A Naturally Occurring Single Nucleotide Polymorphism in the *Salmonella* SPI-2 Type III Effector *srfH/sseI* Controls Early Extraintestinal Dissemination

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Abstract

CD18 expressing phagocytes associated with the gastro-intestinal (GI) epithelium can shuttle *Salmonella* directly into the bloodstream within a few minutes following microbial ingestion. We have previously demonstrated that *Salmonella* controls the CD18 pathway to deeper tissue, manipulating the migratory properties of infected cells as an unappreciated component of its pathogenesis. We have observed that one type III effector, SrfH (also called SseI) that Salmonella secretes into infected phagocytes manipulates the host protein TRIP6 to stimulate their migration. Paradoxically, SrfH was shown in another study to subvert a different host protein, IQGAP1, in a manner that inhibits the productive motility of such cells, perhaps to avoid interactions with T cells. Here, we resolve the discrepancy. We report that one naturally occurring allele of srfH promotes the migration of infected phagocytes into the bloodstream, while another naturally occurring allele that differs by only a single nucleotide polymorphism (SNP) does not. This SNP determines if the protein contains an aspartic acid or a glycine residue at position 103 and may determine if SrfH binds TRIP6. SrfH Gly103 is a rare allele, but is present in the highly invasive strain *Salmonella enterica* serovar Typhimurium UK-1 (stands for universal killer). It is also present in the genome of the only sequenced strain belonging to the emerging pandemic *Salmonella enterica* serovar 4, [5],12:i:-, which is frequently associated with septicemia. Finally, we present evidence that suggests that Gifsy-2, the bacteriophage upon which *srfH* resides, is present in a clinical isolate of the human-specific pathogen, *Salmonella enterica* serovar Typhi. These observations may have interesting implications for our understanding of *Salmonella* pathogenesis.

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Introduction

*Salmonella* is a bacterial pathogen of humans and both warm and cold-blooded animals that can actively invade host cells and proliferate within ones that are normally microbicidal. *Salmonella* is a major public health problem, which leads to more than three million deaths per year [1]. *Salmonella enterica* serovar Typhimurium (S. Typhimurium) usually causes a self-limiting gastroenteritis in humans, but septicemia associated with non-typhoidal *Salmonella* is a growing public health problem, which can affect otherwise healthy individuals, and is especially troublesome in certain immunodeficient people, including those infected with HIV. The closely related *Salmonella enterica* serovar Typhi (S. Typhi) causes typhoid fever, a systemic disease. In addition to public health concerns, S. Typhimurium is also studied because it is a model pathogen without parallel for dissecting basic pathogenic processes, due to its genetic tractability and the availability of excellent murine models of infection. S. Typhimurium produces an acute, systemic disease in BALB/c mice and produces a chronic carrier state in wild type 129X1/Sv mice, similar to the two types of disease observed with S. Typhi infections of humans. The acute phase is often fatal whereas in the carrier state the infection becomes largely asymptomatic, but the bacteria can be shed from internal organs, potentially for the lifetime of the host.

*S. enterica* utilizes two independent type III secretion systems encoded by *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2) to promote its virulence. The bacteria utilize SPI-1 in the gastrointestinal (GI) stage of disease to invade cells and to invoke the inflammatory response [2–4]. *S. enterica* is traditionally thought to only deploy SPI-2 in the systemic phase of disease, to facilitate intracellular survival and growth [5–7]. However, it was shown in one study that S. Typhimurium expresses SPI-2 associated genes in as little as 15 minutes within the GI tract, prior to penetrating the intestine [8].

