEM (n=8).

4. Discussion

4.1. Single versus double endpoint titrations for measuring titratable alkalinity in low pH waters and implications for fish net H^+ transport

The measurement of whole fish acid–base flux in Patrick and Wood (1999) and many other studies is accomplished by measuring titratable alkalinity (base) and total ammonia flux, where influx (uptake) is typically reported as positive and efflux (excretion) is reported as negative values. The titratable base flux (normally efflux) can also be considered titratable acid (TA) influx by changing the sign (e.g., a titratable base efflux of −100 units is a titratable acid (TA) influx of +100 units). The sum of the TA flux and total ammonia (NH_3 and NH_4^+) flux (normally efflux), taking into account signs, represents the net H^+ flux of the fish (McDonald and Wood, 1981). Measurement of titratable alkalinity in physiological studies can be accomplished by either single or double endpoint titration and there are several variations of the method which was originally developed for measuring titratable acidity in blood and urine (Davies et al., 1920; Burton, 1980). The double endpoint method specifically was developed for renal physiology studies to measure TA-HCO_3 in urine as a single value rather than measuring each parameter separately (Hills, 1973). For both methods, a sample is initially sparged with N_2 to remove CO_2 from the system and the sample pH is recorded. In the single endpoint methodology, the sample is initially titrated to pH 4.2 with standardized HCl, sparged for 15 min to ensure removal of all CO_2 (derived from the conversion of HCO_3^- to CO_2 at this pH), and then titrated to pH 3.8/4.0. In the single endpoint methodology, the acid equivalents needed to make this titration to the fixed endpoint (pH 3.8/4.0) are equal to the titratable alkalinity of the sample. In the double endpoint methodology, the sample is titrated to pH 3.8/4.0 using standardized HCl, the sample is sparged with N_2 or CO_2-free air for 15 min to remove the CO_2 generated by the titration of HCO_3^- with HCl. The sample is then titrated back to the initial pH (pH_i) of the sample (after the initial sparge) using standardized NaOH. The titratable alkalinity of the sample is then calculated as the acid equivalents used to titrate to pH 3.8/4.0 minus the base equivalents used to titrate back to pH_i.

The single endpoint methodology assumes that samples analyzed from the beginning and end of a flux experiment have the same buffering capacity and that any measured change in alkalinity is strictly the result of net H^+ transport by the organism. In contrast, back titration of samples to pH_i in the double endpoint methodology provides an absolute measurement of alkalinity at each time, independent of water buffer capacity. Therefore, the difference in titratable alkalinity (measured by the double endpoint method) from the beginning to the end sample should provide an accurate measurement of net H^+ transport by the organism, independent from any change in water buffering capacity that may result from substances released by an animal during a flux experiment (e.g., phosphate, mucus). Thus double endpoint titration should theoretically eliminate the potential error of the single endpoint method that may arise from changing buffer capacity of the water. Hence, it can be argued that it is important to always use the double endpoint methodology when determining net H^+ transport in aquatic organisms. However, conceptually, if the buffering capacity of the water is not changed during a flux experiment, the single and double endpoint titrations should produce the same answer. Under these conditions, use of single endpoint methodology is preferred given that it is considerably less time-intensive to perform. Indeed, the single endpoint methodology is used much more frequently in fish physiology studies because of the reduced level of effort.

The analysis of net H^+ transport in *C. v. variegatus* and *F. heteroclitus* exposed to low pH raises an interesting methodological issue regarding the measurement of titratable acid flux in physiological studies. Based on single endpoint titrations, exposure to low pH water resulted in a reversal of the calculated net H^+ flux from excretion to uptake (Figs. 2B and 3B). This apparent reversal might be the result of a H^+ gradient that would favor the influx of H^+ via an NHE at low water pH (Parks et al., 2008). For *C. v. variegatus*, the presumed reversal of the NHE was much more pronounced than observed for *F. heteroclitus*. The apparent change in calculated net H^+ transport for *C. v. variegatus* was strictly a result of an apparent increase in titratable acid uptake at low pH, as ammonia efflux was unchanged by the low pH exposure. In

