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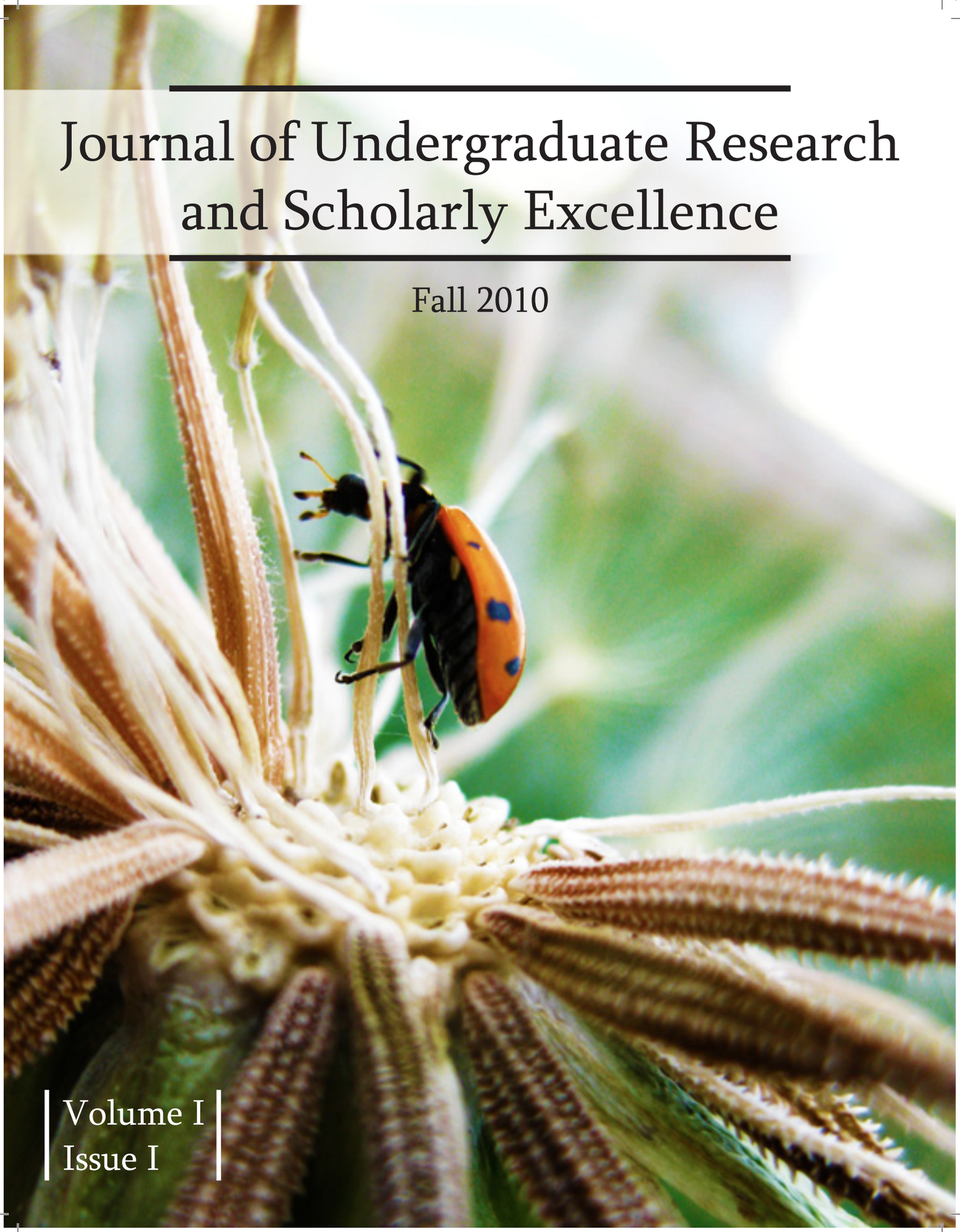

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A letter from the Editor

The first issue has arrived! It has been almost a year since we founded JUR, and after months of marketing, sorting through submissions, and working with referees, the premier issue is ready at last. With its publication, I understand that JUR has finally left the nest, so to speak. What started out as a small group of undergraduate students trying to fill an important niche in publishing has finally, finally taken off to become something greater than they ever expected.

I am proud to say that JUR truly is a journal for undergraduates by undergraduates. It provides the opportunity for students to publish their own work and showcase their talents in any academic subject. This is a journal where research, poetry, reviews, and art can be featured side-by-side as a testament to the scholarly power of undergraduate students.

I'd like to thank the staff of JUR for their hard work that has finally come to fruition. Also, I have to once again recognize our referees; without their help, JUR would not be possible. And thank you, kind reader, because whoever and wherever you are, by having this issue in your hands you are helping us deliver on our promise to undergraduate students.

Most Sincerely,

Jessica Egner
Editor in Chief
Journal of Undergraduate Research and Scholarly Excellence

JUR is housed at Colorado State University in the Office for Undergraduate Research and Artistry at the Institute for Learning and Teaching.

Journal of Undergraduate Research and Scholarly Excellence Staff

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Antibiotic Resistance Profiles for the Opportunistic Pathogens *Burkholderia oklahomensis*, *Burkholderia ubonensis* and *Burkholderia vietnamensis*

By Erin Breland with Herbert P. Schweizer and RoxAnn Karkhoff-Schweizer
Colorado State University

Abstract

Various bacteria belonging to the genus *Burkholderia* are recognized as emerging pathogens. Some of these have not yet been well studied. Here we determined the antibiotic susceptibility profiles of the three opportunistic pathogens *Burkholderia oklahomensis, B. ubonensis* and *B. vietnamensis*. All three bacterial species show resistance to carbenicillin, erythromycin and gentamicin and, with the exception of *B. ubonensis*, are most susceptible to tetracycline, trimethoprim and the carbapenems imipenem and meropenem. *B. ubonensis* was consistently the most resistant of the three bacteria and also exhibits increased resistance to tetracycline and carbapenems. Availability of antibiotic resistance profiles for these bacteria will facilitate future clinical, environmental and genetic studies with these opportunistic pathogens.

Introduction

There are over 40 different species of *Burkholderia* commonly found in surface soils and groundwater worldwide.[1] Although many of these species exhibit intrinsic antibiotic resistance, few have been studied for their antibiotic resistance profile. Understanding bacterial antibiotic resistance is a key factor in understanding the resistance mechanisms innate to bacteria. Developing antibiotic resistance profiles is also crucial for clinical, environmental and genetic studies.

Like many Gram-negative bacteria, mounting evidence indicates that multidrug efflux pumps of the resistance nodulation cell division (RND) superfamily play an important role in the multidrug resistance of *Burkholderia* species. *Burkholderia cenocepacia* expresses several RND pumps that contribute to drug resistance [2, 3]. Likewise, most *B. pseudomallei* strains are intrinsically antibiotic resistant due to AmrAB-OprA [4,5] and BpeAB-OprB [6, 7] efflux pump expression. *Burkholderia pseudomallei* is classified by the Centers for Disease Control and Prevention (CDC) as a bio-safety level (BSL) 3 organism and category B biothreat agent. *Burkholderia pseudomallei* is endemic to Southeast Asia, Northern Australia and other tropical and subtropical regions of the world.[8] In endemic regions it is of clinical importance as the etiologic agent of human melioidosis, a progressive disease with high mortality rates.[9,10]

Other *Burkholderia* species that have been studied include the BSL-2 *B. gladioli* and the *B. cepacia* complex (BCC). As opportunistic pathogens, these soil and water pathogens typically only affect immunocompromised or cystic fibrosis patients. [11, 12] The BCC contains at least ten closely related strains of *Burkholderia* species that are phylogenetically differentiable, but are phenotypically indistinguishable. Other species of *Burkholderia* are also intrinsically antibiotic resistant but there is little known or published for these organisms. The CDC lists the three *Burkholderia* species, *B. oklahomensis, B. ubonensis,* and *B. vietnamensis* as BSL-2 opportunistic pathogens. *B. oklahomensis* C6786 was isolated in 1973 from a wound infection after a farming accident in Oklahoma and initially named as the "Oklahoma" strain of *B. pseudomallei*. It was later determined, through gene sequencing, to be a novel species, *B. oklahomensis*.[13] Three more identical isolates were identified as sharing the same typical *Burkholderia* phenotypical features.[13] Four isolates have been obtained for an environmentally important species *B. ubonensis* that has been speculated to be the tenth genomovar of the BCC. Little is known about this bacterium other than it is found in surface soils and has not to date been associated with human infections.[14] The fifth genomovar of the BCC is known to be *B. vietnamensis*. *Burkholderia vietnamensis* is commonly isolated from surface soils and ground water and has been studied as a plant growth promoting bacterium and bioremediation agent for aromatic hydrocarbons.[15] It is a Gram-negative rod, motile and aerobic. *Burkholderia vietnamensis* is an opportunistic pathogen in humans often affecting cystic fibrosis patients.[16] As a prelude to future studies, we determined the antibiotic susceptibility profiles of *B. oklahomensis, B. ubonensis and B. vietnamensis*.

Materials and Methods

Bacterial strains and growth.

The strains used in this study were the clinical *B. oklahomensis* isolate C6786 (laboratory stock number B94),[13] the environmental *B. ubonensis isolate* A1301 (laboratory stock number B180),[14] and *B. vietnamensis* H4102 (laboratory stock number B122)(obtained from Dr. Alex Hoffmaster, CDC Atlanta). All strains were grown at 37oC. Before use, these strains were struck for single-colonies on Lennox Luria-Bertani (LB) [17] agar (MO BIO Laboratories, Carlsbad, CA) plates. Single colony isolates were inoculated into Lennox LB broth in preparation for minimum inhibitory concentration (MIC) tests. For MIC tests, bacteria were then inoculated into 4 ml of Mueller-Hinton broth (MHB; Becton Dickinson, Sparks, MD) and grown overnight. The next day, the overnight culture was diluted into MHB and grown to log phase (A600nm~0.7). This culture was then diluted in sterile saline and adjusted to the density of a 0.5 McFarland equivalence turbidity standard (Remel, Lenexa, KS).