SrfH was first identified as a gene regulated by the SPI-2 encoded transcription factor SsrB, even though SrfH is located outside of SPI-2 [9]. It was subsequently shown to be a SPI-2 secreted type III effector [10]. SrfH was reported to facilitate the rapid penetration of the bloodstream by infected phagocytes [11]. Another seemingly contradictory study demonstrated that SrfH repressed the productive motility of such cells [12]. The former study utilized S. Typhimurium 14o28s. The latter study showed that *in vitro*, SrfH from a different strain, S. Typhimurium SL1344, causes infected macrophages and dendritic cells to...
migrate aberrantly, not productively responding to chemotactic gradients composed of microbial components or CCL19 respectively [12]. CCL19 gradients normally facilitate dendritic cell–T cell interactions. This behavior requires productively interacting with IQGAP1 via a critical cysteine residue at position 170 [12]. Mutating this residue to alanine does not affect SrfH secretion or subsequent binding to IQGAP1, but blocks a productive interaction. A productive SrfH/IQGAP1 interaction increases bacterial numbers in both the spleen and liver at 45 days post-infection of wild type mice [12]. It was shown that when roughly the same number of S. Typhimurium SL1344 bacteria or a srfH mutant were present in the spleen, the presence of an intact copy of srfH correlated with lower numbers of CD4⁺ T cells. It was concluded that SrfH interferes with the productive motility of the phagocytes harboring it to suppress an immune response. In this study, the authors reported that SrfH does not bind TRIP6 [12].

We demonstrate here that the srfH alleles from the S. Typhimurium strains 14028s and SL1344 are not identical. A single base pair (bp) difference between the two alleles produces proteins containing different amino acids at position 103. The SL1344 SNP eliminates TRIP6 binding in a yeast two-hybrid assay as well as the early travel of infected phagocytes to the bloodstream. This study provides a remarkable example of naturally occurring allelic variants differing by only a SNP, having seemingly antagonistic effects on the same host cell process.

Results

The Two srfH Alleles are not Identical

We have sequenced multiple, independent PCR products containing the srfH allele from an isolate of S. Typhimurium SL1344 obtained from the Salmonella genetic stock center and determined that it is one bp different than all three independent Genbank versions of S. Typhimurium 14028s srfH (Fig. S1), which are all identical (Sam Miller’s group (AF236075.1), Lionello Bossi’s group (AF254763) and ourselves (AF231470.2)). This SNP changes Gly103 (relative to 14028s) to Asp103 (SL1344). In support of our observation, the published S. Typhimurium 14028s [13] and unpublished S. Typhimurium SL1344 (http://www.sanger.ac.uk/Projects/Salmonella/) genome sequences contain this SNP.

SrfH Gly103 but not SrfH Asp103 Promotes Early Travel to the Bloodstream

S. Typhimurium travels from the GI tract to the bloodstream exclusively inside of CD18⁺ phagocytes in the first 30 minutes following oral inoculation [11,14]. To determine if SrfH Asp103, is as capable as SrfH Gly103 in promoting rapid travel to the bloodstream in such cells, we generated two new strains. In one, we deleted the entire srfH open reading frame from S. Typhimurium SL1344. In another, with allelic exchange, we generated a S. Typhimurium SL1344 knock-in mutant that carries the SrfH Gly103 allele instead of its native srfH allele. Throughout the genome, the parent strain and the derivative differ by only a SNP. To assess the impact of residue 103 of SrfH on virulence in an animal model, we introduced either wild-type S. Typhimurium SL1344 SrfH Asp103 or the derivative S. Typhimurium SL1344 SrfH Gly103, or the srfH deletion into BALB/c mice by oral gavage. Thirty minutes after inoculation, significantly more colony forming units (CFUs) were recovered from the blood of the mice infected with the knock-in mutant than from the blood of mice infected with the parent strain (p-value < 0.05) (Fig. 1). This experiment was performed on groups of eight mice on four independent occasions. Although a few wild type S. Typhimurium SL1344 bacteria were recovered, the number was not significantly different from zero (p-value = 0.06). No bacteria could be recovered from the blood of any mice infected with the srfH deletion strain. The srfH deletion strain was similarly tested on groups of eight mice, but only on three independent occasions. The difference in the number of blood-borne bacteria recovered from the mice infected with the knock-in mutant versus the srfH deletion strain was highly significant (p-value = 0.01).
obtained with the two srfH alleles is that the encoded proteins are secreted into phagocytes differently. To address this possibility, we generated three fusion proteins. One was composed of SrfH Asp103 fused to the adenylate cyclase gene (Cya) from Bordetella pertussis, one was composed of SrfH Gly103 fused to Cya, and the final one was composed of LacZ fused to Cya (to serve as a negative control). B. pertussis Cya only generates cAMP in the presence of calmodulin, which is not found in bacteria. This allows for very sensitive and precise detection of protein secretion by S. Typhimurium into infected macrophage cytosol [10,15]. Bone marrow derived macrophages (BMDMs) were separately infected with bacteria expressing the three fusion proteins and cAMP levels measured by ELISA. As can be seen in Figure 2, the two srfH alleles are secreted by SPI-2 into infected macrophage cytosol similarly. Another explanation for the results of the murine dissemination experiment might be that SrfH Asp103 is unstable within mammalian cells. This seems unlikely as a different study observed SrfH Asp103-mediated phenotypes over extended periods of time within BMDMs and dendritic cells [12].