contrast, our results indicate the change in calculated net H^+ transport in *F. heteroclitus* was the combined result of reduced T_{amm} excretion and increased titratable acid uptake. The previous study by Patrick and Wood (1999) on *F. heteroclitus* did not observe a reduction in T_{amm} efflux at low pH, although a reduction T_{amm} efflux has been observed in several other species (Ultsch et al., 1981; McDonald et al., 1983; Hobe et al., 1984; Wright and Wood, 1985). In studies where T_{amm} efflux at low pH was monitored for an extended time, this initial inhibition is always followed by recovery and sometimes stimulation of T_{amm} efflux by 24 h of exposure to low pH (Ultsch et al., 1981; McDonald et al., 1983; Hobe et al., 1984). The mechanism underlying this short-term inhibition followed by recovery is unknown.

An alternative perspective, based on results using double endpoint titrations, is that when exposed to low pH, the net H^+ transport for *C. v. variegatus* changed from near zero to an excretion rate of 371 nEq g^{-1} h^{-1}. In this case, the calculated change in net H^+ transport was the result of a significant reduction in titratable acid uptake (or decreased base excretion) from 325 to 23 nEq g^{-1} h^{-1} rather than the observed increase in the same parameter based on single endpoint titrations. For *F. heteroclitus*, the response was qualitatively similar, although the increase in net H^+ excretion was attenuated somewhat by the observed reduction in T_{amm} efflux.

Which of these perspectives is correct? We postulated four possible confounding factors could contribute to the inconsistent results between the two methodologies. First, we hypothesized that observed differences were simply the result of inter-specific differences between *C. v. variegatus* and *F. heteroclitus* in response to low pH exposure. As we just described, although there do appear to be differences in the magnitude of change in net H^+ transport between the two species, qualitatively, they responded similarly based on both single and double endpoint methodologies.

A second hypothesis was that fish increased mucus excretion or excreted mucus of a different composition as a stress response and that the mucus increased the buffer capacity of the water during the titrations. The proton binding sites on fish mucus added to the water by the fish during the flux period would consume additional acid during titrations and be interpreted as an increase in titratable alkalinity using the single endpoint methodology, but not by the double endpoint methodology. Our results indicate this was probably not the case, as mucus production appears to be reduced in low pH water (Fig. 4). This of course does not rule out the possibility that fish were secreting some other buffer into the water or that the mucus produced at low pH had a greater buffering capacity. For example, urinary phosphate excretion has been shown to be stimulated in rainbow trout exposed to pH 4.2 water (McDonald and Wood, 1981). However, careful titration of initial and final water samples after a 3 h flux at pH 4.5 for *C. v. variegatus* indicated there was no significant change in buffering capacity between the two sampling periods, although there was a trend of increasing buffering capacity at pH 7.5 and higher (Fig. 5). While this may suggest release of a buffer by the fish, the pK of this unknown substance appears to be relatively high and would not influence single versus double endpoint titrations in pH 4.5 water.

A third hypothesis was that ammonia secretion by fish, a large fraction of which would be present as NH_4^+ in the sample, was being titrated by NaOH during the double endpoint titration (Lemann and Lennon, 1966). This could lead to an erroneously low measurement of titratable alkalinity at the end of the flux period, resulting in a correspondingly lower estimate of titratable acid uptake. However, both the single and double endpoint titration methods were relatively insensitive to experimental NH_4^+ additions (as NH_4Cl) to water samples even at concentrations well above those typically measured during a 3-h flux period. There was no significant increase in measured titratable alkalinity concentrations even in the highest ammonia treatment (137 μM) using the double endpoint method (Fig. 6). Given that the maximum measured ammonia concentration in our experiment was 22 μM, it is unlikely that titration of NH_4^+ explains the discrepancy between the two titration methods.

The final hypothesis was that at low pH, the double endpoint methodology would fail to properly account for ammonia excretion if it occurred in the form of NH_3. The theoretical basis for this hypothesis is as follows. In the calculation of net H^+ flux, H^+ and NH_4^+ excretion by the fish contribute to net H^+ efflux, but only H^+ excretion is measured by the titration technique. Ammonia excretion as NH_3 is not H^+ excretion but is measured as titratable alkalinity by the titration technique. In the subsequent calculation of net H^+ transport, the fraction of T_{amm} excretion which is NH_3 is cancelled out by the measurement of NH_3 as titratable alkalinity.