Antibiotics.

Table 1 lists the antibiotics used in the study by function, class and common name. Stock concentrations of antibiotics were made following standard protocol at concentrations of either 4,096 µg/ml or 32,768 µg/ml depending on the strain being tested and antibiotic. Antibiotics were either purchased as powders from Sigma, St. Louis, MO (carbenicillin, gentamicin, erythromycin, and tetracycline) or immobilized on Etest® strips from AB BIODISK, Solna, Sweden (trimethoprim, imipenem, meropenem).

MIC determinations.

A set of standard conditions set by the Clinical and Laboratory Standards Institute (CLSI) [18] must be followed when defining antibiotic resistance profiles. All procedures were performed in a biosafety cabinet (BSL-2+ conditions). The two methods used for MIC determinations were two-fold serial dilution in microtiter plates and Etest®.

Bactericidal			Bacteriostatic	
Class	**Representative tested**		**Class**	**Representative tested**
Aminoglycosides	Gentamicin		Tetracyclines	Tetracycline
Penicillins	Carbenicillin		Sulfonamides	Trimethoprim
Carbapenems	Meropenem Imipenem		Macrolides:	Erythromycin[1]

Table 1. Antibiotics tested in this study. Antibiotics are listed by function, class, and common name.

The two-fold serial dilution method utilizes 96-well plates and two-fold serial dilutions of antibiotic concentrations. Each test was performed in triplicate with positive and negative controls. An antibiotic stock was made at twice the highest desired initial concentration of antibiotic to be tested in the dilutions. The antibiotic stock solution was distributed in 100 µl aliquots into the first column of the first four rows of a 96-well plate. Rows one through three constituted one triplicate experiment for one MIC test of a specific antibiotic. Rows four and five were controls to test the antibiotic stock and bacterial growth respectively to verify negative and positive growth controls. These controls allow visualization of any random growth that may occur in the wells and characteristics of the antibiotics (precipitate, color change, etc.).[18]

Mueller-Hinton broth was distributed (50 µl per well) to columns 2-12 of rows 1-5 and column 1 row 5. The antibiotic was then diluted two-fold throughout the plate. This was achieved by taking 50 µl from the first well in column 1, rows 1-4, into the second column and mixing. This was then followed by taking 50 µl from this well into the next well, mixing and so on. Lastly, 50µl aliquots were removed from the last wells in rows 1-4. Next, prepared bacterial inoculant (50 µl; 0.5 McFarland turbidity standard) was added to each well in rows 1-3 and row 5. The plates were incubated for 24 h 37°C and wells visually examined for growth. Growth in any well is considered a button of growth. The first concentration of antibiotic where no button of growth is visible is regarded the MIC in µg/ml.

Etest® strips are pre-loaded with a gradient of decreasing antibiotic concentrations. Each test was done in triplicate with three plates per test. Bacterial inoculant was prepared the same way as in the two-fold serial dilution method but once adjusted to a 0.5 McFarland standard, a sterile cotton swab was used to transfer the saline inoculant to MHA plates. The plates were struck for confluency (inoculant fully covers the plate), which produces a lawn of growth covering the agar. Etest® strips were placed carefully on the plate with sterile forceps avoiding bubbles and displacement. The plates were then incubated at

37°C for 24 hours. Etest® strips depict a different form of susceptibility showing an area of inhibition on a confluent lawn of growth around the strip labeled with antibiotic concentrations. Etest® MIC results are read by determining the end point of growth adjacent to the strip as seen by the naked eye for bactericidal antibiotics. Trimethoprim is bacteriostatic and thus Etest® protocols require that the results be read at 80% inhibition or the first point of significant inhibition as judged by the naked eye and not where the lawn is completely cleared.[18]

Results and Discussion

The results of multiple trials done in triplicate for accuracy have been condensed to arrive at the estimated minimum inhibitory concentration (MIC) values shown in Table 2. Carbenicillin was the only antibiotic for which MIC tests gave moderately varied results. However, the results were always greater or equal to the value listed in Table 2. For Etest® values that were between two markings on the strip, the upper value was used in accordance to the Etest® reading guide.

The CLSI determines breakpoints based on organism and antibiotic. Bacteria can be susceptible, intermediate, or resistant to antibiotics at different concentrations. After multiple trials (three to four) of each test performed in triplicate, we were able to confidently assign MIC values to each strain for all seven antibiotics used. Similar patterns of resistance and susceptibility can be seen between the three strains with respect to the different antibiotics tested. According to the CLSI breakpoint values for *Burkholderia* species, it can be concluded that *B. oklahomensis* exhibits resistance to carbenicillin, gentamicin and erythromycin, but susceptibility to tetracycline, trimethoprim, imipenem and meropenem. *Burkholderia ubonensis* exhibits resistance to carbenicillin, gentamicin, erythromycin, tetracycline, imipenem and meropenem, and is only susceptible to trimethoprim. *Burkholderia vietnamensis* exhibits resistance to carbenicillin and erythromycin, and is susceptible to tetracycline, trimethoprim, imipenem and meropenem, with possible resistance to gentamicin.

Drug	MIC (µg/mL)[1]		
	B. oklahomensis C6786	*B. ubonensis* H4102	*B. vietnamensis* A1301
Carbenicillin	256	1,024	>512
Gentamicin	32	256	4
Erythromycin	128	64	32
Tetracycline	2	64	2
Trimethoprim	0.5	0.19	0.38
Imipenem	0.094	8	0.19
Meropenem	0.19	3	0.38

Figure 2. Antibiotic Resistance Profiles for *B. oklahomensis*, *B. ubonensis* and *B.vietnamensis*. From the results of multiple trials, the following MIC values were determined. The protocols for MIC determination were performed as listed in the Materials and Methods section.
[1] MICs for trimethoprim, imipenem, meropenem and trimethoprim were determined using Etest®; all others established using the two-fold serial dilution method.

The values obtained were compared to previously determined values in our laboratory for *B. gladioli* pathovar *cocovenenans* and *B. pseudomallei*, where *B. gladioli* pathovar *cocovenenans* was found to be resistant to carbenicillin, erythromycin, tetracycline and imipenem and susceptible to gentamicin, trimethoprim and meropenem (unpublished observations). Burkholderia pseudomallei was determined to be resistant to carbenicillin, gentamicin and erythromycin and susceptible to tetracycline, trimethoprim, imipenem and meropenem.[7]

Generally, each species tested was resistant to older forms of penicillin drugs (carbenicillin) and appears to be susceptible to newer β-lactam antibiotics (imipenem and meropenem). However, *B. ubonensis* also shows resistance to imipenem and meropenem. In *B. pseudomallei*, resistance to older β-lactams is due to expression of the chromosomally encoded PenA β-lactamase, which shows little activity against imipenem and meropenem.[19, 20] (D.A. Rholl and H.P. Schweizer, unpublished observations). *Burkholderia ubonensis* either encodes a similar enzyme with an extended substrate spectrum or the observed increased imipenem and meropenem resistance is due to another mechanism.

Of the three species examined in this study, *B. ubonensis* was consistently more resistant. All three bacterial species show resistance to carbenicillin, erythromycin and gentamicin and this resistance pattern is also observed with *B. pseudomallei* and *B. gladioli* pathovar *cocovenenans*. It has been well established that intrinsic aminoglycoside and macrolide resistance in *B. pseudomallei* is due to expression of the AmrAB-OprA efflux pump.[4, 5] While tempting to speculate that

the same pump also operates in the *Burkholderia* species examined in this study, this remains to be experimentally confirmed.

Availability of the antibiotic resistance profiles determined in this study will facilitate future clinical, environmental and genetic studies with these opportunistic pathogens.

Acknowledgements

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Man, Woman, and Nature, Now

By Matthew J. Young

St. Lawrence University,NY, RMSSN Academy Conference

During the third week of this past May, something original took shape in the shortgrass steppe east of Fort Collins, Colorado. Yes, a funnel cloud that could have crafted itself into a tornado did form during the week, darkening the whole Western sky. However, the other momentous event of the week involved the gathering of 30 undergraduate students ranging from California to New Hampshire, brought together to the steppe. They came not only to learn about the ecology and relationships of the landscape surrounding them, but also to learn about each other and about implementing holistic scientific and policy practices towards sustainability issues of the future.

From the smallest of entities in the pine bark beetle killing off acre upon acre of coniferous forests to the systemically complex realm of global climate change, no issue was ignored. Arguably most key of all, this gathering, the 2010 Summer Academy of the Rocky Mountain Science & Sustainability Network (RMSSN) represented a new path to grow in new modes of environmental leadership and ecosystem awareness. With a January 2010 grant from the National Science Foundation to further bolster the mission of the RMSSN, the Summer Academy out on the Colorado steppe could come to full fruition. Ultimately, we are at the threshold of bridging humans and nature now with exciting possibilities in the months and years to come, cultivating relationships in a human ecosystem all its own that will feed into the one unifying ecosystem all around us: Earth.

One of the powers of the Rocky Mountain Science & Sustainability Network visible in the moments of brainstorming sessions, field trips, or dance parties during the third week of May on the Colorado rangelands continues to grow on me here in the rolling green mountains and vibrant verdancy of Vermont. In no small way of group dynamics and interconnectedness, RMSSN weeks later and thousands of miles away still stands out as an ecosystem all its own. I imagine that even alluding to each individual's niche (NT, NF and various other temperaments fused with differing personalities), interrelationships across these niches, and even our own distinctive and now global biomes, this whole notion slowly coalesces.