SrfH Gly103 Binds TRIP6 in a Yeast Two-hybrid Assay but SrfH Asp103 does not

Considering that SrfH Gly103 promotes the spread of infected phagocytes into the bloodstream and SrfH Asp103 does not, we next tested if the SL1344 SNP interfered with TRIP6 binding. To test this possibility, we cloned SrfH Gly103 and SrfH Asp103 into the bait vector of a yeast two-hybrid system, and cloned full-length TRIP6 into the prey vector. Both bait constructs were sequenced to exclude the possibility that an error was inadvertently introduced in the PCR reactions. We introduced both constructs into a yeast strain engineered to use β-galactosidase activity as readout for bait/prey binding. As can be seen in Figure 3, SrfH Asp103 is unable to bind TRIP6 in this assay, while SrfH Gly103 is, even though both alleles can be expressed stably in yeast. This result is only suggestive as yeast two-hybrid assays can produce both false positives and false negatives. A more definitive answer as to whether or not SrfH Gly103 binds TRIP6 awaits the results of ongoing biochemical clarification.

Natural Distribution of the Two Alleles

We analyzed srfH sequences from all of the completed, publicly available Salmonella genomes and found that SrfH Gly103 is a very rare allele, being present in only three Salmonella strains in addition to S. Typhimurium 14028s. It is present in S. Typhimurium strain UK-1, S. enterica serovar Newport strain SL254 and in S. enterica serovar 4, [5],12i:- (S. 4, [5],12i:--) strain CVM23701. S. Typhimurium UK-1 is interesting in that it is the most invasive Salmonella strain commonly studied [16,17]. Attenuated mutants of this strain have accordingly been used extensively in vaccine development [18]. S. 4, [5],12i:- is an emerging pandemic serovar. It was only rarely associated with disease in the mid-1990s but is now one of the most common serotypes associated with human disease in many countries around the world [19]. It was the fourth most common serovar associated with human disease in the European Union in 2006 and infections caused by it are often severe [19]. An outbreak in New York city in 1998 resulted in a 70% hospitalization rate [20]. The significance of this serovar is heightened by the fact that its members are usually multi-drug resistant.

Figure 2. Both srfH alleles encode proteins that are secreted similarly. BMDMs were infected with S. Typhimurium SL1344 or S. Typhimurium SL1344 sskA::cm (a mutant defective in all SPI-2 secretion) expressing fusion proteins composed of one or the other variants or LacZ (a negative control) fused to Cya. At six hours post-infection, the infected BMDMs were lysed and cAMP levels measured by ELISA. There was no significant difference in the secretion of the two variants. This experiment was performed in triplicate on two independent occasions. Error bars represent the standard error of the mean.

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amplify two other genes believed to be specific to Gifsy-2: in *S. Dakar* (Fig. S2). Both genes can be readily PCR amplified from *srfH* isolated in Dakar in 1988 [25]. We confirmed this report by PCR indicating that there was a strain CT18. One report however presented a zoo-blot, which match the *PLOS ONE* | www.plosone.org 4 September 2012 | Volume 7 | Issue 9 | e45245

...amplifying *srfH* produced septicemia [24].

**Discussion**

This study describes a unique effect of *srfH* allelic variants on *Salmonella* pathogenesis and provides us with new insights into the complex, sometimes strain specific, interactions of pathogens with host cells. Remarkably, a SNP is all that separates the two *srfH* alleles, causing them to have seemingly antagonistic effects on the migration of infected phagocytes. We have demonstrated that a glycine versus an aspartic acid residue at position 103 of *SrfH* is not present in the laboratory strain *S. Typhi Ty2* or in strain CT18. One report however presented a zoo-blot, which indicated that there was a *srfH* homolog in a strain of *S. Typhi* isolated in Dakar in 1993 [25]. We confirmed this report by PCR amplifying *srfH* from *S. Typhi Dakar* with primers designed to match the *srfH* allele of *S. Typhimurium* (Fig. S2). The *srfH* allele in *S. Typhi* Dakar encodes *Asp103*. We next attempted to PCR amplify two other genes believed to be specific to Gifsy-2: *gtgA* and *solC*. Both genes can be readily PCR amplified from *S. Typhi Dakar* (Fig. S2).