Using the single endpoint methodology, initial and final samples collected from the flux experiment are titrated to pH = 3.8. The NH_3 excreted by the fish into the water in the final sample is accounted for by the titratable alkalinity measurement regardless of the pH range. If the flux is performed at circumneutral pH, NH_3 reacts with CO_2 and H_2O to form NH_4HCO_3. The HCO_3 is actually what is titrated. If the flux is performed at acidic pH, well below the pK (6.1) of the CO_2/HCO_3 equilibrium, then NH_4HCO_3 cannot form, instead NH_3 is simply protonated, removing an H^+ ion from the water and essentially forming NH_4OH. In this case, the OH^- is actually what is titrated. In either scenario, NH_3 excretion is measured as titratable alkalinity by the single endpoint method.

In contrast, using the double endpoint methodology, initial and final samples are titrated to pH 3.8, sparged with N_2 to remove CO_2 generated by the titration of HCO_3^- at low pH, and then titrated back to starting pH to measure any changes in water buffering capacity. If the flux is performed at circumneutral pH, NH_3 excreted by the fish is again measured as titratable alkalinity and properly accounted for in calculation of net H^+ excretion as described above. However, if the flux is performed at acidic pH, NH_4OH rather than NH_4HCO_3 is titrated. Because, OH^- will not be converted to CO_2 and therefore not removed by N_2 sparging at pH 3.8, the NH_3 excreted by the fish will not be measured as titratable alkalinity using the double endpoint methodology.

We supported these theoretical differences between the two methodologies by measuring samples spiked with 10 μM NH_4OH into pH 4.5 water. As would be expected if the above hypothesis is correct, the mean measured alkalinity of this water using the single endpoint method was 10 μM higher (not statistically significant), while there was no change (<1 μM) in alkalinity using the double endpoint method (Fig. 7).

Given the above, it is possible to correct for this problem with the double endpoint methodology, if one makes an assumption regarding the fraction of T_{amm} excretion that is NH_3. The maximum possible correction would be to assume 100% of T_{amm} excretion occurred as NH_3 at low pH and was therefore not detected by the double endpoint titration technique. This assumption is based on recent studies indicating that Rh glycoproteins are the principal mechanism of T_{amm} excretion in fish gills (Nawata et al., 2007) and that they transport ammonia as NH_3, not NH_4^+ (Nawata et al., 2010). If we make this maximum possible correction, the corrected double endpoint titration results and corresponding recalculated net H^+ transport estimates for *C. v. variegatus* (23 ± 53 nEq g^{-1} h^{-1}) and *F. heteroclitus* (-37 ± 35 nmol g^{-1} h^{-1}) are similar and indicate no significant net H^+ transport at pH 4.5.

This result still differs significantly from the single endpoint titration results (Figs. 2B and 3B), which indicated net H^+ excretion is not only inhibited, but was actually reversed to significant net H^+ uptake at low pH. There are two possible sources for this apparent net H^+ uptake. First, it is possible that NHE was operating in reverse, facilitating H^+ uptake (Parks et al., 2008). However, when one considers that operation of an apical NHE in reverse would rapidly deplete intracellular Na^+, inhibition rather than reversal of the NHE seems more likely. Inhibition of NHE at low pH agrees with observations of net H^+ transport using the recalculated double endpoint titration results.

Alternatively, it is also possible that while NHE is not reversed, base (e.g. HCO_3^-) excretion is increased or up-regulated at low pH, which again would appear as net H^+ uptake. This scenario would be consistent with observations using the single endpoint methodology and indicate there is still an unidentified problem with the double endpoint methodology. While this is possible, we know that in *Fundulus*, branchial Cl^- uptake and Cl^-:HCO_3^- exchange are negligible in the ~1 mM NaCl water used in our experiment, but Cl^- uptake increases at >2 mM Cl^- in the water (Patrick et al., 1997; Patrick and Wood, 1999). It is unknown whether *Cyprinodon* are capable of Cl^-:HCO_3^- exchange, although we do know that when water NaCl concentration is increased from 2 to 7 mM, an increasing fraction (from 10 to 40%) of Na^+ uptake appears to occur via an apical NKCC (Brix and Grosell, 2012). This is at least suggestive that branchial Cl^-:HCO_3^- exchange is limited in both species.