With members working from Oregon forests to New Hampshire cities to Latin American islands this summer, RMSSN presents all of us in the community a timely yet timeless challenge. Yes, a challenge that even I working in such a diverse, partnership-driven, and community-focused place as Marsh-Billings-Rockefeller National Historical Park (MBRNHP) in Woodstock, Vermont am facing. Ultimately, how can we bridge different stakeholders, different values systems together in such a way to not just bolster eco-literacy, but to synthesize elements of organic leadership and environmental stewardship in the myriad communities we are working within? If I may, I'd like to pose a thought experiment from a book I found in the depths of a research library at MBRNHP a few days ago and tying back to how RMSSN just might fit in.

Envision a country where erratic weather patterns induced by climate change alter sleepily flowing rivers into sheer conduits of silt-laced water, carrying away soil and people alike. Envision a country where political factions and corporate conglomerates find themselves in a perpetual tug-of-war over their responsibilities to saving and restoring the health of their communities. Envision a country where the populace is divided over the future direction of the country's livelihoods. Welcome to the United States...in 1847. Intriguing how some things never change, whatever temporal landscapes our ecosystems are in.

George Perkins Marsh, author of Man and Nature (arguable America's first conservation book) and one of the founding figures at MBRNHP, witnessed all the above trends. From hillside floods coming off of clear-cut ranges in Vermont to the federal government's role in homesteading on the ever burgeoning frontier to growing dissension on how to extract or preserve tracts of land for future generations, Marsh worried. Yet, he transformed his initial worries even before writing Man and Nature in 1864 into incisive speeches given before crowd of hundreds, if not thousands, of people. In 1847, Marsh delivered these lines to a Cambridge, Massachusetts Phi Beta Kappa society:

> "We need not be recluses devoted to quiet literary research, but rather live and act in the busy whirl of the great world, share the anxieties and the hazards of commerce, the toils and the rivalries of the learned pro-fessions, or the fierce strife of contending politi-

cal factions...to then be refreshed by the voice of the Muses." [1]

A call to action by a man of action. Yet, reading through these lines, I wondered over what Marsh could have alluded to by "the voice of the Muses." Then, over the last couple of days, corresponding with fellow RMSSN members, the members themselves became the muses, their stories & voices of adapting to different environments and starting to connect to their summer communities. Individually, our abilities to inspire and drive ourselves and others in our leadership missions, Muse-like, manifested in a variety of ways. Some of our colleagues spoke with conviction on climate change science, challenging us to build confidence in our voices for sustainability. Other colleagues knew how best to lay out transects for wildlife surveys, engaging some of us with not so analytical minds to be more deliberate, calculating, sharp in our thinking.

Yet, the most tangible example of ecological Musing in action came during our last full day of team-building dynamics at Pingree Park, at the foot of snow-laced Comanche Peak. One of our colleagues, Eric from Mexico, came reluctantly at first to the high ropes course, with climbing walls and other wooden structures rising story after story from the ground below. However over the course of the sunny, gusty May afternoon, even Eric ascended to the "Leap of Faith," where one essentially climbs a +30-40 foot telephone pole— only to jump no less than ten feet across open air to grasp onto a metal bar suspended in very trapeze-artist fashion. Granted, harnesses, helmets, and nylon ropes are involved, yet even such equipment never fully compensates the need for trust, for self-confidence, for a leap of faith to reach that bar.

Eric proved no exception, and for almost an hour, he stood anxiously on top of the telephone pole. Meanwhile, many of us broke away from the other rope challenges and began cheering him on in English and Spanish. Collective, rallying trust to hopefully inspire Eric to build in himself his own self-confidence, in those operating the "leap," in all of us, and in his own niche of the moment to jump. Eventually, Eric jumped...and not only jumped, but grasped onto the bar. As one Zen quotes reads, leap and the net will appear— mentally and psychologically, if not physically. Whether or not Eric would have jumped of his own volition without all of us encouraging him on is debatable, but the reciprocity of us hopefully realizing his potential and niche to jump also inspired us, in our own potential and niches in conservation leadership in the future, whatever challenges arise. Leap and the net will appear.

This appears to be the beauty of RMSSN as a sustainable ecosystem with all of its niches the globe over— the ability to continue to evolve, morph, and build upon confidently what is arguably the most solid, human-based homeostasis I've seen anywhere. In nature, homeostasis seeks nothing less than "regulation of an internal environment and always working towards a stable, constant condition."[2] Granted, such homeostasis is also built upon illuminating moments of dynamism, however brief or prolonged they may be. Furthermore, such homeostasis in dynamic niches can be realized through recognizing and building relationships with muses all around us, be they fellow friends & colleagues, birds flying and feeding in the Colorado Rockies, or in the ecosystems of the Colorado Rockies themselves and beyond.

To highlight such homeostasis weaving in times of dynamism, here's some of my summer agenda in Vermont. At MBRNHP, I'll be juggling tasks ranging from teaching environmental education to Woodstock high school students through a newfound Use-Intake program, drafting and creating new website articles and pages for outreach for the park to the rest of the cyber-world, and even getting down and dirty in community gardens with the Ottauquechee Community Partnership, amongst other day-to-day possibilities. Here, dynamic agendas work within the homeostatic whole of connecting communities to realize their full leadership, stewardship, and organic potential. Furthermore, to have a network like RMSSN for critiques, for support, or for just welcome perspective during especially longer days reinforces ecosystem themes working and living this summer and beyond. To take the lessons from Marsh, Man and Nature, and RMSSN, I'd like to take one quote to meditate on from Vermont nature writer, John Elder, who wrote about a local landmark, Mount Tom, immediately before the park's inception. He wrote:

"We must conceive of stewardship not simply as one individual's practice, but rather as the mutual and intimate relationship extending across generations, between a human community and its place on earth." [3]

Wherever we are, with RMSSN all around us, let us conceive and build such a place.

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Elucidation of Phosphoproteins Involved in the Renal Cellular Response to Acute Metabolic Acidosis

By Emi Okayasu with Dana M. Freund, Jessica E. Prenni, and Norman P. Curthoys
Calvin College, Grand Rapids,MI with CSU Department of Biochemistry

Abstract

Metabolic acidosis is caused by a decrease in plasma pH and the concentration of bicarbonate buffer in the blood. The proximal tubule cells of the kidney mediate the body's response to this pH stress by increasing the catabolism of glutamine, effectively producing bicarbonate for export to the bloodstream.[1] Though this response is well characterized, the mechanisms by which this response is activated are unknown.[2] In order to investigate the various pathways which might be involved in initiating this response, a proteomic analysis of the phosphoproteins expressed by proximal tubule cells modeling metabolic acidosis was done. The phosphoproteins expressed by these cells after a 24 hour treatment at pH 6.9 were run on 2D gels, and stained with a quantitative phosphoprotein stain and total protein stain. Analysis was performed with imaging software, and finally proteins were identified by LC-MS/MS and studied for their functional characteristics. Using this approach, both phosphoproteins abundant in the proteome of proximal tubule cells as well as many proteins changing in degree of phosphorylation were found.

Introduction

Metabolic acidosis is a condition characterized by a decrease in blood pH due to the overproduction of acid or a decrease in bicarbonate recovery by the kidney. Under normal physiological conditions, the bicarbonate ions act as a buffer, maintaining an equilibrium blood pH level of approximately 7.4. During metabolic acidosis, the higher concentrations of acid in the blood and decreased recovery of bicarbonate stimulate a pH response in the proximal tubule cells of the kidney.[2] This cellular response restores blood pH by increasing renal uptake and catabolism of glutamine (Figure 1). Once in the cell, glutamine is transported into the mitochondria and converted into the citric acid cycle intermediate α-ketoglutarate by the enzymes glutaminase (GA) and glutamate dehydrogenase (GDH). Intermediates of the citric acid cycle are exported to the cytoplasm, where the cytosolic phoshoenolpyruvate carboxykinase (PEPCK) converts oxaloacetate to phosphoenolpyruvate. Stimulation of the citric acid cycle and the enzymatic activity of PEPCK produce bicarbonate ions.[2] Two key transporters which are up-regulated in this response are the lumenal Na+/H+ antiporter (NHE3), which acidifies the urine bound for excretion, and the basolateral Na+/3HCO3- symporter (NBC1), which releases the bicarbonate ions into the bloodstream to increase the pH.[3]

Although this pH-sensitive cellular response has been well characterized, the signaling pathways which stimulate this response remain unknown. The possible pathways remain difficult to elucidate because of complex regulatory interactions between mRNA and the proteins they encode. While some of the key enzymes are up-regulated during the response by increased levels of transcription, other enzymes are regulated by increased stabilization of their respective mRNA transcripts.[4]

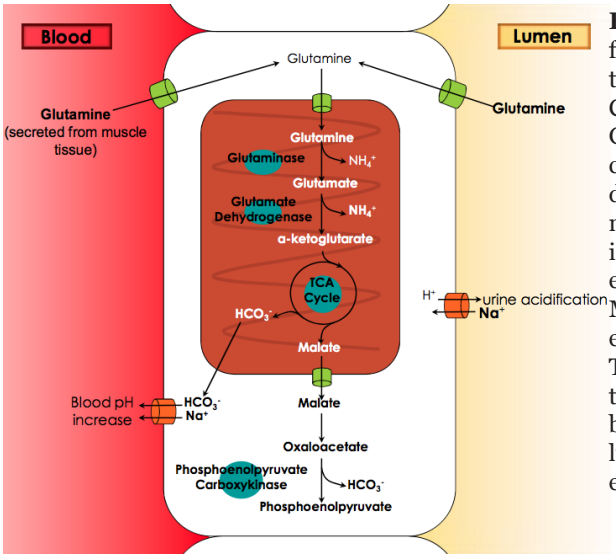

Figure 1: Response Mechanism for Increased Catabolism of Glutamine by the Proximal Tubule Cell During Metabolic Acidosis. Glutamine is imported into the cell and reduced in the mitochondria to the citric acid cycle intermediate, α-ketoglutarate, which is then converted to malate by the enzymes of the citric acid cycle. Malate is converted to phosphoenolpyruvate in the cytoplasm. The 2 HCO3- ions produced in this process are exported to the bloodstream, while H+ enter the lumen of the kidney bound for excretion in the urine.