**srFH and Perhaps Gifsy-2 are Present in a Clinical Isolate of S. Typhi**

*srfH* is not present in the laboratory strain *S. Typhi Ty2* or in strain CT18. Typhi Ty2 or in strain CT18. One report however presented a zoo-blot, which indicated that there was a *srfH* homolog in a strain of *S. Typhi* isolated in Dakar in 1993 [25]. We confirmed this report by PCR amplifying *srfH* from *S. Typhi Dakar* with primers designed to match the *srfH* allele of *S. Typhimurium* (Fig. S2). The *srfH* allele in *S. Typhi* Dakar encodes *Asp103*. We next attempted to PCR amplify two other genes believed to be specific to Gifsy-2: *gtgA* and *solC*. Both genes can be readily PCR amplified from *S. Typhi Dakar* (Fig. S2).

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It is interesting to speculate about the mechanism through which *SrfH* Gly103 promotes the migration of infected phagocytes into the bloodstream. TRIP6 is a member of a sub-family of adaptor proteins that localize to focal adhesions upon activation and govern adhesion and motility. This family of proteins contains amino terminal proline rich regions and three copies of the zinc finger LIM domain motif at their carboxyl terminal ends, which function as molecular platforms to mediate protein-protein interactions [26]. The coupling of the focal adhesion adaptor protein p130<sup>cas</sup> (CAS) with the focal adhesion regulatory molecule Crk serves as a ‘molecular switch’ for the induction of migration through the Rac signaling pathway [27,28]. Interestingly, TRIP6 is capable of mediating CAS/Crk coupling [29], and host directed S-palmitoylation of residue 9 of SrfH targets the protein to the plasma membrane of host cells [30]. Thus, one model for the ability of *SrfH* Gly103 to accelerate cellular motility is that *SrfH* binds TRIP6 in the cytosol and enriches it within focal adhesions where CAS and Crk are in sufficient quantities to be coupled. If this model were correct, *SrfH* Gly103 would only be causing a kinetic change in the migration of infected phagocytes and this implies that a chemokine receptor is up regulated either indirectly as a result of infection, or perhaps more likely, deliberately by the microbe. As attractive as this model may be, we cannot of course exclude at this point the possibility that TRIP6 binding interferes with IQGAP1 binding and/or manipulation.

Much further molecular epidemiological work will be required to answer several lingering and intriguing questions about the natural distribution of the two *srfH* alleles. It is noteworthy that *SrfH* Gly103 is a rare allele, but is present in two highly invasive *Salmonella* strains, as we observed that this residue is important for the extraintestinal dissemination of *Salmonella* at early time points in mice. Whether or not *SrfH* Gly103 contributes to the high invasiveness of *S. Typhimurium* UK-1 and *Salmonella* 4, [5],12:i:- *CVM23701* needs to be determined. It will also be important in future work to determine the distribution of the two alleles in *S. Typhi* [5],12:i:- and perhaps, if supportive data is uncovered, to address the intriguing possibility that the acquisition of *SrfH* Gly103 by members of this serovar influenced the rate of septicaemia associated with it and its pandemic rise over the last 15 years. It is possible that *SrfH* Gly103 is an emerging allele that will become resistant [21]. Interestingly, in Thailand and Brazil it is frequently associated with septicemia [22–24]. In fact, in Sao Paulo Brazil, between 1991 and 2000, 25% of human infections by *S. Typhi* [22–24]. The *srfH* allele of *S. Typhi Ty2* or in strain CT18. One report however presented a zoo-blot, which indicated that there was a *srfH* homolog in a strain of *S. Typhi* isolated in Dakar in 1993 [25]. We confirmed this report by PCR amplifying *srfH* from *S. Typhi Dakar* with primers designed to match the *srfH* allele of *S. Typhimurium* (Fig. S2). The *srfH* allele in *S. Typhi* Dakar encodes *Asp103*. We next attempted to PCR amplify two other genes believed to be specific to Gifsy-2: *gtgA* and *solC*. Both genes can be readily PCR amplified from *S. Typhi Dakar* (Fig. S2).
more prevalent over time, but it could also of course be an anti-

virulence factor. Unfortunately, the sfH alleles appear to be clonal
outside of residue 103 making it difficult to determine which of
the two alleles is newer.