4.2. Effect of low pH on Na^+ uptake and ammonia excretion in *C. v. variegatus*

Although ultimately not the main focus of this study, our initial objective was to evaluate the effects of low pH on Na^+ uptake, ammonia excretion, and acid–base balance in *C. v. variegatus*. Studies on zebrafish and several other fish species (*Oryzias latipes*, *Oreochromis mossambicus*, *Tribolodon hakonensis*) have indicated that they utilize an apical NHE-3/Rh glycoprotein metabolon for Na^+ uptake in freshwater (Hirata et al., 2003; Wu et al., 2010; Furukawa et al., 2011; Kumai and Perry, 2011; Kumai et al., 2011; Shih et al., 2012). Unlike *F. heteroclitus* where Na^+ uptake is inhibited at low pH, Na^+ uptake is enhanced when these species are exposed to low pH water. It is hypothesized that increased NH_3 excretion at low pH shifts the intracellular NH_3-NH_4^+ equilibrium toward the formation of NH_3 and H^+, providing a relatively high concentration of protons for the continued function of NHE-3. Hence, measurement of Na^+ uptake at low pH potentially provides a diagnostic of the presence of NHE-2 versus NHE-3.

Our results indicate *C. v. variegatus* is similar to *F. heteroclitus* as low pH water significantly reduced Na^+ uptake but had no significant effect on ammonia excretion (Figs. 1 and 2). Considering that we previously demonstrated that *C. v. variegatus*, like *F. heteroclitus*, lack an apical H^+ pump (Brix and Grosell, 2012), these results are consistent with NHE-2 being the NHE isoform involved in apical Na^+ uptake for this species.

5. Conclusions

This study demonstrated that when *C. v. variegatus* is exposed to low pH, Na^+ uptake is inhibited and ammonia excretion unchanged, suggesting this species is likely using NHE-2 to facilitate apical Na^+ uptake in freshwater. This tentative conclusion awaits confirmation through gene expression and/or immunohistochemistry. More importantly, our study revealed a significant discrepancy between the single and double endpoint titratable alkalinity methods when used to characterize net H^+ transport at low pH in both *C. v. variegatus* and *F. heteroclitus*. Part of this discrepancy can be accounted for by the inability of the double endpoint methodology to measure NH_3 excretion at low pH, but even after making the maximum possible correction for this error, a significant discrepancy remains between the two methods.

Historically, most studies have relied on either single endpoint titrations (McDonald and Wood, 1981; Ultsch et al., 1981; Evans, 1982, 1984; Goss and Wood, 1990; Patrick et al., 1997; Patrick and Wood, 1999; Edwards et al., 2005; Georgalis et al., 2006) or double endpoint titrations (Taylor et al., 2007; Genz et al., 2008; Brix et al., 2011) to characterize net H^+ transport in aquatic organisms. To the best of our knowledge only two previous studies compared the two methods (Bucking and Wood, 2008; Cooper and Wilson, 2008) and both found congruence between the methods. Both of these studies were performed on rainbow trout in freshwater at circumneutral pH and after demonstrating close agreement between the two methods, used single endpoint titrations for their analyses.

We suggest that comparing results from single and double endpoint titrations may be a reasonable standard practice to adopt when conducting studies on a new species or under environmental conditions that depart significantly from previously validated conditions. Conditions of extreme pH and conditions where compounds with buffer capacity (e.g., fecal matter, regurgitated food, mucus, etc.) may be released by the experimental animals call for a careful comparison of the two methods. With respect to fish exposed to acidic conditions, the methodological issues identified in the current study are problematic and not fully resolved. Additional research is needed to identify the most appropriate method for measuring TA flux in aquatic organisms at low pH before we can reliably characterize net H^+ transport under these conditions.

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