This characterization of the proteome of renal proximal tubule cells focuses on protein regulation and post-translational modifications. As phosphorylation is one of the most important post-translational modifications in the regulation of proteins, this project will focus on the differences in the phosphoproteomes between control cells (grown in pH 7.4 media) and cells modeling metabolic acidosis (pH 6.9 media, 24 h treatment). Identification of phosphoproteins that are present during metabolic acidosis will elucidate how the adaptive response is mediated by proximal tubule cells. Characterization of expression changes that occur specifically in the phosphoprotein fraction will enable a more targeted investigation of how the signals are mediated specifically at the protein level.

Materials and Methods

Mammalian cell culture: Wistar-Kyoto rat proximal tubule (WKPT) cells were grown from stocks stored at -80°C. The WKPT cell line was used because the rat genome has been sequenced, and comprehensive knowledge of the genome is necessary for subsequent identification by mass spectrometry. Cells were plated and grown in a 37°C incubator with pH 7.4 media to confluence. Once confluent, the cells were split onto seven new plates. 24 h before harvesting the proteins for phosphoprotein fractionation, three plates were treated with pH 6.9 media and three plates were incubated with pH 7.4 media to model acute metabolic acidosis. 12 h before harvesting, the media was replenished in order to ensure that any changes observed were due to the pH treatment rather than stress due to starvation.[5]

Phosphoprotein Fractionation

24 h treated cells were lysed and proteins were purified with CHAPS detergent, nucleases, and protease inhibitors. The whole cell lysate from the three control (pH 7.4 media) plates was pooled and approximately 2.5 mg of protein was applied to a QIAGEN phosphoprotein purification column, which binds the phosphate groups of phosphorylated amino acid residues. This process was repeated for the plates treated with pH 6.9 media. The non-phosphoprotein flow through was collected and analyzed on a western blot to ensure that the non-phosphorylated fraction did not contain a significant amount of phosphorylated protein. The columns were washed, and phosphoproteins were eluted with a phosphate buffer. Halt phosphatase inhibitor cocktail was added to the fractions as they were collected. [5] Four sets of phosphoprotein columns were run in order to obtain enough phosphoprotein for three technical replicates of 6.9 and 7.4 2D gels.

Bradford Assays: Bradford assays were performed both before and after phosphoprotein fractionation to quantify the amount of protein initially being applied to the column and to calculate the yield of phosphorylated protein collected from the column. A standard curve was generated using known concentrations (0.5, 1.0, 2.0, 5.0, 7.5, 10.0 mg/ml) of BSA. Absorbance was measured on a Beckman DU 640 UV/Vis Spectrophotometer at 595 nm.[6] The protein samples collected during fractionation were concentrated on NanoSep ultrafiltration columns (MWCO 10 kDa), and the final concentration of phosphoprotein was interpolated using the standard curve generated on the spectrophotometer.

2-Dimensional (2D) Gel Electrophoresis and Protein Staining: The concentrated fractions of phosphoprotein were precipitated and salts removed with a Bio-rad Ready Prep 2D clean-up kit. The protein was then dissolved in rehydration buffer and used to rehydrate an immobilized pH gradient (IPG) gel strip (11 cm, pH range 3-6) for isoelectric focusing. Phosphorylation is a post-translational modification that lowers the isoelectric point (pI) of a protein, thus IPG gel strips with a narrow pH range of 3-6 were used to allow for optimal resolution of phosphoprotein spots. Each IPG strip for the isoelectric focusing was rehydrated with 100 µg of phosphoprotein. Pre-cast SDS-PAGE gels (8x11 cm) loaded with 2 µl Peppermint stick protein marker containing both phosphorylated and non-phosphorylated protein standards were used for separation along the second dimension. For imaging, the gels were first stained with ProQ Diamond phosphoprotein fluorescent gel stain (Invitrogen) which only binds phosphate groups, enabling quantification of phosphoproteins by comparison of spot volume intensities. In addition to staining with Pro-Q Diamond Phosphoprotein Gel Stain, the gels were also stained with SYPRO Ruby Protein Gel Stain (Invitrogen), which non-covalently interacts with the peptide backbone of all proteins [5].

Image Analysis: Gel images were analyzed using Delta 2D software to align gel spots, both between technical replicates and between pH 6.9 and pH 7.4 conditions. Spots that exhibited significant changes in intensity between the conditions were excised from gels with a Genomic Propic II robot for identification by mass spectrometry.

Protein identification

Protein spots of interest were digested using the ProteaseMax (Promega) in-gel tryptic digest procedure, which includes reduction of disulfide bonds with dithiothreitol (DTT), alkylation with iodoacetamide (IAA), and 3 h tryptic diges-

tion in ProteaseMax surfactant. 2 µl of the peptide mixture for each sample was analyzed by LC-MS/MS (Thermo Scientific LTQ linear ion trap) using a 42 min linear gradient from 25%-55% buffer B (90% ACN, 0.1% formic acid) ACN gradient. MS2 scans were collected for all samples and MS3 scans were triggered upon detection of a neutral loss of phosphoric acid.

Bioinformatics: MS2 spectra were searched against the Rat protein sequence database (maintained by the International Protein Index) using the Mascot and Sorcerer/Sequest database search engines. The protein identifications were compiled into Scaffold software (Proteome Software), which was used to manually confirm and validate the protein identifications. Identified proteins were investigated on the online databases www.uniprot.org and www.phosphosite.org to determine biological function.

Results

Protein Fractionation and Separation

Gels were imaged both after the application of the phospho-specific ProQ Diamond stain and after total protein staining with Sypro Ruby (Figure 2). In total, six 2D gels were run (6.9 and 7.4 in triplicate) and 12 images were collected. However, only the first set of gels was used for image analysis and subsequent protein spot identification due to poor resolution of spots in the second and third sets.

Western Blot Verification of Phosphoprotein Fractionation

Verification of the purity of the phosphoprotein fraction eluted from the column was carried out by western blotting. The non-phosphoprotein fractions collected from both the pH 6.9 column and the pH 7.4 column were concentrated on a 10 kDa MWCO spin column and run on a gel alongside phosphoprotein fractions leftover after rehydration of the IPG strip. The resulting gel was transferred to a polyvinylidene fluoride membrane and blotted with an antibody for the known abundant phosphoprotein p44/42 MAPK (44 and 42 kDa) as well as an antibody for the non-phosphoprotein GAPDH (36 kDa). Figure 3 shows that the phosphoprotein fractionations were successful in purifying the phosphoproteins from the non-phosphoproteins. Though there is a small amount of non-phosphoprotein in the phosphoprotein sample, this blot shows that there is no loss of phosphoprotein in the flow-through during fractionation. Relative intensity of the probes was not quantified as different amounts of protein were loaded into each lane.

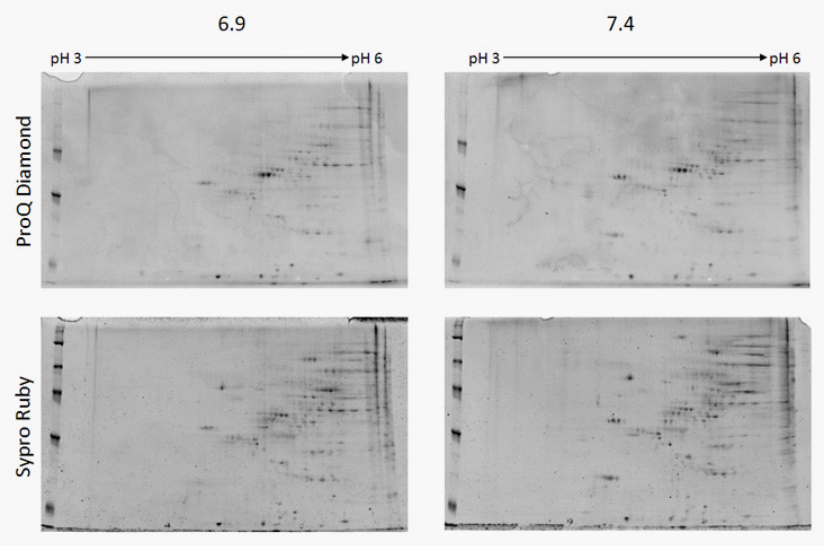

Figure 2: Comparison of 2-Dimentsional Gel Images. Gels were stained with the phosphoprotein stain ProQ Diamond, then re-stained and re-imaged with the total protein stain Sypro Ruby. The peppermint stick molecular weight marker (on the left of each gel) contains two phosphoproteins, which are visible in the ProQ stained gels. All six bands of the marker are visible in the Sypro-stained gels.

Delta 2D Quantitation Analysis

Of the 3 gel sets that were run,

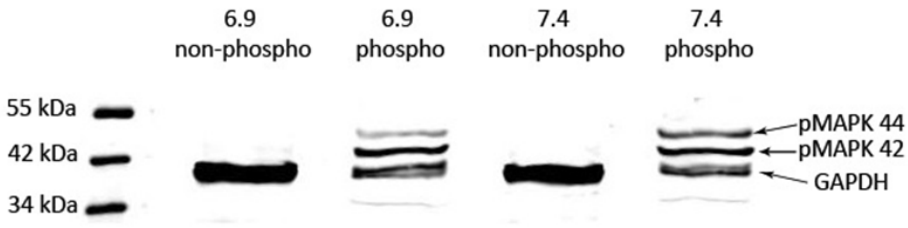

Figure 3: Western Blot of Fractions Collected from the Phosphoprotein Columns. Probing with the antibody for phospho-p44/42 MAPK, it is apparent that the second and fourth lanes with the phosphoprotein fractions from 6.9 and 7.4 contain mainly phosphoprotein. GAPDH is found mostly in the first and third lanes, which contain the non-phosphoprotein fractions from 6.9 and 7.4, respectively.