What effects, if any, that sfH, ggtA and sodCI have on S. Typhi
pathogenesis warrant consideration. Inactivating ggtA in S.
Typhimurium does not have a detectable impact on virulence in
the mouse model of typhoid fever [31]; however, it was identified
along with sfH as contributing to virulence in the murine, long-
term Salmonella infection model [32]. Its function remains to be
determined. SodCI on the other hand has a well-established role
in promoting Salmonella virulence in differentiated macrophages.
SodCII is encoded on the chromosome, outside of Gifsy-2, and has
14028s produces two periplasmic Cu/Zn superoxide dismutases.
SodCII is encoded on the chromosome, outside of Gifsy-2, and has
its absence does not confer a virulence defect. However, sodCI provides a 7 to 10 fold survival advantage [33,34]. SodCI in fact, possesses one of highest catalytic rates ever measured for superoxide dismutases [35]. In future work, it will be interesting to assess if SrfH assists S. Typhi Dakar in manipulating the migratory properties of infected phagocytes. It will be of further interest to determine, what, if any, effect SodCI has on the ability of S. Typhi Dakar to survive within
macrophages.

It is also interesting to consider the origins of ggtA, sodCI and sfH in S. Typhi Dakar. These three genes could have become associated with mobile elements other than Gifsy-2 and acquired, potentially, independently of each other by S. Typhi Dakar; however, the simplest explanation would of course be that S. Typhi Dakar somehow acquired Gifsy-2. Gifsy-2 is a fully functional bacteriophage capable of excising itself from the S. Typhimurium chromosome and transferring itself to non-immune recipient Salmonella strains [31]. However, the attachment site in most strains of S. Typhi is occupied by a related phage that confers immunity to Gifsy-2 [31]. Thus, more work will be required to determine if Gifsy-2 is indeed present in S. Typhi Dakar, and if so, if it has managed to replace the Gifsy-2 prophage, or alternatively has integrated into the chromosome somewhere other than at its normal attachment site.

In addition to shedding light on one pathway through which non-typhoidal Salmonella can enter the bloodstream, studying SrfH may also have implications for understanding S. Typhi pathogenesis. While sfH is not present in the genomes of all strains of S. Typhi, most of them probably have sfH functional analogs, and there will likely be additional strains found to encode SrfH, perhaps including ones that possess Gly103. Pathogen directed host cell migration might prove to be a common feature of invasive pathogens of humans. Thus, unraveling on a molecular level exactly how SrfH subverts host cell motility to promote virulence will perhaps not only enhance our knowledge of Salmonella pathogenesis but also reveal some of the underlying shared logic through which pathogens manipulate host cells.

Materials and Methods

Ethics Statement

Animals were housed, cared for, and used strictly in accordance
with the USDA regulations and the NIH guide for the care and
use of laboratory animals (NIH publication no. 85–23, 1983). The
University of Louisville is fully accredited by the American
Association for the Accreditation of Laboratory Animal Care. A
full-time, specialty-trained veterinarian directs the program of
animal care. The protocol was approved by the University of
Louisville Institutional Animal Care and Use Committee (protocol
# 11032). All reasonable efforts were made to alleviate discomfort.