Spot	Protein	Change in expression in 6.9	Fold change	Function
1	Hsc70-interacting protein Nuclear Ribonuclear Protein Protein Disulfide Isomerase A6	↑Phosphorylation	1.90	ER Chaperone mRNA Processing Protein Folding
2	Hepatoma Derived Growth Factor	↑Phosphorylation	1.66	Nephrogenesis
3	Transcription elongation factor Astrocytic Phosphoprotein	↑Phosphorylation	1.68	Protein Synthesis Anti-apoptosis
4	60S acidic ribosomal protein*	↑Phosphorylation	1.57	Protein Synthesis
5, 6	78 kDa GRP*	↓Phosphorylation	0.44, 0.49	ER Chaperone
7	Transcriptional activator Calponin-3	↑ total protein abundance	1.81	Nucleotide Binding Cytoskeletal Organization
8, 9	Tropomyosin*	↓ total protein abundance	0.62, 0.63	Cytoskeletal Organization
10	Proteasome subunit alpha	↓ total protein abundance	0.48	Protein Degradation
11	Endoplasmin*	↓ total protein abundance	0.31	ER Chaperone
12	Naca Protein	↓ total protein abundance	0.60	Subcellular Protein Localization

Table 1: Phosphoproteins Changing during the Response to Metabolic Acidosis. Changes in phosphorylation were calculated based on the ratio of spot intensity in the ProQ-Diamond stained gels from pH 7.4 to pH 6.9. Changes in total protein abundance were calculated based on the ratio of spot intensity in Sypro stained gels from 7.4 to 6.9. Proteins denoted with * have been previously identified as changing during a 24 h response to metabolic acidosis.[5]

only the first set of gels was used for analysis and quantification due to insufficient resolution and problems with the IEF in the second and third sets. Spot intensity ratios were determined by Delta 2D based on the volume of corresponding gel spots. The spot intensity ratio of the 6.9 gel to the 7.4 gel was calculated individually for all spots on both the SYPRO stained image and the ProQ Diamond stained image. Comparison of the change in total phosphoprotein (6.9 Sypro/7.4 Sypro) to the change in phosphorylation state of the protein (6.9 ProQ Diamond/7.4 ProQ Diamond) indicated whether the observed spot change was due to a change in total protein abundance or a change in phosphorylation. By comparing these two ratios, 12 protein spots were found to be significantly changing (Table 1). Only one set of gel images was used to calculate quantitative changes in protein expression. Thus, in order to be counted as significantly changing, only spots that showed a fold change of magnitude greater than 1.5 were considered.

Protein Identification by LC-MS/MS

In total, 30 spots were picked from each gel and pooled with the corresponding spot before undergoing tryptic digestion. From these 30 pairs of matching spots, 34 proteins were identified (Table 2). In addition to identification of proteins from each of the 30 protein spots, in one of these spots, a phosphorylation site was detected by neutral loss scanning. A neutral loss peak was detected in the MS2 scan, indicating a loss of phosphoric acid (H_3PO_4) from a serine residue on the phosphopeptide (Figure 4a).

Protein	Unique peptides	Function
Astrocytic phosphoprotein	3	Anti-apoptotic Protein
Catechol O-methyltransferase	3	Cell Signaling
Similar to Chromobox protein	2	Chromatin Rearrangement
Tropomyosin	14,21,17	Cytoskeletal Organization
Calponin-3	5,6,7	Cytoskeletal Organization
WAS protein family, member 2	4	Cytoskeletal Organization
Na(+)/H(+) exchange regulatory cofactor	2	Cytoskeletal Organization
NSFL1 cofactor p47	14,14	Golgi Organization
RNA-binding protein	3	mRNA processing
Nuclear ribonucleoprotein F	2,2	mRNA processing
Hepatoma-derived growth factor	3,3,3,3	Nephrogenesis
Transcriptional activator protein Pur-beta	11,9,5	Nucleotide Binding
Proteasome subunit alpha	3	Protein Degradation
Thioredoxin-like protein	2	Protein Degradation
78 kDa GRP	32, 35, 2	Protein Folding in ER
Calreticulin	15	Protein Folding in ER
Endoplasmin	20	Protein Folding in ER
Hsc70-interacting protein	4,6	Protein Folding in ER
Protein disulfide-isomerase A4	4	Protein Folding in ER
Tetratricopeptide repeat-containing protein	4	Protein Folding in ER
Protein disulfide-isomerase A6	2	Protein Folding in ER
Hsp90 co-chaperone Cdc37	2	Protein Folding in ER
Elongation factor 1-delta	10,11	Protein Synthesis
Nucleophosmin	3,2,3	Protein Synthesis
60S acidic ribosomal protein	6	Protein Synthesis
Transcription elongation factor B	3	Protein Synthesis
Cortactin	7,8	Receptor Mediated Endocytosis
Naca Protein	8	Subcellular Protein Localization
Chromobox homolog 3	3,3	Transcriptional Regulation
Small ubiquitin-related modifier	2	Transcriptional Regulation
Similar to chromobox homolog 3	2	Transcriptional Regulation

Table 2: Proteins Identified by LC-MS/MS. 30 spots were excised from the gels, digested with trypsin, and identified by LC-MS/MS analysis. The number of unique peptides indicates how many of the peptides unique to a given protein were found and used to match the peptide to the protein.

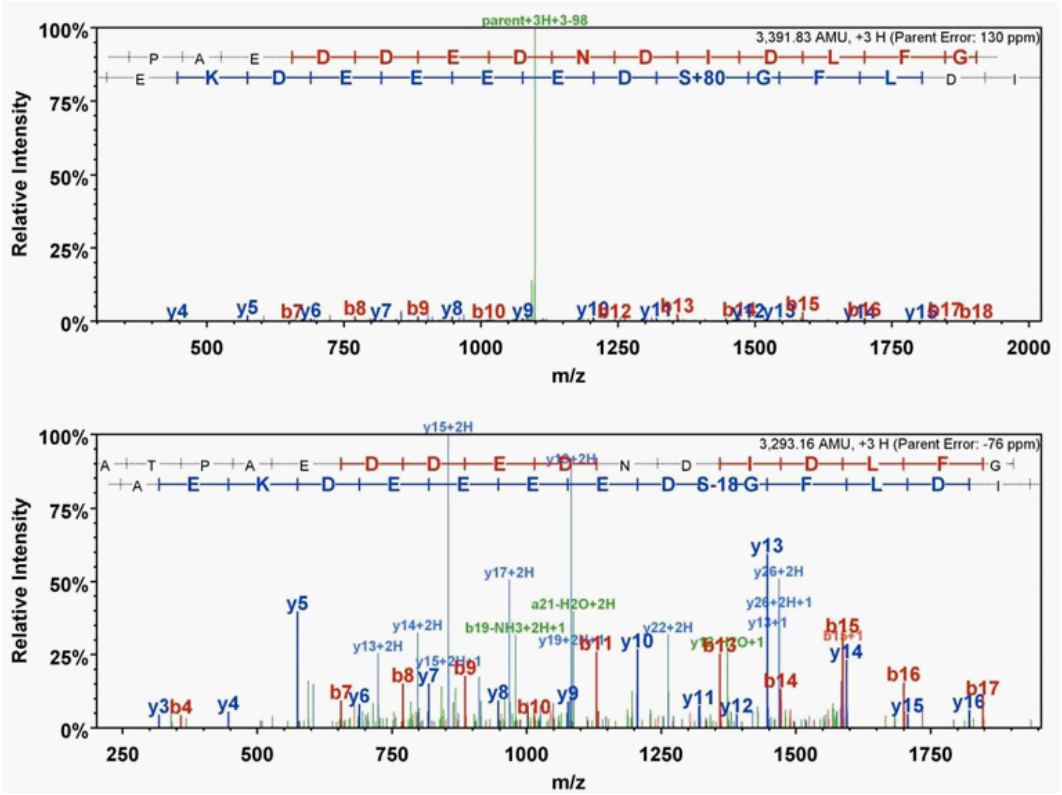

Figure 4: Identification of Phosphorylation site by Neutral Loss Scanning. 4A shows the MS2 scan of a phosphopeptide. The neutral loss of phosphoric acid causes decreased fragmentation along the peptide backbone, as seen by the low relative intensity of the b and y ions. During the neutral loss scanning method, the de-phosphorylated peptide is selected and fragmented in a third MS3 scan (4B). This spectrum shows greater fragmentation along the peptide backbone, as indicated by the higher intensity of the b and y ions. This enables greater confidence in peptide identification as well as identification of the exact residue of phosphorylation.

When intense enough, this neutral loss peak triggers a third MS3 scan in which the peptide is further fragmented in order to obtain a spectrum from which the primary sequence of amino acid residues can be confirmed and the specific phosphorylated residue identified. One phosphorylation site was confirmed by the subsequent MS3 scan. Phosphorylation of S531 on Isoform 2 of Elongation factor 1-delta was detected by the neutral loss peak in the MS2 scan and confirmed by the improved fragmentation in the MS3 scan (Figure 4b).

Discussion

A complete characterization of the proteomic response to metabolic acidosis would require a more comprehensive and replicable data set than provided by the scope of this investigation. The proteins identified as changing during metabolic acidosis in this experiment confirm and support previous findings.[5] Of the 12 proteins identified as changing from pH 7.4 to pH 6.9, five were previously identified as changing in either phosphorylation or total abundance in a prior 2D gel analysis of WKPT cells during 24 h metabolic acidosis.[5] These common proteins are denoted by an asterisk in Table 1. Also, many of the proteins identified in this study have been shown to interact with the proteins found to be changing in this prior study.[5] Most notably, the cytoskeletal protein actin, which previously showed a 2.1 fold increase in phosphorylation, is known to interact with three of the proteins identified in this study: cortactin isoform C, Na+/H+ exchange regulatory cofactor NHE RF-1, and Isoform 1 of troppopmyosin alpha-3 chain. Another protein that was previously found to be increasing in phosphorylation is the stress response chaperone protein, Heat shock cognate 71 (also denoted Hsc 70). Though Hsc 70 itself was not identified in any of the spots analyzed in this study, Hsc 70 interacting protein was found in one of the spots. Hsc 70 interacting protein stabilizes Hsc 70 in its ADP-bound state, thereby increasing its affinity for substrate proteins that require the chaperone activity of Hsc 70 in order to fold correctly. An additional 7 proteins associated with protein folding in the ER were also identified, four of which were found previously to be changing in either phosphorylation or total abundance during metabolic acidosis.

The prevalence of ER lumenal chaperone and folding proteins could be a result of the changes in

the proteome that the cell must undergo in response to the change in pH. In order to restore acid-base balance in the blood, the proximal tubule cell must increase the catabolism of glutamine, which requires increased expression of the enzymes involved in this pathway. Thus, chaperone proteins are essential in mediating the adaptive response. The various functions of these proteins related to protein folding in the ER include co-chaperones that maintain activity of Hsp70 and Hsc70, disulfide isomerases which are essential to stabilizing the tertiary and quaternary structures of mature proteins, and quality control proteins that are associated with degradation of misfolded proteins found in the ER.

Though there appears to be a prevalence of proteins associated with folding and chaperone activity among the various proteins identified in this study, it is difficult to quantify statistically significant changes in the proteome with a limited number of replicates and the low reproducibility rate of 2D gel data in this study. However, the preliminary results of this study do correlate with findings from previous studies. Thus, potential future directions for characterizing the response of proximal tubule cells to metabolic acidosis include running more replicates of 2D gels in order gain statistical p-values for fold changes based on spot intensity between the two gels. Finally, one aspect of this study that was not fully exploited was the use of neutral-loss scanning on the LC-MS/MS to detect specific amino acid residues that were phosphorylated. The neutral loss of phosphoric acid triggered an MS3 scan for a small minority of samples that were run, and only one phosphosite was definitively identified using this technique. By enriching for phosphopeptides before scanning, however, it may be possible to confirm phosphosites on a greater number of peptides, and even detect novel phosphosites on previously uncharacterized phosphoproteins.

Acknowledgements

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Landscapes in Their Dreams

By Courtney Klein
Colorado State University

"I first met Franco Magnani in the summer of 1988, when the Exploratorium in San Francisco held a symposium and an exhibit on memory. The exhibit included fifty paintings and drawings by him – all of Pontito, the little Tuscan hill town where he was born but had not seen for more than thirty years. Next to them, in astounding apposition were photographs of Pontito taken by the Exploratorium's photographer, Susan Schwartzenberg, form exactly the same viewpoints as Magnani's whenever possible…Magnani was billed as 'A Memory Artist,' and one had only to glance at the exhibit to see that he indeed possessed a prodigious memory – a memory that could seemingly reproduce with almost photographic accuracy every building, every street, every stone of Pontito, far away, close up, from any possible angle. It was as if Magnani held in his head an infinitely detailed three-dimensional model of his village, which he could turn around and examine, or explore mentally, and then reproduce on canvas with total fidelity.

"My first thought when I saw the resemblance between the paintings and the photographs was that here was the rare phenomenon, an eidetic artist: an artist able to hold in memory, for hours or days (perhaps for years), an entire scene that has been glimpsed in a flash; the commander (or slave) of a prodigious native power of imagery and memory. But an eidetic artist would scarcely confine himself to a single theme or subject; on the contrary, he would exploit his memory, or display it, in a huge range of subjects, to show that nothing lay beyond its grasp – whereas Magnani seemingly wanted to concentrate it exclusively upon Pontito…"

-Oliver Sacks, An Anthropologist on Mars, 1996

Quite correct is Sacks in saying that Franco Magnani is, in fact, not an eidetic artist, and for very good reason. Magnani, as Sacks divulges later, actually characteristically displays a controversial syndrome known as Gestaut-Geschwind syndrome (also referred to as Dostoyevsky or Waxman-Geschiwnd Syndrome). As a child this old-world artist was uprooted from his beloved hometown by imminent Nazi incursion. He was forced to leave behind the life that he had dearly come to love and abandon all that was familiar, normal and right to him. This would later play a major role not only in his syndrome's disposition, but his life and the personal philosophy by which he would live the rest of his life.

Gestaut-Geschwind Syndrome is a characteristic personality syndrome that is particularly associated with left-hemisphere Temporal Lobe Epilepsy (TLE). It is important, before anything else, to understand Gestaut-Geschwind Syndrome does not always or even often accompany TLE, and many patients who present focal epilepsy are completely devoid of every symptom which accompanies this personality syndrome.[1] However, exact statistics as to the occurrence of Gestaut-Geschwind Syndrome amongst TLE patients are not available, as the exact details and existence of Gestaut-Geschwind Syndrome is still a topic of hot debate. Nonetheless, there is a specific set of symptoms often associated with Gestaut-Geschwind Syndrome such as circumstantiality (excessive verbal output), hypergraphia, altered sexuality (often expressed as hypo sexuality), an intensified mental life (deepened cognitive and emotional responses), hyper religiosity and/or hyper-morality or moral ideas.[2] In many cases, patients with Gestaut-Geschwind Syndrome only display a small number of these symptoms, which is a primary reason many neurologists argue against the existence of this syndrome. For example, Magnani only displays intensified mental responses. His cognitive representation of Pontito is not only uniquely vivid, but its infinitesimal details, down to exact minutiae of specific stones used to build the towns beloved church, are so exact that when compared to photos taken from the same perspective Magnani's works of art are often times indiscernible from the reality of Pontito.

TLE as its own disorder is still in the infancy of research, but it is known to manifest in two different expressions: Mesial Temporal Lobe Epilepsy (MTLE) and Lateral Temporal Lobe Epilepsy (LTLE). A link between ferbile seizures (those coinciding with episodes of fever in young children) and subsequent MTLE has been suggested, but given the nature of such a cause further examination is difficult, and an exact role remains unclear.[3] Some studies have suggested abnormalities in the hippocampus may contribute to status

epilepticus.[1] Such findings support the theory that prolonged seizures damage the brain, resulting in the death of important brain cell structures such as the neuronal dendrite (the conductile regions which propagate the electrical impulses that allow neurons to communicate with one another). MTLE can be hereditary, but is more often related to brain tumors, spinal meningitis, encephalitis, head injury or blood vessel malformations.[4]

LTLE is less common, but more likely to be hereditary, such as in Autosomal Dominant Lateral Lobe Epilepsy, which is generally accompanied by auditory or visual features,[5] but can also be associated with tumors, trauma, encephalitis, meningitis, and vascular or congenial brain malformations.[6] Most often, the cause of any TLE cannot be discerned with any significant degree of certainty.[5]

Given the many uncertainties surrounding the more "tangible" or "observable" elements which construct Gestaut-Geschwind, it's no surprise that exact causes of the syndrome are unknown. These complications also contribute to the general controversy surrounding the existence of this disorder. It is hypothesized, however, that during the seizures common in TLE the emotional centers of the brain (such as the amygdale and ventromedial hypothalamus) and memory centers (such as the hippocampus and parahippocampal and rinal cortices) are over stimulated, essentially purging what can be considered random information.[2]

A study that partially investigated the bilateral hippocampal atrophy (BHA) theory of Gestaut-Geschwind Syndrome has recently offered experimental evidence for its existence. This joint study, headed by L. Tebartz van Elst and funded by the Institute of Neurology in London and the Department of Psychiatry and the Albert-Ludwigs-Universität in Freiburg, Germany specifically sought to examine if "particular bilateral hippocampal atrophy is associated with various psychiatric disorders,"[1] namely those of affect (such as Gestaut-Geschwind Syndrome), depression and schizophrenia. Bilateral hippocampal volume loss has been correlated with different psychiatric disorders such as major depressive disorder, post-traumatic stress disorder (PTSD) and schizophrenia. Similar amygdale volume abnormalities have been observed in schizophrenia and TLE patients.[1] The team hypothesized that a correlation would be found between such atrophies and an increased rate of psychopathology in the sample population. Furthermore, it was hypothesized that TLE patients with very severe hippocampal and amygdalar loss (operationally defined as three or more standard deviations away from the mean) would exhibit Gestaut-Geschwind Syndrome at a greater rate than those with average volume (operationally defined as less than half a standard deviation away from the mean).

The many minutiae of this study's methods are largely irrelevant for the discussion at hand; it his, however, important to note that the patients were exposed to rigorous qualification criteria at several points throughout the study. After the initial disqualification period researchers we able to identify 33 patients with BHA as well as 34 control patients with TLE who did not exhibit severe volume loss. After this patient pool was acquired, participants were matched on basis of age, gender, duration of epilepsy, frequency of seizures, incidence of ferbile convulsions, encephalitis, head trauma, status epilepticus and intelligence. Dozens of other measures were taken throughout the course of this study to ensure the utmost accuracy during assessment (see van Elst, et al 2008, for a detailed discussion), and the neuroscientific rigor was outstanding.

The final data presented no correlation between BHA and disorders such as depression and PTSD. However, significant correlation between hippocampal volume on both sides and left amygdale were found. Furthermore, there were undeniable and significant differences in terms of an increased prevalence of hypergraphia and hypo sexuality in the patient group. This suggests that BHA has a may play a role in the occurrence of Gestaut-Geschwind, and begins to shed light on a physiological explanation of the mysterious syndrome.