General Methods

All molecular biology and genetic manipulations were performed
with established protocols [36,37]. It was determined that SrfH Asp103 does not bind TRIP6 with the Hybrid Hunter yeast two-hybrid assay kit (Invitrogen, Carlsbad, CA), following the manufacturer’s directions, which can be found online at: http://

www.tcd.ie/Genetics/Staff/Noel.Murphy/recombinant

%20dna%20ge4021/hybridman.pdf. β-Gal assays were performed on cultures grown with vigorous shaking at 30°C using a protocol available at: http://labs.fhcrc.org/gottschling/

Yeast%20Protocols/Bgal.html, which was adapted from Current
Protocols in Molecular Biology [36]. It was determined that both
sfH alleles could be stably expressed in yeast by lysing the yeast
correcting the kit’s directions and then subjecting the lysates to SDS-PAGE and visualizing a hybrid protein of the correct molecular weight after performing a Western blot with an anti-

LexA antibody (Invitrogen). LexA was the DNA binding domain that SrfH was fused to in the yeast two-hybrid assay.

Mice, Cell Culture and Bacterial Strains

Four-week-old female Balb/Cj mice were obtained from Jackson labs (Bar Harbor, ME). Bone marrow was harvested and differentiated into primary macrophages, and cultured in RPMI (ATCC, Manassas, VA), supplemented with 20% FBS (Sigma, St. Louis, Mo) and 30% L929 (ATCC) conditioned media as previously described [38]. S. Typhimurium SL1344 was obtained from the Salmonella genetic stock center (Salmonella genetic stock center # 438), originally described in Hoiseth and Stocker [39]. This was the parent strain used in this study which is also the same one used by McLaughlin et. al [12]. The entire sfH open reading frame was deleted with the method of Datsenko and Wanner [40]. To generate a SrfH Gly103 knock-in mutant in an SL1344 background, the SrfH Gly103 allele was PCR amplified from the chromosome of S. Typhimurium 14028s, treated with T4 polynucleotide kinase, and blunt end ligated into the suicide plasmid pRE112 [41], which had been digested with SmaI and dephosphorylated, yielding pMJW2150. This construct was recovered in the Esherichia coli strain MFDpir, which has been engineered to allow the propagation of suicide plasmids [42], pRE112 contains the counter-selectable marker sacB1 as well as a chloramphenicol cassette. S. Typhimurium SL1344 was conjugated with MFDpir + pMJW2150 and merodiploids at the sfH locus recovered on agar plates supplemented with chloramphenicol, but lacking diaminopimelic acid (DAP). The chloramphenicol killed the S. Typhimurium SL1344 cells which did not become merodiploids and the lack of DAP prevented MFDpir cells from growing. Ten merodiploids were passaged serially three times in LB broth without DAP or antibiotics to give them a chance to resolve the duplicated allele. They were then grown to mid-

exponential phase and plated on LB-agar plates without NaCl,

containing 5% sucrose. The sucrose killed the remaining

merodiploids. The strains in which SrfH Gly103 had replaced
SrfH Asp103 were identified by PCR and sequencing. The new
allele was then PCR amplified from one such strain, MJW2163,

with oligonucleotides that flanked the exchanged material, and the entire area sequenced to ensure that no secondary mutations had been inadvertently introduced.

Mouse Dissemination Assay

Mice were orally administered an inoculum of 1×109 bacteria
suspended in 100 μl of phosphate buffered saline. Food was
withheld for 12 hours prior to infection. Thirty minutes after oral infection, mice were euthanized with CO2 and blood recovered by heart puncture with a 25 G needle attached to a 1 mL syringe.

**Supporting Information**

**Figure S1** Sequencing chromatograms of internal regions of the srfH open reading frames. A) strain 14028s and B) strain SL1344. The arrows indicate the G A SNP. (TIF)

**Figure S2** Three Gifsy-2-specific genes are present in a clinical isolate of *S. typhi*. Multiplex PCR with primers specific to internal regions of the *s*. Typhimurium strain 14028s srfH, sodCl, and ggd alleles were PCR amplified from the genome of an isolate of *S. Typhi Dakar*. The first lane contains a DNA ladder with bands corresponding to (from top to bottom) 1 Kb, 850 bp, 650 bp, 500 bp, 400 bp, 300 bp, 200 bp and finally 100 bp. The next lane contained genomic DNA from *S. Typhi Dakar*. The final lane was a no template, negative control. The predicted sizes of the PCR products are 708 bp for *srfH*, 481 bp for *sodCl*, and 329 bp for *ggd*. (TIF)

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**Author Contributions**

Conceived and designed the experiments: MJW. Performed the experiments: JMT MJW. Analyzed the data: MJW. Wrote the paper: MJW.

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