Patients who primarily express symptoms similar to Magnani's (the most widely researched group) generally report a constant theme throughout their hallucinations – in this instance, it is quite important to note that Gestaut-Geschwind Syndrome can be seen in both inter-ictal (between seizures) and ictal (during seizures) states.

In Sacks' account, Magnani most often found himself experiencing his visions of Pontito when in a calm and relaxed state, such as experiencing them with friends. These vivid, inter-ictal hallucinations would settle upon him and cause no other discernable disturbances. On at least one occasion whilst visiting Magnani, Sacks was privy to one of these episodes during which he would reportedly cease his line of conversation and simply lean forward, gazing into a nothingness, periodically turning his head from one side to the next, as if trying to orient himself in his hallucinations. Magnani later explained that by re-orienting himself in real space he could quite accurately view his beloved Pontito as though it actually surrounded him, which, for all intents and purposes, in Magnani's reality it certainly did.

Yet, the question still remains, why would the brain purge the

same information time after time? More recent investigations into this particular oddity suggest that while there are repetitive or reiterative elements prevalent throughout Gestaut-Geschwind patient's visions there are always elements of a fantastic or dreamlike state as well.[7] Another patient, unrelated to Fredo Magnani, explained that she would always see "a sudden vision of London in ruins, herself the sole spectator in this scene of desolation."[8] Magnani himself was acutely aware that his visions, as well as the resulting paintings, were habitually devoid of people, and possessed a post nuclear quality and an air of deeper, more spiritual stillness, despite the fact that every vision, every memory that came to his mind's eyes was keenly attached to an emotional reflection that was connected with a personal interaction with a close friend or relative.[9]

The single most widely researched Gestaut-Geschwind patient is Kumagasu Minakata, a Japanese genius devoted to natural history and folklore famous for his immense range of works. MRI scans of Minakata's post-mortem brain found evidence of right hippocampal atrophy, which correlated with his history of TLE. Many features of Gestaut-Geschwind were identified in a detailed study of his diaries, including a tremendous number of articles, a tendency to write in miniscule characters in compact space (hypergraphia), a lack of interest in sex (hypo sexuality), peculiar ethical concerns, a proclivity to become angry on slight provocation and a notably extraordinary interest in religious matters.[10] While Minakata himself did not express the vivid hallucinations so common to many Gestaut-Geschwind patients, he did fit many of the other criteria.

Most of the research done on this particular syndrome has been aimed at verifying or denying its existence, which, unfortunately for many Gestaut-Geschwind patients, is largely inapplicable in helping "control" or "treat" these patients. However, such a statement makes the gross assumption that these patients feel that help is necessary – Minakata lived a long, productive life in lieu of (or perhaps due to) his Gestaut-Geschwind diagnosis. Likewise, Magnani has admitted on many occasions that he would be lost without his Pontito – in fact, early in his life, before experiencing hallucinations Magnani spent most of his days yearning to return to Pontito, but was terrified at such a prospect. After finally returning late in life, Magnani, after a series of traumatic events related to his return, eventually found himself growing fonder of his beloved hometown, despite living across the ocean.

For individuals unacquainted with Gestaut-Geschwind Syndrome, the disposition may seem debilitating. However, as Sacks concluded his account of his friendship with Fredo Magnani it becomes quite evidence that his may not be the case:

"Franco feels he has twenty, thirty years of work still ahead of him, for the thousand-odd paintings he has done since 1970 convey only a small part of the reality he seeks to portray. He has to have paintings, or simulations, of every detail, from every viewpoint – from the village in the distance, as one drives up to it from Pistoia, to the finest details of the lichened stones in the church. He envisions the building of a museum overlooking the town, which will house a vast archive of Pontito, his Pontito – the thousands of paintings he has made, and the thousands more he still intends to make. It will be the culmination of his life's work, and the redemption of his promise to his mother: 'I shall create it again for you.'"

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Take a Break!

By Rachel Knoshaug
Colorado State University

Luminescence

By Jessica Egner
Colorado State University

Art

Couchman

By Kasey Broscheit
Colorado State University

Plan, Meet Enemy

By Johnathan Holmberg
Colorado State University

I had a plan, you know.

I'd worked it out in advance,
scouring the details,
stalking the arrangements.
You'd believe my pretense —
it's a surprise vacation,
a trip just for us to get out of our tiny studio
away from our tiny town
free from our not-so-tiny problems,
for now.

We'd have spent the night in a room made bigger by not being ours
We'd have explored the city, the past, each other
We'd have reservations for a fancy-but-not-too-fancy dinner
We'd have gone for a walk down the 16th Street Mall
to see the Christmas lights you love so much

The proposal, the cliché:
bended knee
amongst the trees
the shining replacement stars all around us.

Not here,
the one ceiling light
burning too brightly.
Not after a long drive
from a long day at work
after an hour of violent tending
to an old, seeping wound.
Not now.

But now,
now the fight is over,
and we're in our home,
and you're brushing your hair,
and you're smiling at me,
and your hair is draped over your shoulder,
and your ring weighs heavy in my pocket.

Wes Hempel's New Male Gaze in *Fatherhood*

By Samson Eberhart
University of Denver

When wandering through the Modern and Contemporary Art exhibit at the Denver Art Museum, one will undoubtedly stumble upon, and be perplexed by, a particular painting entitled Fatherhood. A young man, muscular and dressed in a toga-like outfit, is surrounded by five naked children climbing upon him as if he was a tree. Surrounding the group is as the balcony of a palace or mansion with huge stone pillars on each side and a breathtaking view of the neighboring countryside, a place to just stand and gaze at the scenery for hours upon hours. The attraction in the painting is not, however, to the scenic balcony view. It is to the man's facial expression: one of shock, awe, and confusion. His blank stare with his look of astonishment captivates the viewer and raises multiple questions. As Devon Jackson argues in Personal Mythologies, Hempel's art "exist[s] on a plane between the very personal and the very historical....His paintings are like a memory....One can feel it, one knows it, but the exactness of what it is stands just outside one's mental reach."[1] It is, in fact, self-reflection that makes Hempel's paintings both interesting and compelling to a wide range of audiences.

An accomplished artist who started his career in Colorado as a writer, Wes Hempel is a man who borrows much of his style and perspective from traditional artists, yet adds obvious variations to his work to create an alternative view on art and common reality. In his work Fatherhood, for example, he borrowed from the traditional idea of maternity in William Bouguereau's La Charite, and transformed a well-known idealistic portrait by forcing the audience to re-consider a not-so-ideal paternal head, namely that of a single male parent.[2] One who gazes upon this overwhelmed man is compelled to re-think the "subversion of gender," to re-consider how "subtle shifts [in] traditional stereotypes currently take place," and to ask how the responsibility of parenthood exists regardless of gender.[3] The father is clothed in a Roman-era red robe and surrounded by marble pillars, forming a setting of structure, order, and strength, but the setting is contrasted with the man's look of confusion, shock, and seclusion. By incorporating Bouguereau into his own work, Hempel accents the contrast between the classical and the contemporary, "conflating the ancient and the new," as he offers up a new male gaze.[4]

To start, Wes Hempel juxtaposes a female Charity figure in the ideal form against a male caretaker in real form, a direct association to reality in a modern society. The Madonna figure in La Charite symbolizes the "Christian virtue of charity" with the children in need of protection, as well as the representation of them being "human souls needing spiritual sustenance."[5] Fatherhood, however, symbolizes, if not indirectly, the own artist's dismay as he realized at age 40 that he would never have children [3]. Convention of a mother caring for young babies has been mastered again and again by countless artists, but few have attempted to place the father within the woman's stereotypical and sacred role. The ideal social structure of the family and of commonality is skewed as a new kind of parenthood, becoming more and more popular in recent years, is introduced. Here is the father as the homemaker, or the contemporary Mr. Mom, a figure who has become more and more common in an ever-changing world as mothers find their place within the workforce.

Hempel has revised gender roles within mainstream society, while also conjoining very separate periods of art, and has formed his own artistic style in the process. Critics note that the majority of Hempel's images are either replicas of classic paintings or are inspired by famous techniques. A self-taught artist who learned his artistic style from influential art images, Hempel repeatedly gives honor to the old masters, respecting their style and own motivations. As Michael Paglia describes in his article Go Figure, "On one level, Hempel's work is traditional and conventional, his painterly techniques right out of the nineteenth century. But on another, his work delivers ironic and contradictory messages, making it crisp, cogent and relevant—relentlessly addressing the concept of the alienation of the individual." This fusion of Hempel's style with that of the Master Painters does build Hempel's credibility and acceptance within the art world, for he is able to take the works of Turner, Van Ruisdael, Hals, Parrish, and Bouguereau to "create his own brand of contemporary realism, reconciling all of it in the same painting."[6]

On the surface, Fatherhood introduces a distressed persona of the male figure, who is alone and in shock because of the five dependents looking to him for protection. The man's face, the focus of the painting, creates an uncomfortable and distraught tone to the entire piece. Here the realism of parenthood comes to a climax. Other than the bright red toga that drapes the man, the natural grays and dull tones throughout the painting suggest the man's strength and power despite the dreary setting. The stone pillars and elevated position of the portrait against the vast background landscape further alludes to order and prosperity. Yet, the man's resounding expression of dismay thwarts any emotion of victory and success. Even the sky, lacking a sun or normal bright blue color, presents the flat situation of fatherhood not as a sunrise or a sunset, but just as a daily order of being as a paternal figure.

A viewer sees that the children hold much greater importance to the overall appeal of the painting than was originally perceived. To begin, all the children represent different personalities, reflecting human nature and a variety of social characters. Whereas the three children directly in contact with the father are content, hopeful, even sleeping within his arms, the two children at his feet are independent and autonomous. The one on the lower left leans upon stacks of books and writes on pieces of paper, symbolic to his interest and curiosity of scholarly endeavors. He does not, however, look to the father for any type of support, acknowledgement, or encouragement. The young baby to the left is also self-sufficient, even using his own body heat, compared to his father's, to keep himself warm. It is striking that as one continues to gaze at the portrait of the family, the young face of the child in the fetal position becomes more and more piercing and expressive than the man's. Not the light, but rather the shadow, that surrounds the young child in the lower right of the painting possibly suggests that he lacks a sense of being able to create his own light, and it as if he is calling out to the audience for support.

Hempel utilizes intriguing tactics to engage the viewer, both in his character's stares and in the interpretation of his pieces of art. Ironically, Hempel imposes a sense of detachment from the actual subject of the painting upon the viewer, a distance very prevalent in Fatherhood. The father's line of sight moves out toward the audience, but he does not look directly at his audience. Rather, it is as if the man is looking behind or even through the spectator, questioning the rights of the people to gaze upon him, for "Hempel creates an icy, palpable distance between the subject and the viewer....Even as they appear to be looking out, they are actually looking right past you."[7] In addition, Hempel stresses that there are countless numbers of possible interpretations of his artwork by his audiences. He believes that "a painting succeeds if viewers are able to attach their own stories to it."[7] Even in his own correspondence, he commends and encourages the questioning and interpretation of his art; and, further, he states that his true intentions are beside the point, that he is pleased that viewers react both with laughter and with uncertainty to this particular painting.[3]

The gender of the two subjects within Fatherhood and La Charite is not the sole feature that differentiates the paintings from each other, as one can notice other details that are thoughtfully changed to transform the interpretation. One glaring difference between the two parental paintings is the soccer ball that rests under the man's left foot. Bouguereau's Madonna figure pours out a jar full of coins down the marble steps. Hempel defines the differences between the old and the contemporary, with a soccer ball in the same position. Whereas the woman pours out money freely and selflessly, possibly to the poor or needy, the soccer ball references a contemporary notion of parenting as the Soccer Mom, or more precisely put the Soccer Dad, shuttling kids to practice after practice after practice. Whereas Hempel's canvas alludes to an ever-so-common parent with unnecessary competitive endeavors to keep his family up with the Jones's, Bouguereau's painting reflects servitude, humility, and compassion from an iconic woman who apparently is more capable of caring for the young ones who surround her.

Wes Hempel, unlike Bouguereau, chose to use the male figure as a center point of his paintings; in nearly all of his works of art, muscular and visually appealing men take center stage. In doing so, Hempel goes against the widely used trend of attracting male audiences into gazing at art via the female figure. Instead he replaces the "iconographic female nude in art history with that of the male."[4] Bouguereau has painted pieces of art that specifically appeal to the male audience, for the naked women, poised and exposed, truly attracts the male gaze. Although Bouguereau did not decide to pursue this sort of male gaze in La Charite, John Berger does provide a poignant example from Bouguereau's often times excessive use of feminine beauty to draw in certain spectators in Ways of Seeing. The painting consists of dozens of naked women, floating into the sky, and three male creatures captivated as they watch this clearly heavenly event. The painter's personal vision was to "seize upon [the nakedness] – sometimes quite regardless of whether it is the first time or

the hundredth."[8] It is interesting, then, to see that Wes Hempel has chosen to walk down a very different path, transforming the common male gaze into the emerging "new male gaze."[4]

The new male gaze, a phrase that Rafael Risemberg coins in his article, is about the up-and-coming homosexual gaze within contemporary art. On the basis of Wes Hempel's own statements, he offers testimony that his paintings correlate to his own homosexuality. Within nearly all of Wes Hempel's paintings, he expresses his own individuality as a gay man, and further extenuates "men in the physical prime of their lives, and simultaneously, as a metaphor for the modern gay man, who feels vulnerable and thwarted even as he makes advance in society."[4] Wes Hempel has done something extraordinary with this masterpiece: he successfully created a piece of art that transformed the idea of the male gaze with a female center point into the new male gaze. He connected both the heterosexuals and the homosexuals within one painting, and within one glare of a man's face. Perhaps the most profound inspiration of the painting Fatherhood is equally saddening, as Hempel realizes that, due to his homosexuality, he will not have the joy and opportunity to have children of his own.[3] Whereas La Charite is forever able to gaze upon her own children, nurturing them tenderly and lovingly, Hempel will live the remainder of his life pondering what that would feel like. The face of the father in Fatherhood is, in real terms, the face of Hempel.

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A Russian Formalist Reading of Shadow of the Colossus

By Jonathan Holmberg
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Shadow of the Colossus is a game for the Playstation 2 that follows a boy's quest to resurrect a girl sacrificed because of a curse. The boy trespasses in a forbidden land and speaks to the spirit residing in an ancient temple. The spirit, named Dormin, offers to revive the girl, so long as the boy destroys the sixteen colossi wandering about the land.

Dormin, though, is evil, and the colossi were created as seals to keep Dormin from manifesting in the real world. So, by the end of the story, the player has spent all of the game working for the evil being, and the girl ends up trapped in the forbidden land with what's left of the boy. While the story clearly wants to challenge our notion of what it means to be a hero, the style of the game does something much more subtle, and much more interesting. Through the use of passive opponents, dramatic irony, and the withholding of information, Shadow of the Colossus defamiliarizes our automatic perceptions about audience participation and culpability.

When talking about Shadow of the Colossus, it's almost easier to talk about what the player doesn't know, rather than what they do. Unlike most other games, where we're given a clear background of the characters and the world and the situation we're playing through, Shadow of the Colossus gives us next to nothing. The game opens on the boy making his way to the temple; we have no idea where he came from. Who sealed Dormin away is never discussed, and neither is the curse which doomed the girl, Mono, to be sacrificed. We, as the audience, never even know what kind of relationship the boy has with Mono. As such, as we play through the game, we hardly have any story-based motivation for our actions–especially as those actions begin to take on a sinister edge. This ties the character's in-game actions directly to our own personal motivations, be they to see the story through to the end, to best the next monster, to explore more of the world, or whatever else.

We're also never given the boy's name, which ties us even more closely to what happens onscreen. Players love to claim personal credit for the great, heroic deeds their characters perform, but in games where a character may do something despicable, players can distance themselves from the action by saying, "Did you just see Rourke detonate that nuke in New York City?" That's not an option here. Every bit of praise, every command, every accusation, every invective is directed at "you." "Your next target is–" "You stole the ancient sword." "You freed the demon." "You" are the constant target of what little dialogue there is in the game, and it is very difficult to avoid shifting that to "I." "I stole the sword." "I killed the colossus." "I freed the demon." If "you" are responsible for all this, how responsible are you?

Player responsibility for what happens might be avoided if the player is as clueless about the ramifications of his actions as the character is. However the game's use of dramatic irony makes it clear that the path you've chosen is a negative one. When fighting a colossus, the fight typical consists of two phases, and the music during the second phase ramps up and becomes much more urgent than during the first. This is fairly common across most video games, and in other video games, when the battle has been won, the music turns celebratory. When the colossus has been defeated, though, the music takes an extreme shift, with a somber and quiet tone. It's almost mournful.

And the music isn't the only thing that clues the player into the wrongness of their behavior. From the corpse of each colossus, black tendrils of... something stream into the air and then seek out and impale the character's body. You can try to run from them, but no matter how far you get, they always catch you. The character grunts in pain, falls to the ground, and reappears in the temple a bit later surrounded by an ever-growing crowd of shadowy, black figures. These are the same figures that, at the beginning of the game, threatened the main character and were destroyed simply by the unsheathing of the ancient sword he carries with him. These creatures were also the main enemies in Ico, Shadow of the Colossus's sister game. Their peaceful presence is not a positive thing. Even if it's not abundantly clear that you're participating in a Bad Thing, suspicions ought to at least be running rampant through your mind.

Perhaps most damning of all, though, are the battles with the colossi. In most games, the levels are full of minor enemies to fight, and the Big Bad Guy attacks you on sight, and does so relentlessly until one of you is dead. In addition, the

Big Bad is typically specifically targeting your character for some reason or another. Not so here. The forbidden land is practically devoid of life. There a few lizards and birds, but the colossi are the closest thing the land has to intelligent life. In addition, all the colossi just hang out in their designated areas. They seem to feel no need to wander. As such, it is up to you, the player to seek out and destroy these essentially docile creatures, for no other reason than because a voice from the heavens told you to.

And they truly are essentially docile. While the colossi do attack you, they only do so when you have invaded their areas, and sometimes they will ignore you, even then, until you actively seek to provoke them. One colossus, shaped like a giant bird, sits on a perch and grooms itself, leaving you free to wander around its area as much as you like. It's not until you shoot it with an arrow that it takes notice of you and tries to get rid of you. Even then, if you leave the bird alone for a while, it will return to its perch and stay there until you provoke it again. This holds true for the other colossi, as well. Leave their area or ignore them for a while and they will leave you be. You are the aggressor, the invader, the destroyer. They just seek to defend themselves and their homes.

How much, then, are you, the player, to blame for the destruction that happens onscreen? If you are watching someone else play the game, can you be held accountable for not stepping up and putting a stop to the train wreck in progress? Those questions may seem too much to ask with regards to a video game, but if we ask it about the events in a video game, maybe then we can start asking those questions about other events where we serve as the audience.

Our only recourse in Shadow of the Colossus is to turn the game off. The world will forever be suspended in the state in which we left it, but we can tell ourselves that the boy abandoned his dangerous course of action and went home. It's just a story, after all. In the real world, though, we have more options. How culpable are we when we watch a report about starvation in Africa or civil unrest in Iran or human rights abuse in China, but don't try to do anything to fix it?

We've become automaticized to think that we are only passive observers of what we watch. Even if we simply turn off sensationalist media, perhaps fewer people will commit heinous acts simply to get their fifteen minutes of fame. Play through Shadow of the Colossus a few times, and maybe you'll be defamiliarized enough to ask, "Just how responsible are we, the audience, for what we see?"

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